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The Molecular Basis for Natural Competence in *Acinetobacter*

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THE MOLECULAR BASIS FOR NATURAL COMPETENCE IN *ACINETOBACTER*

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Bacteria use Horizontal Gene Transfer (HGT) to acquire genetic material, leading to the development of novel traits, such as the spread and development of antibiotic resistance. Natural competence is one type of HGT accomplished by DNA uptake from the environment and incorporation into the genome.

Bacteria from *Acinetobacter* are wildly distributed in the environment and are naturally competent. This propensity is a key factor to the steady increase in drug resistance in *Acinetobacter,* which is a cause for concern in human health as *Acinetobacter* is a major source of nosocomial infections.

During natural competence, type IV pili (T4P) and related filaments are essential for DNA uptake. T4P are extracellular appendages composed of protein subunits (pilins) polymerized into helical fibers which can be extended into the extracellular space and retracted through depolymerization. T4P are required for diverse physiological processes, both retraction-dependent (HGT, twitching motility) and retraction-independent (biofilm formation, host cell adherence). While deletions of *pilA* (the primary pilin) and *pilT* (a cytosolic enzyme necessary for retraction) have been found to abrogate natural competence, the mechanism and DNA receptor remains unclear in *Acinetobacter*.

In this thesis, we investigate the molecular basis for natural competence in

Acinetobacter. Specific aim 1 is to identify extracellular DNA receptor(s) in *Acinetobacter*. To accomplish this, we proposed to identify DNA-receptors incorporated into T4P by directly measuring binding affinity of recombinantly-expressed pilin subunits to plasmid double-stranded DNA. Our data demonstrates that two pilin proteins, PilE1 and PilE2, of *Acinetobacter baumannii* are capable of binding plasmid DNA *in vitro*. Our other specific aim is to quantify the impact of allelic variation in *pilA* on DNA-uptake in *A. baumannii*. We measure natural competence using a common Δ*pilA* strain complemented with *pilA* genes from *A. baumannii* strains from distinct T4P subtypes to assess the impact of T4P *pilA* variation on natural competence. Our data shows an impact on natural competence from T4P subtype, which indicates that pilus subtype is one of the components influencing transformability in different strains of *A.baumannii*, potentially through T4P-subtype-depenent differences in pilus retraction. Our results provide a molecular description of HGT in *Acinetobacter* through natural competence.

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1.1. Natural competence in bacteria

Horizontal gene transfer (the transfer of genetic material between organisms) can occur through multiple mechanisms in bacteria; mechanisms include conjugation between bacterial cells of distinct species, cross-species bacteriophage infection and DNA-uptake through natural competence. Naturally competent bacteria actively pull free dsDNA or ssDNA fragments from the extracellular environment into their own cells depends on the DNA receptor and the size of secretion pores. This genetic material can then potentially be incorporated into the host genome as a mobile element such as a plasmid or through recombination into a bacterial chromosome. However, the likelihood that plasmids integrate into the chromosome is not high, most of the incoming DNA is degraded even if the plasmids have homologous or identical to the chromosome (Barouki & Smith, 1985).

1.1.1. Fitness advantages of natural competence

The potential fitness advantage of natural competence can be mainly divided into three parts. The first, or direct effect, of natural competence is that bacteria use DNA as essential nutrients and building blocks. DNA ingested by natural transformation can serve as a “food” source for bacteria, especially if the ingested DNA is not homologous to the bacterial genome and could not be used for recombination or repair. Studies have shown that DNA molecules ingested by bacteria can provide carbon, phosphate, and energy

sources for natural transformation (Pinchuk et al., 2008; Rosemary J. Redfield, 2001), especially for DNA and RNA synthesis. For example, *Haemophilus influenzae* can only synthesize purines and pyrimidines by acquiring precursors from its environment (Pifer & Smith, 1985; R. J. Redfield, 1993), which means *H. influenzae* can reuse the nucleotides released by degradation of transforming DNA for their own DNA synthesis. Bacterial cells that take up DNA acquire the individual nucleotides that make up the fragments of the DNA polymer. Because nucleotides are needed for DNA and RNA synthesis, require significant nutrient use, and are energetically expensive to synthesize, this contribution of nucleotides significantly reduces the cell's energy requirements for synthesizing new nucleotides for essential cell function. The second function is helping the bacteria to repair DNA damage. If the ingested DNA is homologous to the bacterial genome, the DNA is potentially used for recombination or repair. It has been found that in *Bacillus subtilis* cultures, bacterial cells exposed to DNA-damaging UV irradiation, that undergo transformation, survive better than cells exposed to DNA damage that did not undergo transformation (Hoelzer & Michod, 1991). These data indicate that bacteria can use DNA acquired from the environment to reconstitute their DNA under high stress conditions. The immediate consequences of DNA uptake provide a framework for thinking about its possible evolutionary function. Natural competence contributes to the formation of new species and new forms of resistance that then play an essential role in the development of bacterial pathogenicity (Mell & Redfield, 2014). Studies have shown that when homologous recombination occurs on genes that encode harmful traits in

alleles in the genome, the probability of bacterial elimination of these harmful mutations is greatly increased (De Visser & Elena, 2007). In addition, natural transformation plays an important role in transforming harmless bacteria into major human pathogens. Rasko (2011) found that natural transformation resulted in the recombination of two different *Escherichia coli* pathogens into highly pathogenic *E. coli* (Rasko et al., 2011). Natural transformation may also be involved in the transfer of drug-resistance gene clusters and pathogenicity islands. For example, Perron (2012) found that high frequency recombination of drug-resistance genes in *Acinetobacter baumannii* resulted in the generation of multidrug-resistant strains (MDR) (Perron et al., 2012). The ability of bacteria to freely take up DNA represents a substantial obstacle for the treatment of bacterial infections through antibiotic drugs.

1.1.2. The mechanism of DNA uptake in natural competence

Natural competence occurs in both Gram-positive and Gram-negative bacteria. However, mechanisms differ somewhat due to structural differences in the membranes and surface structures across bacterial taxa. Both Gram-positive and Gram-negative strains use related pilus systems (described in detail in section 1.3 below) to take up DNA. In Gram-positive bacteria, these extracellular appendages are exclusively called competence (com) pili, which bind DNA and bring it through the peptidoglycan layer to the cell surface and are generally thought to serve no other function (Lam et al., 2021; Sheppard et al., 2020; Ellison et al., 2018). In Gram-negative bacteria, type IV pili, which serve multiple adhesive functions (host cell adhesion, biofilm formation, aggregation)

bring DNA through the outer membrane into the periplasm (Dubnau, 1999; Mell & Redfield, 2014).

To cross the plasma membrane and bring DNA into the cytoplasm of Gram-positive bacteria, proteins encoded by *comEA* and *comGB* genes are essential for natural transformation. Briefly, taking *Bacillus subtilis* as an example, ComEA has been identified as the DNA receptor of *Bacillus subtilis*, whose C-terminal region is located outside the cell membrane and has DNA-binding affinity (Chung & Dubnau, 1995; R. Provvedi & Dubnau, 1999). Then ComGB protein can modify the cell wall to form a channel, enabling foreign DNA to bind to the membrane-binding receptor ComEA through the cell wall (Dubnau & Provvedi, 2000). When the exogenous DNA molecule passes through the cell wall and binds to the DNA binding protein ComEA, ComEA presents it to the transmembrane protein ComEC located on the plasma membrane (Hahn et al., 1993); ATPase ComFA is required for this translocation process (Londoño-Vallejo & Dubnau, 1993). Subsequently, the endonuclease NucA will shear the double-stranded DNA molecule located on ComEC into single chains, and then one chain will enter the cytoplasm through ComEC, and the other chain will be degraded into nucleotides or other acid-soluble products (Roberta Provvedi et al., 2001).

Natural competence in Gram-negative bacteria is complicated by the presence of an outer membrane which must also traversed. In Gram-negative bacteria, the uptake of DNA into cells is similar to Gram-positive, conceptually divided into a two-step process, DNA uptake (across the outer membrane) and DNA translocation (across the inner

membrane) (Chen & Dubnau, 2004; Krüger & Stingl, 2011). The major differences of natural competence between Gram-positive and Gram-negative bacteria are in the mechanism of DNA uptake. The DNA translocation process is relatively similar.

The first step in DNA uptake is the recognition and binding of foreign DNA, to be more specific, it is the passage of exogenously exposed DNA from the cell surface through the extracellular membrane into the periplasm. Most bacteria that are able to achieve natural competence can bind DNA from any source, but for some Gram-negative bacteria such as *Neisseria*, DNA that contain a specific DNA uptake sequence (DUS) are much more likely to be recognized and transported (Maughan et al., 2010). DUS is a short sequence of bases widely existing in bacterial genomes, for example in *Neisseria meningitidis*, the DUS is such as 5'-GCCGTCTGAA-3' (Tettelin et al., 2000). DNA that contains DUS is recognized by DUS receptor (DUS-R) and then delivered to secretin PilQ or its homologous protein, a transmembrane protein located on the outer membrane of the cell with a central cavity about 6 nm in diameter in the center, allowing double-stranded DNA (about 2.4 nm) crosses the outer membrane and cell wall and enters the cell to be recognized by ComE protein (Cehovin et al., 2013).

This DNA-uptake process is mediated by a range of related extracellular filaments across the range of bacterial taxa, including type IV pili (T4P), Com pili, and Tad/Flp pili, broadly referred to as type IV filaments (T4F) (Figure 1.1, adapted from (Piepenbrink, 2019)). Studies have shown that most Gram-negative bacteria use type IV pili (T4P) to transport DNA molecules (Averhoff, 2004). For example, in *Neisseria gonorrhoeae*, PilD,

the pilin precursor, processes and assembles the pilin precursor into a pilin, and then enters the periplasm through a cellular membrane protein PilG. PilF and PilT are ATPases, which provide the required energy for assembly and disassembly of pili proteins (Averhoff, 2004). With the assistance of the pilus biogenesis lipoprotein PilP (Drake et al., 1997), DNA molecules cross the outer membrane and cell wall and enter the periplasm through PilQ. DNA molecules with DUS are recognized by the DNA receptor ComP, and then the DNA molecules are transferred to the membrane channel protein ComA by the DNA binding protein ComE, and then the double-stranded DNA are separated into single chains. One strand enters the cytoplasm, and the other is degraded into nucleotides or other acid-soluble products that are released into the extracellular environment or periplasm (Averhoff, 2004; Hepp & Maier, 2016), which may further provide nutrients for the bacteria cell.

1.2. Bacteria from the genus *Acinetobacter* are naturally competent

Acinetobacter is a genus of Gram-negative bacteria belonging to the wider class Gamma-proteobacteria. *Acinetobacter* species are not motile, oxidase-negative, strictly aerobic, non-fermentative, and occur in pairs under magnification (Bitrian et al., 2013). They are commonly found in soil and water, where they contribute to the mineralization of, for example, aromatic compounds. On nonselective agar, they have a coccobacillary morphology. Rods predominate in fluid media, particularly during early growth. The genus *Acinetobacter* includes environmental strains, commensals, and pathogens.

Acinetobacter species are a prominent source of opportunistic nosocomial infections

in the US, in particular the species *Acinetobacter baumannii*. The infection of *Acinetobacter* will cause pneumonia and wound, bloodstream, and urinary tract infections (Center for Disease Control and Prevention). In recent years, the drug resistance rate of *Acinetobacter* species has been increasing year by year, and even pan-resistant or fully resistant strains have emerged (Chakravarty, 2020; Fournier et al., 2006; Giamarellou et al., 2008; Hsueh et al., 2002; Palmen et al., 1993). It has been found that most of the drug-resistant *Acinetobacter* strains contain drug-resistant plasmids (Fondi et al., 2010). After sequencing the drug-resistant plasmids, they also found that most of the plasmids lacked the transfer gene *tra* and the mobilization gene *mob* needed for plasmid conjugation or mobilization, respectively. This data indicated that some of the drug-resistant plasmids of *Acinetobacter* were not disseminated through conjugation and transduction but may, in fact, have spread through natural transformation. These results clearly indicate that bacteria from genus *Acinetobacter* are able to achieve HGT through natural transformation in their natural environment. Under laboratory conditions, natural competence has been observed for several species of *Acinetobacter* including *A. nosocomialis, A. baumannii* and *A. baylyi*. Among those, *Acinetobacter baylyi* BD413 (formerly known as *Acinetobacter calcoaceticus* BD413) is the most common strain used to study the natural competence in *Acinetobacter* (Young et al., 2005) because it has a high natural transformation efficiency, about 0.1% to 0.7% than *A. baumannii* (Juni & Janik, 1969), and the naked DNA taken by this bacterium has no sequence specificity (Harding et al., 2013; Lorenz et al., 1992; Palmen et al., 1993).

1.3. Natural Competence in *Acinetobacter* is dependent upon type IV pili1

T4P are filamentous hair-like appendages, exposed on the bacterial surface (Ligthart et al., 2020; Rozman et al., 2021) and polymerized by thousands of copies of a major pilin subunit and many minor pilin subunits (Craig et al., 2019). Although they are found in both Gram-positive (Melville & Craig, 2013; Piepenbrink & Sundberg, 2016) and Gram-negative species (Craig & Li, 2008; Giltner et al., 2012), only the Gram-negative bacterial systems have been extensively studied. T4P are usually in a state of dynamic equilibrium and can be rapidly polymerized and depolymerized, which provide bacteria with a diverse set of functional adaptations, for example, mediate host-cell adhesion, biofilm formation, twitching motility and natural competence (Chiang & Burrows, 2003; Das et al., 2017; Piepenbrink & Sundberg, 2016). However, they have only been found to mediate natural competence in Gram-negative species. The requirement of T4P in natural competence has been studied by many scientists in different species, like *Acinetobacter* (Leong et al., 2017), *Neisseria* (Aas et al., 2002), *Pseudomonas* (Graupner et al., 2000), *Vibrio* (Ellison et al., 2018) and so on.

Acinetobacter type IV pili (T4P) can incorporate several different subunits. PilA is referred to as the major pilin because it makes up the vast majority of the pilus (~99%) and six other gene products have been identified as potential minor pilins FimU, FimT, PilV, PilW, PilX, PilE1 and PilE2. *Acinetobacter* T4P are known to be required for competence. In 2013, Harding et al. (Harding et al., 2013) and Wilharm et al. (Wilharm et al., 2013) sequenced genomes of *Acinetobacter*. They found the whole genome of *A.*

baumannii contains genes required for natural transformation competence and homologs of genes whose products are known to be critical in TFP biogenesis in other organisms. From that, they hypothesized the participation of T4P in the natural transformation of *Acinetobacter baumannii*. To further confirm the results, Harding *et al* deleted the *pilA* and *pilT* genes in *Acinetobacter baumannii* M2 strain (later recategorized as *Acinetobacter nosocomialis* M2) to inactivate T4P functions. Deletion of *pilA* allowed for the identification of the pili structure by observing the surfaces of *Acinetobacter* by electron microscopy*,* comparing the WT strain with the *pilA* deficient strain. They found that *Acinetobacter* T4P are long, narrow fibers, generally 8 nm in diameter under transmission electron microscopy. Then, they tested the transformation efficiency of the WT strain and the mutated strain, the strains that lost the *pilA* or *pilT* genes also lost the natural transformation ability, indicating that the T4P were involved in the natural transformation process of *Acinetobacter baumannii.* Harding *et al*. also reported that the main subunit of T4P in *Acinetobacter baumannii* M2 strain was encoded by the *pilA* gene. *pilB*, *pilC*, and *pilD* genes are also involved in the synthesis and assembly of T4P. Although T4P play an important role in natural transformation, the structural mechanism by which T4P mediate DNA uptake is not clearly defined. Later in 2014, Cehovin *et al* (Cehovin et al., 2013) identified that ComP is the T4P DNA receptor in *Neisseria meningitidis*. While *Acinetobacter* has no homolog of ComP, we hypothesize that a pilin protein serves as a DNA-receptor in a similar manner.

1.4. T4P subtypes in *A. baumannii* promote differential phenotypes

T4P are essential for many functions as diverse as natural competence, twitching motility, biofilm formation and adherence. Some of the functions are retraction dependent while some are not. In T4P systems, PilB is an extension ATPase motor that mediate pilus extension while PilT and PilU are the ATPases which mediate pilus retraction (Harding et al., 2013; Ligthart et al., 2020). Some T4P systems show all four of these functions (DNA uptake, motility, host cell adhesion, bacterial aggregation) while others only appear to be capable of a subset. This diversity of function is reflected in the sequence of the major pilin proteins which typically have little or no sequence identity (Piepenbrink et al., 2016). By sequencing the T4P systems in different bacteria, it was found that the minor pilins are relatively well conserved and the major pilin showed high variability (Cehovin et al., 2010; Criss et al., 2005; Toma et al., 2002).

Recent work by our group has shown that *A. baumannii* strains produce structurally diverse PilA proteins. Surface electrostatics of *A. baumannii* PilA is variable in strains representative of three prominent subtypes: AB5075, ACICU, and BIDMC57 (Ronish et al., 2019). These strains are all clinical isolates from patients that were infected and treated for antibiotic resistant *Acinetobacter* infections in a clinical setting. The minor pilin proteins FimU, FimT, PilX, PilW, PilV, PilE1 and PilE2 are almost identical in these three strains of *Acinetobacter baumannii*, but the PilA is divergent. Models of the pilus fibers from these three strains of *A. baumannii* indicated an obvious difference in surface electrostatics, especially $PilA^{AB5075}$, which showed an unusual concentration of acidic groups. The electrostatic repulsion would disfavor pilus–pilus contacts, then

further affect the pilus function (Jude & Taylor, 2011). To understand the impact caused by the surface electrostatics, Ronish *et al* complemented a *ΔpilA* knockout strain with divergent *pilA* genes from ACICU, BIDMC57, and AB5075 strains. They tested the twitching motility and biofilm formation of these three strains and found these three strains show different phenotypes in motility and biofilm (Fig. 1.2). The complementary +*ppilA* AB5075 shows a high twitching motility but low biofilm formation, while +*ppilA* ACICU shows a low twitching motility but high biofilm formation. The +*ppilA* BIDMC57 has a similar property with the wild type of *Acinetobacter* M2 strain. That indicates variations in pilus electrostatics affect the equilibrium of microcolony formation, which further alters the stability among motility and biofilm formation in *Acinetobacter*. Ronish *et al* hypothesized that these differences stem from differences in pilus-pilus association between the pili of adjacent cells. However twitching motility is dependent upon retraction while biofilm formation is not (Chiang & Burrows, 2003). Hence, differences in retraction between the complements (variants of *pilA*) could also explain the observed motility and biofilm formation phenotypes. By measuring the ability of these *pilA* variants to take up DNA, we can independently assess their ability to retract as competence also requires retraction (as evidenced by the absence of competence in the pilT mutant).

1.5. Summary

It is known that T4P and related pili play an important part in natural competence through DNA uptake. Although there are many mechanisms of natural competence that

have been studied in different bacteria, little is known about the DNA receptors incorporated into pili. The only DNA receptor that has been discovered is in *Neisseria,* and ComP is a minor pilin. Based on that, our hypothesis is that the DNA receptor(s) in *Acinetobacter* is one of the minor T4P subunits.

The variation of PilA proteins in different *A. baumannii* strains have different surface electrostatics, which may impact the pilus function. In fact, we saw different phenomena in twitching mobility and biofilm formation of three strains, but we have no data on their relative natural competence. T4P variation may impact natural competence through variation in retraction between these T4P subtypes.

To elucidate the mechanism of natural competence in *Acinetobacter*, we will identify the DNA-receptor(s) incorporated into T4P. Additionally, we will use phenotypic differences caused by natural variation in the *pilA* gene to probe the relationship between natural competence and other pilus functions.

1.6. References

Aas, F. E., Wolfgang, M., Frye, S., Dunham, S., Løvold, C., & Koomey, M. (2002). Competence for natural transformation in Neisseria gonorrhoeae: Components of DNA binding and uptake linked to type IV pilus expression. *Molecular Microbiology*, *46*(3). https://doi.org/10.1046/j.1365-2958.2002.03193.x

Averhoff, B. (2004). DNA Transport and Natural Transformation in Mesophilic and Thermophilic Bacteria. In *Journal of Bioenergetics and Biomembranes* (Vol. 36, Issue 1). https://doi.org/10.1023/B:JOBB.0000019595.66733.fa

Barouki, R., & Smith, H. O. (1985). Reexamination of phenotypic defects in rec-1 and rec-2 mutants of Haemophilus influenzae Rd. Journal of Bacteriology, 163(2). https://doi.org/10.1128/jb.163.2.629-634.1985

Bitrian, M., González, R. H., Paris, G., Hellingwerf, K. J., & Nudel, C. B. (2013). Blue-light-dependent inhibition of twitching motility in Acinetobacter baylyi ADP1: Additive involvement of three BLUF-domain-containing proteins. *Microbiology (United Kingdom)*, *159*(PART 9). https://doi.org/10.1099/mic.0.069153-0

Cehovin, A., Simpson, P. J., McDowell, M. A., Brown, D. R., Noschese, R., Pallett, M., Brady, J., Baldwin, G. S., Lea, S. M., Matthews, S. J., & Pelicic, V. (2013). Specific DNA recognition mediated by a type IV pilin. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(8). https://doi.org/10.1073/pnas.1218832110

Cehovin, A., Winterbotham, M., Lucidarme, J., Borrow, R., Tang, C. M., Exley, R. M., &

Pelicic, V. (2010). Sequence conservation of pilus subunits in Neisseria meningitidis. *Vaccine*, *28*(30). https://doi.org/10.1016/j.vaccine.2010.04.065

Chakravarty, B. (2020). Genetic mechanisms of antibiotic resistance and virulence in Acinetobacter baumannii: background, challenges and future prospects. In *Molecular Biology Reports* (Vol. 47, Issue 5). https://doi.org/10.1007/s11033-020-05389-4

Chen, I., & Dubnau, D. (2004). DNA uptake during bacterial transformation. In *Nature Reviews Microbiology* (Vol. 2, Issue 3). https://doi.org/10.1038/nrmicro844

Chiang, P., & Burrows, L. L. (2003). Biofilm formation by hyperpiliated mutants of Pseudomonas aeruginosa. *Journal of Bacteriology*, *185*(7). https://doi.org/10.1128/JB.185.7.2374-2378.2003

Chung, Y. S., & Dubnau, D. (1995). ComC is required for the processing and translocation of ComGC, a pilin-like competence protein of Bacillus subtilis. *Molecular Microbiology*, *15*(3). https://doi.org/10.1111/j.1365-2958.1995.tb02267.x

Craig, L., Forest, K. T., & Maier, B. (2019). Type IV pili: dynamics, biophysics and functional consequences. In *Nature Reviews Microbiology* (Vol. 17, Issue 7). https://doi.org/10.1038/s41579-019-0195-4

Craig, L., & Li, J. (2008). Type IV pili: paradoxes in form and function. In *Current Opinion in Structural Biology* (Vol. 18, Issue 2). https://doi.org/10.1016/j.sbi.2007.12.009

Criss, A. K., Kline, K. A., & Seifert, H. S. (2005). The frequency and rate of pilin

antigenic variation in Neisseria gonorrhoeae. *Molecular Microbiology*, *58*(2). https://doi.org/10.1111/j.1365-2958.2005.04838.x

Das, J., Mokrzan, E., Lakhani, V., Rosas, L., Jurcisek, J. A., Ray, W. C., & Bakaletz, L. O. (2017). Extracellular DNA and type IV pilus expression regulate the structure and kinetics of biofilm formation by nontypeable haemophilus influenzae. *MBio*, *8*(6). https://doi.org/10.1128/mBio.01466-17

De Visser, J. A. G. M., & Elena, S. F. (2007). The evolution of sex: Empirical insights into the roles of epistasis and drift. In *Nature Reviews Genetics* (Vol. 8, Issue 2). https://doi.org/10.1038/nrg1985

Drake, S. L., Sandstedt, S. A., & Koomey, M. (1997). PilP, a pilus biogenesis lipoprotein in Neisseria gonorrhoeae, affects expression of PilQ as a high-molecular-mass multimer. *Molecular Microbiology*, *23*(4). https://doi.org/10.1046/j.1365-2958.1997.2511618.x

Dubnau, D. (1999). DNA uptake in bacteria. In *Annual Review of Microbiology* (Vol. 53). https://doi.org/10.1146/annurev.micro.53.1.217

Dubnau, D., & Provvedi, R. (2000). Internalizing DNA. *Research in Microbiology*, *151*(6). https://doi.org/10.1016/S0923-2508(00)00166-2

Ellison, C. K., Dalia, T. N., Vidal Ceballos, A., Wang, J. C. Y., Biais, N., Brun, Y. V., & Dalia, A. B. (2018). Retraction of DNA-bound type IV competence pili initiates DNA uptake during natural transformation in Vibrio cholerae. *Nature Microbiology*, *3*(7). https://doi.org/10.1038/s41564-018-0174-y

Fondi, M., Bacci, G., Brilli, M., Papaleo, M. C., Mengoni, A., Vaneechoutte, M., Dijkshoorn, L., & Fani, R. (2010). Exploring the evolutionary dynamics of plasmids: The Acinetobacter pan-plasmidome. *BMC Evolutionary Biology*, *10*(1). https://doi.org/10.1186/1471-2148-10-59

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D., & Claverie, J. M. (2006). Comparative genomics of multidrug resistance in Acinetobacter baumannii. *PLoS Genetics*, *2*(1). https://doi.org/10.1371/journal.pgen.0020007

Giamarellou, H., Antoniadou, A., & Kanellakopoulou, K. (2008). Acinetobacter baumannii: a universal threat to public health? In *International Journal of Antimicrobial Agents* (Vol. 32, Issue 2). https://doi.org/10.1016/j.ijantimicag.2008.02.013

Giltner, C. L., Nguyen, Y., & Burrows, L. L. (2012). Type IV Pilin Proteins: Versatile Molecular Modules. *Microbiology and Molecular Biology Reviews*, *76*(4). https://doi.org/10.1128/mmbr.00035-12

Graupner, S., Frey, V., Hashemi, R., Lorenz, M. G., Brandes, G., & Wackernagel, W. (2000). Type IV pilus genes pilA and pilC of Pseudomonas stutzeri are required for natural genetic transformation, and pilA can be replaced by corresponding genes from nontransformable species. *Journal of Bacteriology*, *182*(8). https://doi.org/10.1128/JB.182.8.2184-2190.2000

Hahn, J., Inamine, G., Kozlov, Y., & Dubnau, D. (1993). Characterization of comE, a late competence operon of Bacillus subtilis required for the binding and uptake of transforming DNA. *Molecular Microbiology*, *10*(1). https://doi.org/10.1111/j.1365-2958.1993.tb00907.x

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S. (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). https://doi.org/10.1128/mBio.00360-13

Hepp, C., & Maier, B. (2016). Kinetics of DNA uptake during transformation provide evidence for a translocation ratchet mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(44). https://doi.org/10.1073/pnas.1608110113

Hoelzer, M. A., & Michod, R. E. (1991). DNA repair and the evolution of transformation in Bacillus subtilis. III. Sex with damaged DNA. *Genetics*, *128*(2). https://doi.org/10.1093/genetics/128.2.215

Hsueh, P. R., Teng, L. J., Chen, C. Y., Chen, W. H., Yu, C. J., Ho, S. W., & Luh, K. T. (2002). Pandrug-resistant Acinetobacter baumannii causing nosocomial infections in a University Hospital, Taiwan. *Emerging Infectious Diseases*, *8*(8). https://doi.org/10.3201/eid0805.020014

Jude, B. A., & Taylor, R. K. (2011). The physical basis of type 4 pilus-mediated microcolony formation by Vibrio cholerae O1. *Journal of Structural Biology*, *175*(1).

https://doi.org/10.1016/j.jsb.2011.04.008

Juni, E., & Janik, A. (1969). Transformation of Acinetobacter calco-aceticus (Bacterium anitratum). *Journal of Bacteriology*, *98*(1). https://doi.org/10.1128/jb.98.1.281-288.1969

Krüger, N. J., & Stingl, K. (2011). Two steps away from novelty - principles of bacterial DNA uptake. In *Molecular Microbiology* (Vol. 80, Issue 4). https://doi.org/10.1111/j.1365-2958.2011.07647.x

Lam, T., Ellison, C. K., Eddington, D. T., Brun, Y. V., Dalia, A. B., & Morrison, D. A. (2021). Competence pili in Streptococcus pneumoniae are highly dynamic structures that retract to promote DNA uptake. *Molecular Microbiology*, *116*(2). https://doi.org/10.1111/mmi.14718

Leong, C. G., Bloomfield, R. A., Boyd, C. A., Dornbusch, A. J., Lieber, L., Liu, F., Owen, A., Slay, E., Lang, K. M., & Lostroh, C. P. (2017). The role of core and accessory type IV pilus genes in natural transformation and twitching motility in the bacterium Acinetobacter baylyi. *PLoS ONE*, *12*(8). https://doi.org/10.1371/journal.pone.0182139

Ligthart, K., Belzer, C., de Vos, W. M., & Tytgat, H. L. P. (2020). Bridging Bacteria and the Gut: Functional Aspects of Type IV Pili. In *Trends in Microbiology* (Vol. 28, Issue 5). https://doi.org/10.1016/j.tim.2020.02.003

Londoño-Vallejo, J. A., & Dubnau, D. (1993). comF, a Bacillus subtilis late competence locus, encodes a protein similar to ATP-dependent RNA/DNA helicases. *Molecular*

Microbiology, *9*(1). https://doi.org/10.1111/j.1365-2958.1993.tb01674.x

Lorenz, M. G., Reipschläger, K., & Wackernagel, W. (1992). Plasmid transformation of naturally competent Acinetobacter calcoaceticus in non-sterile soil extract and groundwater. *Archives of Microbiology*, *157*(4). https://doi.org/10.1007/BF00248681

Maughan, H., Wilson, L. A., & Redfield, R. J. (2010). Bacterial DNA uptake sequences can accumulate by molecular drive alone. *Genetics*, *186*(2). https://doi.org/10.1534/genetics.110.119438

Mell, J. C., & Redfield, R. J. (2014). Natural competence and the evolution of DNA uptake specificity. In *Journal of Bacteriology* (Vol. 196, Issue 8). https://doi.org/10.1128/JB.01293-13

Melville, S., & Craig, L. (2013). Type IV Pili in Gram-Positive Bacteria. *Microbiology and Molecular Biology Reviews*, *77*(3), 323–341. https://doi.org/10.1128/mmbr.00063-12

Palmen, R., Vosman, B., Buijsman, P., Breek, C. K. D., & Hellingwerf, K. J. (1993). Physiological characterization of natural transformation in Acinetobacter calcoaceticus. *Journal of General Microbiology*, *139*(2). https://doi.org/10.1099/00221287-139-2-295

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A.,

Rutherford, K. M., Van Vliet, A. H. M., … Barrell, B. G. (2000). The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. *Nature*, *403*(6770). https://doi.org/10.1038/35001088

Perron, G. G., Lee, A. E. G., Wang, Y., Huang, W. E., & Barraclough, T. G. (2012). Bacterial recombination promotes the evolution of multi-drug-resistance in functionally diverse populations. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1733). https://doi.org/10.1098/rspb.2011.1933

Piepenbrink, K. H. (2019). DNA uptake by type IV filaments. In *Frontiers in Molecular Biosciences* (Vol. 6, Issue FEB). https://doi.org/10.3389/fmolb.2019.00001

Piepenbrink, K. H., Lillehoj, E., Harding, C. M., Labonte, J. W., Zuo, X., Rapp, C. A., Munson, R. S., Goldblum, S. E., Feldman, M. F., Gray, J. J., & Sundberg, E. J. (2016). Structural diversity in the type IV Pili of multidrug-resistant acinetobacter. *Journal of Biological Chemistry*, *291*(44). https://doi.org/10.1074/jbc.M116.751099

Piepenbrink, K. H., & Sundberg, E. J. (2016). Motility and adhesion through type IV pili in Gram-positive bacteria. *Biochemical Society Transactions*, *44*(6). https://doi.org/10.1042/BST20160221

Pifer, M. L., & Smith, H. O. (1985). Processing of donor DNA during Haemophilus influenzae transformation: Analysis using a model plasmid system. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(11). https://doi.org/10.1073/pnas.82.11.3731

Pinchuk, G. E., Ammons, C., Culley, D. E., Li, S. M. W., McLean, J. S., Romine, M. F.,

Nealson, K. H., Fredrickson, J. K., & Beliaev, A. S. (2008). Utilization of DNA as a sole source of phosphorus, carbon, and energy by Shewanella spp.: Ecological and physiological implications for dissimilatory metal reduction. *Applied and Environmental Microbiology*, *74*(4). https://doi.org/10.1128/AEM.02026-07

Provvedi, R., & Dubnau, D. (1999). ComEA is a DNA receptor for transformation of competent Bacillus subtilis. *Molecular Microbiology*, *31*(1). https://doi.org/10.1046/j.1365-2958.1999.01170.x

Provvedi, Roberta, Chen, I., & Dubnau, D. (2001). NucA is required for DNA cleavage during transformation of Bacillus subtilis. *Molecular Microbiology*, *40*(3). https://doi.org/10.1046/j.1365-2958.2001.02406.x

Quinn, B., Martinez, J., Liu, C., Nguyen, M., & Ramirez, M. S. (2018). The effect of sub-inhibitory concentrations of antibiotics on natural transformation in Acinetobacter baumannii. In *International Journal of Antimicrobial Agents* (Vol. 51, Issue 5). https://doi.org/10.1016/j.ijantimicag.2018.01.026

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E. E., Sebra, R., Chin, C.-S., Iliopoulos, D., Klammer, A., Peluso, P., Lee, L., Kislyuk, A. O., Bullard, J., Kasarskis, A., Wang, S., Eid, J., Rank, D., … Waldor, M. K. (2011). Origins of the E. coli Strain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany . *New England Journal of Medicine*, *365*(8). https://doi.org/10.1056/nejmoa1106920

Redfield, R. J. (1993). Genes for breakfast: The have-your-cake and-eat-lt-too of

bacterial transformation. *Journal of Heredity*, *84*(5). https://doi.org/10.1093/oxfordjournals.jhered.a111361

Redfield, Rosemary J. (2001). Do bacteria have sex? *Nature Reviews Genetics*, *2*(8). https://doi.org/10.1038/35084593

Ronish, L. A., Lillehoj, E., Fields, J. K., Sundberg, E. J., & Piepenbrink, K. H. (2019). The structure of PilA from Acinetobacter baumannii AB5075 suggests a mechanism for functional specialization in Acinetobacter type IV pili. *Journal of Biological Chemistry*, *294*(1). https://doi.org/10.1074/jbc.RA118.005814

Rozman, V., Accetto, T., Duncan, S. H., Flint, H. J., & Vodovnik, M. (2021). Type IV pili are widespread among non-pathogenic Gram-positive gut bacteria with diverse carbohydrate utilization patterns. *Environmental Microbiology*, *23*(3). https://doi.org/10.1111/1462-2920.15362

Seitz, P., & Blokesch, M. (2013). Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. In *FEMS Microbiology Reviews* (Vol. 37, Issue 3). https://doi.org/10.1111/j.1574-6976.2012.00353.x

Sheppard, D., Berry, J. L., Denise, R., Rocha, E. P. C., Matthews, S., & Pelicic, V. (2020). The major subunit of widespread competence pili exhibits a novel and conserved type IV pilin fold. *Journal of Biological Chemistry*, *295*(19). https://doi.org/10.1074/jbc.RA120.013316

Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A.,

Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J., Nelson, W. C., Gwinn, M. L., DeBoy, R., Peterson, J. D., Hickey, E. K., Haft, D. H., Salzberg, S. L., White, O., Fleischmann, R. D., … Craig Venter, J. (2000). Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. *Science*, *287*(5459). https://doi.org/10.1126/science.287.5459.1809

Toma, C., Kuroki, H., Nakasone, N., Ehara, M., & Iwanaga, M. (2002). Minor pilin subunits are conserved in Vibrio cholerae type IV pili. *FEMS Immunology and Medical Microbiology*, *33*(1). https://doi.org/10.1016/S0928-8244(02)00273-0

Vegge, C. S., Brøndsted, L., Ligowska-Marzeta, M., & Ingmer, H. (2012). Natural Transformation of Campylobacter jejuni Occurs Beyond Limits of Growth. *PLoS ONE*, *7*(9). https://doi.org/10.1371/journal.pone.0045467

Vesel, N., & Blokesch, M. (2021). Pilus production in acinetobacter baumannii is growth phase dependent and essential for natural transformation. *Journal of Bacteriology*, *203*(8). https://doi.org/10.1128/JB.00034-21

Wilharm, G., Piesker, J., Laue, M., & Skiebe, E. (2013). DNA Uptake by the nosocomial pathogen acinetobacter baumannii occurs during movement along wet surfaces. *Journal of Bacteriology*, *195*(18). https://doi.org/10.1128/JB.00754-13

Young, D. M., Parke, D., & Ornston, L. N. (2005). Opportunities for genetic investigation afforded by Acinetobacter baylyi, a nutritionally versatile bacterial species that is highly competent for natural transformation. In *Annual Review of Microbiology* (Vol. 59). https://doi.org/10.1146/annurev.micro.59.051905.105823

Figures

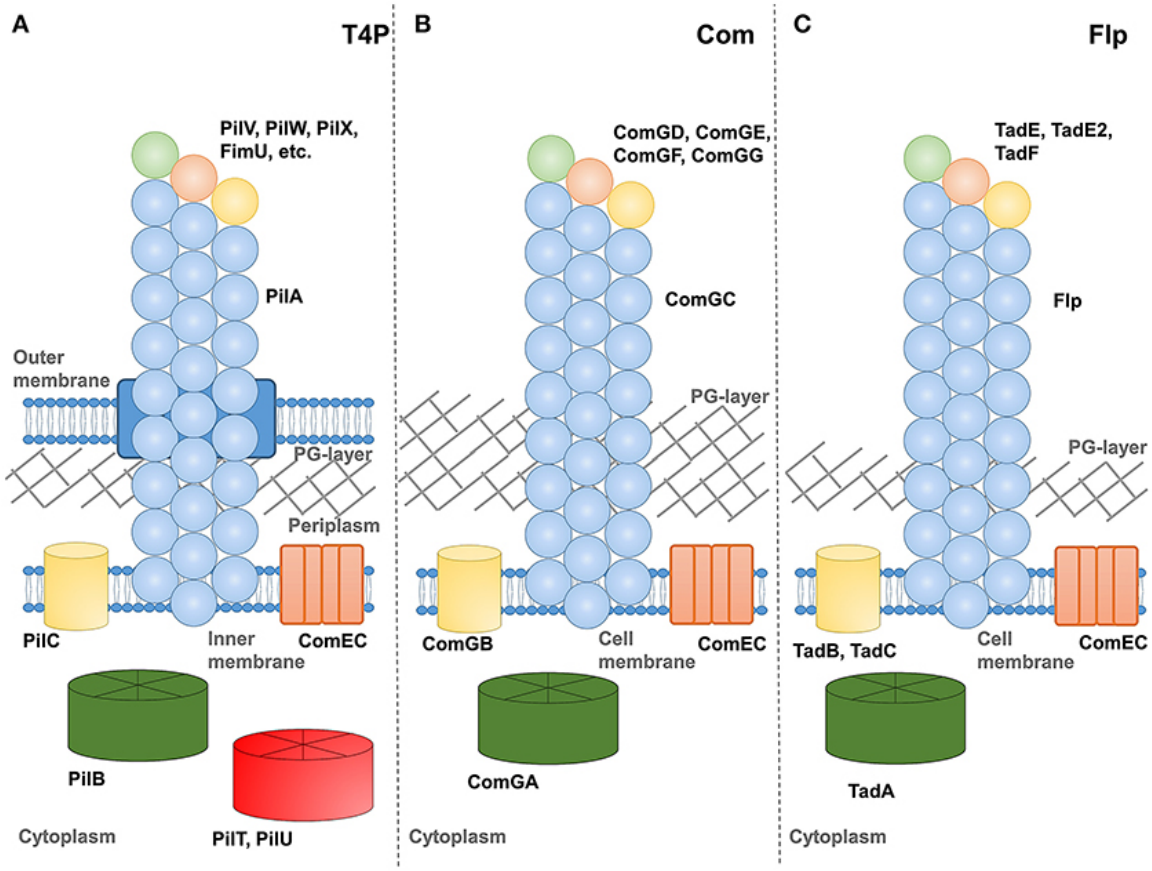


Figure 1.1. Schematic of pilus fibers utilized for DNA uptake.

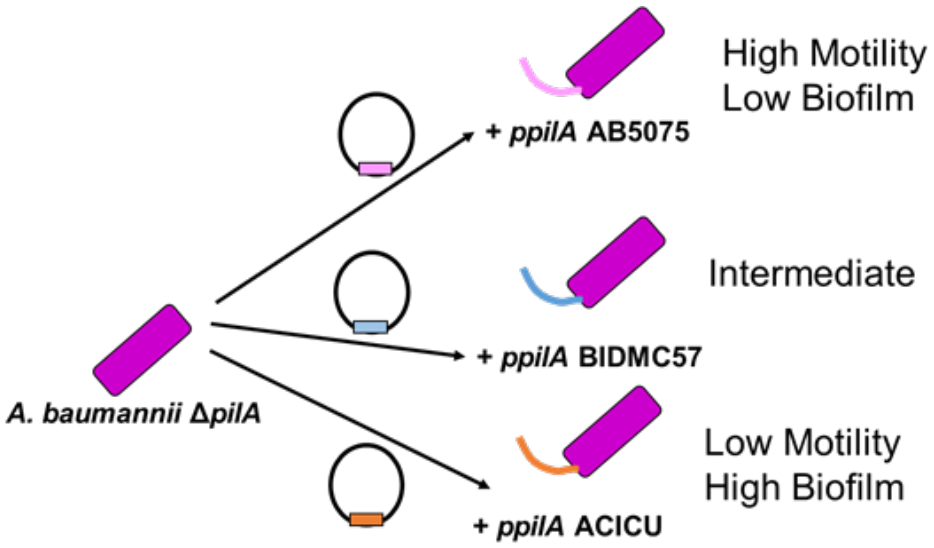


Figure 1.2. Phenotypes of *A. baumannii pilA* complements from distinct T4P subtypes.

CHAPTER 2: IDENTIFYING DNA-UPTAKE RECEPTORS IN *ACINETOBACTER* TYPE IV PILI

2.1 Abstract

Naturally competent bacteria take up DNA from the environment, which allows the bacteria to obtain nutrients, repair DNA damage and incorporate novel genetic material into their genomes. This process of horizontal gene transfer leads to increased genetic diversity, including the spread and development of antibiotic resistance.

Bacteria from the genus *Acinetobacter* are gram-negative, aerobic, non-flagellated bacteria, including environmental strains, commensals, and opportunistic pathogens. The resistance of *Acinetobacter* species to antibiotic drugs has been increasing year by year, and multidrug-resistant strains now account for more than half of terminal *Acinetobacter* infections. As in many other bacterial genera, natural competence in *Acinetobacter* is dependent upon DNA-uptake mediated by type IV pili (T4P). T4P are extracellular appendages composed of protein subunits (pilins) polymerized into a helical fiber.

While genetic deletions of PilA (the primary T4P subunit) and PilT (a cytosolic enzyme necessary for T4P retraction) have been found to abrogate natural competence, the mechanisms through which these mutations disrupt competence remains unclear. One proposed mechanism for these proteins is as the T4P DNA-receptor, which has not been identified in *Acinetobacter*. Indeed, despite the requirement of T4P for natural competence in dozens of bacterial species, only one T4P DNA-receptor has been identified in *Neisseria meningitidis*. Furthermore, that gene, *comP*, has no homolog in

Acinetobacter or related gamma-proteobacteria (*Pseudomonas, Dichelobacter, Moraxella*). Thus, further investigations are necessary to determine the T4P DNA receptor for *Acinetobacter*.

To identify the structural mechanism that T4P in *Acinetobacter* uses to take up DNA, we proposed to identify DNA-receptors by measuring direct binding of recombinantly-expressed soluble pilin subunits to plasmid DNA. We purified several *Acinetobacter* T4P subunits and assessed their ability to bind plasmid DNA using EMSA (electrophoretic mobility shift assays). Furthermore, additional studies using a combination of structural biology and biochemistry will be used to elucidate the structural basis and specificity of these DNA binding interactions. Preliminary data indicates that two minor pilins, PilE1 and PilE2, are capable of binding double-stranded DNA *in vitro*.

2.2 Introduction

Natural competence, or natural transformation, is one mechanism that bacteria use to incorporate novel genetic material through horizontal gene transfer (HGT). HGT plays an essential role in microbial evolution by spreading beneficial genes across phyla both distantly and closely related. (De Visser & Elena, 2007; Mell & Redfield, 2014; Rasko et al., 2011). In natural competence, bacteria can take up DNA from the environment and integrate it into their genomes (Chen & Dubnau, 2004; Dubnau, 1999). As an example, it plays a prominent role in the spread of antibiotic resistance via β-lactamases in *Acinetobacter* and other bacterial genera. (Seitz & Blokesch, 2013).

Bacteria from the genus *Acinetobacter* have the ability to adapt to environmental

changes by HGT, which leads to an increase in drug resistance for *Acinetobacter*. Different terms like multidrug resistant (MDR), extensive drug resistant (XDR), and pan drug resistant (PDR) have been used with varied definitions to describe the extent of antimicrobial resistance among *Acinetobacter spp* (Fournier et al., 2006; Giamarellou et al., 2008; Hsueh et al., 2002) *Acinetobacter* is wildly distributed in the environment and infections typically occur in people in healthcare settings. The increasing drug resistance poses a great challenge for physicians and clinical microbiologists.

Acinetobacter uses T4P to be naturally competent as seen by the inability to take up plasmid DNA when T4P are knocked out. T4P are extracellular appendages that consist of thousands of copies of a major pilin protein and many minor pilin proteins (Craig et al., 2019). *Acinetobacter* species encode a major pilin PilA and putative minor pilins FimU, FimT, PilV, PilW, PilX, PilE1and PilE2 (Ronish et al., 2019). The specific DNA receptor in *Acinetobacter* remains unclear but is hypothesized to be a minor pilin. There are multiple potential mechanisms for the DNA-binding ability of T4P, including binding to the major pilin, minor pilins in a complex at the pilus tip or minor pilins incorporated along the length of the pilus (Figure 2.1. adapted from (Piepenbrink, 2019)). In *Neisseria meningitidis,* the T4P as the DNA-receptor has been identified as the minor pilin ComP; (Cehovin et al., 2013), However ComP, has no homolog in *Acinetobacter* (Figure 2.2. adapted from (Piepenbrink, 2019)).

We hypothesize that one or more of the minor pilin subunits of *Acinetobacter* baumannii (PilX, PilW, PilV, FimU, FimT, PilE1, PilE2) is an extracellular DNA

receptor. Therefore, our aim is to determine which *Acinetobacter* pilins bind to DNA. There is a close relationship between natural transformation and the worsening of clinical bacterial infections. Once we understand the mechanism, we can find ways to interrupt the binding of T4P and DNA, so that we can potentially interrupt the process of *Acinetobacter* gaining beneficial genes from the environment to become more pathogenic, which will improve our ability to treat the infections caused by *Acinetobacter*. In addition, *Acinetobacter* has similar type IV pilus locus to *Pseudomonas*, *Dichelobacter*, *Moraxella*. The DNA uptake model of *Acinetobacter* will provide a direction to investigate the DNA receptor in these related gammaproteobacterial.

2.3 Materials and Methods

2.3.1 Protein samples

In this study, we will use all the T4P subunits of *A. baumannii*, which is the major pilin PilA and the minor pilin FimU, FimT, PilV, PilW, PilX, PilE1 and PilE2.

2.3.2 Protein expression and purification

Codon-optimized sequences of all eight subunits from *A. baumannii*, starting with residue 21 (removing the N-terminal TM-helix), will be cloned into a pETM44 vector with an N-terminal His6 tag separately. These plasmids will be transformed into NiCo21(DE3) competence cells separately and grow to saturation overnight with shaking at 37 °C in LB medium with 50 μg/ml ampicillin. These saturation cultures are then diluted into fresh LB-ampicillin and grown to an optical density (OD) of 0.4–0.6 at 37 °C. These flasks are cooled to 18 °C before induction with 30 mM isopropyl β-d-1-

thiogalactopyranoside and then grow overnight with shaking at 18 °C before being harvested by centrifugation at 7,500◊g for 20 min. The cells are then lysed using 30 ml lysis buffer (50 mM Tris-HCl pH 8.3, 0.7 M sucrose, 1 mM NaEDTA, 15 mM NaN_3), 1 ml lysozyme (0.25 mg/ml final concentration), 1 ml DNase (0.02 mg/ml), 1 ml PMSF (0.1 M), 1 ml $MgCl_2$ (2 M) for 5 minutes and 80 ml lysis buffer (50 mM Tris-HCl pH 8.3, 0.1 M NaCl, Triton X-100(0.5%), 25 mM sodium deoxycholate) for 10-15 min, and the resulting lysate is centrifuged again, this time at 20,000 × g for 30 min. If the protein is soluble, the supernatant is purified using a nickel-nitrilotriacetic acid column, and the elution is further purified by size exclusion chromatography over a GE Healthcare S200 Superdex column using an ÅKTA Purifier FPLC.

If the protein is insoluble, the precipitate or the inclusion body is washed using inclusion body wash buffer (50 mM Tris-HCl pH 8.3, 100 mM NaCl, 1 mM EDTA, Triton X-100 (0.25%), 15 mM NaN3,) and is centrifuged at 7,500 × g for 20 min. This procedure is repeated 4 to 5 times until the supernatant was clear. The pellet is then resuspended in 10-20ml final buffer (5.12 mg/ml MES, 0.48 g/ml urea, 10 mM NaEDTA, 1 mM DDT, pH 6.0). Collect the supernatant and stored at -80℃. The guandine solution (twice the volume of insoluble protein) is combined with the protein and then inject into the refolding buffer (100 mM Tris-HCl, 400 mM L-Arg-HCl, 2mM NaEDTA, 6.3mM Cysteamine, 3.7 mM Cystamine, 0.2 mM PMSF, pH 8.3) through a needle. The mixture is stirred slowly at 4℃ overnight. In the morning, dialysis will begin by pouring the refold solution into a dialysis bag and submerging in 10L of 10 mM Tris-HCl solution at

4°C with stirring for three days. After that, the mixture is purified using a nickel-nitrilotriacetic acid column, and the elution is further purified by size exclusion chromatography over a GE Healthcare S200 Superdex column using an ÅKTA Purifier FPLC.

2.3.3 Biophysical binding measurements

Electrophoretic mobility shift assays (EMSA) are carried out by mixing proteins with recombinantly expressed plasmid DNA (200 ng), incubating at 4°C for 1 h in DNA binding buffer (25 mM Tris-HCl, pH 8.5, 100 mM NaCl), and separation by electrophoresis on a 1.5% agarose gel at either 4C or 25C.

2.4 Results and Discussion

We use EMSA (electrophoretic mobility shift assays) DNA binding assay to determine which *Acinetobacter* pilins bind to DNA. If the protein is able to bind DNA, the complex of protein and DNA will have a lower migration rate than the free unbounded DNA. Thus, we will see a shift on the polyacrylamide gel once the protein is bind to DNA. ComP is the DNA receptor of *Neisseria meningitidis*. Cehovin used a specific DNA uptake sequence that *Neisseria* species favors to determine the DNA receptor. We just used plasmids available to us from other experiments to do the DNA binding experiment and found it sufficient for investigating DNA binding (Fig. 2.3.). Based on that, our experimental design will screen for the competence receptor even though we do not know if the specificity of the *Acinetobacter* DNA receptor, if any.

To validate our experimental approach, we purified *N. meningitidis* ComP and tested

its ability to bind DNA using identical experimental parameters to the ones we propose (Fig. 2.3.). Our results showed that with the increase of protein concentration, PilE1 and PilE2 shows a shift of bands while PilA does not, which leads us to conclude that the major pilin PilA is not able to bind DNA while the minor pilins PilE1 and PilE2 are able to bind DNA *in vitro* (Fig. 2.4.). For four other minor pilins, we tried to purify them individually, however, all of them were insoluble. Therefore, our hypothesis is that the four minor pilins functions as a complex. We have succeeded in refolding a minor pilin complex (based on SDS page and SEC, Figure 2.5.) and then performed the EMSA with the complex, no shift was observed on the agarose gel, which concludes that the minor pilin complex PilV, PilW, PilX and FimU cannot directly bind to DNA (Fig. 2.6.). We are currently in the process of testing the DNA-binding of a PilV/PilW/PilX/FimT complex based on recent data indicating DNA-binding by the FimT homologue in *Legionella pneumoniae*.

2.5 Conclusion

In this experiment, we tested the DNA binding affinity of seven of the eight subunits of *Acinetobacter baumannii* T4P. Our results showed that the major pilin protein is not the DNA receptor of *A. baumannii,* and PilE1 and PilE2 are capable of directly binding to DNA, which indicates that PilE1 and PilE2 may function as DNA receptors in *A. baumannii.*

2.6 References

Cehovin, A., Simpson, P. J., McDowell, M. A., Brown, D. R., Noschese, R., Pallett, M., Brady, J., Baldwin, G. S., Lea, S. M., Matthews, S. J., & Pelicic, V. (2013). Specific DNA recognition mediated by a type IV pilin. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(8). https://doi.org/10.1073/pnas.1218832110

Chen, I., & Dubnau, D. (2004). DNA uptake during bacterial transformation. In *Nature Reviews Microbiology* (Vol. 2, Issue 3). https://doi.org/10.1038/nrmicro844

Craig, L., Forest, K. T., & Maier, B. (2019). Type IV pili: dynamics, biophysics and functional consequences. In *Nature Reviews Microbiology* (Vol. 17, Issue 7). https://doi.org/10.1038/s41579-019-0195-4

De Visser, J. A. G. M., & Elena, S. F. (2007). The evolution of sex: Empirical insights into the roles of epistasis and drift. In *Nature Reviews Genetics* (Vol. 8, Issue 2). https://doi.org/10.1038/nrg1985

Dubnau, D. (1999). DNA uptake in bacteria. In *Annual Review of Microbiology* (Vol. 53). https://doi.org/10.1146/annurev.micro.53.1.217

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D., & Claverie, J. M. (2006). Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genetics*, *2*(1). https://doi.org/10.1371/journal.pgen.0020007

Giamarellou, H., Antoniadou, A., & Kanellakopoulou, K. (2008). *Acinetobacter baumannii*: a universal threat to public health? In *International Journal of Antimicrobial Agents* (Vol. 32, Issue 2). https://doi.org/10.1016/j.ijantimicag.2008.02.013

Hsueh, P. R., Teng, L. J., Chen, C. Y., Chen, W. H., Yu, C. J., Ho, S. W., & Luh, K. T. (2002). Pandrug-resistant *Acinetobacter baumannii* causing nosocomial infections in a University Hospital, Taiwan. *Emerging Infectious Diseases*, *8*(8). https://doi.org/10.3201/eid0805.020014

Mell, J. C., & Redfield, R. J. (2014). Natural competence and the evolution of DNA uptake specificity. In *Journal of Bacteriology* (Vol. 196, Issue 8). https://doi.org/10.1128/JB.01293-13

Piepenbrink, K. H. (2019). DNA uptake by type IV filaments. In *Frontiers in Molecular Biosciences* (Vol. 6, Issue FEB). https://doi.org/10.3389/fmolb.2019.00001

Rasko, D. A., Webster, D. R., Sahl, J. W., Bashir, A., Boisen, N., Scheutz, F., Paxinos, E. E., Sebra, R., Chin, C.-S., Iliopoulos, D., Klammer, A., Peluso, P., Lee, L., Kislyuk, A. O., Bullard, J., Kasarskis, A., Wang, S., Eid, J., Rank, D., … Waldor, M. K. (2011). Origins of the E. coli Strain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany . *New England Journal of Medicine*, *365*(8). https://doi.org/10.1056/nejmoa1106920

Ronish, L. A., Lillehoj, E., Fields, J. K., Sundberg, E. J., & Piepenbrink, K. H. (2019). The structure of PilA from *Acinetobacter baumannii* AB5075 suggests a mechanism

for functional specialization in Acinetobacter type IV pili. *Journal of Biological Chemistry*, *294*(1). https://doi.org/10.1074/jbc.RA118.005814

Seitz, P., & Blokesch, M. (2013). Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. In *FEMS Microbiology Reviews* (Vol. 37, Issue 3). https://doi.org/10.1111/j.1574-6976.2012.00353.x

Figures

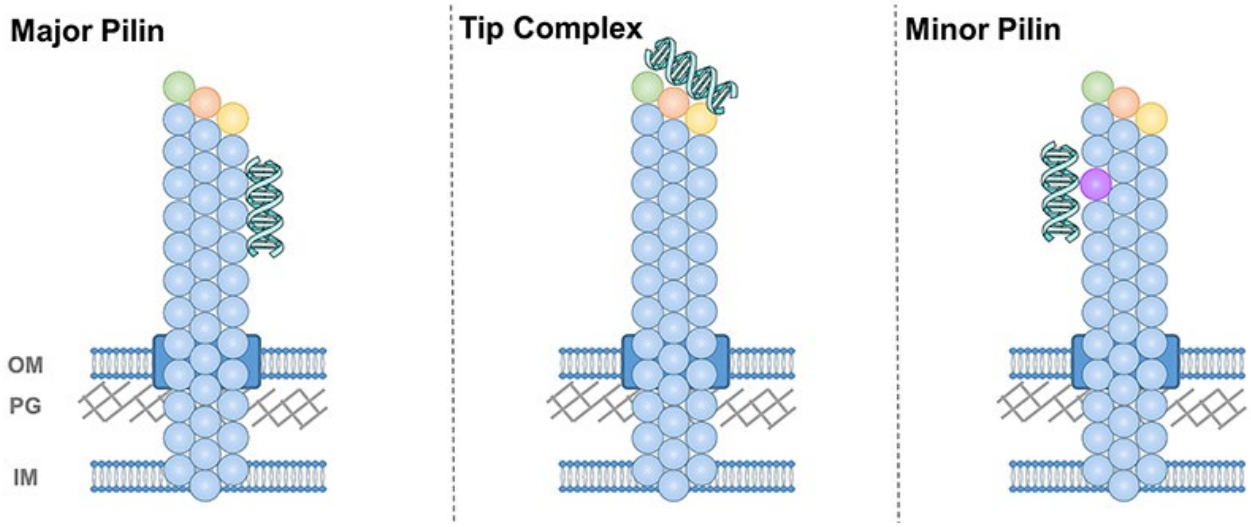


Figure 2.1. Models of T4P DNA binding.

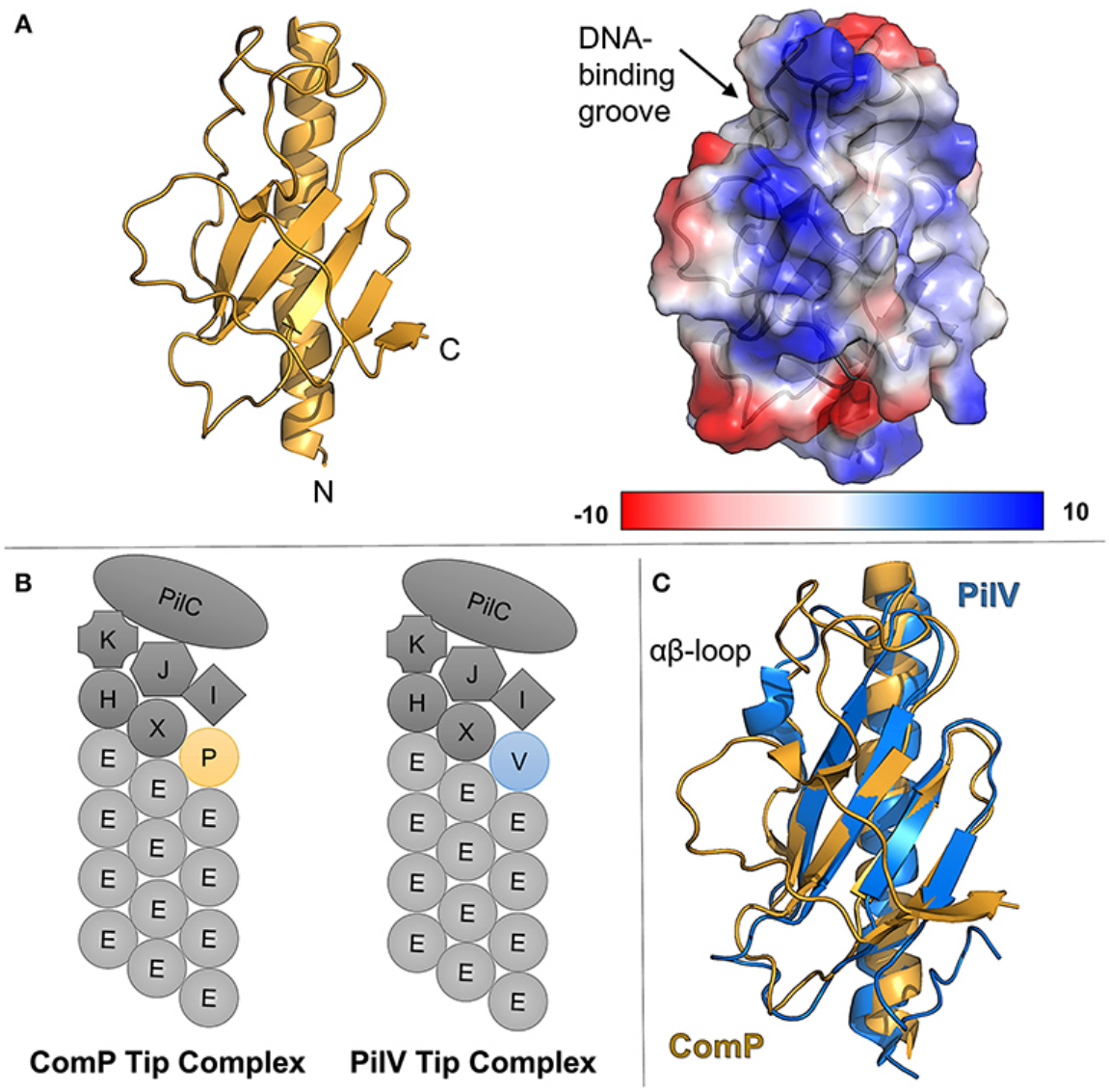


Figure 2.2. Neisseria ComP.

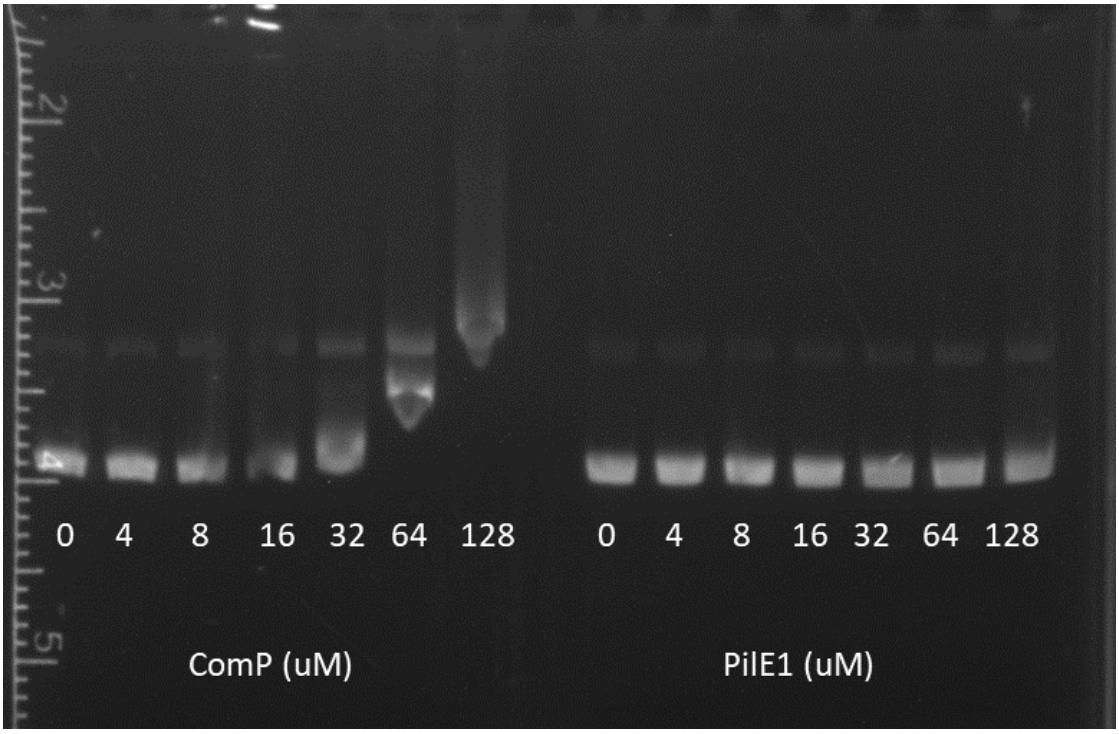


Figure 2.3. DNA binding propensity of *Neisseria meningitidis* minor pilin protein ComP and *Acinetobacter baumannii* minor pilin protein PilE1.

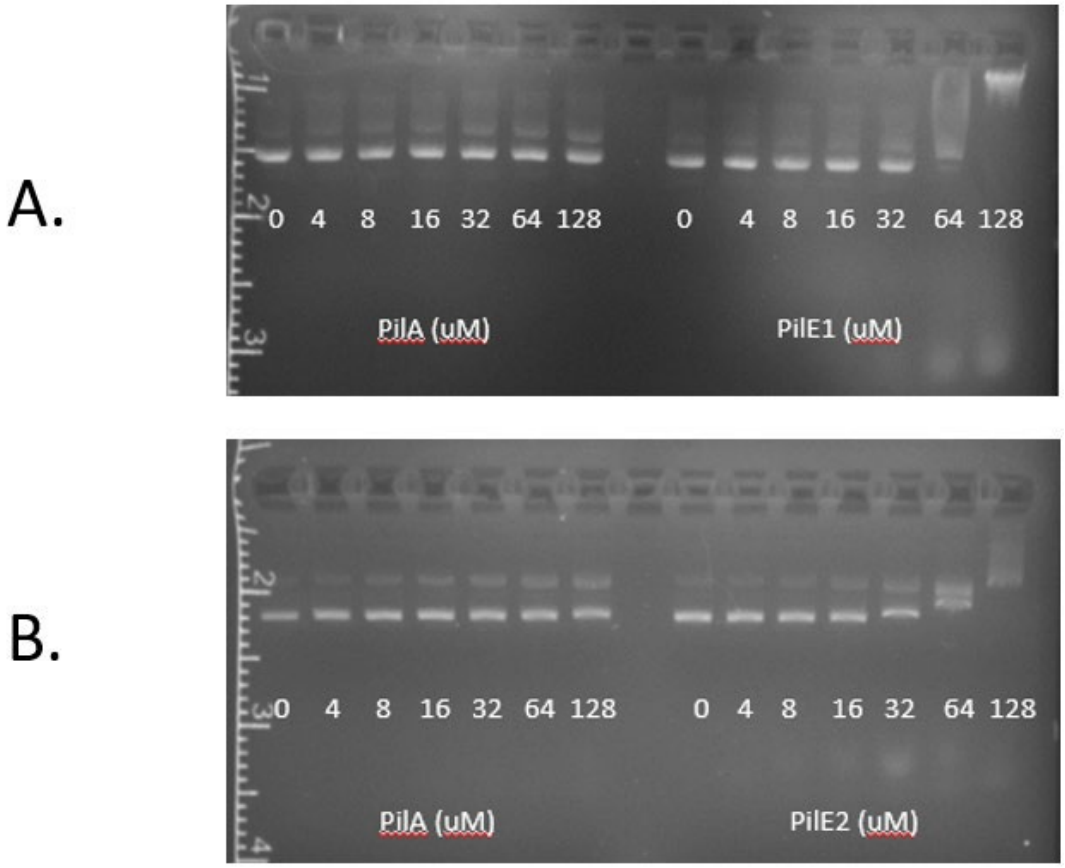


Figure 2.4. The DNA binding propensity of Acinetobacter baumannii major pilin protein PilA and minor pilin protein PilE1 and PilE2.

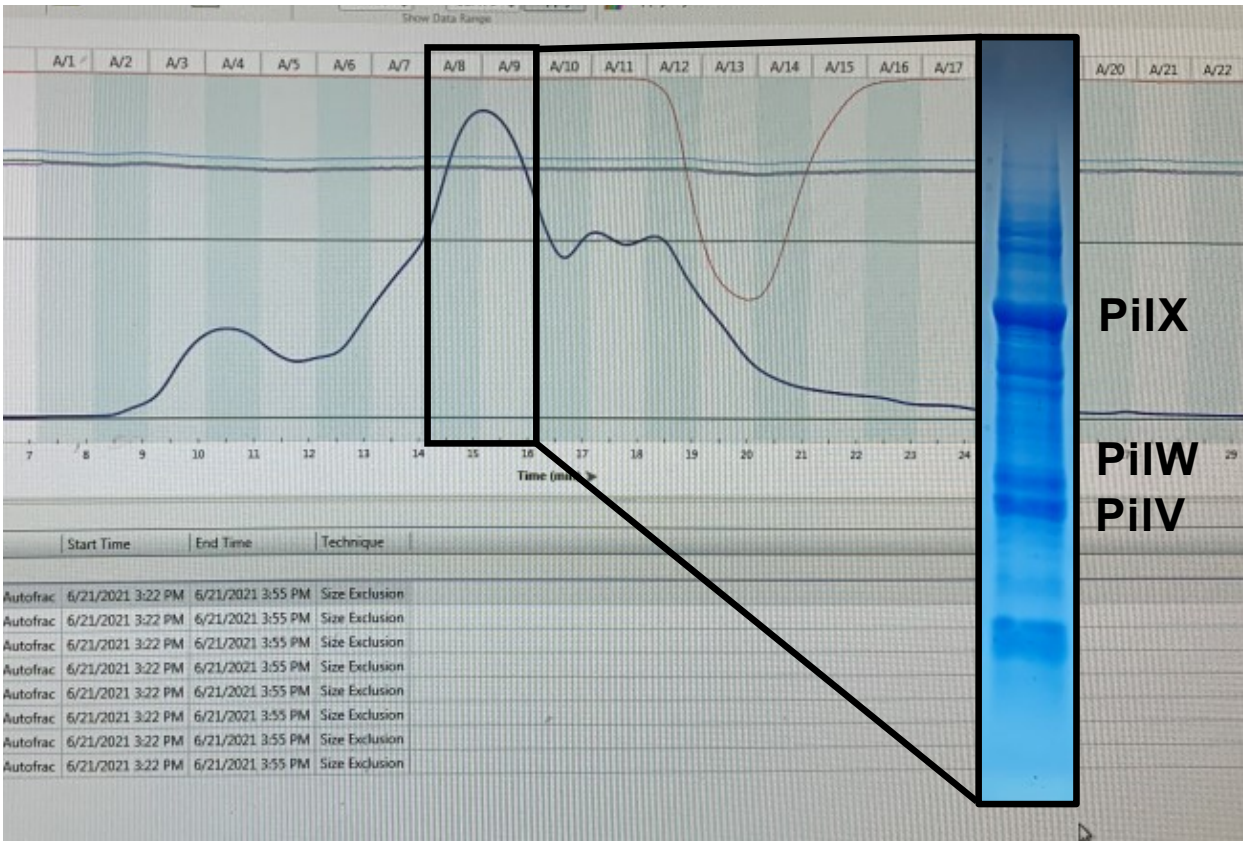


Figure 2.5. Refolding the *Acinetobacter* T4P Tip complex.

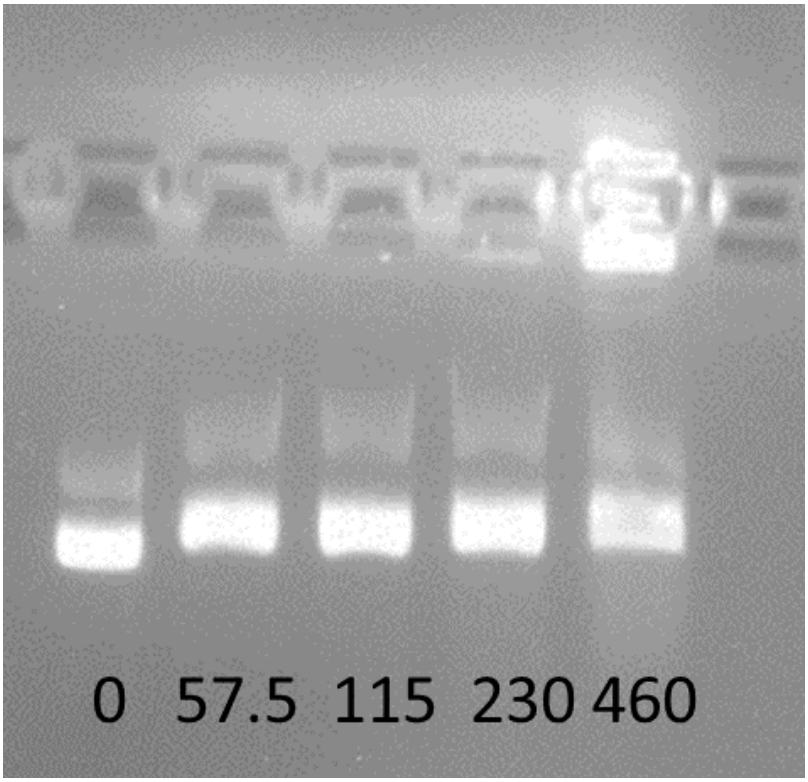


Figure 2.6. The DNA binding propensity of the complex of minor pilin proteins PilV, PilW, PilX and FimU in *Acinetobacter baumannii*.

CHAPTER 3: ASSESSING THE IMPACT OF *ACINETOBACTER* T4P SUBTYPES ON NATURAL COMPETENCE

3.1 Abstract

T4P polymers consist primarily of a single subunit protein (the major pilin) but also incorporate small numbers of other pilin proteins (minor pilins) and are involved in a wide range of physiological processes. Typically, the minor pilins are well conserved while the major pilin is highly variable. Previous work from our group found that allelic variants of *pilA* from diverse strains of *Acinetobacter baumannii* show functional variations in biofilm formation and twitching motility. Based on this functional variation, we also performed natural competence assays in different strains of *Acinetobacter baumannii* to assess the impact of T4P variation in natural competence. Our results show that the structural divergence of PilA also affect the natural competence.

3.2 Introduction

Type IV pili are protein fibers which many species of bacteria can, through polymerization, extend out from their cell surface and, through depolymerization, retract with significant force (Giltner et al., 2012). *Acinetobacter* T4P are involved in several distinct physiological processes, some are retraction-dependent functions (horizontal gene transfer, twitching motility) while others are retraction-independent function (biofilm formation, host cell adherence). Although all strains of *Acinetobacter* carry the genes necessary to produce type IV pili, and the majority of those genes are highly conserved, the protein sequence of the major pilin, PilA, exhibits considerable variation

in the soluble domain (Piepenbrink et al., 2016). Different strains of *Acinetobacter baumannii* show structural differences in PilA which we categorize into three subtypes based on the amino-acid level variation in sequence. These differing *pilA* alleles encode proteins with variations in surface electrostatics resulting in different functions in terms of twitching motility and biofilm formation (Ronish et al., 2019). It remains unknown whether these variations impact natural competence. One important unknown is the speed and force with which each of these T4P subtypes is retracted. Although Ronish et al found no increase in host cell adhesion for the *pilA* complements (characteristic of a *pilT* deletion), differences in retraction could explain some of the observed phenotypic differences as the promotion of biofilm formation is retraction independent while twitching motility is retraction dependent. PilT is the putative retraction ATPase. The usage of *pilT* deletion is because the *pilT* deletion cells still remain piliated, but lose the pilus function, they are unable to mediate twitching motility or DNA uptake (Adams et al., 2019; Chlebek et al., 2019). To evaluate the hypothesis that the various PilA subtypes do have different propensities for pilus retraction, we evaluated the impact of PilA subtype on natural competence, which, like twitching motility, requires retraction. We assessed the impact of T4P variation in natural competence directly by measuring the uptake of plasmid DNA through acquired antibiotic resistance.

3.3 Materials and Methods

3.3.1 Samples

In this study, we will determine how the variation in *pilA* impacts natural

competence or natural transformation ability of *Acinetobacter*. The three *pilA* sequences we used are from strains AB5075 (International Clone I), ACICU (International Clone II), and BIDMC57 (see figure 3.1 adapted from (Ronish et al., 2019)). They are all clinical isolates from the patients that were infected and treated for antibiotic resistant *Acinetobacter* infections. The six samples we used are wild type (WT), pilA knock out (ΔpilA), chromosomal pilA complement (+ppilA), AB5075 (+ppilA^{AB5075}), ACICU (+ppilA^{ACICU}) and BIDMC57 (+ppilA^{BIDMC57}).

3.3.2 Natural transformation assay

All transformation assay were performed with the six samples, similar to transformation assays performed by others (Harding et al., 2013; Vesel & Blokesch, 2021). To be brief, the bacteria were grown overnight in 10 ml LB broth at 37°C while shaking at 200 rpm. The starter cultures were back diluted 1:100 into 2 ml of fresh LB broth and let them grow for 6 h or different time frame. Next, 1 ng of plasmid DNA, which has a streptomycin resistance was added to 20 µl of the bacterial culture, and 20 µl of this mixture was spotted onto LB agar plates. Then the plates were incubated at 37°C for 2 h. After 2h, the bacterial cells were scraped off from the plates using a sterile loop and then resuspended in 200 µl of LB broth. 100 µl of the culture were plated on LB and LB with selective marker specific to DNA used (streptomycin resistance). Serial dilutions will be performed to get a countable colony forming units (CFUs). The plates were incubated at 37 °C overnight and CFUs were counted. The transformation efficiency or transformation frequency was the ratio of the CFU number of transformants obtained on

selective medium by the CFU number of transformants obtained on non-selective medium. For this experiment, usually three biologically independent experiments are performed.

3.4 Results and Discussion

Our results show that in the absence of PilA, no DNA was taken up (no colonies grow on the plate). For wild type and the strains complemented with pilA (+ppilA), we see the growth of colonies. These preliminary data recapitulate the results from Harding *et al* (Harding et al., 2013) and demonstrate that natural competence can be readily observed under our experimental conditions. (Fig. 3.2).

In 2021, Vesel *et al* found that transformation is growth phase-dependent in *A. baumannii*. They grew the bacteria for different time frame, compared the transformation efficiency and concluded that the highest levels of transformation were observed for bacteria grown to the exponential phase. To refine our protocol, we performed the same experiment to investigate the better growth time frame for our *A. baumannii*. We tested the wild type, pilA knock out mutant and complementary pilA from AB5075. Our results showed that transformation start occurs when the time frame is 150min and the countable colonies occurs when the time frame is 240min (Fig. 3.4). Based on the growth cure of Acinetobacter, these two time frames are both in the log phase (Fig. 3.3). Next, we repeated the experiment and checked the transformation for 4-9h growth time frame. The results demonstrated that 6h growth is the best time frame to get a clear and higher transformation (Fig. 3.5). We then performed the refined natural transformation assay

with all the Δ*pilA* deletion and complemented strains; figure 3.6 shows the results of that experiment. All three complements show an increase in natural transformation over the Δ*pilA* strain, but only the AB5075 and ACICU strains show significance ($p = 0.0002$ and $p = 0.002$ respectively). The ACICU complement shows a defect in natural competence compared to the AB5075 complement ($p=0.0018$) approximately equal to the defect observed in twitching motility. These data suggest that pilus retraction is reduced in the pilAAB5075 complement and that this reduction impacts all retraction-dependent functions, including twitching motility and DNA-uptake.

3.5 Conclusions

Our data show that while all T4P subtypes are capable of some level of twitching motility and natural competence (through DNA-uptake), profound differences can be found between subtypes, particularly between those from the International Clone I and International Clone II subtypes. Recent data from other groups implies that transformation efficiency varies widely between *A. baumannii* strains (Hu et al., 2019); we conclude form our data that pilus subtype is one of the components influencing transformability in these strains.

3.6 Reference

Adams, D. W., Pereira, J. M., Stoudmann, C., Stutzmann, S., & Blokesch, M. (2019). The type IV pilus protein PilU functions as a PilT-dependent retraction ATPase. PLoS Genetics, 15(9). https://doi.org/10.1371/journal.pgen.1008393

Chlebek, J. L., Hughes, H. Q., Ratkiewicz, A. S., Rayyan, R., Wang, J. C. Y., Herrin, B. E., Dalia, T. N., Biais, N., & Dalia, A. B. (2019). PilT and PilU are homohexameric ATPases that coordinate to retract type IVa pili. PLoS Genetics, 15(10). https://doi.org/10.1371/journal.pgen.1008448

Giltner, C. L., Nguyen, Y., & Burrows, L. L. (2012). Type IV Pilin Proteins: Versatile Molecular Modules. Microbiology and Molecular Biology Reviews, 76(4). https://doi.org/10.1128/mmbr.00035-12

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S. (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). https://doi.org/10.1128/mBio.00360-13

Hu, Y., He, L., Tao, X., Meng, F., & Zhang, J. (2019). High DNA Uptake Capacity of International Clone II Acinetobacter baumannii Detected by a Novel Planktonic Natural Transformation Assay. Frontiers in Microbiology, 10. https://doi.org/10.3389/fmicb.2019.02165

Piepenbrink, K. H., Lillehoj, E., Harding, C. M., Labonte, J. W., Zuo, X., Rapp, C. A.,

Munson, R. S., Goldblum, S. E., Feldman, M. F., Gray, J. J., & Sundberg, E. J. (2016). Structural diversity in the type IV Pili of multidrug-resistant acinetobacter. *Journal of Biological Chemistry*, *291*(44). https://doi.org/10.1074/jbc.M116.751099

Ronish, L. A., Lillehoj, E., Fields, J. K., Sundberg, E. J., & Piepenbrink, K. H. (2019). The structure of PilA from Acinetobacter baumannii AB5075 suggests a mechanism for functional specialization in Acinetobacter type IV pili. *Journal of Biological Chemistry*, *294*(1). https://doi.org/10.1074/jbc.RA118.005814

Vesel, N., & Blokesch, M. (2021). Pilus production in acinetobacter baumannii is growth phase dependent and essential for natural transformation. *Journal of Bacteriology*, *203*(8). https://doi.org/10.1128/JB.00034-21

Figures

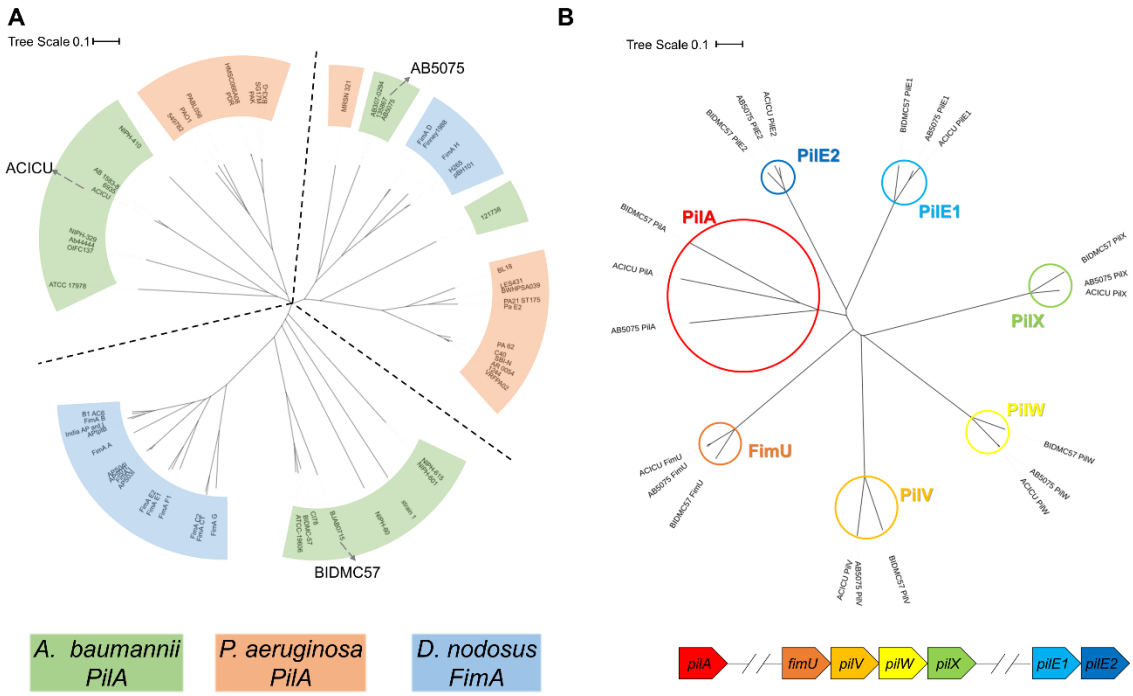


Figure 3.1. Variation in *Acinetobacter baumannii* PilA

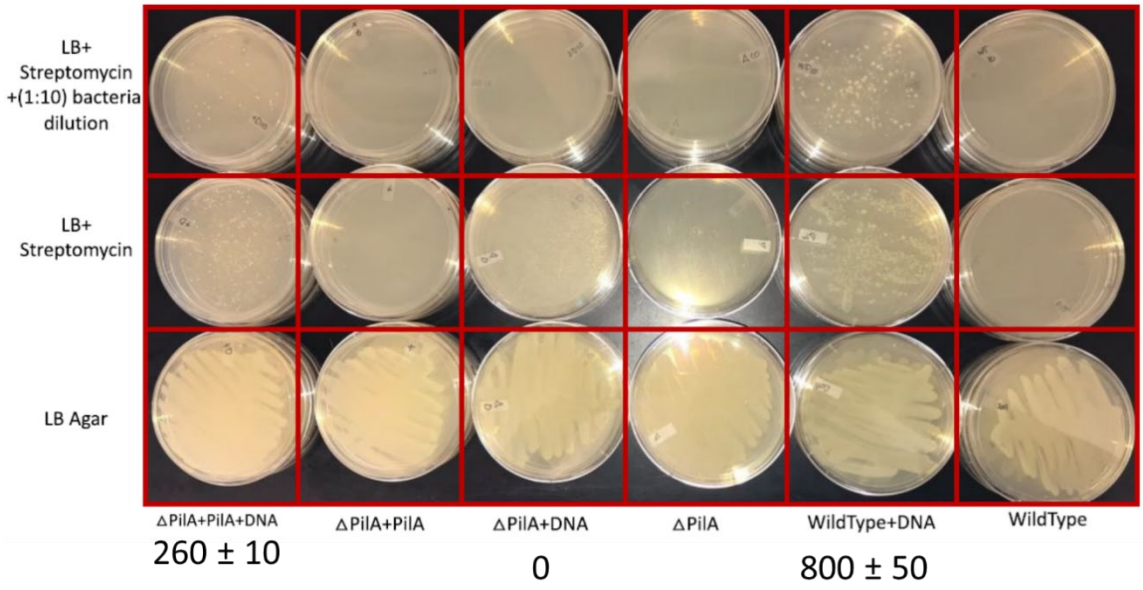


Figure 3.2: Natural competence ability of *Acinetobacter*.

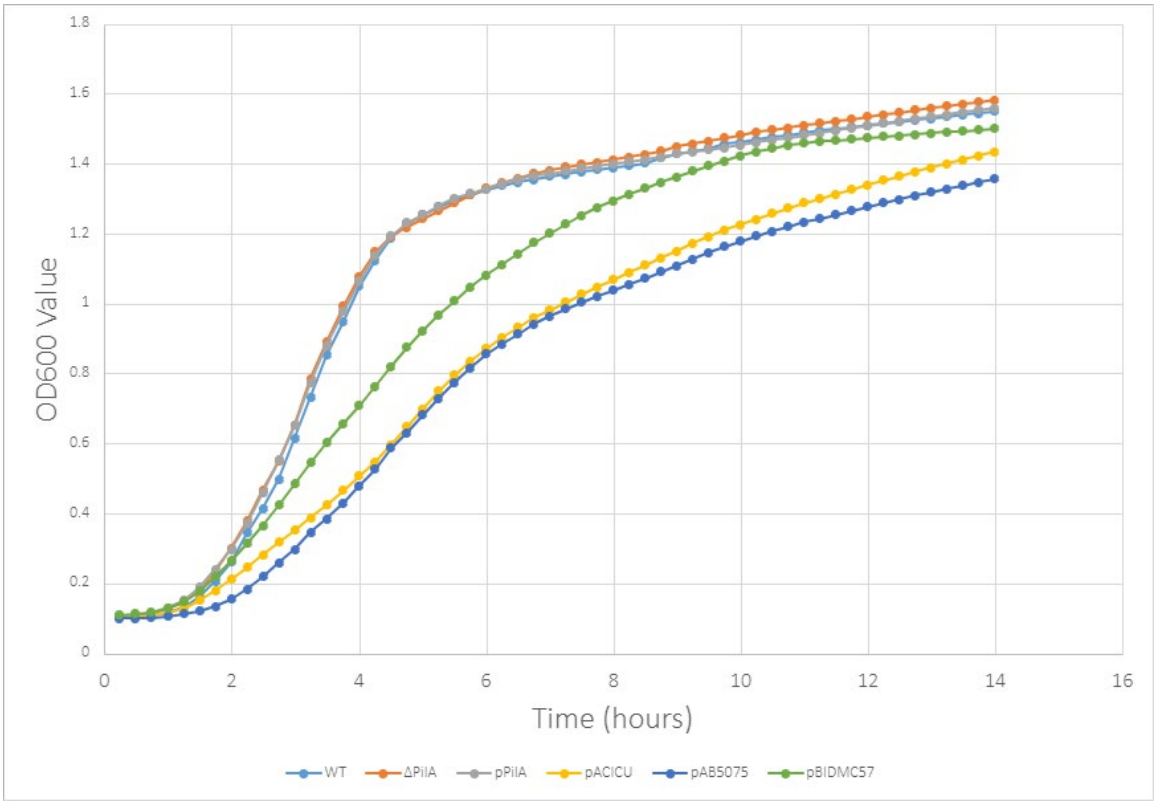


Figure 3.3. Growth curve of *A. baumannii*. The OD600 value is measured every fifteen minutes by plate reader. The total measure period is 14 hours.

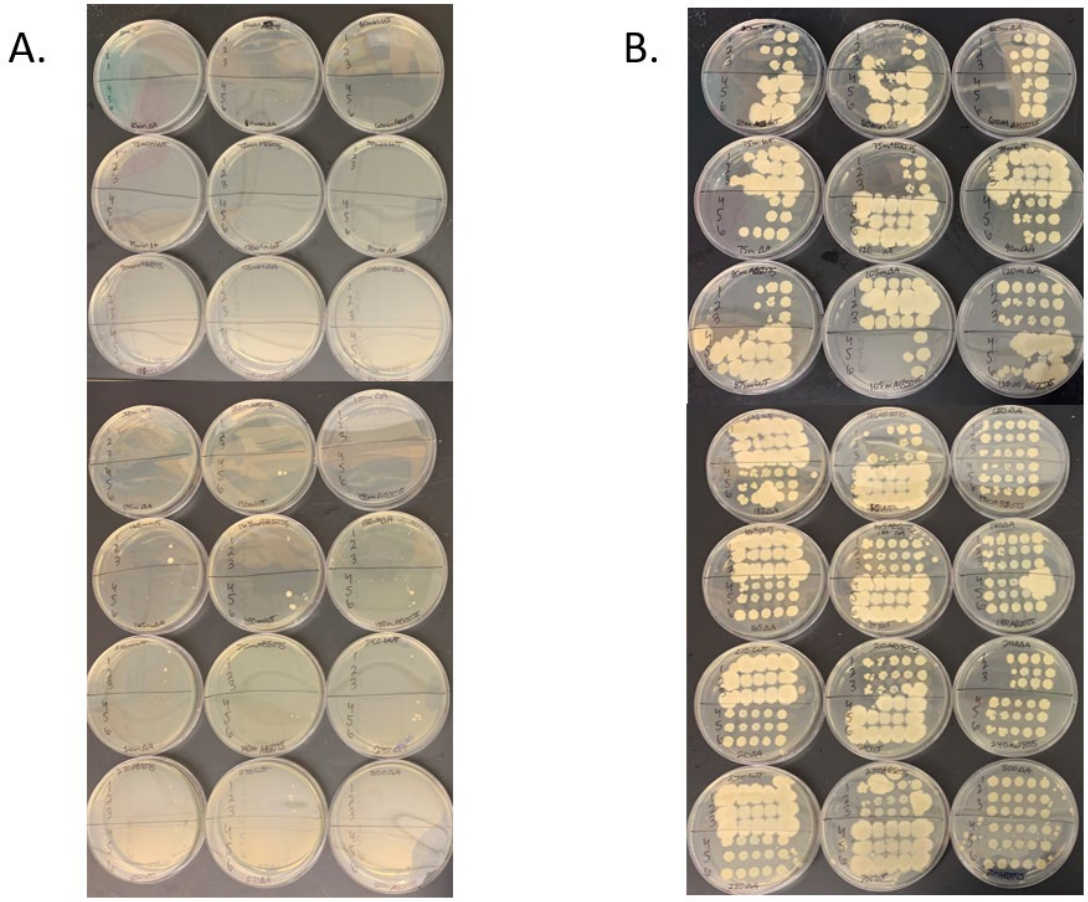
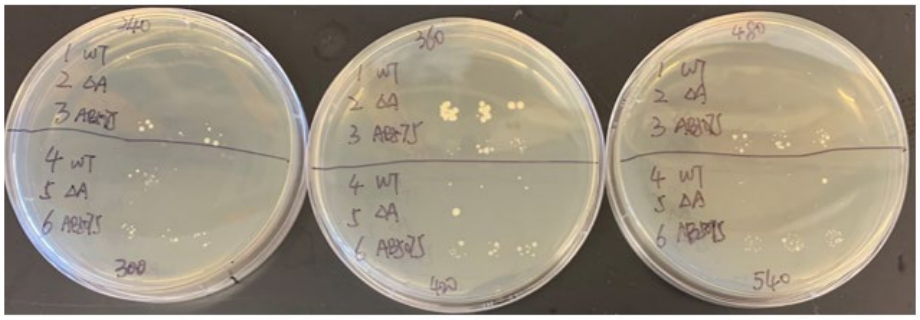


Figure 3.4. Natural transformability of *A. baumannii* over time (30min, 60min, 75min, 90min, 105min, 120min, 135min, 150min, 180min, 210min, 240min, 270min, 300min). (A) Colonies growth on LB with streptomycin agar plate. (B) Colonies growth on LB without streptomycin agar plate.

A.



B.

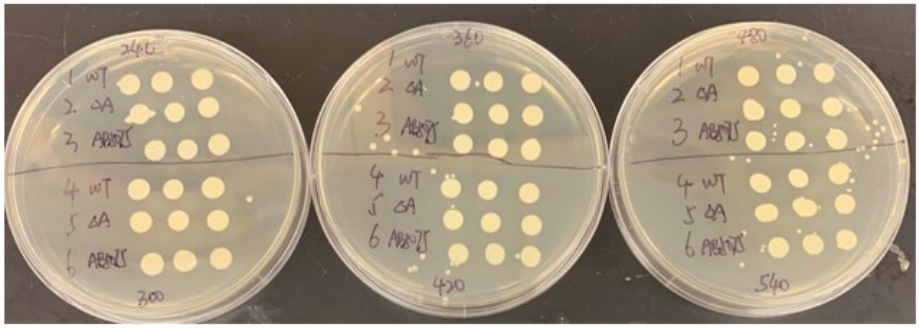


Figure 3.5. Natural transformability of *A. baumannii* over time (240min, 300min, 360min, 420min, 480min, 540min). (A) Colonies growth on LB with streptomycin agar plate. (B) Colonies growth on LB without streptomycin agar plate.

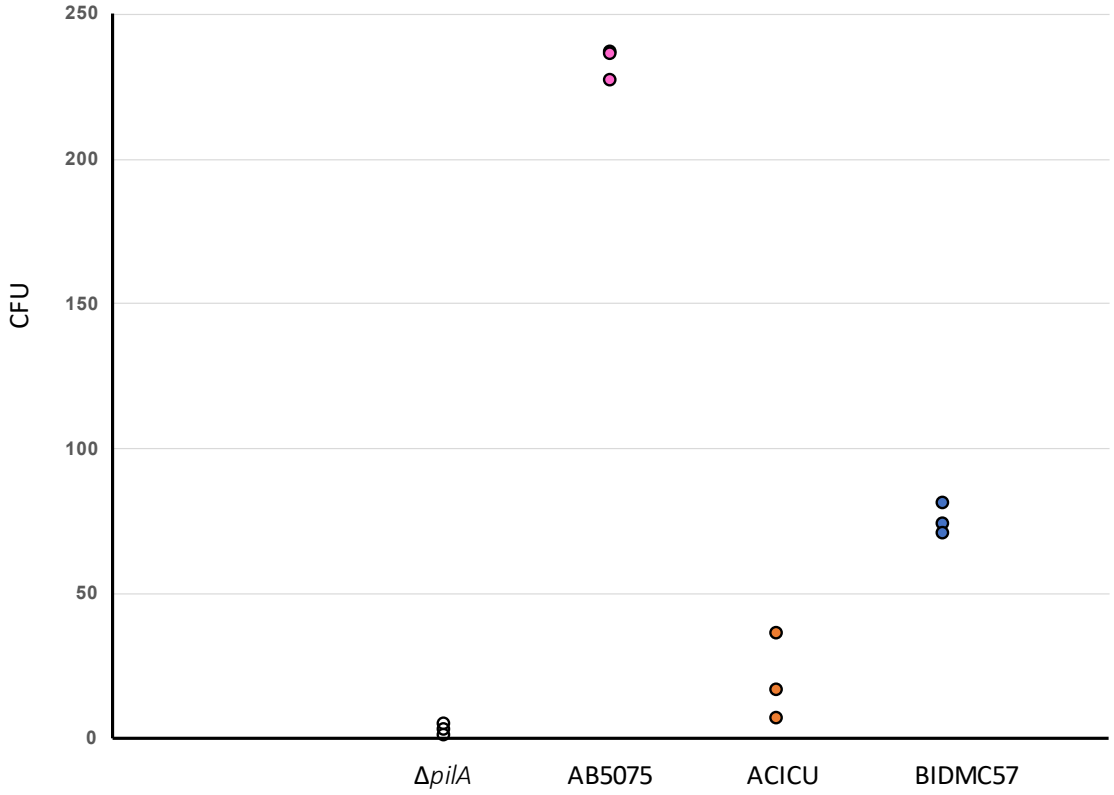


Figure 3.6. Natural competence of *pilA* complements. ΔpilA is the pilA knock out bacteria. AB5075, ACICU and BIDMC57 means these are the chromosomal pilA complement from these three strains. Shown are the CFU value of three independent biological replicates (n=3). CFU value is obtained by counting the colonies that grow on LB with streptomycin plate, the antibiotic selective marker taken up via HGT.

CHAPTER 4: CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

4.1 Conclusion

In this thesis, we investigated the DNA receptor(s) in *Acinetobacter baumanni* and also showed how variations in PilA proteins affected natural transformation. By checking the DNA binding affinity of all T4P subunits, we conclude that the major pilin protein is not the DNA receptor. Similarly, the major pilin in *Neisseria meningitidis* is not the DNA receptor. Rather, the DNA receptor(s) in both *Acinetobacter* and *Neisseria* are minor pilin proteins: PilE1 and PilE2 in *Acinetobacter* as described in this thesis, and ComP in *Neisseria* as was previously described.

For the natural competence assay, we do see natural competence requires T4P. As obserbed for twitching motility, which is also a pilin-mediated process that is retraction dependent, +ppilA AB5075 shows a higher natural competence efficiency, followed by +ppilA BIDMC57. +ppilA ACICU shows a defect in natural competence. Our data illustrate that pilus subtype from different strains impact the natural competence.

4.2 Recommendations for future research

A prepublication manuscript found FimT mediated DNA uptake during bacterial transformation in *Legionella pneumophila*, so as described in chapter 2, we are currently evaluationg the DNA-binding of *A. baumannii* FimT.

Since PilE1 and PilE2 show the ability to bind to DNA, additional studies using a combination of structural biology and biochemistry will be used to elucidate the

structural basis and specificity of these DNA binding interactions. We have identified reproducible crystallization conditions for chimeric fusions of PilE1 and PilE2 to Maltose-binding Protein (MBP). Structural studies using X-ray crystallography are ongoing in order to obtain consistent diffraction patterns. Other methods like NMR structure or homology models can be used to further elucidate the structural basis of natural competence in *Acinetobacter*.

The differences of PilA from different *A. baumannii* strains does have an impact on natural competence. We refined the natural transformation assay by the growth time; however, the same growth time still leads to different OD600 value in six samples. To make the natural transformation efficiency more physiologically-relevant, we need to examine conditions beypnd planktonic growth. In addition, *Pseudomonas* shows natural competence only when it is growth in biofilm, which indicate the biofilm affect competence. Hence, it is also worth investigating how biofilm formation affects competence in *Acinetobacter*, specially whether competence occurs in early (microcolony) or late-stage (mature) biofilms.