Characterization of the Type VI Secretion System in the Bacterial Pathogen *Acinetobacter baumannii*

by

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Abstract

Antibiotic resistant bacteria that cause hospital-acquired infections are a mounting concern for healthcare systems globally. Multidrug resistant (MDR) *Acinetobacter baumannii* has emerged as a common cause of nosocomial infections, and some isolates are now pandrug resistant. These infections can be polymicrobial, with one or several other different bacteria being co-isolated with *A. baumannii*, suggesting interactions with other microorganisms is common for this pathogen. The type VI secretion system (T6SS) has recently been described as an offensive molecular weapon used by many Gram-negative bacteria to kill competing bacteria. This multi-component apparatus facilitates the contact-dependent injection of toxic effector proteins into nearby prey cells and can allow the bacterium expressing the T6SS to dominate a particular environment. Production of cognate immunity proteins prevents self-inflicted intoxication by binding to and inactivating the toxins, which provides a means of discriminating self from non-self. The T6SS is dynamic and energetically costly, and therefore appears to be exquisitely regulated in most bacteria.

Apart from increased antibiotic resistance, little is known about the factors that have contributed to the rapid rise of *A. baumannii* as a nosocomial pathogen. In this thesis, we combined bioinformatic and genetic analyses to experimentally characterize the T6SS in *A. baumannii*. We found that many species of *Acinetobacter* secrete a conserved T6SS protein called Hcp, indicating a functional secretory system. Through phenotypic screens and genome sequencing of clinical isolates, we identified a novel regulatory system that controls expression of the T6SS, which confers an anti-bacterial phenotype. This regulatory mechanism resulted in the loss of antibiotic resistance in those cells which activated their T6SS, indicating an incompatibility between these

two phenotypes. We found that this regulation was controlled by a conjugative MDR plasmid, with proteins expressed by the plasmid repressing T6SS. We present the hypothesis that *A. baumannii* differentiate into antibiotic resistant cells or bacterial killers, with the two phenotypes being mutually exclusive.

The biogenesis of the T6SS has been intensely studied during the past decade, but has not been investigated in detail for *A. baumannii*. We screened mutants in the model organism *A. baylyi* to uncover the genetic requirements for elaborating a functional T6SS. These experiments revealed several essential genes that were required for T6SS function, but had not been characterized in other bacteria. This led to the biochemical characterization of the novel T6SS component TagX, which hydrolyzes peptidoglycan, that we propose facilitates the transit of the T6SS through the producing organism's peptidoglycan layer. Furthermore, we describe the first T6SS-dependent anti-bacterial effectors of *A. baumannii*, and elucidate the requirement of VgrG proteins for their effector activity. Overall, the studies presented in this thesis provide a comprehensive understanding of the T6SS in this emerging pathogen and inform future studies on the role of this secretory system in the pathobiology of *A. baumannii*.

Preface

Portions of Chapter 1 of this thesis have been published as "Weber, B.S., Harding, C.M., and Feldman, M.F. (2016). Pathogenic *Acinetobacter*: from the cell surface to infinity and beyond. Journal of Bacteriology 198 (6) 880-887". Harding, CM was responsible for preparation of sections on pili, type II secretion, and autotransporters. I was responsible for preparing the remaining sections and organizing and editing the manuscript under the supervision of Feldman MF.

Chapter 2 of this thesis has been published as "Weber, B.S., Miyata, S.T., Iwashkiw, J.A., Mortenson, B.L., Skaar, E.P., Pukatzki, S., and Feldman, M.F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. PLoS ONE 8 (1): e55142. doi:10.1371/journal.pone.0055142". Miyata ST assisted with bacterial killing assays under the supervision of Pukatzki S. Iwashkiw JA performed initial annotation of the type VI secretion system gene cluster. Mortenson BL performed mice experiments under the supervision of Skaar EP. I was responsible for performing and designing the remaining experiments, as well as data analysis and preparation of the manuscript. Conclusions were drawn under the supervision of Feldman MF.

Chapter 3 of this thesis has been published as "Weber, B.S., Ly, P., Irwin, J.N., Pukatzki, S., and Feldman, M.F. (2015). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. PNAS 112 (30) 9442-9447". Ly, P cloned and expressed TetR regulatory proteins. Irwin JN analyzed bioinformatics data. Pukatzki S assisted with genome assembly. I was responsible for performing and designing the remaining experiments, as well as data analysis and preparation of the manuscript. Conclusions were drawn under the supervision of Feldman MF.

Chapter 4 of this thesis has been accepted for publication as "Weber, B.S., Hennon, S.W., Wright, M.S., Scott, N.E., de Berardinis, V., Foster, L.J., Ayala, J.A., Adams, M.D., and Feldman, M.F. (2016). Genetic dissection of the type VI secretion system in *Acinetobacter* and identification of a novel peptidoglycan hydrolase TagX required for its biogenesis. mBio (Manuscript number: mBio01253-16R1)". Hennon SW performed enzymatic assays with TagX. Wright MS performed RNA sequencing under the supervision of Adams MD. Scott NE performed differential mass spectrometry under the supervision of Foster LJ. de Berardinis V provided *Acinetobacter baylyi* strains. Ayala JA performed HPLC analyses. I was responsible for performing and designing the remaining experiments, as well as data analysis and preparation of the manuscript. Conclusions were drawn under the supervision of Feldman MF.

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I am indebted to members of the Feldman lab, both past and present, for their support, advice, and training throughout the years. Those that have passed through the lab have all contributed towards my development. Nico and Wael remain constant sources of advice, inspiration, and coffee-chats, and we have developed a close friendship that I truly value. The group in St. Louis made my time there genuinely enjoyable, and I thank Amy, Christian, Ezequiel, Rachel, and Seth for their friendship and scientific advice during my short stay. The support staff at both the University of Alberta and Washington University in St. Louis provided experimental assistance and training crucial to the progress of this thesis.

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List of Abbreviations

Acb	*Acinetobacter calcoaceticus-baumannii* complex
Ata	*Acinetobacter* timeric autotransporter
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
GFP	Green fluorescent protein
Hcp	Hemolysin-coregulated protein
HPLC	High performance liquid chromatography
IM	Inner membrane
INSeq	Insertion sequencing
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MS	Mass spectrometry
MS/MS	Tandem MS
LB	Lysogeny broth/Luria-bertani broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MDR	Multidrug resistance

NaCl	Sodium chloride
OM	Outer membrane
OMV	Outer membrane vesicles
OXA	Oxacillinase
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween-20
PG	Peptidoglycan
PNAG	poly-beta-1-6-N-acetylglucosamine
Rhs	Recombination hot-spot
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNAseq	RNA sequencing
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S/T	Sulfamethoxazole/trimethoprim
TMB	3,3',5,5'-Tetramethylbenzidine
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol Hydrochloride
TssA-M	Type six secretion protein A-M
TEAB	Triethylammonium bicarbonate
TPM	Transcripts per million
T2SS	Type II secretion system

T3SS	Type III secretion system
T4SS	Type IV secretion system
T5SS	Type V secretion system
T6SS	Type VI secretion system
T6+	T6SS positive
T6-	T6SS negative
Und-P	Undecaprenyl phosphate
VgrG	Valine-glycine repeat protein G

1 Introduction

Portions of Chapter 1 of this thesis have been published

Weber, B.S., Harding, C.M., and Feldman, M.F. (2016). Pathogenic *Acinetobacter*: from the Cell Surface to Infinity and Beyond. Journal of Bacteriology 198 (6) 880-887.

Hospital-acquired infections caused by opportunistic bacterial pathogens are a significant contributor to patient death and a major healthcare burden (Boucher *et al.*, 2009). Coupled with an alarming increase in antibiotic resistance, and few new antibiotics in development, drug-resistant bacterial pathogens have quickly established themselves as a global threat to healthcare security. These infections are estimated to directly or indirectly claim the lives of 100,000 people and result in an additional $34 billion in health care costs each year in the US alone (Infectious Diseases Society of *et al.*, 2011). Due to the evolutionary capabilities of microorganisms, resistance to antibiotics is expected to occur; indeed, resistance has been detected since the beginning of widespread antibiotic use (Fowler *et al.*, 2014). However, the shear number of bacterial species that are now displaying multidrug resistance to some of the 'last line of defense' antibiotics is of great concern (Spellberg *et al.*, 2008). In contrast to past decades, there is no longer a fresh supply of novel antibiotics in the developmental pipeline, and thus the threat of a 'post-antibiotic era' is a real concern (Spellberg *et al.*, 2008; Talbot *et al.*, 2006). Pandrug resistant bacteria, which are resistant to every clinically relevant antibiotic, have already been isolated (Falagas *et al.*, 2008; Valencia *et al.*, 2009). In the absence of clear progress in advancing antimicrobial discovery, it is imperative to understand the molecular mechanisms used by bacteria to persist in hospital environments so that alternative treatment strategies can be designed.

1.1 Organism studied in this thesis

1.1.1 *Acinetobacter baumannii*

1.1.1.1 Relevant characteristics of *Acinetobacter* spp.

Members of the genus *Acinetobacter* are Gram-negative, strictly aerobic, oxidase-negative, catalase-positive, and non-lactose fermenting. The genus *Acinetobacter* was initially proposed in

1954, and widely adopted following studies conducted by Baumann et al. later (Baumann *et al.*, 1968; Brisou *et al.*, 1954). Although only a few species of *Acinetobacter* were initially recognized, DNA-DNA hybridization analysis resulted in a total of 12 distinct genomic species of *Acinetobacter* (Bouvet, 1986). The list of genomic species was further extended by others (Bouvet *et al.*, 1989; Tjernberg *et al.*, 1989), and currently includes approximately 30 distinct genomic species (Peleg *et al.*, 2008a). Four of these genomic species, *A. calcoaceticus*, *A. baumannii*, *A. pittii*, and *A. nosocomialis*, have previously been grouped together as the '*Acinetobacter calcoaceticus-baumannii* (Acb) complex' due to the difficulty in phenotypically differentiating these species (Gerner-Smidt *et al.*, 1991). This complex contains the three most medically relevant *Acinetobacter* spp. (*A. baumannii*, *A. pittii*, and *A. nosocomialis*), as well as an environmental species (*A. calcoaceticus*), and thus may be misleading in a healthcare setting and therefore has generally fallen out of favor (Peleg *et al.*, 2008a). While existing literature has often failed to differentiate the species of this complex, new typing methods have been developed to address this concern (Chen *et al.*, 2014). This is particularly relevant considering the differences in antibiotic susceptibilities and infection outcomes associated with the medically relevant members of the Acb complex, with *A. baumannii* often presenting more of a challenge from a clinical perspective (Lim *et al.*, 2007; Park *et al.*, 2014).

1.1.1.2 Infections caused by *A. baumannii*

A. baumannii is generally considered the most clinically relevant species of the genus, as it is commonly isolated in hospital acquired infections (Dijkshoorn *et al.*, 2007). Although infrequently encountered outside of the healthcare environment, *A. baumannii* has been found with various other sources such as food, water, human skin, and veterinary animals (Dijkshoorn *et al.*,

2007; Peleg *et al.*, 2008a). Carriage rates in the community are considered to be relatively low (Seifert *et al.*, 1997). This organism has recently emerged as one of the most concerning Gram-negative pathogens to infiltrate the hospital setting, and is among the few pathogens that have significantly increased as a cause of infection in US hospitals (Sievert *et al.*, 2013). *A. baumannii* is responsible for more than 12,000 infections per year in US and is considered a "serious" threat to healthcare systems (Centers for Disease Control and Prevention, 2013). Immunocompromised patients are at high risk for infection, typically causing ventilator-associated pneumonia and bloodstream infections. Mortality rates can approach 50%, although this varies considerably between studies, and patients with prolonged hospital stays are more susceptible to infection (Garnacho-Montero *et al.*, 2005; Seifert *et al.*, 1995). *A. baumannii* can also cause a wide-range of other diseases, include soft-tissue infections, urinary tract infections, meningitis, and community acquired pneumonia, demonstrating the versatility of this pathogen (Figure 1.1A) (Peleg *et al.*, 2008a). Soft tissue infections were common in military personnel injured in the Iraq and Afghanistan conflicts (Sebeny *et al.*, 2008).

Patients colonized or infected with *A. baumannii* can serve as a source for spreading this organism in the healthcare setting. Indeed, outbreaks are frequently caused by *A. baumannii* and are notoriously difficult to control, being attributed to its extreme dessication and disinfection tolerance (Ahmed-Bentley *et al.*, 2013; Bernards *et al.*, 1998; Bernards *et al.*, 2004; Fournier *et al.*, 2006; Jawad *et al.*, 1998; Melamed *et al.*, 2003). Moreover, healthcare workers are often suspected of spreading *A. baumannii* through poor hygienic practices (Ahmed-Bentley *et al.*, 2013; Maragakis *et al.*, 2008a). While these outbreaks are often caused by a single source strain of *A. baumannii*, introduction of multiple strains at the same institution has been reported, causing concurrent outbreaks and leading to horizontal gene transfer between isolates (Snitkin *et al.*, 2011).

A. baumannii is often isolated from polymicrobial infections with other organisms like *Escherichia coli* and *Klebsiella pneumoniae*, complicating treatment strategies and suggesting that interactions occur among these microorganisms (Ahmed-Bentley *et al.*, 2013; Liao *et al.*, 2014).

Infections caused by *A. baumannii* are becoming increasingly difficult to treat, owing to this pathogen's ability to acquire multi-drug resistance (MDR), and pan-drug resistant isolates have now been reported (Valencia *et al.*, 2009). New strategies for treating and managing MDR *A. baumannii* infections are urgently needed, which requires a detailed understanding of this organism's pathobiology. Unfortunately, relatively little is known about the basic biological processes utilized by this organism to cause disease and persist in the hospital environment.

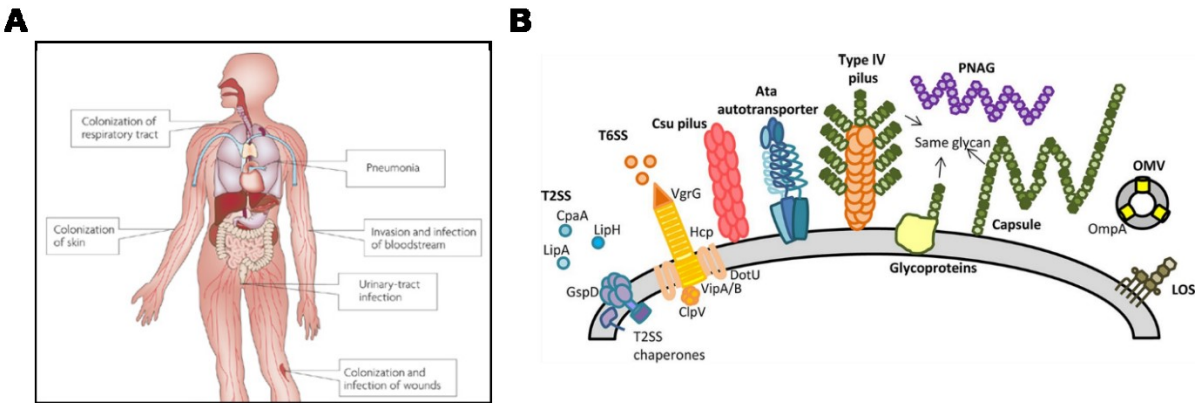

Figure 1.1 Pathogenic and molecular features of *A. baumannii*

A) Schematic depiction of the range of infections caused by *A. baumannii*. B) Summary of experimentally studied structures present on the cell surface of *A. baumannii.* Part A of this figure is adapted from (Dijkshoorn *et al.*, 2007).

1.1.1.3 Antibiotic resistance

The rise in antibiotic resistance among *A. baumannii* isolates is of worldwide concern, and oversight bodies are beginning to take note of this salient threat (Boucher *et al.*, 2009; Centers for Disease Control and Prevention, 2013). Once considered a relatively benign infectious agent, the

rapid evolution of multidrug resistant *A. baumannii* has made many antibiotics obsolete against this organism (Garcia-Quintanilla *et al.*, 2013). *A. baumannii* encode intrinsic resistance to some β-lactams through the overexpression of a chromosomal AmpC-type cephalosporinase, and point mutants of this protein can lead to resistance to extended spectrum β-lactams (Bou *et al.*, 2000; Corvec *et al.*, 2003). Resistance to carbapenems, the antibiotics of choice for treating *A. baumannii* infections, is often mediated by acquisition of class D oxacillinases such as OXA-23 in transposon structures (Bonnin *et al.*, 2013; Poirel *et al.*, 2010; Poirel *et al.*, 2006). New Delhi metallo-β-lactamases are increasingly being reported in *A. baumannii*, providing high level of resistance to the important carbapenem imipenem (Bonnin *et al.*, 2012). Overexpression of endogenous efflux pumps are further contributors to antibiotic resistance in *A. baumannii,* especially the intrinsic AdeABC tripartite resistance-nodulation-cell division pump which also provides resistance to fluoroquinolones (Coyne *et al.*, 2011; Magnet *et al.*, 2001). Resistance to aminoglycosides is most often mediated by the ArmA protein, a 16S rRNA methylase that is horizontally acquired, and is commonly identified in strains that also produce OXA-23 (Doi *et al.*, 2007).

The identification of strains resistant to all front-line antibiotics has forced health care providers to re-evaluate antibiotics seldom used in the clinic. The polymixin antibiotic colistin, discovered in the 1950's, has become the last line of defence for treating *A. baumannii* infections refractory to treatment with standard antimicrobials (Falagas *et al.*, 2005; Li *et al.*, 2005). Owing to acute renal toxicity, use of colistin has been sparse since its discovery, but has now been increasingly used for the treatment of MDR infections (Falagas *et al.*, 2005). Not surprisingly, *A. baumannii* isolates resistant to colistin have subsequently been described. Mutations affecting the *pmrAB* two-component system, resulting in the addition of a phosphoethanolamine group to lipid A, or complete loss of lipid A, are the most common mechanisms of resistance (Adams *et al.*,

2009; Beceiro *et al.*, 2011; Moffatt *et al.*, 2010). The emergence of colistin resistance has ushered in an age of pandrug resistant *A. baumannii,* and these isolates are already beginning to emerge in the healthcare setting (Valencia *et al.*, 2009).

1.1.2 Virulence mechanisms

The emergence of *A. baumannii* isolates resistant to all standard therapies has led to a pressing need to elucidate the mechanisms used by pathogenic *Acinetobacter* to cause disease. Despite decades of work addressing the molecular underpinnings of antibiotic resistance and its spread, relatively little attention has been focused on understanding the virulence traits that are responsible for this pathogen's success in the healthcare setting.

The bacterial cell surface plays essential roles in sensing of the environment, interactions with the host, and maintaining cellular homeostasis (Silhavy *et al.*, 2010). The molecular structures present on the cell surface, and those that extend beyond the surface, are of central importance to understanding the pathogenesis of an organism. These are often the key determinants that mediate bacterial virulence and thus represent important targets for novel antimicrobials and vaccines (Grandi, 2010). While all bacterial cell surfaces are composed primarily of lipids, carbohydrates, and proteins, the diversity in the molecular composition and arrangement of these structures has vast implications for virulence in pathogenic bacteria. Recent experimental investigation into the arrangement and composition of these structures on *Acinetobacter* cell surfaces has provided important insights into their role in the pathobiology of these important human pathogens (Figure 1.1B).

1.1.2.1 Lipooligosaccharide (LOS)

Lipopolysaccharide (LPS), the major component of the outer leaflet of the outer membrane of many Gram-negative bacteria, is an immunostimulatory molecule that plays an important role in bacterial resistance to external stresses (Raetz *et al.*, 2002). LPS is composed of the endotoxic lipid A, a core oligosaccharide, and a repeating sugar structure called the O antigen (Raetz *et al.*, 2002). During biosynthesis of LPS, the core oligosaccharide is built onto the lipid A moiety in the cytoplasm and flipped into the periplasmic space. The repeat subunit of the O antigen is synthesized separately onto an undecaprenyl phosphate (Und-P) carrier, which is then flipped to the periplasm, and ligated to the lipid A core by the WaaL ligase enzyme (Whitfield *et al.*, 1997); thus, the WaaL ligase is essential for the production of O antigen. Many pathogens, such as *E.coli* and *Salmonella* spp., produce diverse O antigen structures that form the basis for serotyping schemes (Liu *et al.*, 2014; Orskov *et al.*, 1977; Stenutz *et al.*, 2006). However other important bacterial pathogens, such as *Neisseria* and *Campylobacter*, lack a WaaL ligase ortholog and do not produce O antigen, elaborating only lipooligosaccharide (LOS) (Preston *et al.*, 1996).

Whether *Acinetobacter* spp. elaborate LPS or LOS on their cell surface has been a topic of considerable debate in the field. Several reports have described the presence of LPS in *Acinetobacter*, many of which have used structural or antibody-based methods to detect the O antigen carbohydrate moieties (Haseley *et al.*, 1997a; Haseley *et al.*, 1997b; Haseley *et al.*, 1994, 1998; MacLean *et al.*, 2009; Pantophlet *et al.*, 1999; Traub, 1989; Vinogradov *et al.*, 2003; Vinogradov *et al.*, 2002; Vinogradov *et al.*, 1996). However, most *Acinetobacter* spp. do not show a "typical" LPS laddering upon silver staining of isolated LPS (Fregolino *et al.*, 2011; Pantophlet, 2008), leading to doubts as to whether *Acinetobacter* spp. actually produce a true O antigen. Intriguingly, *Acinetobacter* spp. possess either one or two genes that encode proteins with similar

domains found in WaaL ligase orthologs depending on the strain (Harding *et al.*, 2015; Iwashkiw *et al.*, 2012b; Kenyon *et al.*, 2013; Schulz *et al.*, 2013); however, these domains are also found in PglL, the enzyme responsible for *O*-linked protein glycosylation (see below) (Iwashkiw *et al.*, 2012b). Bioinformatic analysis alone is not sufficient to distinguish between WaaL and PglL orthologs, and careful experimental analysis is required to determine the true function of proteins possessing these domains. Recent work has now conclusively demonstrated that in *Acinetobacter* spp. with a single "*waaL*-like" gene that this gene actually encodes a PglL enzyme, and possesses no O antigen ligase activity (Iwashkiw *et al.*, 2012b). While *Acinetobacter* spp. encoding two "*waaL*-like" genes were initially suggested to encode one PglL enzyme and one WaaL enzyme (Schulz *et al.*, 2013), experimental analysis has identified both enzymes as being exclusively involved in protein *O*-linked glycosylation, with no role in O antigen biosynthesis (Harding *et al.*, 2015). In light of these recent data, and in the absence of experimental data showing a protein possessing divergent O antigen ligase activity, it seems most likely that *Acinetobacter* spp. produce LOS but not LPS. In any case, targeted and random mutagenesis of genes involved in synthesis of the LOS core oligosaccharide has shown that this component is a major contributor to *Acinetobacter* survival and virulence (Lin *et al.*, 2012; Luke *et al.*, 2010; McQueary *et al.*, 2012). The cluster of genes which synthesize the sugar component of LOS are extremely diverse among *Acinetobacter* spp., and a number of different structures have been determined or predicted (Kenyon *et al.*, 2014). Modification of the LOS has also been shown to impart resistance to antimicrobials, similar to what has been found in other bacterial species. Specifically, these modifications occur on the lipid A structure of *Acinetobacter* spp. and leads to decreased susceptibility to antibiotic and antimicrobial peptides, and increased survival during desiccation (Arroyo *et al.*, 2011; Boll *et al.*, 2015; Chin *et al.*, 2015). *Acinetobacter* spp. have also been shown

to acquire mutations in the lipid A biosynthetic pathway when treated with colistin, resulting in resistance to the antibiotic (Beceiro *et al.*, 2011; Hood *et al.*, 2013; Moffatt *et al.*, 2010; Pelletier *et al.*, 2013). Interestingly, these studies found that mutations in certain lipid A biosynthetisis genes resulted in complete loss of LOS.

1.1.2.2 Glycoproteins

The post translational modification of proteins with glycans, once thought to be exclusive to eukaryotes, has been identified in all forms of life. In bacteria, carbohydrates can be attached via the amide of asparagine (*N*-linked) or the hydroxyl of serine/threonine residues (*O*-linked). In a series of steps analogous to O antigen biosynthesis, glycans are assembled onto the Und-P lipid carrier and flipped to the periplasmic face of the inner membrane, where an *O*-oligosaccharyltransferase (OTase) enzyme catalyzes the transfer of the complete carbohydrate structure to a serine or threonine residue on the cognate acceptor protein (Hug *et al.*, 2011). Bioinformatic analysis identified a protein in *A. baumannii* that showed homology to the *O*-OTase from *Neisseria meningitidis*, named PglL (Iwashkiw *et al.*, 2012b). PglL proteins often contain domains similar to WaaL ligases (Wzy_C domain), and bioinformatic identification of a PglL-like protein is not sufficient to differentiate it from WaaL ligases, thus necessitating experimental characterization. When the *pglL*-like gene from *A. baumannii* was deleted, a loss of a carbohydrate specific band was detected after SDS-PAGE analysis with no change in LOS profile (Iwashkiw *et al.*, 2012b). Further in-depth characterization of the *A. baumannii pglL* mutant by mass spectrometry determined that the *pglL*-deficient strain lacked a total of seven glycoproteins, which were glycosylated with a pentasaccharide in the wild type strain. Loss of protein glycosylation in *A. baumannii* resulted in pleiotropic effects on several virulence-associated phenotypes, including

biofilm formation and survival in a mouse model of systemic infection (Iwashkiw *et al.*, 2012b). Protein glycosylation has now been shown to be a conserved modification present throughout the genus *Acinetobacter*, but the composition of the glycan moiety and the number and identity of the modified proteins varies among different strains (Scott *et al.*, 2014b). Interestingly, the carbohydrate structure attached to glycoproteins is identical to the repeat units found in capsular polysaccharide (Lees-Miller *et al.*, 2013). Although the phenotypes associated with complete loss of the protein glycosylation have been studied, the contribution of individual glycoproteins to these phenotypes remains unknown.

The gene encoding *pglL* in *A. baumannii* is located immediately downstream of the predicted major type IV pilin gene, *pilA*, a common target for glycosylation in several bacteria. In *A. baumannii* ATCC 17978, this protein was not found to be glycosylated under laboratory conditions, but it was glycosylated upon overexpression in the presence of PglL (Harding *et al.*, 2015; Iwashkiw *et al.*, 2012b). Intriguingly, most *Acinetobacter* actually encode for two proteins with a domain from the Wzy_C superfamily common to PglL and WaaL orthologs. It was originally suggested that this second gene could be a WaaL ligase involved in O antigen biosynthesis (Schulz *et al.*, 2013). Through mutagenesis and functional studies, however, it was determined that both genes actually encode *O*-OTases (Harding *et al.*, 2015). The *O*-OTase encoded nearby the pilin was found to exclusively glycosylate the cognate pilin protein, similar to the PilO/TfpO protein from *P. aeruginosa* (Castric, 1995), while the second gene encoded a general *O*-OTase responsible for the glycosylation of multiple proteins, similar to PglL from *Neisseria* (Faridmoayer *et al.*, 2008; Faridmoayer *et al.*, 2007). Although the functional significance of having two glycosylation systems remains to be determined, this represents the first known case of multiple *O*-OTases present in a single bacterium.

1.1.2.3 Capsule

Like many other pathogens, *A. baumannii*. produce an extracellular capsule that provides a layer of protection from external threats such as complement-mediated killing (Russo *et al.*, 2010). Capsule production and protein glycosylation are exquisitely linked in this bacterium, as the carbohydrate repeat unit found in capsule is the same as the single repeat unit attached to proteins (Lees-Miller *et al.*, 2013). The sugar subunits for capsule and protein glycosylation are derived from the same pathway, where an initiating glycosyltransferase PglC/ItrA attaches the first carbohydrate to Und-P, followed by the addition of other sugar monomers by glycosyltransferase enzymes to complete the repeat unit (Lees-Miller *et al.*, 2013). This repeat unit is then flipped to the periplasm, and at this point the capsule and protein glycosylation pathways diverge. In the case of protein glycosylation, this single repeat unit is attached to the target protein by the *O*-OTase (Iwashkiw *et al.*, 2012b). For capsule production, individual sugar repeat units are instead polymerized and exported to the cell surface. This bifurcated pathway represents a novel mechanism that illustrates the evolutionary connections between capsule and protein glycosylation that may allow *A. baumannii* to rapidly adapt to changing environments. How *A. baumannii* partitions a given carbohydrate repeat unit to the protein glycosylation pathway or capsule production remains to be determined. Although the carbohydrate structures produced by different strains are highly variable, functional studies have shown that the production of capsule is essential for survival during infection and growth in serum (Kenyon *et al.*, 2013; Lees-Miller *et al.*, 2013; Russo *et al.*, 2010; Wang *et al.*, 2014). Recently, it was reported that production of capsule can be augmented by the presence of sub-inhibitory levels of antibiotics, which increases resistance to complement mediated killing and leads to a hyper-virulent phenotype in a mouse model of

systemic infection (Geisinger *et al.*, 2015). This capsule hyper-production phenotype was shown to be controlled by the BfmRS two-component system, which regulates several other important virulence factors in this organism (Tomaras *et al.*, 2008). *A baumannii* also produces a surface associated poly-beta-1-6-N-acetylglucosamine (PNAG) polysaccharide, which is important for virulence and biofilm formation (Choi *et al.*, 2009).

1.1.2.4 Pili

Filamentous bacterial surface appendages, termed pili, mediate interactions between the producing organism and their environment. *Acinetobacter* pili have been studied as far back as 1975, when Henrichsen identified that *A. calcoaceticus* strains displaying surface fimbrial structures were shown to exhibit twitching motility (Henrichsen, 1975; Henrichsen *et al.*, 1975), a form of bacterial locomotion now known to be dependent on functioning type IV pili (Merz *et al.*, 2000). Furthermore, it has been shown that the non-pathogenic, model organism *A. baylyi* ADP1 produces both thin and thick pili (Gohl *et al.*, 2006); however, the roles pili play in pathogenic *Acinetobacter* species' biology and pathobiology have only partially been uncovered.

A chaperone/usher pili system, designated Csu pili, has been identified in all sequenced pathogenic *Acinetobacter* spp.; however, the Csu pili have been primarily studied in *A. baumannii* (Tomaras *et al.*, 2003). The Csu pili are required for biofilm formation/maintenance in *A. baumannii* ATCC 19606, however, were found to not play a role in adherence to human epithelial cells (de Breij *et al.*, 2009). Another study found that the CsuA/B pilin subunit was the most abundant protein identified within the pellicle matrix of multiple *A. baumannii* strains (Nait Chabane *et al.*, 2014), further strengthening the role of Csu pili in biofilm formation and maintenance. Previous reports also identified a single nucleotide insertion in the *csuB* gene of *A.*

baumannii ATCC 17978 suggesting that this system may be non-functional in this strain (Eijkelkamp *et al.*, 2011; Eijkelkamp *et al.*, 2014); however, recent re-sequencing of the *A. baumannii* ATCC 17978 genome did not find the same insertion event (Weber *et al.*, 2015b). Lastly, other chaperone/usher pili-like systems have been bioinformatically identified in many *A. baumannii* strains, but none of these pili systems have been functionally characterized (Eijkelkamp *et al.*, 2014; Nait Chabane *et al.*, 2014). Mass spectrometric characterization of pellicle-associated proteins did however find that non Csu-pilin subunits were present indicating that these systems may be functional.

Medically relevant *Acinetobacter* spp. have also been shown to produce type IV pili (Tfp), which are dynamic bacterial surface appendages known to mediate twitching motility, horizontal gene transfer, and biofilm formation (Burrows, 2012). Although bioinformatic studies have identified genes predicted to encode proteins required for the biogenesis of Tfp in *A. baumannii*, only *A. nosocomialis* strain M2 has been conclusively shown to produce functioning Tfp (Carruthers *et al.*, 2013b; Harding *et al.*, 2013), which is glycosylated by a TfpO-like oligosaccharyltransferase (Harding *et al.*, 2015). Many *A. baumannii* isolates have been found to be naturally transformable and exhibit twitching motility (Eijkelkamp *et al.*, 2011; Ramirez *et al.*, 2010; Wilharm *et al.*, 2013; Yoon *et al.*, 2015), two classical Tfp-associated phenotypes, which strongly indicate their presence. Tfp-like structures were also identified on *A. baumannii* ATCC 17978 (Tucker *et al.*, 2014); furthermore, mutants in predicted Tfp biogenesis components of *A. baumannii* ATCC 17978 had impaired biofilm formation (Tucker *et al.*, 2014), yet, the major pilin subunit, PilA, has not been shown to be surface-exposed and/or associated with the pilin structures observed. Although the role of Tfp in *Acinetobacter* motility and natural transformation has

emerged as a possible virulence factor, no studies have conclusively linked Tfp to the pathobiology of *Acinetobacter,* as is the case for *Pseudomonas* and *Neisseria.*

1.1.2.5 Protein Secretion

Extracellular export of proteins is a fundamental process for all forms of life. Protein secretion systems of Gram-negative bacteria are extremely diverse in function and composition, and are often important mediators of virulence. Recent research has uncovered several of the mechanisms *A. baumannii* uses to secrete proteins and the role they play in the biology of this organism.

1.1.2.6 Type II secretion

The most recently described secretion system is a functional type II secretion system (T2SS) identified in both *A. nosocomialis* strain M2 (Harding *et al.*, 2015) and *A. baumannii* ATCC 17978 (Johnson, 2015); moreover, it was shown that clinical isolates of *A. pittii*, *A. baumannii*, *A. calcoaceticus*, and *A. junnii* all were able to secrete type II substrates indicating that functioning type II secretion systems seems to be the rule and not the exception. In regards to the T2SS of *A. nosocomialis* strain M2, a two-dimensional differential gel electrophoresis approach was utilized and identified multiple putative type II substrates; however, the LipA and LipH lipases as well as the CpaA metallopeptidase were validated as *bona fide* type II secretion substrates. Interestingly, both LipA and CpaA required specific membrane-associated chaperones for secretion, which indicates that T2SS chaperones are more widespread than previously recognized. Importantly, it was shown that the *A. nosocomialis* strain M2 *gspD* mutant, lacking the outer-membrane secretin of the T2SS, was severely attenuated in both the *Galleria mellonella* and

murine pulmonary infections models. Specifically, mice intranasally infected with the *gspD* mutant strain had approximately two logs less bacterial burden in both the lungs and spleen after 36 hours when compared to both the parental strain and the complemented mutant. In *A. baumannii* ATCC 17978, Johnson et al identified a lipase, LipA, secreted in a T2SS-dependent manner that was required for growth on media containing lipids as a sole carbon source (Johnson, 2015). Mutants in LipA or the T2SS-structural gene GspD were less competitive than wild type in a mixed infection murine model of bacteremia. Collectively, these findings indicate the *Acinetobacter* T2SS is a previously unrecognized virulence factor mediating pathogenesis in a relevant mammalian model. Interestingly, a recent study by Wang *et al* utilized a mutant in the *gspN* gene in *A. baumannii* ATCC 17978 for the validation of their INSeq murine pulmonary infection studies and subsequently found that the *gspN* mutant did not display any virulence defect in survival or competition models as compared to the parental strain (Wang *et al.*, 2014). Although these data are contradictory to the newly defined role of type II secretion in *Acinetobacter*, it has been previously demonstrated that *gspN* homologs were not required for a functioning T2SS in *Klebsiella* oxytoca (Possot *et al.*, 2000); furthermore, *gspN* homologs are absent from numerous known T2SS in other Gram-negative bacteria (Campos *et al.*, 2013), thus, indicating the dispensable nature of GspN from functioning T2SSs.

1.1.2.7 Autotransporters

A type V autotransporter has been characterized in *A. baumannii*. The *Acinetobacter* trimeric autotransporter (Ata) was found to be crucial for certain *A. baumannii* strains' ability to adhere to extracellular matrix components including collagen I, III, IV, and V (Bentancor *et al.*, 2012). Ata is also an important mediator of *A. baumannii* biofilm

formation/maintenance as an *ata* mutant in *A. baumannii* ATCC 17978 had a significantly diminished biofilm and was less virulent in a murine intraperitoneal infection model when compared to the parental and complemented strains (Bentancor *et al.*, 2012).

1.1.2.8 Outer membrane vesicles

A special case of protein secretion is the production of outer membrane vesicles (OMVs), which are blebs of outer membrane (OM) released from the bacterial cell surface (Haurat *et al.*, 2014). There is significant debate as to whether OMVs are produced by a directed process or simply represent cellular debris. Proteomic comparisons between the OM and OMVs of some bacteria has shown that the protein profile differs between these two fractions, indicating that some OM proteins are excluded from OMV recruitment and suggesting OMV formation is a directed process (Haurat *et al.*, 2014). However, many studies also detect cytoplasmic proteins in OMV preparations, indicating that cell lysis could also be a major contributor to OMV formation (Haurat *et al.*, 2014). In spite of this, OMVs have been implicated in numerous biological functions, with particular attention devoted to their role in virulence (Ellis *et al.*, 2010). Several studies on OMVs in *Acinetobacter* have suggested they have many functions, including roles in horizontal gene transfer, antibiotic resistance, and virulence. A wide variety of cargo have been identified in OMVs from different *Acinetobacter* strains, including virulence proteins, antibiotic resistant determinants, and DNA (Dallo *et al.*, 2012; Fulsundar *et al.*, 2014; Fulsundar *et al.*, 2015; Kwon *et al.*, 2009; Li *et al.*, 2015; Liao *et al.*, 2015; Rumbo *et al.*, 2011). An important virulence factor of *A. baumannii*, OmpA, has also been found associated with OMVs, and OMVs have been suggested to act as a delivery mechanism for this protein to host cells (Jin *et al.*, 2011). Furthermore, OmpA has also been suggested to be involved in the biogenesis of OMVs (Moon *et*

al., 2012). OMVs from *Acinetobacter* may have an important role for developing novel therapeutics, as they can stimulate a strong immune response and are protective when administered as a vaccine (Jun *et al.*, 2013; McConnell *et al.*, 2011; Nho *et al.*, 2015).

1.2 The bacterial T6SS

The ability to transport proteins across biological membranes requires dedicated machinery, and Gram-negative bacteria have evolved a number of different mechanisms to achieve this (Costa *et al.*, 2015). Cell surface structures and secreted proteins of bacteria are essential for interactions with the environment and host tissues, and therefore represent attractive targets for antibiotics and vaccines (Grandi, 2010). The T6SS was formally described in 2006, with two publications detailing the requirement of a conserved gene cluster in *Vibrio cholerae* and *Pseudomonas aeruginosa* for secretion of a protein called Hcp. In *V. cholerae*, this gene cluster was shown to be essential for resisting predation by the amoebae *Dictyostelium discoideum,* and was responsible for the secretion of another protein called VgrG (Pukatzki *et al.*, 2006). Similarly, Hcp was secreted in a T6SS-dependent manner in *P. aeruginosa* and could be detected in the sputum from chronically infected cystic fibrosis patients (Mougous *et al.*, 2006). Both studies strongly implicated the T6SS as a virulence factor in these organisms, and bioinformatic analyses suggested the T6SS was widespread among Gram-negative bacteria (Boyer *et al.*, 2009).

1.2.1 Core structural components

Through bioinformatic and mutagenesis studies, the T6SS is now known to be composed of 13 conserved ‘core’ genes present in a variably arranged gene cluster, all of which are essential for assembly and function (Boyer *et al.*, 2009; Zheng *et al.*, 2011; Zheng *et al.*, 2007). These 13

core proteins are designated as TssA-M, for Type six secretion proteins A-M. For historical reasons, some proteins retain their original designation (Hcp: TssD, VgrG: TssI, and ClpV: TssH), and their common names will be used here.

For other secretion systems, such as T3SS and T4SS, high resolution structures of the assembled apparatuses have yielded important insights into the molecular processes governing their biogenesis and function (Fronzes *et al.*, 2009; Kubori *et al.*, 1998). Accordingly, early structural analysis of T6SS components resulted in a rapid increase in our perception of how this secretory machine assembles and performs its function. Crystal structures of Hcp and truncated VgrG proteins showed they share homology with T4 bacteriophage proteins: Hcp showed sequence and structural similarity with both gp19 tail tube and gp27 proteins, while VgrG was homologous to the needle/spike protein gp5-gp27 (Leiman *et al.*, 2009; Mougous *et al.*, 2007; Pell *et al.*, 2009). The VgrG-dependent head to tail stacking of tubular Hcp hexamers has been demonstrated *in vivo*, similar to phage tail tubes (Brunet *et al.*, 2014). TssE was also predicted to share homology with components of the bacteriophage base-plate (Leiman *et al.*, 2009; Lossi *et al.*, 2011). TssB and TssC were found to assemble into tubular sheath structures *in vitro*, analogous to a bacteriophage sheath (Bonemann *et al.*, 2009). These structures were subsequently visualized *in vivo* using cryo-electron tomography of whole *V. cholera* cells (Basler *et al.*, 2012b). Furthermore, they were determined to display dynamic rounds of elongation and contraction, with the TssB subunits of contracted sheaths being recognized and recycled through the pore of the ATPase ClpV, which has been demonstrated to localize to T6SS assemblies (Basler *et al.*, 2012a; Basler *et al.*, 2012b; Bonemann *et al.*, 2009; Kapitein *et al.*, 2013; Mougous *et al.*, 2006; Pietrosiuk *et al.*, 2011). Structural analyses have suggested that conformational changes upon contraction of the TssBC sheath are responsible for ClpV recognition of TssB (Clemens *et al.*, 2015; Kube *et al.*,

2014; Kudryashev *et al.*, 2015). Experimentally determined interactions between Hcp and TssB have supported a model whereby the Hcp tube is surrounded by a TssBC sheath; indeed, Hcp is essential for TssBC sheath formation, wherease TssBC are dispensible for Hcp tube assembly (Basler *et al.*, 2012b; Brunet *et al.*, 2014). The similarities of several T6SS components with bacteriophage proteins, and the dynamic nature of the TssB/C sheath assembly and contraction mechanism, favored a model whereby the T6SS resembles an inverted bacteriophage (Zoued *et al.*, 2014).

The T6SS is anchored to the membrane by a complex formed by the essential components TssJ, TssL, and TssM. An electron microscopy structure of this 1.7-megadalton assembly showed it possessed five-fold symmetry and contains domains in the cytoplasm, inner membrane, and outer membrane (Durand *et al.*, 2015). Fluorescence microscopy analysis determined that the outer membrane anchored lipoprotein TssJ recruits the inner membrane proteins TssM and TssL, forming a large cytoplasmic base that extends through the periplasm to the outer membrane as a rocket-shaped ring structure (Durand *et al.*, 2015). Six additional essential proteins, TssAEFGK and VgrG were recently shown to comprise a bacteriophage-like baseplate component of the T6SS, which is recruited to the TssJLM membrane complex and serves as the nucleation site for Hcp and TssBC polymerization (Brunet *et al.*, 2015). Several of these baseplate components have homology with bacteriophage baseplate components. TssA specifically recruits other members of the baseplate structure, and serves as the priming site for Hcp and TssBC polymerization by binding the distal end of the tubular structure (Brunet *et al.*, 2015; Planamente *et al.*, 2016; Zoued *et al.*, 2016). Together, the studies undertaken over the past decade have led to a coherent model of the T6SS structure and dynamics (Figure 1.2), and have facilitated the equally impressive advancement in our knowledge of the functional aspects of this apparatus.

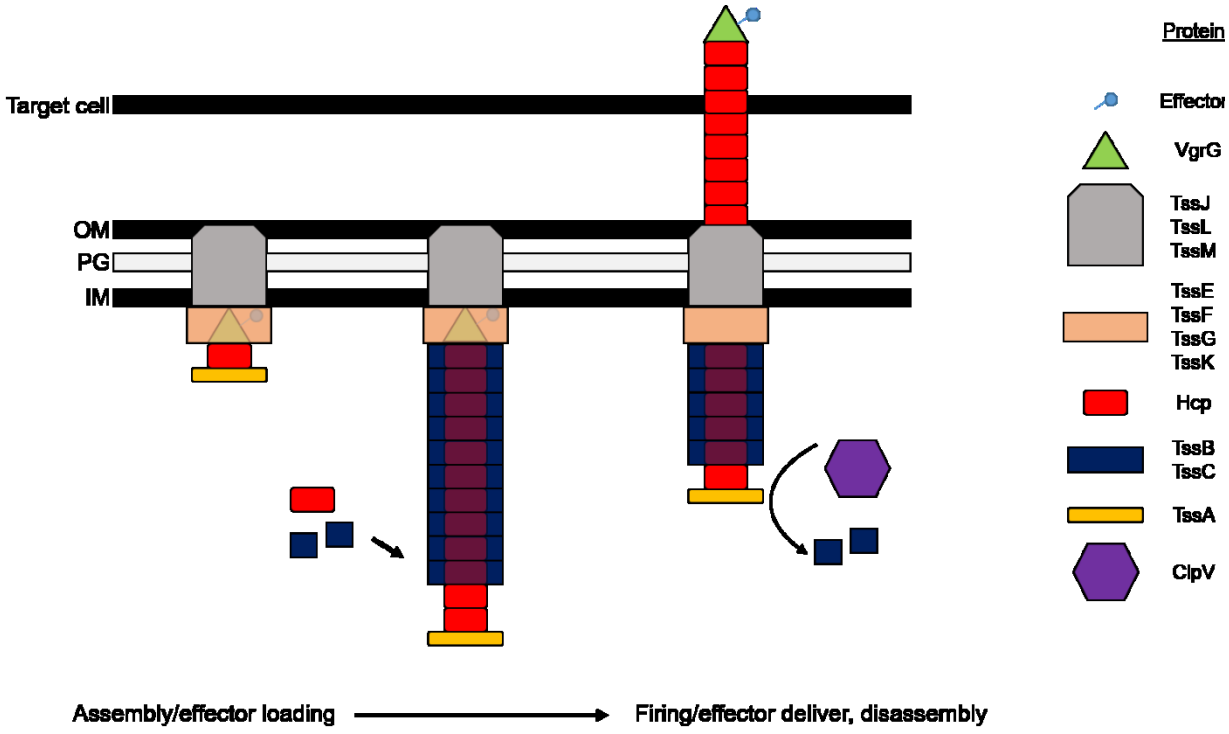

Figure 1.2 Biogenesis of the bacterial T6SS

The T6SS undergoes dynamic rounds of assembly followed by rapid contraction and effector release inside host cells in a contact dependent manner. At least 13 core components are required for function.

1.2.2 T6SS function and effectors

The T6SS mediates the export of numerous effector proteins which are responsible for carrying out a variety of biological functions (Russell *et al.*, 2014). Several studies have described effectors which target eukaryotic cells and play a role in virulence. In *V. cholerae*, the VgrG1 protein contains a C-terminal actin cross-linking domain and was shown to be secreted into phagocytic cells, thereby blocking bacterial uptake and causing host-cell death (Ma *et al.*, 2009; Ma *et al.*, 2010; Pukatzki *et al.*, 2007). VgrG proteins with additional effector domains have subsequently been described in several other bacteria; A VgrG from *Aeromonas hydrophila* also targets actin by ADP-ribosylation and VgrG-5 of *Burkholderia pseudomallei* mediates membrane

fusion and cell-cell spread of this organism (Schwarz *et al.*, 2014; Suarez *et al.*, 2010; Toesca *et al.*, 2014). Non-VgrG effector toxins targeting membrane components, such as VasX from *V. cholera* and a phospholipase from *P. aeruginosa* are also important for host cell toxicity (Jiang *et al.*, 2014; Miyata *et al.*, 2011). Other bacteria require a functional T6SS for survival or toxicity towards host cells, however the molecular basis for this requirement has not been fully characterized (Bleumink-Pluym *et al.*, 2013; Burtnick *et al.*, 2011; Burtnick *et al.*, 2010; Hachani *et al.*, 2016; Schell *et al.*, 2007).

A fundamental shift in our understanding of T6SS function came when it was discovered that a major role of this secretory system is to transport toxins to kill other bacteria in a contact-dependent manner (Hood *et al.*, 2010). Three effectors of *P. aeruginosa*, Tse1-3, were found to be delivered to other bacteria in a T6SS-dependent manner, with Tse1 and Tse3 having peptidoglycan hydrolase activity (Russell *et al.*, 2011). Immunity proteins encoded near the effectors provided protection from self-intoxication, allowing *P. aeruginosa* to kill other bacteria but avoid affecting kin cells. *B. thailandensis* requires one of its five T6SSs, T6SS-1, to outcompete other bacteria and proliferate in mixed biofilms (Schwarz *et al.*, 2010). Similar findings of an anti-bacterial role for the T6SS have subsequently been reported for numerous bacterial species (Carruthers *et al.*, 2013c; Gueguen *et al.*, 2012; MacIntyre *et al.*, 2010; Murdoch *et al.*, 2011; Weber *et al.*, 2015b), indicating this phenotype represents a key physiological function of the T6SS (Russell *et al.*, 2014). These antagonistic interactions likely play an important role in relevant physiological environments, such as in the mammalian gut, and may facilitate efficient horizontal gene transfer (Borgeaud *et al.*, 2015; Chatzidaki-Livanis *et al.*, 2016; Fu *et al.*, 2013; Hecht *et al.*, 2016; Wexler *et al.*, 2016).

A number of anti-bacterial effectors have now been characterized, along with their cognate protective immunity proteins, that generally target conserved and essential aspects of bacterial physiology. The bacterial-specific peptidoglycan layer is a common target for several T6SS effectors, which cleave linkages in both the peptide and glycan moieties (Durand *et al.*, 2014; Ho *et al.*, 2014; Russell *et al.*, 2014). Effectors cleaving the glycan subunits have been termed Tge (type VI secretion glycoside hydrolase effectors), while Tae (type VI secretion amidase effector) target the peptide units of peptidoglycan, with producing cells being protected by Tgi and Tai immunity proteins, respectively (Russell *et al.*, 2011; Russell *et al.*, 2012; Whitney *et al.*, 2013). VgrG3 from *V. cholerae* was found to encode a C-terminal extension with peptidoglycan-degrading activity (Brooks *et al.*, 2013; Dong *et al.*, 2013), and other peptidoglycan-degrading effectors have been found based on their toxicity towards *E. coli* and T6SS-dependent secretion (Fritsch *et al.*, 2013). Interestingly, although purified peptidoglycan from Gram-positive bacteria can be cleaved by peptidoglycan hydrolyzing effectors, Gram-positive bacteria have not been found to be susceptible to T6SS-mediated killing (Chou *et al.*, 2012; MacIntyre *et al.*, 2010). Another class of effectors have been determined to function as lipases (Russell *et al.*, 2013). These effectors target various bonds in phospholipids, and their immunity proteins contain signal peptides, indicating these lipases primarily act in the periplasm (Dong *et al.*, 2013; Jiang *et al.*, 2014; Russell *et al.*, 2013). Interestingly, several lipase effectors have also been shown to be important for eukaryotic pathogenicity, perhaps indicating a dual role for these enzymes, which is the case for the pore-forming toxin VasX from *V. cholerae* (Dong *et al.*, 2013; Jiang *et al.*, 2016; Jiang *et al.*, 2014; Miyata *et al.*, 2011). Effectors functioning as nucleases have also been identified in several bacteria. These include recombination hot-spot proteins (Rhs) fused to nuclease domains in *Dickeya dadantii* and *Serratia marcescans*, and the Tde DNase from *Agrobacterium*

tumefaciens, as well as several other predicted nucleases (Alcoforado Diniz *et al.*, 2015; Durand *et al.*, 2014; Koskiniemi *et al.*, 2013; Ma *et al.*, 2014). A recently identified effector from *P. aeruginosa* was shown to deplete intracellular pools of NAD(P)+ (Whitney *et al.*, 2015).

Multiple routes of effector delivery by the T6SS have been identified. As mentioned, effector domains fused to VgrG proteins are delivered based on the fact that VgrG's are themselves components of the secretory apparatus (Pukatzki *et al.*, 2006). Hcp, the hallmark secreted protein of all T6SS studied, has recently been identified as a receptor for T6SS effectors (Silverman *et al.*, 2013; Whitney *et al.*, 2014b). Electron microscopy imaging showed that small globular proteins <20kDa could be accommodated into the 40 Å lumen of Hcp hexamers, and this interaction stabilizes the effector-Hcp complex (Silverman *et al.*, 2013; Whitney *et al.*, 2014b). Recently identified PAAR proteins, which can themselves contain effector domains, have been proposed to sit atop VgrG proteins in the T6SS structure and facilitate effector delivery (Bondage *et al.*, 2016; Cianfanelli *et al.*, 2016; Shneider *et al.*, 2013). More recently, the requirement of VgrG proteins for secretion of effectors has been demonstrated, with adaptor proteins mediating interactions between them (Hachani *et al.*, 2014; Hachani *et al.*, 2011b; Liang *et al.*, 2015; Unterweger *et al.*, 2015; Whitney *et al.*, 2014b) .

1.2.3 Regulation of expression

In most bacteria, expression of the T6SS is tightly controlled by numerous different mechanisms (Miyata *et al.*, 2013a; Silverman *et al.*, 2012). Extensive work in *P. aeruginosa* has revealed that this species fires its T6SS in response to external attacks or upon detecting lysis of kin cells (Basler *et al.*, 2013; Ho *et al.*, 2013; LeRoux *et al.*, 2012; LeRoux *et al.*, 2015). A complex post-translational signalling and phosphorylation cascade results in T6SS activation upon

detection of external threats or surface growth, which is integrated into the global Gac/Rsm regulatory network of this organism (Basler *et al.*, 2013; Hsu *et al.*, 2009; LeRoux *et al.*, 2015; Mougous *et al.*, 2007; Silverman *et al.*, 2011). Several regulatory proteins and environmental signals regulate T6SS in other bacteria, including iron, zinc, osmolarity, quorum sensing and temperature (Brunet *et al.*, 2011; Burtnick *et al.*, 2013; Dong *et al.*, 2012; Gueguen *et al.*, 2013; Ishikawa *et al.*, 2012; Pieper *et al.*, 2009; Zheng *et al.*, 2010). In addition, regulation of T6SS often varies between different strains of the same species, and may reflect the particular niche those strains inhabit (Bernardy *et al.*, 2016). The dynamic nature of the T6SS requires a large input of energy, and therefore constitutive expression of the system is likely a costly process (Basler, 2015).

1.3 Thesis Objectives

Defining the genetic and regulatory requirements for a functional T6SS in *A. baumannii* are the main objectives of this thesis. Overall, the goal was to identify and characterize factors that may influence the success of *A. baumannii* as a pathogen, and to potentially identify new therapeutic strategies for treating infections caused by this organism.

1.3.1 Functional analysis of the T6SS in *Acinetobacter*

Previous bioinformatic studies suggested the T6SS is widespread among Gram-negative bacteria (Bingle *et al.*, 2008). The second chapter of this thesis details studies aimed at bioinformatic detection of the T6SS in *Acinetobacter* spp., and determining the functionality of this system in *A. baumannii* and other *Acinetobacter* spp. Development of an anti-Hcp antibody, genetic knockouts, and complementation were used to experimentally validate our *in silico* predictions. Phenotypic tests were used in an attempt to uncover the role of T6SS in *A. baumannii*.

1.3.2 Role of a resistance plasmid in regulating T6SS in clinical *A. baumannii* isolates

Many bacterial strains used to study T6SS in the laboratory are selected for their constitutive T6SS or are genetically manipulated to stably express the T6SS (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). Most isolates of *A. baumannii* do not secrete Hcp under standard laboratory conditions, many of which are clinical specimens, suggesting some form of regulation. In chapter three, screening and genome sequencing experiments are presented that uncover a novel plasmid-based regulatory mechanism that controls the anti-bacterial T6SS of *A. baumannii*, and detail a link between T6SS and antibiotic resistance. This analysis is extended to other *A. baumannii* strains, and molecular methods are used dissect the regulatory pathway.

1.3.3 Genetic requirements for a functional T6SS in *Acinetobacter* spp.

The complement of genes required for a functional T6SS have been studied in detail for a few bacterial species (Zheng *et al.*, 2011; Zheng *et al.*, 2007). While many of the core T6SS components are conserved, regulatory and effector genes often differ between bacteria. Chapter four of this thesis examines the genes necessary for T6SS in the model strain *A. baylyi*, and presents the first experimental evidence of T6SS-dependent effectors in *A. baumannii*. Additionally, a novel and essential T6SS component uncovered by this screen is functionally characterized by biochemical methods

1.4 References

Adams, M. D., Nickel, G. C., Bajaksouzian, S., Lavender, H., Murthy, A. R., Jacobs, M. R., & Bonomo, R. A. (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrobial agents and chemotherapy, 53*(9), 3628-3634.

Ahmed-Bentley, J., Chandran, A. U., Joffe, A. M., French, D., Peirano, G., & Pitout, J. D. (2013). Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. *Antimicrobial agents and chemotherapy, 57*(7), 3085-3091.

Alcoforado Diniz, J., & Coulthurst, S. J. (2015). Intraspecies Competition in *Serratia marcescens* Is Mediated by Type VI-Secreted Rhs Effectors and a Conserved Effector-Associated Accessory Protein. *Journal of bacteriology, 197*(14), 2350-2360.

Arroyo, L. A., Herrera, C. M., Fernandez, L., Hankins, J. V., Trent, M. S., & Hancock, R. E. (2011). The pmrCAB operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrobial agents and chemotherapy, 55*(8), 3743-3751.

Basler, M. (2015). Type VI secretion system: secretion by a contractile nanomachine. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 370*(1679).

Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell, 152*(4), 884-894.

Basler, M., & Mekalanos, J. J. (2012a). Type 6 secretion dynamics within and between bacterial cells. *Science, 337*(6096), 815.

Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012b). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186.

Baumann, P., Doudoroff, M., & Stanier, R. Y. (1968). A study of the *Moraxella* group. II. Oxidative-negative species (genus *Acinetobacter*). *Journal of bacteriology, 95*(5), 1520-1541.

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., . . . Woodford, N. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. *Antimicrobial agents and chemotherapy, 55*(7), 3370-3379.

Bentancor, L. V., Camacho-Peiro, A., Bozkurt-Guzel, C., Pier, G. B., & Maira-Litran, T. (2012). Identification of Ata, a multifunctional trimeric autotransporter of *Acinetobacter baumannii*. *J Bacteriol, 194*(15), 3950-3960.

Bernards, A. T., Frenay, H. M., Lim, B. T., Hendriks, W. D., Dijkshoorn, L., & van Boven, C. P. (1998). Methicillin-resistant Staphylococcus aureus and *Acinetobacter baumannii*: an unexpected difference in epidemiologic behavior. *American journal of infection control, 26*(6), 544-551.

Bernards, A. T., Harinck, H. I., Dijkshoorn, L., van der Reijden, T. J., & van den Broek, P. J. (2004). Persistent *Acinetobacter baumannii*? Look inside your medical equipment. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 25*(11), 1002-1004.

Bernardy, E. E., Turnsek, M. A., Wilson, S. K., Tarr, C. L., & Hammer, B. K. (2016). Diversity of Clinical and Environmental Isolates of *Vibrio cholerae* in Natural Transformation and

Contact-Dependent Bacterial Killing Indicative of Type VI Secretion System Activity. *Applied and environmental microbiology, 82*(9), 2833-2842.
Bingle, L. E., Bailey, C. M., & Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Current opinion in microbiology, 11*(1), 3-8.
Bleumink-Pluym, N. M., van Alphen, L. B., Bouwman, L. I., Wosten, M. M., & van Putten, J. P. (2013). Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity. *PLoS pathogens, 9*(5), e1003393.
Boll, J. M., Tucker, A. T., Klein, D. R., Beltran, A. M., Brodbelt, J. S., Davies, B. W., & Trent, M. S. (2015). Reinforcing Lipid A Acylation on the Cell Surface of *Acinetobacter baumannii* Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival. *mBio, 6*(3), e00478-00415.
Bondage, D. D., Lin, J. S., Ma, L. S., Kuo, C. H., & Lai, E. M. (2016). VgrG C terminus confers the type VI effector transport specificity and is required for binding with PAAR and adaptor-effector complex. *Proceedings of the National Academy of Sciences of the United States of America, 113*(27), E3931-3940.
Bonemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., & Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *The EMBO journal, 28*(4), 315-325.
Bonnin, R. A., Nordmann, P., & Poirel, L. (2013). Screening and deciphering antibiotic resistance in *Acinetobacter baumannii*: a state of the art. *Expert review of anti-infective therapy, 11*(6), 571-583.
Bonnin, R. A., Poirel, L., Naas, T., Pirs, M., Seme, K., Schrenzel, J., & Nordmann, P. (2012). Dissemination of New Delhi metallo-beta-lactamase-1-producing *Acinetobacter baumannii* in Europe. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(9), E362-365.
Borgeaud, S., Metzger, L. C., Scrignari, T., & Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science, 347*(6217), 63-67.
Bou, G., & Martinez-Beltran, J. (2000). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 44*(2), 428-432.
Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., . . . Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 48*(1), 1-12.
Bouvet, P. J., & Jeanjean, S. (1989). Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Research in microbiology, 140*(4-5), 291-299.
Bouvet, P. J. M. G., P. A. D. . (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol*(36), 228-240.
Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC genomics, 10*, 104.
Brisou, J., & Prevot, A. R. (1954). [Studies on bacterial taxonomy. X. The revision of species under *Acromobacter* group]. *Annales de l'Institut Pasteur, 86*(6), 722-728.

Brooks, T. M., Unterweger, D., Bachmann, V., Kostiuk, B., & Pukatzki, S. (2013). Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *The Journal of biological chemistry, 288*(11), 7618-7625.

Brunet, Y. R., Bernard, C. S., Gavioli, M., Lloubes, R., & Cascales, E. (2011). An epigenetic switch involving overlapping fur and DNA methylation optimizes expression of a type VI secretion gene cluster. *PLoS genetics, 7*(7), e1002205.

Brunet, Y. R., Henin, J., Celia, H., & Cascales, E. (2014). Type VI secretion and bacteriophage tail tubes share a common assembly pathway. *EMBO reports, 15*(3), 315-321.

Brunet, Y. R., Zoued, A., Boyer, F., Douzi, B., & Cascales, E. (2015). The Type VI Secretion TssEFGK-VgrG Phage-Like Baseplate Is Recruited to the TssJLM Membrane Complex via Multiple Contacts and Serves As Assembly Platform for Tail Tube/Sheath Polymerization. *PLoS genetics, 11*(10), e1005545.

Burrows, L. L. (2012). *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol, 66*, 493-520.

Burtnick, M. N., & Brett, P. J. (2013). *Burkholderia mallei* and *Burkholderia pseudomallei* cluster 1 type VI secretion system gene expression is negatively regulated by iron and zinc. *PloS one, 8*(10), e76767.

Burtnick, M. N., Brett, P. J., Harding, S. V., Ngugi, S. A., Ribot, W. J., Chantratita, N., . . . Deshazer, D. (2011). The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. *Infection and immunity, 79*(4), 1512-1525.

Burtnick, M. N., DeShazer, D., Nair, V., Gherardini, F. C., & Brett, P. J. (2010). *Burkholderia mallei* cluster 1 type VI secretion mutants exhibit growth and actin polymerization defects in RAW 264.7 murine macrophages. *Infection and immunity, 78*(1), 88-99.

Campos, M., Cisneros, D. A., Nivaskumar, M., & Francetic, O. (2013). The type II secretion system - a dynamic fiber assembly nanomachine. *Res Microbiol, 164*(6), 545-555.

Carruthers, M. D., Harding, C. M., Baker, B. D., Bonomo, R. A., Hujer, K. M., Rather, P. N., & Munson, R. S., Jr. (2013b). Draft Genome Sequence of the Clinical Isolate *Acinetobacter nosocomialis* Strain M2. *Genome Announc, 1*(6).

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S., Jr. (2013c). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PloS one, 8*(3), e59388.

Castric, P. (1995). pilO, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology, 141 (Pt 5)*, 1247-1254.

Centers for Disease Control and Prevention, O. o. I. D. (2013). Antibiotic Resistance Threats in the United States, 2013. Retrieved from http://www.cdc.gov/drugresistance/threat-report-2013

Chatzidaki-Livanis, M., Geva-Zatorsky, N., & Comstock, L. E. (2016). *Bacteroides fragilis* type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. *Proceedings of the National Academy of Sciences of the United States of America, 113*(13), 3627-3632.

Chen, T. L., Lee, Y. T., Kuo, S. C., Yang, S. P., Fung, C. P., & Lee, S. D. (2014). Rapid identification of *Acinetobacter baumannii*, *Acinetobacter nosocomialis* and *Acinetobacter pittii* with a multiplex PCR assay. *Journal of medical microbiology, 63*(Pt 9), 1154-1159.

Chin, C. Y., Gregg, K. A., Napier, B. A., Ernst, R. K., & Weiss, D. S. (2015). A PmrB-regulated Deacetylase Required for Lipid A Modification and Polymyxin Resistance in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*.

Choi, A. H., Slamti, L., Avci, F. Y., Pier, G. B., & Maira-Litran, T. (2009). The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of bacteriology, 191*(19), 5953-5963.

Chou, S., Bui, N. K., Russell, A. B., Lexa, K. W., Gardiner, T. E., LeRoux, M., . . . Mougous, J. D. (2012). Structure of a peptidoglycan amidase effector targeted to Gram-negative bacteria by the type VI secretion system. *Cell reports, 1*(6), 656-664.

Cianfanelli, F. R., Alcoforado Diniz, J., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion System. *PLoS pathogens, 12*(6), e1005735.

Clemens, D. L., Ge, P., Lee, B. Y., Horwitz, M. A., & Zhou, Z. H. (2015). Atomic structure of T6SS reveals interlaced array essential to function. *Cell, 160*(5), 940-951.

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H., & Reynaud, A. (2003). AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *The Journal of antimicrobial chemotherapy, 52*(4), 629-635.

Costa, T. R., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature reviews. Microbiology, 13*(6), 343-359.

Coyne, S., Courvalin, P., & Perichon, B. (2011). Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrobial agents and chemotherapy, 55*(3), 947-953.

Dallo, S. F., Zhang, B., Denno, J., Hong, S., Tsai, A., Haskins, W., . . . Weitao, T. (2012). Association of *Acinetobacter baumannii* EF-Tu with cell surface, outer membrane vesicles, and fibronectin. *TheScientificWorldJournal, 2012*, 128705.

de Breij, A., Gaddy, J., van der Meer, J., Koning, R., Koster, A., van den Broek, P., . . . Dijkshoorn, L. (2009). CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606(T) to human airway epithelial cells and their inflammatory response. *Res Microbiol, 160*(3), 213-218.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature reviews. Microbiology, 5*(12), 939-951.

Doi, Y., Adams, J. M., Yamane, K., & Paterson, D. L. (2007). Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. *Antimicrobial agents and chemotherapy, 51*(11), 4209-4210.

Dong, T. G., Ho, B. T., Yoder-Himes, D. R., & Mekalanos, J. J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America, 110*(7), 2623-2628.

Dong, T. G., & Mekalanos, J. J. (2012). Characterization of the RpoN regulon reveals differential regulation of T6SS and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic acids research, 40*(16), 7766-7775.

Durand, E., Cambillau, C., Cascales, E., & Journet, L. (2014). VgrG, Tae, Tle, and beyond: the versatile arsenal of Type VI secretion effectors. *Trends in microbiology, 22*(9), 498-507.

Durand, E., Nguyen, V. S., Zoued, A., Logger, L., Pehau-Arnaudet, G., Aschtgen, M. S., . . . Fronzes, R. (2015). Biogenesis and structure of a type VI secretion membrane core complex. *Nature, 523*(7562), 555-560.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., & Brown, M. H. (2011). Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS microbiology letters, 323*(1), 44-51.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Paulsen, I. T., & Brown, M. H. (2014). Comparative analysis of surface-exposed virulence factors of *Acinetobacter baumannii*. *BMC Genomics, 15*, 1020.

Ellis, T. N., & Kuehn, M. J. (2010). Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiology and molecular biology reviews : MMBR, 74*(1), 81-94.

Falagas, M. E., & Kasiakou, S. K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 40*(9), 1333-1341.

Falagas, M. E., Rafailidis, P. I., Matthaiou, D. K., Virtzili, S., Nikita, D., & Michalopoulos, A. (2008). Pandrug-resistant *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections: characteristics and outcome in a series of 28 patients. *International journal of antimicrobial agents, 32*(5), 450-454.

Faridmoayer, A., Fentabil, M. A., Haurat, M. F., Yi, W., Woodward, R., Wang, P. G., & Feldman, M. F. (2008). Extreme substrate promiscuity of the *Neisseria* oligosaccharyl transferase involved in protein O-glycosylation. *The Journal of biological chemistry, 283*(50), 34596-34604.

Faridmoayer, A., Fentabil, M. A., Mills, D. C., Klassen, J. S., & Feldman, M. F. (2007). Functional characterization of bacterial oligosaccharyltransferases involved in O-linked protein glycosylation. *Journal of bacteriology, 189*(22), 8088-8098.

Fournier, P. E., & Richet, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 42*(5), 692-699.

Fowler, T., Walker, D., & Davies, S. C. (2014). The risk/benefit of predicting a post-antibiotic era: is the alarm working? *Annals of the New York Academy of Sciences, 1323*, 1-10.

Fregolino, E., Gargiulo, V., Lanzetta, R., Parrilli, M., Holst, O., & Castro, C. D. (2011). Identification and structural determination of the capsular polysaccharides from two *Acinetobacter baumannii* clinical isolates, MG1 and SMAL. *Carbohydrate research, 346*(7), 973-977.

Fritsch, M. J., Trunk, K., Diniz, J. A., Guo, M., Trost, M., & Coulthurst, S. J. (2013). Proteomic identification of novel secreted antibacterial toxins of the *Serratia marcescens* type VI secretion system. *Molecular & cellular proteomics : MCP, 12*(10), 2735-2749.

Fronzes, R., Schafer, E., Wang, L., Saibil, H. R., Orlova, E. V., & Waksman, G. (2009). Structure of a type IV secretion system core complex. *Science, 323*(5911), 266-268.

Fu, Y., Waldor, M. K., & Mekalanos, J. J. (2013). Tn-Seq analysis of *Vibrio cholerae* intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. *Cell host & microbe, 14*(6), 652-663.

Fulsundar, S., Harms, K., Flaten, G. E., Johnsen, P. J., Chopade, B. A., & Nielsen, K. M. (2014). Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation. *Applied and environmental microbiology, 80*(11), 3469-3483.

Fulsundar, S., Kulkarni, H. M., Jagannadham, M. V., Nair, R., Keerthi, S., Sant, P., . . . Chopade, B. A. (2015). Molecular characterization of outer membrane vesicles released from *Acinetobacter radioresistens* and their potential roles in pathogenesis. *Microbial pathogenesis, 83-84*, 12-22.

Garcia-Quintanilla, M., Pulido, M. R., Lopez-Rojas, R., Pachon, J., & McConnell, M. J. (2013). Emerging therapies for multidrug resistant *Acinetobacter baumannii*. *Trends in microbiology, 21*(3), 157-163.

Garnacho-Montero, J., Ortiz-Leyba, C., Fernandez-Hinojosa, E., Aldabo-Pallas, T., Cayuela, A., Marquez-Vacaro, J. A., . . . Jimenez-Jimenez, F. J. (2005). *Acinetobacter baumannii* ventilator-associated pneumonia: epidemiological and clinical findings. *Intensive care medicine, 31*(5), 649-655.

Geisinger, E., & Isberg, R. R. (2015). Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS pathogens, 11*(2), e1004691.

Gerner-Smidt, P., Tjernberg, I., & Ursing, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *Journal of clinical microbiology, 29*(2), 277-282.

Gohl, O., Friedrich, A., Hoppert, M., & Averhoff, B. (2006). The thin pili of *Acinetobacter* sp. strain BD413 mediate adhesion to biotic and abiotic surfaces. *Applied and environmental microbiology, 72*(2), 1394-1401.

Grandi, G. (2010). Bacterial surface proteins and vaccines. *F1000 Biol Rep, 2*.

Gueguen, E., & Cascales, E. (2012). Promoter swapping unveils the role of the *Citrobacter rodentium* CTS1 Type VI secretion system in inter-bacterial competition. *Applied and environmental microbiology*.

Gueguen, E., Durand, E., Zhang, X. Y., d'Amalric, Q., Journet, L., & Cascales, E. (2013). Expression of a *Yersinia pseudotuberculosis* Type VI Secretion System Is Responsive to Envelope Stresses through the OmpR Transcriptional Activator. *PloS one, 8*(6), e66615.

Hachani, A., Allsopp, L. P., Oduko, Y., & Filloux, A. (2014). The VgrG proteins are "a la carte" delivery systems for bacterial type VI effectors. *The Journal of biological chemistry, 289*(25), 17872-17884.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011b). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Hachani, A., Wood, T. E., & Filloux, A. (2016). Type VI secretion and anti-host effectors. *Current opinion in microbiology, 29*, 81-93.

Harding, C. M., Nasr, M. A., Kinsella, R. L., Scott, N. E., Foster, L. J., Weber, B. S., . . . Feldman, M. F. (2015). *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol Microbiol, 96*(5), 1023-1041.

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S., Jr. (2013). *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio, 4*(4).

Haseley, S., & Wilkinson, S. G. (1997a). Structure of the O18 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 301*(3-4), 187-192.

Haseley, S. R., Traub, W. H., & Wilkinson, S. G. (1997b). Structures of polymeric products isolated from the lipopolysaccharides of reference strains for *Acinetobacter baumannii* O23 and O12. *European journal of biochemistry / FEBS, 244*(1), 147-154.

Haseley, S. R., & Wilkinson, S. G. (1994). Structure of the putative O10 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 264*(1), 73-81.

Haseley, S. R., & Wilkinson, S. G. (1998). Structure of the O-7 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 306*(1-2), 257-263.
Haurat, M. F., Elhenawy, W., & Feldman, M. F. (2014). Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. *Biological chemistry*.
Hecht, A. L., Casterline, B. W., Earley, Z. M., Goo, Y. A., Goodlett, D. R., & Bubeck Wardenburg, J. (2016). Strain competition restricts colonization of an enteric pathogen and prevents colitis. *EMBO reports*.
Henrichsen, J. (1975). The occurrence of twitching motility among Gram-negative bacteria. *Acta Pathol Microbiol Scand B, 83*(3), 171-178.
Henrichsen, J., & Blom, J. (1975). Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. *Acta Pathol Microbiol Scand B, 83*(2), 103-115.
Ho, B. T., Basler, M., & Mekalanos, J. J. (2013). Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science, 342*(6155), 250-253.
Ho, B. T., Dong, T. G., & Mekalanos, J. J. (2014). A view to a kill: the bacterial type VI secretion system. *Cell host & microbe, 15*(1), 9-21.
Hood, M. I., Becker, K. W., Roux, C. M., Dunman, P. M., & Skaar, E. P. (2013). genetic determinants of intrinsic colistin tolerance in *Acinetobacter baumannii*. *Infection and immunity, 81*(2), 542-551.
Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., . . . Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell host & microbe, 7*(1), 25-37.
Hsu, F., Schwarz, S., & Mougous, J. D. (2009). TagR promotes PpkA-catalysed type VI secretion activation in *Pseudomonas aeruginosa*. *Molecular microbiology, 72*(5), 1111-1125.
Hug, I., & Feldman, M. F. (2011). Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology, 21*(2), 138-151.
Infectious Diseases Society of, A., Spellberg, B., Blaser, M., Guidos, R. J., Boucher, H. W., Bradley, J. S., . . . Gilbert, D. N. (2011). Combating antimicrobial resistance: policy recommendations to save lives. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 52 Suppl 5*, S397-428.
Ishikawa, T., Sabharwal, D., Broms, J., Milton, D. L., Sjostedt, A., Uhlin, B. E., & Wai, S. N. (2012). Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infection and immunity, 80*(2), 575-584.
Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., . . . Feldman, M. F. (2012b). Identification of a general O-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS pathogens, 8*(6), e1002758.
Jawad, A., Seifert, H., Snelling, A. M., Heritage, J., & Hawkey, P. M. (1998). Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates. *Journal of clinical microbiology, 36*(7), 1938-1941.
Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N. R., . . . Jin, Q. (2016). The *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host Autophagy by Activating Endoplasmic Reticulum Stress. *Cell reports*.

Jiang, F., Waterfield, N. R., Yang, J., Yang, G., & Jin, Q. (2014). A *Pseudomonas aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic cells. *Cell host & microbe, 15*(5), 600-610.

Jin, J. S., Kwon, S. O., Moon, D. C., Gurung, M., Lee, J. H., Kim, S. I., & Lee, J. C. (2011). *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. *PloS one, 6*(2), e17027.

Johnson, T. L., Waack, U., Smith, S., Mobley, H., Sandkvist, M. . (2015). *Acinetobacter baumannii* is dependent on the type II secretion system and its substrate LipA for lipid utilization and in vivo fitness. *Journal of bacteriology*.

Jun, S. H., Lee, J. H., Kim, B. R., Kim, S. I., Park, T. I., Lee, J. C., & Lee, Y. C. (2013). *Acinetobacter baumannii* outer membrane vesicles elicit a potent innate immune response via membrane proteins. *PloS one, 8*(8), e71751.

Kapitein, N., Bonemann, G., Pietrosiuk, A., Seyffer, F., Hausser, I., Locker, J. K., & Mogk, A. (2013). ClpV recycles VipA/VipB tubules and prevents non-productive tubule formation to ensure efficient type VI protein secretion. *Molecular microbiology, 87*(5), 1013-1028.

Kenyon, J. J., & Hall, R. M. (2013). Variation in the complex carbohydrate biosynthesis loci of *Acinetobacter baumannii* genomes. *PloS one, 8*(4), e62160.

Kenyon, J. J., Nigro, S. J., & Hall, R. M. (2014). Variation in the OC locus of *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipooligosaccharide. *PloS one, 9*(9), e107833.

Koskiniemi, S., Lamoureux, J. G., Nikolakakis, K. C., t'Kint de Roodenbeke, C., Kaplan, M. D., Low, D. A., & Hayes, C. S. (2013). Rhs proteins from diverse bacteria mediate intercellular competition. *Proceedings of the National Academy of Sciences of the United States of America, 110*(17), 7032-7037.

Kube, S., Kapitein, N., Zimniak, T., Herzog, F., Mogk, A., & Wendler, P. (2014). Structure of the VipA/B type VI secretion complex suggests a contraction-state-specific recycling mechanism. *Cell reports, 8*(1), 20-30.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., . . . Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science, 280*(5363), 602-605.

Kudryashev, M., Wang, R. Y., Brackmann, M., Scherer, S., Maier, T., Baker, D., . . . Basler, M. (2015). Structure of the type VI secretion system contractile sheath. *Cell, 160*(5), 952-962.

Kwon, S. O., Gho, Y. S., Lee, J. C., & Kim, S. I. (2009). Proteome analysis of outer membrane vesicles from a clinical *Acinetobacter baumannii* isolate. *FEMS microbiology letters, 297*(2), 150-156.

Lees-Miller, R. G., Iwashkiw, J. A., Scott, N. E., Seper, A., Vinogradov, E., Schild, S., & Feldman, M. F. (2013). A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Molecular microbiology, 89*(5), 816-830.

Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., . . . Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4154-4159.

LeRoux, M., De Leon, J. A., Kuwada, N. J., Russell, A. B., Pinto-Santini, D., Hood, R. D., . . . Mougous, J. D. (2012). Quantitative single-cell characterization of bacterial interactions

reveals type VI secretion is a double-edged sword. *Proceedings of the National Academy of Sciences of the United States of America, 109*(48), 19804-19809.
LeRoux, M., Kirkpatrick, R. L., Montauti, E. I., Tran, B. Q., Peterson, S. B., Harding, B. N., . . . Mougous, J. D. (2015). Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. *eLife, 4*.
Li, J., Nation, R. L., Milne, R. W., Turnidge, J. D., & Coulthard, K. (2005). Evaluation of colistin as an agent against multi-resistant in Gram-negative bacteria. *International journal of antimicrobial agents, 25*(1), 11-25.
Li, Z. T., Zhang, R. L., Bi, X. G., Xu, L., Fan, M., Xie, D., . . . Zhang, K. X. (2015). Outer membrane vesicles isolated from two clinical *Acinetobacter baumannii* strains exhibit different toxicity and proteome characteristics. *Microbial pathogenesis, 81*, 46-52.
Liang, X., Moore, R., Wilton, M., Wong, M. J., Lam, L., & Dong, T. G. (2015). Identification of divergent type VI secretion effectors using a conserved chaperone domain. *Proceedings of the National Academy of Sciences of the United States of America, 112*(29), 9106-9111.
Liao, Y. T., Kuo, S. C., Chiang, M. H., Lee, Y. T., Sung, W. C., Chen, Y. H., . . . Fung, C. P. (2015). *Acinetobacter baumannii* extracellular OXA-58 is primarily and selectively released via outer membrane vesicles after Sec-dependent periplasmic translocation. *Antimicrobial agents and chemotherapy*.
Liao, Y. T., Kuo, S. C., Lee, Y. T., Chen, C. P., Lin, S. W., Shen, L. J., . . . Chen, T. L. (2014). Sheltering effect and indirect pathogenesis of carbapenem-resistant *Acinetobacter baumannii* in polymicrobial infection. *Antimicrobial agents and chemotherapy, 58*(7), 3983-3990.
Lim, Y. M., Shin, K. S., & Kim, J. (2007). Distinct antimicrobial resistance patterns and antimicrobial resistance-harboring genes according to genomic species of *Acinetobacter* isolates. *Journal of clinical microbiology, 45*(3), 902-905.
Lin, L., Tan, B., Pantapalangkoor, P., Ho, T., Baquir, B., Tomaras, A., . . . Spellberg, B. (2012). Inhibition of LpxC protects mice from resistant *Acinetobacter baumannii* by modulating inflammation and enhancing phagocytosis. *mBio, 3*(5).
Liu, B., Knirel, Y. A., Feng, L., Perepelov, A. V., Senchenkova, S. N., Reeves, P. R., & Wang, L. (2014). Structural diversity in *Salmonella* O antigens and its genetic basis. *FEMS microbiology reviews, 38*(1), 56-89.
Lossi, N. S., Dajani, R., Freemont, P., & Filloux, A. (2011). Structure-function analysis of HsiF, a gp25-like component of the type VI secretion system, in *Pseudomonas aeruginosa*. *Microbiology, 157*(Pt 12), 3292-3305.
Luke, N. R., Sauberan, S. L., Russo, T. A., Beanan, J. M., Olson, R., Loehfelm, T. W., . . . Campagnari, A. A. (2010). Identification and characterization of a glycosyltransferase involved in *Acinetobacter baumannii* lipopolysaccharide core biosynthesis. *Infection and immunity, 78*(5), 2017-2023.
Ma, A. T., McAuley, S., Pukatzki, S., & Mekalanos, J. J. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell host & microbe, 5*(3), 234-243.
Ma, A. T., & Mekalanos, J. J. (2010). In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. *Proceedings of the National Academy of Sciences of the United States of America, 107*(9), 4365-4370.

Ma, L. S., Hachani, A., Lin, J. S., Filloux, A., & Lai, E. M. (2014). Agrobacterium tumefaciens deploys a superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in planta. *Cell host & microbe, 16*(1), 94-104.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America, 107*(45), 19520-19524.

MacLean, L. L., Perry, M. B., Chen, W., & Vinogradov, E. (2009). The structure of the polysaccharide O-chain of the LPS from *Acinetobacter baumannii* strain ATCC 17961. *Carbohydrate research, 344*(4), 474-478.

Magnet, S., Courvalin, P., & Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial agents and chemotherapy, 45*(12), 3375-3380.

Maragakis, L. L., & Perl, T. M. (2008a). *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 46*(8), 1254-1263.

McConnell, M. J., Rumbo, C., Bou, G., & Pachon, J. (2011). Outer membrane vesicles as an acellular vaccine against *Acinetobacter baumannii*. *Vaccine, 29*(34), 5705-5710.

McQueary, C. N., Kirkup, B. C., Si, Y., Barlow, M., Actis, L. A., Craft, D. W., & Zurawski, D. V. (2012). Extracellular stress and lipopolysaccharide modulate *Acinetobacter baumannii* surface-associated motility. *Journal of microbiology, 50*(3), 434-443.

Melamed, R., Greenberg, D., Porat, N., Karplus, M., Zmora, E., Golan, A., . . . Dagan, R. (2003). Successful control of an *Acinetobacter baumannii* outbreak in a neonatal intensive care unit. *The Journal of hospital infection, 53*(1), 31-38.

Merz, A. J., So, M., & Sheetz, M. P. (2000). Pilus retraction powers bacterial twitching motility. *Nature, 407*(6800), 98-102.

Miyata, S. T., Bachmann, V., & Pukatzki, S. (2013a). Type VI secretion system regulation as a consequence of evolutionary pressure. *Journal of medical microbiology, 62*(Pt 5), 663-676.

Miyata, S. T., Kitaoka, M., Brooks, T. M., McAuley, S. B., & Pukatzki, S. (2011). *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill Dictyostelium discoideum. *Infection and immunity, 79*(7), 2941-2949.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., . . . Boyce, J. D. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial agents and chemotherapy, 54*(12), 4971-4977.

Moon, D. C., Choi, C. H., Lee, J. H., Choi, C. W., Kim, H. Y., Park, J. S., . . . Lee, J. C. (2012). *Acinetobacter baumannii* outer membrane protein A modulates the biogenesis of outer membrane vesicles. *Journal of microbiology, 50*(1), 155-160.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., . . . Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science, 312*(5779), 1526-1530.

Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nature cell biology, 9*(7), 797-803.

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., & Coulthurst, S. J. (2011). The opportunistic pathogen Serratia marcescens utilizes type VI secretion to target bacterial competitors. *Journal of bacteriology, 193*(21), 6057-6069.

Nait Chabane, Y., Marti, S., Rihouey, C., Alexandre, S., Hardouin, J., Lesouhaitier, O., . . . De, E. (2014). Characterisation of pellicles formed by *Acinetobacter baumannii* at the air-liquid interface. *PLoS One, 9*(10), e111660.

Nho, J. S., Jun, S. H., Oh, M. H., Park, T. I., Choi, C. W., Kim, S. I., . . . Lee, J. C. (2015). *Acinetobacter* nosocomialis secretes outer membrane vesicles that induce epithelial cell death and host inflammatory responses. *Microbial pathogenesis, 81*, 39-45.

Orskov, I., Orskov, F., Jann, B., & Jann, K. (1977). Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. *Bacteriol Rev, 41*(3), 667-710.

Pantophlet, R. (2008). *Lipopolysaccharides of Acinetobacter* (E. Gerischer Ed.). Norfolk, UK: Caister Academic Press.

Pantophlet, R., Brade, L., & Brade, H. (1999). Identification of *Acinetobacter baumannii* strains with monoclonal antibodies against the O antigens of their lipopolysaccharides. *Clinical and diagnostic laboratory immunology, 6*(3), 323-329.

Park, K. H., Shin, J. H., Lee, S. Y., Kim, S. H., Jang, M. O., Kang, S. J., . . . Jang, H. C. (2014). The clinical characteristics, carbapenem resistance, and outcome of *Acinetobacter* bacteremia according to genospecies. *PloS one, 8*(6), e65026.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008a). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical microbiology reviews, 21*(3), 538-582.

Pell, L. G., Kanelis, V., Donaldson, L. W., Howell, P. L., & Davidson, A. R. (2009). The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4160-4165.

Pelletier, M. R., Casella, L. G., Jones, J. W., Adams, M. D., Zurawski, D. V., Hazlett, K. R., . . . Ernst, R. K. (2013). Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 57*(10), 4831-4840.

Pieper, R., Huang, S. T., Robinson, J. M., Clark, D. J., Alami, H., Parmar, P. P., . . . Peterson, S. N. (2009). Temperature and growth phase influence the outer-membrane proteome and the expression of a type VI secretion system in Yersinia pestis. *Microbiology, 155*(Pt 2), 498-512.

Pietrosiuk, A., Lenherr, E. D., Falk, S., Bonemann, G., Kopp, J., Zentgraf, H., . . . Mogk, A. (2011). Molecular basis for the unique role of the AAA+ chaperone ClpV in type VI protein secretion. *The Journal of biological chemistry, 286*(34), 30010-30021.

Planamente, S., Salih, O., Manoli, E., Albesa-Jove, D., Freemont, P. S., & Filloux, A. (2016). TssA forms a gp6-like ring attached to the type VI secretion sheath. *The EMBO journal*.

Poirel, L., Naas, T., & Nordmann, P. (2010). Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrobial agents and chemotherapy, 54*(1), 24-38.

Poirel, L., & Nordmann, P. (2006). Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 12*(9), 826-836.

Possot, O. M., Vignon, G., Bomchil, N., Ebel, F., & Pugsley, A. P. (2000). Multiple interactions between pullulanase secreton components involved in stabilization and cytoplasmic membrane association of PulE. *J Bacteriol, 182*(8), 2142-2152.

Preston, A., Mandrell, R. E., Gibson, B. W., & Apicella, M. A. (1996). The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol, 22*(3), 139-180.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15508-15513.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.

Raetz, C. R., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem, 71*, 635-700.

Ramirez, M. S., Don, M., Merkier, A. K., Bistue, A. J., Zorreguieta, A., Centron, D., & Tolmasky, M. E. (2010). Naturally competent *Acinetobacter baumannii* clinical isolate as a convenient model for genetic studies. *J Clin Microbiol, 48*(4), 1488-1490.

Rumbo, C., Fernandez-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., . . . Bou, G. (2011). Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 55*(7), 3084-3090.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature, 475*(7356), 343-347.

Russell, A. B., LeRoux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., . . . Mougous, J. D. (2013). Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature, 496*(7446), 508-512.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature reviews. Microbiology, 12*(2), 137-148.

Russell, A. B., Singh, P., Brittnacher, M., Bui, N. K., Hood, R. D., Carl, M. A., . . . Mougous, J. D. (2012). A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. *Cell host & microbe, 11*(5), 538-549.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., . . . Campagnari, A. A. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and immunity, 78*(9), 3993-4000.

Schell, M. A., Ulrich, R. L., Ribot, W. J., Brueggemann, E. E., Hines, H. B., Chen, D., . . . Deshazer, D. (2007). Type VI secretion is a major virulence determinant in *Burkholderia* mallei. *Molecular microbiology, 64*(6), 1466-1485.

Schulz, B. L., Jen, F. E., Power, P. M., Jones, C. E., Fox, K. L., Ku, S. C., . . . Jennings, M. P. (2013). Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PloS one, 8*(5), e62768.

Schwarz, S., Singh, P., Robertson, J. D., LeRoux, M., Skerrett, S. J., Goodlett, D. R., . . . Mougous, J. D. (2014). VgrG-5 is a *Burkholderia* type VI secretion system-exported protein required for multinucleated giant cell formation and virulence. *Infection and immunity, 82*(4), 1445-1452.

Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., . . . Mougous, J. D. (2010). *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS pathogens, 6*(8), e1001068.

Scott, N. E., Kinsella, R. L., Edwards, A. V., Larsen, M. R., Dutta, S., Saba, J., . . . Feldman, M. F. (2014b). Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Molecular & cellular proteomics : MCP, 13*(9), 2354-2370.

Sebeny, P. J., Riddle, M. S., & Petersen, K. (2008). *Acinetobacter baumannii* skin and soft-tissue infection associated with war trauma. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 47*(4), 444-449.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I., & Vaneechoutte, M. (1997). Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *Journal of clinical microbiology, 35*(11), 2819-2825.

Seifert, H., Strate, A., & Pulverer, G. (1995). Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. *Medicine, 74*(6), 340-349.

Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature, 500*(7462), 350-353.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., . . . Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 34*(1), 1-14.

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harb Perspect Biol, 2*(5), a000414.

Silverman, J. M., Agnello, D. M., Zheng, H., Andrews, B. T., Li, M., Catalano, C. E., . . . Mougous, J. D. (2013). Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. *Molecular cell, 51*(5), 584-593.

Silverman, J. M., Austin, L. S., Hsu, F., Hicks, K. G., Hood, R. D., & Mougous, J. D. (2011). Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. *Molecular microbiology, 82*(5), 1277-1290.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology, 66*, 453-472.

Snitkin, E. S., Zelazny, A. M., Montero, C. I., Stock, F., Mijares, L., Murray, P. R., & Segre, J. A. (2011). Genome-wide recombination drives diversification of epidemic strains of *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 108*(33), 13758-13763.

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., . . . Infectious Diseases Society of, A. (2008). The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 46*(2), 155-164.

Stenutz, R., Weintraub, A., & Widmalm, G. (2006). The structures of *Escherichia coli* O-polysaccharide antigens. *FEMS microbiology reviews, 30*(3), 382-403.

Suarez, G., Sierra, J. C., Erova, T. E., Sha, J., Horneman, A. J., & Chopra, A. K. (2010). A type VI secretion system effector protein, VgrG1, from Aeromonas hydrophila that induces host cell toxicity by ADP ribosylation of actin. *Journal of bacteriology, 192*(1), 155-168.

Talbot, G. H., Bradley, J., Edwards, J. E., Jr., Gilbert, D., Scheld, M., Bartlett, J. G., & Antimicrobial Availability Task Force of the Infectious Diseases Society of, A. (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 42*(5), 657-668.

Tjernberg, I., & Ursing, J. (1989). Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 97*(7), 595-605.

Toesca, I. J., French, C. T., & Miller, J. F. (2014). The Type VI secretion system spike protein VgrG5 mediates membrane fusion during intercellular spread by pseudomallei group *Burkholderia* species. *Infection and immunity, 82*(4), 1436-1444.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473-3484.

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., & Actis, L. A. (2008). Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology, 154*(Pt 11), 3398-3409.

Traub, W. H. (1989). *Acinetobacter baumannii* serotyping for delineation of outbreaks of nosocomial cross-infection. *Journal of clinical microbiology, 27*(12), 2713-2716.

Tucker, A. T., Nowicki, E. M., Boll, J. M., Knauf, G. A., Burdis, N. C., Trent, M. S., & Davies, B. W. (2014). Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio, 5*(4), e01313-01314.

Unterweger, D., Kostiuk, B., Otjengerdes, R., Wilton, A., Diaz-Satizabal, L., & Pukatzki, S. (2015). Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *The EMBO journal, 34*(16), 2198-2210.

Valencia, R., Arroyo, L. A., Conde, M., Aldana, J. M., Torres, M. J., Fernandez-Cuenca, F., . . . Aznar, J. (2009). Nosocomial outbreak of infection with pan-drug-resistant *Acinetobacter baumannii* in a tertiary care university hospital. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 30*(3), 257-263.

Vinogradov, E. V., Brade, L., Brade, H., & Holst, O. (2003). Structural and serological characterisation of the O-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter baumannii* strain 24. *Carbohydrate research, 338*(23), 2751-2756.

Vinogradov, E. V., Duus, J. O., Brade, H., & Holst, O. (2002). The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter baumannii* strain ATCC 19606. *European journal of biochemistry / FEBS, 269*(2), 422-430.

Vinogradov, E. V., Pantophlet, R., Dijkshoorn, L., Brade, L., Holst, O., & Brade, H. (1996). Structural and serological characterisation of two O-specific polysaccharides of *Acinetobacter*. *European journal of biochemistry / FEBS, 239*(3), 602-610.

Wang, N., Ozer, E. A., Mandel, M. J., & Hauser, A. R. (2014). Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *mBio, 5*(3), e01163-01114.

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015b). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 112*(30), 9442-9447.

Wexler, A. G., Bao, Y., Whitney, J. C., Bobay, L. M., Xavier, J. B., Schofield, W. B., . . . Goodman, A. L. (2016). Human symbionts inject and neutralize antibacterial toxins to persist in the gut. *Proceedings of the National Academy of Sciences of the United States of America, 113*(13), 3639-3644.

Whitfield, C., Amor, P. A., & Koplin, R. (1997). Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. *Molecular microbiology, 23*(4), 629-638.

Whitney, J. C., Beck, C. M., Goo, Y. A., Russell, A. B., Harding, B. N., De Leon, J. A., . . . Mougous, J. D. (2014b). Genetically distinct pathways guide effector export through the type VI secretion system. *Molecular microbiology, 92*(3), 529-542.

Whitney, J. C., Chou, S., Russell, A. B., Biboy, J., Gardiner, T. E., Ferrin, M. A., . . . Mougous, J. D. (2013). Identification, structure, and function of a novel type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. *The Journal of biological chemistry, 288*(37), 26616-26624.

Whitney, J. C., Quentin, D., Sawai, S., LeRoux, M., Harding, B. N., Ledvina, H. E., . . . Mougous, J. D. (2015). An interbacterial NAD(P)(+) glycohydrolase toxin requires elongation factor Tu for delivery to target cells. *Cell, 163*(3), 607-619.

Wilharm, G., Piesker, J., Laue, M., & Skiebe, E. (2013). DNA uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs during movement along wet surfaces. *J Bacteriol, 195*(18), 4146-4153.

Yoon, E. J., Chabane, Y. N., Goussard, S., Snesrud, E., Courvalin, P., De, E., & Grillot-Courvalin, C. (2015). Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*. *MBio, 6*(2).

Zheng, J., Ho, B., & Mekalanos, J. J. (2011). Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PloS one, 6*(8), e23876.

Zheng, J., & Leung, K. Y. (2007). Dissection of a type VI secretion system in Edwardsiella tarda. *Molecular microbiology, 66*(5), 1192-1206.

Zheng, J., Shin, O. S., Cameron, D. E., & Mekalanos, J. J. (2010). Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America, 107*(49), 21128-21133.

Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M. S., Logger, L., Douzi, B., . . . Cascales, E. (2014). Architecture and assembly of the Type VI secretion system. *Biochimica et biophysica acta, 1843*(8), 1664-1673.

Zoued, A., Durand, E., Brunet, Y. R., Spinelli, S., Douzi, B., Guzzo, M., . . . Cascales, E. (2016). Priming and polymerization of a bacterial contractile tail structure. *Nature, 531*(7592), 59-63.

2 Genomic and functional analysis of the type VI secretion system in *Acinetobacter baumannii*

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2.1 Introduction

The diversity of the Gram-negative *Acinetobacter* spp. is exemplified by the wide range of environments from which these bacteria can be isolated from. These environments include soils, (Houang *et al.*, 2001), activated sludge (Carr *et al.*, 2003), food (Berlau *et al.*, 1999), and colonized human carriers (Seifert *et al.*, 1997). The traits of several species of this genus have been recognized as potentially having important implications to the field of biotechnology, including roles for degradation of hydrocarbons (Mara *et al.*, 2012) and plant growth-promoting traits (Rokhbakhsh-Zamin *et al.*, 2011). *A. baumannii* is recognized as one of the most clinically important species of *Acinetobacter* (Peleg *et al.*, 2008a); thus, much attention has been directed towards the ability of some members of this genus to cause severe infections. As a primarily nosocomial pathogen, *A. baumannii* causes a wide-range of infections in immunocompromised people, most often pneumonia and bloodstream infections (Dijkshoorn *et al.*, 2007), and, in contrast with most other *Acinetobacter* spp., it is rarely isolated outside of the hospital environment (Peleg *et al.*, 2008a). The treatment of *A. baumannii* infections has become increasingly difficult due to the widespread dissemination of multi- and pan-drug resistant strains (Perez *et al.*, 2007). Antibiotic resistance and epidemiology have been the focus of much of the scientific work on *A. baumannii*, but little is known about the strategies this bacterium uses for pathogenesis. Potential virulence mechanisms employed by *A. baumannii* are, however, beginning to be uncovered (March *et al.*, 2010; McConnell *et al.*, 2012; Mortensen *et al.*, 2012). Well characterized iron-and zinc-acquisition systems are involved in *A. baumannii* persistence within the host (Gaddy *et al.*, 2012; Hood *et al.*, 2012b; Zimbler *et al.*, 2009), and capsule has been shown to be essential for resistance to serum killing and for survival in a rat model of infection (Russo *et al.*, 2010). *A. baumannii* phospholipases have also been implicated in interactions with epithelial cells and serum resistance

(Camarena *et al.*, 2010; Jacobs *et al.*, 2010). Furthermore, the propensity of *A. baumannii* to resist desiccation and form biofilms may contribute to endemic disease within a healthcare setting (Jawad *et al.*, 1998; Tomaras *et al.*, 2003; Vidal *et al.*, 1996; Wendt *et al.*, 1997). An outer membrane protein A (OmpA) has been proposed to mediate interactions with epithelial cells and induce dendritic cell death (Choi *et al.*, 2005; Choi *et al.*, 2008; Lee *et al.*, 2010). It was recently shown that a conserved protein glycosylation system in *A. baumannii* is critical for full virulence in several infection models, as well as for biofilm formation (Iwashkiw *et al.*, 2012b).

Bacteria use several secretory mechanisms to export effector molecules into the surrounding environment or, in some cases, directly into neighbouring cells (Gerlach *et al.*, 2007; Hayes *et al.*, 2010). It has been proposed that *A. baumannii* is able to transport the aforementioned OmpA via outer membrane vesicles to host cells, ultimately resulting in cytotoxicity towards the host (Jin *et al.*, 2011). Sequencing of the *A. baumannii* genome identified a set of genes homologous to those involved in the *Legionella/Coxiella* type IV secretion system (T4SS), and although the exact role of these genes remains to be determined, mutation of the locus resulted in virulence defects (Smith *et al.*, 2007). Another bacterial secretion system, the type VI secretion system (T6SS), was recently described as a novel secretion system in Gram-negative bacteria (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). The T6SS is structurally related to the cell-puncturing device of the T4 bacteriophage (Leiman *et al.*, 2009; Pell *et al.*, 2009; Pukatzki *et al.*, 2007), and the complement of genes encoding this system have been identified in the genomes of numerous bacteria through *in silico* analysis, including *A. baumannii* (Bingle *et al.*, 2008; Boyer *et al.*, 2009).

The T6SS has been implicated in the interaction between bacteria and between bacteria and their hosts. In *V. cholerae*, the T6SS is involved in host cell actin crosslinking, cytotoxicity

towards amoeba, and interbacterial killing (Ma *et al.*, 2010; MacIntyre *et al.*, 2010; Pukatzki *et al.*, 2006). *P. aeruginosa* activates a T6SS during infection of cystic fibrosis patients (Mougous *et al.*, 2006), and also uses T6SS-delivered toxins to actively kill competing bacteria (Hood *et al.*, 2010; Russell *et al.*, 2011). Several *Burkholderia* species encode T6SSs as virulence factors, and play a major role in the intracellular lifecycle of these organisms (French *et al.*, 2011; Rosales-Reyes *et al.*, 2012) . *B. mallei*, a biothreat agent, requires a T6SS for full virulence in the hamster meliodosis model (Schell *et al.*, 2007). Interestingly, *B. thailandensis*, which encodes five T6SS gene clusters (1-5), uses T6SS-5 and T6SS-1 to mediate interactions with eukaryotic and prokaryotic organisms, respectively (Schwarz *et al.*, 2010). *Helicobacter hepaticus* was found to use its T6SS to limit host inflammation and maintain a balanced relationship between host and microbe (Chow *et al.*, 2010). Thus, it seems that the T6SS is a mechanism that can be adapted by individual bacterial species to interact with other prokaryotes, eukaryotes, or both.

T6SSs incorporate characteristic components (Boyer *et al.*, 2009; Shalom *et al.*, 2007), including the secreted proteins Hcp and VgrG, and structural proteins ClpV, TssM, and TssL (Boyer *et al.*, 2009; Filloux *et al.*, 2008). Hcp secretion is considered a molecular marker of a functional T6SS, and has been used extensively to evaluate activity of the T6SS (Pukatzki *et al.*, 2009). Hcp forms hexamers that assemble as tubular structures and resemble the bacteriophage T4 tail tube (Ballister *et al.*, 2008; Leiman *et al.*, 2009; Mougous *et al.*, 2006). VgrG proteins, some of which have evolved to contain virulence activity in their C-termini (Ma *et al.*, 2009; Pukatzki *et al.*, 2007), are structurally similar to the puncturing device of T4 bacteriophage (Leiman *et al.*, 2009; Pukatzki *et al.*, 2007). The AAA^+ protein ClpV utilizes ATP hydrolysis in order to dissemble another T6SS tubular structure composed of interacting TssB/TssC proteins (Bonemann *et al.*, 2009; Mougous *et al.*, 2006; Pietrosiuk *et al.*, 2011). TssB/TssC tubules, which

are critical for a functional T6SS, are homologous to the tail sheath of T4 bacteriophage (Leiman *et al.*, 2009) and provide dynamic contractile structures that assemble within the cytoplasm and may drive T6SS components outside the cell (Basler *et al.*, 2012b). TssM and TssL, homologs of the T4SS IcmF and DotU proteins (Sexton *et al.*, 2004), respectively, physically interact (de Bruin *et al.*, 2011; Zheng *et al.*, 2007) and are required for secretion of conserved T6SS components (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006).

In this work we present genomic and experimental data showing widespread T6SS distribution and activity in several species from the genus *Acinetobacter*, and, in particular, *A. baumannii*. We report that under standard laboratory conditions, *A. baumannii* ATCC 17978 encodes a constitutively active T6SS that secretes the conserved component Hcp via a T6SS-dependent mechanism.

2.2 Experimental Procedures

2.2.1 Bacterial strains and growth conditions

The *A. baumannii* reference strains used in this study were obtained from American Type Culture Collection. All strains and plasmids used are listed in Table 2.1. Strains were grown in Luria-Bertani (LB) medium at 37°C with shaking. Where necessary, antibiotics were added to the medium at the following concentrations: gentamicin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and tetracycline (50 μg ml^{-1}).

Table 2.1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. baumannii		
ATCC 17978	Reference strain	(Piechaud *et al.*, 1951)
Δ*hcp*	A1S_1296 minus derivative of ATCC 17978	This study

Δ*hcp* / pHcp	A1S_1296 minus derivative of ATCC 17978 complement	This study
Δ*hcp* / pWH1266	A1S_1296 minus derivative of ATCC 17978 vector control	This study
Δ*tssM*	A1S_1302 minus derivative of ATCC 17978	This study
Δ*tssM* / pTssM	A1S_1302 minus derivative of ATCC 17978 complement	This study
Δ*tssM* / pWH1266	A1S_1302 minus derivative of ATCC 17978, vector control	This study
pWH1266	Wild type vector control	This study
SDF	Body lice isolate	(Vallenet *et al.*, 2008)
AYE	Human isolate	(Vallenet *et al.*, 2008)
ATCC 19606	Reference strain	(Bouvet, 1986)
1375	Clinical isolate, blood	This study
1224	Clinical isolate, thigh	(Iwashkiw *et al.*, 2012b)
1225	Clinical isolate, coccyx	This study
A. calcoaceticus A	Clinical isolate , urine	(Iwashkiw *et al.*, 2012b)
A. calcoaceticus B	Clinical isolate,leg	(Iwashkiw *et al.*, 2012b)
A. pittii A	Clinical isolate, urine	(Iwashkiw *et al.*, 2012b)
A. pittii B	Clinical isolate, bronchial wash	(Iwashkiw *et al.*, 2012b)
A. junii	Clinical isolate, tracheal tube	(Iwashkiw *et al.*, 2012b)
A. baylyi ADP1	Soil isolate	(Juni, 1972)
A. nosocomialis 1221	Clinical isolate, gentamicin resistant prey for killing assay	(Iwashkiw *et al.*, 2012b)
E. coli		
DH5α	General cloning and plasmid propagation	Invitrogen
MG1655R	Rifampicin resistant K-12 strain, bacterial prey	(MacIntyre *et al.*, 2010)
V. cholerae		
V52	*hlyA*$^-$, *hapA*$^-$, and *rtxA*$^-$	(Pukatzki *et al.*, 2006)
V52 Δ*tssM*	*tssM* minus derivative of V52	(Pukatzki *et al.*, 2006)
Plasmids		
pEXT20	Cloning and expression vector, IPTG inducible, Amp^r	(Dykxhoorn *et al.*, 1996)
pSPG1	Source of *aacC1* Gm^r cassette	(Ugalde *et al.*, 2000)
pFLP2	*sacB,* suicide vector	(Hoang *et al.*, 1998)
pABK	Kan cassette cloned into NheI sites of pFLP2	This study
pEXT22	Source of Kan^r cassette	(Dykxhoorn *et al.*, 1996)
pWH1266	Amp^r Tet^r	(Hunger *et al.*, 1990)

pBAV1K-T5-gfp	Plasmid used to confer kanamycin resistance in bacterial killing assay	(Bryksin *et al.*, 2010)
pWEB01	*hcp* (A1S_1296) in pEXT20, 10His tag for purification, Amp^r	This study
pWEB02	Flanking regions of *hcp* in pEXT20, Amp^r	This study
pWEB03	Flanking regions of *hcp* with internal Gm^r cassette from pSPG1, in pEXT20	This study
pWEB04	*hcp* knockout construct from pWEB3 in pFLP2, Gm^r	This study
pWEB05	*tssM* knockout construct in pABK, Kan^r	This study
pWEB06	*hcp* cloned into pEXT20, Amp^r	This study
pWEB07	*tssM* cloned into pEXT20, Amp^r	This study
pHcp	*hcp* in pWH1266, Tet^r	This study
pTssM	*tssM* in pWH1266, Tet^r	This study

2.2.2 Purification of *A. baumannii* Hcp for antibody development

Purification of the histidine tagged Hcp was performed essentially as described elsewhere (Iwashkiw *et al.*, 2012a). Briefly, the *A. baumannii hcp* gene (A1S_1296) was cloned into pEXT20 with a 10 histidine tag using HcpFwd and HcpRev10His, creating pEXT20-Hcp10His, and electroporated into *E. coli* DH5α. 1L of fresh LB was inoculated with 20mL of an overnight culture of *E. coli* containing this vector, and grown for 4h with 1h induction by addition of 1mM IPTG. Cells were harvested and resuspended in binding buffer (10mM imidazole, 300mM NaCl, 20mM Tris-HCl, pH 8.0) and lysed using a French pressure cell, followed by centrifugation. Supernatants were collected, and pellets were resuspended in binding buffer for a second round of lysis followed by centrifugation. Inclusion bodies were solubilized as previously described (Margetts *et al.*, 2000) by resuspending the pellets obtained above in binding buffer containing 6M urea. Supernatants and solubilized inclusion bodies were mixed and loaded onto a HisTrap HP column (Amersham

Pharma Biosciences) equilibrated with 10 column volumes of binding buffer with a flow rate of 1mL min^{-1} for Ni^{2+}-affinity chromatography. The column was washed with 25 column volumes of washing buffer (20mM imidazole, 300mM NaCl, 20mM Tris-HCl, 6M urea pH 8.0). Bound protein was eluted using elution buffer (250mM imidazole, 300mM NaCl, 20mM Tris-HCl, 6M urea pH 8.0). Protein purity was determined by Coomassie stain following SDS-PAGE, and mass spectrometry analysis was performed to confirm protein ID. Sample was then transferred to PBS buffer by buffer exchange using a PD-10 column (GE Healthcare). Protein concentration was determined by the Bradford assay (Bio-Rad), and purified protein was sent to SACRI antibody services (University of Calgary, Alberta, Canada) for development of rabbit-derived polyclonal antibodies.

2.2.3 Preparation of cell-free supernatants for SDS-PAGE

The OD_{600} of overnight *Acinetobacter* cultures were determined and fresh LB was inoculated with OD-normalized volumes of bacterial culture. Antibiotics were not added to diluted cultures in order to avoid potential cell lysis. After approximately 4 hours, bacteria were harvested by centrifugation (10 min at 5,000 × g) and supernatants collected and filtered through 0.22 μm syringe filters (Millipore Corporation, Billerica, MA) to obtain cell-free supernatants. Supernatant proteins were precipitated by the addition of 1:4 volumes trichloroacetic acid and incubation at 4°C for 20 min. Protein pellets were obtained by centrifugation at 14,000 × g for 5 min. The samples were then washed twice with ice-cold acetone, centrifuged to pellet, and supernatant removed. The pellets were dried in a heat block at 95°C and resuspended in loading buffer. OD_{600} normalized volumes of whole cells or supernatants were loaded onto 15% SDS-PAGE gels for separation, transferred to a nitrocellulose membrane, and probed by Western immunoblot with polyclonal rabbit anti-Hcp (1:1500) and mouse monoclonal anti-RNA polymerase (1:2500, RNAP

α-subunit; Neoclone). Membranes were then probed with IRDye conjugated anti-mouse and anti-rabbit antibodies and visualized on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.2.4 ELISA assay for Hcp secretion

For ELISA assays, 250µl of LB in a 96-well plate was inoculated in triplicate with individual colonies of the *Acinetobacter* strains used in this study. The plates were incubated in a humidified container (to prevent evaporation) at 37°C in a shaking incubator at 200rpm for ~9h to allow sufficient growth of all strains. Following incubation, the optical density at 600nm was determined for each well by a plate reader, and then plates were centrifuged at 4k rpm for 10min. Fifty µl of supernatants were transferred to high-binding ELISA 96-well plates containing 50µl of binding buffer (100mM sodium bicarbonate/carbonate) and incubated at 4°C overnight. The plates were washed with PBS, blocked with a solution of 5% skim milk in PBS for 1.5h, and then probed with a solution of 2.5% skim milk in PBST containing a 1:7500 dilution of the anti-Hcp antibody for 1h. The plates were washed with PBST and probed with a 1:5000 dilution of horse radish peroxidase conjugated goat anti-rabbit antibody (Bio-Rad) in 2.5% skim milk-PBST solution for 1h. The plates were again washed with PBST, and then 100µl of TMB substrate (Cell Signaling Technology, Danvers, MA) was added to each well. The plates were allowed to develop for ~5mins before absorbance at 650nm was measured by a plate reader. Alternatively, STOP (Cell Signaling Technology, Danvers, MA) solution could be added to end the colorimetric reaction, and absorbance at 450nm measured.

2.2.5 Construction of mutants and complemented strains

Primers are listed in Table 2.2, with restriction sites underlined where relevant. Approximately 1000bp of DNA flanking either side of the 17978 *hcp* gene (A1S_1296) was amplified and individually cloned into pEXT20 using the primers 5'-hcpFwd and 5'-hcpRev for the upstream region, and 3'-hcpwdF and 3'-hcpRev for the downstream region. These segments were subcloned into a single plasmid to generate pWEB02. A gentamicin resistance cassette (*aacC1*) was excised from pSPG1 by *Sma*I digest, and subsequently ligated with pWEB02 to generate pWEB03. An *Eco*RI/*Xba*I double digest removed the entire fragment from pEXT20, and was then ligated to a similarly cut pFLP2 plasmid, which encodes a *sacB* counter selection gene and does not replicate in *A. baumannii*. The resultant pWEB04 plasmid was electroporated into 17978 cells followed by selection for those cells that had integrated the plasmid by plating on gentamicin. Gentamicin resistant colonies were used to inoculate 5 ml of liquid media, and were subculture every day for three days. After three days of growth, 200 µl of this culture was plated onto solid media containing gentamicin and 10% sucrose (w/v) to select for double recombinants. Genomic DNA was isolated from the resulting gentamicin/sucrose resistant 17978 colonies, and PCR and sequencing was performed to confirm the successful replacement of *hcp* with the *aacC1* resistance cassette. For complementation, the *hcp* gene was amplified using primers HcpFwd and HcpRev and cloned into the EcoRI/XbaI sites of pEXT20 generating pWEB06. After digest with *Eco*RI/*Xba*I, the construct was subcloned into similarly digested pWH1266 shuttle plasmid, creating pHcp. pHcp was electroporated into 17978 Δ*hcp* for complementation analysis.

For the unmarked mutation of *tssM*, primer pairs *tssM*UpFwd, *tssM*UpRev, and *tssM*DwFwd, *tssM*DwRev were used to amplify approximately 500bp of DNA upstream and downstream of *tssM*, respectively. The two PCR products were then mixed in equimolar amounts and nested overlap-extension PCR was performed using primers *tssM*FwdNest and *tssM*RevNest.

The product was cloned into pABK, a derivative of pFLP2 with a kanamycin cassette inserted into its *Nhe*I sites, generating pWEB05. The vector was then transformed into wild type 17978 and plated on kanamycin to select for integration. Following the procedure described above, cells were then plated on sucrose containing plates to select for double recombinants. Colonies which were sucrose resistant but kanamycin sensitive were selected for PCR screening and sequencing to confirm generation of 17978 Δ*tssM*. The growth curve of 17978 Δ*tssM* was identical to parental 17978. For complementation, primers *tssM*Fwd and *tssM*Rev10His were used to amplify the full-length *tssM* gene and cloned into pEXT20. The product was then amplified out of pWEB07 plasmid using *tssM*Fwd1 and *tssM*Rev10His primers and cloned into the *Pst*I site of pWH1266, generating p*TssM*.

Table 2.2 Primers used in this study

Primer name	Sequence (5'-3')
5'-hcpFwd	aaagaattcatgaataatactcaatcagcagcaatgccacttgttg
5'-hcpRev	aaacccggggctgaccttgattaatttgaggactgagg
3'-hcpFwd	aaacccgggggtggaggctgacataaagttagcctctgctg
3'-hcpRev	aaatctagactaaatcgagcttaagataactaaactttccgggaaac
HcpFwd	atatggatccatgaaagatatatacgttgag
HcpRev	aatttctagattacgctgcgtaagaagctg
HcpRev10His	aaagtcgacttagtggtggtggtggtggtggtggtggtgcgctgcgtaagaagctg
*tssM*UpFwd	atacaattttaggctacttgtggc
*tssM*UpRev	caagctttaatgagtgttctgg

*tssM*FwdNest	atatgaattcattatcgccttgtcattgttcgtgg
*tssM*DwFwd	ccagaacactcattaaagcttgattagtggtgagtccacttacccaagc
*tssM*DwRev	ttgactgcattgcatattccaatgc
*tssM*RevNest	atattctagaaatgtatgcatcaatgctcctcc
*tssM*Fwd	atatggatccatgcatacaattttaggctacttgtgg
*tssM*Rev10His	atatctgcagtcagtggtggtggtggtggtggtggtggtgtggcttaactcccgc
*tssM*Fwd1	atatctgcagcaggtcgtaaatcactgcataattcg

2.2.6 *D. discoideum* plaque assay and *Galleria mellonella* killing assay

The *D. discoideum* plaque assay was performed essentially as described previously (Pukatzki *et al.*, 2006). Mid logarithmic growth phase amoebae were mixed with overnight cultures of bacteria to a final concentration of 1×10^3 cells ml^{-1}. 0.2 ml of the suspension was then plated on SM/5 agar containing 1% ethanol (Smith *et al.*, 2007). Plates were incubated at room temperature and monitored for *D. discoideum* plaques for up to 7 days. For *G. mellonella* killing assays, the experiments were performed as previously described (Peleg *et al.*, 2009). Briefly, PBS-washed bacterial cells were normalized by OD_{600} and 5-µl aliquots were injected into *G. mellonella* larvae (Dr. Andrew Keddie, University of Alberta). For each group, 10 *G. mellonella* were used, and colony counts on LB agar were used to determine the CFUs injected. Larvae were incubated at 37°C after injection and survival was plotted using the Kaplan-Meier method and analyzed using the log-rank test (Peleg *et al.*, 2009). Experiments comparing wild type and mutant were discarded if the difference in CFU counts were >0.5Log (Peleg *et al.*, 2009). PBS injected *G. mellonella*

were used as a negative control and showed 100% survival for the duration of the experiment. Fig. 4A shows representative results from two separate experiments.

2.2.7 Animal Infections

For assessing pathogenesis *in vivo,* we utilized a murine model of *A. baumannii* pneumonia previously developed in our laboratory with a few modifications (Jacobs *et al.*, 2010). Briefly, 7-week-old female C57BL/6 mice were anesthetized followed by intranasal inoculation with 3-5 x 10^8 CFU *A. baumannii* in 40 µl PBS. At 36 hours post-infection mice were euthanized, and CFU were enumerated in lungs and livers following tissue homogenization and plating serial dilutions on LB agar plates. All of the infection experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Mice were obtained from Jackson Laboratories.

2.2.8 Bacterial killing assay

Killing assays were performed as described previously (MacIntyre *et al.*, 2010). Bacterial strains were grown overnight on LB agar plates with the appropriate antibiotics. *V. cholerae* strains V52 and V52 Δ*tssM* were used as positive and negative controls for bacterial killing, respectively. The *E. coli* K-12 strain MG1655 (rifampicin resistant) was used as prey in initial assays. Intra-species competition assays were performed with *A. baumannii* ATCC 19606 and *A. baylyi* ADP1 transformed with pBAV1K-T5-gfp, a plasmid conferring kanamycin resistance and allowing for selection against *A. baumannii* ATCC 17978, as well as a clinical isolate *A. nosocomialis* 1221 that was naturally gentamicin resistant. Cells were harvested, resuspended in LB and mixed at a 10:1 ratio (predator:prey). Bacterial mixtures were spotted onto LB agar for four hours at 37°C, unless otherwise noted. Cells were harvested and seven serial dilutions were performed. Each

serial dilution was plated in 10µL spots on LB with appropriate antibiotic to select for surviving prey. Plates were incubated overnight at 37°C and the surviving prey were enumerated the following day. Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparisons post-test.

2.3 Results

2.3.1 The T6SS is operational in several species within the *Acinetobacter* genus

Bioinformatic analysis of the genomes from several sequenced species of *Acinetobacter* revealed the presence of genes resembling a typical T6SS gene cluster (Figure 2.1) (Bingle *et al.*, 2008; Boyer *et al.*, 2009) . These putative T6SS loci contain homologs of 12 core T6SS genes (Figure 2.1 and Table 2.3); herein, T6SS genes are referred to by their generic names or by the proposed *tss* nomenclature of Shalom *et al* (Shalom *et al.*, 2007). The gene clusters encode the hallmarks *hcp, clpV*, and *tssM*, as well as accessory components and genes with unknown function. Varying numbers of genes located outside the clusters encode putative VgrG proteins, which are often secreted via the T6SS. Many of the identified VgrG sequences are greater than 750 amino acids in length, indicating they may contain evolved effector domains in their C-termini; however, apart from N-terminal homology to bacteriophage components gp44 and gp5 that is typical of VgrG proteins (Pukatzki *et al.*, 2007), we were unable to identify conserved protein domains that could be indicative of possible functions.

All sequenced *A. baumannii* strains appear to have the core T6SS genes in a syntenic organization. *A. calcoaceticus* RUH2202, *A. oleivorans* DR1, *A. baylyi* ADP1, *A. johnsonii* SH046, *A. radioresistans* SH164, and *A. lwoffii* WJ10621 were all found to possess the same 12 core genes present in *A. baumannii*; however, as shown in Figure 2.1, the organization differed

slightly in some strains, with an opposite orientation of the final four genes in the cluster observed for *A. baylyi*, *A. johnsonii*, *A. radioresistans*, and *A. lwoffii*. The conserved T6SS proteins encoded by these clusters generally show high sequence identity (70% or greater) with *A. baumannii,* although the sequences of the VgrG proteins are slightly more divergent (60% or greater sequence identity) (Table 2.3). Each cluster, however, lacks an obvious homolog of *tssJ*, an outer membrane anchored lipoprotein (Aschtgen *et al.*, 2008). Interestingly, as shown in Table S1, several *Acinetobacter* species are not predicted to encode a functional T6SS (*A. pittii*, *A. nosocomialis*, *A. haemolyticus*, and *A. junii*) due to the absence of several conserved proteins, yet still encode *vgrG* genes and in some cases *tssM* or *tssL* homologs.

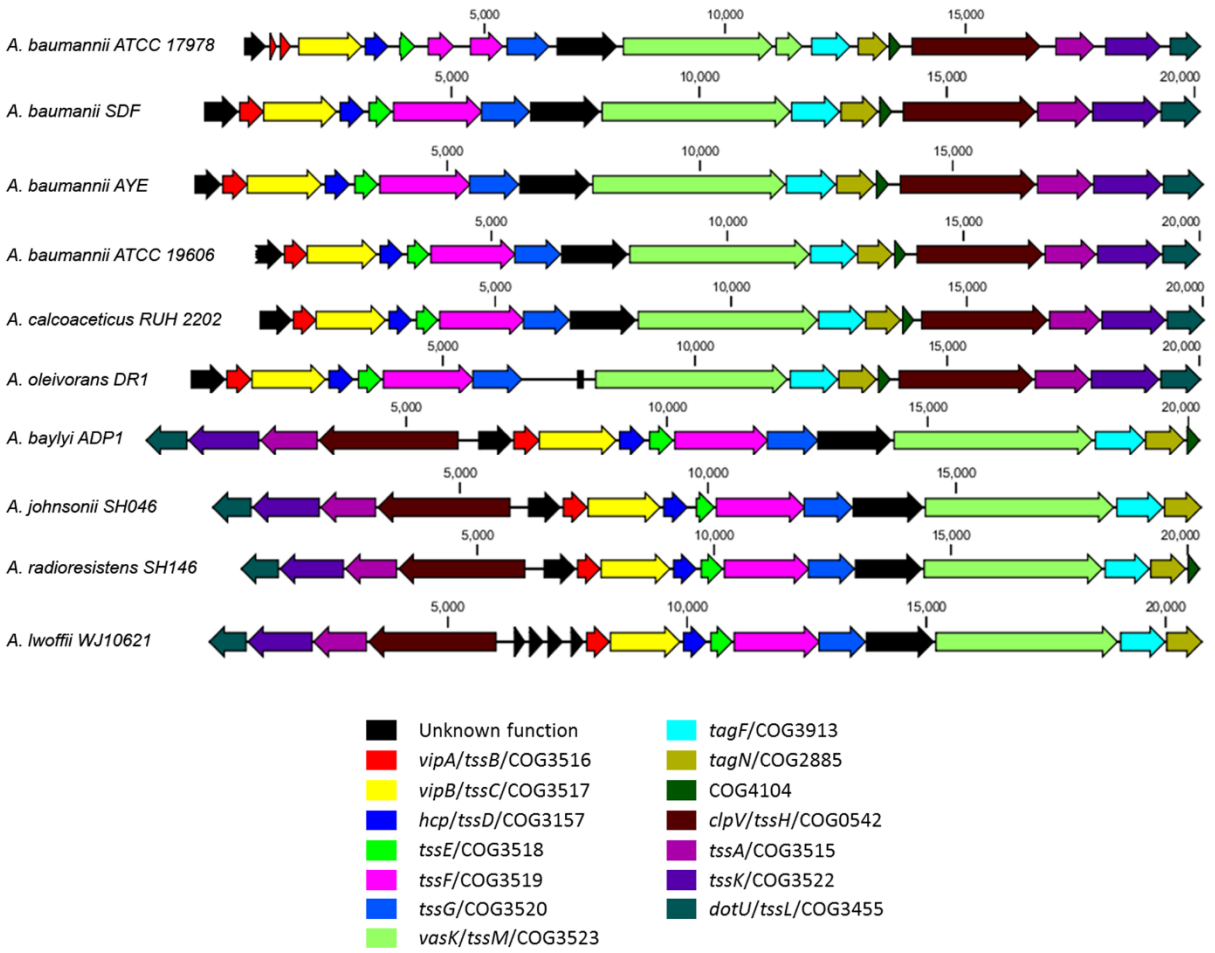


Figure 2.1 Genetic organization of T6SS loci.

Selected genomes of sequenced *Acinetobacter* strains were probed for the presence of T6SS genes, with those genes predicted to be involved in T6SS colored and identified below the figure.

Table 2.3 Identification of conserved T6SS components in selected *Acinetobacter* spp.

tss designation	Gene name/ COG id	*A. baumannii* ATCC 17978	*A. baumannii* ATCC 19606	*A. baumannii* SDF	*A. baumannii* AYE	*A. calcoaceticus* RUH2202	*A. radioresistans* SH164	*A. lwoffii* WJ10621	*A. johnsonii* SH046	*A. oleivorans* DR1	*A. baylyi* ADP1	*V. cholerae* V52	*P. aeruginosa* PAO1 (HSI-I)	*B. pseudomallei* K96243 (T6SS-1)
tssB	*vipA*/3516	A1S_1293, A1S_1294	HMPREF0010_01125	ABSDF2251	ABAYE2415	HMPREF0012_00601	HMPREF0018_00611	AlwoW_010100002240	HMPREF0016_00041	AOLE_12265	ACIAD2691	VCA0107	PA0083	BPSL3107
tssC	*vipB*/3517	A1S_1295	HMPREF0010_01124	ABSDF2250	ABAYE2414	HMPREF0012_00602	HMPREF0018_00610	AlwoW_010100002235	HMPREF0016_00042	AOLE_12260	ACIAD2690	VCA0108	PA0084	BPSL3106
tssD	*hcp*/3157	A1S_1296	HMPREF0010_01123	ABSDF2249	ABAYE2413	HMPREF0012_00603	HMPREF0018_00609	AlwoW_010100002230	HMPREF0016_00043	AOLE_12255	ACIAD2689	VCA0017, VC1264	PA0085	BPSL3105
tssE	3518	A1S_1297	HMPREF0010_01122	ABSDF2248	ABAYE2412	HMPREF0012_00604	HMPREF0018_00608	AlwoW_010100002225	HMPREF0016_00044	AOLE_12250	ACIAD2688	VCA0109	PA0087	BPSL3104
tssF	3519	A1S_1298, A1S_1299	HMPREF0010_01121	ABSDF2247	ABAYE2411	HMPREF0012_00605	HMPREF0018_00607	AlwoW_010100002220	HMPREF0016_00045	AOLE_12245	ACIAD2687	VCA0110	PA0088	BPSL3103
tssG	3520	A1S_1300	HMPREF0010_01120	ABSDF2246	ABAYE2410	HMPREF0012_00606	HMPREF0018_00606	AlwoW_010100002215	HMPREF0016_00046	AOLE_12240	ACIAD2686	VCA0111	PA0089	BPSL3102
-	-	A1S_1301	HMPREF0010_01119	ABSDF2245	ABAYE2409	HMPREF0012_00607	HMPREF0018_00605	AlwoW_010100002210	HMPREF0016_00047	-	ACIAD2685	-	-	-
tssM	*tssM, icmF*/3523	A1S_1302, A1S_1303	HMPREF0010_01118	ABSDF2244	ABAYE2408	HMPREF0012_00608	HMPREF0018_00604	AlwoW_010100002205	HMPREF0016_00048	AOLE_12230	ACIAD2684	VCA0120	PA0077	BPSL3097
tagF	3913	A1S_1304	HMPREF0010_01117	ABSDF2243	ABAYE2407	HMPREF0012_00609	HMPREF0018_00603	AlwoW_010100002200	HMPREF0016_00049	AOLE_12225	ACIAD2683	-	PA0076	BPSL3098
tagN	2885	A1S_1305	HMPREF0010_01116	ABSDF2242	ABAYE2406	HMPREF0012_00610	HMPREF0018_00602	AlwoW_010100002195	HMPREF0016_00050	AOLE_12220	ACIAD2682	-	-	BPSL3099
-	4104	A1S_1306	HMPREF0010_01115	ABSDF2241	ABAYE2405	HMPREF0012_00611	HMPREF0018_00601	AlwoW_010100001175	HMPREF0016_00292	AOLE_12215	ACIAD2681	VCA0105	PA0093	-
tssH	*clpV*/0542	A1S_1307	HMPREF0010_01114	ABSDF2240	ABAYE2404	HMPREF0012_00612	HMPREF0018_00613	AlwoW_010100002265	HMPREF0016_00039	AOLE_12210	ACAID2694	VCA0116	PA0090	BPSL3101
tssA	3515	A1S_1308	HMPREF0010_01113	ABSDF2239	ABAYE2403	HMPREF0012_00613	HMPREF0018_00614	AlwoW_010100002270	HMPREF0016_00038	AOLE_12205	ACAID2695	VCA0119	PA0082	BPSL3100
tssK	3522	A1S_1309	HMPREF0010_01112	ABSDF2238	ABAYE2402	HMPREF0012_00614	HMPREF0018_00615	AlwoW_010100002275	HMPREF0016_00037	AOLE_12200	ACAID2696	VCA0114	PA0079	BPSL3110
tssL	*dotU*/3455	A1S_1310	HMPREF0010_01111	ABSDF2237	ABAYE2401	HMPREF0012_00615	HMPREF0018_00616	AlwoW_010100002280	HMPREF0016_00036	AOLE_12195	ACAID2697	VCA0115	PA0078	BPSL3111
tssI	*vgrG*/3501	A1S_0550, A1S_1288, A1S_1289, A1S_3364	HMPREF0010_03251, HMPREF0010_03005, HMPREF0010_03468, HMPREF0010_01450	ABSDF1392, ABSDF2265	ABAYE0118, ABAYE2454	HMPREF0012_03328, HMPREF0012_00593, HMPREF0012_00597, HMPREF0012_02476	HMPREF0018_02686	AlwoW_010100005395, AlwoW_010100013878, AlwoW_010100014673	HMPREF0016_00553, HMPREF0016_01111	AOLE_18955, AOLE_12340, AOLE_12305, AOLE_00565, AOLE_13955	ACIAD3115, ACIAD1788, ACIAD3427, ACIAD0167	VCA0123, VC1416, VCA0018	PA0091, PA0095	BPSS1503

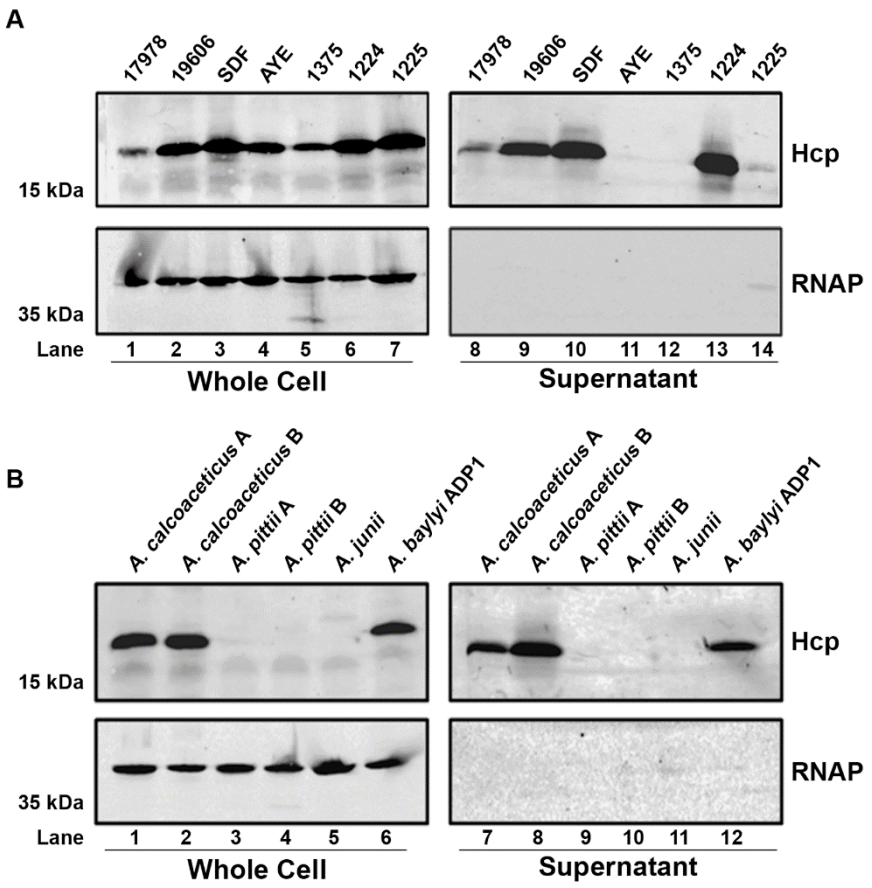
We next wanted to determine whether the T6SSs encoded in these loci were active under laboratory conditions. The presence of Hcp in culture supernatants is used as a reliable indicator of an active T6SS (Pukatzki *et al.*, 2009); therefore, we developed a polyclonal antibody raised against a purified, recombinant Hcp protein from *A. baumannii* ATCC 17978 (17978). Hcp expression and secretion was analyzed in several strains of *A. baumannii* and non-*baumannii* species. The *A. baumannii* strains studied included four well-characterized and sequenced strains (17978, 19606, SDF and AYE; Table 2.3) and three uncharacterized clinical isolates of *A. baumannii* (strains 1375, 1224, and 1225; Table 2.3). Although protein levels varied, Hcp was detected in the whole cell samples of all strains (Figure 2.2). Interestingly, supernatants showed a greater variation; Hcp secretion was more pronounced in strains SDF, 19606, and 1224, compared

to 17978. Strains AYE and 1375 did not show detectable levels of secreted Hcp under the conditions tested. Strain 1225 showed minimal Hcp secretion; however, all supernatant samples prepared from this strain had detectable levels of the cytoplasmic control protein RNA polymerase α-subunit, indicating lysis may account for the small amount of Hcp protein detected. In agreement with available genomic sequence data, our results indicate that the T6SS, and Hcp expression, is conserved among *A. baumannii* strains; however, the secretion of Hcp protein varied among isolates. Furthermore, our results agree with a recent report in which Hcp was found in culture supernatants from strain 19606 (Henry *et al.*, 2012).

The non-*baumannii* strains investigated were clinical isolates of *A. calcoaceticus* (strains A and B), *A. pittii* (strains A and B), and *A. junii*. The sequenced strain of the non-pathogenic soil isolate *A. baylyi* ADP1 (Juni, 1972; Vaneechoutte *et al.*, 2006) was also included. Both *A. calcoaceticus* strains and *A. baylyi* ADP1 showed robust Hcp expression and secretion (Figure 2.2), correlating with the presence of predicted T6SS genes in their respective genomes. The *A. pittii* and *A. junii* strains, which are not predicted to encode T6SSs (Table 2.3) and do not contain a Hcp homolog, did not react against the anti-Hcp antibody. Thus, while the T6SS is not universally conserved among *Acinetobacter* species, all tested strains with a predicted T6SS express and/or secrete Hcp.

To help visualize the differences in Hcp secretion we developed an ELISA assay to detect Hcp in supernatants. The T6SS-positive strains identified in Figure 2.2 were cultured in 96-well plates and supernatants were collected. These supernatants were incubated in 96-well ELISA plate overnight, and the secreted Hcp was detected using an anti-Hcp antibody by an indirect ELISA approach. The results of a typical assay are shown in Figure 2.3. Due to differences in growth of different strains observed in this assay, it is not possible to directly compare secretion rates.

However, this assay clearly separates the strains into “high secretors” and “low secretors” (Figure 2.2C and Figure 2.4A). The high secretor strains generally reach a lower final optical density (Figure 2.4B) and therefore the high levels of Hcp in supernatants can be attributed to higher rates of Hcp secretion and not a larger number of cells. The results from this ELISA are in agreement with the data obtained via Western blots.



C

	17978	19606	SDF	AYE	1375	1224	1225	*A. calcoaceticus* A	*A. calcoaceticus* B	*A. baylyi* ADP1
Growth	++	+	+	++	+	++	++	+	+	+
Hcp secretion	↓	↑	↑	↓	↓	↑	↓	↑	↑	↑

Figure 2.2 The T6SS is active in several species of *Acinetobacter*.

A) Whole cell and supernatant samples prepared from cultures of several A. *baumannii* strains were probed with anti-Hcp (top panels) and the lysis control anti-RNA polymerase (RNAP; bottom panels). B) Whole cell and supernatant samples prepared from cultures of different species within the genus *Acinetobacter* probed as described above. C) Summary of growth and Hcp secretion characteristics, determined by Western blot and ELISA, of all T6SS-positive strains analyzed in this study. “Fast” growing strains (++) and “slow” growing strains (+) were defined as those which reached a high or low optical density, respectively, and set arbitrarily by the indicated line in Figure S2. Hcp secretion is summarized as high (↑) or low (↓) based on Western blots and ELISA.

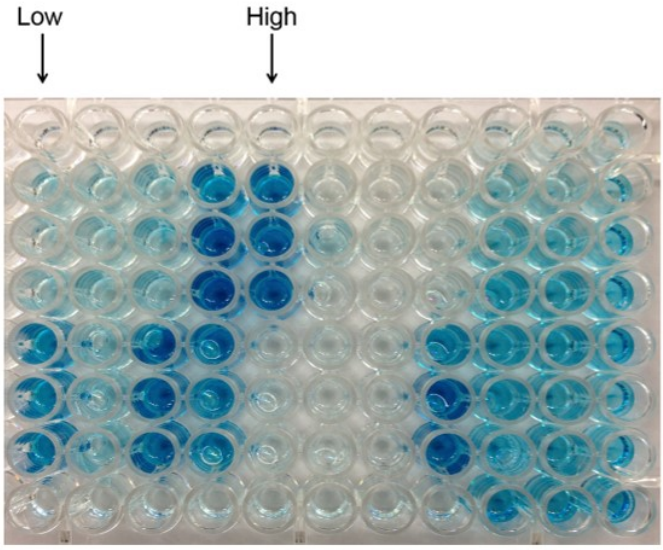


Figure 2.3 Visual results of a typical Hcp secretion ELISA assay with several different strains of *Acinetobacter*

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An example of the distinction between strains that are “high” or “low” Hcp secretors are indicated by arrows.

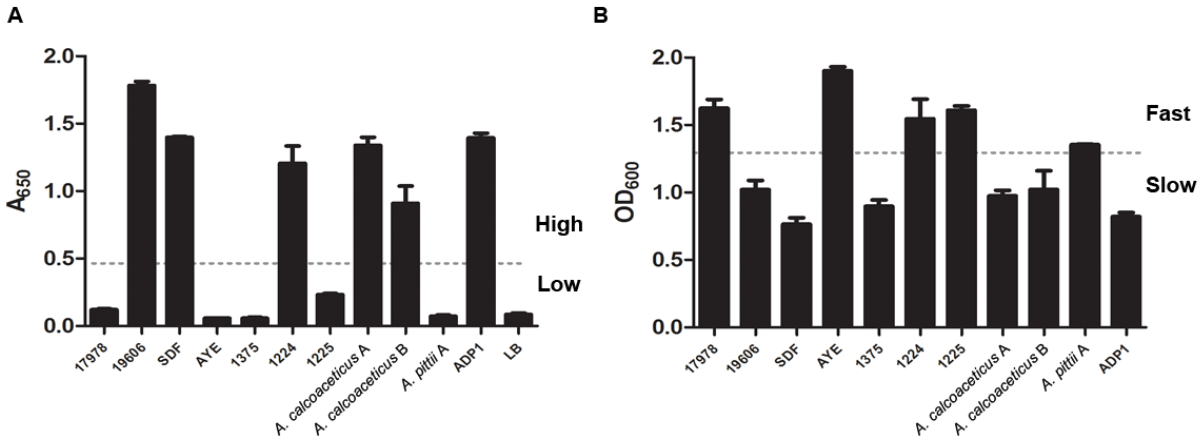

Figure 2.4 Quantification of ELISA results.

A) Absorbance at 650 nm following ELISA assay for Hcp secretion. The grey broken line indicates an arbitrary cut-off between “high” and “low” secreting strains. B) Opitcal density at 600 nm of strains shown in part A prior to isolation of supernatants and ELISA assay. The grey broken line indicates an arbitrary cut-off between “fast” and “slow” growing strains. *A. pittii* A, a strain which does not encode *hcp*, was used as a control.

2.3.2 *A. baumannii* ATCC 17978 secretes Hcp in a T6SS-dependent manner

While Hcp was detected in the supernatants of several species of *Acinetobacter,* we wanted to determine whether this was a process dependent on other genes within the cluster. Due to the importance of *A. baumannii* as a nosocomial pathogen, and because our lab has previously employed 17978 in molecular studies of pathogenesis (Iwashkiw *et al.*, 2012b), we chose to use this strain as our model organism. We generated a *hcp* mutant (17978 Δ*hcp*) by allelic exchange with a gentamicin resistance cassette and probed whole cells and culture supernatants with the anti-Hcp antibody. Hcp was detected by Western blot in the whole cell extract and cell-free supernatant of the wild type strain (Figure 2.5A), but the band corresponding to Hcp was absent from pellet and supernatant fractions from the 17978 Δ*hcp* strain. Constitutive expression of Hcp from a plasmid restored Hcp expression and secretion in the mutant strain. As before, cytoplasmic RNA polymerase was used as a lysis and loading control, and was only seen in whole cell fractions, indicating that the presence of Hcp in culture supernatants was not due to cell lysis, and instead is actively exported by the bacterium.

To determine if Hcp secretion by 17978 is dependent on a functional T6SS, we generated an unmarked *tssM* deletion strain (17978 Δ*tssM*). TssM, a structural component of the T6SS, has been shown to be required for T6SS activity, and is therefore required for Hcp secretion (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). In agreement with these previous results, whole cell samples from the 17978 Δ*tssM* strain contained Hcp, but its secretion was completely abrogated (Figure 2.5B). Expression of TssM from a plasmid complemented secretion of Hcp to the supernatant, indicating the lack of Hcp secretion was due to mutation of *tssM*. Taken together, these results suggest that the T6SS of 17978 is functional, and that its ability to secrete the conserved component Hcp is dependent upon at least one other gene in the cluster.

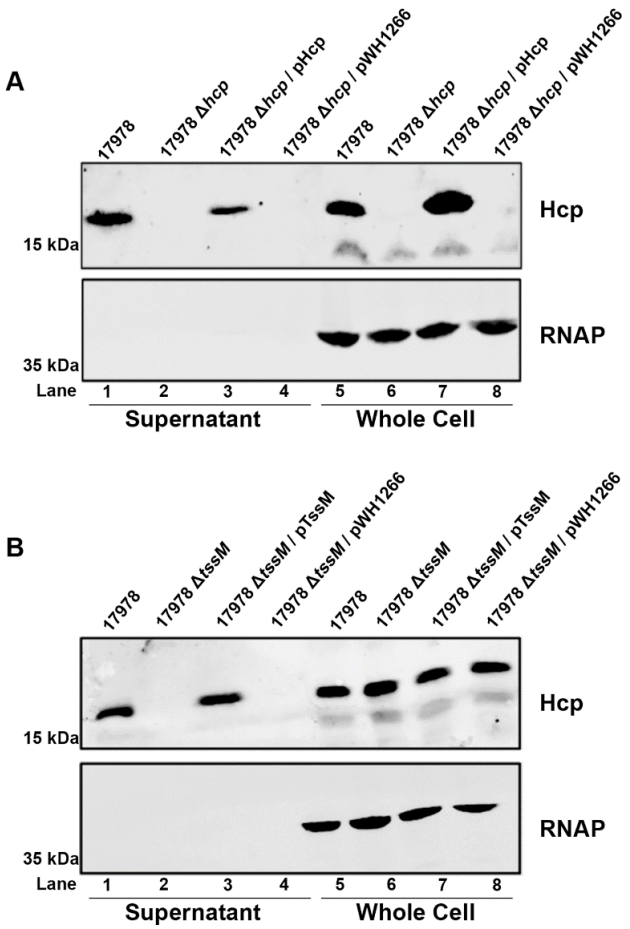

Figure 2.5 *A. baumannii* ATCC 17978 requires the conserved TssM protein for T6SS activity.

A) Whole cell and supernatant samples prepared from cultures of wild type 17978, the T6SS mutant 17978 Δ*hcp,* and its complemented (pHcp) or vector control (pWH1266) derivatives, were separated by SDS-PAGE and probed by Western blot with anti-Hcp (upper panel) or an anti-RNA polymerase (RNAP; lower panel) antibodies. B) Western blot of whole cell and supernatant samples prepared from cultures of wild type 17978, the T6SS mutant 17978 Δ*tssM,* and its complemented (pTssM) or vector control (pWH1266) derivatives probed for Hcp (upper panel) and RNAP (lower panel).

2.3.3 The *tssM* mutant of *A. baumannii* ATCC 17978 is not attenuated for virulence against amoebae, *Galleria mellonella*, or mice

D. discoideum amoebae have been widely used as a host model to study bacterial virulence factors (Steinert *et al.*, 2005), and was used as a model system for assessing T6SS-mediated

virulence in *V. cholerae* (Pukatzki *et al.*, 2006). An active T6SS of *B. cenocepacia* has also been found to be important for mediating resistance to *D. discoideum* and for macrophage actin rearrangements (Aubert *et al.*, 2008). When mixed and plated on agar containing ethanol, *A. baumannii* ATCC 17978 has been shown to kill *D. discoideum* and prevent plaque formation, the indicator of amoeboid feeding on the bacteria (Smith *et al.*, 2007), and has been used to identify *A. baumannii* virulence factors (Iwashkiw *et al.*, 2012b). When we co-plated *D. discoideum* with 17978 or 17978 Δ*tssM* on SM/5 agar, no plaques were observed in the bacterial lawns, indicating the T6SS mutant retained a virulent phenotype towards the amoebae (data not shown).

G. mellonella have also been used as non-mammalian eukaryotic models for assessing virulence defects of *A. baumannii* (Gaddy *et al.*, 2012; Iwashkiw *et al.*, 2012b; Peleg *et al.*, 2009). Injection of *A. baumannii* bacteria into the insect results in a dose-dependent killing, with the inoculum required for efficient killing varying between *A. baumannii* strains and species. We injected *G. mellonella* wax moth larvae with approximately 10^6 and 10^7 CFUs of wild type 17978 and 17978 Δ*tssM*, respectively (Figure 2.6A). As previously reported, we observed a dose-dependent killing of *G. mellonella* by *A. baumannii,* however the *tssM* mutant retained virulence levels comparable to wild type bacteria.

We next assessed whether the T6SS of 17978 played a role in a murine pneumonia model. This model has proven useful to discriminate between wild type and attenuated *A. baumannii* strains (Hood *et al.*, 2012a; Hood *et al.*, 2012b; Jacobs *et al.*, 2010). Mice were intranasally infected with wild type 17978 or Δ*tssM* bacteria. After 36 hours, the bacterial burden in the lungs and liver of infected animals was quantified, which revealed no significant difference in pathogenicity between the two strains (Figure 2.6B). Taken together, these results suggest that the T6SS does not play a role in virulence against eukaryotic systems by *A. baumannii* ATCC 17978.

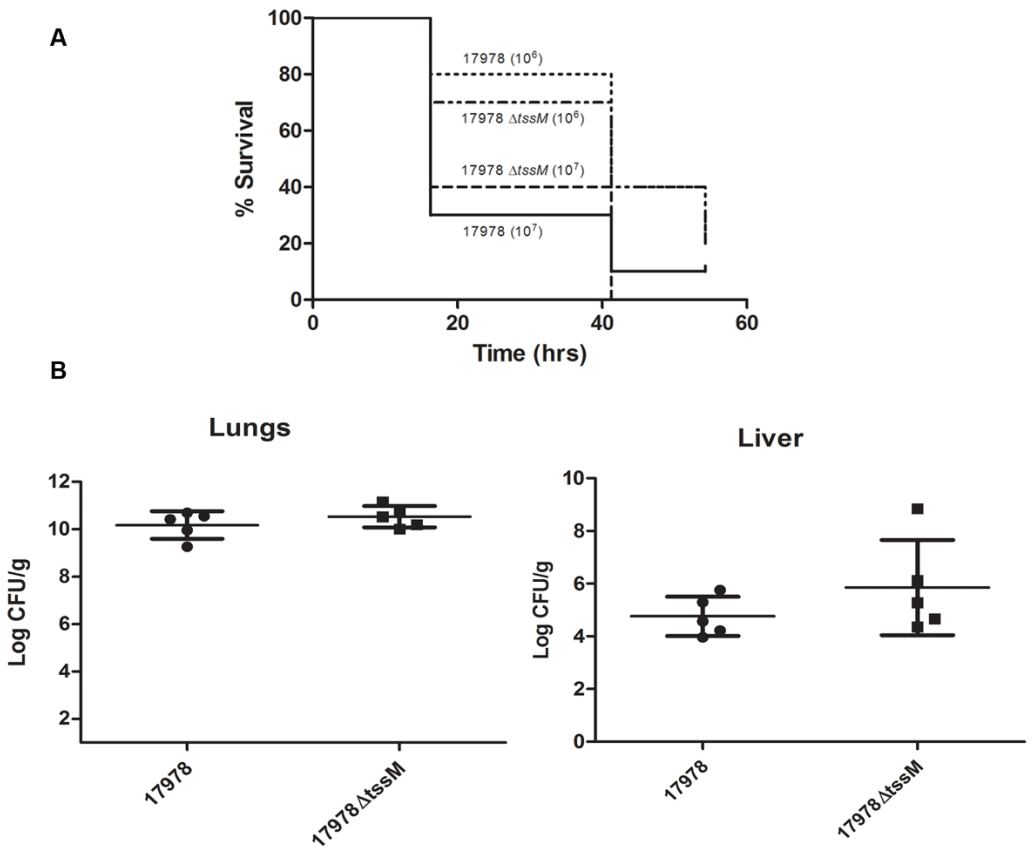

Figure 2.6 The T6SS is not required for virulence towards *G. mellonella* or in a mouse model of pneumonia.

A) Groups of 10 larvae were injected with approximately 10^6 or 10^7 CFU of wild type 17978 or the *tssM* mutant, incubated at 37°C, and monitored for survival. No significant difference ($p>0.05$) in survival was observed (log-rank test). B) Bacterial burden of lung and liver tissue from mice infected intranasally with either wild type 17978 or Δ*tssM* 36h post infection. No significant difference ($p>0.05$; two-tailed, unpaired Student's *t* test) in bacterial burden of the two strains was observed in either tissue.

2.3.4 *A. baumannii* ATCC 17978 appears not to kill other bacteria via the T6SS, nor employs this system for biofilm formation

Recently, the T6SS of several bacterial pathogens has been shown to mediate killing of other bacteria (Hood *et al.*, 2010; MacIntyre *et al.*, 2010; Russell *et al.*, 2011; Schwarz *et al.*, 2010). To determine if *A. baumannii* also exhibits T6SS-mediated antibacterial activity, we

initially used a rifampicin resistant derivative of *E. coli* strain MG1655, a strain susceptible to killing by *V. cholerae* (MacIntyre *et al.*, 2010), as a target in bacterial killing assays. Co-incubation of wild type 17978 or 17978 Δ*tssM* with *E. coli* MG1655 showed no differences in killing of *E. coli*, while a drastic reduction in viable *E. coli* was seen when confronted with *V. cholerae* (Figure 2.7). Of note, 17978 seemed to slightly reduce *E. coli* growth in a T6SS-independent fashion as compared to the avirulent *V. cholerae* strain.

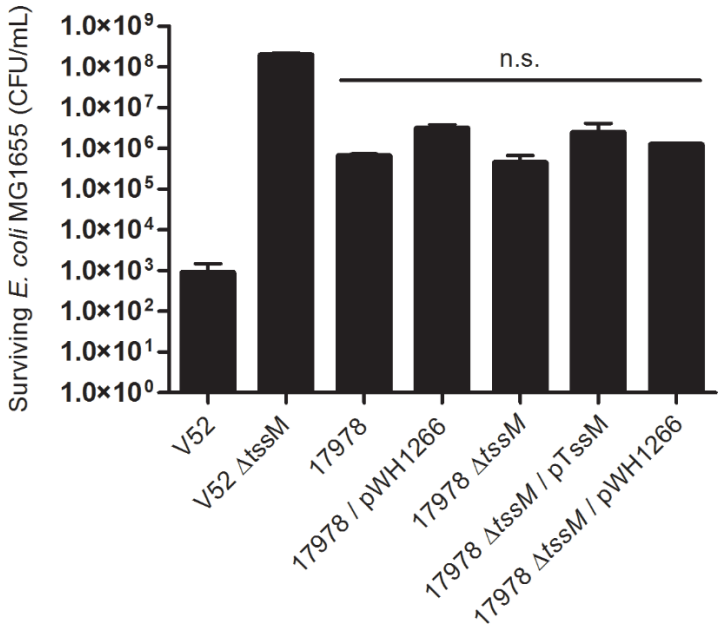

Figure 2.7 The T6SS of 17978 is not used for killing of *E. coli* MG1655.

Survival of *E. coli* was determined by plate counts after exposure to wild type17978, 17978 with vector control (17978/pWH1266), the 17978 Δ*tssM* T6SS mutant, and its complemented (pTssM) and vector control (pWH1266) derivatives. Wild type *V. cholerae* (V52), and the isogenic *tssM* mutant derivative (V52 Δ*tssM*), were used as positive and negative controls for bacterial killing, respectively. The data presented correspond to three independent experiments and are plotted as means ± SD. Comparison of the 17978 strains shows no significant differences in killing (n.s.; $p>0.05$; Tukey's multiple comparison post-test).

The conditions employed in this assay were optimized for *V. cholerae*. It is possible that *A. baumannii* is prey-specific, or that different experimental conditions are needed for bacterial killing. We therefore tested different incubation times (4 hours or 20 hours), agar concentrations (0.5 and 1.5 %), and other bacteria for the killing assays. The alternative prey tested were another *A. baumannii* strain (*A. baumannii* ATCC 19606), and two non-*baumannii Acinetobacter* species, one containing a T6SS (*A. baylyi* ADP1) and the other lacking a T6SS in its genome (*A. nosocomialis* 1221). There were no significant differences in the survival of any of these preys in all the conditions tested (data not shown). These results suggest that *A. baumannii* ATCC 17978 may be highly specific for its target, or that it may require particular conditions to kill other bacteria. Alternatively, *A. baumannii* may use the T6SS for a different function. It has been shown that mutation of the T6SS of enteroaggregative *E. coli* (EAEC) results in diminished biofilm formation (Aschtgen *et al.*, 2008), and the ability of *A. baumannii* to form biofilms may contribute to its pathogenicity and long term survival in hospital environments. Using a continuous flow-cell system, we determined that the biofilms formed by 17978 Δ*tssM* were indistinguishable from wild type 17978 (Figure 2.8), suggesting the T6SS does not play a role in biofilm formation. Similar to our results, *B. thailandensis* does not require its T6SS-1 for biofilm formation (Schwarz *et al.*, 2010).

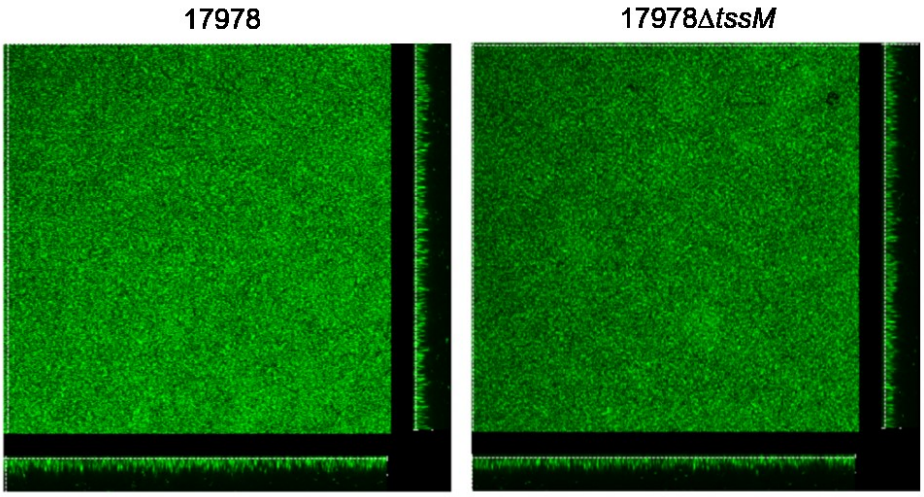

Figure 2.8 Biofilm formation is not affected by loss of *tssM.*

Confocal laser scanning microscopy images of wild type and *tssM* mutant in a flow cell biofilm assay.

2.4 Discussion

Inspection of the genome of several sequenced species of *Acinetobacter* revealed 12 genes conserved in all T6SSs, including the previously identified "hallmarks" of T6SSs (Boyer *et al.*, 2009). Notably, the T6SS cluster of all *Acinetobacter* species lacked obvious homologs to *tssJ*, an outer membrane lipoprotein shown to be essential for Hcp secretion by the EAEC T6SS. However, this lipoprotein is also absent from the *Rhizobium leguminosarum* T6SS, which has demonstrated T6SS activity (Bladergroen *et al.*, 2003), and suggests that the T6SS can still function in the absence of *tssJ*. The organization of the T6SS was identical among all *A. baumannii* genomes analyzed (Fig. 1), with nearly 99% nucleotide sequence identity between strains (data not shown), suggesting that this secretion system is conserved. Our analysis also uncovered the genetic components of T6SSs in several other species of *Acinetobacter*, including *A. calcoaceticus*, *A. oleivorans*, *A. baylyi*, *A. johnsonii*, *A. radioresistens*, and *A. lwoffii*. Additionally, genomic analysis of sequenced *A. pittii, A. junii*, *A. nosocmialis*, and *A.haemolyticus* strains indicate that they lack

homologs to conserved T6SS components, including Hcp (Table 2.3). Interestingly, all genome sequences we analyzed for this study showed the presence of VgrG-like proteins, even those strains not predicted to encode a T6SS. Moreover, *A. pittii* and *A. nosocomialis* seem to have homologs of the T6SS component TssL, and *A. haemolyticus* possesses a homolog of TssM. This may indicate that functionality of the T6SS, as evidenced by a lack of core components, may have been lost in these strains, while the VgrG's, which are located outside the T6SS cluster in *Acinetobacter* species, and TssL or TssM have been retained for an as yet unknown reason.

Through immunoblotting and mutational analysis we showed that Hcp is secreted by 17978, and that the TssM protein is necessary for Hcp secretion. Previous work (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006), has established that TssM is an essential structural component of the secretory apparatus. Similarly, our results showed that TssM is also essential for T6SS activity in *A. baumannii*. While the remaining genes of the cluster remain to be functionally characterized, our results demonstrate that 17978 encodes a *bona-fide* T6SS.

We analyzed Hcp expression and secretion in several *A. baumannii* strains, both sequenced (17978, SDF, AYE, 19606) and unsequenced (1375, 1224, 1225), as well as other species within the genus *Acinetobacter*. We developed an ELISA-based method to detect Hcp in the culture supernatants. With this method, together with Western blot analysis, we observed wide-variation in the actual secretion of Hcp to culture supernatants, with some isolates showing robust Hcp secretion (SDF, 19606, 1224. *A. calcoaceticus* A/B), and others with little (17978) or no (AYE, 1225) secretion. The ELISA method described in this work could be employed in the future for screening of T6SS inhibitors or to identify mutations affecting T6SS functionality. Clinical strains of *P. aeruginosa* have also been shown to display differences in their secretory profiles of Hcp (Mougous *et al.*, 2006; Mougous *et al.*, 2007). In these cases, expression of the T6SS-activating

or T6SS-repressing PpkA or PppA regulatory proteins could induce secretion in non-secreting isolates or repress secretion in Hcp secreting isolates, respectively, indicating that some clinical isolates may undergo mutations in their regulatory components (Mougous *et al.*, 2007). Of note, we were unable to identify homologs of the *P. aeruginosa ppkA*/*pppA* post-translational regulatory system in *A. baumannii,* indicating that a different regulatory mechanism is likely involved. Indeed, other regulatory mechanisms have been described in other bacteria (Silverman *et al.*, 2012). Although the elements which regulate T6SS in *A. baumannii* are not known, in a recent study the transcriptional profile of a LPS-deficient *A. baumannii* ATCC 19606 strain was analyzed (Henry *et al.*, 2012). It was shown that this strain upregulated expression of genes involved in cell-envelope and membrane biogenesis. Interestingly, the authors found that several genes encoding the T6SS locus analyzed in this study were down-regulated, which correlated with a loss of Hcp in culture supernatants. This suggests that the T6SS may be turned off under stress conditions. It is tempting to speculate that the strains that do not secrete Hcp constitutively may sense environmental signals and activate their T6SS.

Several T6SSs have been shown to facilitate killing of competing bacterial species (Hood *et al.*, 2010; MacIntyre *et al.*, 2010; Murdoch *et al.*, 2011; Schwarz *et al.*, 2010). In the case of *P. aeruginosa*, this is mediated by T6SS-directed intoxication of other bacteria with protein effectors as part of a toxin-antitoxin system (Hood *et al.*, 2010; Russell *et al.*, 2011). We determined that 17978 is unable to utilize its T6SS for antibacterial activity against *E. coli* MG1655, a strain previously shown to be susceptible to the *V. cholerae* T6SS (MacIntyre *et al.*, 2010). 17978 Δ*tssM* showed no difference in ability to affect *E. coli* survival compared to wild type. However, compared to the negative control *V. cholerae* Δ*tssM*, *E. coli* survival was decreased more than 100-fold when co-incubated with the *A. baumannii* strains, suggesting inhibition of *E. coli* growth

through an unknown, T6SS-independent mechanism. This may be the result of an unidentified inhibitory factor produced by *A. baumannii*, or alternatively, a consequence of competition for nutrients. We tested other conditions and additional bacterial prey, obtaining the same results. It is possible that the *A. baumannii* ATCC 17978 T6SS is prey-selective or requires specific growth conditions that we were unable to determine. Alternatively, *A. baumannii* ATCC 17978 may not use its T6SS against other bacteria.

In an attempt to determine the biological function of the T6SS in *A. baumannii*, we tested 17978, and its isogenic *tssM* mutant derivative, in non-mammalian infection models. *D. discoideum* are unicellular amoebae which feed on bacteria through phagocytic mechanisms analogous to macrophages (Rupper *et al.*, 2001), and have become a widely used host model for studying bacterial pathogenesis (Steinert *et al.*, 2005). Recently, *A. baumannii* was shown to be virulent towards amoebae, but required the presence of ethanol-stimulated virulence genes to kill *D. discoideum* (Smith *et al.*, 2007). *A. baumannnii* ATCC 17978 and 17978 Δ*tssM* were equally virulent towards *D. discoideum*, indicating that the T6SS of this strain does not play a role in ethanol-stimulated virulence. A previous study identified several genes up-regulated by the presence of ethanol in *A. baumannii*; however none of the genes presumed to be involved in the T6SS, including *vgrGs*, were significantly affected (Camarena *et al.*, 2010). We also tested the wild type and *tssM* mutant in the *G. mellonella* insect infection model, which has previously been used to assess the pathogenesis of *Acinetobacter* (Gaddy *et al.*, 2012; Iwashkiw *et al.*, 2012b; Peleg *et al.*, 2009), and provides an alternative to the challenges associated with mammalian models. In this assay, the killing of *G. mellonella* larvae is dose-dependent (Peleg *et al.*, 2009). We observed no statistically different survival of the insects by the two strains at either inoculum. Our results suggest that the T6SS of 17978 does not contribute to pathogenicity in these two non-mammalian

models of infection. We then utilized an established mouse model of pneumonia to assess any potential role of the T6SS in mammalian infection. The bacterial burden in the lungs and liver was similar between wild type-infected and mutant-infected mice, indicating similar infectivity between the two bacterial strains in this model.

It should be noted that the T6SS is not exclusively harboured by pathogenic *Acinetobacter* species. *A. calcoaceticus* and *A. baylyi*, which we have experimentally demonstrated to have active T6SSs, are rarely implicated in serious human disease (Peleg *et al.*, 2008a), and the specific strain of *A. baylyi* used in this study, ADP1, was derived from a soil isolate (Juni, 1972; Vaneechoutte *et al.*, 2006). Two of the most clinically relevant species of *Acinetobacter*, *A. pittii* and *A. nosocomialis* (formerly *Acinetobacter* genomosp. 3 and *Acinetobacter* genomosp. 13TU, respectively (Nemec *et al.*, 2011)), appear not to have functional T6SSs (Table S1 and Fig 2B). Also, as shown, the T6SS does not play a role in biofilm formation for *A. baumannii* ATCC 17978. Taken together, our results suggest that presence of a T6SS does not correlate with virulence in the genus *Acinetobacter*, at least in the models analyzed. The finding that several non-pathogenic, environmental *Acinetobacter* species possess T6SSs may indicate another function. Most of the proposed roles for T6SS systems of other bacteria do not seem to be applicable to *A. baumannii*. Considering the plasticity of the *A. baumannii* genome (Sahl *et al.*, 2011; Snitkin *et al.*, 2011), it is unlikely that the system has been functionally conserved in so many strains for no reason, and therefore we believe it likely provides some advantage to the bacterium. Future work in our laboratory will attempt to define the role T6SS plays in the *Acinetobacter* genus.

2.5 References

Aschtgen, M. S., Bernard, C. S., De Bentzmann, S., Lloubes, R., & Cascales, E. (2008). SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *Journal of bacteriology, 190*(22), 7523-7531.

Aubert, D. F., Flannagan, R. S., & Valvano, M. A. (2008). A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. *Infection and immunity, 76*(5), 1979-1991.

Ballister, E. R., Lai, A. H., Zuckermann, R. N., Cheng, Y., & Mougous, J. D. (2008). In vitro self-assembly of tailorable nanotubes from a simple protein building block. *Proceedings of the National Academy of Sciences of the United States of America, 105*(10), 3733-3738.

Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012b). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186.

Berlau, J., Aucken, H. M., Houang, E., & Pitt, T. L. (1999). Isolation of *Acinetobacter* spp. including A. *baumannii* from vegetables: implications for hospital-acquired infections. *The Journal of hospital infection, 42*(3), 201-204.

Bingle, L. E., Bailey, C. M., & Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Current opinion in microbiology, 11*(1), 3-8.

Bladergroen, M. R., Badelt, K., & Spaink, H. P. (2003). Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Molecular plant-microbe interactions : MPMI, 16*(1), 53-64.

Bonemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., & Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *The EMBO journal, 28*(4), 315-325.

Bouvet, P. J. M. G., P. A. D. . (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter* haemolyticus sp. nov., *Acinetobacter* johnsonii sp. nov., and *Acinetobacter* junii sp. nov. and emended descriptions of *Acinetobacter* calcoaceticus and *Acinetobacter* lwoffii. *Int J Syst Bacteriol*(36), 228-240.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC genomics, 10*, 104.

Bryksin, A. V., & Matsumura, I. (2010). Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. *PloS one, 5*(10), e13244.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., & Snyder, M. (2010). Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS pathogens, 6*(4), e1000834.

Carr, E. L., Kampfer, P., Patel, B. K., Gurtler, V., & Seviour, R. J. (2003). Seven novel species of *Acinetobacter* isolated from activated sludge. *International journal of systematic and evolutionary microbiology, 53*(Pt 4), 953-963.

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., . . . Lee, J. C. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cellular microbiology, 7*(8), 1127-1138.

Choi, C. H., Lee, J. S., Lee, Y. C., Park, T. I., & Lee, J. C. (2008). *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC microbiology, 8*, 216.

Chow, J., & Mazmanian, S. K. (2010). A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell host & microbe, 7*(4), 265-276.

de Bruin, O. M., Duplantis, B. N., Ludu, J. S., Hare, R. F., Nix, E. B., Schmerk, C. L., . . . Nano, F. E. (2011). The biochemical properties of the Francisella pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in type VI secretion. *Microbiology, 157*(Pt 12), 3483-3491.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature reviews. Microbiology, 5*(12), 939-951.

Dykxhoorn, D. M., St Pierre, R., & Linn, T. (1996). A set of compatible tac promoter expression vectors. *Gene, 177*(1-2), 133-136.

Filloux, A., Hachani, A., & Bleves, S. (2008). The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology, 154*(Pt 6), 1570-1583.

French, C. T., Toesca, I. J., Wu, T. H., Teslaa, T., Beaty, S. M., Wong, W., . . . Miller, J. F. (2011). Dissection of the *Burkholderia* intracellular life cycle using a photothermal nanoblade. *Proceedings of the National Academy of Sciences of the United States of America, 108*(29), 12095-12100.

Gaddy, J. A., Arivett, B. A., McConnell, M. J., Lopez-Rojas, R., Pachon, J., & Actis, L. A. (2012). Role of acinetobactin-mediated iron acquisition functions in the interaction of *Acinetobacter baumannii* strain ATCC 19606T with human lung epithelial cells, Galleria mellonella caterpillars, and mice. *Infection and immunity, 80*(3), 1015-1024.

Gerlach, R. G., & Hensel, M. (2007). Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. *International journal of medical microbiology : IJMM, 297*(6), 401-415.

Hayes, C. S., Aoki, S. K., & Low, D. A. (2010). Bacterial contact-dependent delivery systems. *Annual review of genetics, 44*, 71-90.

Henry, R., Vithanage, N., Harrison, P., Seemann, T., Coutts, S., Moffatt, J. H., . . . Boyce, J. D. (2012). Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglucosamine. *Antimicrobial agents and chemotherapy, 56*(1), 59-69.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., & Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene, 212*(1), 77-86.

Hood, M. I., Becker, K., Roux, C. M., Dunman, P. M., & Skaar, E. P. (2012a). Genetic determinants of intrinsic colistin tolerance in *Acinetobacter baumannii*. *Infection and immunity*.

Hood, M. I., Mortensen, B. L., Moore, J. L., Zhang, Y., Kehl-Fie, T. E., Sugitani, N., . . . Skaar, E. P. (2012b). Identification of an *Acinetobacter baumannii* Zinc Acquisition System that Facilitates Resistance to Calprotectin-mediated Zinc Sequestration. *PLoS pathogens, 8*(12), e1003068.

Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., . . . Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell host & microbe, 7*(1), 25-37.

Houang, E. T., Chu, Y. W., Leung, C. M., Chu, K. Y., Berlau, J., Ng, K. C., & Cheng, A. F. (2001). Epidemiology and infection control implications of *Acinetobacter* spp. in Hong Kong. *Journal of clinical microbiology, 39*(1), 228-234.

Hunger, M., Schmucker, R., Kishan, V., & Hillen, W. (1990). Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter* calcoaceticus and its use for *Escherichia coli* shuttle plasmids. *Gene, 87*(1), 45-51.

Iwashkiw, J. A., Fentabil, M. A., Faridmoayer, A., Mills, D. C., Peppler, M., Czibener, C., . . . Feldman, M. F. (2012a). Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. *Microbial cell factories, 11*, 13.

Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., . . . Feldman, M. F. (2012b). Identification of a general O-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS pathogens, 8*(6), e1002758.

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., . . . Dunman, P. M. (2010). Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infection and immunity, 78*(5), 1952-1962.

Jawad, A., Seifert, H., Snelling, A. M., Heritage, J., & Hawkey, P. M. (1998). Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates. *Journal of clinical microbiology, 36*(7), 1938-1941.

Jin, J. S., Kwon, S. O., Moon, D. C., Gurung, M., Lee, J. H., Kim, S. I., & Lee, J. C. (2011). *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. *PloS one, 6*(2), e17027.

Juni, E. (1972). Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *Journal of bacteriology, 112*(2), 917-931.

Lee, J. S., Choi, C. H., Kim, J. W., & Lee, J. C. (2010). *Acinetobacter baumannii* outer membrane protein A induces dendritic cell death through mitochondrial targeting. *Journal of microbiology, 48*(3), 387-392.

Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., . . . Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4154-4159.

Ma, A. T., McAuley, S., Pukatzki, S., & Mekalanos, J. J. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell host & microbe, 5*(3), 234-243.

Ma, A. T., & Mekalanos, J. J. (2010). In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. *Proceedings of the National Academy of Sciences of the United States of America, 107*(9), 4365-4370.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America, 107*(45), 19520-19524.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M. C., Maida, I., . . . Fani, R. (2012). Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Research in microbiology, 163*(3), 161-172.

March, C., Regueiro, V., Llobet, E., Moranta, D., Morey, P., Garmendia, J., & Bengoechea, J. A. (2010). Dissection of host cell signal transduction during *Acinetobacter baumannii*-triggered inflammatory response. *PloS one, 5*(4), e10033.

Margetts, M. B., Barr, I. G., & Webb, E. A. (2000). Overexpression, purification, and refolding of a Porphyromonas gingivalis cysteine protease from *Escherichia coli*. *Protein expression and purification, 18*(3), 262-268.

McConnell, M. J., Actis, L., & Pachon, J. (2012). *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS microbiology reviews*.

Mortensen, B. L., & Skaar, E. P. (2012). Host-microbe interactions that shape the pathogenesis of *Acinetobacter baumannii* infection. *Cellular microbiology, 14*(9), 1336-1344.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., . . . Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science, 312*(5779), 1526-1530.

Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nature cell biology, 9*(7), 797-803.

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., & Coulthurst, S. J. (2011). The opportunistic pathogen Serratia marcescens utilizes type VI secretion to target bacterial competitors. *Journal of bacteriology, 193*(21), 6057-6069.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., . . . Dijkshoorn, L. (2011). Genotypic and phenotypic characterization of the *Acinetobacter* calcoaceticus-*Acinetobacter baumannii* complex with the proposal of *Acinetobacter* pittii sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter* nosocomialis sp. nov. (formerly *Acinetobacter* genomic species 13TU). *Research in microbiology, 162*(4), 393-404.

Peleg, A. Y., Jara, S., Monga, D., Eliopoulos, G. M., Moellering, R. C., Jr., & Mylonakis, E. (2009). Galleria mellonella as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrobial agents and chemotherapy, 53*(6), 2605-2609.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008a). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical microbiology reviews, 21*(3), 538-582.

Pell, L. G., Kanelis, V., Donaldson, L. W., Howell, P. L., & Davidson, A. R. (2009). The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4160-4165.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., & Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 51*(10), 3471-3484.

Piechaud, M., & Second, L. (1951). [Studies of 26 strains of Moraxella Iwoffi]. *Annales de l'Institut Pasteur, 80*(1), 97-99.

Pietrosiuk, A., Lenherr, E. D., Falk, S., Bonemann, G., Kopp, J., Zentgraf, H., . . . Mogk, A. (2011). Molecular basis for the unique role of the AAA+ chaperone ClpV in type VI protein secretion. *The Journal of biological chemistry, 286*(34), 30010-30021.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it

cross-links actin. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15508-15513.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.

Pukatzki, S., McAuley, S. B., & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Current opinion in microbiology, 12*(1), 11-17.

Rokhbakhsh-Zamin, F., Sachdev, D., Kazemi-Pour, N., Engineer, A., Pardesi, K. R., Zinjarde, S., . . . Chopade, B. A. (2011). Characterization of plant-growth-promoting traits of *Acinetobacter* species isolated from rhizosphere of Pennisetum glaucum. *Journal of microbiology and biotechnology, 21*(6), 556-566.

Rosales-Reyes, R., Skeldon, A. M., Aubert, D. F., & Valvano, M. A. (2012). The Type VI secretion system of *Burkholderia cenocepacia* affects multiple Rho family GTPases disrupting the actin cytoskeleton and the assembly of NADPH oxidase complex in macrophages. *Cellular microbiology, 14*(2), 255-273.

Rupper, A., & Cardelli, J. (2001). Regulation of phagocytosis and endo-phagosomal trafficking pathways in Dictyostelium discoideum. *Biochimica et biophysica acta, 1525*(3), 205-216.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature, 475*(7356), 343-347.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., . . . Campagnari, A. A. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and immunity, 78*(9), 3993-4000.

Sahl, J. W., Johnson, J. K., Harris, A. D., Phillippy, A. M., Hsiao, W. W., Thom, K. A., & Rasko, D. A. (2011). Genomic comparison of multi-drug resistant invasive and colonizing *Acinetobacter baumannii* isolated from diverse human body sites reveals genomic plasticity. *BMC genomics, 12*, 291.

Schell, M. A., Ulrich, R. L., Ribot, W. J., Brueggemann, E. E., Hines, H. B., Chen, D., . . . Deshazer, D. (2007). Type VI secretion is a major virulence determinant in *Burkholderia* mallei. *Molecular microbiology, 64*(6), 1466-1485.

Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., . . . Mougous, J. D. (2010). *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS pathogens, 6*(8), e1001068.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I., & Vaneechoutte, M. (1997). Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *Journal of clinical microbiology, 35*(11), 2819-2825.

Sexton, J. A., Miller, J. L., Yoneda, A., Kehl-Fie, T. E., & Vogel, J. P. (2004). Legionella pneumophila DotU and IcmF are required for stability of the Dot/Icm complex. *Infection and immunity, 72*(10), 5983-5992.

Shalom, G., Shaw, J. G., & Thomas, M. S. (2007). In vivo expression technology identifies a type VI secretion system locus in *Burkholderia* pseudomallei that is induced upon invasion of macrophages. *Microbiology, 153*(Pt 8), 2689-2699.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology, 66*, 453-472.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., & Snyder, M. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes & development, 21*(5), 601-614.

Snitkin, E. S., Zelazny, A. M., Montero, C. I., Stock, F., Mijares, L., Murray, P. R., & Segre, J. A. (2011). Genome-wide recombination drives diversification of epidemic strains of *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 108*(33), 13758-13763.

Steinert, M., & Heuner, K. (2005). Dictyostelium as host model for pathogenesis. *Cellular microbiology, 7*(3), 307-314.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473-3484.

Ugalde, J. E., Czibener, C., Feldman, M. F., & Ugalde, R. A. (2000). Identification and characterization of the Brucella abortus phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. *Infection and immunity, 68*(10), 5716-5723.

Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., . . . Cruveiller, S. (2008). Comparative analysis of *Acinetobacter*s: three genomes for three lifestyles. *PloS one, 3*(3), e1805.

Vaneechoutte, M., Young, D. M., Ornston, L. N., De Baere, T., Nemec, A., Van Der Reijden, T., . . . Dijkshoorn, L. (2006). Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. *Applied and environmental microbiology, 72*(1), 932-936.

Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Gonzalez, G., Garcia, A., & Zemelman, R. (1996). Biofilm formation by *Acinetobacter baumannii*. *Microbios, 86*(346), 49-58.

Wendt, C., Dietze, B., Dietz, E., & Ruden, H. (1997). Survival of *Acinetobacter baumannii* on dry surfaces. *Journal of clinical microbiology, 35*(6), 1394-1397.

Zheng, J., & Leung, K. Y. (2007). Dissection of a type VI secretion system in Edwardsiella tarda. *Molecular microbiology, 66*(5), 1192-1206.

Zimbler, D. L., Penwell, W. F., Gaddy, J. A., Menke, S. M., Tomaras, A. P., Connerly, P. L., & Actis, L. A. (2009). Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, 22*(1), 23-32.

3 A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*

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3.1 Introduction

Antibiotic-resistant bacteria that cause hospital-acquired infections are a mounting concern for health care systems globally (Boucher *et al.*, 2009). MDR *A. baumannii* is emerging as a frequent cause of difficult-to-treat nosocomial infections, and some isolates are resistant to all clinically relevant antibiotics (Sievert *et al.*, 2013; Valencia *et al.*, 2009). *A. baumannii* is often isolated from polymicrobial infections and therefore spends at least a part of its time competing with other bacteria (Maragakis *et al.*, 2008b). Antagonistic interactions between bacteria manifest in a variety of different ways (Hibbing *et al.*, 2010), and the type VI secretion system (T6SS) is a potent weapon used by many Gram-negative bacteria to kill competitors (Boyer *et al.*, 2009; Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). The multicomponent T6SS apparatus facilitates a dynamic contact-dependent injection of toxic effector proteins into prey cells, and expression of cognate immunity proteins prevents self-inflicted intoxication (Basler *et al.*, 2012b; Hood *et al.*, 2010; Russell *et al.*, 2011). The T6SS is composed of several conserved proteins involved in the formation of the secretory apparatus (Bingle *et al.*, 2008; Cascales, 2008). One of these components, hemolysin-coregulated protein (Hcp), forms hexameric tubule structures that are robustly secreted to the culture supernatants in bacteria with an active T6SS, allowing it to be used as a molecular marker for T6SS activity (Mougous *et al.*, 2006; Pukatzki *et al.*, 2009).

The T6SS is a dynamic apparatus (Basler *et al.*, 2012a). Its biogenesis follows energetically costly cycles of assembly/disassembly, and therefore, in most bacteria, T6SS appears to be exquisitely regulated. T6SS is silenced in most strains and only activated under specific conditions, such as an attack from another bacterium or in environments leading to membrane perturbations (Basler *et al.*, 2013; Bernard *et al.*, 2010b; Ho *et al.*, 2013; Silverman *et al.*, 2012). Many

Acinetobacter spp. encode the genes for a T6SS, including *A. noscomialis* and *A. baylyi*, which possess a constitutively active antibacterial T6SS (Carruthers *et al.*, 2013a; Carruthers *et al.*, 2013c; de Berardinis *et al.*, 2008a; Shneider *et al.*, 2013; Weber *et al.*, 2013). *A. baumannii* strains have been shown by us and others to secrete Hcp (Henry *et al.*, 2012; Weber *et al.*, 2013), but to our knowledge a T6SS-dependent phenotype has not been ascribed to this species. Furthermore, our previous results showed that Hcp secretion is highly variable between *A. baumannii* strains, with some isolates carrying an inactive system (Weber *et al.*, 2013). The precise regulatory mechanism(s) underlying T6SS suppression in some *A. baumannii* is unknown.

Here, we show that a large resistance plasmid of *A. baumannii* functions to repress the T6SS by encoding negative regulators of its activity. Analysis of colonies from a clinical isolate showed that the plasmid is readily lost in a subset of the population. This leads to the activation of the T6SS, which imparts the ability to kill other bacteria, with the simultaneous loss of antibiotic resistance. We propose that the differentiation into T6SS+ MDR– and T6SS– MDR+ phenotypes may constitute a novel survival strategy of this organism.

3.2 Experimental Procedures

3.2.1 Strains and Hcp secretion assays.

The bacterial strains used in this study are listed in Table 3.1. Strains were routinely grown in LB medium at 37 °C with shaking. The antibiotics ampicillin (50 µg/mL), kanamycin (50 µg/mL), tetracycline (5 µg/mL, rifampicin (100 µg/mL), gentamicin (15 µg/mL), and S/T (30/6 µg/mL) were added when necessary. Hcp-ELISAs were performed as previously described (Weber *et al.*, 2013). Briefly, individual colonies of a given *A. baumannii* strain struck out on an agar plate were used to inoculate 96-well plates filled with growth medium. After overnight growth, plates

were centrifuged and 75 µL of supernatants were transferred into high-binding ELISA 96-well plates containing 25 µL binding buffer and incubated at 4 °C overnight. The plates were then washed with PBS, blocked with a solution of 5% (wt/vol) skim milk, and then probed with 2.5% (wt/vol) skim milk–phosphate-buffered saline Tween-20 solution containing a 1:7500 dilution of an anti-Hcp antibody. Following washes, HRP-conjugated goat anti-rabbit (Bio-Rad) was added at a 1:5000 dilution to the plates. After another set of washes with PBST, 100 µL 3,3′,5,5′-Tetramethylbenzidine substrate (Cell Signaling Technologies) was added to the wells, and plates were allowed to develop for ∼5–10 min and then photographed. T6+ strains (Ab_{04}T6+, Ab_{17978}T6+, Ab_{1438}T6+) were isolated by Hcp-ELISA. When a well giving rise to a T6+ signal (i.e., blue color after ELISA) was identified, the original 96-well growth plate (the source of supernatant sample) was used to reisolate the cells. These cells were plated on LB agar, and subsequent rounds of Hcp ELISAs were performed using freshly isolated individual colonies to confirm the T6+ phenotype. Further, the final T6+ colonies were tested for antibiotic resistances; Ab_{04}T6+ and Ab_{1438}T6+ were frequently tested for gentamicin (15 µg/mL) and tetracycline (5 µg/mL) sensitivity, and Ab_{17978}T6+ was tested for sensitivity to the combination of S/T (30/6 µg/mL). Similarly, all T6– strains were tested for resistance to the above antibiotics. Western blots to confirm Hcp secretion phenotype were performed on whole cells and supernatants as described (21).

Table 3.1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or Reference
Strains		
A. baumannii Ab_{04}	clinical isolate	(Ahmed-Bentley *et al.*, 2013)
Ab_{04}T6-	Ab04 containing pAB04, antibiotic resistant, T6SS inactive	This study
Ab_{04}T6+	Ab04 lacking pAB04, antibiotic sensitive, T6SS active	This study
A. baumannii ATCC 17978	type strain; wild type	ATCC, (Piechaud *et al.*, 1951)
Ab_{17978}T6-	Ab17978 containing pAB3, sulfamethoxazole/trimethoprim resistant, T6SS inactive	This study

Ab_{17978}T6+	Ab17978 lacking pAB3, sulfamethoxazole/trimethoprim sensitive, T6SS active	This study
Ab_{17978}T6+Δ*hcp*	Ab17978 lacking pAB3, sulfamethoxazole/trimethoprim sensitive, T6SS active, *hcp* mutant	This study, (Weber *et al.*, 2013)
Ab_{17978}T6+Δ*tssM*	Ab17978 lacking pAB3, sulfamethoxazole/trimethoprim sensitive, T6SS active, *tssM* mutant	This study, (Weber *et al.*, 2013)
Ab_{17978}T6+ (Rif^R; pBAVMCS)	Spontaneous rifampicin resistant mutant of Ab17978T6+, harbours pBAVMCS for kanamycin resistance, used for conjugation study	This study
A. baumannii 1438	clinical isolate	This study
Ab_{1438}T6-	Ab1438 containing plasmid, antibiotic resistant, T6SS inactive	This study
Ab_{1438}T6+	Ab1438 lacking plasmid, antibiotic sensitive, T6SS active	This study
A. baumannii ATCC 19606	type strain; wild type	ATCC
A. baylyi ADP1	wild type	(Juni, 1972)
V. cholerae V52	$hlyA^-$, $hapA^-$, and $rtxA^-$	(MacIntyre *et al.*, 2010)
V. cholerae V52vasK	vasK minus derivative of V52	(MacIntyre *et al.*, 2010)
E. coli MG1655R	Rifampicin resistant K-12 strain, bacterial prey for Ab04T6- for surviving cell number enumeration	(MacIntyre *et al.*, 2010)
E. coli Ec01	Clinical isolate, from patient who was also infected with *A. baumannii* and *K. pneumoniae*	(Ahmed-Bentley *et al.*, 2013)
K. pneumoniae Kp01	Clinical isolate, from patient who was also infected with *A. baumannii* and *E. coli*	(Ahmed-Bentley *et al.*, 2013)
E. coli DH5α	general cloning and plasmid propagation	Invitrogen
E. coli TG1	killing assay prey (with pBAV1K-T5-gfp)	Stratagene
E. coli Top10	general cloning and plasmid propagation, killing assay prey (with pHC60)	Invitrogen
Plasmids		
pBAV1K-t5-gfp	GFP expressing plasmid, used for killing assays	(Bryksin *et al.*, 2010)
pEXT20	general cloning plasmid; Amp^R	(Dykxhoorn *et al.*, 1996)
pBAVMCS	pBAV1K-t5-gfp with GFP gene removed, *E. coli-Acinetobacter* shuttle plasmid; Kan^R	(Scott *et al.*, 2014b)
pBAVMCS-tet	pBAVMCS with tetracycline gene from pWH1266 inserted at SacI site	This study
pWH1266	Expression plasmid, *E. coli-Acinetobacter* shuttle plasmid; Tet^r	(Hunger *et al.*, 1990)
pTetR1-1	tetR1 in pEXT20	This study
pTetR1-2	tetR1 in pBAVMCS	This study
pTetR1-3	tetR1 in pWH1266	This study
pTetR2-1	tetR2 in pEXT20	This study
pTetR2-2	tetR2 in pBAVMCS	This study
pTetR2-3	tetR2 in pBAVMCS-tet	This study
pHNS-1	hns in pEXT20	This study
pHNS-2	hns in pWH1266	This study

3.2.2 Bacterial killing assays

Competition assays for Ab_{04}T6–/T6+ were performed using either *E. coli* MG1655R or *E. coli* pBAV1K-t5- gfp as prey. The former strain was used to produce the serial dilution image shown in Figure 3.1 and the latter for the GFP image. Assays were set up using overnight cultures that were normalized to an OD600 of 1 and done in triplicate. Strains grown with antibiotics were first washed before resuspension. *A. baumannii* and *E. coli* were mixed at a 1:1 ratio, and 5 µL

was spotted on a dry LB agar plate. After 4h, the spot was either cut out and resuspended in 1 mL liquid LB and 10 µL serial dilutions plated on rifampicin (100 µg·mL−1) to select for *E. coli* (when MG1655R was prey), or the plates were imaged directly on a FujiFilm FLA-5000 imaging system (Fuji Photo Film Co.) using the 473 nm SHG (secondharmonic generation) blue laser and LPB filter, with images acquired using a voltage between 300 and 400 (when *E. coli* containing pBAV1K-t5-gfp was prey). Representative images were used for figures. Controls consist of the *E. coli* prey incubated with a nonresistant or non–GFP-expressing *E. coli* parental strain. For killing assays using the clinical Ec01 and Kp01 strains as prey, a spontaneous rifampicin-resistant mutant of Ec01 was obtained by plating an overnight culture on rifampicin (100 µg/mL). Kp01 was naturally rifampicin resistant. The Ec01 parental strain and a rifampicin-sensitive laboratory strain of *K. pneumoniae* were used as controls, and the killing assay was performed as for *E. coli* MG1655R, with the exception that the killing assay with *K. pneumoniae* as prey was done at a predator-to-prey ratio of 10:1 to clearly illustrate the killing phenotype of Ab_{04}T6+. Subsequent killing assays shown in Figure 3.4, Figure 3.5 and Figure 3.13 and were performed using *E. coli* pBAV1K-t5-gfp as prey and done as described above, with representative images shown. The assays shown in Figure 3.6 and Figure 3.7 were performed by using inherent antibiotic resistance differences between strains

3.2.3 Disk Diffusion Assays

Antibiotic resistance profiles were determined by disk diffusion. Overnight cultures were resuspended to OD 1in fresh media, 300 µL was plated onto LB agar, and discs were overlaid. After overnight incubation, plate images were acquired using a Molecular Imager Gel Doc XR system (Bio-Rad), and the provided software was used to measure inhibition zone diameter. All experiments were done in triplicate, and data were analyzed by two-way ANOVA with Bonferroni

posttests. Antibiotics tested were amikacin (AK), ampicillin (AMP), aztreonam (ATM), chloramphenicol (C), ceftazidime (CAZ), ciprofloxacin (CIP), gentamicin (CN), erythromicin (E), imipenem (IMP), kanamycin (K), streptomycin (S), spectinomycin (SPT), S/T (SXT), tetracycline (TE), tobramycin (TOB), piperacillin/tazobactam (TZP), and trimethoprim (W).

3.2.4 Genome Sequencing

The DNA libraries for genomic DNA extracted from Ab_{04}T6–, Ab_{04}T6+, Ab_{17978}T6–, and Ab_{17978}T6+ (DNeasy Blood and Tissue Kit, Qiagen) were prepared following the Pacific Biosciences 20 kb Template Preparation Using BluePippin Size-Selection System protocol. We sheared 7.5 µg of high-molecular-weight genomic DNA (final volume of 100 µL) using the Covaris g-TUBES (Covaris Inc.) at 4,500 rpm (1,900 × g) for 60 s on each side on an Eppendorf centrifuge 5424 (Eppendorf). The sheared DNA was size selected on a BluePippin system (Sage Science Inc.) using a cutoff range of 7 kb to 50 kb. The DNA damage repair, end repair, and SMRTbell ligation steps were performed as described in the template preparation protocol with the SMRTbell Template Prep Kit 1.0 reagents (Pacific Biosciences). The sequencing primer was annealed at a final concentration of 0.8333 nM, and the P4 polymerase was bound at 0.500 nM. The libraries were sequenced on a PacBio RSII instrument at a loading concentration (on-plate) of 80 pM using the MagBead loading protocol, DNA sequencing kit 2.0, SMRT cells v3, and 3-h movies. Sequencing was performed at the McGill University and Genome Quebec Innovation Center. Illumina sequencing was carried out at the McMaster Genomics Facility, and DNA libraries were prepared using the Nextera XT kit (Illumina) as per the manufacturer's instructions and sequenced on an Illumina MiSeq with 2× 250 bp paired-end reads. Fold coverage ranged from ~150–205×, and initially assembly was carried out using SPAdes (Bankevich *et al.*, 2012).

3.2.5 Identification of Other Strains Harboring Plasmids

We screened our *Acinetobacter* strain library (~15 isolates) using primer pairs Node_182F/Node_182R (Table 3.2) to identify other strains carrying plasmids similar to pAB04-1/pAB3. These primers target a conserved region on the plasmid. We bioinformatically identified other strains by BLAST, using the full pAB04-1/pAB3 plasmids as the query against both the nucleotide collection (nr) database and the whole genome shotgun (wgs) database.

3.2.6 Recombinant Plasmid Construction

All primers are listed in Table 3.2. The hns gene was amplified from Ab_{04}T6– by PCR with primers H-nsFwdBamHI and H-nsRevSalI6His and then cloned into pEXT20 using BamHI and SalI cut sites to produce pHNS1. hns was then reisolated and amplified by PCR using primers H-nsFwdPstI and H-nsRevPstI6His and inserted into pWH1266 by PstI to make pHNS2. The genes encoding tetR1 and tetR2 were PCR amplified from Ab_{04}T6– by PCR with primers TetR18-FwdBamHI, TetR18- RevPstI, TetR22-FwdEcoRI, and TetR22-RevPstI and then cloned into pEXT20 by BamHI/PstI and EcoRI/PstI (respectively) to construct pTetR1-1 and pTetR2-1. pTetR1-2 was constructed by subcloning tetR1 from pTetR1-1 into pBAVMCS using BamHI/ PstI. pTetR1-3 was similarly constructed by isolation of tetR1 from pTetR1-1 with EcoRI/PstI and cloning into pWH1266. pTetR2-2 was constructed by PCR amplification of tetR2 with the pEXT20 promoter of pTetR2-1 using primers pEXTMCSFwdPstI and TetR22-RevPstI and cloning into the PstI site of pBAVMCS. The tetracycline resistance gene from pWH1266 was inserted into pTetR2-2 using SacI to construct pTetR2-3. Plasmids were sequenced and transformed into strains by electroporation, and gene expression was confirmed by Western blot. For testing the effect of recombinant protein expression on Hcp expression/secretion, Ab04T6+ was transformed with pHNS-2, pTetR1-3, and pTetR2-3 and their respective empty vectors. Ab1438T6+, Ab17978T6+, and *A. baumannii* ATCC 19606 were transformed with pTetR1-2 and

pTetR2-2 or empty pBAVMCS. *A. baylyi* ADP1 was transformed with pTetR1-3, pTetR2-2, or their respective empty vectors, whereas *V. cholerae* V52 was transformed with pTetR1-1, pTetR2-1, and pEXT20 empty vector.

Table 3.2 Primers used in this study

Primer name	Sequence (5'-3')
Node_182F	TTACAACAAGTATCTGTAGGTCCTGACC
Node_182R	AAGTGTCTGAATGTCTATCAATGCC
T6SSF	AATGTTGTACAGCAAGTTGATCC
T6SSR	AATTGCTTGTGAACTATCTTCTGG
H-nsFwdBamHI	AAAGGATCCTATACTCAGATTATTAGTGATCC
H-nsRevSalI6His	TATGTCGACTTAGTGGTGGTGGTGGTGGTGTTTAATCAGGAATTCACTCAGC
H-nsFwdPstI	AAACTGCAGTATACTCAGATTATTAGTGATCC
H-nsRevPstI6His	TATCTGCAGTTAGTGGTGGTGGTGGTGGTGTTTAATCAGGAATTCACTCAGC
pEXTMCSFwdPstI	ATATCTGCAGCAGGTCGTAAATCACTGCATAATTCG
TetR18-FwdBamHI	ATATGGATCCATGACTAAAGTTATTTCAAAAAGAAAAACC
TetR18-RevPstI	ATTACTGCAGTTAGTGGTGGTGGTGGTGGTGAGCCTCAAATGTCTGAATTAGACTC
TetR22-FwdEcoRI	AATAGAATTCATGACAAATAAAGCTTCACAGCCTAG
TetR22-RevPstI6His	ATAACTGCAGTCAGTGGTGGTGGTGGTGGTGAAATTGCTCACCATATAAACTTTCAATATC
rpoB-1441F	GAGCGTGCTGTTAAAGAGCG
rpoB-2095R	CTGCCTGACGTTGCATGT

3.2.7 Conjugation Experiments

For conjugation experiments, Ab_{17978}T6– was used as the donor and a modified strain of Ab_{17978}T6+ was used as the recipient. A spontaneous rifampicin-resistant mutant of Ab_{17978}T6+ was isolated, into which pBAVMCS (providing kanamycin resistance) was introduced by electroporation, generating Ab_{17978}T6+ (RifR; pBAVMCS). Overnight cultures of Ab_{17978}T6–, grown in S/T (to ensure plasmid maintenance), and Ab_{17978}T6+ (RifR; pBAVMCS), grown in rifampicin (100 μg/mL) and kanamycin, were pelleted, washed three times in fresh media, and resuspended at an OD600 of 1. The cultures were mixed at a 1:1 ratio, and 25 μL was spotted onto

an LB agar plate. After overnight incubation, the spots were harvested, resuspended in liquid LB, and plated onto media containing S/T and kanamycin. Colonies were only obtained from experiments where both strains were mixed together. Colonies were then replica plated onto several different antibiotic-containing media to ensure obtained colonies were indeed Ab_{17978}T6+ (RifR; pBAVMCS), which obtained pAB3 plasmid. Growth on rifampicin, PCR for presence of plasmid with primers Node_182F and Node_182R, as well as sequencing of the rpoB gene with primers rpoB-1441F and rpoB-2095R (Norton *et al.*, 2013) confirmed that all colonies tested were true transconjugants. Hcp expression and secretion was used as a final confirmation of pAB3 mobilization.

3.3 Results

3.3.1 Individual colonies of a MDR clinical *A. baumannii* isolate show an on–off T6SS phenotype that correlates with a loss of DNA and antibiotic resistance.

To determine the regulatory mechanisms involved in *A. baumannii* T6SS, the Hcp secretion profile of an MDR clinical isolate that caused a recent outbreak (Ahmed-Bentley *et al.*, 2013) was assessed by an Hcp-ELISA (Weber *et al.*, 2013). We found that individual colonies from a single patient isolate (Ab_{04}) displayed two contrasting Hcp secretion profiles (Figure 3.1A), which were verified by Western blot (Figure 3.1B). Colonies displaying robust Hcp secretion profiles were considered T6SS+ (Ab_{04}T6+), whereas those with no detectable Hcp secretion were considered T6SS– (Ab_{04}T6–). Ab_{04} caused a clonal outbreak that originated from an index patient who was also coinfected with *E. coli* and *K. pneumoniae* (Ahmed-Bentley *et al.*, 2013). Ab_{04}T6+, but not Ab_{04}T6–, caused a considerable reduction in survival of these *E. coli* and *K. pneumoniae* coisolates in competition assays (Figure 3.1C). The killing ability of Ab_{04}T6+ was further confirmed with common laboratory *E. coli* strains as prey (Figure 3.1D). Note that, although bactericidal activity is not formally shown in the competition assays, we use the term “killing,” as

broadly used for *Acinetobacter* T6SS activity in previous studies (Basler *et al.*, 2013; Shneider *et al.*, 2013).

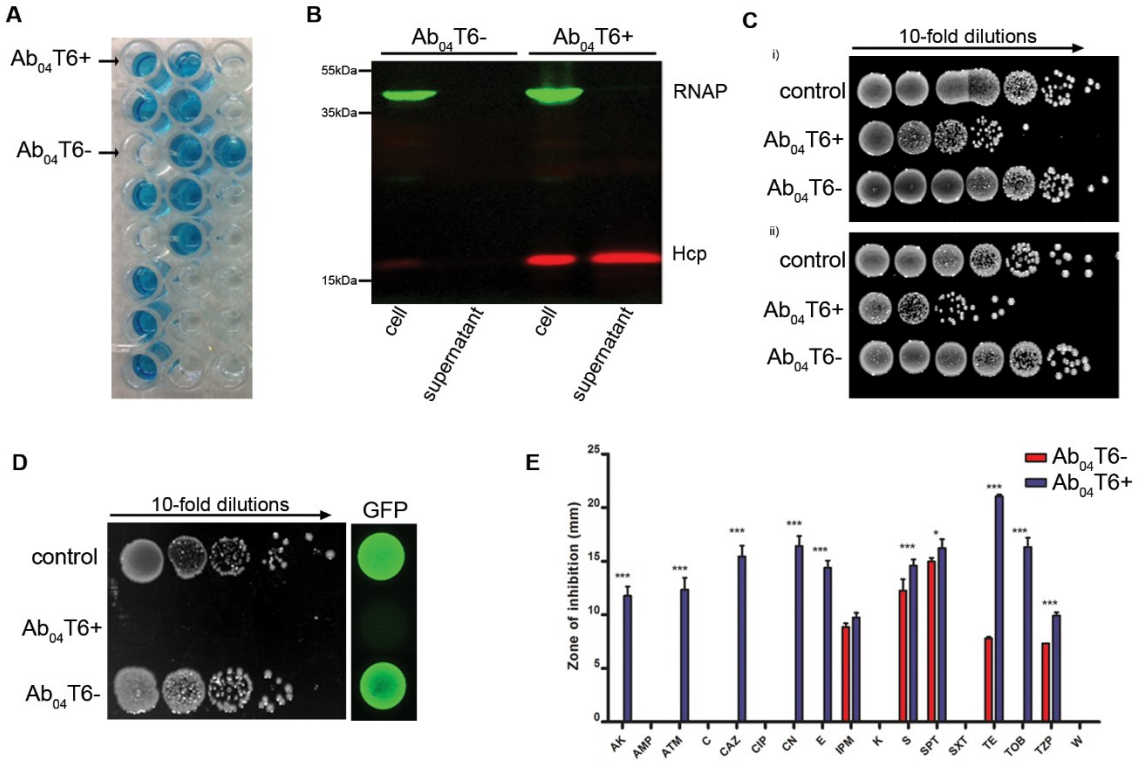

Figure 3.1 Outbreak isolate *A. baumannii* Ab_{04} displays an on/off T6SS phenotype concomitant with a loss of antibiotic resistance.

A) Detection of Hcp secretion from individual colonies of *A. baumannii* Ab04 (Ab_{04}) by Hcp-ELISA. Ab_{04}T6+ and Ab_{04}T6– labels indicate the typical readout of colonies giving rise to robust or undetectable levels of Hcp secretion, respectively. B) Hcp secretion (red) profiles of Ab_{04}T6+ and Ab_{04}T6– colonies were confirmed by Western blot on whole cells and supernatants, with RNA polymerase (RNAP; green) as the lysis control. C) Recovery of surviving clinical isolates of *E. coli* (i) or *K. pneumoniae* (ii), coisolated during *A. baumannii* outbreak, after coincubation with Ab_{04}T6+, Ab_{04}T6–, or control strain. D) Recovery of surviving *E. coli* MG1655R after incubation with Ab_{04}T6–, Ab_{04}T6+, or a rifampicin-sensitive *E. coli* strain (control). GFP images were produced using an *E. coli* strain constitutively expressing GFP as prey, and representative images from a single experiment are shown. E) Antibiotic susceptibilities of Ab_{04}T6– and Ab_{04}T6+ as assessed by disk diffusion assay. Antibiotic abbreviations are listed in Experimental Procedures. ***$P < 0.001$, *$P < 0.05$.

To identify the genetic difference(s) responsible for the discrepancy in T6SS phenotypes, we used Illumina sequencing to generate draft genomes for Ab_{04}T6– and Ab_{04}T6+. Analysis of the

de novo assembled genomes revealed that Ab_{04}T6+ contained a noticeably smaller genome than Ab_{04}T6–, lacking a total of ~170 kb of DNA. Some of the genes encoded by this DNA contained putative antibiotic resistance genes. We determined that Ab_{04}T6+ lost resistance to several classes of clinically important antibiotics, including β-lactams (aztreonam and ceftazidime), aminoglycosides (gentamicin, amikacin, and tobramycin), the macrolide erythromycin, and tetracycline (Figure 3.1E and Figure 3.2). These results suggested that Ab_{04}T6+ had undergone some form of DNA loss, leading to antibiotic susceptibility and T6SS activation.

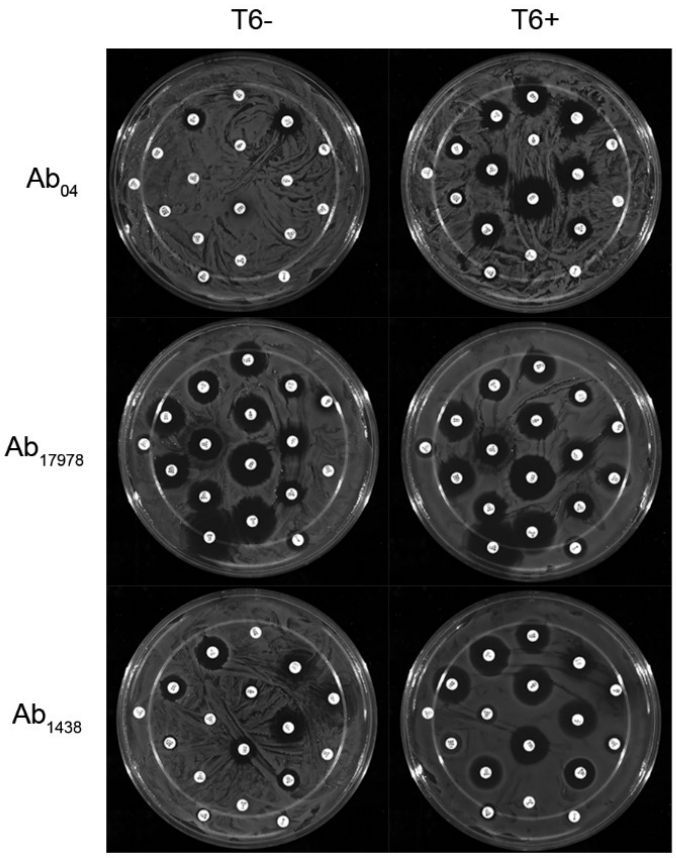

Figure 3.2 Antibiotic resistance loss by T6+ strains.

Antibiotic resistance was assessed by disk diffusion for T6– and T6+ variants of the three strains. Images here show typical results, which are presented in graphical form in Figure 3.1 and Figure 3.4.

3.3.2 DNA loss leading to T6SS activation and antibiotic susceptibility is widespread in *A. baumannii*.

We speculated that the DNA missing in Ab_{04}T6+ may be present in other *A. baumannii* strains. We used a combination of PCR and bioinformatic methods to identify other *A. baumannii* strains harboring this additional DNA. Two strains were identified by a positive PCR specific for the missing DNA (Figure 3.3). These were the sequenced and well-characterized reference strain *A. baumannii* ATCC 17978 (Ab_{17978}), which was isolated in the early 1950s, before the introduction of many common antibiotics, and is considered a relatively drug-sensitive

strain, and a recent MDR clinical isolate from Argentina, *A. baumannii* 1438 (Ab_{1438}). In agreement with the PCR results, homology searches of *A. baumannii* genome sequences revealed several other strains possessing this DNA, including Ab_{17978} (Table 3.3). Using the Hcp-ELISA, bacteria from Ab_{17978} and Ab_{1438} displaying an on/off phenotype for T6SS were isolated (Figure 3.4). Although the T6SS locus was present in both cell types, the T6+ variants did not yield the PCR product detectable in the T6– strains (Figure 3.3). In contrast to their T6– counterparts, Ab_{17978}T6+ and Ab_{1438}T6+ efficiently killed *E. coli* in competition assays (Figure 3.4). This killing was dependent on a functional T6SS, as Ab_{17978}T6+ lacking essential T6SS components *hcp* or *tssM* did not kill *E. coli* (Figure 3.5). Because Ab_{04}T6+ had lost antibiotic resistance, we compared the resistance profiles of Ab_{1438}T6–/T6+ and Ab_{17978}T6–/T6+. Ab_{1438}T6+ showed a significant decrease in resistance to several antibiotics compared with Ab_{1438}T6–, and Ab_{17978}T6+ lost resistance to the combination of sulfamethoxazole/trimethoprim (S/T) compared with Ab_{17978}T6– (Figure 3.2 and Figure 3.4).

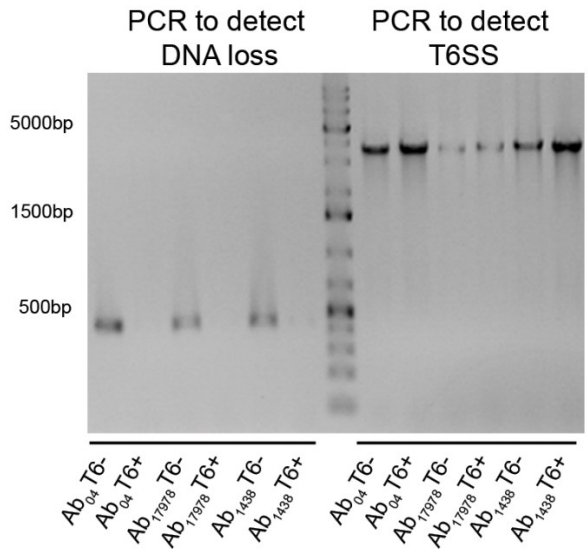

Figure 3.3 PCR detection of DNA present in T6– but not T6+ strains indicates DNA loss in Ab_{17978} and Ab_{1438}.

Primers (Node_182F and Node_182R) were designed, based on Illumina data from Ab_{04}T6–, to amplify a ~400-bp fragment of DNA present in Ab_{04}T6– but not Ab_{04}T6+ (left side of gel). Strains Ab_{17978} and Ab_{1438} also yielded this PCR product, which was only present in the T6– variants. Primers T6SSF and T6SSR were used to confirm that all strains still possessed the T6SS locus (right side of gel).

Table 3.3 *A. baumannii* strains that contain DNA sequences with homology to pAB3 and pAB04-1

Strain	Plasmid?	GenBank accession
A. baumannii ATCC 17978	No/Yes; plasmid pAB3*	CP000521.1*
A. baumannii IOMTU433	Yes; plasmid pIOMTU433	AP014650.1
A. baumannii OIFC109	Yes; plasmid pOIFC109-122	ALAL01000013.1
A. baumannii OIFC137	Yes; plasmid pOIFC137-122	AFDK01000004.1
A. baumannii OIFC143	Yes; plasmid pOIFC143-128	AFDL01000008.1
A. baumannii Naval-18	Yes; plasmid pNaval18-131	AFDA02000009.1
A. baumannii 233846	Not indicated	JMOG01000034.1
A. baumannii 318814	Not indicated	JFEQ01000014.1
A. baumannii 1419130	Not indicated	JEWL01000060.1
A. baumannii CI79	Not indicated	AVOD01000009.1
A. baumannii CI86	Not indicated	AVOB01000033.1
A. baumannii NIPH 527	Not indicated	APQW01000030.1
A. baumannii WC-348	Not indicated	AMZT01000015.1
A. baumannii IS-116	Not indicated	AMGF01000021.1
A. baumannii OIFC065	Not indicated	AMFV01000043.1

A. baumannii OIFC0162	Not indicated	AMFH01000054.1
A. baumannii Naval-13	Not indicated	AMDR01000015.1
Acinetobacter sp. NCTC 10304	Not indicated	AIEE01000255.1
A. baumannii AB5256	Not indicated	AHAI01000050.1

*- Integrated into chromosome in original sequence

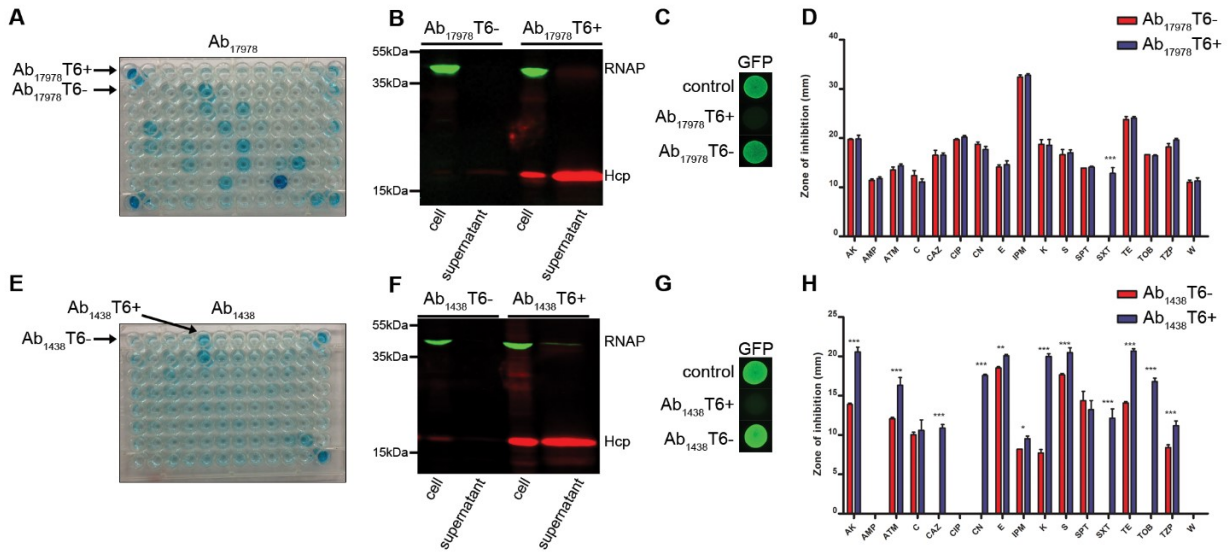

Figure 3.4 Intercolony variation in T6SS and antibiotic resistance is common in *A. baumannii*.

A) and E) Detection of Hcp secretion from individual colonies of *A. baumannii* ATCC 17978 (Ab_{17978}) and *A. baumannii* 1438 (Ab_{1438}) by Hcp-ELISA, with colonies giving rise to a T6+ (Ab_{17978}T6+, Ab_{1438}T6+) or T6– (Ab_{17978}T6–, Ab_{1438}T6–) readout indicated. B) and F) ELISA results confirmed by Western blot. C) and G) Survival of GFP-expressing *E. coli*, as assessed by fluorescence, after incubation with T6+ or T6– variants of Ab_{17978} and Ab_{1438}, or with non-GFP parental strain (control). Representative images from a single experiment are shown. D) and H) Antibiotic susceptibilities of Ab_{17978}T6+/T6– and Ab_{1438}T6+/T6– as assessed by a disk diffusion assay. Antibiotic abbreviations are listed in Experimental procedures. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

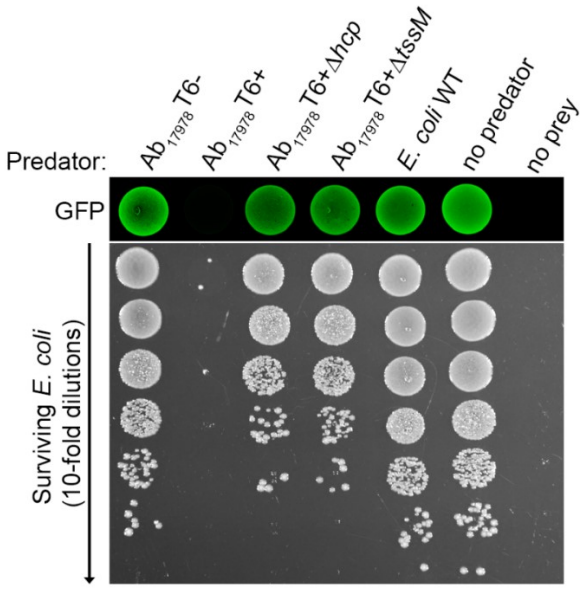

Figure 3.5 Bacterial killing is dependent on a functional T6SS.

Survival of GFP-expressing *E. coli*, as assessed by fluorescence and antibiotic selection, after incubation with various strains of Ab_{17978}, including the T6SS mutants Ab_{17978}T6+Δ*hcp* and Ab_{17978}T6+Δ*tssM*. *E. coli* WT indicates the parental strain lacking the GFP expression vector and is the no prey control. *E. coli* was selected for in serial dilutions using kanamycin.

Although the *A. baumannii* T6SS effector–immunity pairs have not been characterized, activation of the T6SS in the T6+ strains could pose a threat to neighboring T6– sister cells. Through competition assays between T6+/T6– counterparts, we found that T6– cells were not affected by their T6+ kin (Figure 3.6). This could indicate that *A. baumannii* is not capable of self-targeting or that the immunity proteins protecting from T6SS-mediated attacks are produced even under T6– conditions. Given findings in *V.cholerae*, in which immunity proteins are transcribed constitutively and independently of other T6SS genes (Miyata *et al.*, 2013b), we favor the latter hypothesis. This is further supported by our experiments showing that T6+ *A. baumannii* are able to kill nonkin T6– cells (Figure 3.7), which implies that lack of kin-cell killing is due to immunity

and not an inability to target another *A. baumannii* cell. Furthermore, this suggests that effector–immunity pairs are diverse among *A. baumannii* isolates.

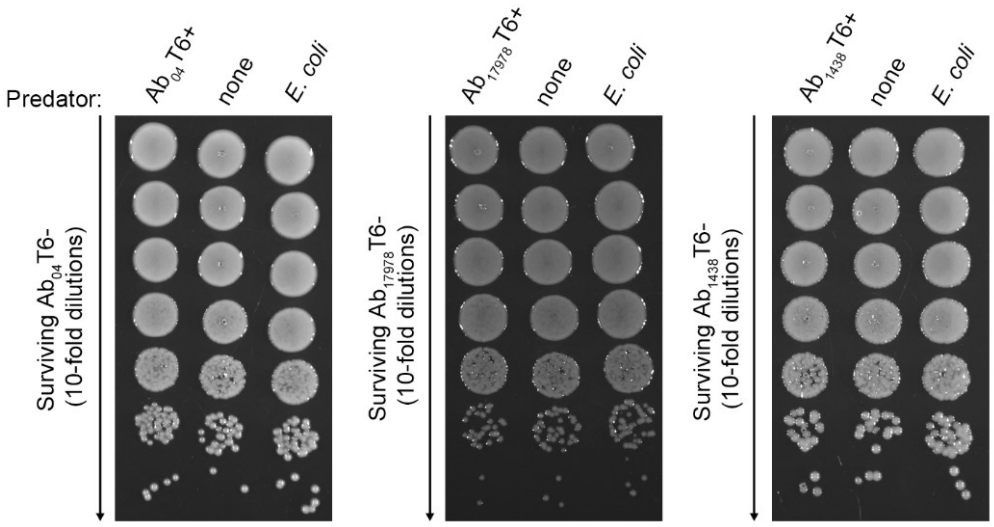

Figure 3.6 T6– *A. baumannii* are not killed by T6+ sister cells.

Survival of indicated T6– *A. baumannii* after incubation with T6+ counterpart or controls is shown. T6– strains were selected based on difference in antibiotic resistance with their T6+ kin.

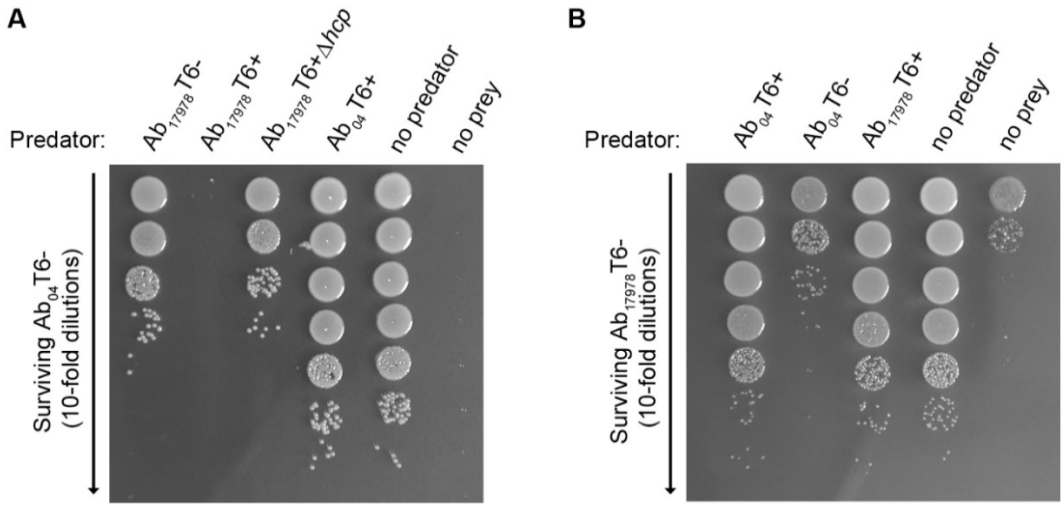

Figure 3.7 T6+ *A. baumannii* kill nonkin T6– *A. baumannii.*

A) Coincubation of Ab_{04}T6– with indicated predator strains at a 1:2 predator-to-prey ratio. Surviving Ab_{04}T6– was determined by serial dilutions on gentamicin-containing plates. The no predator and no prey controls indicate Ab_{04}T6– or Ab_{17978}T6– plated without the competitor, respectively. B) Coincubation of a rifampicin-resistant derivative of Ab_{17978}T6– with indicated predator strains at a 5:1 predator-to-prey ratio. Surviving Ab_{17978}T6– was determined by plating on rifampicin. The no predator and no prey controls indicate rifampicin-resistant Ab_{17978}T6– or Ab_{04}T6– plated without the competitor, respectively. Note that Ab_{04}T6– displays a low level of spontaneous resistance.

3.3.3 Loss of a Conserved, Conjugative Resistance Plasmid Results in T6SS Activation.

To fully elucidate the genetic changes underlying the observed phenotypes, we sequenced the genomes of Ab_{04}T6+, Ab_{04}T6–, Ab_{17978}T6+, and Ab_{17978}T6– using PacBio long read technology (Eid *et al.*, 2009). Ab_{04}T6– and Ab_{04}T6+ genomes were completely closed and identical, except for the presence of a 170-kb plasmid (pAB04-1) present only in Ab_{04}T6– (Figure 3.8A). Similarly, the only detectable difference between Ab_{17978}T6– and Ab_{17978}T6+ was the presence of a 150-kbp plasmid (pAB3) in Ab_{17978}T6– (Figure 3.8A). Interestingly, pAB3 was assembled as part of the chromosome in the original genome sequencing of Ab_{17978} (Smith *et al.*, 2007). This may be the result of prior genome assembly errors or possibly plasmid integration in the chromosome, although we observed no evidence of integration in our sequence data.

Additionally, Illumina sequencing reads from Ab_{1438}T6–, but not from Ab_{1438}T6+, aligned onto pAB04-1 and pAB3 with considerable sequence coverage (Figure 3.9).

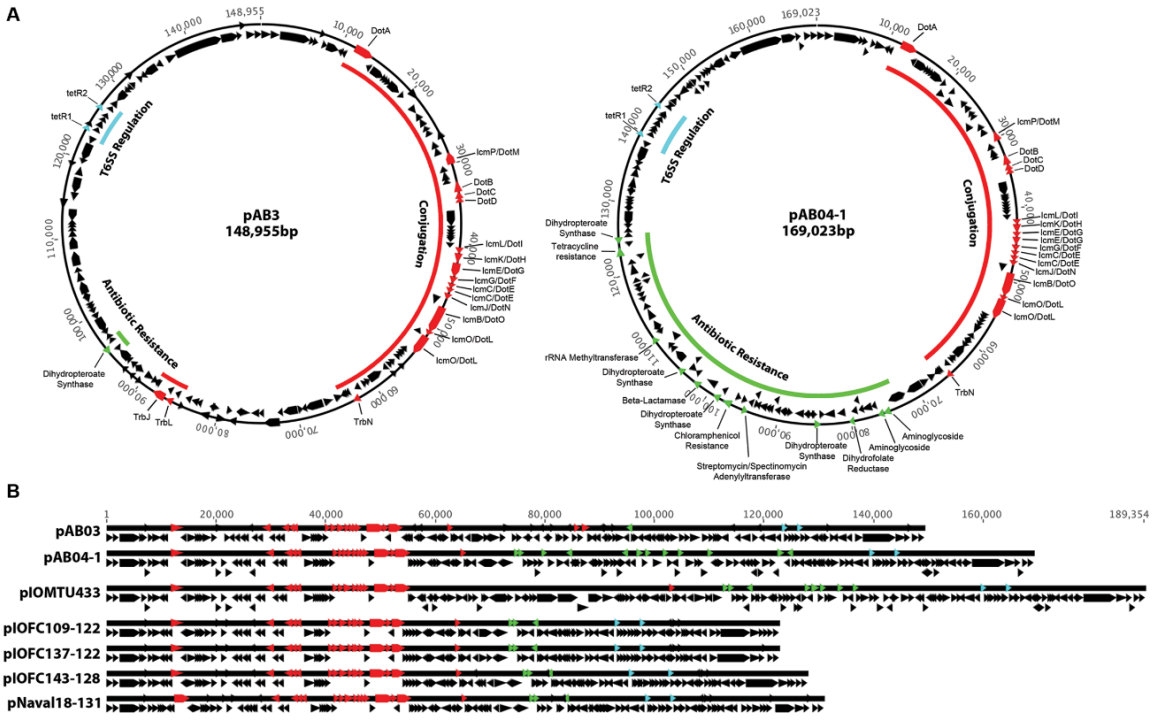

Figure 3.8 *A. baumannii* plasmids share common structural features.

A) Assembled plasmids from PacBio sequencing of Ab_{17978}T6– (pAB3, accession no. CP012005) and Ab_{04}T6– (pAB04-1, accession no. CP012007) and other plasmids taken from GenBank (B) highlighting common conjugation (red), antibiotic resistance (green), and T6SS regulation (blue) loci.

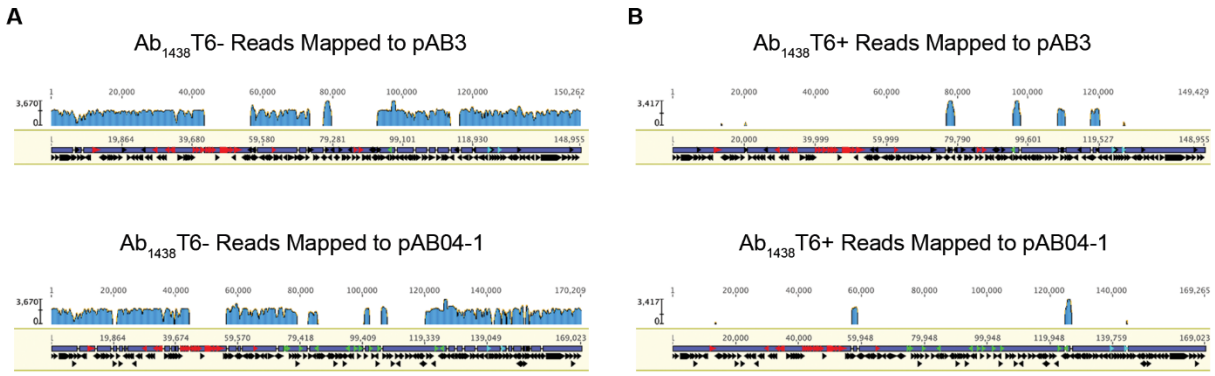

Figure 3.9 Illumina reads from Ab_{1438}T6–, but not Ab_{1438}T6+, map onto pAB03 and pAB04-1.

A) and B) Sequencing reads from Ab_{1438}T6– map well onto both pAB03 and pAB04-1, whereas very few reads from Ab_{1438}T6+ do. The small regions of mapped reads of Ab_{1438}T6+ are mobile element regions that are also found elsewhere in the genomes.

pAB04-1 and pAB3 are highly similar over much of their sequence and seem to share a common backbone that includes a putative conjugative T4SS. pAB04-1 contains a large island with several antibiotic resistance genes that are absent in pAB3. DNA sequences corresponding to similar plasmids are present in many other recent MDR isolates of *A. baumannii* (Figure 3.8B). Considering that pAB3 is from an "old" isolate, this suggests that pAB3 could be considered as an ancestral form of these current plasmids, which encode more resistance genes (Figure 3.8 and Table 3.3). Through conjugation experiments, we determined that pAB3 could be transferred from Ab_{17978}T6– to Ab_{17978}T6+, resulting in gain of antibiotic resistance and suppression of the T6SS in transconjugants (Figure 3.10). This indicates that the T4SS contained in pAB3 is functional and that this plasmid can be disseminated among *Acinetobacter* strains.

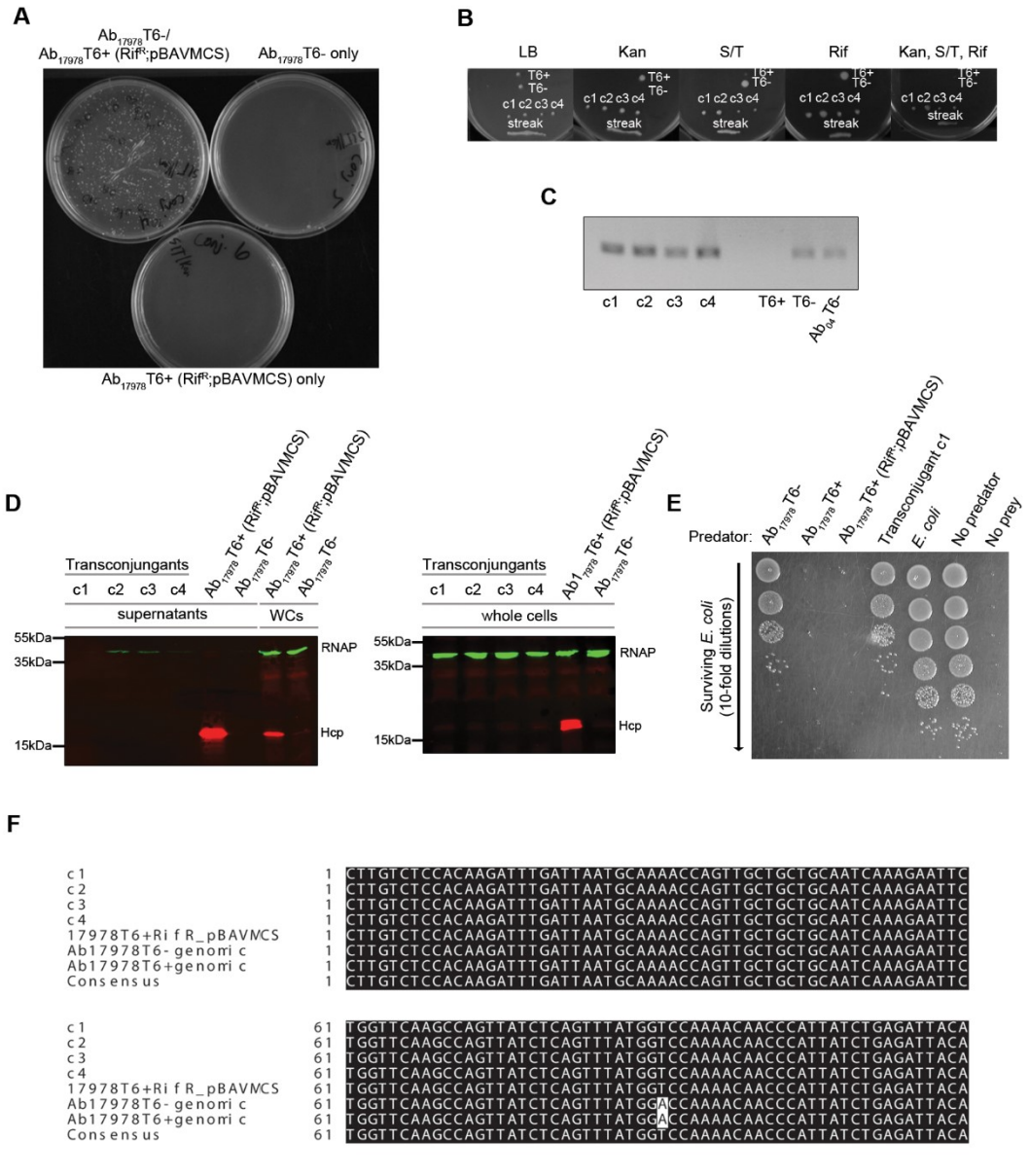

Figure 3.10 pAB3 is self-transmissible from Ab_{17978}T6– to Ab_{17978}T6+ and shuts down T6SS in transconjugants.

A) Putative transconjugant colonies were obtained on plates containing kanamycin (Kan) and S/T when Ab_{17978}T6– was coincubated with a spontaneous rifampicin-resistant strain of Ab_{17978}T6+ harboring the Kan resistance plasmid pBAVMCS [Ab_{17978}T6+ (Rif^R; pBAVMCS)], and the mixture was plated after overnight incubation, but not when either was incubated alone. Only Ab_{17978}T6+ (RifR; pBAVMCS) that obtained pAB3, which imparts S/T resistance, is expected to grow. B) Individual transconjugant colonies (c1–c4) or a loop full of transconjugants (streak) were replica plated onto various antibiotics. Only

transconjugants grew on the combination plate of Kan, S/T, and Rif. C) PCR detection of pAB3 using primer Node_182F/R. D) Hcp expression and secretion is repressed in transconjugants. E) Transconjugants are unable to kill *E. coli* in a bacterial killing assay. Predators are as listed, with an antibiotic sensitive *E. coli* strain used as a control. F) Confirmation that c1–c4 are true transconjugants by PCR and sequencing of the *rpoB* gene. Ab_{17978}T6+ (Rif^R; pBAVMCS) and c1–c4 all have a nucleotide difference in their rpoB gene, giving rise to Rif^R, whereas the parental Ab_{17978}T6+ genomic and Ab_{17978}T6– genomic are identical, indicating that c1–c4 are all truly Ab_{17978}T6+ (Rif^R; pBAVMCS) harboring pAB3.

To estimate the frequency of plasmid loss, we plated Ab_{17978} in the presence of S/T (to ensure plasmid maintenance) and then inoculated single colonies into 96-well plates lacking antibiotics. Wells exhibiting diverse Hcp secretion levels were observed (Figure 3.11). About 100 clones of the bacteria contained in representative wells were plated in the presence and absence of antibiotics, and the number of antibiotic-sensitive bacteria was counted, with plasmid loss confirmed by PCR. We estimated that, in our experimental conditions, the percentage of cells lacking the plasmid ranges from 0% to 5% of a given population.

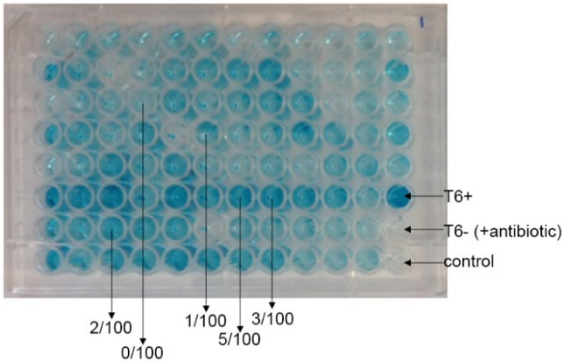

Figure 3.11 Estimating plasmid loss rates in Ab17978

Plasmid loss rates in Ab_{17978}. Individual colonies of plasmid-containing Ab_{17978}T6– were inoculated into 96-well plates containing antibiotic-free growth medium. Supernatants were collected for Hcp-ELISA, which identified a wide range of Hcp secretion phenotypes. We selected several representative wells and determined the proportion of Ab_{17978} that had lost the plasmid by replica plating and PCR.

3.3.4 Two Plasmid-Encoded TetR-Like Regulators Suppress the *A. baumannii* T6SS.

Because of their similar backbone, we reasoned that the plasmids possessed by T6– *A. baumannii* strains likely contained a genetic element that was responsible for T6SS repression. Each plasmid encodes several conserved predicted regulator genes, including an *hns*-like gene (locus tag ACX61_19730) and two *tetR*-like regulators, *tetR*-like1 and *tetR*-like2 (*tetR1* and *tetR2*, locus tags ACX61_19730 and ACX61_19655, respectively) (Figure 3.8). H-NS proteins are a family of global regulators that bind A-T–rich regions and are usually used to silence horizontally acquired genes (Fang *et al.*, 2008). Proteins within the H-NS family have been suggested to be involved in T6SS regulation (Castang *et al.*, 2008; Salomon *et al.*, 2014). TetR-like regulators play a broad role in many aspects of prokaryotic physiology (Cuthbertson *et al.*, 2013), and members of this family have been implicated in T6SS regulation (Ishikawa *et al.*, 2009). Close homologs of TetR1 and TetR2 are only present in *A. baumannii* harboring pAB04-1/pAB3-like plasmids. The *hns*, *tetR1*, and *tetR2* genes were cloned and ectopically expressed in the three T6+ strains to test their effect on T6SS. We observed no change in Hcp expression or secretion in Ab_{04}T6+ carrying the *hns*-like gene (Figure 3.12). In contrast, introduction of TetR1 or TetR2 dramatically decreased expression and secretion of Hcp in all T6+ strains (Figure 3.13A). Furthermore, TetR1 and TetR2 abolished Hcp expression and secretion in *A. baumannii* ATCC 19606 and *A. baylyi* ADP1, both of which possess a constitutively active T6SS under laboratory conditions but had no effect on *V. cholerae* T6SS (Figure 3.12). In killing assays, T6+ strains expressing either TetR1 or TetR2 showed an impaired ability to kill *E. coli*, consistent with a defect in Hcp secretion (Figure 3.13B).

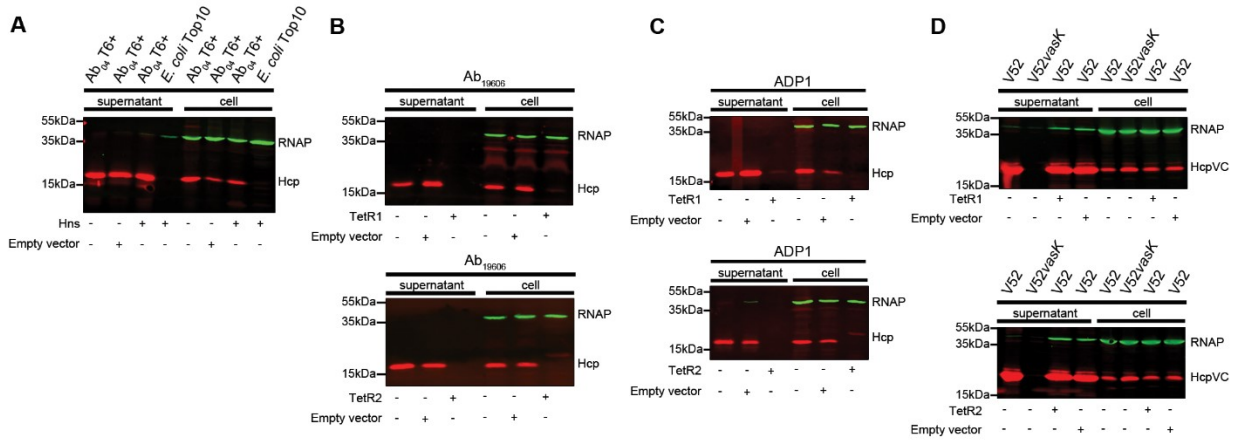

Figure 3.12 Plasmid-encoded TetR1 and TetR2, but not an Hns-like protein, repress the T6SS in *Acinetobacter* but have no effect on *V. cholerae*.

A) Detection of Hcp expression (cell) and secretion (supernatant) in Ab_{04}T6+ expressing an Hns-like (Hns) protein encoded on the pAB04-1, with RNA polymerase (RNAP) as a loading and lysis control. B-D) Expression and secretion of Hcp by TetR1 (*Upper*) and TetR2 (*Lower*) expressing *A. baumannii* ATCC 19606 (B), *A. baylyi* ADP1 (C), and *V. cholerae* V52 (D). The *V. cholerae* Hcp (HcpVC) was detected using an anti-HcpVC antibody, and the T6SS *vasK* mutant strain was included as a secretion control.

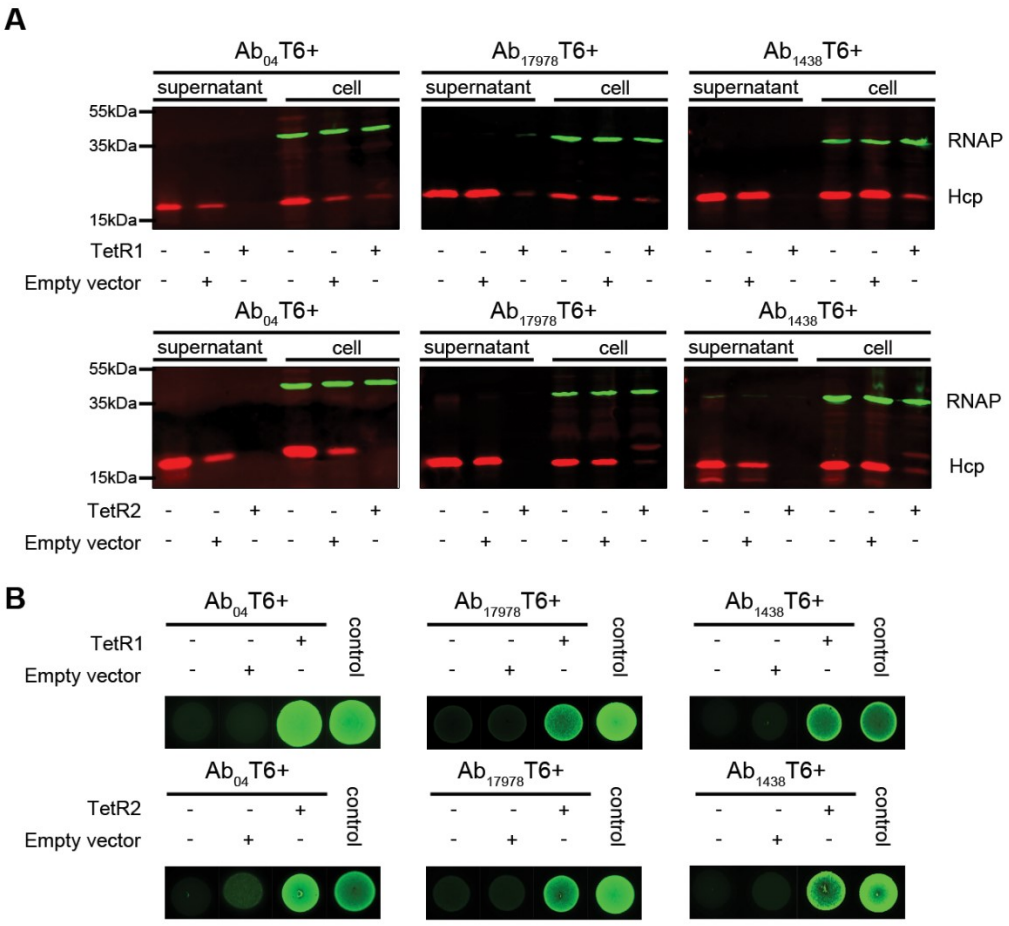

Figure 3.13 Plasmid-encoded TetR1 and TetR2 repress the T6SS.

A) Detection of Hcp (Red) expression and secretion in Ab_{04}T6+, Ab_{17978}T6+, and Ab_{11438}T6+ expressing TetR1 (upper panels) or TetR2 (lower panels), with RNA polymerase (RNAP; green) as a lysis control. B) Survival of GFP-expressing *E. coli*, as assessed by fluorescence, after coincubation with TetR1 (upper panels) or TetR2 (lower panels) expressing *A. baumannii* strains. Control spot consists of GFP-expressing *E. coli* incubated with non-GFP parental strain, and representative images from a single experiment are shown.

3.4 Discussion

In this study, we uncovered the molecular mechanisms leading to the repression of T6SS in *A. baumannii.* Our work suggests an explanation for the differences in T6SS activity observed among different strains of *A. baumannii* (Weber *et al.*, 2013). By screening different colonies from a MDR clinical isolate, we discovered that *A. baumannii* cells can present an on/off T6SS

phenotype that is controlled by the presence or absence of a resistance plasmid that carries the repressors of the secretion system. Indeed, two repressors belonging to the TetR family, both individually capable of repressing the T6SS, are encoded in the plasmid. We have identified this plasmid in clinical isolates from geographically diverse locations around the world and also in the Ab_{17978}, one of the most commonly used strains in laboratories that was isolated in the 1950s. Upon plasmid loss, which occurs frequently in the absence of selection, the cells differentiate into T6+ bacteria specialized in the elimination of competing bacteria. Those cells that keep the plasmid maintain the T6SS in a silent state but retain the ability to resist antibiotics. It is conceivable that a ligand(s) for the TetR proteins exists that would relieve T6SS repression without the loss of the resistance plasmid. However, our data suggest that *A. baumannii* loses this plasmid readily without antibiotic selection, indicating plasmid loss is a true mechanism for T6SS activation. In the human host, plasmid loss has been documented in *A. baumannii* strains isolated from the same patient over time (Wright *et al.*, 2014). Furthermore, the finding that this plasmid can be conjugated from a T6– cell to a T6+ cell raises the possibility that the plasmid can be disseminated back into those cells that have lost it. In a previous work, we reported that although Hcp secretion was detectable in *A. baumannii* 17978, T6SS was not used to kill other bacteria (Weber *et al.*, 2013). We believe that the use of a mixed T6SS+/T6SS– population in that study masked the activity of the T6SS in this strain. It is conceivable that other reports using strains harboring the plasmid may also be biased by the use of mixed populations.

The observation that the TetR repressors can act on T6SS in a wide range of *A. baumannii* strains and species suggests they operate on a conserved component found across *Acinetobacters.* This may not be surprising, given the high sequence conservation of T6SS loci in these organisms (Carruthers *et al.*, 2013c; Weber *et al.*, 2013). It remains unknown

whether *A. baumannii* strains with a constitutively active T6SS, like *A. baumannii* ATCC 19606 and *A. baumannii* SDF (Henry *et al.*, 2012; Weber *et al.*, 2013) (which do not harbor a similar plasmid), at some point (whether during laboratory culture or before isolation) lost an analogous plasmid to those described here, leading to T6SS activation, or whether this plasmid was independently acquired by strains like Ab_{17978} and Ab_{04}, silencing their previously active T6SS. The finding that these plasmids are highly conserved and apparently only present in *A. baumannii* suggests that this method of regulation is restricted to *Acinetobacter*. Our data also show that T6– *A. baumannii* are resistant to T6SS-mediated attack from T6+ sister cells but not from nonkin T6+ *A. baumannii*. This indicates that the immunity proteins involved in preventing self-intoxication are produced even when the secretory apparatus itself is not expressed, similar to the scenario described in *V. cholerae* (Miyata *et al.*, 2013b). However, at this time the effector–immunity pairs of *A. baumannii* have not been characterized, and as such direct experimental evidence of this remains to be seen.

It has been demonstrated that antibiotic resistance-carrying plasmids can impose a fitness cost on their bacterial hosts (Andersson *et al.*, 2010). Furthermore, as has been shown for *Salmonella* Typhimurium, expression of a secretion system can be costly and reduce the competitive fitness of an organism in environments where the secretory apparatus is not beneficial (Sturm *et al.*, 2011). Our results suggest that *A. baumannii* has partitioned two phenotypes: an ability to resist being killed by antibiotics, and an ability to kill using its T6SS. For the strains and conditions tested in this study, these phenotypes are mutually exclusive. It is tempting to speculate that *A. baumannii* has evolved the strategy of carrying the T6SS repressors in a frequently lost MDR plasmid as a response to fitness defects imposed by harboring both a large resistance plasmid and a constitutively active T6SS. We suggest a model for the relationship between MDR and T6SS

that allows *A. baumannii* to maintain both systems while avoiding potential deleterious effects (Figure 3.14). When MDR *A. baumannii* is not under the threat of antibiotics, such as in the inanimate hospital environment or an untreated polymicrobial infection, there is an increased likelihood of encountering competitors. In this instance, repression of T6SS is relieved in a subset of the bacterial population by plasmid loss, allowing *A. baumannii* to actively attack other bacteria. Under conditions where antibiotics are present, MDR *A. baumannii* may derive enough of a survival advantage from antibiotic resistance alone that an active T6SS is neither necessary nor beneficial (Wright *et al.*, 2014). In fact, genome sequencing of several recent MDR *A. baumannii* isolates revealed that some lack a full T6SS locus, suggesting some strains have inactivated their secretion system in favor of antibiotic resistance (Eijkelkamp *et al.*, 2013; Hornsey *et al.*, 2011; Wright *et al.*, 2014). The plasmid present in the old isolate Ab_{17978} strain encodes a single antibiotic resistance. The fitness cost of this does not seem to justify the use of the plasmid as a molecular switch for T6SS; however, it is possible that loss of the T6SS-repressing plasmid provides other advantage(s). The plasmids encode about 150 genes, including several other regulators such as H-NS, whose functions remain to be elucidated. It is conceivable that these control other important metabolic pathways or virulence mechanisms in *A. baumannii*. The antibiotic cassettes appear to be a later addition to the plasmid, and the fact that in only a few decades the number of antibiotic resistance cassettes has increased from 1 up to 11 demonstrates that insertion of resistance cassettes in an "easy-to-lose" plasmid containing the repressors of T6SS is a very efficient strategy to accumulate MDR. An alternative view is that encoding T6SS repressors in the plasmid prevents T6SS-mediated killing of potential recipients, facilitating plasmid propagation among different *Acinetobacter* strains. In the context of hospital environments, the encounter of *A. baumannii* with antibiotics is inevitable, and therefore plasmid loss could be regarded as an

altruistic mechanism to differentiate cells specialized for elimination of competing bacteria. The interplay between T6SS and antibiotic resistance may constitute an important survival strategy for this nosocomial pathogen.

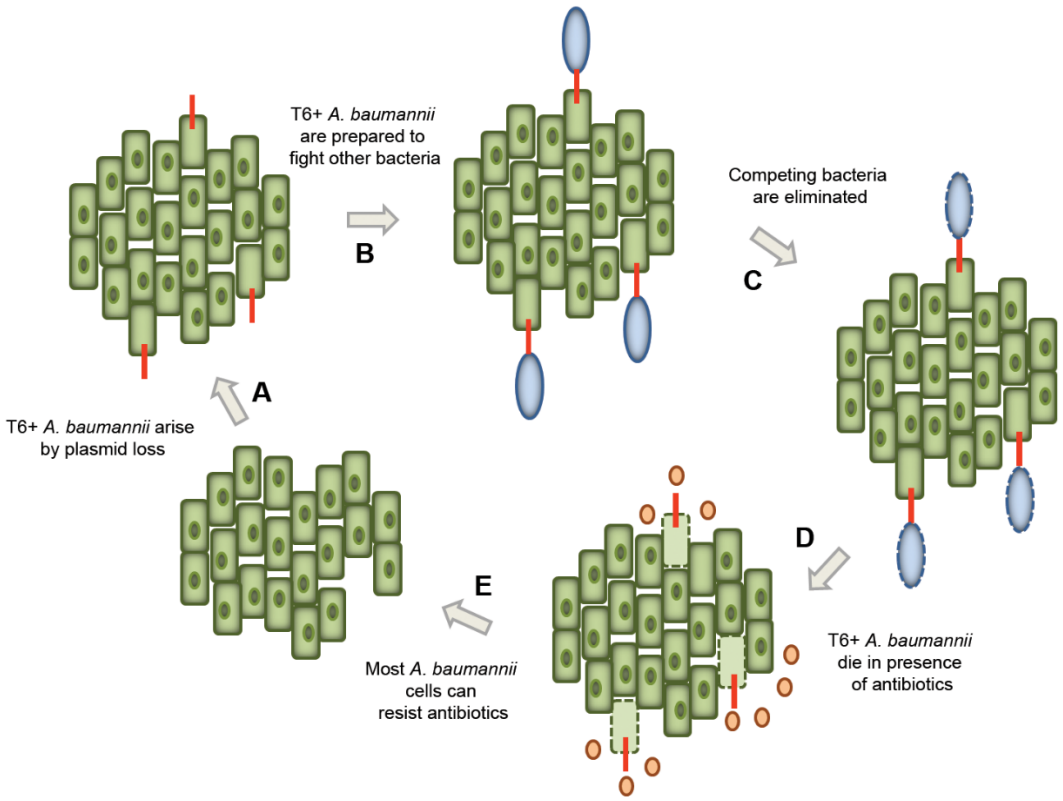

Figure 3.14 A model for MDR and T6SS in *A. baumannii*.

A. baumannii harbors a MDR plasmid that encodes repressors of T6SS. In the absence of antibiotics, this plasmid is lost in a subset of the population and results in T6SS activation (A). The activation of the T6SS prepares *A. baumannii* for competition (B) and imparts the ability to kill other bacteria that may try to enter the same environment (C). Upon (re)introduction of antibiotics, plasmid-less *A. baumannii* will die (D), and the rest of the *A. baumannii* cells will be resistant and ensure survival of the population (E).

3.5 References

Ahmed-Bentley, J., Chandran, A. U., Joffe, A. M., French, D., Peirano, G., & Pitout, J. D. (2013). Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. *Antimicrobial agents and chemotherapy, 57*(7), 3085-3091.

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews. Microbiology, 8*(4), 260-271.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., . . . Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology, 19*(5), 455-477.

Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell, 152*(4), 884-894.

Basler, M., & Mekalanos, J. J. (2012a). Type 6 secretion dynamics within and between bacterial cells. *Science, 337*(6096), 815.

Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012b). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186.

Bernard, C. S., Brunet, Y. R., Gueguen, E., & Cascales, E. (2010b). Nooks and crannies in type VI secretion regulation. *Journal of bacteriology, 192*(15), 3850-3860.

Bingle, L. E., Bailey, C. M., & Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Current opinion in microbiology, 11*(1), 3-8.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., . . . Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 48*(1), 1-12.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC genomics, 10*, 104.

Bryksin, A. V., & Matsumura, I. (2010). Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. *PloS one, 5*(10), e13244.

Carruthers, M. D., Harding, C. M., Baker, B. D., Bonomo, R. A., Hujer, K. M., Rather, P. N., & Munson, R. S., Jr. (2013a). Draft Genome Sequence of the Clinical Isolate *Acinetobacter* nosocomialis Strain M2. *Genome announcements, 1*(6).

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S., Jr. (2013c). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PloS one, 8*(3), e59388.

Cascales, E. (2008). The type VI secretion toolkit. *EMBO reports, 9*(8), 735-741.

Castang, S., McManus, H. R., Turner, K. H., & Dove, S. L. (2008). H-NS family members function coordinately in an opportunistic pathogen. *Proceedings of the National Academy of Sciences of the United States of America, 105*(48), 18947-18952.

Cuthbertson, L., & Nodwell, J. R. (2013). The TetR family of regulators. *Microbiology and molecular biology reviews : MMBR, 77*(3), 440-475.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008a). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

Dykxhoorn, D. M., St Pierre, R., & Linn, T. (1996). A set of compatible tac promoter expression vectors. *Gene, 177*(1-2), 133-136.
Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., . . . Turner, S. (2009). Real-time DNA sequencing from single polymerase molecules. *Science, 323*(5910), 133-138.
Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Elbourne, L. D., Paulsen, I. T., & Brown, M. H. (2013). H-NS plays a role in expression of *Acinetobacter baumannii* virulence features. *Infection and immunity, 81*(7), 2574-2583.
Fang, F. C., & Rimsky, S. (2008). New insights into transcriptional regulation by H-NS. *Current opinion in microbiology, 11*(2), 113-120.
Henry, R., Vithanage, N., Harrison, P., Seemann, T., Coutts, S., Moffatt, J. H., . . . Boyce, J. D. (2012). Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglucosamine. *Antimicrobial agents and chemotherapy, 56*(1), 59-69.
Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology, 8*(1), 15-25.
Ho, B. T., Basler, M., & Mekalanos, J. J. (2013). Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science, 342*(6155), 250-253.
Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., . . . Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell host & microbe, 7*(1), 25-37.
Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., . . . Woodford, N. (2011). Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *The Journal of antimicrobial chemotherapy, 66*(7), 1499-1503.
Hunger, M., Schmucker, R., Kishan, V., & Hillen, W. (1990). Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter* calcoaceticus and its use for *Escherichia coli* shuttle plasmids. *Gene, 87*(1), 45-51.
Ishikawa, T., Rompikuntal, P. K., Lindmark, B., Milton, D. L., & Wai, S. N. (2009). Quorum sensing regulation of the two hcp alleles in *Vibrio cholerae* O1 strains. *PloS one, 4*(8), e6734.
Juni, E. (1972). Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *Journal of bacteriology, 112*(2), 917-931.
MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America, 107*(45), 19520-19524.
Maragakis, L. L., Tucker, M. G., Miller, R. G., Carroll, K. C., & Perl, T. M. (2008b). Incidence and prevalence of multidrug-resistant *Acinetobacter* using targeted active surveillance cultures. *JAMA : the journal of the American Medical Association, 299*(21), 2513-2514.
Miyata, S. T., Unterweger, D., Rudko, S. P., & Pukatzki, S. (2013b). Dual expression profile of type VI secretion system immunity genes protects pandemic *Vibrio cholerae*. *PLoS pathogens, 9*(12), e1003752.
Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., . . . Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science, 312*(5779), 1526-1530.

Norton, M. D., Spilkia, A. J., & Godoy, V. G. (2013). Antibiotic resistance acquired through a DNA damage-inducible response in *Acinetobacter baumannii*. *Journal of bacteriology, 195*(6), 1335-1345.

Piechaud, M., & Second, L. (1951). [Studies of 26 strains of Moraxella Iwoffi]. *Annales de l'Institut Pasteur, 80*(1), 97-99.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.

Pukatzki, S., McAuley, S. B., & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Current opinion in microbiology, 12*(1), 11-17.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature, 475*(7356), 343-347.

Salomon, D., Klimko, J. A., & Orth, K. (2014). H-NS regulates the Vibrio parahaemolyticus type VI secretion system 1. *Microbiology, 160*(Pt 9), 1867-1873.

Scott, N. E., Kinsella, R. L., Edwards, A. V., Larsen, M. R., Dutta, S., Saba, J., . . . Feldman, M. F. (2014b). Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Molecular & cellular proteomics : MCP, 13*(9), 2354-2370.

Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature, 500*(7462), 350-353.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., . . . Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 34*(1), 1-14.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology, 66*, 453-472.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., & Snyder, M. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes & development, 21*(5), 601-614.

Sturm, A., Heinemann, M., Arnoldini, M., Benecke, A., Ackermann, M., Benz, M., . . . Hardt, W. D. (2011). The cost of virulence: retarded growth of Salmonella Typhimurium cells expressing type III secretion system 1. *PLoS pathogens, 7*(7), e1002143.

Valencia, R., Arroyo, L. A., Conde, M., Aldana, J. M., Torres, M. J., Fernandez-Cuenca, F., . . . Aznar, J. (2009). Nosocomial outbreak of infection with pan-drug-resistant *Acinetobacter baumannii* in a tertiary care university hospital. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 30*(3), 257-263.

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., & Feldman, M. F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PloS one, 8*(1), e55142.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., . . . Adams, M. D. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *mBio, 5*(1), e00963-00913.

4 Genetic dissection of the type VI secretion system in *Acinetobacter* and identification of a novel peptidoglycan hydrolase TagX required for its biogenesis

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4.1 Introduction

While many species of *Acinetobacter*, a genus of Gram-negative γ-proteobacteria, are commonly cultivated from environments such as soil and water, several *Acinetobacter* species (spp.) are important opportunistic pathogens (Baumann, 1968). The most clinically relevant member of the genus, *A. baumannii*, is rarely isolated outside of the hospital setting and is a major healthcare threat due to extensive drug resistance (Peleg *et al.*, 2008a; Perez *et al.*, 2007). Environmental and pathogenic strains of *Acinetobacter* spp. are likely to encounter, and interact with, other microbes. The factors mediating such interactions are not well known, although cross-kingdom signaling has been shown to affect the outcome of *A. baumannii* interactions with some eukaryotes (Peleg *et al.*, 2008b). Furthermore, the ability to withstand desiccation and form biofilms may contribute to *Acinetobacter* spp. persistence under hostile conditions in their various niches (Jawad *et al.*, 1998; Tomaras *et al.*, 2003; Vidal *et al.*, 1996). The multitude of molecular structures present on the cell surface contribute in a variety of ways to survival and pathogenicity in these organisms (Weber *et al.*, 2015a).

Secretion systems of Gram-negative bacteria are diverse in terms of both structural components used to assemble the secretion system and the proteins exported by each apparatus. Among the different secretory systems employed by Gram-negative bacteria, the type VI secretion system (T6SS) has emerged as a widespread mechanism for protein export (Boyer *et al.*, 2009; Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). The T6SS delivers effector proteins to both bacterial and eukaryotic cells in a contact dependent manner, with the exported toxins harbouring enzymatic activity deleterious to the target cell (Russell *et al.*, 2014). There exists a significant diversity in the effector repertoire among T6SS-containing organisms, and delivery depends on the assembly of a core set of 13 conserved T6SS genes (Boyer *et al.*, 2009), called *tss* genes, as well as a variable number of T6SS-associated genes (*tag* genes), which are accessory components that often

contribute to regulation. Many T6SS components resemble bacteriophage proteins, leading to models whereby the T6SS apparatus mimics an inverted bacteriophage that assembles in the cytoplasm and contracts, eventually releasing its cargo (Basler *et al.*, 2012b). Indeed, structural and functional studies have provided support for this model (Basler *et al.*, 2012a; Pell *et al.*, 2009). In addition to secretion of effector proteins, contraction of the T6SS also leads to the export of two hallmark proteins, Hcp and VgrG. Hcp, one of the main components secreted by all functional T6SSs (Pukatzki *et al.*, 2009), assembles as a hexamer and bears similarity to phage λ tail protein gpV (Leiman *et al.*, 2009; Mougous *et al.*, 2006; Pell *et al.*, 2009). Detection of secreted Hcp in culture supernatants is a well established molecular marker for a functional T6SS (Pukatzki *et al.*, 2009). VgrG proteins are similar to the complex formed by gp27/gp5 of T4 phage (Leiman *et al.*, 2009) and can contain effector activity in extended C-terminal domains (Pukatzki *et al.*, 2007). Although Hcp and VgrG are often co-dependent for secretion and required for T6SS activity, multiple similar VgrG proteins are usually possessed by a single organism and each may not be essential for Hcp secretion (Brunet *et al.*, 2015; Cianfanelli *et al.*, 2016; Hachani *et al.*, 2011a; Pukatzki *et al.*, 2007). Recently, Hcp and VgrG have been shown to be necessary for distinct effector export pathways (Whitney *et al.*, 2014a). Hcp can interact with effector substrates via the internal residues of its ring-shaped structure (Silverman *et al.*, 2013), and PAAR-domain containing proteins can interact with the tip of some VgrG trimers (Shneider *et al.*, 2013). Furthermore, effectors are usually encoded nearby *vgrG* genes that are essential for secretion of that effector (Hachani *et al.*, 2014). These effectors may interact directly with the cognate VgrG or utilize adaptor proteins to facilitate their secretion (Flaugnatti *et al.*, 2015; Hachani *et al.*, 2014; Liang *et al.*, 2015; Unterweger *et al.*, 2015). Other important components include TssB and TssC proteins, which assemble cytoplasmic structures resembling a bacteriophage sheath (Bonemann *et*

al., 2009), and interchange rapidly between an extended and contracted state (Basler *et al.*, 2012b). The sheath formed by TssB/TssC, which requires a gp25-like baseplate protein TssE for assembly (Basler *et al.*, 2012b; Lossi *et al.*, 2011), may physically accommodate the Hcp tubule (Cascales *et al.*, 2012) and is recycled by another essential T6SS component, ClpV (TssH), upon contraction (Bonemann *et al.*, 2009). Mutagenesis and functional studies have confirmed the importance of the remaining T6SS components for apparatus function (Zheng *et al.*, 2011; Zheng *et al.*, 2007), including a membrane complex and other essential structural elements (Aschtgen *et al.*, 2010a; Brunet *et al.*, 2015; Durand *et al.*, 2015; Zoued *et al.*, 2016). Structures spanning the bacterial cell wall, for example flagella and type III secretion systems, often require dedicated peptidoglycan remodelling enzymes for their assembly (Scheurwater *et al.*, 2011). Despite the tremendous advances in our understanding of T6SS biogenesis in the past decade, it remains to be determined how this secretory system passes through the peptidoglycan (PG) layer of the T6SS-encoding organism.

It has recently become appreciated that many *Acinetobacter* spp. possess an antibacterial T6SS (Basler *et al.*, 2013; Carruthers *et al.*, 2013c; Repizo *et al.*, 2015; Weber *et al.*, 2015b; Weber *et al.*, 2013). However, individual strains vary in the expression of the T6SS, with some exhibiting robust T6SS activity and others possessing an apparently inactive system (Repizo *et al.*, 2015; Weber *et al.*, 2015b; Weber *et al.*, 2013). The T6SS of several *A. baumannii* strains is controlled by plasmid-encoded regulators, and, generally, multi-drug resistant strains do not show T6SS activity under laboratory conditions (Repizo *et al.*, 2015; Weber *et al.*, 2015b). Bioinformatic analysis has suggested the core T6SS components in *Acinetobacter* spp. are encoded in a single conserved locus, with *vgrG* genes being distributed in varying numbers throughout the genome (de Berardinis *et al.*, 2008b; Henry *et al.*, 2012; Weber *et al.*, 2013). While the *tssM, tssB,* and *hcp*

genes have been mutated and confirmed as essential for T6SS activity in *Acinetobacter*, the remaining genes in the T6SS cluster, as well as the *vgrG*'s, have not been experimentally tested for their contribution to T6SS (Carruthers *et al.*, 2013c; Repizo *et al.*, 2015; Weber *et al.*, 2013).

In this work, we analyzed the genetic requirements for the elaboration of a functional T6SS in *Acinetobacter* spp. Furthermore, we evaluate the contribution of *vgrG* proteins and effectors to apparatus function and antibacterial activity. Finally, we describe a novel structural component of the T6SS, a peptidoglycan degrading enzyme we termed TagX. We found that this enzyme is conserved across several genera and is essential for extracellular export of Hcp. By functioning as an L,D-endopeptidase, we propose that TagX performs the essential enzymatic step of cell wall degradation, allowing transit of the T6SS tubule.

4.2 Experimental Procedures

4.2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used for this study are listed in Table 4.1. Strains were grown in LB medium at 37 °C with shaking. The antibiotics ampicillin (50 µg/mL), kanamycin (50 µg/mL), rifampicin (150 µg/mL), and tetracycline (5 µg/mL) were added where necessary.

Table 4.1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
A. baylyi ADP1	wild type strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2678	mutant strain	
A. baylyi ADP1 ΔACIAD2680	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2681	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2682	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2683	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2684	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2685	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2686	mutant strain	(de Berardinis *et al.*, 2008a)

A. baylyi ADP1 ΔACIAD2687	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2688	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2689	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2690	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2691	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2693	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2694	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2695	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2696	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2697	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2698	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2699	mutant strain (*tagX*)	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2700	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2704	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2708	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2716	mutant strain	(de Berardinis *et al.*, 2008a)
A. baylyi ADP1 ΔACIAD2699 pEVL17-TagX	mutant strain, expressing *A. baylyi* TagX (ACIAD2699)	This study
A. baumannii ATCC17978 T6+	T6SS active strain	(Weber *et al.*, 2015b)
A. baumannii ATCC17978 T6-	T6SS inactive strain	(Weber *et al.*, 2015b)
A. baumannii ATCC17978 T6+ Δ*tssM*	mutant strain	(Weber *et al.*, 2015b)
A. baumannii ATCC17978 T6+ Δ*vgrG1*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG2*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG3*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,3*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG2,3*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG2,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG3,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2,3*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,3,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG2,3,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2,3,4*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG2,3,4*Δ*tse3*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,* pBAVMCS	mutant strain, vector control	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,* pBAVMCS-VgrG1	mutant strain, expressing VgrG1	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2,4,* pBAVMCS	mutant strain, vector control	This study
A. baumannii ATCC17978 T6+ Δ*vgrG1,2,4,* pBAVMCS-VgrG1	mutant strain, expressing VgrG1	This study
A. baumannii ATCC17978 T6+ Δ*tagX*	mutant strain	This study
A. baumannii ATCC17978 T6+ Δ*tagX* pBAVMCS	mutant strain with vector, Kan^R	This study
A. baumannii ATCC17978 T6+ Δ*tagX* pBAVMCS-TagX	mutant strain expressing TagX, Kan^R	This study
E. coli MG1655R	*E. coli* strain used for killing assays, Rif^R	(MacIntyre *et al.*, 2010)
pBAVMCS	cloning vector, Kan^R	(Scott *et al.*, 2014a)
pBAVMCS-VgrG1	Construct expressing 10His-tagged VgrG1	This study
pEXT20	Cloning Vector, Amp^R, IPTG inducible	(Dykxhoorn *et al.*, 1996)
pTdi	pEXT20 construct expressing *A. baumannii* Tdi, Amp^R, IPTG inducible	This study
pTagX	pEXT20 construct expressing *A. baumannii* TagX, Amp^R, IPTG inducible	This study
$pTagX^{D287N}$	pEXT20 construct expressing *A. baumannii* TagX mutant, Amp^R, IPTG inducible	This study
pBAVMCS-TagX	Construct expressing *A. baumannii* 6His-tagged TagX with pEXT20 promoter, Kan^R	This study
pEVL17-TagX	Construct expressing *A. baylyi* TagX, Amp^R	This study

4.2.2 RNAseq of T6+ and T6- cells

Triplicate cultures of T6+ and T6- *A. baumannii* ATCC 17978 were grown overnight, diluted into fresh medium and grown to OD_{600} 0.5. 300 µl of cells were lysed and RNA stabilized using the protocols and buffers for the RNAprotect Bacteria Reagent kit (Qiagen). RNA was purified using the RNeasy Mini Kit (Qiagen) and ribosomal RNA was depleted using the RiboZero kit (Illumina). Directional RNASeq libraries were constructed using the PrepX RNA-Seq for Illumina library kit (Wafergen) and sequenced using an Illumina NextSeq 500 as paired-end 75 base reads. Reads were mapped as paired-ends to the *A. baumannii* ATCC 17978-mff genome; raw counts and transcripts per million (TPM) values were calculated, and differentially expressed genes were identified using the DESeq2 package in R (Love *et al.*, 2014). RNAseq reads were deposited to the SRA with the following accession numbers: SRR3017659, SRR3017662, SRR3017665, SRR3017667, SRR3017669, SRR3017671

4.2.3 Sample preparation for whole cell proteomics

10mg of freeze dried bacterial cellular material was suspended in 4% SDS, 100mM TEAB pH8.0, 20mM DTT and boiled at 95˚C at 2000rpm for 10 min. Dried protein pellets were resuspended in 6 M urea, 2 M thiourea, 40 mM NH_4HCO_3 and reduced / alkylated prior to digestion with Lys-C (1/200 w/w) and then trypsin (1/50 w/w) overnight as previously described (Scott *et al.*, 2011). Digested samples were acidified to a final concentration of 0.5% formic acid and desalted with 50mg tC18 SEP-PAK (Waters corporation, Milford, USA) according to manufactures instructions. Briefly, tC18 SEP-PAKs were conditioned with buffer B (80% ACN,

0.1% formic acid), washed with 10 volumes of Buffer A* (0.1% TFA, 2% ACN), sample loaded, column washed with 10 volumes of Buffer A* and bound peptides eluted with buffer B then dried. For TMT labelling samples were resuspended in 100mM TEAB and 100ug of peptide used according to the manufacturer's instructions. Samples T6 + B1, T6 + B2, T6 + B3, T6 - B1, T6 – B2 and T6 – B3 were labelled with TMT label 126, 127, 128, 129, 130 and 131 respectively. After labelling samples were pooled and C18 StAGE Tips[2] were used to desalt the peptides prior to fractionation by basic reversed phase chromatography. Briefly, peptides were separated using an 1100 series high-performance liquid chromatography (HPLC) instrument with a Zorbax Extend-C18 column (1.0 x 50 mm, 3.5 µm, Agilent) and a flow rate of 100 µl/min. Following gradient was run: initial 5 min from 100% buffer A (5 mM ammonium formate, 2% acetonitrile, pH 10) to 6% buffer B (5 mM ammonium formate, 90% acetonitrile, pH 10), then in 2 min to 8% buffer B, followed by an increase to 27% buffer B in 38 min, to 31% B in 4 min, to 39% B in 4 min, to 60% B in 7 min and completed with a 4 min run at 100% buffer B and a 26 min gradient back to 100% buffer A. 100 µl fractions were collected in a 96 well plate with every eighth fraction combined to generate a total of 8 fractions which were concentrated by vacuum centrifugation and subjected to mass spectrometric analysis.

4.2.4 LC-MS/MS analysis

Purified peptides were resuspended in Buffer A* and separated on a EASY-nLC1000 system coupled to a LTQ-Orbitrap Velos (Thermo Scientific). Briefly, samples were loaded directly onto an in house packed 30 cm, 75 µm inner diameter, 360 µm outer diameter, ReproSil – Pur C_{18} AQ 3 µm (Dr. Maisch, Ammerbuch-Entringen, Germany). RP analytical separation was performed at 350 nl/min over a 180 min gradients altering the buffer B concentration from 0% to

32% B over 150 min, then from 32% B to 40% B in the next 5 min, then increased to 100% B over 2.5 min period, held at 100% B for 2.5 min, and then dropped to 0% B for another 20 min. The LTQ-Orbitrap Velos was operated using Xcalibur v2.2 (Thermo Scientific) with a capillary temperature of 275°C with data-dependent acquisition utilized switching between MS, and CID (NCE 35, activation Q, 0.25; activation time, 10 ms, AGC of 4×10^4) and HCD MS-MS (resolution 7.5k, NCE 45, AGC 2e5 and a maximum fill time of 200ms).

4.2.5 MS Data analysis

MS data were processed using MaxQuant (v1.5.3.28 (Cox *et al.*, 2008)). Database searching was carried out against the reference *A. baumannii* ATCC 17978 proteome (downloaded from uniprot, 16-11-14, 3799 proteins) and our recent re-sequencing genome *A. baumannii* ATCC 17978-mff proteome (Weber *et al.*, 2015b) with the following search parameters: carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, acetylation of protein N-terminal trypsin/P cleavage with a maximum of 2 missed cleavages. TMT-6 based quantitation was enable using the default TMT-6 settings with a MS tolerance of 6ppm, HCD MS/MS tolerance of 20 ppm CID MS/MS tolerance of 0.5 Da and a maximum false discovery rate of 1.0% for protein and peptide identifications were used. The resulting protein group output was processed within the Perseus (v1.5.0.9) analysis environment to remove reverse matches and common proteins contaminates.

4.2.6 Hcp secretion and bacterial killing

Detection of Hcp in normalized samples of whole cells and culture supernatants by Western blot was performed as described previously, using an anti-Hcp and anti-RNA polymerase

antibody, which was used as a lysis control (Biolegend) (Weber *et al.*, 2015b). Except where indicated, samples were collected from cells grown for 4 hours after 1:50 back dilution into fresh medium after overnight growth. Detection of Hcp in the strains complement with the VgrG1 expressing plasmids was done on strains after overnight growth.

Bacterial killing assays were performed as follows. Cultures of *A. baumannii* and *E. coli* MG1655R were grown overnight, and the *E. coli* were washed three times with fresh LB to remove rifampicin. The cells were then resuspended to OD_{600} 1.0, and 100µl of *E. coli* was mixed with 10µl of *A. baumannii*, and a 10µl sample was spotted onto a dry LB agar plate. After 4 hours incubation at 37°C, spots were cut from the agar and resuspended by mixing with 500µl LB broth. This mixture was 10-fold serially diluted and dilutions plated on rifampicin-containing LB agar to determine the colony forming units of *E. coli* remaining. Controls consisted of *E. coli* MG1655R mixed with rifampicin-sensitive *E. coli* top10 or LB medium. Experiments were performed twice in technical triplicate. For analysis of Tsi2, experiments were performed as above but using a tetracycline resistant *E. coli* strain containing either pEXT20 vector control or expressing pTdi. Competitions for these experiments were done at a 1:20 *A. baumannii* - *E. coli* ratio and *E. coli* enumerated on agar plates containing tetracycline.

4.2.7 Construction of *A. baumannii* mutants, cloning and Western blotting

Primers used in this study are listed in Table 4.2. Mutants were constructed using the method described previously (Tucker *et al.*, 2014). Briefly, an antibiotic resistance cassette was amplified using ~110bp oligonucleotide primers (Integrated DNA Technologies) with homology to the flanking regions of the targeted gene with an additional 3' 18-25 nucleotides of homology to the FRT site-flanked kanamycin cassette from plasmid pKD4. This PCR product was

electroporated into competent T6+ *A. baumannii* ATCC 17978 carrying pAT04, which expresses the Rec_{Ab} recombinase. Mutants were selected on 7.5 µg/ml kanamycin, and integration of the resistance marker was confirmed by PCR. To remove the kanamycin cassette, electrocompetent mutants were transformed with pAT03, which expresses the FLP recombinase to remove the FRT-flanked resistance cassette. Additional mutations were made by repeating this process, with PCR and sequencing used to confirm the validity of all mutants.

The *vgrG1* gene from *A. baumannii* ATCC 17978 was cloned with a 10-His tag into the BamHI and PstI sites of pBAVMCS using primers vgrG1FwdBamHI and vgrG1RevPstI10His. The *tagX* gene from *A. baylyi* was cloned into the PacI and NotI sites of pEVL174 vector (de Berardinis *et al.*, 2008b) using primers ACIAD2699-pacI and ACIAD2699-notI, and the *tagX* gene from *A. baumannii* ATCC 17978 was cloned into the BamHI and SalI sites of pEXT20 using primers tagXFwdBamHI and tagXFwdSalI. This construct was used as a template for point mutations. Cloning of the *A. baumannii tagX* into the expression vector pBAVMCS was performed using the primers tagXpromFwdPstI and tagXRevPstI6His with the pTagX vector used as template, in order to maintain the pEXT20 promoter for constitutive expression. The *tdi* gene was cloned into the KpnI/SalI sites of pEXT20 with a C-terminal FLAG tag using the primer pairs tdiFwd and tdiRev. Expression of proteins was confirmed by Western blot using a rabbit polyclonal anti-His and (Pierce) anti-FLAG (Sigma). Detection of primary antibodies was done with fluorescent secondary antibodies as previously described (Weber *et al.*, 2013).

Table 4.2 Primers used in this study

Primer name	sequence (5'-3')	Purpose
tagXFwdBamHI	ATA TGG ATC CAT GAT GAT TTT TCT AAT TTT CTT TTG TTT TGC	clone tagX into pEXT20 with 6-His tag
tagXRevSalI6His	ATA GTC GAC TTA GTG GTG ATG GTG ATG ATG ACT GAT GTC ATT TAA GCT TTC GGC	clone tagX into pEXT20 with 6-His tag
tagXpromFwdPstI	ATA CTG CAG TTG CGC CGA CAT CAT AAC GGT TCT GG	clong tagX into pBAVMCS with pEXT20 promoter, 6-His tag

tagXRevPstI6His	ATA CTG CAG TTA GTG GTG ATG GTG ATG ATG ACT GAT GTC ATT TAA GCT TTC GGC	clong tagX into pBAVMCS with pEXT20 promoter, 6-His tag
ACIAD2699-pac	CCCTTAATTAATGTTTAAAGCGCTACTTCCTCAGAGTAAACAAAAA C	clone *A. baylyi tagX* into pEVL17
ACIAD2699-not	TTTGCGGCCGCTTAACTGGCTGCATTGGCAGTTAATTGG	clone *A. baylyi tagX* into pEVL17
vgrG1FwdBamHI	ATA GGA TCC ATG CAG ATG AGT GTG TCG AGT ATA TTG G	clone vgrG1 into pBAVMCS with 10-His tag
vgrG1RevPstI10His	ATA CTG CAG TCA GTG GTG ATG ATG GTG GTG ATG GTG ATG ATG ATT TAC CAC ATG TAT TTT ATA TTG C	clone vgrG1 into pBAVMCS with 10-His tag
vgrG1KOFwd	TTA CTG GCG GCA CAT TAC TTA CTT TTC CAT GTG CTT TTT TTA ACA ATT CAG TCG CTT GTT TTT TTA TAT CTT TAT TGG CAT CTA TTA CAT TAT CCA TTT CAA TTA GCG ATT GTG TAG GCT GGA GCT GCT TCG	mutate vgrG1
vgrG1KORev	TTA GAC TAG TTA CAT TTT GAA AAT GAG TAT AGA TAG ACA GAT TAG TCT TTT TTT AAT TAA AAT ATG GCT TAA ATA TAA GTT TGA TTG ATA ATA AAA ATG CAG CAT ATG AAT ATC CTC CTT AGT TCC TAT TCC G	mutate vgrG1
vgrG2KOFwd	TTT CAG CGC CAT ACC AAA ACT GCT GTT GTA TTT CCG TAG GTC GTT CTG CGA TCT TTG ACC AGA ATT GTT GAT TTT GAG GAT CTG GCA TAA TTC TTT ATT CAG CGA TTG TGT AGG CTG GAG CTG CTT CG	mutate vgrG2
vgrG2KORev	TAG CCA TTA ACA TTG CAA TTG ATA TAA AAA ATA GAC AGA TCT GTA CGC ATT TTA TAA AAT ATA ATC GAT TAA GTC TCA ATT TAT AAC AAA CGT AAT GTT TCA TAT GAA TAT CCT CCT TAG TTC CTA TTC CG	mutate vgrG2
vgrG3KOFwd	AAG ATT ATA CTA TAT TTT ACA TTT GGT AAT TCT TTA TTT CTT TGA TTA TAA AAG ACT ATT GTA TAT TCA AAA ATA GTA TAA GGA TTC ATT ATA TCT TTA ACC AGC GAT TGT GTA GGC TGG AGC TGC TTC G	mutate vgrG3
vgrG3KORev	TAA GTT AGG GGA AGA TAA GCT AAT TTA TAT AAA TTA GAC AAT TCT GTC TGT TTT TTA TAA AAT ATC GAT GAA TAT GAC TCA GTT TTT ATA ATA ATG ATG CAT ATG AAT ATC CTC CTT AGT TCC TAT TCC G	mutate vgrG3
vgrG4KOFwd	AAA TTG ATA GAA ATA ACG CAT AAT TTT GTA GAC AGA TAG TTC TGT TTT TTA TAA AGT GCA GCC GTT TAT ATA TCC TTA TGA TTA TAA AAA AAT GCT TAG CGA TTG TGT AGG CTG GAG CTG CTT CG	mutate vgrG4
vgrG4KORev	ATA AGA TTA ACC CGT TAG GTC AGG TGG CCT AAC TTA AAT AAA AAA GTC TCC GAC AAA CTC GGT ACG GTT CAA TTT ATA ACT ATC ATT TAT TTT TTC ATA CAT ATG AAT ATC CTC CTT AGT TCC TAT TCC G	mutate vgrG4
tse2KOFwd	ATG ATG TCC TCT GTC ACC TCA ACT TTA TCA ATT GGT TGA CCA TAT TTC TCA TAA AAG TAA TCA ATA TCT TCT TGG TTC ATC TTA CTA CCC TCT ATT ACT TAG CGA TTG TGT AGG CTG GAG CTG CTT CG	mutate tse2
tse2KORev	AAG ACC AAA GTA AAG ATG AAT TTC CAG CTT TAG AAG TAG ATG ACT GGT TTA CTC AAC TTG GAA GCA GTA CAA AGA CTG GAA AAG AGG AAT AAA GAA TTA TGC CAC ATA TGA ATA TCC TCC TTA GTT CCT ATT CCG	mutate tse2
tse3KOFwd	TTA ACA AAA AAA CAT CAT CTT GAC TTA AAC CAA AAT AAT TCT GTC AGA TGT GCG GTT AAT TAA ACC AAA TCC TAT AGA AAC ATA TAA TAT AAT TAC ACA GCG ATT GTG TAG GCT GGA GCT GCT TCG	mutate tse3
tse3KORev	AAG ATT TGA AAC TGA TGG CTC AGA ACA AAT TTC CAT TCA TAT TTG TGA TGA AAA TGC TTC TAA ATA TAA ACT AGC GGC AAA AGG TTA AAG ATA TAA TGA ATC ATA TGA ATA TCC TCC TTA GTT CCT ATT CCG	mutate tse3
tagXKOFwd	TCC GGT TGA ACA AGA GGC TGA TGT GGA ACC ACC TAA AGC TTC GGT TGA TAT TAA GAT TAC ACG AAG TCC ATT TGC TGT GAA TTA GTT AAG TCA TGA TGA TTA GCG ATT GTG TAG GCT GGA GCT GCT TCG	mutate tagX
tagXKORev	AAT TTA TAC ATG GGA AAA AGC TGG GTT GAT TTC CCA GCT TGT CGA TCA CAT GAC TAC CGT TTT ACA TGA TCG CTT CCA ATC AGG CTC CTA GGT TTA ACT CAT ATG AAT ATC CTC CTT AGT TCC TAT TCC G	mutate tagX
$tagX^{D287N}$	GCAAAAGGGTATCAAAGTTATTTTCAATTTGGTTTAGCGGCAAATG TGGCCTTTAAGCG	point mutant of tagX

tdiFwd	ATT GGT ACC ATG AAC CAA GAA GAT ATT GAT TAC	cloning tdi
tdiRev	ATG TCG ACT CAC TTG TCG TCA TCG TCT TTG TAG TCG TTC TCA CCA TCC CAA TAC CAA TAC	cloning tdi

4.2.8 Point Mutations

The QuickChange site directed mutagenesis strategy was utilized to make the TagX point mutants. Briefly, the pEXT20 plasmid containing the 6x-His tagged version of TagX was used as the template for the PCR reaction. Complimentary oligonucleotides containing the desired mutation were used as primers and Pfu turbo was utilized for the extension reaction. After completion of the PCR, the products were digested with DpnI to degrade the template DNA and were then transformed into TOP10 cells. Sequencing was used to confirm mutations.

4.2.9 Purification of TagX and TagX point mutants

Overnight cultures of BL21 cells bearing the pEXT20 plasmid with TagX containing a C-terminal 6x-His tag were back diluted 1:100 into 1L of Luria Broth media and grown at 37^{o}C until OD_{600} ~0.6. The cultures were induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 3 hours and the cells were collected by centrifugation at 5,000 xg for 10 minutes at 4^{O}C. The cells were resuspended in purification buffer (25mM Tris, pH 7.5, 200mM NaCl, 10% glycerol) lysed and cell debris was removed by low speed centrifugation. The supernatant was collected and membranes were isolated by ultracentrifugation at 100,000 xg for one hour. The supernatant was removed and membranes were solubilized in resuspension buffer (purification buffer + 2% Triton X-100) to extract TagX from the membrane. The solution was ultracentrifuged again to remove the extracted membranes and the supernatant was loaded onto a Ni-NTA agarose column, washed

with purification buffer, buffer supplemented with 10 mM and 40 mM imidazole, and eluted with buffer supplemented with 300 mM imidazole.

4.2.10 Purification of peptidoglycan and zymograms

Peptidoglycan (PG) was isolated from *A. baumannii* ATCC 17978 according to the method described by Hoyle and Beveridge (Hoyle *et al.*, 1984). After inoculation into 1L cultures and growth overnight, cells were pelleted and resuspended in water at a density of 200 g/L followed by dropwise addition to an equal volume of boiling 8% SDS. Once all of the cell suspension was added, the mixture was boiled for three hours and then ultracentrifuged at 100,000 x g for one hour at room temperature in order to pellet the PG. The supernatant was removed and the pellet was washed five times with deionized water in order to remove residual SDS. The washed PG was lyophilized overnight until dry and then weighed in order to determine the yield. Purified TagX samples resusended in 2x Laemmli buffer were separated using 12% Bis-Tris SDS-PAGE containing 0.1% (w/v) peptidoglycan followed by washing with water for one hour at room temperature using multiple changes of the water in order to remove residual SDS. The gel was then equilibrated in renaturation buffer (10 mM Tris-HCl, pH 7, 0.1% Triton X-100, 10mM $ZnCl_2$) for one hour followed by the addition of fresh buffer and incubation at 37°C with agitation. Water was used to wash the gel, methylene blue stain (0.1% methylene blue, 0.01% KOH) was added, and the gel was incubated for one hour. Water washes were used to destain the gel until the bands of degraded PG were visible.

4.2.11 Limited proteolysis of TagX

Samples of purified TagX in purification buffer at a concentration of 15 μM were digested with proteinase K (200 μg/mL final concentration) on ice. The reaction was quenched after 0.25, 0.5, 0.75, 1, 2.5, 5, 10, 15, 20, 30, 40, 50, and 60 minutes by adding 5mM PMSF and 1x SDS-PAGE loading buffer. A control sample was added to 5mM PMSF and 1x SDS-PAGE buffer before the addition of proteinase K. Once the digestion was complete, the samples were analyzed by western blot and zymogram as described above.

4.2.12 Cellular Localization of TagX

Overnight cultures of *A. baumannii* ATCC 17978 cells expressing TagX from pBAVMCS were diluted 1:100 and grown to OD_{600}0.6 was reached. The cells were then pelleted and 2 OD's were resuspended in 100 μL of lysis buffer (20% sucrose, 30mM Tris-HCl, pH8.5, 1 mM EDTA, 1 mg/mL lysozyme) and incubated on ice for one hour. The samples were centrifuged at 21,000 x g for five minutes to pellet spheroplasts containing the cytoplasmic and inner membrane fractions. The periplasmic and outer membrane fraction (supernatant) was removed and the spheroplasts were resuspended in 100 μL of 0.1M Tris-HCl pH=8.5 followed by freezing in liquid nitrogen and then thawing for ten minutes at 37°C. This step was repeated two more times in order to efficiently lyse the spheroplasts. Following lysis, centrifugation was performed for five minutes at 2,000 x g in order to pellet whole cells. The supernatant was removed and then centrifuged for five minutes at 21,000 x g to separate the membrane fraction (pellet) from the cytoplasmic fraction (supernatant). The samples were resuspended in 2x Laemmli buffer and western blot analysis was performed using antibody against the 6x-His tag to detect TagX and RNA polymerase as a control.

4.2.13 Analysis of muropeptides

Purified peptidoglycan or PG digested with TagX was suspended in 10 mL of 10 mM Tris-HCl (pH 7.2) and digested with 100 mg/L of α-amylase (Sigma-Aldrich, St. Louis, MO) for one hour at 37°C. This was followed by digestion with 100 mg/L of pre-activated pronase E (Merck, Darmstadt, Germany) at 60°C for 90 min. Amylase and pronase were inactivated by boiling for 20 minutes in 1% SDS and the peptidoglycan was collected and washed as described above for the purification. The collected peptidoglycan was digested with 100 g/mL cellosyl muramidase (Hoechst AG, Frankfurt, Germany) in 50 mM phosphate buffer (pH 4.9) at 37°C for 16 hours. The enzyme was inactivated by boiling for 10 minutes in a water bath and was then centrifuged at 14,000 rpm for 5 minutes to remove insoluble debris. The supernatant was mixed with 1/5 volume of 0.5 M sodium borate buffer (pH 9.0) and reduced with excess sodium borohydride (NaBH4) for 30 min at room temperature. The pH was measured with indicator strips (Acilit; Merck) and was adjusted to pH 3.0 with orthophosphoric acid. The samples were filtered (Millex-GV filters, 0.22 μm pore size, 2.5-mm diameter; Millipore, Cork, Ireland) and were injected into the HPLC. Separations were performed on a Breeze 2 HPLC system, consisting of a 1525 binary HPLC pump model code 5CH (Waters), a UV-visible detector 2489 (Waters), a manual injector model 7725i (Rheodyne), and an Aeris Peptide XB-C18, 3.6 μm, 250 by 4.6 mm reverse-phase column (Phenomenex). The column was equilibrated at 45°C and separation of individual muropeptides (detection wavelength of 204 nm) was performed in a linear gradient. The mobile-phase (A 50 mM sodium phosphate [pH 4.35]; B 75 mM sodium phosphate, 15% methanol [pH 4.95]) gradient consisted of elution at 1.0 ml/min with 100% A for 5 minutes, followed by a 60-minute linear gradient to 0% A/100% B and then 100% B for 5 minutes.

Molecular weight analysis of isolated muropeptides by MS with a Lineal Ionic Tramp Model LTQ-VELOS from Thermo-Scientific was carried out in the CBMSO Protein Chemistry Facility that belongs to ProteoRed, PRB2-ISCIII.

4.2.14 Data Availability

RNAseq data was deposited to the NCBI Short Read Archive with the following accession numbers: SRR3017659, SRR3017662, SRR3017665, SRR3017667, SRR3017669, SRR3017671

4.3 Results

4.3.1 Transcriptomic and genetic analysis reveals novel genes required for T6SS in *Acinetobacter*

While the proteins required for assembly and function of the T6SS have been investigated in detail in some bacteria, little is known about the requirements for T6SS in *Acinetobacter*. We recently described a novel mechanism of T6SS regulation possessed by several strains of *A. baumannii* (Weber *et al.*, 2015b). In these strains, plasmid-encoded regulators repressed the chromosomally encoded T6SS. Upon spontaneous plasmid loss, and therefore loss of the repressors, the T6SS was activated and functioned as an antibacterial apparatus. However, the precise transcriptional changes that occurred upon plasmid loss were not investigated. We used RNAseq to probe the changes in gene expression between plasmid-containing (T6-) and plasmid-less (T6+) *A. baumannii* ATCC 17978 cells in order to define the components required for a functional T6SS. This transcriptomic data revealed the genes in the previously predicted T6SS cluster that were upregulated in T6+ cells (Table 4.3). The transcriptomic changes were significant, with up to nearly 50-fold upregulation of some genes. Furthermore, distantly encoded *vgrG* gene clusters also showed significant changes in their transcriptional profile (see below). We then

confirmed these changes at the protein level by a whole cell differential proteomics approach (Table 4.3). Of the 21 genes identified as statistically differentially expressed by RNAseq in the main T6SS cluster, we identified and quantified, based on at least two unique peptides, 18 of these proteins, all of which were statistically different for T6+ cells compared to T6-. These data prompted us to genetically dissect the contribution of these proteins to the assembly of the *Acinetobacter* T6SS.

Table 4.3 Transcriptomic and differential proteomic analysis of T6SS and VgrG clusters in T6+ and T6- *A. baumannii* ATCC 17978

Gene locus	T6SS gene	T6+ TPM (std. dev)	T6- TPM (std. dev)	Log_2 fold-change T6+ vs T6- (p-value)	RNAseq Transcript Fold-change T6+ vs T6-	Corresponding gene in *A. baylyi* ADP1	TMT based protein Log_2 fold changes T6+ vs T6- (p-value)	Number of peptides identified (Andromeda Identification score)
T6SS cluster								
ACX60_11600	*tagX*	37.2 (3.2)	3.4 (1.0)	3.1 (3.3E-28)	8.3	ACIAD2699	NaN	NaN
ACX60_11605	hypothetical	63.0 (17.0)	2.7 (0.5)	4.0 (2.8E-32)	15.5	ACIAD2698	1.8 (0.00041)	3 (22.204)
ACX60_11610	*tssL*	91.3 (18.4)	4.5 (2.1)	3.9 (6.5E-46)	14.7	ACIAD2697	1.2 (0.028232)	1 (2.4722)
ACX60_11615	*tssK*	259.6 (14.1)	10.8 (1.6)	4.3 (7.8E-158)	19.8	ACIAD2696	1.7 (0.011007)	6 (76.533)
ACX60_11620	*tssA*	221.9 (26.5)	5.2 (0.8)	5.0 (3.8E-134)	32.8	ACIAD2695	2.6 (0.001157)	5 (21.411)
ACX60_11625	*clpV*	258.7 (8.6)	23.1 (2.0)	3.3 (4.8E-144	9.5	ACIAD2694	2.5 (0.002065)	35 (323.31)
ACX60_11630	PAAR	39.2 (15.7)	5.0 (0.9)	2.3 (8.0E-08)	5.1	ACIAD2681	0.9 (0.066403)	1 (12.532)
ACX60_11635	*tagN*	99.4 (9.3)	8.9 (1.5)	3.2 (1.9E-48)	9.0	ACIAD2682	1.5 (0.004644)	6 (54.441)
ACX60_11640	*tagF*	44.4 (12.6)	3.3 (0.8)	3.3 (2.6E-28)	9.9	ACIAD2683	1.7 (0.005088)	4 (22.213)
ACX60_11645	*tssM*	320.3 (16.8)	23.3 (0.8)	3.5 (3.2E-194)	11.7	ACIAD2684	1.8 (0.029757)	35 (323.31)
ACX60_11650	hypothetical	89.3 (13.9)	4.1(1.2)	4.1 (6.8E-72)	17.1	ACIAD2685	1.2 (0.000429)	2 (28.15)
ACX60_11655	*tssG*	179.8 (32.2)	4.5 (0.3)	4.9 (3.2E-99)	30.1	ACIAD2686	1.4 (0.000083)	2 (4.6976)
ACX60_11660	*tssF*	134.9 (28.3)	4.5 (0.5)	4.6 (1.1E-103)	23.5	ACIAD2687	2.7 (0.018475)	4 (23.9)
ACX60_11665	*tssE*	1078.5 (165.7)	30.2 (2.3)	4.8 (3.2E-167)	28.7	ACIAD2688	NaN	NaN
ACX60_11670	*hcp*	11801.2 (588.2)	198.8 (18.9)	5.6 (0)	49.4	ACIAD2689	2.8 (0.000359)	11 (139.41)
ACX60_11675	*tssC*	1823.6 (258.4)	30.5 (1.6)	5.6 (0)	49.0	ACIAD2690	2.7 (0.000598)	23 (323.31)
ACX60_11680	*tssB*	1516.7 (145.5)	34.7 (7.8)	5.1(3.3E-206)	35.2	ACIAD2691	3.1 (0.000546)	14 (253.32)
ACX60_11685	hypothetical	545.2 (6.2)	9.3 (0.6)	5.5 (8.2E-202)	46.3	ACIAD2693	2.7 (0.001271)	9 (235.49)
ACX60_11690	hypothetical	600.3 (50.0)	125.4 (19.9)	2.0 (8.7E-43)	4.0	ACIAD3112/3113	1.1 (0.002987)	18 (321.94)
ACX60_11695	*tse3* effector	167.0 (37.0)	14.6 (0.2)	3.2 (2.6E-77)	9.5	ACIAD3114	NaN	NaN
ACX60_11700	*vgrG3*	96.3 (10.7)	47.8 (1.4)	0.8 (1.2E-08)	1.8	ACIAD3115	1.2 (0.013778)	13 (1.2162)

ACX60_11705	hypothetical	6.0 (2.3)	2.7 (0.6)	0.7 (3.0E-01)	1.6	none	0.1 (0.646629)	1 (1.8381)
VgrG1 cluster								
ACX60_17640	hypothetical	16.2 (3.2)	3.4 (2.3)	1.7 (2.8E-4)	3.2	none	NaN	NaN
ACX60_17645	hypothetical	9.6 (40)	0.4 (0.4)	2.6 (7.9E-6)	6.0	none	NaN	NaN
ACX60_17650	hypothetical	34.8 (12.5)	1.5 (1.0)	3.5 (4.8E-15)	11.6	none	NaN	NaN
ACX60_17655	hypothetical	37.6 (5.3)	2.0 (1.2)	3.5 (3.8E-19)	11.1	none	1.7(0.010153)	7 (67.368)
ACX60_17660	*tse1*	328.8 (53.3)	12.0 (1.7)	4.4 (2.0E-117)	21.6	none	2.3 (0.003947)	1 (1.6312)
ACX60_17665	*vgrG1*	203.4 (27.4)	36.5 (1.0)	2.2 (9.11E-59)	4.8	multiple *vgrG*'s	2.4 (0.011138)	16 (222.15)
ACX60_17670	hypothetical	6.7 (2.9)	1.4 (0.3)	1.6 (2.7E-3)	3.0	none	NaN	NaN
VgrG2 cluster								
ACX60_15360	*tsi2* (*tdi*)	192.3 (17.0)	103.2 (2.9)	0.7 (2.3E-7)	1.6	none	0.1 (0.350265)	9 (114.72)
ACX60_15365	*tse2* (*tde*)	158.9 (17.2)	64.0 (3.3)	1.1 (1.8E-15)	2.1	none	0.4 (0.135599)	8 (65.825)
ACX60_15370	*vgrG2*	143.1 (15.3)	200.4 (13.8)	-0.7 (2.7E-7)	-1.6	multiple *vgrG*'s	1.0 (0.049891)	12 (33.815)
VgrG4 cluster								
ACX60_00585	*vgrG4*	65.5 (9.4)	23.2 (2.6)	1.3	2.4	multiple *vgrG*'s	NaN	NaN
ACX60_00590	hypothetical	1.3 (0.2)	3.9 (0.1)	-0.7	-1.6	none	NaN	NaN
ACX60_00595	hypothetical	1.8 (1.8)	6.2 (4.1)	-0.3	-1.2	none	NaN	NaN
ACX60_00600	hypothetical	0.4 (0.7)	0.0 (0.0)	0.2	1.1	none	NaN	NaN
ACX60_00605	*tse4* (*tae*)	13.0 (1.6)	8.2 (2.1)	0.5	1.4	none	NaN	NaN
ACX60_00610	*tsi4*	19.2 (8.6)	8.8 (3.1)	0.8	1.8	none	0.2 (0.283492)	10 (105.54)
ACX60_00615	hypothetical	5.4 (0.7)	3.2 (1.4)	0.5	1.4	none	NaN	NaN

Recently, a complete collection of null mutants was constructed in non-pathogenic *A. baylyi* ADP1, which included genes that encode proteins expected to be involved in T6SS apparatus assembly and activity (de Berardinis *et al.*, 2008a). *A. baylyi* ADP1 possesses a T6SS that secretes the conserved Hcp protein under standard laboratory growth conditions (Weber *et al.*, 2013), and it was previously demonstrated that this secretion system contributes towards antibacterial activity against *E. coli* (Basler *et al.*, 2013). Furthermore, the genes in this cluster are highly conserved across all T6SS-encoding *Acinetobacter* spp. (Weber *et al.*, 2013). Using our transcriptomic data as a guide, we tested several mutants within, and surrounding, the predicted T6SS cluster, for ADP1 T6SS function (Fig. 1A). Hcp secretion was abolished in 14 of the mutants,

which clustered in a 15-gene tract from ACIAD2684-ACIAD2699 (Fig. 1B) and confirms that this cluster comprises the core T6SS genes in this organism. The *tagN* (ACIAD2682), *tagF* (ACIAD2683) and ACIAD2698 mutants secreted Hcp to a greater extent than wild type. While ACIAD2698 has no homologs outside of the genus *Acinetobacter*, TagN contains a PG binding domain and is present in *Burkholderia* T6SS-1 (Aschtgen *et al.*, 2010b) and TagF is a repressor of the *Pseudomonas aeruginosa* H1-T6SS (Silverman *et al.*, 2011).

Excluding the *hcp* mutant itself, most mutant strains expressed levels of Hcp comparable to wild type ADP1 when analyzed by Western blot, indicating that a lack of expression was not a factor in the loss of Hcp secretion (Fig. 1B). However, *tssB and tssC* mutants expressed a detectably lower level of Hcp, which could account for the loss of Hcp secretion. As *tssB* and *tssC* are located upstream of *hcp*, it is possible that mutation of these genes affected downstream expression of *hcp*. However, the *tssB* and *tssC* genes are both essential for Hcp secretion in other bacteria (Zheng *et al.*, 2011). ACIAD2685, ACIAD2693, and ACIAD2699 do not have characterized homologs in other T6SS-encoding organisms, indicating these represent novel genetic components of the *Acinetobacter* T6SS. ACIAD2685 is predicted to be a membrane protein, and ACIAD2693 is predicted to encode a signal peptide, and therefore likely functions in the periplasm. Further analysis of ACIAD2699 indicated that the protein encoded by this gene may play an important role for T6SS transit through the peptidoglycan layer (see below).

These results establish the core components of the T6SS in *A. baylyi* ADP1, and, considering their conserved nature in this genus (Weber *et al.*, 2013), offer insight into necessary T6SS components in other *Acinetobacter* spp.

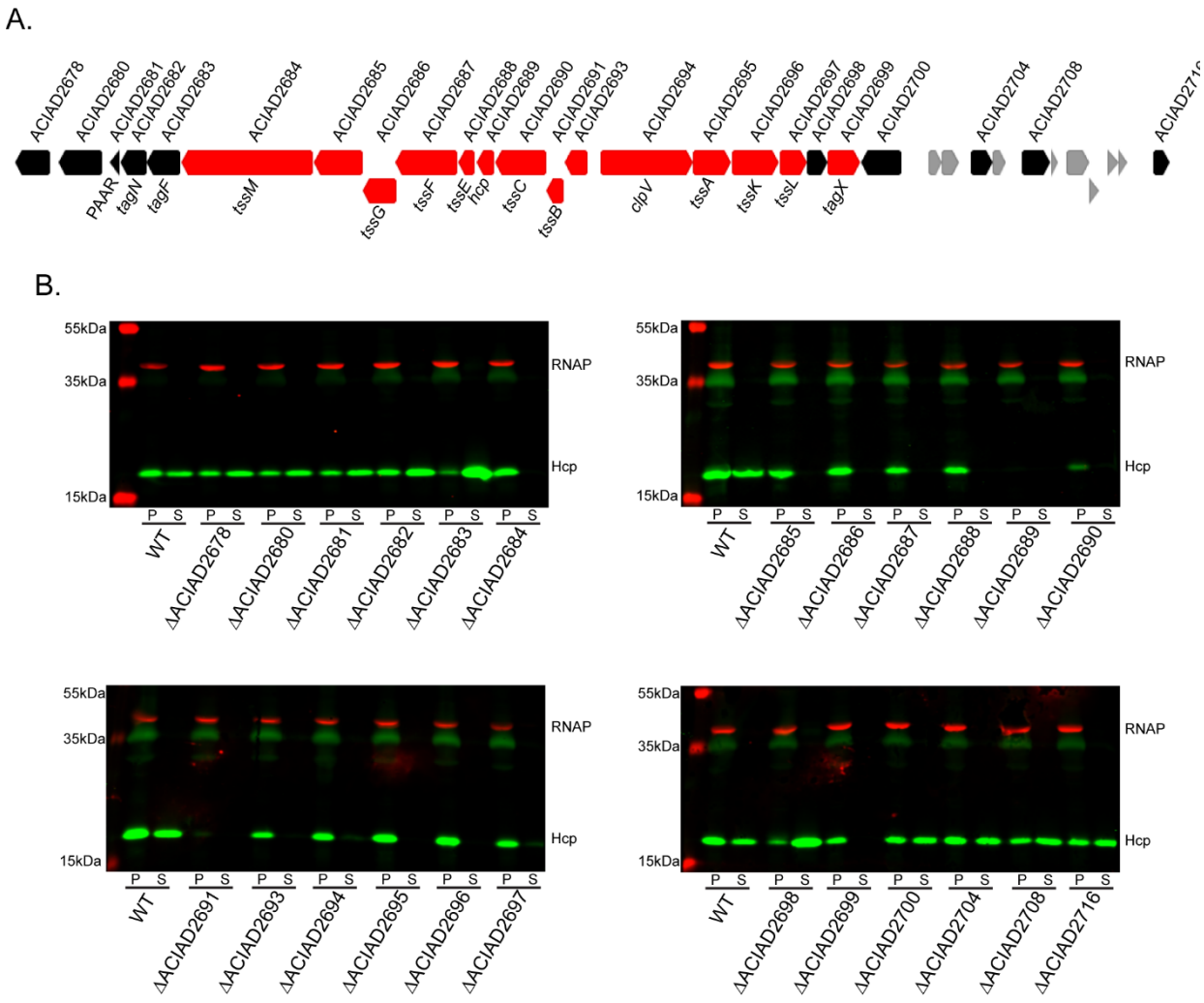

Figure 4.1 Genetic requirements for T6SS in *A. baylyi* ADP1

A) Genetic organization of the T6SS locus in *A. baylyi* ADP1. Locus numbers are indicated above with relevant T6SS nomenclature shown below. Genes highlighted in red are essential for Hcp secretion, and those in black are not essential or act to enhance secretion. Genes in grey were not tested. B) Western blots probing for Hcp expression and secretion in whole cell pellets (P) or supernatants (Sup) of the indicated *A. baylyi* ADP1 strains. Numbers represent the ACIAD gene numbers. RNA polymerase (RNAP) is used as a loading and lysis control.

4.3.2 Establishing the contribution of VgrG proteins to apparatus formation and bacterial killing identifies important antibacterial effectors

VgrG proteins are secreted T6SS components that can be essential for forming a functional T6SS, but also play a role in secretion of other components (Hachani *et al.*, 2014; Pukatzki *et al.*, 2006; Shneider *et al.*, 2013). These proteins can mediate secretion of downstream encoded effectors or encode effector functions in extended C-terminal domains (Hachani *et al.*, 2014; Pukatzki *et al.*, 2007; Pukatzki *et al.*, 2009). The number of *vgrG* genes encoded by *Acinetobacter* spp. varies but is generally between two and four (Fig. 2) (Weber *et al.*, 2013). *A. baumannii* ATCC 17978 contains four VgrG proteins (VgrG1-4), none of which contain an identifiable effector domain. However, near each *vgrG* gene is a putative effector, suggesting that these VgrG's act as adaptors for the secretion of cognate effectors (Figure 4.2). Indeed, all VgrG proteins in *A. baumannii* ATCC 17978 contain a C-terminal DUF2345 domain, which has been shown to play a role in stabilizing VgrG-effector interactions (Flaugnatti *et al.*, 2015). Our bioinformatic analysis indicated that *A. baumannii* possess a diverse repertoire of effectors encoded in *vgrG* clusters, with some seemingly susceptible to transposase integration (ex. strain AYE) (Figure 4.2). The predicted effector downstream of *A. baumannii* ATCC 17978 *vgrG1* contains domains associated with lipases, and several type VI lipase effectors (Tle) have been previously shown to target lipidic substrates accessible from the periplasm, with cognate immunity proteins located in the periplasm (Russell *et al.*, 2013). Furthermore, this putative effector contains a GXSXG motif typical of some Tle family members (Russell *et al.*, 2013). This suggests that the enzyme encoded downstream of *vgrG1* is a Tle, and immunity would be encoded by a nearby protein. Immunity proteins are typically more difficult to predict *in silico*; however, the protein of unknown function encoded by ACX60_17670 contains a signal peptide directing it to the periplasm, which is the expected site of activity for Tle (Figure 4.2). The predicted effector encoded near *vgrG2* contains a nuclease domain and the downstream immunity protein contains a GAD-like and DUF1851 domain. A

similar nuclease (type VI Dnase effector, Tde) and immunity pair were found in *Agrobacterium tumefaciens,* with the Tde effector being delivered in a T6SS-dependent manner (Ma *et al.*, 2014). Indeed, these authors bioinformatically identified this *A. baumannii* effector as a member of the Tde superfamily, containing the requisite HXXD catalytic motif. The predicted effector protein in the *vgrG3* cluster did not show homology to any known effector domains, but our experimental data suggest it plays an important role in bacterial killing (see below). While T6SS effectors are generally named based on their characterized biochemical activity, in the absence of such data we have tentatively identified this gene as a *tse* (type six effector). The effector downstream of *vgrG4* contains a LysM peptidoglycan binding motif and a M23 peptidase family domain, indicating it likely targets the peptidoglycan, which is a common target for T6SS Tae effectors (Russell *et al.*, 2011). Furthermore, the predicted immunity protein contains a signal peptide, which would indicate it interacts with an effector targeted to the periplasm.

From our RNAseq and differential proteomic analysis, we found that most of the *vgrG* genes and their associated cluster were upregulated in T6+ *A. baumannii* (Table 4.3). The *vgrG3* gene, which is encoded upstream of the main T6SS cluster, was upregulated 1.8-fold, while *tse* transcripts increased 9.5-fold. Similarly, transcripts of *vgrG1* and its associate *tle* increased by 4.8- and 21.6-fold, respectively. Interestingly, *vgrG2* expression decreased by 1.6-fold, while the effector and immunity genes were upregulated by 2.1- and 1.6-fold, respectively. However, expression of this cluster was robust in both T6+ and T6- cells, as assessed by transcripts per million (TPM) counts, which may suggest the cluster containing *vgrG2* is regulated in a different manner. We found a significant increase in transcription of *vgrG4* (2.4-fold upregulated) in T6+ cells, however the changes in expression of the predicted downstream effector and immunity proteins, *tae* and *tai* (increased 1.4- and 1.8-fold, respectively) did not reach statistical significance.

Collectively, these data support the notion that many of these *vgrG* clusters are co-regulated with the core T6SS in this organism.

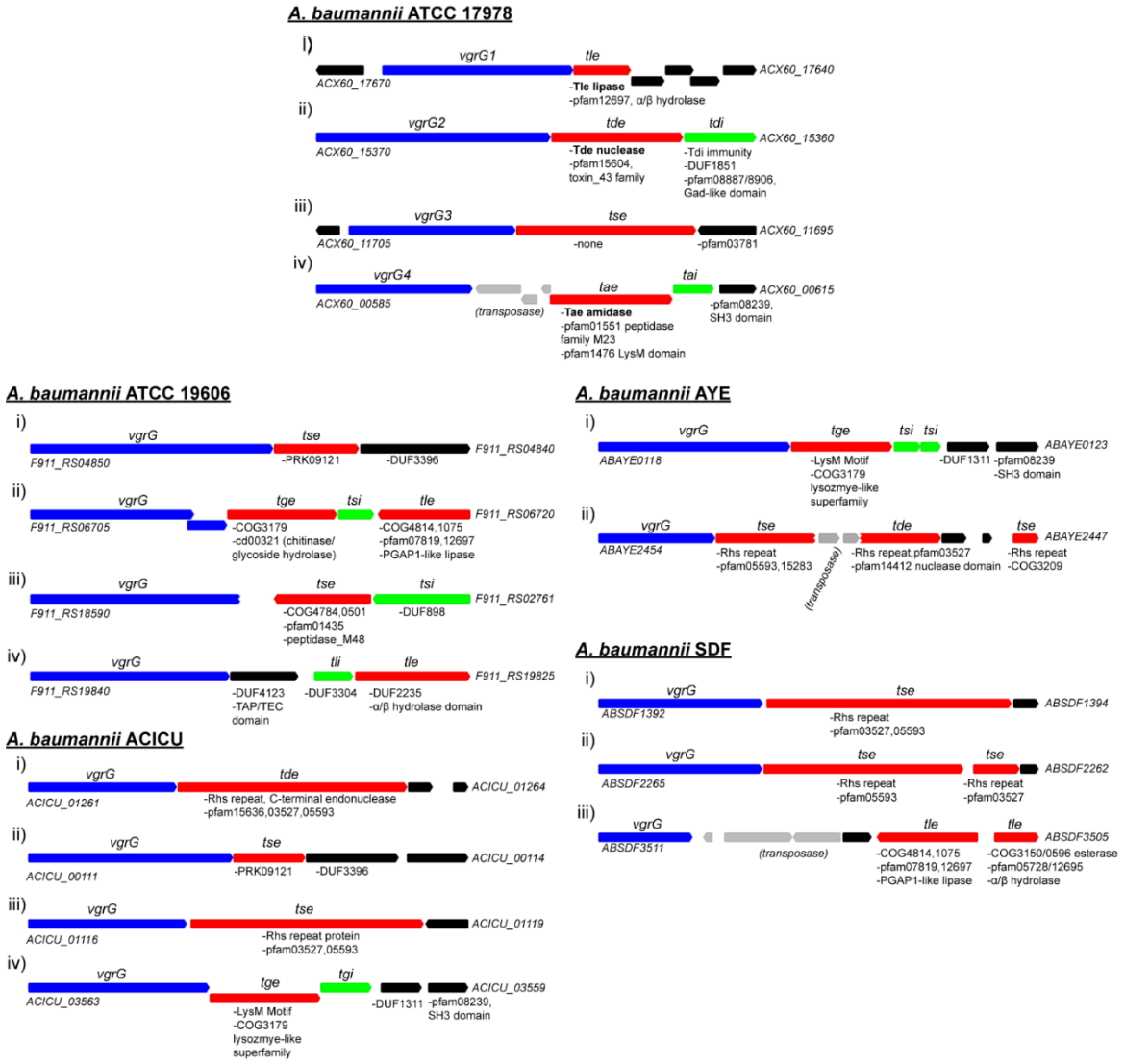

Figure 4.2 Schematic of the *vgrG* clusters present in selected *A. baumannii* strains.

The *vgrG* genes are shown in blue, the putative *tse* effectors in red, and the *tsi* immunity genes in green. Locus tags are shown for the first and last gene of each cluster, and conserved domains are indicated below genes where relevant.

With the core components of the T6SS delineated, we sought to determine the role of these VgrG proteins in *A. baumannii* T6SS function. Using a recently described mutagenesis strategy (Tucker *et al.*, 2014), we generated a complete series of mutants lacking one, two, three, or all four *vgrG* genes in every possible combination in the *A. baumannii* ATCC 17978 T6+ background. These strains were then assessed for their ability to express and secrete Hcp, as well as their ability to kill *E. coli* prey cells (Figure 4.3). As a control for Hcp secretion and *E. coli* killing, we used a mutant in the essential *tssM* gene, which prevents apparatus assembly and T6SS-mediated bacterial killing (Weber *et al.*, 2013).

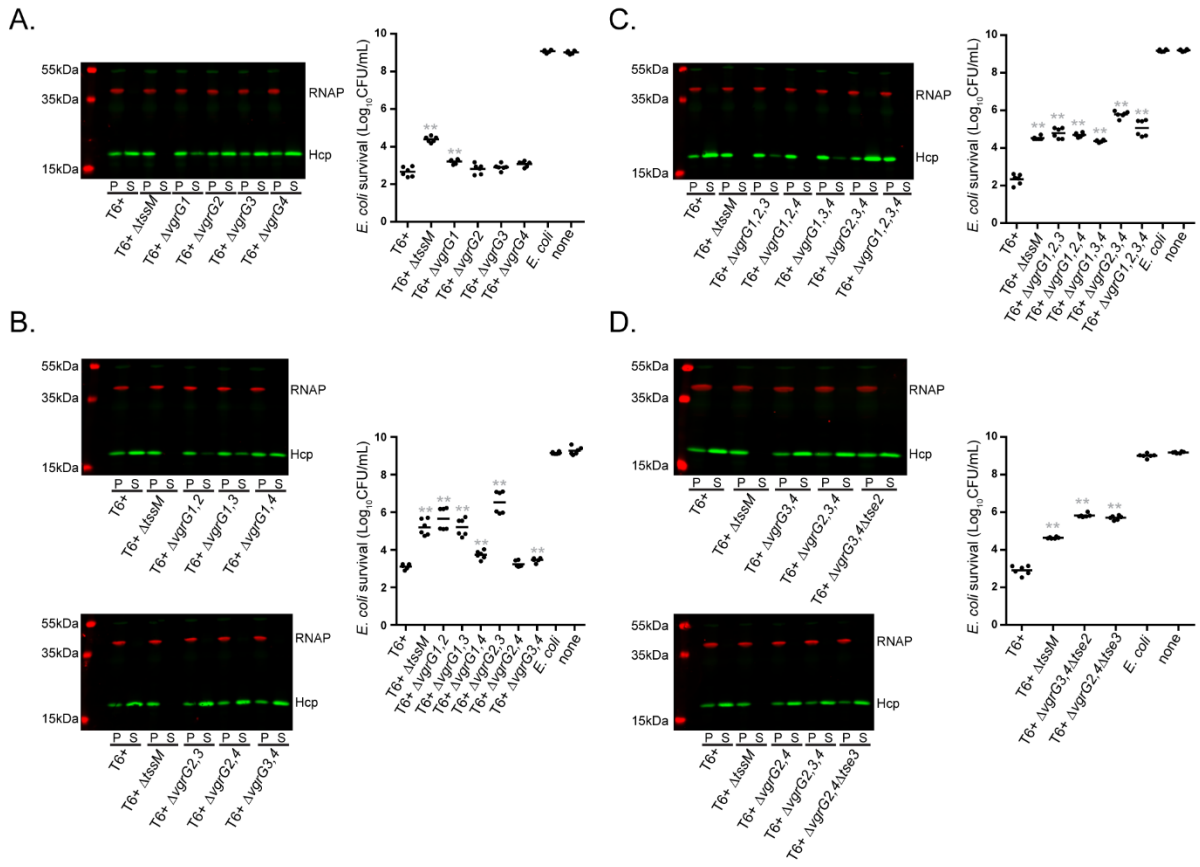

Figure 4.3 Hcp secretion and bacterial killing by T6+ *A. baumannii* ATCC 17978 *vgrG* mutants.

Western blots probing for Hcp and RNA polymerase (RNAP), and *E. coli* killing assays were performed with A) single *vgrG* deletion mutants, B) double *vgrG* deletion mutants, C) triple and quadruple *vgrG* deletion mutants, and D) *tse2* and *tse3* effector mutants in the Δ*vgrG3,4* or Δ*vgrG2,4* backgrounds, respectively. For the bacterial killing assay graphs, the indicated *A. baumannii* predator strains are listed on the x-axis, with the log-transformed surviving *E. coli* MG1655R CFU's on the y-axis. A drug sensitive strain of *E. coli*, or no predator (none), were used as controls. The results shown are from two independent experiments, performed in triplicate. Stars indicate statistical significance compared to wild typeT6+ (unpaired, two-tailed Student's t-test: **P<0.01)

The results of our Hcp secretion and bacterial killing experiments with the full suite of *vgrG* mutants are summarized in Table 4.4. Based on our data, *vgrG1* can be considered the most significant contributor to Hcp secretion in T6+ *A. baumannii*. When *vgrG1* was mutated, secretion of Hcp was severely impaired; in all other single *vgrG* mutants, Hcp was secreted to wild type

levels (Figure 4.3A). Double *vgrG* mutants in which *vgrG1* was deleted secreted markedly less Hcp than wild type, although there were subtle differences between Δ*vgrG1,2,* Δ*vgrG1,3,* and Δ*vgrG1,4* (Figure 4.3B). The remaining double mutants, all of which contained a functional copy of *vgrG1*, did not show any Hcp secretion defect. Minimal amounts of Hcp were detectable in the supernatants from the Δ*vgrG1,2,3* and Δ*vgrG1,3,4* triple mutants, which suggests that VgrG4 or VgrG2 are required for low levels of Hcp secretion observed in the absence of VgrG1 (Figure 4.3C). A total lack of Hcp in supernatants from the Δ*vgrG1,2,4* mutant indicated that VgrG3 does not play a role in Hcp secretion. Interestingly, Hcp secretion was identical to wild type bacteria in the Δ*vgrG2,3,4* strain, which solely contains VgrG1. Expression of VgrG1 from a plasmid in either the Δ*vgrG1* or Δ*vgrG1vgrG2vgrG4* background reconstituted Hcp secretion, confirming the essential role of VgrG1 for Hcp export (Figure 4.4). As expected, no Hcp was secreted by the strain lacking all four *vgrG* genes (Figure 4.3C).

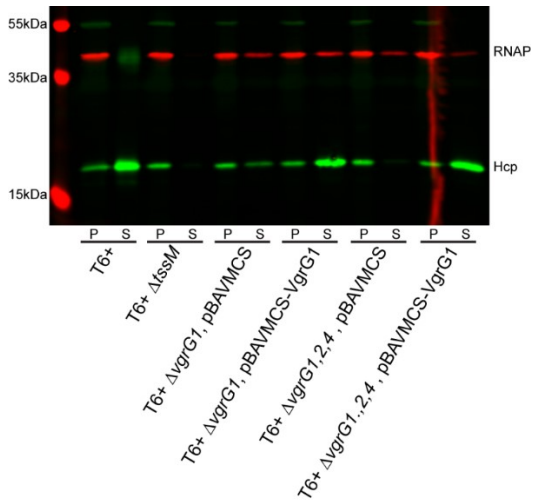

Figure 4.4 Complementation of Δ*vgrG1* mutation

Whole cell pellets (P) and supernatants (S) from overnight grown samples were separated by SDS-PAGE and probed with anti-His and anti-RNAP antibodies.

Assessment of antibacterial activity in the various *vgrG* mutant strains revealed that deletion of *vgrG1* had a small but significant effect on *E. coli* survival, while the remaining single *vgrG* mutants had no appreciable bacterial killing defect when compared to wild type (Figure 4.3A). This result is consistent with the Hcp secretion defect in the Δ*vgrG1* mutant strain. Many of the double *vgrG* mutants were severely impaired in their ability to kill *E. coli*. The Δ*vgrG1,2* and Δ*vgrG1,3* mutants were attenuated to the level of the Δ*tssM* strain, while Δ*vgrG1,4* had a less pronounced yet still significant defect (Figure 4.3B). This result mirrors their corresponding Hcp secretion phenotypes. Of the remaining double mutants, each of which retained wild type levels of Hcp secretion, Δ*vgrG2,3* was particularly defective in bacterial killing. In fact, *E. coli* survived to a greater extent when incubated with this mutant than with the Δ*tssM* strain. We found a small but statistically relevant loss of bacterial killing by the Δ*vgrG3,4* strain, whereas no phenotypic difference was detected between Δ*vgrG2,4* and wild type bacteria. All triple mutants, regardless of the combination, were unable to kill *E. coli* in a T6SS-dependent manner (Figure 4.3C). Of particular note is the loss of bacterial killing by the Δ*vgrG2,3,4* strain, which was the only triple mutant able to secrete Hcp to wild type levels, suggesting that any effectors requiring VgrG1 for export are not sufficient for killing *E. coli.* Predictably, mutation of all four *vgrG* genes abrogated bacterial killing (Figure 4.3C).

The above experiments showed that Δ*vgrG2,4* and Δ*vgrG3,4* mutant strains retained wild type (or near wild type) levels of Hcp secretion and antibacterial activity, while the Δ*vgrG2,3,4* strain secreted Hcp to similar levels but was unable to kill *E. coli*. This suggested that the putative effectors encoded near *vgrG2* and *vgrG3*, Tde and Tse, respectively, may play a key role in bacterial killing. We therefore constructed the Δ*vgrG3,4*Δ*tde* and Δ*vgrG2,4*Δ*tse* mutants, and

assessed the ability of these strains to secrete Hcp and kill *E. coli*. While these mutants secreted wild type levels of Hcp, neither was able to kill *E. coli*, indicating that Tse2 and Tse3 are important mediators of antibacterial activity (Figure 4.3D). Furthermore, expression of Tdi in *E. coli* was protective against killing by the Δ*vgrG3,4* strain, confirming the Tde/Tdi as an effector/immunity pair (Figure 4.5)

Table 4.4 Summary of Hcp secretion and bacterial killing data in Figure 4.3

A. baumannii ATCC 17978 Strain*	Hcp secretion*	Bacterial killing*
T6+	++++	++++
T6+ Δ*tssM*	-	+
T6+ *Δ*vgrG1*	++	+++
T6+ Δ*vgrG2*	++++	++++
T6+ Δ*vgrG3*	++++	++++
T6+ Δ*vgrG4*	++++	++++
T6+ Δ*vgrG1,2*	+	+
T6+ Δ*vgrG1,3*	+	+
T6+ Δ*vgrG1,4*	++	++
T6+ Δ*vgrG2,3*	++++	-
T6+ Δ*vgrG2,4*	++++	++++
T6+ Δ*vgrG3,4*	++++	+++
T6+ Δ*vgrG1,2,3*	+	+
T6+ Δ*vgrG1,2,4*	-	+
T6+ Δ*vgrG1,3,4*	+	+
T6+ Δ*vgrG2,3,4*	++++	-
T6+ Δ*vgrG1,2,3,4*	-	+
T6+ Δ*vgrG3,4*Δ*tse2*	++++	-
T6+ Δ*vgrG2,4*Δ*tse3*	++++	-

*-Hcp secretion is considered as "-", and wild type T6+ levels are considered "++++". Wild type T6+ levels of bacterial killing are considered "++++", whereas the T6+ Δ*tssM* mutant is considered "+" as there are differences between T6+ Δ*tssM* and the least virulent *vgrG* mutants (ex. T6+ Δ*vgrG2,3*), which are considered "-".

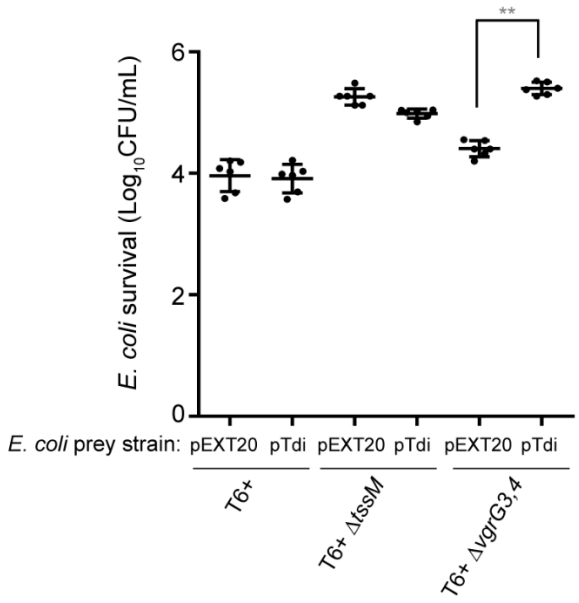

Figure 4.5 Expression of Tdi protects *E. coli* from killing by T6+ Δ*vgrG3,4*.

Competition assays with *E. coli* prey strains containing either empty vector (pEXT20) or expressing the putative Tsi2 immunity protein (pTdi). The predator *A. baumannii* strains are listed below on the x-axis. The results shown are from three independent experiments, performed in duplicate. Stars indicate statistical significance (unpaired, two-tailed Student's t-test: **P<0.01).

4.3.3 Identification of a peptidoglycan hydrolyzing enzyme essential for T6SS function

While much progress has been made in understanding the biogenesis and formation of a functional T6SS, it remains unknown how the assembled apparatus transits the peptidoglycan layer of the T6SS-encoding organism (Silverman *et al.*, 2012). In our analysis of the *A. baylyi* ADP1 T6SS, we identified ACIAD2699 as essential for Hcp secretion. The homolog of this gene in *A. baumannii* ATCC 17978 (ACX60_11600) was co-regulated with the T6SS (Table 4.3). This gene encodes a protein with two predicted transmembrane domains and a C-terminal peptidase domain found in the peptidoglycan-cleaving VanY peptidase superfamily of proteins (pfam13539), but does not resemble any characterized T6SS component from other bacteria (Figure 4.6A and Figure 4.7). Analysis of protein charge distribution revealed a stretch of positive charges directly after the

first predicted transmembrane domain, and based on the 'positive inside rule', would position the C-terminal enzymatic domain in the periplasm (Figure 4.7) (von Heijne, 1992). Bioinformatic searches, alignments, and *in silico* modelling showed that this C-terminal domain shared metal coordinating and active site residues with a *Listeria* phage endolysin, Ply500, an L-alanoyl-D-glutamate endopeptidase that was previously characterized and crystallized (Korndorfer *et al.*, 2008; Loessner *et al.*, 1995) (Figure 4.6B). Furthermore, this gene is conserved in the T6SS clusters of all *Acinetobacter* spp., and a homolog is also present in the T6SS clusters of *Burkholderia thailandensis* and *Ralstonia solanacearum* (Figure 4.8A and B). We reasoned that this enzyme, which we have termed TagX (type VI associated gene X) in keeping with previous nomenclature, could play an essential role in T6SS tubule transit through the peptidoglycan layer of the T6SS-producing *Acinetobacter*.

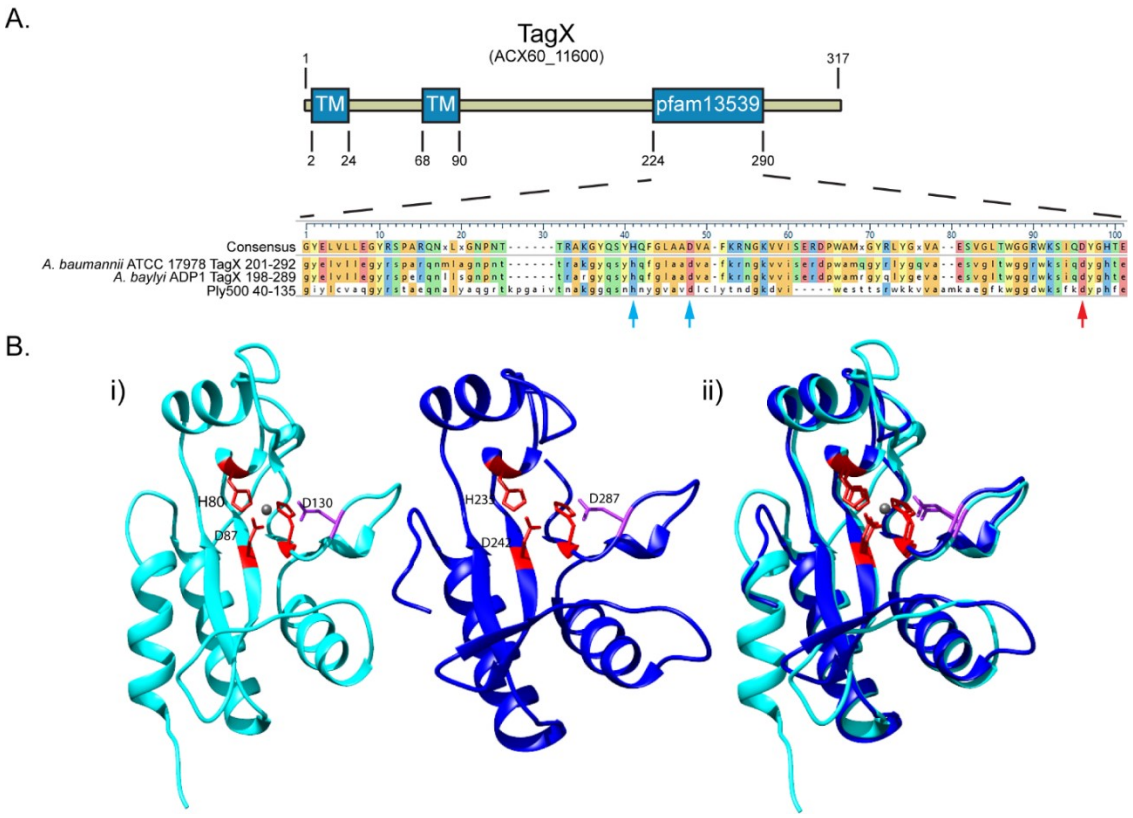

Figure 4.6 The TagX protein of *Acinetobacter*.

A) Schematic of TagX, showing the location of the transmembrane domains (TM) and the pfam13539 peptidase domain which resembles members of the VanY superfamily. Shown below is an alignment of the *A. baumannii* and *A. baylyi* TagX pfam13539 domains with the *Listeria* phage endolysin PlyA500 (accession: AAY52812). Relevant metal coordinating residues are indicated by the blue arrows, and the red arrow shows the general base in the active site of the characterized Ply500 enzyme. B) i) The crystal structure of the enzymatically active domain Ply500 (PDB: 2VO9) (Korndorfer *et al.*, 2008; Loessner *et al.*, 1995) (left) and the modelled structure of the C-terminal end of *A. baumannii* TagX. The metal coordinating residues are colored in red and the general base residue is purple. ii) Overlay of the crystal structure of Ply500 and the modelled structure of TagX, Modelling was performed using SWISS-MODEL (Schwede *et al.*, 2003).

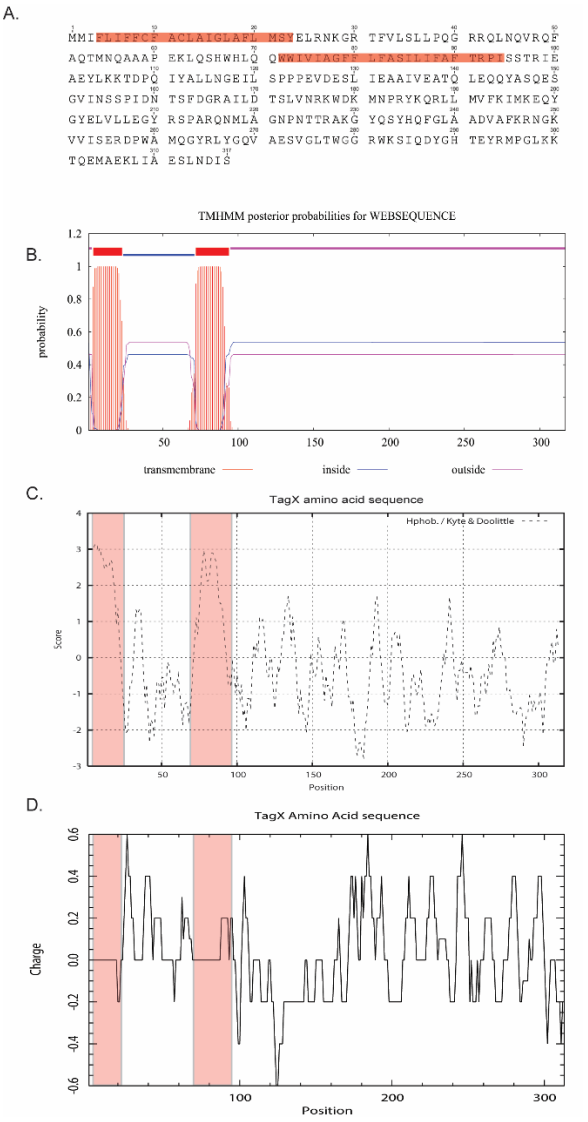

Figure 4.7 The Bioinformatic analysis of TagX.

A) Amino acid sequence of TagX, with predicted transmembrane domains highlighted. B) Graphical view of the predicted transmembrane domains and orientation of TagX, showing C-terminal position in periplasm, using the online tool TMHMM (Krogh *et al.*, 2001). C) Hydrophobicity (http://web.expasy.org/protscale/) and D) charge (http://www.bioinformatics.nl/cgi-bin/emboss/charge) plots of TagX, showing positive charges located after the first transmembrane segment.

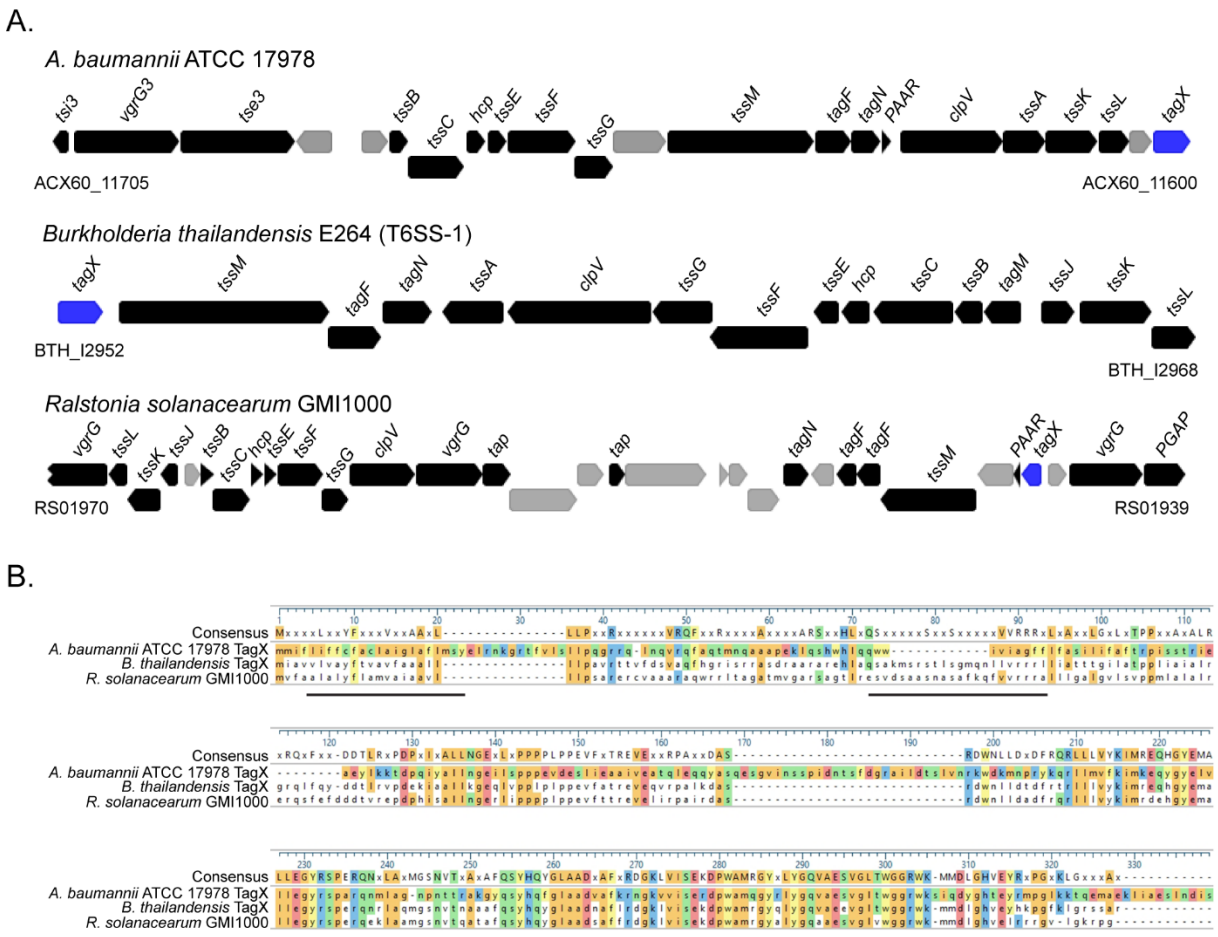

Figure 4.8 The TagX protein is present in the T6SS of several bacterial species

A) Schematic of the core T6SS clusters from *A. baumannii* ATCC17978, *B. thailandensis* E264, and *R. solanacearum* GMI1000. Black arrows represent T6SS-related genes, and *tagX* is shown in blue. Genes with unknown function are shown in grey. B) Alignment of the full length TagX proteins from these three bacteria.

We mutated the *tagX* homolog in *A. baumannii* ATCC 17978, and confirmed that this strain was unable to secrete Hcp (Figure 4.9A). Importantly, Hcp secretion was restored in *A. baumannii* and *A. baylyi tagX* mutants upon expression of their respective TagX protein from a plasmid (Figure 4.9A). Using the alignment against the Ply500 peptidoglycan hydrolase as a guide, we identified conserved amino acids comprising the putative active site and metal coordinating residues of TagX. We reasoned that, if TagX were a peptidoglycan hydrolase, mutation of these

sites would abrogate peptidoglycan hydrolysis by this enzyme. We cloned, expressed, and purified C-terminally His-tagged full length TagX and a TagX mutant in the putative active site ($TagX^{D287N}$) from *E. coli* (Figure 4.9B and Figure 4.10). Expression and purification of the wild type and $TagX^{D287N}$ were similar. Western blot analysis of the purified TagX showed that the protein ran as two predominant bands at a molecular weight of ~40kDa, near the predicted molecular weight of 36.2 kDa, and also contained a higher molecular weight species at ~80kDa, possibly a dimer. Several degradation products that maintained anti-His reactivity were observed when the protein was purified from *E. coli* without protease inhibitors (to prevent inhibition of TagX enzymatic activity), indicating they contained the predicted catalytic domains (Figure 4.9B). Addition of protease inhibitors lacking EDTA prevented this degradation (Figure 4.9B).

To assess the ability of TagX to cleave peptidoglycan, we performed peptidoglycan zymogram assays using gels impregnated with peptidoglycan isolated from *A. baumannii.* After renaturing and staining the peptidoglycan zymograms, only lanes containing TagX, but not $TagX^{D287N}$, resulted in clear bands of peptidoglycan hydrolysis (Figure 4.9B). Interestingly, of the two predominant protein bands detected by Western blot, only the lower molecular weight species showed activity, which may suggest the full-length enzyme is inactive. Further, despite being present in low quantities, TagX degradation products were fully active, degrading PG comparably to the higher molecular weight species. We further assessed the stability and activity of TagX by limited proteolysis with Proteinase K (Musumeci *et al.*, 2014). In these time-course experiments, full-length enzyme was almost fully degraded by the Proteinase K treatment (Figure 4.9C). In contrast, the C-terminal fragments were completely resistant to protease activity and accumulated throughout the experiment, presumably from the cleaved full-length TagX. The accumulation of the protease-resistant C-terminal fragment correlated with very significant increase in

peptidoglycan hydrolysis by these samples as seen by zymogram (Figure 4.9C). At 60 minutes, the C-terminal fragment was almost exclusively present. The protein levels of full-length TagX at time 0 and the C-terminal fragment at 60 minutes were not markedly different, yet the C-terminal fragment cleaved peptidoglycan to a much greater extent than the full-length TagX at these respective time points. This suggests that the C-terminal may display higher enzymatic activity than the full length protein, and considering its resistance to protease digestion, may suggest a possible regulatory mechanism.

We performed protein localization studies by fractioning *A. baumannii* cells expressing TagX. As expected, TagX could be detected in the whole cell fraction, along with the inner membrane fraction, and to a lesser extent in the cytosol (Figure 4.9D). No TagX was detected in the fraction containing both the periplasm and the outer membrane. TagX was not found to be secreted to culture supernatants (Figure 4.9E), indicating that TagX is a bona-fide structural element of the T6SS.

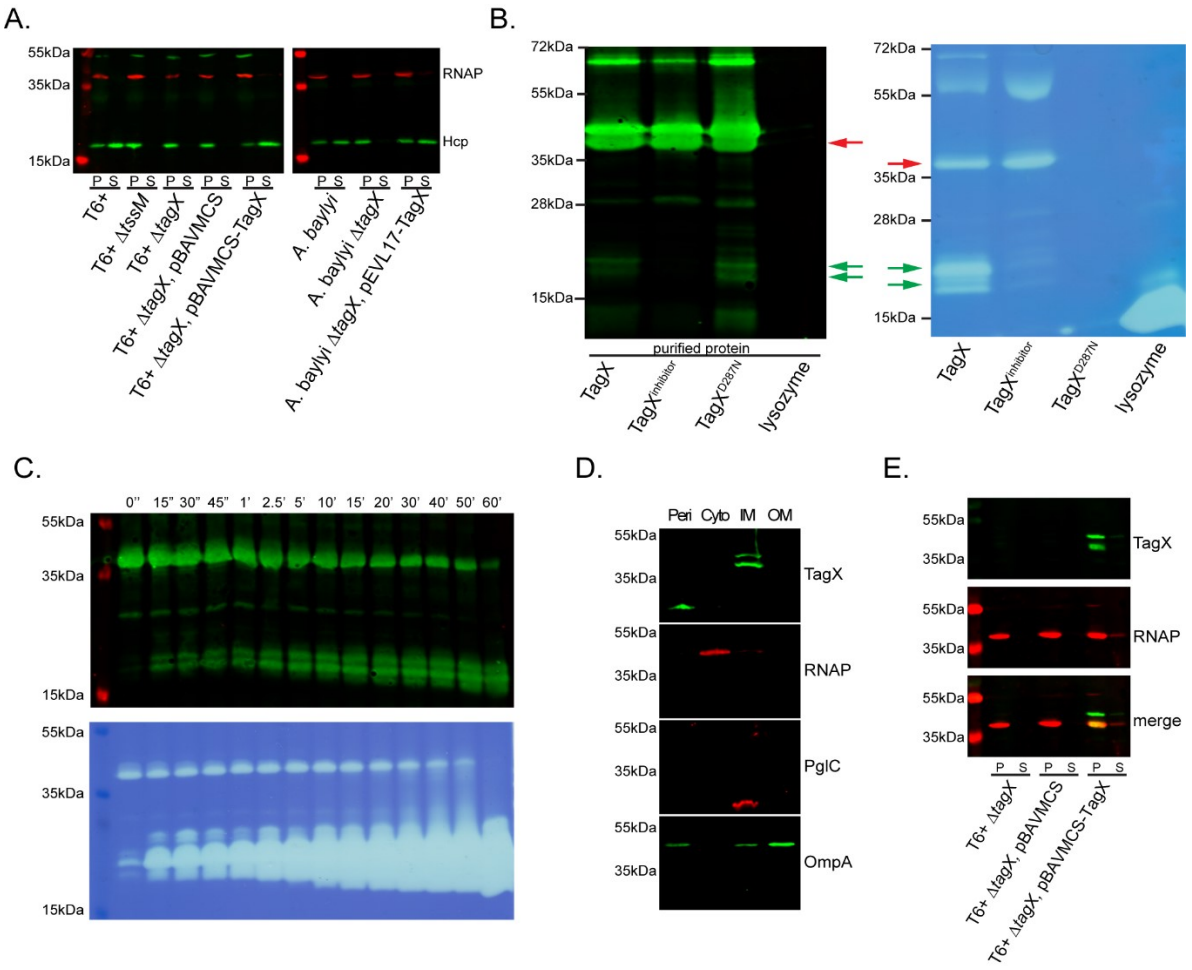

Figure 4.9 TagX is a membrane anchored peptidoglycan hydrolase

TagX is a membrane anchored peptidoglycan hydrolase. A) Expression and secretion of Hcp and RNA polymerase (RNAP) in pellet (P) and supernatant (S) samples of *A. baumannii* wild type (T6+), Δ*tssM*, Δ*tagX*, the Δ*tagX* vector control and the complemented Δ*tagX* mutant. B) Expression and secretion of Hcp in pellet (P) and supernatant (S) samples of wild type *A. baylyi* and the Δ*tagX* mutant and the complemented strain. B) Left gel: Western blot analysis of purified TagX, TagX plus protease inhibitor ($TagX^{inhibitor}$) and $TagX^{D287N}$ mutant. Proteins were detected using a polyclonal anti-His antibody. Right gel: Zymogram with purified TagX proteins and lysozyme as a control. Red and green arrows indicate areas of peptidoglycan degradation, and the corresponding bands in the Western blot. C) Limited proteolysis of purified TagX. TagX was treated with Proteinase K for various times (0-60min) and run on an SDS-PAGE gel and analyzed by Western blot (upper gel) or loaded onto a gel impregnated with peptidoglycan for zymogram analysis (lower gel). D) Cellular fractionation studies of TagX in periplasmic (Peri), cytoplasmic (Cyto), inner membrane (IM), or outer membrane (OM) fractions of *A. baumannii* with RNA polymerase, PglC, and OmpA as localization controls. E) Pellet (P) and supernatant (S) samples of the T6+ *A. baumannii tagX* mutant, the *tagX* mutant containing vector control, or expressing TagX were analyzed by Western blot for TagX expression. Low

levels of TagX in the supernatant correspond to leakage of RNA polymerase, indicating some lysis occurs.

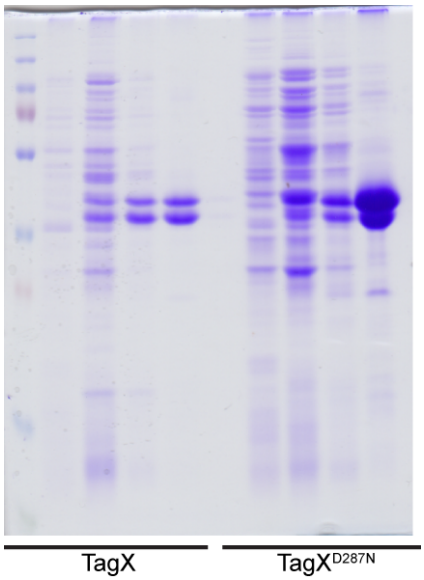

Figure 4.10 Purification of TagX and TagX^{D287N} point mutant.

Coomassie gel of flow through, wash and elution fractions from the purifications.

In order to determine its site of cleavage, we incubated purified TagX and TagX^{D287N} with peptidoglycan from *A. baumannii*, and performed HPLC and mass spectrometry (MS) to assess cleavage products. Peptidoglycan incubated with TagX^{D287N} or buffer alone looked similar by HPLC (Figure 4.11A). Conversely, peptidoglycan partially digested with TagX showed evidence of degradation, with a large peak of a monomeric species eluting at ~7min. Upon complete digestion, this was the only peak remaining. MS analysis of this peak showed it was composed of the peptidoglycan fragment *N*-acetyl-glucosamine-*N*-acetyl-muramic acid attached to L-Ala, which is the first amino acid residue of the peptide stem (Figure 4.11B). These results indicate the cleavage site of TagX to be between the L-Ala-D-Glu residues of the peptidoglycan pentapeptide, enzymatically acting as an L,D-endopeptidase (Figure 4.11B, inset).

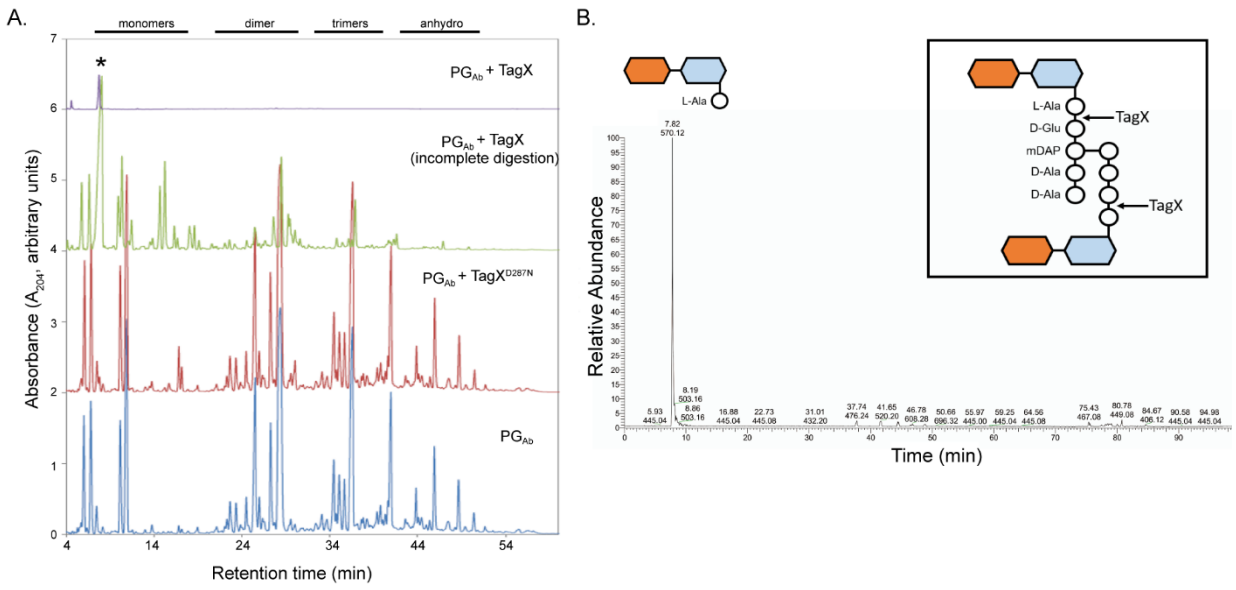

Figure 4.11 TagX is an L,D-endopeptidase.

A) Chromatographic analysis by HPLC of isolated *A. baumannii* peptidoglycan (PG_{Ab}). Peptidolgycan was digested with TagX, an incomplete digestion with TagX, with $TagX^{D287N}$, or without addition of enzyme. After treatment the PG was subsequently digested with muramidase and the resulting muropeptides were analyzed by HPLC. Peaks corresponding to peptidoglycan monomers, dimer, trimers, and anhydromuropeptides are indicated above. The asterisk (*) indicates the peak in the PG_{Ab} + TagX sample that was further selected for MS characterization. B) MS analysis of the peak selected from HPLC trace. The major peak at m/z 570 corresponds to a peptidoglycan monomer attached to L-alanine. The inset shows the proposed TagX cleavage site on a peptidoglycan dimer. mDAP (*meso*-diaminopimelic acid).

4.4 Discussion

There have been great strides made in our understanding of the function, biogenesis, and regulation of the bacterial T6SS since its formal description a decade ago (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). However, recent studies have also made it clear that the T6SS can display variations among different species, and even among strains of a species or cells of a population.

Functionally, the T6SS can be used as either an anti-bacterial or anti-eukaryotic weapon, or both, depending on the organism (Ho *et al.*, 2014). While many T6SS components are conserved across bacteria, others are species-specific. Finally, a diverse range of strategies have evolved to regulate T6SS activity among different organisms (Bernard *et al.*, 2010a; Silverman *et al.*, 2012). For these reasons, it is important to experimentally study the T6SS in the organism of choice in order to delineate the distinctive aspects of this secretory apparatus that may differ from other bacteria. Our results further emphasize this point, as we have shown that the T6SS of *Acinetobacter* spp., while encoding many components already shown to be important for T6SS function in other bacteria, contains several novel genes that are essential for Hcp secretion but not encoded by other characterized T6SS. Our analysis showed that 14 genes, *ACIAD2684-2697* and *ACIAD2699,* are essential for Hcp secretion in *A. baylyi*. The systematic mutagenesis of the T6SS in *Edwardsiella tarda*, *Agrobacterium tumefaciens,* and *Vibrio cholerae* has yielded important insights into the core genes required for T6SS activity (Lin *et al.*, 2013; Zheng *et al.*, 2011; Zheng *et al.*, 2007). In general, our results are consistent with the previous studies in other bacterial species. This includes a PAAR protein (ACIAD2681), that when mutated by itself has no effect on Hcp secretion, but if mutated combination with two other PAAR proteins, located elsewhere on the chromosome, blocks T6SS (Shneider *et al.*, 2013). Most *A. baylyi* genes required for Hcp secretion, and thus T6SS activity, are also required for T6SS in *E. tarda*, *V. cholerae*, and *A. tumefaciens*. However, our analysis has uncovered three essential T6SS genes (ACIAD2685, ACIAD2693, and ACIAD2699) which, to our knowledge, have not previously been implicated in T6SS activity and are conserved across *Acinetobacter* spp. This indicates that these proteins play an important role in T6SS biogenesis in this genus, but their precise role remains to be determined. *Acinetobacter* spp. do not encode a readily identifiable homolog of TssJ (Weber *et al.*, 2013), a lipoprotein

required for T6SS activity in some organisms, and thus these proteins may compensate for this. The mutation of ACIAD2698 gene increased Hcp secretion, indicating that it may perform some regulatory function. Finally, we have identified a novel role for ACIAD2699 (*tagX*), as discussed below, which highlights the importance of examining individual T6SS clusters.

VgrG proteins are secreted in a T6SS-dependent manner and can either contain toxic domains or facilitate the secretion of other effectors (Hachani *et al.*, 2014; Pukatzki *et al.*, 2007; Unterweger *et al.*, 2015). These proteins can also be essential for Hcp secretion, and thus T6SS function. In *V. cholerae*, mutation of *vgrG-1* or *vgrG-2,* but not *vgrG-3*, prevents Hcp secretion (Pukatzki *et al.*, 2007), although *vgrG-3* mutation results in a detectable Hcp secretion defect (Brooks *et al.*, 2013). Similarly, in *P. aeruginosa*, *vgrG1a* and *vgrG1c* are required for efficient Hcp secretion, and Hcp was undetectable in the double mutant (Hachani *et al.*, 2011a). A third *vgrG*, *vgrG1b*, was dispensable for secretion of Hcp (Hachani *et al.*, 2011a). In addition to their importance in apparatus assembly, it has now become apparent that many VgrG proteins facilitate the secretion of cognate effectors (Hachani *et al.*, 2014; Unterweger *et al.*, 2015). Our RNAseq, differential proteomics, and bioinformatic analyses identified the putative *vgrG* clusters in several strains of *A. baumannii*, and, although the VgrG proteins were themselves not predicted to encode effector domains, all were nearby predicted effectors encoding various enzymatic functions. In *A. baumannii* ATCC 17978, we identified the presence of four *vgrG* genes which were scattered throughout the genome. Their proximity to proteins with predicted toxin domains suggested that these VgrG proteins may facilitate effector secretion. While we did not assess the secretion of these proteins in this study, our mutational strategy showed that VgrG1 is necessary for efficient Hcp secretion, similar to what was seen in *P. aeruginosa*, and deletion of *vgrG1* resulted in a modest bacterial killing defect. Furthermore, the sole presence of VgrG1 was sufficient for wild-

type levels of Hcp secretion in the Δ*vgrG2,3,4* mutant, confirming its importance for Hcp export. Because mutation of *vgrG1* affected T6SS apparatus formation, mutants lacking this gene in combination with other *vgrG* genes are difficult to assess for their role in bacterial killing. However, our finding that several double mutants (which contained a functional *vgrG1*) lost the ability to kill *E. coli* provides important insights in the role of these other *vgrG* genes in anti-bacterial activity. For example, the Δ*vgrG2,3* mutant secreted wild type levels of Hcp but did not kill *E. coli*, which suggests that the presence of VgrG1 and VgrG4 is not sufficient to kill *E. coli*. One hypothesis for this is that the effectors downstream of VgrG1 and VgrG4 are not active against bacteria. Tle encodes a putative lipase, and Tge has a LysM peptidoglycan-binding and a peptidase domain, indicating it acts on peptidoglycan. Both lipase and murein hydrolase effectors have been implicated in T6SS-mediated bacterial killing (Russell *et al.*, 2014), suggesting Tle and Tge could be active against *E. coli*. Alternatively, Tle and Tge action may not be sufficient for a bactericidal effect. In contrast, the Δ*vgrG2,4* and Δ*vgrG3,4* mutants secreted Hcp and killed *E. coli* similarly to wild-type, which implies effectors secreted in a VgrG2- and VgrG3-dependent manner are important for mediating bacterial killing. Indeed, mutation of *tde* or *tse* in these backgrounds, respectively, completely abrogated killing. The genetic makeup of the *vgrG2* gene cluster, including the *tde* effector and *tdi* immunity genes, is strikingly similar to the nuclease effector/immunity pair described in *A. tumefaciens*, suggesting Tde functions as a nuclease (Ma *et al.*, 2014). Supporting this is our finding that *E. coli* were rescued from Tde killing by expression of the Tdi immunity protein. Despite its clear importance for anti-bacterial activity, we were unable to detect any obvious toxin domains associated with Tse, and thus this protein may represent a new family of T6SS-delivered toxins. One interesting finding is that significant amounts of *E. coli* killing by the Δ*tssM* mutant of *A. baumannii* ATCC 17978 are detected when compared to *E. coli*

controls. This has been described previously by us and others, and suggests additional anti-bacterial mechanisms exist in this strain apart from T6SS (Repizo *et al.*, 2015; Weber *et al.*, 2013). Furthermore, our assays showed that several combinations of *vgrG* mutants, for example Δ*vgrG2,3,* were attenuated for *E. coli* killing to an even greater extent than the Δ*tssM* mutant. While the mechanistic basis for this is not known, VgrG's from *P. aeruginosa* and *Francisella tularensis* have been demonstrated to be secreted in a T6SS-independent manner, which may explain some of the observed T6SS-independent killing (Barker *et al.*, 2009; Hachani *et al.*, 2011b).

Dedicated peptidoglycan hydrolases are often genetically linked with macromolecular complexes that have components that cross the peptidoglycan layer (Scheurwater *et al.*, 2011). Biochemically, these enzymes are frequently lytic transglycosylases that cleave the glycosidic linkage between the dissacharide backbone of peptidoglycan (Koraimann, 2003). In many cases these hydrolases physically interact with core components of the membrane spanning complexes, allowing them to alter the peptidoglycan layer at precise cellular locations. Examples of this include VirB1 from *Brucella suis*, which interacts with other VirB T4SS components, FlgJ from *Salmonell*a, which interacts with other flagellar components, and the lytic transglycosylase EtgA from *E. coli*, which is essential for T3SS (Burkinshaw *et al.*, 2015; Hirano *et al.*, 2001; Hoppner *et al.*, 2005; Nambu *et al.*, 1999). Bacteriophage, although entering the cell from the external environment, must also possess a mechanism to cross the peptidoglycan when puncturing a bacterial cell. For T4 phage, this is accomplished by the lysozyme domain of the gp5 protein (Takeda *et al.*, 1998). Interestingly, VgrG proteins share sequence and structural homology to gp5, but lack this lysozyme domain (Kanamaru *et al.*, 2002; Leiman *et al.*, 2009; Lossi *et al.*, 2011; Pukatzki *et al.*, 2007; Uchida *et al.*, 2014). Despite the requirement of a peptidoglycan hydrolase

for these systems, an enzyme performing this function for T6SS has yet to be described. Our genetic analysis of the *A. baumannii* ATCC 17978 and *A. baylyi* ADP1 T6SS clusters identified TagX as an essential component of the T6SS machinery, as *tagX* mutants failed to secrete Hcp. The C-terminal end of TagX shares homology with enzymatically active domains of a Ply500 from *L. monocytogenes* phage A500 and CwlK from *Bacillus subtilis*. Both enzymes are part of the 'LAS' family of peptidases, and are L-alanoyl-D-glutamate endopeptidases that cleave the peptidoglycan peptide stem (Fukushima *et al.*, 2007; Korndorfer *et al.*, 2008; Loessner *et al.*, 1995). The finding that TagX and the phage protein Ply500 share sequence similarity agrees with the evolutionary connection between T6SS and many bacteriophage components (Leiman *et al.*, 2009). Our experiments confirmed TagX as an inner membrane L,D-endopeptidase, requiring conserved amino acid residues for activity encoded in its C-terminal end. Interestingly, TagX expressed and purified as two major protein bands, but only the smaller molecular weight protein was active, indicating that the full length TagX protein lacks activity. As the protein was expressed with a C-terminal His-tag, the N-terminal domain may possess some form of autoinhibitory activity. Autoinhibition of peptidoglycan hydrolases has been previously demonstrated, and thus TagX may be regulated in a similar fashion (Bublitz *et al.*, 2009). Furthermore, the C-terminal end is highly resistant to proteinase K digestion, and although the biological relevance of this is unknown, may play a role in regulation of TagX. It is tempting to speculate the interaction with other components of the T6SS apparatus may control the enzymatic activity of TagX, allowing for precise spatial regulation of peptidoglycan degradation. TagX is present in the T6SS cluster in several other organisms, including many β-proteobacteria, but is notably absent from other well characterized T6SS, such as those of *P. aeruginosa* and *V. cholerae*. We hypothesize that T6SS-

encoding organisms have evolved other mechanisms for enabling T6SS passage through the peptidoglycan layer, and we predict additional enzymes playing this role remain to be discovered.

The data presented here provides a framework and direction for future studies of the T6SS in *Acinetobacter* spp. We have established the basic requirements for elaborating a functional T6SS in these organisms, and demonstrated that VgrG proteins show marked differences in their relative contributions to Hcp secretion and bacterial killing. Furthermore, we have delineated the role of a putative nuclease/immunity pair, Tde/Tdi, and an effector of unknown activity, Tse, in anti-bacterial activity. Our experiments suggest these effectors require their cognate VgrG for secretion by the T6SS. Future studies on the nature of effectors secreted by this system, as well as the basis for their secretion, should further uncover the precise physiological role for T6SS these organisms. Finally, our discovery of TagX presents a more complete picture of T6SS biogenesis, and suggests that similar enzymes should be identifiable in other organisms.

4.5 References

Aschtgen, M. S., Gavioli, M., Dessen, A., Lloubes, R., & Cascales, E. (2010a). The SciZ protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the cell wall. *Molecular microbiology, 75*(4), 886-899.

Aschtgen, M. S., Thomas, M. S., & Cascales, E. (2010b). Anchoring the type VI secretion system to the peptidoglycan: TssL, TagL, TagP... what else? *Virulence, 1*(6), 535-540.

Barker, J. R., Chong, A., Wehrly, T. D., Yu, J. J., Rodriguez, S. A., Liu, J., . . . Klose, K. E. (2009). The Francisella tularensis pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. *Molecular microbiology, 74*(6), 1459-1470.

Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell, 152*(4), 884-894.

Basler, M., & Mekalanos, J. J. (2012a). Type 6 secretion dynamics within and between bacterial cells. *Science, 337*(6096), 815.

Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012b). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186.

Baumann, P. (1968). Isolation of *Acinetobacter* from soil and water. *Journal of bacteriology, 96*(1), 39-42.

Bernard, C. S., Brunet, Y. R., Gueguen, E., & Cascales, E. (2010a). Nooks and crannies in type VI secretion regulation. *Journal of bacteriology, 192*(15), 3850-3860.

Bonemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., & Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *The EMBO journal, 28*(4), 315-325.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC genomics, 10*, 104.

Brooks, T. M., Unterweger, D., Bachmann, V., Kostiuk, B., & Pukatzki, S. (2013). Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *The Journal of biological chemistry, 288*(11), 7618-7625.

Brunet, Y. R., Zoued, A., Boyer, F., Douzi, B., & Cascales, E. (2015). The Type VI Secretion TssEFGK-VgrG Phage-Like Baseplate Is Recruited to the TssJLM Membrane Complex via Multiple Contacts and Serves As Assembly Platform for Tail Tube/Sheath Polymerization. *PLoS genetics, 11*(10), e1005545.

Bublitz, M., Polle, L., Holland, C., Heinz, D. W., Nimtz, M., & Schubert, W. D. (2009). Structural basis for autoinhibition and activation of Auto, a virulence-associated peptidoglycan hydrolase of Listeria monocytogenes. *Molecular microbiology, 71*(6), 1509-1522.

Burkinshaw, B. J., Deng, W., Lameignere, E., Wasney, G. A., Zhu, H., Worrall, L. J., . . . Strynadka, N. C. (2015). Structural analysis of a specialized type III secretion system peptidoglycan-cleaving enzyme. *The Journal of biological chemistry, 290*(16), 10406-10417.

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S., Jr. (2013c). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PloS one, 8*(3), e59388.

Cascales, E., & Cambillau, C. (2012). Structural biology of type VI secretion systems. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 367*(1592), 1102-1111.

Cianfanelli, F. R., Alcoforado Diniz, J., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion System. *PLoS pathogens, 12*(6), e1005735.

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol, 26*(12), 1367-1372.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008a). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008b). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

Durand, E., Nguyen, V. S., Zoued, A., Logger, L., Pehau-Arnaudet, G., Aschtgen, M. S., . . . Fronzes, R. (2015). Biogenesis and structure of a type VI secretion membrane core complex. *Nature, 523*(7562), 555-560.

Dykxhoorn, D. M., St Pierre, R., & Linn, T. (1996). A set of compatible tac promoter expression vectors. *Gene, 177*(1-2), 133-136.

Flaugnatti, N., Le, T. T., Canaan, S., Aschtgen, M. S., Nguyen, V. S., Blangy, S., . . . Journet, L. (2015). A phospholipase A antibacterial Type VI secretion effector interacts directly with the C-terminal domain of the VgrG spike protein for delivery. *Molecular microbiology*.

Fukushima, T., Yao, Y., Kitajima, T., Yamamoto, H., & Sekiguchi, J. (2007). Characterization of new L,D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in Bacillus subtilis. *Mol Genet Genomics, 278*(4), 371-383.

Hachani, A., Allsopp, L. P., Oduko, Y., & Filloux, A. (2014). The VgrG proteins are "a la carte" delivery systems for bacterial type VI effectors. *The Journal of biological chemistry, 289*(25), 17872-17884.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011a). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011b). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Henry, R., Vithanage, N., Harrison, P., Seemann, T., Coutts, S., Moffatt, J. H., . . . Boyce, J. D. (2012). Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglucosamine. *Antimicrobial agents and chemotherapy, 56*(1), 59-69.

Hirano, T., Minamino, T., & Macnab, R. M. (2001). The role in flagellar rod assembly of the N-terminal domain of Salmonella FlgJ, a flagellum-specific muramidase. *Journal of molecular biology, 312*(2), 359-369.

Ho, B. T., Dong, T. G., & Mekalanos, J. J. (2014). A view to a kill: the bacterial type VI secretion system. *Cell host & microbe, 15*(1), 9-21.

Hoppner, C., Carle, A., Sivanesan, D., Hoeppner, S., & Baron, C. (2005). The putative lytic transglycosylase VirB1 from Brucella suis interacts with the type IV secretion system core components VirB8, VirB9 and VirB11. *Microbiology, 151*(Pt 11), 3469-3482.

Hoyle, B. D., & Beveridge, T. J. (1984). Metal binding by the peptidoglycan sacculus of *Escherichia coli* K-12. *Canadian journal of microbiology, 30*(2), 204-211.

Jawad, A., Seifert, H., Snelling, A. M., Heritage, J., & Hawkey, P. M. (1998). Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates. *Journal of clinical microbiology, 36*(7), 1938-1941.

Kanamaru, S., Leiman, P. G., Kostyuchenko, V. A., Chipman, P. R., Mesyanzhinov, V. V., Arisaka, F., & Rossmann, M. G. (2002). Structure of the cell-puncturing device of bacteriophage T4. *Nature, 415*(6871), 553-557.

Koraimann, G. (2003). Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. *Cellular and molecular life sciences : CMLS, 60*(11), 2371-2388.

Korndorfer, I. P., Kanitz, A., Danzer, J., Zimmer, M., Loessner, M. J., & Skerra, A. (2008). Structural analysis of the L-alanoyl-D-glutamate endopeptidase domain of Listeria bacteriophage endolysin Ply500 reveals a new member of the LAS peptidase family. *Acta crystallographica. Section D, Biological crystallography, 64*(Pt 6), 644-650.

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology, 305*(3), 567-580.

Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., . . . Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4154-4159.

Liang, X., Moore, R., Wilton, M., Wong, M. J., Lam, L., & Dong, T. G. (2015). Identification of divergent type VI secretion effectors using a conserved chaperone domain. *Proceedings of the National Academy of Sciences of the United States of America, 112*(29), 9106-9111.

Lin, J. S., Ma, L. S., & Lai, E. M. (2013). Systematic dissection of the agrobacterium type VI secretion system reveals machinery and secreted components for subcomplex formation. *PloS one, 8*(7), e67647.

Loessner, M. J., Wendlinger, G., & Scherer, S. (1995). Heterogeneous endolysins in Listeria monocytogenes bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Molecular microbiology, 16*(6), 1231-1241.

Lossi, N. S., Dajani, R., Freemont, P., & Filloux, A. (2011). Structure-function analysis of HsiF, a gp25-like component of the type VI secretion system, in *Pseudomonas aeruginosa*. *Microbiology, 157*(Pt 12), 3292-3305.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology, 15*(12), 550.

Ma, L. S., Hachani, A., Lin, J. S., Filloux, A., & Lai, E. M. (2014). Agrobacterium tumefaciens deploys a superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in planta. *Cell host & microbe, 16*(1), 94-104.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America, 107*(45), 19520-19524.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., . . . Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science, 312*(5779), 1526-1530.

Musumeci, M. A., Faridmoayer, A., Watanabe, Y., & Feldman, M. F. (2014). Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology, 24*(1), 39-50.

Nambu, T., Minamino, T., Macnab, R. M., & Kutsukake, K. (1999). Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in Salmonella typhimurium. *Journal of bacteriology, 181*(5), 1555-1561.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008a). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical microbiology reviews, 21*(3), 538-582.

Peleg, A. Y., Tampakakis, E., Fuchs, B. B., Eliopoulos, G. M., Moellering, R. C., Jr., & Mylonakis, E. (2008b). Prokaryote-eukaryote interactions identified by using Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 105*(38), 14585-14590.

Pell, L. G., Kanelis, V., Donaldson, L. W., Howell, P. L., & Davidson, A. R. (2009). The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4160-4165.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., & Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 51*(10), 3471-3484.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15508-15513.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.

Pukatzki, S., McAuley, S. B., & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Current opinion in microbiology, 12*(1), 11-17.

Repizo, G. D., Gagne, S., Foucault-Grunenwald, M. L., Borges, V., Charpentier, X., Limansky, A. S., . . . Salcedo, S. P. (2015). Differential Role of the T6SS in *Acinetobacter baumannii* Virulence. *PloS one, 10*(9), e0138265.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature, 475*(7356), 343-347.

Russell, A. B., LeRoux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., . . . Mougous, J. D. (2013). Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature, 496*(7446), 508-512.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature reviews. Microbiology, 12*(2), 137-148.
Scheurwater, E. M., & Burrows, L. L. (2011). Maintaining network security: how macromolecular structures cross the peptidoglycan layer. *FEMS microbiology letters, 318*(1), 1-9.
Schwede, T., Kopp, J., Guex, N., & Peitsch, M. C. (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic acids research, 31*(13), 3381-3385.
Scott, N. E., Kinsella, R. L., Edwards, A. V., Larsen, M. R., Dutta, S., Saba, J., . . . Feldman, M. F. (2014a). Diversity within the O-linked protein glycosylation systems of *Acinetobacter* species. *Molecular & cellular proteomics : MCP, 13*(9), 2354-2370.
Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., Edwards, A. V., Crossett, B., . . . Cordwell, S. J. (2011). Simultaneous Glycan-Peptide Characterization Using Hydrophilic Interaction Chromatography and Parallel Fragmentation by CID, Higher Energy Collisional Dissociation, and Electron Transfer Dissociation MS Applied to the N-Linked Glycoproteome of *Campylobacter jejuni*. *Mol Cell Proteomics, 10*(2), M000031MCP000201.
Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature, 500*(7462), 350-353.
Silverman, J. M., Agnello, D. M., Zheng, H., Andrews, B. T., Li, M., Catalano, C. E., . . . Mougous, J. D. (2013). Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. *Molecular cell, 51*(5), 584-593.
Silverman, J. M., Austin, L. S., Hsu, F., Hicks, K. G., Hood, R. D., & Mougous, J. D. (2011). Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. *Molecular microbiology, 82*(5), 1277-1290.
Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology, 66*, 453-472.
Takeda, S., Hoshida, K., & Arisaka, F. (1998). Mapping of functional sites on the primary structure of the tail lysozyme of bacteriophage T4 by mutational analysis. *Biochimica et biophysica acta, 1384*(2), 243-252.
Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473-3484.
Tucker, A. T., Nowicki, E. M., Boll, J. M., Knauf, G. A., Burdis, N. C., Trent, M. S., & Davies, B. W. (2014). Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio, 5*(4), e01313-01314.
Uchida, K., Leiman, P. G., Arisaka, F., & Kanamaru, S. (2014). Structure and properties of the C-terminal beta-helical domain of VgrG protein from *Escherichia coli* O157. *J Biochem, 155*(3), 173-182.
Unterweger, D., Kostiuk, B., Otjengerdes, R., Wilton, A., Diaz-Satizabal, L., & Pukatzki, S. (2015). Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *The EMBO journal, 34*(16), 2198-2210.
Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Gonzalez, G., Garcia, A., & Zemelman, R. (1996). Biofilm formation by *Acinetobacter baumannii*. *Microbios, 86*(346), 49-58.
von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *Journal of molecular biology, 225*(2), 487-494.

Weber, B. S., Harding, C. M., & Feldman, M. F. (2015a). Pathogenic *Acinetobacter*: from the Cell Surface to Infinity and Beyond. *Journal of bacteriology, 198*(6), 880-887.

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015b). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 112*(30), 9442-9447.

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., & Feldman, M. F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PloS one, 8*(1), e55142.

Whitney, J. C., Beck, C. M., Goo, Y. A., Russell, A. B., Harding, B., De Leon, J. A., . . . Mougous, J. D. (2014a). Genetically distinct pathways guide effector export through the type VI secretion system. *Molecular microbiology*.

Zheng, J., Ho, B., & Mekalanos, J. J. (2011). Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PloS one, 6*(8), e23876.

Zheng, J., & Leung, K. Y. (2007). Dissection of a type VI secretion system in Edwardsiella tarda. *Molecular microbiology, 66*(5), 1192-1206.

Zoued, A., Durand, E., Brunet, Y. R., Spinelli, S., Douzi, B., Guzzo, M., . . . Cascales, E. (2016). Priming and polymerization of a bacterial contractile tail structure. *Nature, 531*(7592), 59-63.

5 Discussion

The rise of bacterial pathogens resistant to all clinically useful antibiotics represents one of the most significant threats facing modern medicine. With no apparent reprieve in the form of new antibiotics seeming to be near, there is critical need to understand the factors that these organisms require for persistence in the hospital environment. The studies presented in this thesis provide new and important insights into one of the least understood but most problematic opportunistic pathogens, *A. baumannii*.

5.1 Description of the T6SS in *Acinetobacter*

Chapter two of this thesis presented a genomic analysis and description of the T6SS in several species of *Acinetobacter*. The similarity in sequence and organization of the cluster in all these species suggests that the T6SS was acquired early on in the evolution of this genus. The core cluster was highly conserved across species, while VgrG proteins differed in both number and sequence even within the same species. This may reflect the fact that different strains are predicted to produce different effectors, which require a VgrG protein for their delivery, as is described in Chapter four. It is interesting to note that TssJ, which forms part of the membrane complex with TssL and TssM, seems to be absent in *Acinetobacter* spp. TssJ is an outer membrane lipoprotein that has been proposed to serve as an anchor point for the membrane complex (Durand *et al.*, 2015). How *Acinetobacter* spp. compensate for the lack of TssJ remains to be seen, but it is interesting to note that *Burkholerdia* spp. also seem to lack this key component, indicating anchorage to the outer membrane by another mechanism (Aschtgen *et al.*, 2010b). Furthermore, in Chapter four we show that there are at least two essential genes in the T6SS cluster of

Acinetobacter spp. that show no homology to characterized components, and therefore these may represent functional homologs of TssJ.

By producing an antibody against the Hcp protein of *A. baumannii*, we were able to analyze the expression level of this key T6SS component. The production and secretion of Hcp has long been considered a molecular marker for an active T6SS (Pukatzki *et al.*, 2006; Pukatzki *et al.*, 2009). Hcp was expressed by all *A. baumannii* strains, but secretion varied considerably. For *A. baumannii* ATCC 17978, Chapter three describes the regulatory mechanisms governing T6SS activity and shows that two population of cells (T6+ and T6-) exist with this strain. For the other *A. baumannii* strains, the regulatory mechanisms preventing Hcp secretion remain unknown. For some of the unsequenced isolates tested, it is possible that mutations in the other T6SS genes are responsible for that lack of Hcp secretion. Deleterious T6SS mutations in *A. baumannii* have been previously described by genome sequencing (Eijkelkamp *et al.*, 2013; Hornsey *et al.*, 2011; Wright *et al.*, 2014). However, *A. baumannii* AYE is a sequenced strain that does not secrete Hcp, and inspection of the T6SS cluster did not show any obvious mutations explaining this phenotype. While Hcp was not secreted in this strain, it was still expressed, perhaps suggesting a 'pre-loaded' state of the T6SS, allowing a quick response to some as yet unknown signal. The relatively few strictly environmental *Acinetobacter* spp. we tested (*A. calcoaceticus* and *A. baylyi*) both expressed and secreted Hcp. While many more strains should be tested to draw any firm conclusions, environmental species of *Acinetobacter* may encounter competitors more often and therefore maintain an always-active T6SS.

Despite showing that Hcp is secreted in a T6SS-dependent manner, we were unable to define a phenotypic role for the system in *A. baumannii* ATCC 17978. The subsequent reports that *A. baylyi* ADP1 and *A. nosocomialis* M2 (formerly called *A. baumannii* M2) were capable of T6SS-

mediated antibacterial were surprising considering we did not detect this activity for *A. baumannii* ATCC 17978 (Basler *et al.*, 2013; Carruthers *et al.*, 2013c). Our work provided the first genetic and functional analysis of the T6SS in *Acinetobacter* spp., described the presence of the core T6SS gene structure, and showed that the system was active in many strains of the important pathogenic species *A. baumannii*. Clearly, however, more in-depth experimental analysis was required to decipher the regulatory mechanisms and phenotypic role of T6SS in *A. baumannii*.

5.2 Regulation and role of T6SS in *A. baumannii*

The significance of *A. baumannii* as a nosocomial pathogen has made it an important member of the *Acinetobacter* genus to study. As with many organisms, laboratories worldwide have focused on one or two "model" strains of *A. baumannii,* developing genetic tools and undertaking genome sequencing efforts in order to create a standardized system for study. Even under these controlled conditions, the 'same' strain can diverge when cultured in different laboratories over decades of work and no longer behave the same phenotypically (Tang *et al.*, 2015). *A. baumannii* ATCC 17978 has been considered one of the model strains in the field for many years, is genetically tractable, and exhibits low levels of antibiotic resistance. This strain was isolated from a clinical case in the 1950's, and thus has been grown and shipped to laboratories worldwide for more than half a century. Considering the rise in antibiotic resistance among new clinical isolates of *A. baumannii*, and the fact that we saw variation in secretion of Hcp among *A. baumannii*, we sought to explore T6SS expression in more recently isolated MDR strains.

The 2013 outbreak of *A. baumannii* at a local Edmonton hospital provided an opportunity to test a low-passage, MDR clinical isolate for T6SS activity (Ahmed-Bentley *et al.*, 2013). Our key technical innovation to perform Hcp secretion tests in 96-well plates by ELISA allowed us to

discover that individual colonies of this single hospital isolate showed contrasting Hcp secretion profiles. The T6+ colonies that secreted Hcp robustly were able to kill *E. coli*, whereas T6- were not. This was the first report showing that the T6SS of *A. baumannii* could be used to kill other bacteria. We also made the interesting finding that these T6+ cells could kill *E. coli* and *K. pneumoniae* strains that were isolated from the same infection site of the index patient as was the *A. baumannii* strain. The finding that the *A. baumannii*, but not the *E. coli* or *K.* pneumoniae, went on to infect additional patients (causing the outbreak) may suggest that *A. baumannii* outcompeted the other bacteria at the infection site, although this is purely speculative (Ahmed-Bentley *et al.*, 2013).

We used short-read sequencing methods to characterized the genetic differences between T6+ and T6-. This revealed that the genome of T6+ cells was smaller than that of T6- by about 170kb. However, the genetic context of this difference was difficult to understand as the short reads did not allow for the *de novo* assembly of a fully closed chromosome, and the DNA apparently missing from T6+ cells was assembled as several fragments. We were, however, able to determine that in fact DNA was "lost" from T6+ cells, and this DNA loss corresponded with T6SS activation in T6+ cells. Furthermore, genes in these fragments were predicted to encode antibiotic resistance enzymes, which was confirmed by phenotypically testing T6+ and T6- cells. This established an inverse relationship between T6SS and antibiotic resistance in this strain. PCR screening of our isolate collection revealed that other strains contained similar fragments of DNA, and we could recapitulate T6SS activation, loss of DNA, and loss of antibiotic resistance in these strains by testing single colonies.

In order to fully understand the genetic differences, we re-sequenced the strains using long-read technologies. This method allowed for the complete assembly of a fully closed, circular

chromosomes, and yielded a complete 170kb plasmid from the T6- cells. The use of long-read sequencing was crucial for the identification of this multidrug resistance plasmid. The fully assembled plasmid from the outbreak strain *A. baumannii* Ab04 contained an large cluster of antibiotic resistance genes, conjugative transfer elements, and large number of genes encoding hypothetical proteins. We found that *A. baumannii* ATCC 17978 contained an ancestral form of the T6SS-controlling plasmid, called pAB3, lacking most of the antibiotic resistance genes acquired by the outbreak strain. This made it clear that the studies done on this strain in Chapter two were likely done with a mixed population of T6+ and T6-, which is likely why we were unable to identify a clear phenotype. Indeed, using T6SS mutants in the defined T6+/T6- backgrounds, we showed that this strain does in fact have an anti-bacterial T6SS. An interesting, unintentional consequence of using long-read sequencing on *A. baumannii* ATCC 17978 was that we drastically improved the existing genome sequence of this strain (Smith *et al.*, 2007). In the original effort, pAB3 was assembled as part of the chromosome. This was likely because pAB3 has a 14kb stretch of DNA that is identical to a 14kb section in the chromosome, which would have been difficult for short read assemblers to differentiate. Furthermore, cursory comparison of our genome with the original showed our version corrected many other errors, particularly in SNPs in homopolymer runs. At least one other group has independently identified pAB3 as a plasmid rather than a chromosomal element, indicating that the original sequence was flawed rather than differences between laboratory strains (Dr. Ruth Hall, personal communication). Furthermore, we did not identify any sequencing reads (both long and short) that suggested the plasmid may transiently integrate into the chromosome. Considering the widespread use of *A. baumannii* ATCC 17978 as a model laboratory strain, this new genome sequence should prove valuable to many researchers. We did not perform long-read sequencing on the MDR *A. baumannii* 1438 strain from Argentina,

but short read sequencing confirmed the sequence reads from the T6- cells of this strain mapped well to pAB3 and pAB04-1, confirming the worldwide dissemination of this plasmid.

While several putative regulatory proteins are encoded by genes present on the plasmid, we found that two TetR-like proteins repressed T6SS expression and function when expressed *in trans*. It is well established that plasmids not only regulate their own gene expression but can also have wide-ranging effects on chromosomal genes (Takahashi *et al.*, 2015). The finding that these TetR-like repressors can inhibit T6SS in a range of *Acinetobacter* spp., but not in *V. cholerae*, indicates that they have evolved specificity for *Acinetobacter.* In the appendix section of this thesis, we make an interesting connection between these TetR1 and Csu pili expression, but further work is required to explain the regulatory role in this case. Plasmid-based repressors of T6SS have not been previously identified, and plasmid loss as a mechanism of transcriptional control represents a new paradigm for *A. baumannii* gene expression. It remains to be tested whether plasmid loss occurs in more biologically relevant conditions, such as in the human host or in the inanimate hospital environment. Several lines of evidence suggest that plasmid loss may represent a genuine mechanism of regulation. First, genome sequencing of consecutive isolates from a single patient has previously suggested that plasmid loss may occur *in vivo* (Wright *et al.*, 2014). Second, at least one high-throughput screen for virulence factors in *A. baumannii* ATCC 17978 using a mouse model of infection identified many of the pAB3 plasmid genes as being important for pathogenesis (Subashchandrabose *et al.*, 2016). This study used transposon-directed insertion-site sequencing, in which a sequenced pooled transposon mutants were inoculated into mice, allowed to cause infection, and then organs collected and surviving *A. baumannii* collected and re-sequenced to determine the relative abundance of each mutant. Mutants under-represented in the cells collected after infection were suggested to be less fit, and therefore important virulence

factors. Many genes on pAB3 were identified as putative virulence factors by this method. An alternative explanation is that the plasmid may have been lost during infection, or perhaps during laboratory culture. Clearly, validation of these genes in mono-infections and complementation would be required to confirm the results, but further emphasize the fact that our discovery of pAB3 may have more important implications in the broader context of the literature than solely as a regulator of T6SS. A previous study suggesting that the regulator HNS plays a role in T6SS expression in *A. baumannii* ATCC 17978 should also be revisited in light of our findings (Eijkelkamp *et al.*, 2013)

We established a link between antibiotic resistance and T6SS that suggests these two processes may not be able to coexist in a single cell. The findings are based on the fact that antibiotic resistance has accumulated in the regulatory plasmid over the past six decades, and that all strains harbouring this plasmid repress T6SS function. Furthermore, our previous work and that of others has established a general correlation that MDR *A. baumannii* strains repress T6SS, which can also be plasmid independent (Repizo *et al.*, 2015; Weber *et al.*, 2013). Several recently sequenced clinical isolates have also been found to either lack a T6SS or possess a degraded cluster (Eijkelkamp *et al.*, 2013; Hornsey *et al.*, 2011; Wright *et al.*, 2014). This may suggest that these MDR clinical isolates derive more of a fitness benefit by being antibiotic resistant and do not require the T6SS. Some strains may have evolved to tightly regulate the system, such as the MDR-plasmid mechanism we described, while others have completely inactivated their T6SS and permanently differentiated into MDR strains. Based on the recent findings of T6SS loss in some MDR *A. baumannii*, a hypothetical model is proposed illustrating a potential pathway to permanent T6SS inactivation (Figure 5.1). The absence of a T6SS these clinical isolates implies that this secretion system is not essential for virulence; indeed, high-throughput screens have failed to

identify T6SS components as important for pathogenicity in mouse models (Subashchandrabose *et al.*, 2016; Umland *et al.*, 2012; Wang *et al.*, 2014). The fitness cost of antibiotic resistance and of secretion systems have been well documented, and thus may provide an explanation between the apparent incompatibility of these two systems in *A. baumannii* (Andersson *et al.*, 2010; Andersson *et al.*, 1999; Sturm *et al.*, 2011). Environmental strains like *A. baylyi* and *A. calcoaceticus* constitutively express their T6SS, as does *A. baumannii* SDF isolated from body lice, which suggests the T6SS primarily functions in a context outside of infection, likely for competing with other bacteria. We found that *A. baumannii* strains with active T6SS are able to outcompete each other, suggesting that intra-species competition may be frequent. Additional studies on low-passage, non-laboratory derived strains are warranted to delineate the regulatory and functional differences for T6SS among *Acinetobacter* spp.

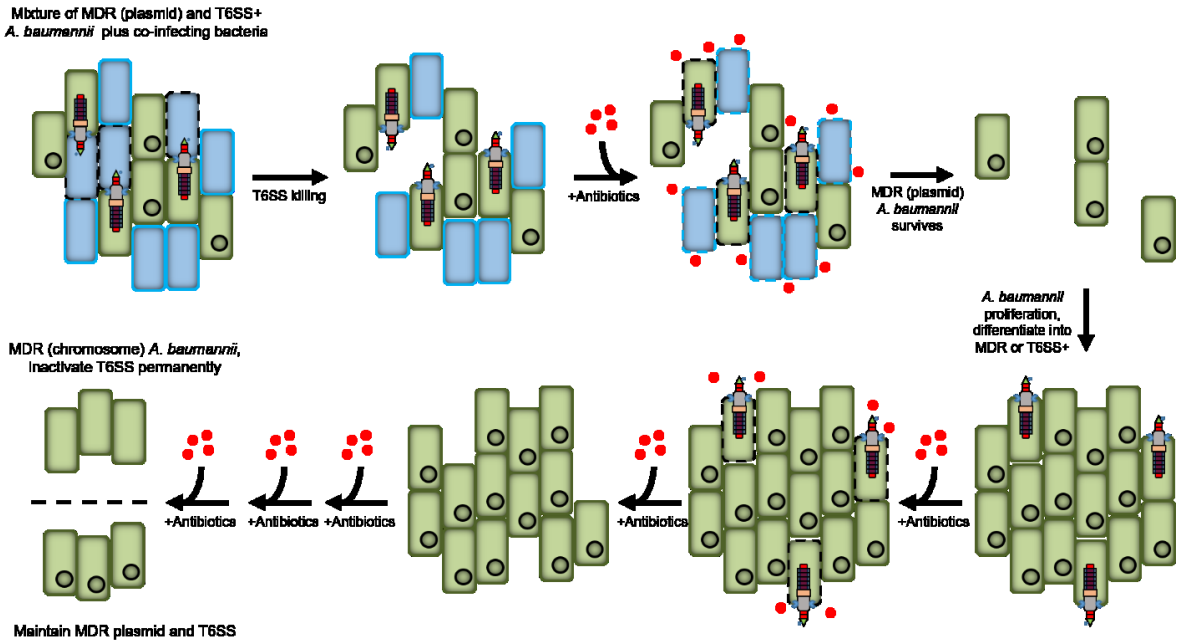

Figure 5.1 Model for T6SS loss during prolonged antibiotic use

MDR *A. baumannii* which contain most of their antibiotic resistance genes on the T6SS regulatory plasmid may experience competition from other bacteria during initial stages of infection. Prior to initiation of antibiotic therapy, a subset of cells will spontaneously differentiate by plasmid loss (and therefore loss of antibiotic resistance), express their T6SS, and kill other bacteria in order to compete for scarce nutrients. Upon treatment of the infected patient with antibiotics, the cells which have turned on their T6SS (by plasmid loss) are killed, as are the drug sensitive co-infecting bacteria. The MDR cells which maintained the plasmid survive and are selected for, and can proliferate. If the antibiotic concentration lowers, these cells will again undergo spontaneous plasmid loss, differentiating into T6SS+ or MDR+. If antibiotics are re-introduced, the T6SS+ cells will be killed, selecting for those that maintained their MDR+ phenotype. In this model, if antibiotics are constantly present for a prolonged period of time, MDR cells may insert their antibiotic resistance genes into the chromosome, while at the same time deleting the T6SS cluster as it is no longer beneficial for survival. Alternatively, the MDR plasmid may persist, allowing maintenance of both antibiotic resistance and T6SS.

5.3 Genetic requirements for T6SS activity in *Acinetobacter*

In Chapter Four, we undertook a genetic approach to define the requirements for T6SS in *Acinetobacter*. The conservation in genetic sequence and cluster organization of the T6SS among different species of *Acinetobacter* allowed us to utilize a previously constructed mutant library of

A. baylyi to test the contribution of numerous genes to a functional T6SS (de Berardinis *et al.*, 2008b). While similar studies have been performed in *V. cholerae* and *E. tarda*, based on our previous bioinformatics analysis we hypothesized that *Acinetobacter* spp. may carry additional novel genes required for T6SS biogenesis (Zheng *et al.*, 2007). While we confirmed that genes previously reported to be essential for T6SS assembly in other bacteria were also required in *A. baylyi*, we identified an additional three essential hypothetical genes lacking homologs in other organisms. Furthermore, three genes playing an apparent regulatory role were identified. These analyses led us to propose TagX as a novel feature of the bacterial T6SS that allows transit of the apparatus through the peptidoglycan layer (Figure 5.2).

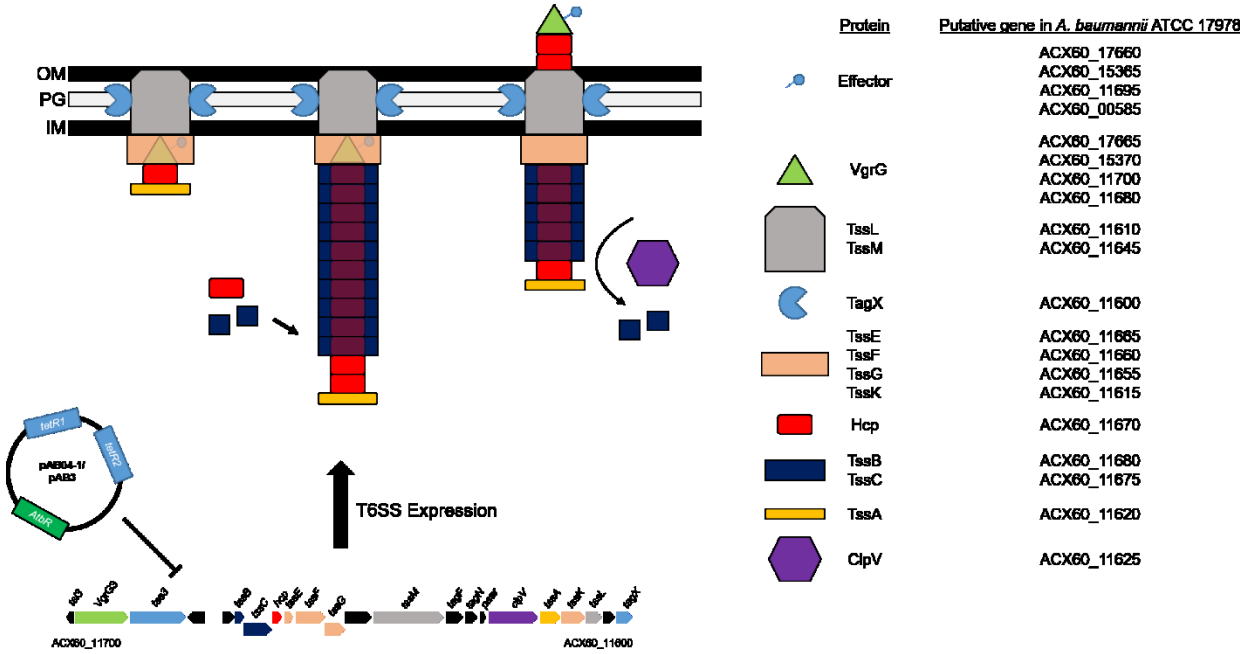

Figure 5.2 Model for T6SS assembly and regulation in *A. baumannii* ATCC 17978

While similar to other organisms, the *A. baumannii* T6SS requires previously unidentified components for biogenesis, including the novel peptidoglycan hydrolase TagX. Regulation in this strain and others is performed by a resistance plasmid which encodes repressors and is regularly lost in cells cultured under laboratory conditions.

We also sought to establish the role of VgrG and effector proteins in the anti-bacterial activity of *A. baumannii*. The number of double, triple and quadruple mutants constructed for this aspect are unprecedented for *A. baumannii,* and relied on a recently described mutational strategy (Tucker *et al.*, 2014). The combination of VgrG mutants was essential to validating their role in transport of anti-bacterial effectors as single deletions of VgrG/effectors does not always lead to an observable phenotype (Dong *et al.*, 2013). Our experiments identified VgrG1 as being important for T6SS biogenesis, while VgrG2 and VgrG3 were important for effector delivery to competing bacteria. These experiments suggest a similar role for VgrG proteins as has been described in other bacteria, and extend the more recent discovery that VgrG proteins are important for delivery of effectors (Bondage *et al.*, 2016; Brooks *et al.*, 2013; Cianfanelli *et al.*, 2016; Hachani *et al.*, 2014; Hachani *et al.*, 2011b; Liang *et al.*, 2015; Pukatzki *et al.*, 2007; Unterweger *et al.*, 2015). We confirmed the VgrG-dependent role of a putative nuclease, Tde, and its antitoxin, Tdi, to anti-bacterial killing and self-protection, as well as the anti-bacterial role of a putative effector named Tse3. These are the first descriptions of T6SS effector proteins and their VgrG-dependency in *A. baumannii*, and provide important insights for future studies of the role of this secretion system in inter-bacterial interactions of this organism. Future experiments explicitly showing effector secretion, and biochemically characterizing their activity, will further serve to unravel the role of these toxins in inter-bacterial interactions.

Our screen for essential genes indicated that a previously undescribed protein with predicted peptidoglycan hydrolase activity was required for biogenesis of the *A. baylyi* T6SS. We subsequently mutated the homolog in *A. baumannii* ATCC 17978 and showed it was also essential and could be complement in both species. Bioinformatic predictions suggested this protein localized to the inner membrane, and because it had predicted enzymatic activity, we decided to

study it further. We confirmed the *in silico* predictions by showing that this protein, now named TagX, localized to the inner membrane and was able to degrade peptidoglycan. We hypothesized that TagX could allow for T6SS passage through the encoding-organisms peptidoglycan layer, an essential activity that has been described for other secretion systems but not for the T6SS (Scheurwater *et al.*, 2011). By HPLC and MS, we confirmed that TagX possessed LD-endopeptidase activity, cleaving between the L-Ala and D-Glu residues in the peptidoglycan peptide stem. While soluble effectors that cleave peptidoglycan have been described, this was the first membrane-associated protein with hydrolase activity described for T6SS, and the linkage cleaved by TagX had also not been described (Russell *et al.*, 2014) . Based on the additional finding that TagX was not secreted, we concluded that this enzyme represented a novel component of the T6SS machinery; indeed, homologs were found in T6SS clusters from other organisms. Interestingly, TagX is a sequence and functional homolog of an enzyme characterized from a *Listeria monocytogenes* bacteriophage required for cell entry, further indicating an evolutionary relationship between bacteriophages and the T6SS. Follow-up studies to identify binding partners, and at which point in the assembly process TagX is required for, are now being undertaken in the laboratory. In total, these studies provide a significantly enhanced understanding of the T6SS in *A. baumannii*, and will guide future studies on this secretion system in this increasingly important pathogen.

5.4 References

Ahmed-Bentley, J., Chandran, A. U., Joffe, A. M., French, D., Peirano, G., & Pitout, J. D. (2013). Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. *Antimicrobial agents and chemotherapy, 57*(7), 3085-3091.

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews. Microbiology, 8*(4), 260-271.

Andersson, D. I., & Levin, B. R. (1999). The biological cost of antibiotic resistance. *Current opinion in microbiology, 2*(5), 489-493.

Aschtgen, M. S., Thomas, M. S., & Cascales, E. (2010b). Anchoring the type VI secretion system to the peptidoglycan: TssL, TagL, TagP... what else? *Virulence, 1*(6), 535-540.

Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell, 152*(4), 884-894.

Bondage, D. D., Lin, J. S., Ma, L. S., Kuo, C. H., & Lai, E. M. (2016). VgrG C terminus confers the type VI effector transport specificity and is required for binding with PAAR and adaptor-effector complex. *Proceedings of the National Academy of Sciences of the United States of America, 113*(27), E3931-3940.

Brooks, T. M., Unterweger, D., Bachmann, V., Kostiuk, B., & Pukatzki, S. (2013). Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *The Journal of biological chemistry, 288*(11), 7618-7625.

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S., Jr. (2013c). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PloS one, 8*(3), e59388.

Cianfanelli, F. R., Alcoforado Diniz, J., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion System. *PLoS pathogens, 12*(6), e1005735.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008b). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

Dong, T. G., Ho, B. T., Yoder-Himes, D. R., & Mekalanos, J. J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America, 110*(7), 2623-2628.

Durand, E., Nguyen, V. S., Zoued, A., Logger, L., Pehau-Arnaudet, G., Aschtgen, M. S., . . . Fronzes, R. (2015). Biogenesis and structure of a type VI secretion membrane core complex. *Nature, 523*(7562), 555-560.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Elbourne, L. D., Paulsen, I. T., & Brown, M. H. (2013). H-NS plays a role in expression of *Acinetobacter baumannii* virulence features. *Infection and immunity, 81*(7), 2574-2583.

Hachani, A., Allsopp, L. P., Oduko, Y., & Filloux, A. (2014). The VgrG proteins are "a la carte" delivery systems for bacterial type VI effectors. *The Journal of biological chemistry, 289*(25), 17872-17884.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011b). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and

multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., . . . Woodford, N. (2011). Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *The Journal of antimicrobial chemotherapy, 66*(7), 1499-1503.

Liang, X., Moore, R., Wilton, M., Wong, M. J., Lam, L., & Dong, T. G. (2015). Identification of divergent type VI secretion effectors using a conserved chaperone domain. *Proceedings of the National Academy of Sciences of the United States of America, 112*(29), 9106-9111.

Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15508-15513.

Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.

Pukatzki, S., McAuley, S. B., & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Current opinion in microbiology, 12*(1), 11-17.

Repizo, G. D., Gagne, S., Foucault-Grunenwald, M. L., Borges, V., Charpentier, X., Limansky, A. S., . . . Salcedo, S. P. (2015). Differential Role of the T6SS in *Acinetobacter baumannii* Virulence. *PloS one, 10*(9), e0138265.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature reviews. Microbiology, 12*(2), 137-148.

Scheurwater, E. M., & Burrows, L. L. (2011). Maintaining network security: how macromolecular structures cross the peptidoglycan layer. *FEMS microbiology letters, 318*(1), 1-9.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., & Snyder, M. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes & development, 21*(5), 601-614.

Sturm, A., Heinemann, M., Arnoldini, M., Benecke, A., Ackermann, M., Benz, M., . . . Hardt, W. D. (2011). The cost of virulence: retarded growth of Salmonella Typhimurium cells expressing type III secretion system 1. *PLoS pathogens, 7*(7), e1002143.

Subashchandrabose, S., Smith, S., DeOrnellas, V., Crepin, S., Kole, M., Zahdeh, C., & Mobley, H. L. (2016). *Acinetobacter baumannii* Genes Required for Bacterial Survival during Bloodstream Infection. *mSphere, 1*(1).

Takahashi, Y., Shintani, M., Takase, N., Kazo, Y., Kawamura, F., Hara, H., . . . Nojiri, H. (2015). Modulation of primary cell function of host Pseudomonas bacteria by the conjugative plasmid pCAR1. *Environmental microbiology, 17*(1), 134-155.

Tang, L., Liang, X., Moore, R., & Dong, T. G. (2015). Commentary: The icmF3 Locus is Involved in Multiple Adaptation- and Virulence-related Characteristics in *Pseudomonas aeruginosa* PAO1. *Frontiers in cellular and infection microbiology, 5*, 83.

Tucker, A. T., Nowicki, E. M., Boll, J. M., Knauf, G. A., Burdis, N. C., Trent, M. S., & Davies, B. W. (2014). Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio, 5*(4), e01313-01314.

Umland, T. C., Schultz, L. W., MacDonald, U., Beanan, J. M., Olson, R., & Russo, T. A. (2012). In vivo-validated essential genes identified in *Acinetobacter baumannii* by using human ascites overlap poorly with essential genes detected on laboratory media. *mBio, 3*(4).

Unterweger, D., Kostiuk, B., Otjengerdes, R., Wilton, A., Diaz-Satizabal, L., & Pukatzki, S. (2015). Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *The EMBO journal, 34*(16), 2198-2210.

Wang, N., Ozer, E. A., Mandel, M. J., & Hauser, A. R. (2014). Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *mBio, 5*(3), e01163-01114.

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., & Feldman, M. F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PloS one, 8*(1), e55142.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., . . . Adams, M. D. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *mBio, 5*(1), e00963-00913.

Zheng, J., & Leung, K. Y. (2007). Dissection of a type VI secretion system in Edwardsiella tarda. *Molecular microbiology, 66*(5), 1192-1206.

6 Appendix: Preliminary Characterization of the Csu pilus in *A. baumannii* ATCC 17978

6.1 Introduction

Pili formed by Gram-negative bacteria play an important role in these organisms ability to adhere to surfaces and form biofilms, invade host cells, and can impart motility (Waksman *et al.*, 2009). The chaperone-usher family of pili have been the focus of many studies, and the P-pile and type I pili of uropathogenic *E. coli* play a particularly important role in establishing infection (Anderson *et al.*, 2003). In *A. baumannii*, a chaperone-usher pathway for pili biogenesis has been determined to play an important role in mediating binding to abiotic surfaces (Tomaras *et al.*, 2003). The genes for this chaperone-usher pathway are encoded in a single five-gene operon, termed Csu, and includes, CsuA/B, CsuA, CsuB, CsuC, CsuD, and CsuE (Tomaras *et al.*, 2008). Although the precise molecular contribution of each protein has not been determined, bioinformatics analysis has suggested that CsuA/B is the major pilin, CsuD is the usher, and CsuC functions as the chaperone (Pakharukova *et al.*, 2015; Tomaras *et al.*, 2003; Tomaras *et al.*, 2008). CsuE has been suggested to function as a tip adhesion, while the role of CsuA, CsuB, although these may represent additional minor pilin subunits (Pakharukova *et al.*, 2015; Tomaras *et al.*, 2003; Tomaras *et al.*, 2008). Despite it's presence in many sequenced *A. baumannii* strains, the role of Csu has only been described for *A. baumannii* ATCC 19606, a strain widely used in the laboratory. Furthermore, previous sequence analysis has suggested that other strains, including *A. baumannii* ATCC 17978, encode non-functional Csu operon due to inactivating single-nucleaotide polymorphisms (Eijkelkamp *et al.*, 2011; Eijkelkamp *et al.*, 2014).

Based on our RNAseq analysis performed in Chapter four of this thesis, we found that T6+ cells of *A. baumannii* ATCC 17978 significantly upregulated transcripts of the entire Csu operon, suggesting a putative plasmid-based regulation similar to T6SS in this strain. Here we present data showing that, despite previous reports, Csu are expressed and functional in *A. baumannii* ATCC

17978. Furthermore, our analysis suggests that Csu expression is sensitive to the antibiotics sulfamethoxazole and trimethoprim. T6+ cells of this strain do not express Csu nor form biofilms when in the presence of these antibiotics, while T6- cells are completely resistant to the effects of sulfamethoxazole and partially resistant to the effects of trimethoprim. The combination of the two antibiotics is able to completely inhibit Csu expression and biofilm formation even in the resistant T6- strain. We show that this resistance is due to the dihydropteroate synthase gene present on the pAB3 plasmid that controls T6SS. Finally, our studies indicate that genes present on pAB3 may playa role in regulating Csu expression.

6.2 Transcriptomic and functional analysis of Csu pili in *A. baumannii* ATCC 17978 T6+/T6- cells

As described in Chapter four, we performed RNAseq analysis on T6+/T6- cells of *A. baumannii* ATCC 17978. Along with the described changes in T6SS expression, we found a significant upregulation in transcript levels of all *csu* genes in T6+ cells (Fig. 1A and B), suggesting that loss of pAB3 in T6+ cells may influence Csu expression. Our resequencing of this strain has now shown that the original genome sequence of this strain contained several errors in this region, and is in fact fully intact (Weber *et al.*, 2015b). We constructed a complete knockout of the full *csu* cluster (*csuFull*), as well as a single gene deletion of *csuD* (Fig. 1C). We detected expression of CsuA/B, the major pilin, in T6+ cells but not the two mutant strains, and this expression was not dependent on a functional T6SS as the *tssM* mutant expressed wild-type levels (Fig. 1D). Consistent with our RNAseq, T6- cells expressed low levels of CsuA/B. We could detect surface pili by electron microscopy in wild type T6+ cells but not those in the *csu* mutant strains, and T6- cells expressed visibly lower levels of this surface appendage (Fig. 1E). Loss of CsuA/B expression in the *csuD* mutant is consistent with previous reports that mutating components of this

pathway results in a feedback inhibition of the entire system (Tomaras *et al.*, 2003). The *csuFull* and *csuD* mutants did not affect Hcp expression or secretion in T6+ cells (Fig. 1F). These results show that *A. baumannii* ATCC 17978 produces Csu pili, and that expression of this pili depends on a functional Csu pathway and may be regulated by the presence of the pAB3 plasmid.

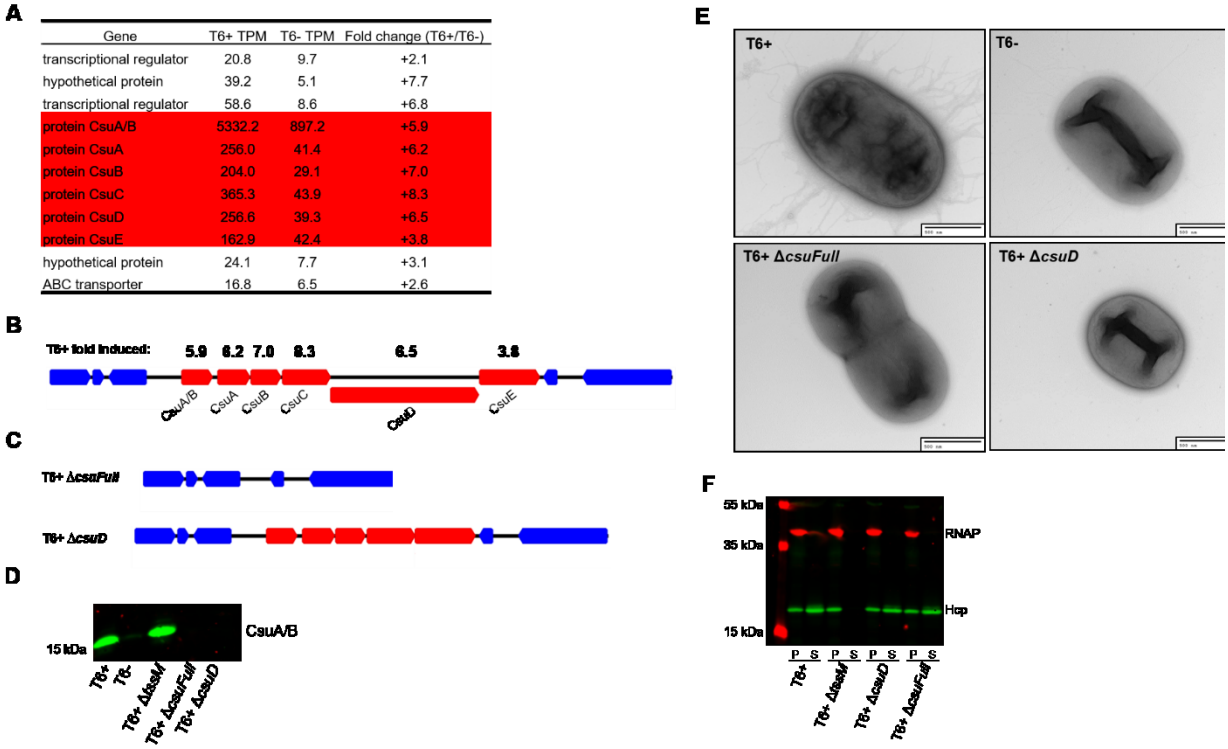

Figure 6.1 Analysis of Csu expression in *A. baumannii* ATCC 17978

A) RNAseq data showing raw transcripts per million (TPM) for T6+ and T6- cells and the fold difference between the two cells types. B) Csu cluster present in *A. baumannii* ATCC 17978. C) Construction of the full *csu* cluster (*csuFull)* and the *csuD* mutant strains. D) CsuA/B expression in various strains as determined by Western blot with an anti-CsuA/B antibody. D) Electron micrograph images of the indicated strains shows visible surface pili in wild type but not *csu* mutant strains. F) Hcp expression and secretion is not affected in the *csu* mutant strains.

To confirm our results, we undertook complementation experiments. The entire Csu cluster was cloned with it's native promoter in the *E. coli-A. baumannii* shuttle vector pBAV (Fig. 2A). In *E. coli*, we detected expression of CsuA/B by western blot, suggesting the complementation

vector was functional (Fig. 2B). The vector was introduced into the *csuFull* mutant, where robust CsuA/B expression was detected by Western blot (Fig. 2C). We attempted to visualize the Csu pilu on the surface of the complemented cells by electron microscopy, but did not see a restoration of the wild-type phenotype (Fig. 2D). Instead, complemented cells appeared to produce short structures on the cells surface that were clearly absent in the mutant but did not resemble wild-type cells. Western blots and coomassie stained SDS-PAGE gels confirmed that very little CsuA/B was on the cell surface (Fig. 2E).

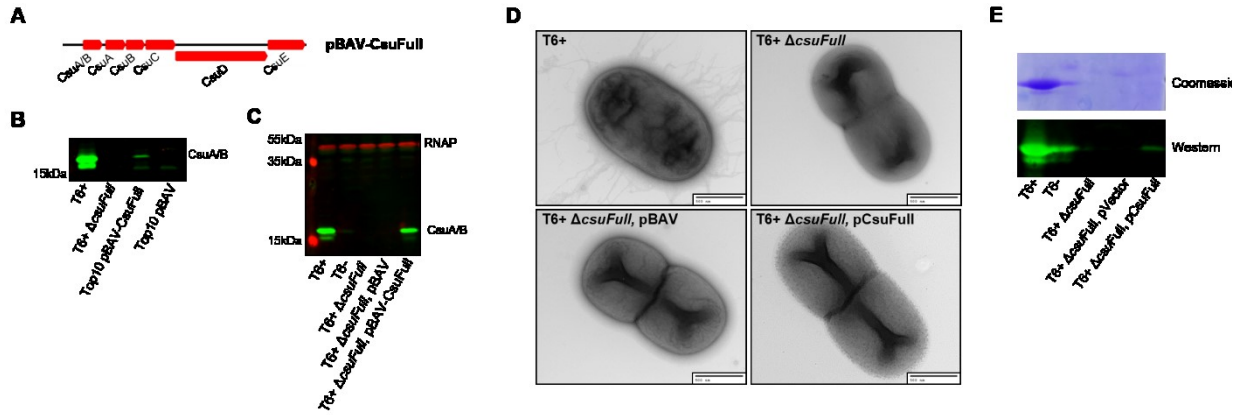

Figure 6.2 The *csuFull* mutant is not complemented by heterologous *csu* cluster expression

A) Schematic of the *csu* cluster cloned into pBAV with its native promtoer. B) CsuA/B is expressed in *E. coli* Top10 cells from pBAV-CsuFull. C) CsuA/B is expressed in the *csuFull* mutant. D) Pili are not visible on the surface of the complemented strain, but small structure distinct from the wild type can be seen. E) Coomassie stained SDS-PAGE gel (top) and Western blot (bottom) showing CsuA/B present in shear preps from T6+ cells but not the complemented strain

We next attempted complementation of the *csuD* mutant, and found by Western blot that CsuA/B was expressed and present on the cells surface of these mutants when expressing the full Csu cluster *in trans* (Fig. 3A and B). This was confirmed by the presence of pili structures on the cell surface of this strain by electron microscopy (Fig. 3C). We further confirmed the role of Csu

pili for attachment and biofilm formation on polystyrene surfaces, with the *csuD* mutant forming no detectable biofilm in these assays (Fig. 3D). Importantly, this phenotype could be complemented. Our previous report showed that two plasmid-based regulators were responsible for repressing T6SS in T6- cells. Expression of TetR1, but not TetR2, completely abolished biofilm formation in T6+ cells, suggesting a potential regulatory role of the pAB3 plasmid (Fig. 3E).

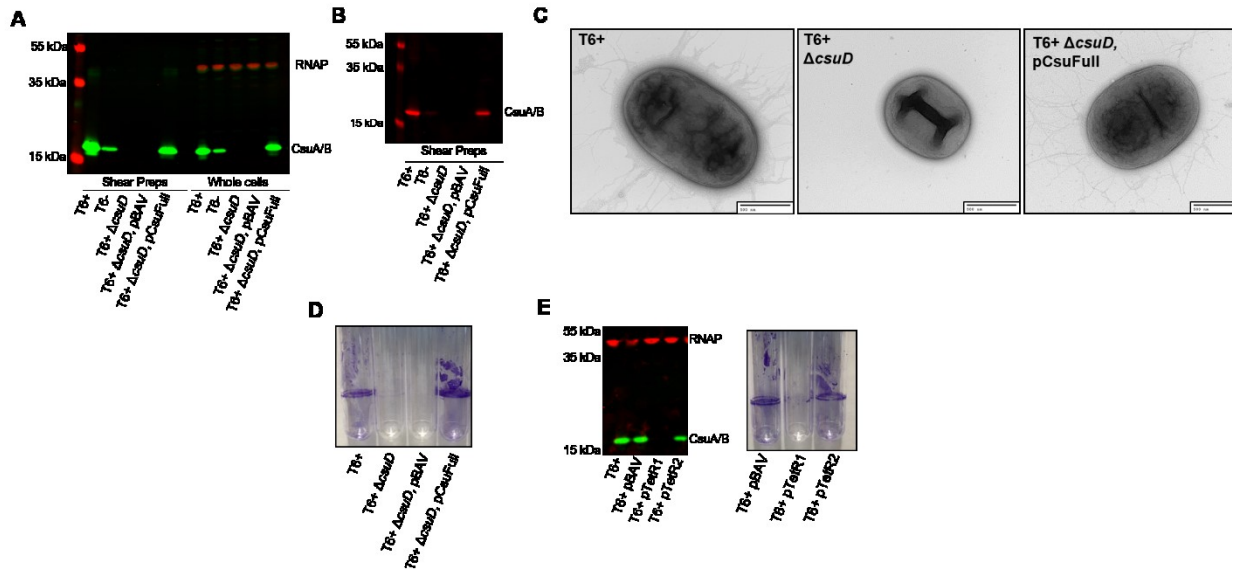

Figure 6.3 The *csuD* mutant can be complemented and is does not form biofilms

A) Western blot analysis of CsuA/B expression in the indicated strains shows robust CsuA/B expression in whole cells and its presence in shear preps in the complemented *csuD* strain. B) Coomassie stained SDS-PAGE showing CsuA/B in shear preps. C) Electron micrographs of the wild type, *csuD* mutant, and complemented strain shows a return to wild type phenotype when complemented. D) The *csuD* mutant does not form biofilms and can be complemented. E) TetR1, but not TetR2, represses CsuA/B expression and biofilm formation when overexpressed in wild type T6+ cells.

6.3 Antibiotics inhibit Csu expression

The T6- cells contain the pAB3 plasmid, which can be selected for with a combination of 30µg/mL sulfamethoxazole and 6µg/ml trimethoprim (sulf/tri) (Weber *et al.*, 2015b). Plasmid

pAB3 confers resistance to sulfamethoxazole only, with the sulfamethoxazole miniumum inhibitory concentration (MIC) being approximately 200μg/mL of T6+ cells. However, utilizing the combination of the two antibiotics, which target the same pathway, reduces the amount of antibiotic needed for selection. Both strains exhibit similar MICs to trimethoprim. The samples used for RNAseq analysis of T6+ and T6- cells were prepared by inoculating overnight cultures, which were subsequently used to inoculate fresh media the following day for collection of RNA from mid-log phase cells. T6- were grown overnight with antibiotic selection to ensure plasmid maintenance, but antibiotics were not used in the subsequent culture for RNA extraction to avoid inadvertent effects from their presence. We sought to examine the effects of these antibiotics on Csu expression and biofilm formation. Surprisingly, we found that T6- cells formed robust biofilms when cultured in the absence of sulf/tri (Fig. 4A and B). Biofilm formation was dependent on Csu pili, as a T6- *csu* mutant did not form biofilms regardless of the presence or absence of antibiotic (Fig. 4A and B). It is worth noting that loss of *csuD* in either T6+ or T6- cells resulted in the exclusive accumulation of cells in the bottom of the 96-well plates used for the biofilm assay (Fig. 4A). Western blot analysis confirmed that T6- cells expressed CsuA/B to the same level as T6+ cells in the absence of antibiotic selection (Fig. 4C). Importantly, the solvent used to solubilize the sulf/tri (DMSO) had no effect on biofilm formation, indicating the loss of CsuA/B expression and biofilm formation was the result of the antibiotic (Fig. 4D).

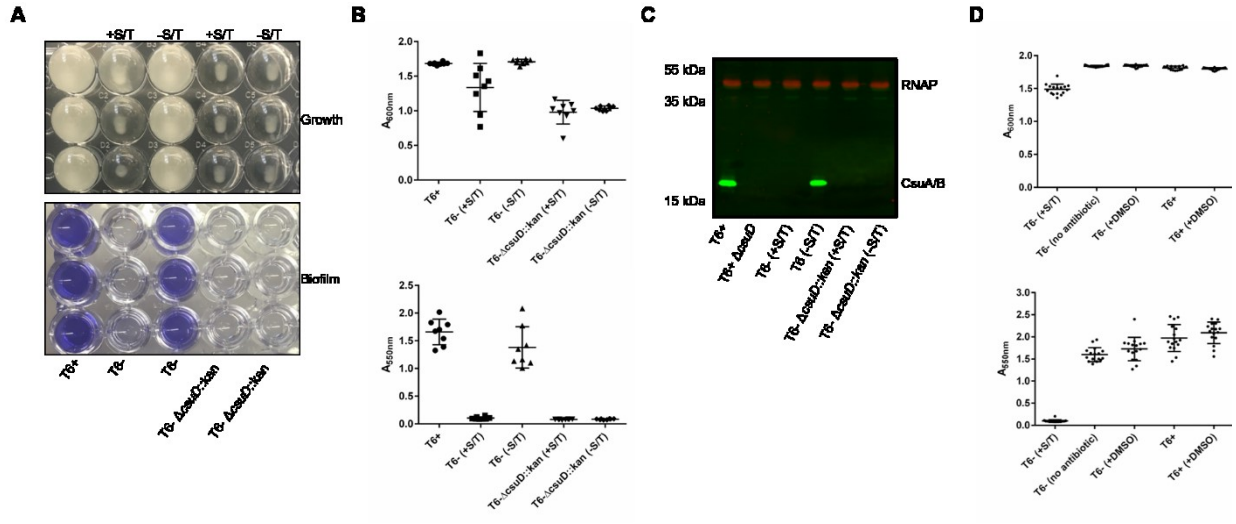

Figure 6.4 Antibiotics sulf/tri inhibit biofilm formation and CsuA/B expression in T6- cells

A) Visual results depicting growth and biofilm formation by the indicated strains in the presence or absence of sulf/tri antibiotic. B) Quantification of data in part A for growth (A600) and biofilm formation (A550). C) Western blot showing CsuA/B expression in the various strains with or without the addition of sulf/tri. D) DMSO does not affect biofilm formation

Addition of sub-inhibitory concentration of trimethoprim or sulfamethoxazole to T6+ cells resulted in a loss of biofilm formation (Fig. 5A and B). Addition of trimethoprim to T6- cells resulted in a similar biofilm defect, but sulfamethoxazole had no effect (Fig. 5A and B). Inactivation of the *dhps* gene present on pAB3 resulted in complete sensitivity to the effects of sulf/tri (Fig. 5A and B). Growth of this strain in the presence of kanamycin had little effect on biofilm formation, which suggest that the presence of pAB3 may only play a minor role in regulating Csu expression.

Analysis of CsuA/B in T6+ cells exposed to trimethoprim or sulfamethoxazole showed a complete loss of CsuA/B expression (Fig. 5C). CsuA/B expression was reduced in T6- cells exposed to trimethoprim, but unaffected in those grown in the presence of sulfamethoxazole. Insertional inactivation of the *dhps* gene on pAB3 in T6- cells resulted in complete sensitivity to

the effects on CsuA/B expression from both trimethoprim and sulfamethoxazole (Fig. 5C). As in the biofilm assays, selection for pAB3 in this strain with kanamycin did not affect CsuA/B expression. These results indicate that the presence of DHPS not only confers high-level resistance to sulfamethoxazole, but also enables resistance to the repressive effects of this antibiotic on Csu expression. It is likely that the reduced, but not absent, levels of CsuA/B in the T6- cells treated with trimethoprim is a result of the *dhps* gene functioning in the same pathway as is inhibited by trimethoprim. Importantly, non of the above conditions affected the expression or secretion of the hallmark T6SS protein Hcp (Fig. 5D).

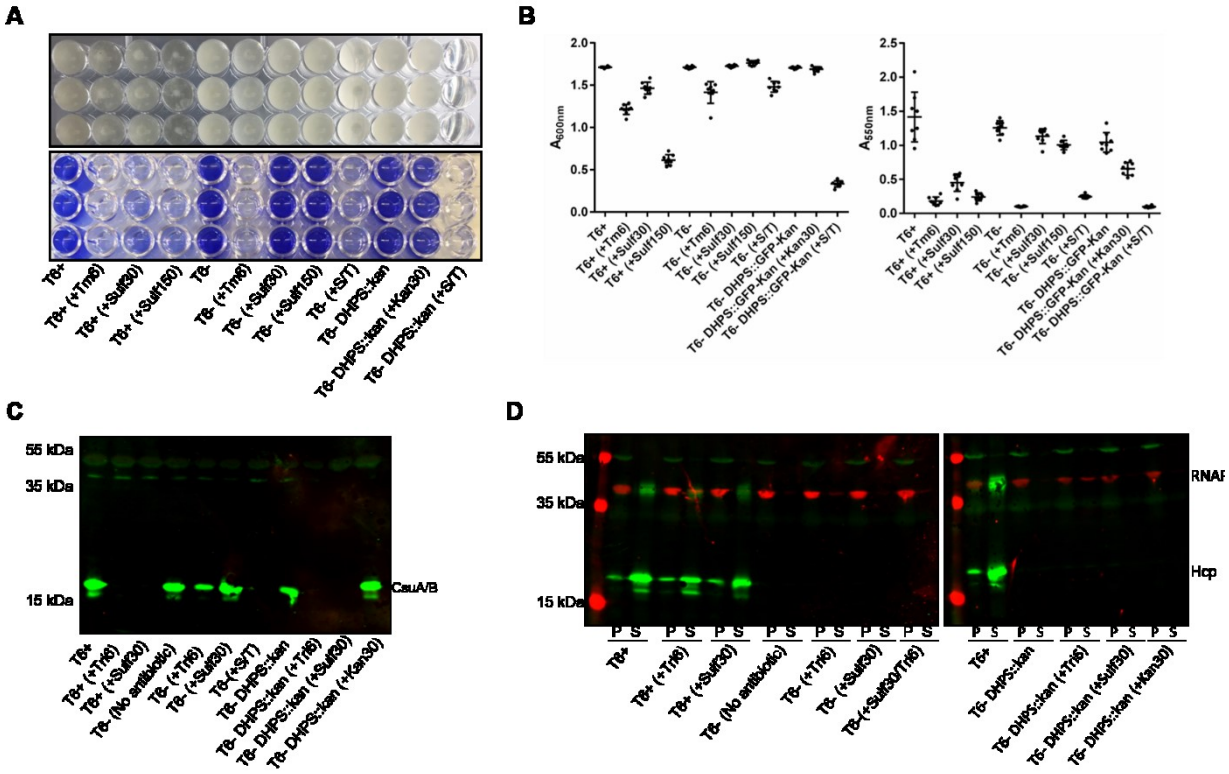

Figure 6.5 Sulfamethoxazole and Trimethoprim differentially affect biofilm formation and CsuA/B expression in T6+ and T6- cells

A) Visual results of growth and biofilm formation by the indicated strains in the presence or absence of sulfamethoxazole, trimethoprim, sulf/tri or kanamycin. B) Quantification of data in part A for growth (A600) and biofilm formation (A550). C) Western blot showing CsuA/B expression in the various strains with or without the addition of antibiotic. D) Western blot of Hcp expression and secretion showing no effect on T6SS under the indicated conditions

6.4 Conclusions

Our comparative RNAseq analysis of T6+ (lacking pAB3) and T6- (containing pAB3) cells of *A. baumannii* ATCC 17978 suggested that loss of pAB3 resulted in the upregulation of the Csu pilin cluster. We discovered for the first time that this strain produces Csu pili, in contrast to bioinformatic predictions published previous. Furthermore, our analysis showed that expression

of pili is sensitive to antibiotics which target the folic acid biosynthetic pathway, with both sulfamethoxazole and trimethoprim able to inhibit Csu expression.

By constructing in-frame deletions of the *csuD* gene, we found that the Csu pili is a critical mediatior of biofilm formation in *A. baumannii* ATCC 17978, which was confirmed by our complementation experiments. The presence of pili in shear preps and EM images confirmed there presence on the cell surface. Furthermore, we showed that a regulator present on pAB3, TetR1, could inhibit the expression of Csu. TetR1, and a second regulator TetR2, both repress expression of the T6SS in T6- cells containing the pAB3 plasmid (Weber *et al.*, 2015b). This may reflect a complex regulatory connection between these two pathways. It is interesting to note that recent genomic sequence analysis has shown that the genes encoding Csu and T6SS are absent in some recent clinical isolates (Wright *et al.*, 2014).

The transcriptomic data suggested that pAB3 played a role in Csu regulation, repressing Csu expression in T6- cells containing the plasmid. From the data presented here, however, it seems more likely that pAB3 plays a more minor role in regulating expression of this pili. It may be more likely that the cultures used for growth of T6-cells for RNA extraction, although not containing any antibiotic themselves, may have been influence by antibiotic present in the inoculum. However, these cultures were seeded at a 1:100 dilution, and therefore the sulf/tri used to select for pAB3 in the overnights would have been diluted. Alternatively, the cells used to seed the starter culture may have not fully recovered Csu expression during their growth to mid-log phase and RNA extraction. Although further analysis and RNAseq is warranted to explore all possibilities, we note that T6SS was not affected by the presence of antibiotic. The fact that TetR1 expression

in T6+ repressed Csu expression and biofilm formation raises the possibility that pAB3 has some role in regulation.

Despite the unresolved role of pAB3 in Csu regulation, our data clearly demonstrates the effect of sulfamethoxazole and trimethoprim on Csu expression. T6+ cells are unable to express CsuA/B in the presence of either antibiotic, which results in a loss of biofilm formation. CsuA/B expression in T6- cells is reduced by trimethoprim but not sulfamethoxazole, which is due to the presence of the *dhps* gene in pAB3. Inactivation of *dhps* resulted in complete sensitivity in this background. When the combination of sulf/tri is added to T6- cells during growth, CsuA/B expression is completely inhibited, indicating that despite being able to grow under these conditions, they are not permissive for biofilm formation and CsuA/B expression. Previous work done nearly three decades ago has shown that antibiotics, including trimethoprim and sulfamethoxazole, can have effects on pili expression in *E. coli*, raising the possibility that these antibiotics could be used as an adjunctive therapy to reduce problematic biofilms formed by *A. baumannii* (Kovarik *et al.*, 1989; Schifferli *et al.*, 1986).

6.5 Experimental Procedures

6.5.1 Sample preparation and Western blotting

Strains are listed in Table 1. Bacteria were routinely grown in LB broth or agar, with antibiotics added to cultures where appropriate at the indicated concentrations. Whole cell and supernatant samples were prepared as previously described (Weber *et al.*, 2013). Shear preps were performed by vortexing cells collected from an agar plate and isolating supernatants after centrifugation, as previous (Harding *et al.*, 2013).. Western blotting was performed on the LiCor system as described

previously, using anti-CsuA/B, anti-RNAP, and anti-Hcp antibodies for detection (Tomaras *et al.*, 2003; Weber *et al.*, 2013).

Table 6.1 Strains used in this study

Strain	Plasmid	Relevant characteristics and reference
A. baumannii ATCC 17978 T6+		wild type strain, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6-	pAB3	wild type strain, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6+ Δ*tssM*		T6SS mutant, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6+ Δ*csuFull*		Full *csu* cluster mutant, this study
A. baumannii ATCC 17978 T6+ Δ*csuD*		*csuD* mutant, this study
E. coli top10	pBAV	Shuttle vector
E. coli top10	pBAV-CsuFull	Expressing full Csu cluster, this study
A. baumannii ATCC 17978 T6+ Δ*csuFull*	pBAV	Vector control, this study
A. baumannii ATCC 17978 T6+ Δ*csuFull*	pBAV-CsuFull	Expressing full Csu cluster, this study
A. baumannii ATCC 17978 T6+ Δ*csuD*	pBAV	Vector control, this study
A. baumannii ATCC 17978 T6+ Δ*csuD*	pBAV-CsuFull	Expressing full Csu cluster, this study
A. baumannii ATCC 17978 T6+	pBAV	Vector control, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6+	pTetR1	Expressing TetR1, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6+	PTetR2	Expressing TetR2, (Weber *et al.*, 2015b)
A. baumannii ATCC 17978 T6- Δ*csuD::*kan	pAB3Δ*dhps*	Marked *csuD* mutant, this study
A. baumannii ATCC 17978 T6- Δ*dhps::*kan	pAB3Δ*dhps*	Marked *dhps* mutant, this study

6.5.2 Electron microscopy

Strains were grown overnight on LB agar plates, and the following day a sample was resuspended in 1mL PBS and processed by negative staining at the Washington University School of Medicine Molecular Microbiology Imaging Facility. Representative electron micrograph images are shown.

6.5.3 Biofilm assays

For biofilm assays in conical tubes, 1 mL of LB was inoculated with the indicate strains and incubated overnight without shaking at 37°C. Culture were then poured out and washed with three times with water, followed by the addition of 1.5mL 1% crystal violet. After 10 minutes incubation, crystal violet was removed followed by five water washes. Tubes were then imaged to visualize biofilm formation.A

Assays performed in 96-well plates were performed essentially as described (O'Toole, 2011). Briefly, 200µl of liquid media was added into the wells, with antibiotics added where indicated. Individual colonies of each strain were inoculated into 8 or 16 wells from a previously struck out plates. The 96-well plates were incubated for 24h in a humidified chamber at 37°C. After incubation, the optical density at 600nm was measured with a plate reader. The liquid was then removed by inverting the plate, and washed three times in water by immersion. 300µl crystal violet was then added to stain biofilms for 10min, followed by removal and five water washes. Crystal violet-stained biofilms were then solubilized with a 30% acetic acid solution for quantification at 550nm in a plate reader

6.6 References

Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J., & Hultgren, S. J. (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. *Science, 301*(5629), 105-107.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., & Brown, M. H. (2011). Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS microbiology letters, 323*(1), 44-51.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Paulsen, I. T., & Brown, M. H. (2014). Comparative analysis of surface-exposed virulence factors of *Acinetobacter baumannii*. *BMC Genomics, 15*, 1020.

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S., Jr. (2013). *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio, 4*(4).

Kovarik, J. M., Hoepelman, I. M., & Verhoef, J. (1989). Influence of fluoroquinolones on expression and function of P fimbriae in uropathogenic *Escherichia coli*. *Antimicrobial agents and chemotherapy, 33*(5), 684-688.

O'Toole, G. A. (2011). Microtiter dish biofilm formation assay. *Journal of visualized experiments : JoVE*(47).

Pakharukova, N., Tuittila, M., Paavilainen, S., & Zavialov, A. (2015). Crystallization and preliminary X-ray diffraction analysis of the Csu pili CsuC-CsuA/B chaperone-major subunit pre-assembly complex from *Acinetobacter baumannii*. *Acta Crystallogr F Struct Biol Commun, 71*(Pt 6), 770-774.

Schifferli, D. M., Abraham, S. N., & Beachey, E. H. (1986). Influence of trimethoprim and sulfamethoxazole on the synthesis, expression, and function of type 1 fimbriae of *Escherichia coli*. *The Journal of infectious diseases, 154*(3), 490-496.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473-3484.

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., & Actis, L. A. (2008). Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology, 154*(Pt 11), 3398-3409.

Waksman, G., & Hultgren, S. J. (2009). Structural biology of the chaperone-usher pathway of pilus biogenesis. *Nature reviews. Microbiology, 7*(11), 765-774.

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015b). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 112*(30), 9442-9447.

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., & Feldman, M. F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PloS one, 8*(1), e55142.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., . . . Adams, M. D. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *mBio, 5*(1), e00963-00913.

7 Bibliography

Adams, M. D., Nickel, G. C., Bajaksouzian, S., Lavender, H., Murthy, A. R., Jacobs, M. R., & Bonomo, R. A. (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrobial agents and chemotherapy, 53*(9), 3628-3634.

Ahmed-Bentley, J., Chandran, A. U., Joffe, A. M., French, D., Peirano, G., & Pitout, J. D. (2013). Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. *Antimicrobial agents and chemotherapy, 57*(7), 3085-3091.

Alcoforado Diniz, J., & Coulthurst, S. J. (2015). Intraspecies Competition in Serratia marcescens Is Mediated by Type VI-Secreted Rhs Effectors and a Conserved Effector-Associated Accessory Protein. *Journal of bacteriology, 197*(14), 2350-2360.

Anderson, G. G., Palermo, J. J., Schilling, J. D., Roth, R., Heuser, J., & Hultgren, S. J. (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. *Science, 301*(5629), 105-107.

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews. Microbiology, 8*(4), 260-271.

Andersson, D. I., & Levin, B. R. (1999). The biological cost of antibiotic resistance. *Current opinion in microbiology, 2*(5), 489-493.

Arroyo, L. A., Herrera, C. M., Fernandez, L., Hankins, J. V., Trent, M. S., & Hancock, R. E. (2011). The pmrCAB operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrobial agents and chemotherapy, 55*(8), 3743-3751.

Aschtgen, M. S., Bernard, C. S., De Bentzmann, S., Lloubes, R., & Cascales, E. (2008). SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *Journal of bacteriology, 190*(22), 7523-7531.

Aschtgen, M. S., Gavioli, M., Dessen, A., Lloubes, R., & Cascales, E. (2010a). The SciZ protein anchors the enteroaggregative *Escherichia coli* Type VI secretion system to the cell wall. *Molecular microbiology, 75*(4), 886-899.

Aschtgen, M. S., Thomas, M. S., & Cascales, E. (2010b). Anchoring the type VI secretion system to the peptidoglycan: TssL, TagL, TagP... what else? *Virulence, 1*(6), 535-540.

Aubert, D. F., Flannagan, R. S., & Valvano, M. A. (2008). A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. *Infection and immunity, 76*(5), 1979-1991.

Ballister, E. R., Lai, A. H., Zuckermann, R. N., Cheng, Y., & Mougous, J. D. (2008). In vitro self-assembly of tailorable nanotubes from a simple protein building block. *Proceedings of the National Academy of Sciences of the United States of America, 105*(10), 3733-3738.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., . . . Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to

single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology, 19*(5), 455-477.
Barker, J. R., Chong, A., Wehrly, T. D., Yu, J. J., Rodriguez, S. A., Liu, J., . . . Klose, K. E. (2009). The Francisella tularensis pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. *Molecular microbiology, 74*(6), 1459-1470.
Basler, M. (2015). Type VI secretion system: secretion by a contractile nanomachine. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 370*(1679).
Basler, M., Ho, B. T., & Mekalanos, J. J. (2013). Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. *Cell, 152*(4), 884-894.
Basler, M., & Mekalanos, J. J. (2012a). Type 6 secretion dynamics within and between bacterial cells. *Science, 337*(6096), 815.
Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J., & Mekalanos, J. J. (2012b). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature, 483*(7388), 182-186.
Baumann, P. (1968). Isolation of *Acinetobacter* from soil and water. *Journal of bacteriology, 96*(1), 39-42.
Baumann, P., Doudoroff, M., & Stanier, R. Y. (1968). A study of the Moraxella group. II. Oxidative-negative species (genus *Acinetobacter*). *Journal of bacteriology, 95*(5), 1520-1541.
Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., . . . Woodford, N. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. *Antimicrobial agents and chemotherapy, 55*(7), 3370-3379.
Bentancor, L. V., Camacho-Peiro, A., Bozkurt-Guzel, C., Pier, G. B., & Maira-Litran, T. (2012). Identification of Ata, a multifunctional trimeric autotransporter of *Acinetobacter baumannii*. *J Bacteriol, 194*(15), 3950-3960.
Berlau, J., Aucken, H. M., Houang, E., & Pitt, T. L. (1999). Isolation of *Acinetobacter* spp. including A. *baumannii* from vegetables: implications for hospital-acquired infections. *The Journal of hospital infection, 42*(3), 201-204.
Bernard, C. S., Brunet, Y. R., Gueguen, E., & Cascales, E. (2010a). Nooks and crannies in type VI secretion regulation. *Journal of bacteriology, 192*(15), 3850-3860.
Bernard, C. S., Brunet, Y. R., Gueguen, E., & Cascales, E. (2010b). Nooks and crannies in type VI secretion regulation. *Journal of bacteriology, 192*(15), 3850-3860.
Bernards, A. T., Frenay, H. M., Lim, B. T., Hendriks, W. D., Dijkshoorn, L., & van Boven, C. P. (1998). Methicillin-resistant Staphylococcus aureus and *Acinetobacter baumannii*: an unexpected difference in epidemiologic behavior. *American journal of infection control, 26*(6), 544-551.
Bernards, A. T., Harinck, H. I., Dijkshoorn, L., van der Reijden, T. J., & van den Broek, P. J. (2004). Persistent *Acinetobacter baumannii*? Look inside your medical equipment. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 25*(11), 1002-1004.
Bernardy, E. E., Turnsek, M. A., Wilson, S. K., Tarr, C. L., & Hammer, B. K. (2016). Diversity of Clinical and Environmental Isolates of *Vibrio cholerae* in Natural Transformation and

Contact-Dependent Bacterial Killing Indicative of Type VI Secretion System Activity. *Applied and environmental microbiology, 82*(9), 2833-2842.
Bingle, L. E., Bailey, C. M., & Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Current opinion in microbiology, 11*(1), 3-8.
Bladergroen, M. R., Badelt, K., & Spaink, H. P. (2003). Infection-blocking genes of a symbiotic Rhizobium leguminosarum strain that are involved in temperature-dependent protein secretion. *Molecular plant-microbe interactions : MPMI, 16*(1), 53-64.
Bleumink-Pluym, N. M., van Alphen, L. B., Bouwman, L. I., Wosten, M. M., & van Putten, J. P. (2013). Identification of a functional type VI secretion system in *Campylobacter jejuni* conferring capsule polysaccharide sensitive cytotoxicity. *PLoS pathogens, 9*(5), e1003393.
Boll, J. M., Tucker, A. T., Klein, D. R., Beltran, A. M., Brodbelt, J. S., Davies, B. W., & Trent, M. S. (2015). Reinforcing Lipid A Acylation on the Cell Surface of *Acinetobacter baumannii* Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival. *mBio, 6*(3), e00478-00415.
Bondage, D. D., Lin, J. S., Ma, L. S., Kuo, C. H., & Lai, E. M. (2016). VgrG C terminus confers the type VI effector transport specificity and is required for binding with PAAR and adaptor-effector complex. *Proceedings of the National Academy of Sciences of the United States of America, 113*(27), E3931-3940.
Bonemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., & Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *The EMBO journal, 28*(4), 315-325.
Bonnin, R. A., Nordmann, P., & Poirel, L. (2013). Screening and deciphering antibiotic resistance in *Acinetobacter baumannii*: a state of the art. *Expert review of anti-infective therapy, 11*(6), 571-583.
Bonnin, R. A., Poirel, L., Naas, T., Pirs, M., Seme, K., Schrenzel, J., & Nordmann, P. (2012). Dissemination of New Delhi metallo-beta-lactamase-1-producing *Acinetobacter baumannii* in Europe. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(9), E362-365.
Borgeaud, S., Metzger, L. C., Scrignari, T., & Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science, 347*(6217), 63-67.
Bou, G., & Martinez-Beltran, J. (2000). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 44*(2), 428-432.
Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., . . . Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 48*(1), 1-12.
Bouvet, P. J., & Jeanjean, S. (1989). Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Research in microbiology, 140*(4-5), 291-299.
Bouvet, P. J. M. G., P. A. D. . (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter* haemolyticus sp. nov., *Acinetobacter* johnsonii sp. nov., and *Acinetobacter* junii sp. nov. and emended descriptions of *Acinetobacter* calcoaceticus and *Acinetobacter* lwoffii. *Int J Syst Bacteriol*(36), 228-240.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., & Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC genomics, 10*, 104.

Brisou, J., & Prevot, A. R. (1954). [Studies on bacterial taxonomy. X. The revision of species under Acromobacter group]. *Annales de l'Institut Pasteur, 86*(6), 722-728.

Brooks, T. M., Unterweger, D., Bachmann, V., Kostiuk, B., & Pukatzki, S. (2013). Lytic activity of the *Vibrio cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *The Journal of biological chemistry, 288*(11), 7618-7625.

Brunet, Y. R., Bernard, C. S., Gavioli, M., Lloubes, R., & Cascales, E. (2011). An epigenetic switch involving overlapping fur and DNA methylation optimizes expression of a type VI secretion gene cluster. *PLoS genetics, 7*(7), e1002205.

Brunet, Y. R., Henin, J., Celia, H., & Cascales, E. (2014). Type VI secretion and bacteriophage tail tubes share a common assembly pathway. *EMBO reports, 15*(3), 315-321.

Brunet, Y. R., Zoued, A., Boyer, F., Douzi, B., & Cascales, E. (2015). The Type VI Secretion TssEFGK-VgrG Phage-Like Baseplate Is Recruited to the TssJLM Membrane Complex via Multiple Contacts and Serves As Assembly Platform for Tail Tube/Sheath Polymerization. *PLoS genetics, 11*(10), e1005545.

Bryksin, A. V., & Matsumura, I. (2010). Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. *PloS one, 5*(10), e13244.

Bublitz, M., Polle, L., Holland, C., Heinz, D. W., Nimtz, M., & Schubert, W. D. (2009). Structural basis for autoinhibition and activation of Auto, a virulence-associated peptidoglycan hydrolase of Listeria monocytogenes. *Molecular microbiology, 71*(6), 1509-1522.

Burkinshaw, B. J., Deng, W., Lameignere, E., Wasney, G. A., Zhu, H., Worrall, L. J., . . . Strynadka, N. C. (2015). Structural analysis of a specialized type III secretion system peptidoglycan-cleaving enzyme. *The Journal of biological chemistry, 290*(16), 10406-10417.

Burrows, L. L. (2012). *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol, 66*, 493-520.

Burtnick, M. N., & Brett, P. J. (2013). *Burkholderia* mallei and *Burkholderia* pseudomallei cluster 1 type VI secretion system gene expression is negatively regulated by iron and zinc. *PloS one, 8*(10), e76767.

Burtnick, M. N., Brett, P. J., Harding, S. V., Ngugi, S. A., Ribot, W. J., Chantratita, N., . . . Deshazer, D. (2011). The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia* pseudomallei. *Infection and immunity, 79*(4), 1512-1525.

Burtnick, M. N., DeShazer, D., Nair, V., Gherardini, F. C., & Brett, P. J. (2010). *Burkholderia* mallei cluster 1 type VI secretion mutants exhibit growth and actin polymerization defects in RAW 264.7 murine macrophages. *Infection and immunity, 78*(1), 88-99.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., & Snyder, M. (2010). Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS pathogens, 6*(4), e1000834.

Campos, M., Cisneros, D. A., Nivaskumar, M., & Francetic, O. (2013). The type II secretion system - a dynamic fiber assembly nanomachine. *Res Microbiol, 164*(6), 545-555.

Carr, E. L., Kampfer, P., Patel, B. K., Gurtler, V., & Seviour, R. J. (2003). Seven novel species of *Acinetobacter* isolated from activated sludge. *International journal of systematic and evolutionary microbiology, 53*(Pt 4), 953-963.

Carruthers, M. D., Harding, C. M., Baker, B. D., Bonomo, R. A., Hujer, K. M., Rather, P. N., & Munson, R. S., Jr. (2013a). Draft Genome Sequence of the Clinical Isolate *Acinetobacter* nosocomialis Strain M2. *Genome announcements, 1*(6).

Carruthers, M. D., Harding, C. M., Baker, B. D., Bonomo, R. A., Hujer, K. M., Rather, P. N., & Munson, R. S., Jr. (2013b). Draft Genome Sequence of the Clinical Isolate *Acinetobacter nosocomialis* Strain M2. *Genome Announc, 1*(6).

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S., Jr. (2013c). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PloS one, 8*(3), e59388.

Cascales, E. (2008). The type VI secretion toolkit. *EMBO reports, 9*(8), 735-741.

Cascales, E., & Cambillau, C. (2012). Structural biology of type VI secretion systems. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 367*(1592), 1102-1111.

Castang, S., McManus, H. R., Turner, K. H., & Dove, S. L. (2008). H-NS family members function coordinately in an opportunistic pathogen. *Proceedings of the National Academy of Sciences of the United States of America, 105*(48), 18947-18952.

Castric, P. (1995). pilO, a gene required for glycosylation of *Pseudomonas aeruginosa* 1244 pilin. *Microbiology, 141 (Pt 5)*, 1247-1254.

Centers for Disease Control and Prevention, O. o. I. D. (2013). Antibiotic Resistance Threats in the United States, 2013. Retrieved from http://www.cdc.gov/drugresistance/threat-report-2013

Chatzidaki-Livanis, M., Geva-Zatorsky, N., & Comstock, L. E. (2016). Bacteroides fragilis type VI secretion systems use novel effector and immunity proteins to antagonize human gut Bacteroidales species. *Proceedings of the National Academy of Sciences of the United States of America, 113*(13), 3627-3632.

Chen, T. L., Lee, Y. T., Kuo, S. C., Yang, S. P., Fung, C. P., & Lee, S. D. (2014). Rapid identification of *Acinetobacter baumannii*, *Acinetobacter* nosocomialis and *Acinetobacter* pittii with a multiplex PCR assay. *Journal of medical microbiology, 63*(Pt 9), 1154-1159.

Chin, C. Y., Gregg, K. A., Napier, B. A., Ernst, R. K., & Weiss, D. S. (2015). A PmrB-regulated Deacetylase Required for Lipid A Modification and Polymyxin Resistance in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*.

Choi, A. H., Slamti, L., Avci, F. Y., Pier, G. B., & Maira-Litran, T. (2009). The pgaABCD locus of *Acinetobacter baumannii* encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of bacteriology, 191*(19), 5953-5963.

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., . . . Lee, J. C. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cellular microbiology, 7*(8), 1127-1138.

Choi, C. H., Lee, J. S., Lee, Y. C., Park, T. I., & Lee, J. C. (2008). *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC microbiology, 8*, 216.

Chou, S., Bui, N. K., Russell, A. B., Lexa, K. W., Gardiner, T. E., LeRoux, M., . . . Mougous, J. D. (2012). Structure of a peptidoglycan amidase effector targeted to Gram-negative bacteria by the type VI secretion system. *Cell reports, 1*(6), 656-664.

Chow, J., & Mazmanian, S. K. (2010). A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell host & microbe, 7*(4), 265-276.

Cianfanelli, F. R., Alcoforado Diniz, J., Guo, M., De Cesare, V., Trost, M., & Coulthurst, S. J. (2016). VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion System. *PLoS pathogens, 12*(6), e1005735.

Clemens, D. L., Ge, P., Lee, B. Y., Horwitz, M. A., & Zhou, Z. H. (2015). Atomic structure of T6SS reveals interlaced array essential to function. *Cell, 160*(5), 940-951.

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H., & Reynaud, A. (2003). AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *The Journal of antimicrobial chemotherapy, 52*(4), 629-635.

Costa, T. R., Felisberto-Rodrigues, C., Meir, A., Prevost, M. S., Redzej, A., Trokter, M., & Waksman, G. (2015). Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nature reviews. Microbiology, 13*(6), 343-359.

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol, 26*(12), 1367-1372.

Coyne, S., Courvalin, P., & Perichon, B. (2011). Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrobial agents and chemotherapy, 55*(3), 947-953.

Cuthbertson, L., & Nodwell, J. R. (2013). The TetR family of regulators. *Microbiology and molecular biology reviews : MMBR, 77*(3), 440-475.

Dallo, S. F., Zhang, B., Denno, J., Hong, S., Tsai, A., Haskins, W., . . . Weitao, T. (2012). Association of *Acinetobacter baumannii* EF-Tu with cell surface, outer membrane vesicles, and fibronectin. *TheScientificWorldJournal, 2012*, 128705.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008a). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., . . . Weissenbach, J. (2008b). A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology, 4*, 174.

de Breij, A., Gaddy, J., van der Meer, J., Koning, R., Koster, A., van den Broek, P., . . . Dijkshoorn, L. (2009). CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606(T) to human airway epithelial cells and their inflammatory response. *Res Microbiol, 160*(3), 213-218.

de Bruin, O. M., Duplantis, B. N., Ludu, J. S., Hare, R. F., Nix, E. B., Schmerk, C. L., . . . Nano, F. E. (2011). The biochemical properties of the Francisella pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in type VI secretion. *Microbiology, 157*(Pt 12), 3483-3491.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature reviews. Microbiology, 5*(12), 939-951.

Doi, Y., Adams, J. M., Yamane, K., & Paterson, D. L. (2007). Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. *Antimicrobial agents and chemotherapy, 51*(11), 4209-4210.

Dong, T. G., Ho, B. T., Yoder-Himes, D. R., & Mekalanos, J. J. (2013). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America, 110*(7), 2623-2628.

Dong, T. G., & Mekalanos, J. J. (2012). Characterization of the RpoN regulon reveals differential regulation of T6SS and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic acids research, 40*(16), 7766-7775.

Durand, E., Cambillau, C., Cascales, E., & Journet, L. (2014). VgrG, Tae, Tle, and beyond: the versatile arsenal of Type VI secretion effectors. *Trends in microbiology, 22*(9), 498-507.
Durand, E., Nguyen, V. S., Zoued, A., Logger, L., Pehau-Arnaudet, G., Aschtgen, M. S., . . . Fronzes, R. (2015). Biogenesis and structure of a type VI secretion membrane core complex. *Nature, 523*(7562), 555-560.
Dykxhoorn, D. M., St Pierre, R., & Linn, T. (1996). A set of compatible tac promoter expression vectors. *Gene, 177*(1-2), 133-136.
Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., . . . Turner, S. (2009). Real-time DNA sequencing from single polymerase molecules. *Science, 323*(5910), 133-138.
Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Elbourne, L. D., Paulsen, I. T., & Brown, M. H. (2013). H-NS plays a role in expression of *Acinetobacter baumannii* virulence features. *Infection and immunity, 81*(7), 2574-2583.
Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., & Brown, M. H. (2011). Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS microbiology letters, 323*(1), 44-51.
Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Paulsen, I. T., & Brown, M. H. (2014). Comparative analysis of surface-exposed virulence factors of *Acinetobacter baumannii*. *BMC Genomics, 15*, 1020.
Ellis, T. N., & Kuehn, M. J. (2010). Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiology and molecular biology reviews : MMBR, 74*(1), 81-94.
Falagas, M. E., & Kasiakou, S. K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 40*(9), 1333-1341.
Falagas, M. E., Rafailidis, P. I., Matthaiou, D. K., Virtzili, S., Nikita, D., & Michalopoulos, A. (2008). Pandrug-resistant Klebsiella pneumoniae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections: characteristics and outcome in a series of 28 patients. *International journal of antimicrobial agents, 32*(5), 450-454.
Fang, F. C., & Rimsky, S. (2008). New insights into transcriptional regulation by H-NS. *Current opinion in microbiology, 11*(2), 113-120.
Faridmoayer, A., Fentabil, M. A., Haurat, M. F., Yi, W., Woodward, R., Wang, P. G., & Feldman, M. F. (2008). Extreme substrate promiscuity of the Neisseria oligosaccharyl transferase involved in protein O-glycosylation. *The Journal of biological chemistry, 283*(50), 34596-34604.
Faridmoayer, A., Fentabil, M. A., Mills, D. C., Klassen, J. S., & Feldman, M. F. (2007). Functional characterization of bacterial oligosaccharyltransferases involved in O-linked protein glycosylation. *Journal of bacteriology, 189*(22), 8088-8098.
Filloux, A., Hachani, A., & Bleves, S. (2008). The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology, 154*(Pt 6), 1570-1583.
Flaugnatti, N., Le, T. T., Canaan, S., Aschtgen, M. S., Nguyen, V. S., Blangy, S., . . . Journet, L. (2015). A phospholipase A antibacterial Type VI secretion effector interacts directly with the C-terminal domain of the VgrG spike protein for delivery. *Molecular microbiology*.
Fournier, P. E., & Richet, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 42*(5), 692-699.

Fowler, T., Walker, D., & Davies, S. C. (2014). The risk/benefit of predicting a post-antibiotic era: is the alarm working? *Annals of the New York Academy of Sciences, 1323*, 1-10.
Fregolino, E., Gargiulo, V., Lanzetta, R., Parrilli, M., Holst, O., & Castro, C. D. (2011). Identification and structural determination of the capsular polysaccharides from two *Acinetobacter baumannii* clinical isolates, MG1 and SMAL. *Carbohydrate research, 346*(7), 973-977.
French, C. T., Toesca, I. J., Wu, T. H., Teslaa, T., Beaty, S. M., Wong, W., . . . Miller, J. F. (2011). Dissection of the *Burkholderia* intracellular life cycle using a photothermal nanoblade. *Proceedings of the National Academy of Sciences of the United States of America, 108*(29), 12095-12100.
Fritsch, M. J., Trunk, K., Diniz, J. A., Guo, M., Trost, M., & Coulthurst, S. J. (2013). Proteomic identification of novel secreted antibacterial toxins of the Serratia marcescens type VI secretion system. *Molecular & cellular proteomics : MCP, 12*(10), 2735-2749.
Fronzes, R., Schafer, E., Wang, L., Saibil, H. R., Orlova, E. V., & Waksman, G. (2009). Structure of a type IV secretion system core complex. *Science, 323*(5911), 266-268.
Fu, Y., Waldor, M. K., & Mekalanos, J. J. (2013). Tn-Seq analysis of *Vibrio cholerae* intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. *Cell host & microbe, 14*(6), 652-663.
Fukushima, T., Yao, Y., Kitajima, T., Yamamoto, H., & Sekiguchi, J. (2007). Characterization of new L,D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in Bacillus subtilis. *Mol Genet Genomics, 278*(4), 371-383.
Fulsundar, S., Harms, K., Flaten, G. E., Johnsen, P. J., Chopade, B. A., & Nielsen, K. M. (2014). Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation. *Applied and environmental microbiology, 80*(11), 3469-3483.
Fulsundar, S., Kulkarni, H. M., Jagannadham, M. V., Nair, R., Keerthi, S., Sant, P., . . . Chopade, B. A. (2015). Molecular characterization of outer membrane vesicles released from *Acinetobacter* radioresistens and their potential roles in pathogenesis. *Microbial pathogenesis, 83-84*, 12-22.
Gaddy, J. A., Arivett, B. A., McConnell, M. J., Lopez-Rojas, R., Pachon, J., & Actis, L. A. (2012). Role of acinetobactin-mediated iron acquisition functions in the interaction of *Acinetobacter baumannii* strain ATCC 19606T with human lung epithelial cells, Galleria mellonella caterpillars, and mice. *Infection and immunity, 80*(3), 1015-1024.
Garcia-Quintanilla, M., Pulido, M. R., Lopez-Rojas, R., Pachon, J., & McConnell, M. J. (2013). Emerging therapies for multidrug resistant *Acinetobacter baumannii*. *Trends in microbiology, 21*(3), 157-163.
Garnacho-Montero, J., Ortiz-Leyba, C., Fernandez-Hinojosa, E., Aldabo-Pallas, T., Cayuela, A., Marquez-Vacaro, J. A., . . . Jimenez-Jimenez, F. J. (2005). *Acinetobacter baumannii* ventilator-associated pneumonia: epidemiological and clinical findings. *Intensive care medicine, 31*(5), 649-655.
Geisinger, E., & Isberg, R. R. (2015). Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS pathogens, 11*(2), e1004691.
Gerlach, R. G., & Hensel, M. (2007). Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. *International journal of medical microbiology : IJMM, 297*(6), 401-415.
Gerner-Smidt, P., Tjernberg, I., & Ursing, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *Journal of clinical microbiology, 29*(2), 277-282.

Gohl, O., Friedrich, A., Hoppert, M., & Averhoff, B. (2006). The thin pili of *Acinetobacter* sp. strain BD413 mediate adhesion to biotic and abiotic surfaces. *Applied and environmental microbiology, 72*(2), 1394-1401.

Grandi, G. (2010). Bacterial surface proteins and vaccines. *F1000 Biol Rep, 2*.

Gueguen, E., & Cascales, E. (2012). Promoter swapping unveils the role of the Citrobacter rodentium CTS1 Type VI secretion system in inter-bacterial competition. *Applied and environmental microbiology*.

Gueguen, E., Durand, E., Zhang, X. Y., d'Amalric, Q., Journet, L., & Cascales, E. (2013). Expression of a Yersinia pseudotuberculosis Type VI Secretion System Is Responsive to Envelope Stresses through the OmpR Transcriptional Activator. *PloS one, 8*(6), e66615.

Hachani, A., Allsopp, L. P., Oduko, Y., & Filloux, A. (2014). The VgrG proteins are "a la carte" delivery systems for bacterial type VI effectors. *The Journal of biological chemistry, 289*(25), 17872-17884.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011a). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jove, D., & Filloux, A. (2011b). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *The Journal of biological chemistry, 286*(14), 12317-12327.

Hachani, A., Wood, T. E., & Filloux, A. (2016). Type VI secretion and anti-host effectors. *Current opinion in microbiology, 29*, 81-93.

Harding, C. M., Nasr, M. A., Kinsella, R. L., Scott, N. E., Foster, L. J., Weber, B. S., . . . Feldman, M. F. (2015). *Acinetobacter* strains carry two functional oligosaccharyltransferases, one devoted exclusively to type IV pilin, and the other one dedicated to *O*-glycosylation of multiple proteins. *Mol Microbiol, 96*(5), 1023-1041.

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S., Jr. (2013). *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio, 4*(4).

Haseley, S., & Wilkinson, S. G. (1997a). Structure of the O18 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 301*(3-4), 187-192.

Haseley, S. R., Traub, W. H., & Wilkinson, S. G. (1997b). Structures of polymeric products isolated from the lipopolysaccharides of reference strains for *Acinetobacter baumannii* O23 and O12. *European journal of biochemistry / FEBS, 244*(1), 147-154.

Haseley, S. R., & Wilkinson, S. G. (1994). Structure of the putative O10 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 264*(1), 73-81.

Haseley, S. R., & Wilkinson, S. G. (1998). Structure of the O-7 antigen from *Acinetobacter baumannii*. *Carbohydrate research, 306*(1-2), 257-263.

Haurat, M. F., Elhenawy, W., & Feldman, M. F. (2014). Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. *Biological chemistry*.

Hayes, C. S., Aoki, S. K., & Low, D. A. (2010). Bacterial contact-dependent delivery systems. *Annual review of genetics, 44*, 71-90.

Hecht, A. L., Casterline, B. W., Earley, Z. M., Goo, Y. A., Goodlett, D. R., & Bubeck Wardenburg, J. (2016). Strain competition restricts colonization of an enteric pathogen and prevents colitis. *EMBO reports*.

Henrichsen, J. (1975). The occurrence of twitching motility among Gram-negative bacteria. *Acta Pathol Microbiol Scand B, 83*(3), 171-178.

Henrichsen, J., & Blom, J. (1975). Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. *Acta Pathol Microbiol Scand B, 83*(2), 103-115.

Henry, R., Vithanage, N., Harrison, P., Seemann, T., Coutts, S., Moffatt, J. H., . . . Boyce, J. D. (2012). Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglucosamine. *Antimicrobial agents and chemotherapy, 56*(1), 59-69.

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology, 8*(1), 15-25.

Hirano, T., Minamino, T., & Macnab, R. M. (2001). The role in flagellar rod assembly of the N-terminal domain of Salmonella FlgJ, a flagellum-specific muramidase. *Journal of molecular biology, 312*(2), 359-369.

Ho, B. T., Basler, M., & Mekalanos, J. J. (2013). Type 6 secretion system-mediated immunity to type 4 secretion system-mediated gene transfer. *Science, 342*(6155), 250-253.

Ho, B. T., Dong, T. G., & Mekalanos, J. J. (2014). A view to a kill: the bacterial type VI secretion system. *Cell host & microbe, 15*(1), 9-21.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., & Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene, 212*(1), 77-86.

Hood, M. I., Becker, K., Roux, C. M., Dunman, P. M., & Skaar, E. P. (2012a). Genetic determinants of intrinsic colistin tolerance in *Acinetobacter baumannii*. *Infection and immunity*.

Hood, M. I., Becker, K. W., Roux, C. M., Dunman, P. M., & Skaar, E. P. (2013). genetic determinants of intrinsic colistin tolerance in *Acinetobacter baumannii*. *Infection and immunity, 81*(2), 542-551.

Hood, M. I., Mortensen, B. L., Moore, J. L., Zhang, Y., Kehl-Fie, T. E., Sugitani, N., . . . Skaar, E. P. (2012b). Identification of an *Acinetobacter baumannii* Zinc Acquisition System that Facilitates Resistance to Calprotectin-mediated Zinc Sequestration. *PLoS pathogens, 8*(12), e1003068.

Hood, R. D., Singh, P., Hsu, F., Guvener, T., Carl, M. A., Trinidad, R. R., . . . Mougous, J. D. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell host & microbe, 7*(1), 25-37.

Hoppner, C., Carle, A., Sivanesan, D., Hoeppner, S., & Baron, C. (2005). The putative lytic transglycosylase VirB1 from Brucella suis interacts with the type IV secretion system core components VirB8, VirB9 and VirB11. *Microbiology, 151*(Pt 11), 3469-3482.

Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., . . . Woodford, N. (2011). Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *The Journal of antimicrobial chemotherapy, 66*(7), 1499-1503.

Houang, E. T., Chu, Y. W., Leung, C. M., Chu, K. Y., Berlau, J., Ng, K. C., & Cheng, A. F. (2001). Epidemiology and infection control implications of *Acinetobacter* spp. in Hong Kong. *Journal of clinical microbiology, 39*(1), 228-234.

Hoyle, B. D., & Beveridge, T. J. (1984). Metal binding by the peptidoglycan sacculus of *Escherichia coli* K-12. *Canadian journal of microbiology, 30*(2), 204-211.

Hsu, F., Schwarz, S., & Mougous, J. D. (2009). TagR promotes PpkA-catalysed type VI secretion activation in *Pseudomonas aeruginosa*. *Molecular microbiology, 72*(5), 1111-1125.

Hug, I., & Feldman, M. F. (2011). Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology, 21*(2), 138-151.

Hunger, M., Schmucker, R., Kishan, V., & Hillen, W. (1990). Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter* calcoaceticus and its use for *Escherichia coli* shuttle plasmids. *Gene, 87*(1), 45-51.

Infectious Diseases Society of, A., Spellberg, B., Blaser, M., Guidos, R. J., Boucher, H. W., Bradley, J. S., . . . Gilbert, D. N. (2011). Combating antimicrobial resistance: policy recommendations to save lives. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 52 Suppl 5*, S397-428.

Ishikawa, T., Rompikuntal, P. K., Lindmark, B., Milton, D. L., & Wai, S. N. (2009). Quorum sensing regulation of the two hcp alleles in *Vibrio cholerae* O1 strains. *PloS one, 4*(8), e6734.

Ishikawa, T., Sabharwal, D., Broms, J., Milton, D. L., Sjostedt, A., Uhlin, B. E., & Wai, S. N. (2012). Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infection and immunity, 80*(2), 575-584.

Iwashkiw, J. A., Fentabil, M. A., Faridmoayer, A., Mills, D. C., Peppler, M., Czibener, C., . . . Feldman, M. F. (2012a). Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. *Microbial cell factories, 11*, 13.

Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., . . . Feldman, M. F. (2012b). Identification of a general O-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS pathogens, 8*(6), e1002758.

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., . . . Dunman, P. M. (2010). Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infection and immunity, 78*(5), 1952-1962.

Jawad, A., Seifert, H., Snelling, A. M., Heritage, J., & Hawkey, P. M. (1998). Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates. *Journal of clinical microbiology, 36*(7), 1938-1941.

Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N. R., . . . Jin, Q. (2016). The *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host Autophagy by Activating Endoplasmic Reticulum Stress. *Cell reports*.

Jiang, F., Waterfield, N. R., Yang, J., Yang, G., & Jin, Q. (2014). A *Pseudomonas aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic cells. *Cell host & microbe, 15*(5), 600-610.

Jin, J. S., Kwon, S. O., Moon, D. C., Gurung, M., Lee, J. H., Kim, S. I., & Lee, J. C. (2011). *Acinetobacter baumannii* secretes cytotoxic outer membrane protein A via outer membrane vesicles. *PloS one, 6*(2), e17027.

Johnson, T. L., Waack, U., Smith, S., Mobley, H., Sandkvist, M. . (2015). *Acinetobacter baumannii* is dependent on the type II secretion system and its substrate LipA for lipid utilization and in vivo fitness. *Journal of bacteriology*.

Jun, S. H., Lee, J. H., Kim, B. R., Kim, S. I., Park, T. I., Lee, J. C., & Lee, Y. C. (2013). *Acinetobacter baumannii* outer membrane vesicles elicit a potent innate immune response via membrane proteins. *PloS one, 8*(8), e71751.

Juni, E. (1972). Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *Journal of bacteriology, 112*(2), 917-931.

Kanamaru, S., Leiman, P. G., Kostyuchenko, V. A., Chipman, P. R., Mesyanzhinov, V. V., Arisaka, F., & Rossmann, M. G. (2002). Structure of the cell-puncturing device of bacteriophage T4. *Nature, 415*(6871), 553-557.

Kapitein, N., Bonemann, G., Pietrosiuk, A., Seyffer, F., Hausser, I., Locker, J. K., & Mogk, A. (2013). ClpV recycles VipA/VipB tubules and prevents non-productive tubule formation to ensure efficient type VI protein secretion. *Molecular microbiology, 87*(5), 1013-1028.

Kenyon, J. J., & Hall, R. M. (2013). Variation in the complex carbohydrate biosynthesis loci of *Acinetobacter baumannii* genomes. *PloS one, 8*(4), e62160.

Kenyon, J. J., Nigro, S. J., & Hall, R. M. (2014). Variation in the OC locus of *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipooligosaccharide. *PloS one, 9*(9), e107833.

Koraimann, G. (2003). Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. *Cellular and molecular life sciences : CMLS, 60*(11), 2371-2388.

Korndorfer, I. P., Kanitz, A., Danzer, J., Zimmer, M., Loessner, M. J., & Skerra, A. (2008). Structural analysis of the L-alanoyl-D-glutamate endopeptidase domain of Listeria bacteriophage endolysin Ply500 reveals a new member of the LAS peptidase family. *Acta crystallographica. Section D, Biological crystallography, 64*(Pt 6), 644-650.

Koskiniemi, S., Lamoureux, J. G., Nikolakakis, K. C., t'Kint de Roodenbeke, C., Kaplan, M. D., Low, D. A., & Hayes, C. S. (2013). Rhs proteins from diverse bacteria mediate intercellular competition. *Proceedings of the National Academy of Sciences of the United States of America, 110*(17), 7032-7037.

Kovarik, J. M., Hoepelman, I. M., & Verhoef, J. (1989). Influence of fluoroquinolones on expression and function of P fimbriae in uropathogenic *Escherichia coli*. *Antimicrobial agents and chemotherapy, 33*(5), 684-688.

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology, 305*(3), 567-580.

Kube, S., Kapitein, N., Zimniak, T., Herzog, F., Mogk, A., & Wendler, P. (2014). Structure of the VipA/B type VI secretion complex suggests a contraction-state-specific recycling mechanism. *Cell reports, 8*(1), 20-30.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., . . . Aizawa, S. I. (1998). Supramolecular structure of the Salmonella typhimurium type III protein secretion system. *Science, 280*(5363), 602-605.

Kudryashev, M., Wang, R. Y., Brackmann, M., Scherer, S., Maier, T., Baker, D., . . . Basler, M. (2015). Structure of the type VI secretion system contractile sheath. *Cell, 160*(5), 952-962.

Kwon, S. O., Gho, Y. S., Lee, J. C., & Kim, S. I. (2009). Proteome analysis of outer membrane vesicles from a clinical *Acinetobacter baumannii* isolate. *FEMS microbiology letters, 297*(2), 150-156.

Lee, J. S., Choi, C. H., Kim, J. W., & Lee, J. C. (2010). *Acinetobacter baumannii* outer membrane protein A induces dendritic cell death through mitochondrial targeting. *Journal of microbiology, 48*(3), 387-392.

Lees-Miller, R. G., Iwashkiw, J. A., Scott, N. E., Seper, A., Vinogradov, E., Schild, S., & Feldman, M. F. (2013). A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Molecular microbiology, 89*(5), 816-830.

Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., . . . Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4154-4159.

LeRoux, M., De Leon, J. A., Kuwada, N. J., Russell, A. B., Pinto-Santini, D., Hood, R. D., . . . Mougous, J. D. (2012). Quantitative single-cell characterization of bacterial interactions reveals type VI secretion is a double-edged sword. *Proceedings of the National Academy of Sciences of the United States of America, 109*(48), 19804-19809.

LeRoux, M., Kirkpatrick, R. L., Montauti, E. I., Tran, B. Q., Peterson, S. B., Harding, B. N., . . . Mougous, J. D. (2015). Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. *eLife, 4*.

Li, J., Nation, R. L., Milne, R. W., Turnidge, J. D., & Coulthard, K. (2005). Evaluation of colistin as an agent against multi-resistant in Gram-negative bacteria. *International journal of antimicrobial agents, 25*(1), 11-25.

Li, Z. T., Zhang, R. L., Bi, X. G., Xu, L., Fan, M., Xie, D., . . . Zhang, K. X. (2015). Outer membrane vesicles isolated from two clinical *Acinetobacter baumannii* strains exhibit different toxicity and proteome characteristics. *Microbial pathogenesis, 81*, 46-52.

Liang, X., Moore, R., Wilton, M., Wong, M. J., Lam, L., & Dong, T. G. (2015). Identification of divergent type VI secretion effectors using a conserved chaperone domain. *Proceedings of the National Academy of Sciences of the United States of America, 112*(29), 9106-9111.

Liao, Y. T., Kuo, S. C., Chiang, M. H., Lee, Y. T., Sung, W. C., Chen, Y. H., . . . Fung, C. P. (2015). *Acinetobacter baumannii* extracellular OXA-58 is primarily and selectively released via outer membrane vesicles after Sec-dependent periplasmic translocation. *Antimicrobial agents and chemotherapy*.

Liao, Y. T., Kuo, S. C., Lee, Y. T., Chen, C. P., Lin, S. W., Shen, L. J., . . . Chen, T. L. (2014). Sheltering effect and indirect pathogenesis of carbapenem-resistant *Acinetobacter baumannii* in polymicrobial infection. *Antimicrobial agents and chemotherapy, 58*(7), 3983-3990.

Lim, Y. M., Shin, K. S., & Kim, J. (2007). Distinct antimicrobial resistance patterns and antimicrobial resistance-harboring genes according to genomic species of *Acinetobacter* isolates. *Journal of clinical microbiology, 45*(3), 902-905.

Lin, J. S., Ma, L. S., & Lai, E. M. (2013). Systematic dissection of the agrobacterium type VI secretion system reveals machinery and secreted components for subcomplex formation. *PloS one, 8*(7), e67647.

Lin, L., Tan, B., Pantapalangkoor, P., Ho, T., Baquir, B., Tomaras, A., . . . Spellberg, B. (2012). Inhibition of LpxC protects mice from resistant *Acinetobacter baumannii* by modulating inflammation and enhancing phagocytosis. *mBio, 3*(5).

Liu, B., Knirel, Y. A., Feng, L., Perepelov, A. V., Senchenkova, S. N., Reeves, P. R., & Wang, L. (2014). Structural diversity in Salmonella O antigens and its genetic basis. *FEMS microbiology reviews, 38*(1), 56-89.

Loessner, M. J., Wendlinger, G., & Scherer, S. (1995). Heterogeneous endolysins in Listeria monocytogenes bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Molecular microbiology, 16*(6), 1231-1241.

Lossi, N. S., Dajani, R., Freemont, P., & Filloux, A. (2011). Structure-function analysis of HsiF, a gp25-like component of the type VI secretion system, in *Pseudomonas aeruginosa*. *Microbiology, 157*(Pt 12), 3292-3305.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology, 15*(12), 550.

Luke, N. R., Sauberan, S. L., Russo, T. A., Beanan, J. M., Olson, R., Loehfelm, T. W., . . . Campagnari, A. A. (2010). Identification and characterization of a glycosyltransferase involved in *Acinetobacter baumannii* lipopolysaccharide core biosynthesis. *Infection and immunity, 78*(5), 2017-2023.

Ma, A. T., McAuley, S., Pukatzki, S., & Mekalanos, J. J. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell host & microbe, 5*(3), 234-243.

Ma, A. T., & Mekalanos, J. J. (2010). In vivo actin cross-linking induced by *Vibrio cholerae* type VI secretion system is associated with intestinal inflammation. *Proceedings of the National Academy of Sciences of the United States of America, 107*(9), 4365-4370.

Ma, L. S., Hachani, A., Lin, J. S., Filloux, A., & Lai, E. M. (2014). Agrobacterium tumefaciens deploys a superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in planta. *Cell host & microbe, 16*(1), 94-104.

MacIntyre, D. L., Miyata, S. T., Kitaoka, M., & Pukatzki, S. (2010). The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proceedings of the National Academy of Sciences of the United States of America, 107*(45), 19520-19524.

MacLean, L. L., Perry, M. B., Chen, W., & Vinogradov, E. (2009). The structure of the polysaccharide O-chain of the LPS from *Acinetobacter baumannii* strain ATCC 17961. *Carbohydrate research, 344*(4), 474-478.

Magnet, S., Courvalin, P., & Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial agents and chemotherapy, 45*(12), 3375-3380.

Mara, K., Decorosi, F., Viti, C., Giovannetti, L., Papaleo, M. C., Maida, I., . . . Fani, R. (2012). Molecular and phenotypic characterization of *Acinetobacter* strains able to degrade diesel fuel. *Research in microbiology, 163*(3), 161-172.

Maragakis, L. L., & Perl, T. M. (2008a). *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 46*(8), 1254-1263.

Maragakis, L. L., Tucker, M. G., Miller, R. G., Carroll, K. C., & Perl, T. M. (2008b). Incidence and prevalence of multidrug-resistant *Acinetobacter* using targeted active surveillance cultures. *JAMA : the journal of the American Medical Association, 299*(21), 2513-2514.

March, C., Regueiro, V., Llobet, E., Moranta, D., Morey, P., Garmendia, J., & Bengoechea, J. A. (2010). Dissection of host cell signal transduction during *Acinetobacter baumannii*-triggered inflammatory response. *PloS one, 5*(4), e10033.

Margetts, M. B., Barr, I. G., & Webb, E. A. (2000). Overexpression, purification, and refolding of a Porphyromonas gingivalis cysteine protease from *Escherichia coli*. *Protein expression and purification, 18*(3), 262-268.

McConnell, M. J., Actis, L., & Pachon, J. (2012). *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS microbiology reviews*.

McConnell, M. J., Rumbo, C., Bou, G., & Pachon, J. (2011). Outer membrane vesicles as an acellular vaccine against *Acinetobacter baumannii*. *Vaccine, 29*(34), 5705-5710.

McQueary, C. N., Kirkup, B. C., Si, Y., Barlow, M., Actis, L. A., Craft, D. W., & Zurawski, D. V. (2012). Extracellular stress and lipopolysaccharide modulate *Acinetobacter baumannii* surface-associated motility. *Journal of microbiology, 50*(3), 434-443.

Melamed, R., Greenberg, D., Porat, N., Karplus, M., Zmora, E., Golan, A., . . . Dagan, R. (2003). Successful control of an *Acinetobacter baumannii* outbreak in a neonatal intensive care unit. *The Journal of hospital infection, 53*(1), 31-38.

Merz, A. J., So, M., & Sheetz, M. P. (2000). Pilus retraction powers bacterial twitching motility. *Nature, 407*(6800), 98-102.

Miyata, S. T., Bachmann, V., & Pukatzki, S. (2013a). Type VI secretion system regulation as a consequence of evolutionary pressure. *Journal of medical microbiology, 62*(Pt 5), 663-676.

Miyata, S. T., Kitaoka, M., Brooks, T. M., McAuley, S. B., & Pukatzki, S. (2011). *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill Dictyostelium discoideum. *Infection and immunity, 79*(7), 2941-2949.

Miyata, S. T., Unterweger, D., Rudko, S. P., & Pukatzki, S. (2013b). Dual expression profile of type VI secretion system immunity genes protects pandemic *Vibrio cholerae*. *PLoS pathogens, 9*(12), e1003752.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D., Vinogradov, E., Seemann, T., . . . Boyce, J. D. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial agents and chemotherapy, 54*(12), 4971-4977.

Moon, D. C., Choi, C. H., Lee, J. H., Choi, C. W., Kim, H. Y., Park, J. S., . . . Lee, J. C. (2012). *Acinetobacter baumannii* outer membrane protein A modulates the biogenesis of outer membrane vesicles. *Journal of microbiology, 50*(1), 155-160.

Mortensen, B. L., & Skaar, E. P. (2012). Host-microbe interactions that shape the pathogenesis of *Acinetobacter baumannii* infection. *Cellular microbiology, 14*(9), 1336-1344.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., . . . Mekalanos, J. J. (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science, 312*(5779), 1526-1530.

Mougous, J. D., Gifford, C. A., Ramsdell, T. L., & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nature cell biology, 9*(7), 797-803.

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., & Coulthurst, S. J. (2011). The opportunistic pathogen Serratia marcescens utilizes type VI secretion to target bacterial competitors. *Journal of bacteriology, 193*(21), 6057-6069.

Musumeci, M. A., Faridmoayer, A., Watanabe, Y., & Feldman, M. F. (2014). Evaluating the role of conserved amino acids in bacterial O-oligosaccharyltransferases by in vivo, in vitro and limited proteolysis assays. *Glycobiology, 24*(1), 39-50.

Nait Chabane, Y., Marti, S., Rihouey, C., Alexandre, S., Hardouin, J., Lesouhaitier, O., . . . De, E. (2014). Characterisation of pellicles formed by *Acinetobacter baumannii* at the air-liquid interface. *PLoS One, 9*(10), e111660.

Nambu, T., Minamino, T., Macnab, R. M., & Kutsukake, K. (1999). Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in Salmonella typhimurium. *Journal of bacteriology, 181*(5), 1555-1561.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., . . . Dijkshoorn, L. (2011). Genotypic and phenotypic characterization of the *Acinetobacter* calcoaceticus-*Acinetobacter baumannii* complex with the proposal of *Acinetobacter* pittii sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter* nosocomialis sp. nov. (formerly *Acinetobacter* genomic species 13TU). *Research in microbiology, 162*(4), 393-404.

Nho, J. S., Jun, S. H., Oh, M. H., Park, T. I., Choi, C. W., Kim, S. I., . . . Lee, J. C. (2015). *Acinetobacter* nosocomialis secretes outer membrane vesicles that induce epithelial cell death and host inflammatory responses. *Microbial pathogenesis, 81*, 39-45.

Norton, M. D., Spilkia, A. J., & Godoy, V. G. (2013). Antibiotic resistance acquired through a DNA damage-inducible response in *Acinetobacter baumannii*. *Journal of bacteriology, 195*(6), 1335-1345.

O'Toole, G. A. (2011). Microtiter dish biofilm formation assay. *Journal of visualized experiments : JoVE*(47).

Orskov, I., Orskov, F., Jann, B., & Jann, K. (1977). Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. *Bacteriol Rev, 41*(3), 667-710.

Pakharukova, N., Tuittila, M., Paavilainen, S., & Zavialov, A. (2015). Crystallization and preliminary X-ray diffraction analysis of the Csu pili CsuC-CsuA/B chaperone-major subunit pre-assembly complex from *Acinetobacter baumannii*. *Acta Crystallogr F Struct Biol Commun, 71*(Pt 6), 770-774.

Pantophlet, R. (2008). *Lipopolysaccharides of Acinetobacter* (E. Gerischer Ed.). Norfolk, UK: Caister Academic Press.

Pantophlet, R., Brade, L., & Brade, H. (1999). Identification of *Acinetobacter baumannii* strains with monoclonal antibodies against the O antigens of their lipopolysaccharides. *Clinical and diagnostic laboratory immunology, 6*(3), 323-329.

Park, K. H., Shin, J. H., Lee, S. Y., Kim, S. H., Jang, M. O., Kang, S. J., . . . Jang, H. C. (2014). The clinical characteristics, carbapenem resistance, and outcome of *Acinetobacter* bacteremia according to genospecies. *PloS one, 8*(6), e65026.

Peleg, A. Y., Jara, S., Monga, D., Eliopoulos, G. M., Moellering, R. C., Jr., & Mylonakis, E. (2009). Galleria mellonella as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrobial agents and chemotherapy, 53*(6), 2605-2609.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008a). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical microbiology reviews, 21*(3), 538-582.

Peleg, A. Y., Tampakakis, E., Fuchs, B. B., Eliopoulos, G. M., Moellering, R. C., Jr., & Mylonakis, E. (2008b). Prokaryote-eukaryote interactions identified by using

Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America, 105*(38), 14585-14590.
Pell, L. G., Kanelis, V., Donaldson, L. W., Howell, P. L., & Davidson, A. R. (2009). The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences of the United States of America, 106*(11), 4160-4165.
Pelletier, M. R., Casella, L. G., Jones, J. W., Adams, M. D., Zurawski, D. V., Hazlett, K. R., . . . Ernst, R. K. (2013). Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 57*(10), 4831-4840.
Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., & Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 51*(10), 3471-3484.
Piechaud, M., & Second, L. (1951). [Studies of 26 strains of Moraxella Iwoffi]. *Annales de l'Institut Pasteur, 80*(1), 97-99.
Pieper, R., Huang, S. T., Robinson, J. M., Clark, D. J., Alami, H., Parmar, P. P., . . . Peterson, S. N. (2009). Temperature and growth phase influence the outer-membrane proteome and the expression of a type VI secretion system in Yersinia pestis. *Microbiology, 155*(Pt 2), 498-512.
Pietrosiuk, A., Lenherr, E. D., Falk, S., Bonemann, G., Kopp, J., Zentgraf, H., . . . Mogk, A. (2011). Molecular basis for the unique role of the AAA+ chaperone ClpV in type VI protein secretion. *The Journal of biological chemistry, 286*(34), 30010-30021.
Planamente, S., Salih, O., Manoli, E., Albesa-Jove, D., Freemont, P. S., & Filloux, A. (2016). TssA forms a gp6-like ring attached to the type VI secretion sheath. *The EMBO journal*.
Poirel, L., Naas, T., & Nordmann, P. (2010). Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrobial agents and chemotherapy, 54*(1), 24-38.
Poirel, L., & Nordmann, P. (2006). Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 12*(9), 826-836.
Possot, O. M., Vignon, G., Bomchil, N., Ebel, F., & Pugsley, A. P. (2000). Multiple interactions between pullulanase secreton components involved in stabilization and cytoplasmic membrane association of PulE. *J Bacteriol, 182*(8), 2142-2152.
Preston, A., Mandrell, R. E., Gibson, B. W., & Apicella, M. A. (1996). The lipooligosaccharides of pathogenic gram-negative bacteria. *Crit Rev Microbiol, 22*(3), 139-180.
Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D., & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proceedings of the National Academy of Sciences of the United States of America, 104*(39), 15508-15513.
Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., . . . Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. *Proceedings of the National Academy of Sciences of the United States of America, 103*(5), 1528-1533.
Pukatzki, S., McAuley, S. B., & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Current opinion in microbiology, 12*(1), 11-17.

Raetz, C. R., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem, 71*, 635-700.

Ramirez, M. S., Don, M., Merkier, A. K., Bistue, A. J., Zorreguieta, A., Centron, D., & Tolmasky, M. E. (2010). Naturally competent *Acinetobacter baumannii* clinical isolate as a convenient model for genetic studies. *J Clin Microbiol, 48*(4), 1488-1490.

Repizo, G. D., Gagne, S., Foucault-Grunenwald, M. L., Borges, V., Charpentier, X., Limansky, A. S., . . . Salcedo, S. P. (2015). Differential Role of the T6SS in *Acinetobacter baumannii* Virulence. *PloS one, 10*(9), e0138265.

Rokhbakhsh-Zamin, F., Sachdev, D., Kazemi-Pour, N., Engineer, A., Pardesi, K. R., Zinjarde, S., . . . Chopade, B. A. (2011). Characterization of plant-growth-promoting traits of *Acinetobacter* species isolated from rhizosphere of Pennisetum glaucum. *Journal of microbiology and biotechnology, 21*(6), 556-566.

Rosales-Reyes, R., Skeldon, A. M., Aubert, D. F., & Valvano, M. A. (2012). The Type VI secretion system of *Burkholderia cenocepacia* affects multiple Rho family GTPases disrupting the actin cytoskeleton and the assembly of NADPH oxidase complex in macrophages. *Cellular microbiology, 14*(2), 255-273.

Rumbo, C., Fernandez-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., . . . Bou, G. (2011). Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy, 55*(7), 3084-3090.

Rupper, A., & Cardelli, J. (2001). Regulation of phagocytosis and endo-phagosomal trafficking pathways in Dictyostelium discoideum. *Biochimica et biophysica acta, 1525*(3), 205-216.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., & Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature, 475*(7356), 343-347.

Russell, A. B., LeRoux, M., Hathazi, K., Agnello, D. M., Ishikawa, T., Wiggins, P. A., . . . Mougous, J. D. (2013). Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. *Nature, 496*(7446), 508-512.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature reviews. Microbiology, 12*(2), 137-148.

Russell, A. B., Singh, P., Brittnacher, M., Bui, N. K., Hood, R. D., Carl, M. A., . . . Mougous, J. D. (2012). A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. *Cell host & microbe, 11*(5), 538-549.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., . . . Campagnari, A. A. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and immunity, 78*(9), 3993-4000.

Sahl, J. W., Johnson, J. K., Harris, A. D., Phillippy, A. M., Hsiao, W. W., Thom, K. A., & Rasko, D. A. (2011). Genomic comparison of multi-drug resistant invasive and colonizing *Acinetobacter baumannii* isolated from diverse human body sites reveals genomic plasticity. *BMC genomics, 12*, 291.

Salomon, D., Klimko, J. A., & Orth, K. (2014). H-NS regulates the Vibrio parahaemolyticus type VI secretion system 1. *Microbiology, 160*(Pt 9), 1867-1873.

Schell, M. A., Ulrich, R. L., Ribot, W. J., Brueggemann, E. E., Hines, H. B., Chen, D., . . . Deshazer, D. (2007). Type VI secretion is a major virulence determinant in *Burkholderia* mallei. *Molecular microbiology, 64*(6), 1466-1485.

Scheurwater, E. M., & Burrows, L. L. (2011). Maintaining network security: how macromolecular structures cross the peptidoglycan layer. *FEMS microbiology letters, 318*(1), 1-9.

Schifferli, D. M., Abraham, S. N., & Beachey, E. H. (1986). Influence of trimethoprim and sulfamethoxazole on the synthesis, expression, and function of type 1 fimbriae of *Escherichia coli*. *The Journal of infectious diseases, 154*(3), 490-496.

Schulz, B. L., Jen, F. E., Power, P. M., Jones, C. E., Fox, K. L., Ku, S. C., . . . Jennings, M. P. (2013). Identification of bacterial protein O-oligosaccharyltransferases and their glycoprotein substrates. *PloS one, 8*(5), e62768.

Schwarz, S., Singh, P., Robertson, J. D., LeRoux, M., Skerrett, S. J., Goodlett, D. R., . . . Mougous, J. D. (2014). VgrG-5 is a *Burkholderia* type VI secretion system-exported protein required for multinucleated giant cell formation and virulence. *Infection and immunity, 82*(4), 1445-1452.

Schwarz, S., West, T. E., Boyer, F., Chiang, W. C., Carl, M. A., Hood, R. D., . . . Mougous, J. D. (2010). *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS pathogens, 6*(8), e1001068.

Schwede, T., Kopp, J., Guex, N., & Peitsch, M. C. (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic acids research, 31*(13), 3381-3385.

Scott, N. E., Kinsella, R. L., Edwards, A. V., Larsen, M. R., Dutta, S., Saba, J., . . . Feldman, M. F. (2014a). Diversity within the O-linked protein glycosylation systems of *Acinetobacter* species. *Molecular & cellular proteomics : MCP, 13*(9), 2354-2370.

Scott, N. E., Kinsella, R. L., Edwards, A. V., Larsen, M. R., Dutta, S., Saba, J., . . . Feldman, M. F. (2014b). Diversity Within the O-linked Protein Glycosylation Systems of *Acinetobacter* Species. *Molecular & cellular proteomics : MCP, 13*(9), 2354-2370.

Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., Edwards, A. V., Crossett, B., . . . Cordwell, S. J. (2011). Simultaneous Glycan-Peptide Characterization Using Hydrophilic Interaction Chromatography and Parallel Fragmentation by CID, Higher Energy Collisional Dissociation, and Electron Transfer Dissociation MS Applied to the N-Linked Glycoproteome of *Campylobacter jejuni*. *Mol Cell Proteomics, 10*(2), M000031MCP000201.

Sebeny, P. J., Riddle, M. S., & Petersen, K. (2008). *Acinetobacter baumannii* skin and soft-tissue infection associated with war trauma. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 47*(4), 444-449.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I., & Vaneechoutte, M. (1997). Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *Journal of clinical microbiology, 35*(11), 2819-2825.

Seifert, H., Strate, A., & Pulverer, G. (1995). Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality. *Medicine, 74*(6), 340-349.

Sexton, J. A., Miller, J. L., Yoneda, A., Kehl-Fie, T. E., & Vogel, J. P. (2004). Legionella pneumophila DotU and IcmF are required for stability of the Dot/Icm complex. *Infection and immunity, 72*(10), 5983-5992.

Shalom, G., Shaw, J. G., & Thomas, M. S. (2007). In vivo expression technology identifies a type VI secretion system locus in *Burkholderia* pseudomallei that is induced upon invasion of macrophages. *Microbiology, 153*(Pt 8), 2689-2699.

Shneider, M. M., Buth, S. A., Ho, B. T., Basler, M., Mekalanos, J. J., & Leiman, P. G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature, 500*(7462), 350-353.

Sievert, D. M., Ricks, P., Edwards, J. R., Schneider, A., Patel, J., Srinivasan, A., . . . Fridkin, S. (2013). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 34*(1), 1-14.

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harb Perspect Biol, 2*(5), a000414.

Silverman, J. M., Agnello, D. M., Zheng, H., Andrews, B. T., Li, M., Catalano, C. E., . . . Mougous, J. D. (2013). Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. *Molecular cell, 51*(5), 584-593.

Silverman, J. M., Austin, L. S., Hsu, F., Hicks, K. G., Hood, R. D., & Mougous, J. D. (2011). Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. *Molecular microbiology, 82*(5), 1277-1290.

Silverman, J. M., Brunet, Y. R., Cascales, E., & Mougous, J. D. (2012). Structure and regulation of the type VI secretion system. *Annual review of microbiology, 66*, 453-472.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M., & Snyder, M. (2007). New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes & development, 21*(5), 601-614.

Snitkin, E. S., Zelazny, A. M., Montero, C. I., Stock, F., Mijares, L., Murray, P. R., & Segre, J. A. (2011). Genome-wide recombination drives diversification of epidemic strains of *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 108*(33), 13758-13763.

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H. W., Scheld, W. M., . . . Infectious Diseases Society of, A. (2008). The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 46*(2), 155-164.

Steinert, M., & Heuner, K. (2005). Dictyostelium as host model for pathogenesis. *Cellular microbiology, 7*(3), 307-314.

Stenutz, R., Weintraub, A., & Widmalm, G. (2006). The structures of *Escherichia coli* O-polysaccharide antigens. *FEMS microbiology reviews, 30*(3), 382-403.

Sturm, A., Heinemann, M., Arnoldini, M., Benecke, A., Ackermann, M., Benz, M., . . . Hardt, W. D. (2011). The cost of virulence: retarded growth of Salmonella Typhimurium cells expressing type III secretion system 1. *PLoS pathogens, 7*(7), e1002143.

Suarez, G., Sierra, J. C., Erova, T. E., Sha, J., Horneman, A. J., & Chopra, A. K. (2010). A type VI secretion system effector protein, VgrG1, from Aeromonas hydrophila that induces host cell toxicity by ADP ribosylation of actin. *Journal of bacteriology, 192*(1), 155-168.

Subashchandrabose, S., Smith, S., DeOrnellas, V., Crepin, S., Kole, M., Zahdeh, C., & Mobley, H. L. (2016). *Acinetobacter baumannii* Genes Required for Bacterial Survival during Bloodstream Infection. *mSphere, 1*(1).

Takahashi, Y., Shintani, M., Takase, N., Kazo, Y., Kawamura, F., Hara, H., . . . Nojiri, H. (2015). Modulation of primary cell function of host Pseudomonas bacteria by the conjugative plasmid pCAR1. *Environmental microbiology, 17*(1), 134-155.

Takeda, S., Hoshida, K., & Arisaka, F. (1998). Mapping of functional sites on the primary structure of the tail lysozyme of bacteriophage T4 by mutational analysis. *Biochimica et biophysica acta, 1384*(2), 243-252.

Talbot, G. H., Bradley, J., Edwards, J. E., Jr., Gilbert, D., Scheld, M., Bartlett, J. G., & Antimicrobial Availability Task Force of the Infectious Diseases Society of, A. (2006). Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 42*(5), 657-668.

Tang, L., Liang, X., Moore, R., & Dong, T. G. (2015). Commentary: The icmF3 Locus is Involved in Multiple Adaptation- and Virulence-related Characteristics in *Pseudomonas aeruginosa* PAO1. *Frontiers in cellular and infection microbiology, 5*, 83.

Tjernberg, I., & Ursing, J. (1989). Clinical strains of *Acinetobacter* classified by DNA-DNA hybridization. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica, 97*(7), 595-605.

Toesca, I. J., French, C. T., & Miller, J. F. (2014). The Type VI secretion system spike protein VgrG5 mediates membrane fusion during intercellular spread by pseudomallei group *Burkholderia* species. *Infection and immunity, 82*(4), 1436-1444.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473-3484.

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., & Actis, L. A. (2008). Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology, 154*(Pt 11), 3398-3409.

Traub, W. H. (1989). *Acinetobacter baumannii* serotyping for delineation of outbreaks of nosocomial cross-infection. *Journal of clinical microbiology, 27*(12), 2713-2716.

Tucker, A. T., Nowicki, E. M., Boll, J. M., Knauf, G. A., Burdis, N. C., Trent, M. S., & Davies, B. W. (2014). Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio, 5*(4), e01313-01314.

Uchida, K., Leiman, P. G., Arisaka, F., & Kanamaru, S. (2014). Structure and properties of the C-terminal beta-helical domain of VgrG protein from *Escherichia coli* O157. *J Biochem, 155*(3), 173-182.

Ugalde, J. E., Czibener, C., Feldman, M. F., & Ugalde, R. A. (2000). Identification and characterization of the Brucella abortus phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. *Infection and immunity, 68*(10), 5716-5723.

Umland, T. C., Schultz, L. W., MacDonald, U., Beanan, J. M., Olson, R., & Russo, T. A. (2012). In vivo-validated essential genes identified in *Acinetobacter baumannii* by using human ascites overlap poorly with essential genes detected on laboratory media. *mBio, 3*(4).

Unterweger, D., Kostiuk, B., Otjengerdes, R., Wilton, A., Diaz-Satizabal, L., & Pukatzki, S. (2015). Chimeric adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *The EMBO journal, 34*(16), 2198-2210.

Valencia, R., Arroyo, L. A., Conde, M., Aldana, J. M., Torres, M. J., Fernandez-Cuenca, F., . . . Aznar, J. (2009). Nosocomial outbreak of infection with pan-drug-resistant *Acinetobacter baumannii* in a tertiary care university hospital. *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America, 30*(3), 257-263.

Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., . . . Cruveiller, S. (2008). Comparative analysis of *Acinetobacter*s: three genomes for three lifestyles. *PloS one, 3*(3), e1805.

Vaneechoutte, M., Young, D. M., Ornston, L. N., De Baere, T., Nemec, A., Van Der Reijden, T., . . . Dijkshoorn, L. (2006). Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*. *Applied and environmental microbiology, 72*(1), 932-936.

Vidal, R., Dominguez, M., Urrutia, H., Bello, H., Gonzalez, G., Garcia, A., & Zemelman, R. (1996). Biofilm formation by *Acinetobacter baumannii*. *Microbios, 86*(346), 49-58.

Vinogradov, E. V., Brade, L., Brade, H., & Holst, O. (2003). Structural and serological characterisation of the O-antigenic polysaccharide of the lipopolysaccharide from *Acinetobacter baumannii* strain 24. *Carbohydrate research, 338*(23), 2751-2756.

Vinogradov, E. V., Duus, J. O., Brade, H., & Holst, O. (2002). The structure of the carbohydrate backbone of the lipopolysaccharide from *Acinetobacter baumannii* strain ATCC 19606. *European journal of biochemistry / FEBS, 269*(2), 422-430.

Vinogradov, E. V., Pantophlet, R., Dijkshoorn, L., Brade, L., Holst, O., & Brade, H. (1996). Structural and serological characterisation of two O-specific polysaccharides of *Acinetobacter*. *European journal of biochemistry / FEBS, 239*(3), 602-610.

von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *Journal of molecular biology, 225*(2), 487-494.

Waksman, G., & Hultgren, S. J. (2009). Structural biology of the chaperone-usher pathway of pilus biogenesis. *Nature reviews. Microbiology, 7*(11), 765-774.

Wang, N., Ozer, E. A., Mandel, M. J., & Hauser, A. R. (2014). Genome-wide identification of *Acinetobacter baumannii* genes necessary for persistence in the lung. *mBio, 5*(3), e01163-01114.

Weber, B. S., Harding, C. M., & Feldman, M. F. (2015a). Pathogenic *Acinetobacter*: from the Cell Surface to Infinity and Beyond. *Journal of bacteriology, 198*(6), 880-887.

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015b). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America, 112*(30), 9442-9447.

Weber, B. S., Miyata, S. T., Iwashkiw, J. A., Mortensen, B. L., Skaar, E. P., Pukatzki, S., & Feldman, M. F. (2013). Genomic and functional analysis of the type VI secretion system in *Acinetobacter*. *PloS one, 8*(1), e55142.

Wendt, C., Dietze, B., Dietz, E., & Ruden, H. (1997). Survival of *Acinetobacter baumannii* on dry surfaces. *Journal of clinical microbiology, 35*(6), 1394-1397.

Wexler, A. G., Bao, Y., Whitney, J. C., Bobay, L. M., Xavier, J. B., Schofield, W. B., . . . Goodman, A. L. (2016). Human symbionts inject and neutralize antibacterial toxins to persist in the gut. *Proceedings of the National Academy of Sciences of the United States of America, 113*(13), 3639-3644.

Whitfield, C., Amor, P. A., & Koplin, R. (1997). Modulation of the surface architecture of gram-negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. *Molecular microbiology, 23*(4), 629-638.

Whitney, J. C., Beck, C. M., Goo, Y. A., Russell, A. B., Harding, B., De Leon, J. A., . . . Mougous, J. D. (2014a). Genetically distinct pathways guide effector export through the type VI secretion system. *Molecular microbiology*.

Whitney, J. C., Beck, C. M., Goo, Y. A., Russell, A. B., Harding, B. N., De Leon, J. A., . . . Mougous, J. D. (2014b). Genetically distinct pathways guide effector export through the type VI secretion system. *Molecular microbiology, 92*(3), 529-542.

Whitney, J. C., Chou, S., Russell, A. B., Biboy, J., Gardiner, T. E., Ferrin, M. A., . . . Mougous, J. D. (2013). Identification, structure, and function of a novel type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. *The Journal of biological chemistry, 288*(37), 26616-26624.

Whitney, J. C., Quentin, D., Sawai, S., LeRoux, M., Harding, B. N., Ledvina, H. E., . . . Mougous, J. D. (2015). An interbacterial NAD(P)(+) glycohydrolase toxin requires elongation factor Tu for delivery to target cells. *Cell, 163*(3), 607-619.

Wilharm, G., Piesker, J., Laue, M., & Skiebe, E. (2013). DNA uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs during movement along wet surfaces. *J Bacteriol, 195*(18), 4146-4153.

Wright, M. S., Haft, D. H., Harkins, D. M., Perez, F., Hujer, K. M., Bajaksouzian, S., . . . Adams, M. D. (2014). New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *mBio, 5*(1), e00963-00913.

Yoon, E. J., Chabane, Y. N., Goussard, S., Snesrud, E., Courvalin, P., De, E., & Grillot-Courvalin, C. (2015). Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*. *MBio, 6*(2).

Zheng, J., Ho, B., & Mekalanos, J. J. (2011). Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PloS one, 6*(8), e23876.

Zheng, J., & Leung, K. Y. (2007). Dissection of a type VI secretion system in Edwardsiella tarda. *Molecular microbiology, 66*(5), 1192-1206.

Zheng, J., Shin, O. S., Cameron, D. E., & Mekalanos, J. J. (2010). Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America, 107*(49), 21128-21133.

Zimbler, D. L., Penwell, W. F., Gaddy, J. A., Menke, S. M., Tomaras, A. P., Connerly, P. L., & Actis, L. A. (2009). Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine, 22*(1), 23-32.

Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M. S., Logger, L., Douzi, B., . . . Cascales, E. (2014). Architecture and assembly of the Type VI secretion system. *Biochimica et biophysica acta, 1843*(8), 1664-1673.

Zoued, A., Durand, E., Brunet, Y. R., Spinelli, S., Douzi, B., Guzzo, M., . . . Cascales, E. (2016). Priming and polymerization of a bacterial contractile tail structure. *Nature, 531*(7592), 59-63.