An Investigation of Natural Transformation Phenotype and Type IV Pilus Protein in *Acinetobacter baylyi* ADP1 Mutants

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Abstract

Natural transformation is one of the mechanisms of horizontal gene transfer, wherein bacteria acquire exogenous DNA and integrate it into their genome. For this process to occur, the bacteria must be competent, meaning they express proteins necessary for DNA uptake and internalization. Type IV pili (T4P) constitute one such protein complex that aids bacteria in twitching motility, enabling them to move through their environment by extending and retracting the pili. Additionally, T4P can bind to and take up exogenous DNA for further internalization, making them a crucial component of natural transformation.

Acinetobacter baylyi ADP1 is known to be highly competent and thus serves as a good model organism for studying genes and proteins necessary for DNA uptake and natural transformation. In this study, three *A. baylyi* mutants created by random transposon insertions were tested for twitching motility and transformation efficiency. All mutants were previously found to exhibit altered twitching motility to varying degrees, leading to the hypothesis that they may also have altered transformation efficiency since both motility and transformation are dependent upon the same molecular structure.

In determining transformation efficiency, mutant cells are cultured in LB broth along with isolated streptomycin resistance DNA. The three mutant strains each exhibit distinct natural transformation phenotypes: lower, equivalent, and higher than the ADP1 wild type. This suggests that the transposon may interrupt different sites in the genome for each mutant, resulting in loss, gain, or neutral function of the competence protein. Transposon Insertion Sequencing could determine the exact location of the interrupted gene. Due to time constraints, sequencing results are still inconclusive, but this lays the groundwork for further experimentation.

To further investigate whether altered transformation efficiency is due to malfunctioning T4P or the mechanism by which bacteria integrate DNA, we examined the presence of ComP, the main protein composing the extracellular part of T4P, using Western blotting. This experiment provided inconclusive results due to the presence of a protein band in the negative control. Alternatively, we also examined the pili structure under a scanning electron microscope, which was observable in all mutants. These results suggest that although T4P may remain physically intact, transposon interruption affects either the DNA integration mechanism in some mutants or the T4P DNA uptake system in others, resulting in altered natural transformation.

This research serves as a good starting point for testing the phenotypes of ADP1 mutants generated through transposon mutagenesis and developing experimental procedures for more conclusive results.

Introduction

Horizontal Gene Transfer

A process in which genetic information is passed down from parents to offspring is called vertical gene transfer (Yutin 2013) and is common in all prokaryotes and eukaryotes species. In bacteria, vertical gene transfer occurs during binary fission which is how bacteria maintain their population. A large population is essential for survival but so is the ability to adapt to the environment and reproduce. Binary fission creates opportunities for mutation which lead to genetic diversity and allows some level of adaptation. However, bacteria have the additional ability of acquiring genetic diversity through a process called horizontal gene transfer enabling them to survive in certain environments.

Horizontal gene transfer (HGT) is a process in which bacteria acquire exogenous genes and integrate them into their own genome (Burmeister 2015). HGT is regarded as the process which drives the evolution of bacteria, as such that this very mechanism is one of the processes which promote resistance towards antibiotics. There are three mechanisms considered as HGT: conjugation, transduction and natural transformation. Conjugation refers to the transmission of genetic material via physical contact with another bacterial cell whereas in transduction genetic material is transferred through bacteriophages. Unlike conjugation or transduction, natural transformation relies on the bacteria's own ability to take up extracellular DNA from their environment (Burmeister 2015). In all three mechanisms, DNA is taken up, processed for various conditions and purposes that will be discussed later, and incorporated into the host's genome. This research focuses on the natural transformation of a bacterium *Acinetobacter baylyi*.

Competence, Transformation and Evolution

Naturally transformable bacteria take up exogenous DNA upon entering a physiological state called competence (Leong et al. 2017). In this state of competence, specific proteins required for DNA internalization and processing are expressed. For instance, a transmembrane channel protein ComEA transports single-stranded DNA (ssDNA) through the inner membrane to the cytosol. Subsequently, the DNA processing A protein (DprA) would load recombinase RecA onto the ssDNA which may promote a homology search in the host chromosome and begin the process of DNA recombination (Johnston et al. 2014). Gram negative and Gram positive bacteria that are known to be naturally transformable encode *ComEA* homologs and phylogenetic analysis shows conservation of a functional *DprA* gene. Another important protein complex is the type IV pilus (T4P) proteins which in many bacterial species mediate natural transformation (Leong et al. 2017). Specific pilin subunits would bind to extracellular DNA and the ATP driven disassembling process would reel it into the cell surface. This mechanism is similar to how bacteria "twitch", which is moving through its environment using the pilus as an anchor. Instead of pulling on the DNA and retracting it, twitching requires bacteria to pull on the weight of its own cell to move forward (Leong et al. 2017). Many bacterial species possess a homolog of a type IV pilus gene but there is variation whether type IV pilus bacteria are responsible for both transformation and/or twitching and whether the kind of DNA that can be taken up by the pilin subunit must be species-specific or not.

Despite the fact that many genes enabling this mechanism are well conserved, the environmental and cellular signals which trigger competence vary among transformable species. In *L. pneumophila* and *S. pneumonia* competence is a response to genotoxic stress such as UV radiation or mitomycin C

(Charpentier et al. 2010). However, while starvation coincides with maximal transformability in *H. influenzae* (Redfield 1993), in *A. baylyi* competence is induced by high nutrient levels (Utnes et al. 2015).

Various signals trigger competence across phylogenetically widely divergent bacterial species, but what is the true evolutionary benefit of this process? Even though the genes involved are conserved, the evolutionary importance of competence and transformation is highly controversial. In fact, competence does not necessarily lead to increased fitness. Hülter et al. showed that an *A. baylyi* mutant with dysfunctional DNA uptake gene has higher relative fitness than wild type under the specific conditions they used to culture the cells (2017). This means that even when competence is compromised, *A. baylyi* still managed to survive and grow slightly better even under genotoxic conditions. Their result suggests that exogenous DNA taken up does not link to how these DNA will be recombined and repair DNA damage but instead is utilized as nutrition or as building block for DNA metabolism. As competence and natural transformation are two evolutionary conserved yet separate processes with clearly different proteins and induction cues involved, it is interesting to further research the underlying cost and advantages of these processes.

Acinetobactar baylyi **as Model Organism**

Acinetobacter species are Gram-negative soil bacteria and were recognized in the taxonomic system in 1984 (Juni 1984) with two classified species *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii* (Skerman et al. 1989). Since then this genus now contains 82 species with a validly published and correct name according to www.bacterio.net; as of January 2024. The *Acinetobacter baylyi* strain in this research is referred to as ADP1 or in some published research referred to its originally described name BD413. As this research mainly focuses on bacterial competence and natural transformation, *A. baylyi* ADP1 is a good candidate in addressing this area of research. According to research by Palmen et al, when using chromosomal DNA as donor DNA, 0.1% of total culture is transformed after 1 hour of incubation (1997) and when using an integrative plasmid as donor DNA, 25-30% of the total culture is transformed (1993). This high transformability reflects nearly full competence of the bacteria (since the bacteria has to first be competent before it can transform). Unlike many bacteria that possess competence during specific development phase such as in *Bacillus subtilis* which enters competence at the onset of stationary phase, *A. baylyi* shows continuous competence throughout its growth phase and early into stationary phase (Bedore et al. 2023). This also results from the selectivity of DNA taken up. *H. Influenza* and species in the family Neisseriaceae selectively take up isogenic DNA (Frye et al. 2013; Mell et al. 2012), *A. baylyi* ADP1 does not discriminate, it transfers DNA of any source into the cytoplasm (Porstendörfer et al. 1997).

Type IV Pili and ComP

Many bacterial species possess filamentous appendages called pili which function in surface adherence along with participating in horizontal gene transfer process. There are five classes of pili in Gram negative bacteria: chaperone/usher (CU) pili, curli, type IV pili, type III secretion needle, and type IV secretion pili—each having their own mechanisms for folding, secretion, and ordered assembly (Waksman and Hultgren 2009). This paper focuses on the type IV pili (T4P) which is found throughout many Gram negative bacterial families including *A. baylyi*. Type IV pili system is composed of five central proteins which are (i) major pilin subunit, (ii) pre pilin peptidase, (iii) an assembly ATPase, (iv) inner membrane core proteins and (v) outer membrane secretin channel (Milville and Craig 2013).

T4P is a dynamic structure that extends and retracts through the process of assembling and disassembling (this movement is referred to as "twitching") when coming into contact with extracellular materials. The pilus assembly mechanism proposed by Craig et al. involves iteratively adding three pilin subunits to the growing filament in the periplasm, forming three helical strands of T4P (3-start helix formation)(2006). This process is aided by ATP hydrolysis through ATPase in the cytoplasm. The disassembly mechanism is reversed, involving retraction ATPase such as PilT or PilU, which will dissociate the subunits (Milville and Craig 2013). Furthermore, a positively charged groove is observed during the 3-start helix formation, wide enough to bind the negatively charged backbone of dsDNA, contributing to the role of T4P in DNA uptake and leading to natural transformation (Craig et al. 2006). This positively charged groove was proposed for *Pseudomonas* T4P, which binds and takes up DNA without sequence specificity (van Schaik et al. 2005). This could also be a plausible explanation for how *A. baylyi* DNA uptake is also non specific.

Upon closer examination of *A. baylyi ADP1*, a study conducted by Leong et al. has constructed a model of T4P with associated competence and signal transduction proteins and further identified genes in T4P that are involved in twitching motility and/or natural transformation (2017). Core proteins found to be required for both processes are PilC, ComM, ComQ, PilF, PilU, PilT, ComP, ComB and PilX (Leong et al. 2017). In multiple studies and literature addressing the competence and natural transformation of *A. baylyi*, ComP has garnered significant attention for its crucial role in DNA binding and uptake. ComP is described as a pilin-like competence factor present in the cytoplasmic membrane, and the outer membrane size varies depending on the stage of formation (unmodified 15-kDa, glycosylated 20-kDa, and final modification 23-kDa) (Porstendörfer et al. 2000). Due to its similarity in amino acid sequence compared to other major pilin proteins such as PilE of *N. gonorrhoeae* and PilA of *P. aeruginosa*, ComP is considered closely related and structurally similar to T4P (Porstendörfer et al. 1997). Experiments showed that the *Acinetobacter* sp strain BD413 mutant (T205) exhibited a defective DNA binding and transformation deficiency phenotype due to the loss of operon which contains *comP* gene (Porstendörfer et al. 1997). This finding was confirmed again by Leong et al. demonstrating that the null mutation of *comP* results in defects in twitching motility and a loss in transformation efficiency (2017).

Transposon Mutagenesis

Transposon or transposable elements (TEs) are DNA sequences that can move from one location in the genome to another (Pray 2008). TEs are also known as jumping genes and were discovered in almost all organisms, both prokaryote and eukaryote typically in large numbers. In humans, TEs made up approximately 50% of the human genome and 90% of the maize genome (SanMiguel, 1996). There are two primary types of TEs: retrotransposons and DNA transposons. Retrotransposons rely on the reverse transcriptase enzyme for transposition, while DNA transposons encode an enzyme transposase, allowing them to autonomously remove and insert themselves into a new location (Pray 2008). TEs can generate various genetic alterations and thus have been used as genetic tools to study and analyze gene function and regulation, one most relevant to this research is transposon insertion sequencing.

In the context of insertional transposon mutagenesis, transposon insertion sequencing (TIS) utilizes high throughput sequencing to generate statistical data on the essentiality of each genetic component in the genome (Cain et al. 2020). Transposon sequencing can also be used to determine fitness conferred by single genes and to map genetic interaction in microorganisms. (van Opijnen et al. 2009). In bacteria, the insertion of transposons (with known genetic components) disrupts the genome at random sites, creating a pool of mutants. These mutants are subsequently grown under selective conditions of interest, such as in antibiotic media. High-throughput sequencing is then performed to determine the location in the genome where a transposon is inserted and the frequency of insertion into a particular region. This information reveals which gene is responsible for growth, antibiotic resistance, and other traits depending on the conditions of interest. The most often used method for transposon to be delivered into the genome is by utilizing integrative plasmid which will deliver both transposon and transposase gene into the target genome (Gallagher et al. 2007). There are two main types of transposon for generating the mutants: mariner-based transposons and Tn5-based vectors. Mariner-based transposon targets thymine-adenine (TA) dinucleotides (Chiang and Robin 2002) as an insertion site where Tn5 inserts randomly but does have preference for high GC content regions (Chao et al. 2016). Both types of transposons can be used depending on what model organism is working with and what kind of statistical data is expected. For this research we used a Tn5 type called T26 which has a tetracycline resistance marker that works in ADP1.

The application of transposons has proven to be highly promising in the study of bacterial genetics. While cutting-edge genome editing tools like CRISPR can induce mutations at the targeted region of interest, they involve a lengthy process of design, synthesis, and cloning (Cain et al. 2020). The strength of TIS lies in the simplicity of transposon generation and insertion without the need for prior knowledge of an organism's genetic makeup. The randomness of insertional transposon mutagenesis also allows for the discovery of new genes. Although transposon technology is yet to be fully developed and tested on organisms with greater genetic complexity, it stands out as a potent genetic tool in both the present and the future.

Research Questions

1. Three ADP1 mutants (CCL2508, CCL2511 and CCL2514) are known to exhibit altered twitching motility. The first experiment aims to test whether they also exhibit altered transformation efficiency since both processes (twitching and transforming) depend on the type IV pilus.
2. Two different mechanisms play an important role in *A. baylyi* transformation; DNA uptake via type IV pili and DNA recombination. ComP is a protein composing the extracellular part of the type IV pili and is necessary for DNA uptake. This follow up research aims to test whether transformation efficiency of ADP1 mutants is associated with the DNA uptake mechanism and its key extracellular protein ComP?

Methodology

Table 1. List of ADP1 strains used in this experiment

CCL Number	Original Name	Background	Phenotype	Date Frozen Down
2055	n/a	n/a	ADP1 Wild Type	7/9/2014
1011	n/a	n/a	$streptomycin^R$	3/13/2008
2129	n/a	CCL2055	ComP tag + $trimethoprim^R$	10/7/2014
2508	AM.12.11-B-11	n/a	ADP1 Tn :: $tetracycline^R$	4/24/2023
2511	AM.12.11-B-8	n/a	ADP1 Tn :: $tetracycline^R$	4/24/2023
2514	AM.12.07-B-17	n/a	ADP1 Tn :: $tetracycline^R$	4/24/2023
2521	n/a	CCL2508	ComP tag + $trimethoprim^R$ + Tn::$tetracycline^R$	12/10/2023
2522	n/a	CCL2511	ComP tag + $trimethoprim^R$ + Tn::$tetracycline^R$	12/10/2023
2523	n/a	CCL2514	ComP tag + $trimethoprim^R$ + Tn::$tetracycline^R$	12/10/2023

Solution Preparation

Antibiotic stock solution

Tetracycline (tet) stock solution: 5 mg/mL in 95-100% ethanol. Vortex to dissolve, wrapped in thin foil and stored at -20C.

Streptomycin (str20) stock solution: 20 mg/mL in water, stored at 4 C.

Trimethoprim (Trm) stock solution: 20mg/mL in DMSO, stored at 4C.

LB and LB agar

Luria Bertani media (LB) was prepared using 10 g tryptone (US Biological Life Sciences), 5 g yeast extract (BD and later US Biological Life Sciences yeast extract), and 10 g NaCl (Fisher Scientific) per liter of water. Autoclaved the solution within an hour. LB agar was prepared by adding 7.5 g of bacto agar per 500mL of LB, autoclaved within an hour. Tetracycline, streptomycin and trimethoprim were added to the LB agar after cooling down to 55 C with the final concentration of 5 µg/ml, 20 µg/mL and 20 ug/mL respectively.

Antibiotic Culture Broth

LB-tet: 25 mL plain LB and 25 µL of tetracycline stock solution.

LB-str20: 15 mL plain LB and 15 µL of streptomycin stock solution.

DNA Isolation

Streak plate of CCL1011 cells on LB-str20 was incubated overnight. Used a single colony to inoculate in 2 mL LB-str20 broth. Collect cell pellets by microcentrifuge at maximum speed for 10 minutes. Discarded supernatants and resuspend in 1 mL PBS. Extracted DNA using 500 µL phenol-chloroform-isoamyl alcohol. Alternatively, Qiagen DNeasy Kit was also used to extract chromosomal DNA from ADP1 mutant strains (see appendix 2: Laboratory protocol). Tested for DNA concentration and purity using the nanodrop.

<u>Transformation Experiment</u>

Transformation Puddle

Streaked plates of CCL2055, CCL2508, CCL2511 and CCL2514 and grew colonies overnight at 37 C. Obtained a single colony from each plate and grew overnight culture in 2 mL LB-tet broth (for the mutants) and 2 mL plain LB broth (for the WT) at 37 C in the shaker. To 50 µL of each cell culture, added 5 uL of isolated str^R DNA (CCL1011). Resuspended the solution by pipetting several times and then transferred a total of 50 µL of cell culture to an LB plate, creating a “puddle”. Incubated the plate for 10-12 hours at 37 C.

Determination of Efficiency

Prepared 750uL PBS in each sterile microfuge tube and used P1000 to scrape out the “puddle” into each tube. Used a 96-well plate to do a ten-fold dilution series using 90uL PBS and 10uL cells. Plated out 2 individual droplets of 10 ul of each dilution on 1/8 sections on both a plain LB and LB-str20 plate. Incubated the plates overnight at 37 C. Transformation efficiency was calculated as a ratio of transformed cells/mL (strR) to total cells/mL (Plain LB; see appendix 3 calculation). Transformation efficiency of the mutants were compared to that of the wild type.

<u>PCR and Gel Electrophoresis</u>

DNA was isolated from each mutant and tested for concentration and purity using the nanodrop. DNA was stored at -20 C. PCR round 1 and 2 master mixes (see Appendix 1: Recipe) were prepared and kept at -20 C. For PCR round 1, 1 µL of DNA and 49 µL of the master mix were combined (total volume is 50 µL). For PCR round 2, 3 µL of the round 1 product and 47 µL of the master mix were used (total volume is 50 µL). PCR thermocycler settings referred from Gallagher et al, 2015. Stored the PCR round 2 product at -20 C.

Prepared an agarose gel for electrophoresis by dissolving 5 g agarose in a 50 mL 1X TAE buffer in a microwave, added 5 µL of GelRed and poured the mixture into a casted gel tray. Once the gel solidified, filled the gel tank with 1X TAE buffer. Mixed 10 µL PCR round 2 product with 2 µL loading dye. Loaded 10 µL running samples into each well and 10 µL Quick-Load® Purple 2-Log DNA ladder as a reference in a separate well. The gel was run at 80 Volt for 1.5 hours. Viewed the gel picture using iBright Nucleic acid scan.

ComP Expression experiment

Transformation of strains

Streaked plate of CCL2129 and grew colonies overnight at 37 C. Used a single colony to inoculate in 2 mL LB broth at 37 C in the shaker. 3 overnight cultures of CCL2129 were made. To each of 50 µL cultures added 5 µL DNA of CCL2508, CCL2511 and CCL2514 respectively. Resuspended the solution several times with pipette and then transferred a total of 50 µL of cell culture to an LB plate, creating a "puddle". Incubated the puddle for 10-12 hours at 37 C.

Selecting transformed cells through dilution series

Prepared 750 µL PBS in each sterile microfuge tube and use P1000 to scrape out the "puddle" into each tube. Use a 96-well plate to do a ten-fold dilution in sterile PBS, 90 µL PBS and 10 µL cells. Created 8 sections on the LB-Trm + tet plate and plated the ten-fold dilution 2 of 10 µL droplets into each section. Incubated the plates overnight at 37 C. Single colony growing on the most diluted section on the plate is selected and streaked out in a new LB-Trm + tet plate. Incubated the plate overnight. This process was repeated one more time by selecting a single colony in the previous plate and streaking out in the new LB-Trm + tet plate.

Cryogenic Cell Preservation

Prepared LB-20% glycerol and cryovials for each new transformant. Pipetted 1.5 mL of LB-20% glycerol into each cryovial and used a sterile long-stem Q tip to scrape cells into the cryovial. Labeled the cryovial with the appropriate transformants order (CCL2521, CCL2522 and CCL2523). Inserted into the appropriate freezer box in numerical order and froze down at -70 C.

Measuring cell density by spectrophotometry

Streaked out mutants CCL2521, CCL2522 and CCL2523 on the LB-Trm + tet plate and grew overnight at 37 C. Inoculated single colonies in 2 mL LB broth in the shaker at 37 C. Obtained 50 µL of each culture to create "puddle" on the LB plate and incubate overnight. Prepared a sterile microfuge tube with 1000 µL PBS. Used P1000 to scrape out the "puddle" into the PBS and vortex. Made a 1:6 dilution by adding 200 µL of cells and 1000 µL of PBS in each cuvette and placed into a spectrophotometer. Measured at wavelength 600 nm. Once obtained the absorbance value, calculated (see appendix 3: Calculation) the amount of cells and PBS needed to equalize the cell density of all samples.

SDS-PAGE sample preparation

Cell pellets were collected by microcentrifuge at maximum speed, and supernatant was discarded. Resuspended the pellet with 40 µL PBS and added 20 µL of dark blue 3X SDS-PAGE loading dye. Samples were heated at 100 C for 10 minutes. Two Bio-Rad gels were used to run SDS-PAGE, one was used in Western Blotting, the other for Coomassie Blue Staining. Protein lysates were separated at 100 Volt for 90 minutes in a Carboy 1x SDS running buffer.

Western blot
The separated peptides on the gel were transferred to a PVDF membrane overnight in the cold room at 30 Volt. Western blot was performed using the reagents from the Pierce™ Fast Western Blot Kit, ECL Substrate. First the anti-strii primary antibody was used to incubate. Subsequently the antibody was revealed on the PVDF membrane with the HRP reagents following manufacturer's protocol (see appendix 1: Recipe). The gel was viewed on the iBright with the ChemiBlot scan. Coomassie Blue staining was performed with the second SDS-PAGE gel. The gel was placed in a plastic container and Coomassie Blue solution was added enough to cover it. Placed the plastic container on the rocker overnight. Destaining solution was used to wash off excess Coomassie Blue, the wash was repeated several times in a span of 2 hours to obtain a clear image.

Scanning Electron Microscopy (SEM)
Isolated 2-3 single colonies from each mutant culture plate with sterile loop and mixed the cells with PBS in a microfuge tube. On a cover slip, placed 1-2 droplets of poly-L-lysine and spread out using a pipette, then placed 1 droplet of the cell solution and spread out using a pipette. Specimen fixation was done through a series of solution incubation, starting from 2.5% glutaraldehyde, PBS, and ethanol (50%, 70%, 85%, 95% and 100% respectively). Put the slip through the critical point dryer and afterward through sputter coating. Cover slip was attached to a stub with double sided tape. Painted the underneath area and margin of the cover slip with graphite conductive adhesive. When ready to use, load the stub on the SEM stage and set the machine to begin the scanning.

Results: Raw Data

Table 2. ADP1 strains twitching ratio calculated by previous students

	Trial 1	Trial 2	Average
CCL2508	0.892	0.779	0.835
CCL2511	0.828	0.811	0.819
CCL2514	0.490	0.340	0.415
comP:: kan negative control	0.281	0.230	0.255

Table 3. ADP1 strains transformation efficiency calculation trial 1

Strains	Plain LB Dilution Plate			Str20+LB Dilution Plate			Transformation Efficiency
	Colony counted /20 µL	Dilution Factor	# total cells/ mL	Colony counted /20 µL	Dilution Factor	# transformed cells/ mL	
CCL2055	44/20	10^7	2.2×10^{10}	23/20	10^7	1.15×10^{10}	0.5227
CCL2508	6/20	10^7	3.0×10^9	9/20	10^0	4.5×10^3	1.5×10^{-7}
CCL2511	14/20	10^7	7.0×10^9	2/20	10^5	1.0×10^7	0.001428
CCL2514	5/20	10^7	$2.5 \text{ X } 10^9$	33/20	10^6	1.65×10^9	0.660

Table 4. ADP1 strains transformation efficiency calculation trial 2

Strains	Plain LB Dilution Plate			Str20+LB Dilution Plate			Transformation Efficiency
	Colony counted /20 µL	Dilution Factor	# total cells/ mL	Colony counted /20 µL	Dilution Factor	# transformed cells/ mL	
CCL2055	6/20	10^7	3×10^9	2/20	10^7	1×10^9	0.333
CCL2508	3/20	10^5	1.5×10^7	9/20	10^0	4.5×10^3	3×10^{-4}
CCL2511	24/20	10^5	1.2×10^8	39/20	10^4	1.95×10^7	0.1625
CCL2514	12/20	10^7	6×10^9	4/20	10^7	2×10^9	0.333

Table 5. ADP1 strains transformation efficiency (Data from Block 7)

Strains	Plain LB Dilution Plate			Str20+LB Dilution Plate			Transformation Efficiency
	Colony counted /20 µL	Dilution Factor	# total cells/ mL	Colony counted /20 µL	Dilution Factor	# transformed cells/ mL	
CCL2055	32/20	10^7	3.25×10^{10}	11/20	10^4	1.15×10^7	3.54×10^{-4}
CCL2508	29/20	10^7	2.95×10^{10}	25/20	10^3	2.55×10^6	8.644×10^{-5}
CCL2511	45/20	10^7	4.50×10^{10}	11/20	10^4	1.15×10^7	2.56×10^{-4}
CCL2514	12/20	10^7	6×10^9	6/20	10^7	2×10^9	0.333

Table 6. OD600 Absorbance for Western blot samples trial 1

	OD 600 of 1:6 Dilution	Actual OD600	Fd	Vs (µL)	PBS Added (µL)
CCL2129	1.102 A	6.612	6.612	151.24	848.76
CCL2521	0.746 A	4.476	4.476	223.41	776.59
CCL2522	1.105 A	6.630	6.630	150.83	849.17
CCL2523	1.221 A	7.326	7.326	136.50	863.50

Table 7. OD600 Absorbance for Western blot samples trial 2

	OD 600 of 1:6 Dilution	Actual OD600	Fd	Vs (µL)	PBS Added (µL)
CCL2129	0.963 A	5.778	5.778	173.07	826.93
CCL2521	0.665 A	3.99	3.99	250.62	749.38
CCL2522	1.036 A	6.216	6.216	160.87	839.13
CCL2523	1.084 A	6.504	6.504	153.75	846.25
CCL2055	1.065 A	6.39	6.39	156.49	843.51

Results

ADP1 mutants exhibit altered transformation efficiency

Bacterial transformation is one of the horizontal gene transfer mechanisms in which exogenous DNA is integrated into the host genome. This process is believed to promote antibiotic resistance in bacteria since it enables them to adapt and survive under a genotoxic environment. A Gram negative soil bacterium, *A. baylyi* ADP1's transformation efficiency is regarded as one of the highest and thus a good candidate for studying genes and proteins involved in this mechanism. In this study, I selected three ADP1 mutants (Table 1) which were generated through transposon to test for their transformation efficiency in comparison to the ADP1 wild type. Prior to this study, these mutants were tested for their twitching motility (Table 2), which is the ability of a bacteria to extend and retract their extracellular type IV pilus to move through its environment. According to the result, all ADP1 mutants have altered twitching motility with CCL2514 being more severely affected than the other two. Regardless, all mutants are able to twitch to some degree and their ratio is higher than the comP::kan negative control which lacks the ability to twitch. Altered twitching motility leads to the hypothesis that they would possess altered transformation efficiency as well since this mechanism also requires functional type IV pilus. Mutants were streaked on a LB-tet plate in order to select for cells with tetracycline resistance. Cells are then cultured in a plain LB broth with the addition of CCL1011 isolated DNA which possess streptomycin resistance. The cell mixture was grown in a puddle and incubated overnight at 37 C. Then the puddle was scraped into sterile PBS and subsequently diluted up to 10^7 fold in a 96-well plate dilution series. Diluted cells are placed in a sectioned plain LB and LB-str20 plate ranging from 10^0 dilution to 10^7 dilution (Figure 1). Transformation efficiency was calculated by (transformed cells/mL)/(total cells/mL), transformed cells are obtained from the Str20 LB plate and total cells from the plain LB plate (Table 4,5 and 6).

Two trials of transformation efficiency were performed (Table 3, 4 and Figure 2). This recent data and the data from Block 7, 2023 (Table 5) is combined and averaged (Figure 3). CCL2508 transformation efficiency is almost undetectable with the lowest value of 1.5×10^{-7} . CCL2511 exhibited a comparable transformation efficiency to those of the WT in block 7 last year, but the more recent trials show that it has a transformation efficiency lower than wild type. Interestingly, CCL2514 exhibits higher transformation efficiency than those of WT in all three experiments. The difference between the transformation efficiency measured in Block 7 and the more recent trials could be due to the laboratory condition, diluting solution used (we used saline for Block 7 experiment and PBS for trial 1 and 2) and potentially storage conditions that could differ and impact the quality of the sample.

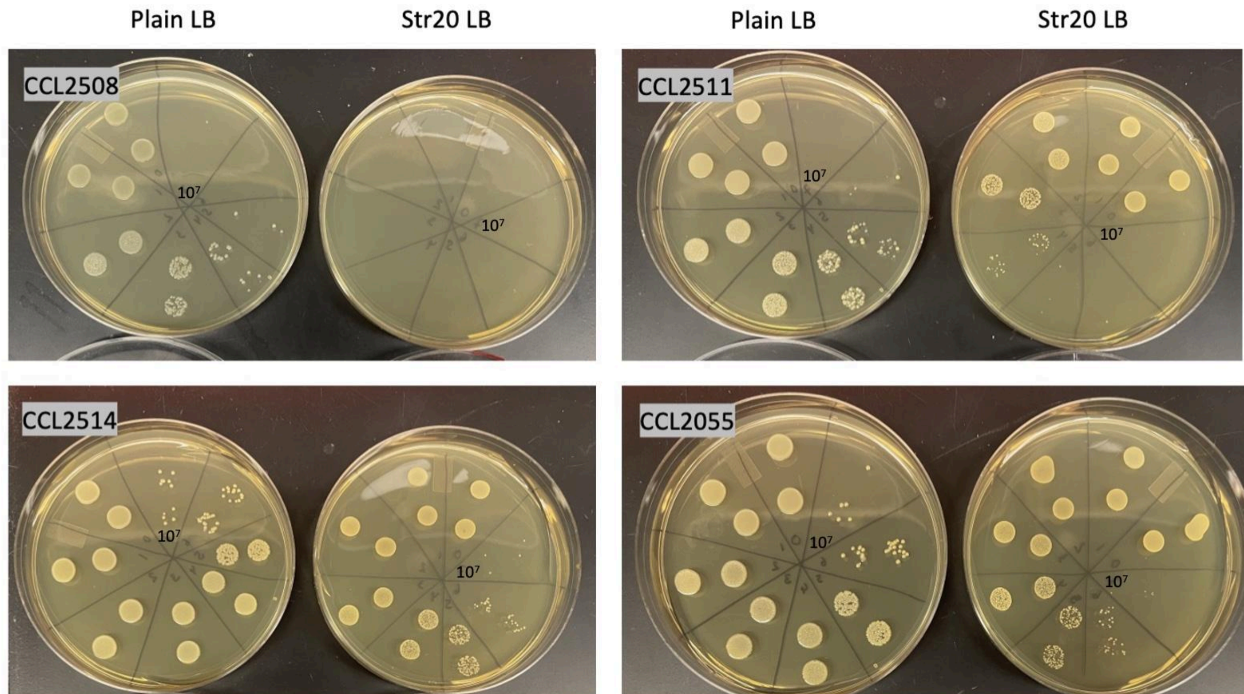

Figure 1. Diluted cells are placed onto both sectioned plain LB and Str20 LB plate
Presence of bacterial colonies decreases as the dilution factor increases for all plates. The colonies are able to grow in a higher dilution factor on a plain LB plate than on the Str20 LB plate. However, those that are able to grow in a high dilution factor on a Str20 plate possess the streptomycin antibiotic resistance phenotype which confirms that transformation is successful. This can be seen for CCL2514 and the CCL2055 (WT). Colonies growth of CCL2508 is the least compared to the other mutants.

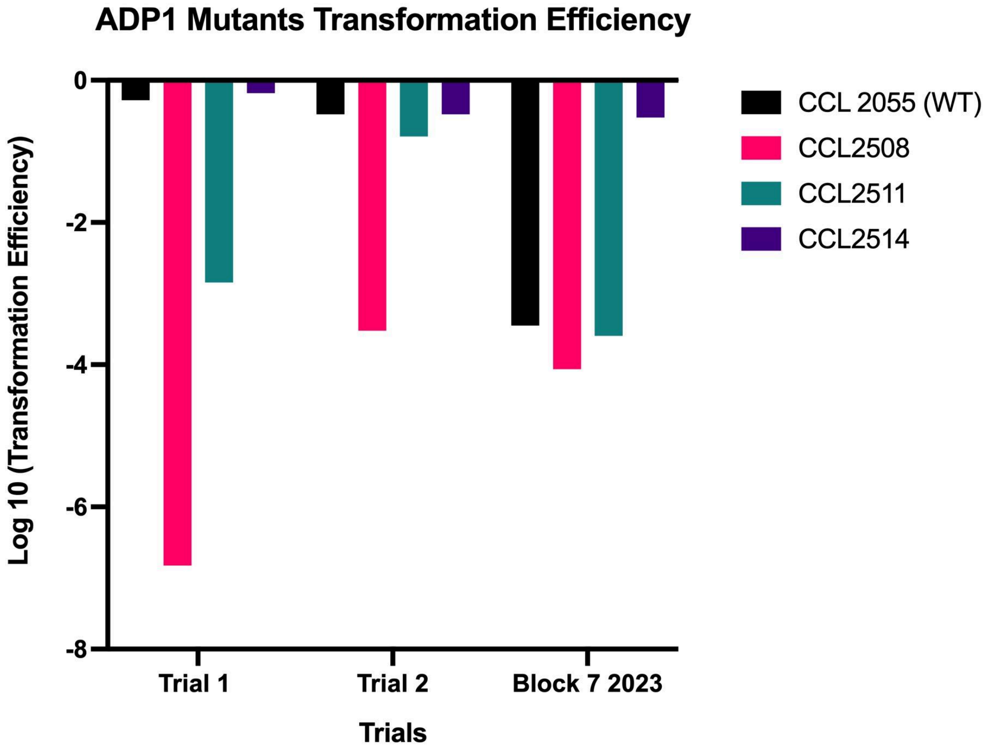

Figure 2. Transformation efficiencies of ADP1 mutants in comparison to the ADP1 WT
Calculated transformation efficiency is plotted using a log-10 scale. Results from trial 1 and 2 exhibit similar transformation phenotypes where CCL2508 has dramatically lower efficiencies than WT. Data from Block 7 2023 which was done 7 months earlier shows that WT, CCL2508 and CCL2511 have almost comparable transformation efficiencies where CCL2514 still possess higher transformation efficiency in all three experiments.

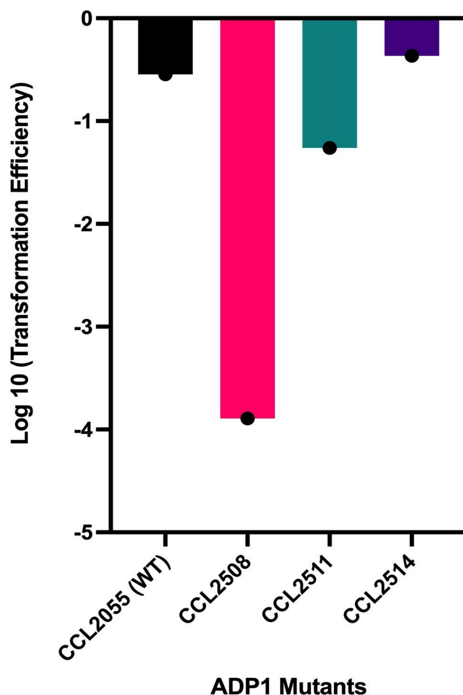

Figure 3. Average transformation efficiencies of ADP1 mutants in comparison to the ADP1 WT Average transformation efficiency of trial 1, 2 and data from Block 7. CCL2508 is approximately 10^4 times less transformable than WT whereas CCL2514 is approximately 5 times more transformale than WT. CCL2511 has relatively comparable transformation efficiency with approximately 5 times lower than WT.

Smears are observed after PCR of ADP1 mutants DNA

The location of transposon insertion in the ADP1 mutants' genome can be determined through a DNA sequencing method known as Transposon Insertion Sequencing (TIS). Prior to this procedure, DNA from each mutant must be isolated and purified. This procedure is done through the use of the Qiagen DNeasy Kit (see DNA isolation). The procedure uses Polymerase Chain Reaction (PCR) with primers designed to amplify the sequence containing the T26 transposon (see PCR methodology). A successful PCR would show some discrete bands and a smear at least 500 bp long. The presence of a smear is highly possible due to the use of degenerate primers (Table 8). Degenerate primers are used during PCR round 1 with the hope that they would bind to the unknown DNA sequence adjacent to the transposon. A well designed PCR mix and thermocycler protocol would be able to bring out discrete bands amongst the smear. Our result is in Figure 4. All the smears with the size approximately between ~0.8 kb to ~2.0 kb are observed in all samples with some slight highlights of the band present among the smearing. These highlights are not strong enough to be considered discrete bands. The presence of dimerized primers at the bottom of the gel indicates that most primers are unused and hence did not bind effectively to the transposon and the adjacent DNA sequence. Overall, this gel electrophoresis scan suggests improvements in PCR mix and thermocycler protocol for a better discrete band result.

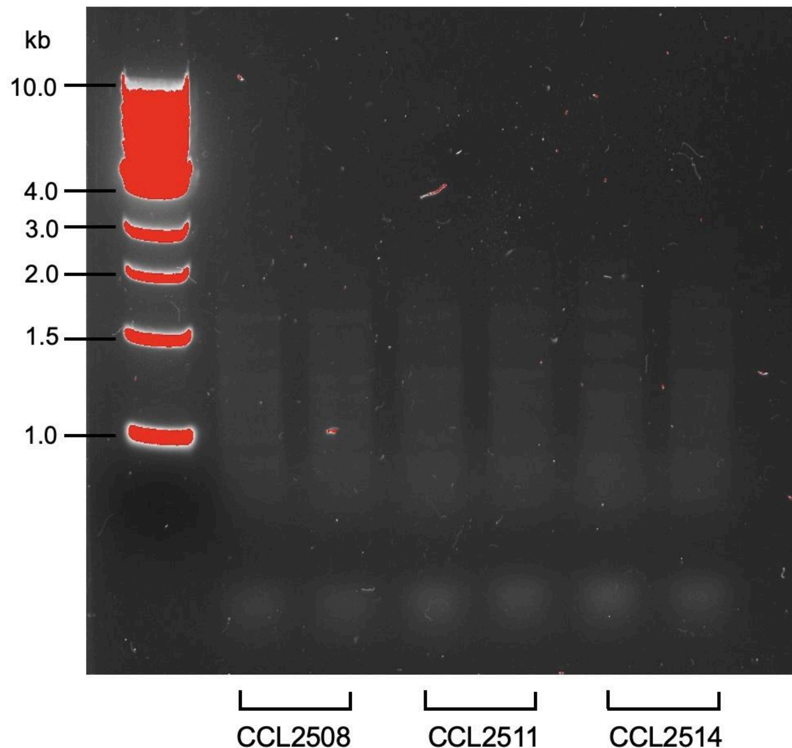

Figure 4. Smears are observed after PCR of ADP1 mutants DNA
DNA ladder (lane 1) and ADP1 mutant DNA (lane 2-7) were loaded in a 1% agarose gel electrophoresis. Two samples of each mutant were loaded accordingly, 2508 (lane 2 & 3), 2511 (lane 4 & 5) and 2514 (lane 6 & 7). Smears from ~ 0.8 kb to ~ 2.0 kb are present in all samples as well as dimerized primers at much less than 0.5 kb near the bottom of the gel. Negative control (WT) was not included in PCR nor in this scan.

Western Blot test for the presence of ComP in a transformed CCL2129 provides inconclusive result

Additionally, we aimed to determine whether the ComP expression is normal in the mutants. ComP is the most abundant pilin (Porstendörfer et al. 1997) in ADP1. ComP differs in size depending on the states of covalent modification options which are: unmodified 15-kDa, glycosylated 20-kDa, and final modification 23-kDa (Porstendörfer, 2000). To do this, new strains were created by transforming the mutant's DNA into wild type ADP1, CCL2129. We looked for the consequence that could potentially affect the synthesis of ComP protein in the transformant. CCL2129 has an epitope-tagged *comP-strii* background and has a trimethoprim resistance phenotype. We performed a 10 fold dilution on the transformed cells and grew them in a Tet+Trm LB plate. Cells were subjected to SDS-PAGE analysis followed by Western blotting against the str-ii tag. In examining the Coomassie stain, all protein samples are present in the correct location with every lane containing relatively similar protein abundance. In the first western blot performed we did not add a blot reducing agent (β-mercaptoethanol or dithiothreitol) when preparing the protein lysate. Blot reducing agent has the ability to break the disulfide bonds that may still exist in the protein and to keep the proteins in their unfolded (not cross-linked) state. Two

protein bands, at 70kDa and at 15kDa are detected in all samples (figure 5B). A second western blot is carried out and β-mercaptoethanol is added to the protein lysate. Additionally we added CCL2055 which is an ADP1 wild type without str-ii tag as a negative control sample. We expect an empty lane from CCL2055 since the primary antibody should not be able to detect and bind to the tag. However, bands are detected for all samples including 2055, at 70 kDa and slightly visible at 15 kDa (fig 6B). Thus, the blots are inconclusive; apparently the primary antibody is staining a protein found in all the cells, including the wild type that do not have tagged ComP.

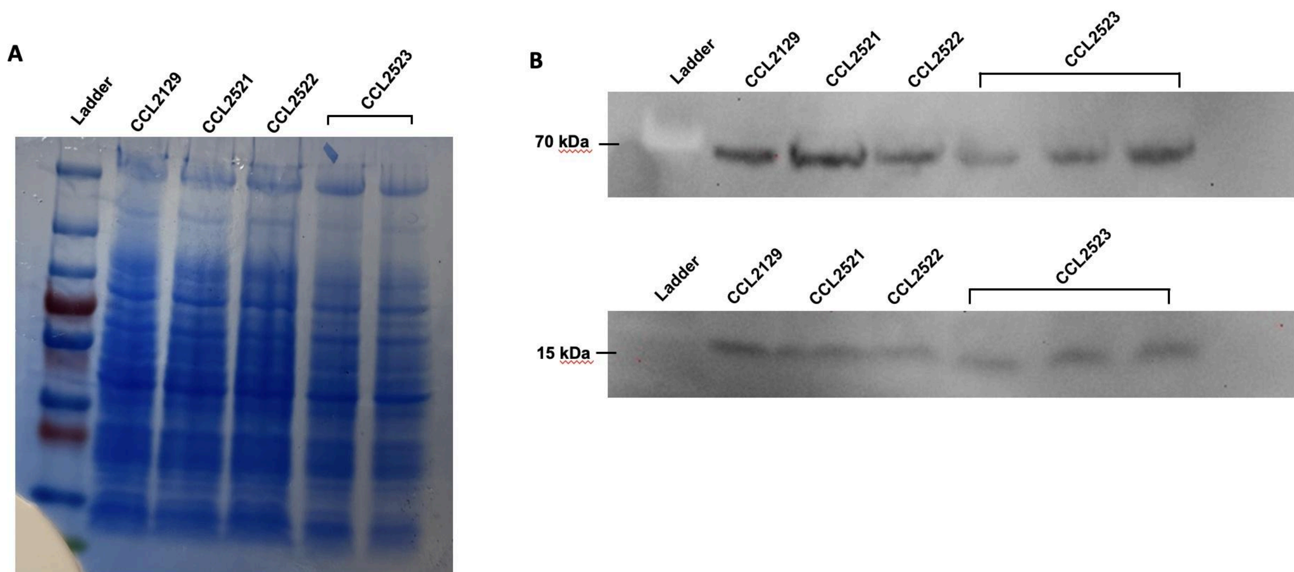

Figure 5. ComP observed in all round 1 (without reducing agent) Western blot samples
Coomassie Blue stain and Western blot membrane viewed from the iBright scan. 5A The purpose of Coomassie Blue in this experiment is to ensure proper loading and protein migration. 5B Protein bands detected on the membrane for all samples (lane 2 CCL2129, lane 3 CCL2521, lane 4 CCL2522, lane 5,6 and 7 are CCL2523) at 70kDa and 15kDa. Negative control (WT) was not included in the Coomassie Blue Stain nor the Western blot membrane.

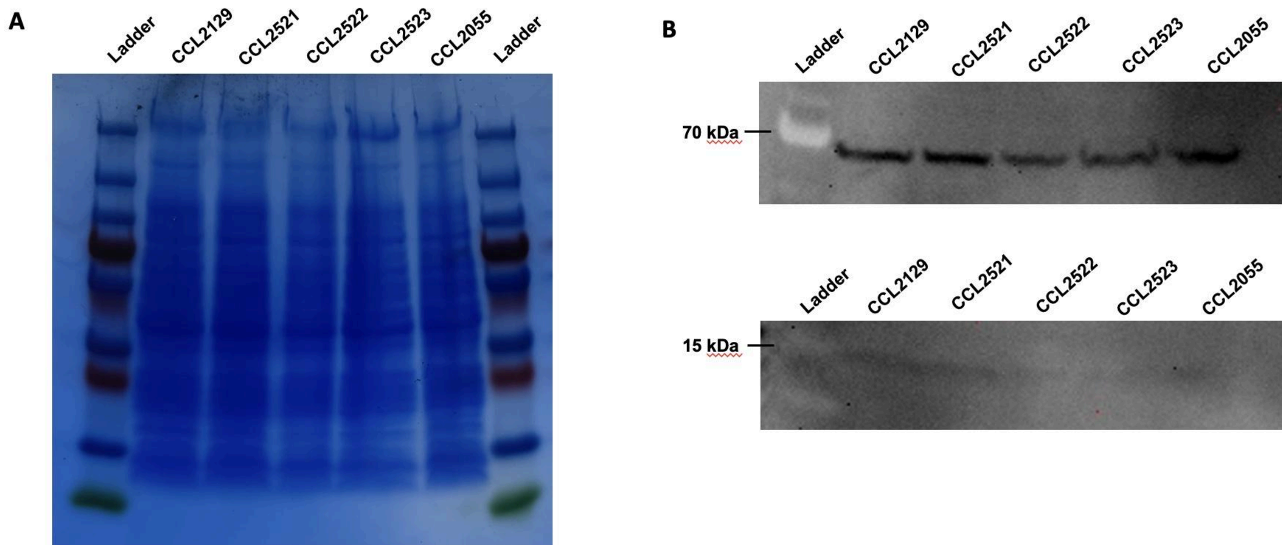

Figure 6. ComP observed in all round 2 (with reducing agent) Western blot samples
Coomassie Blue stain and western blot membrane viewed from the iBright scan. 6A The purpose of Coomassie Blue in this experiment is to ensure proper loading and protein migration. 6B Protein bands detected on the membrane for all samples (lane 2 CCL2129, lane 3 CCL2521, lane 4 CCL2522, lane 5 CCL2523 and lane 6 CCL2055) at 70 kDa and slightly at 15 kDa . CCL2055 is used as a WT control in which it does not have the str-ii tag.

Pili structures in all ADP1 mutant are visible under SEM

Lastly, we carried out a brief side-project to look at the bacteria and pili structures in all ADP1 mutants to get a general idea of the morphology such as size, length and their interactions. Mutant cells are streaked on the plain LB plate and incubate overnight at 37. 2-3 single colonies are scraped out and kept in the PBS solution. No extra solution or DNA are added to the solution prior to the TEM sample preparation procedure. Under SEM, at the magnification of 3,000X many small white clusters are observed. By increasing the magnification to be at least 10,000X, thousands of bacterial cells are seen to be closely packed together forming a big clump. Pili was not observable within the clump therefore we had to look for a very loosely packed cluster to be able to observe the cell individually. A bean-like structure is the most common feature observed with some variations in length and width. For example, a cell in 7A is 1.56 µm long and 0.42 µm wide whereas the cell in 7D is 2.8 µm long and 0.43 µm wide. With a loosely clustered cell, pili are seen as a long, thin fiber-like structure connecting at least 2 cells together. Overall, pili seemed to have a longer length than the cell body and it is quite hard to tell where it originates since multiple cells and pili are stacking on each other. We were able to measure the length of pili in 7A (1.67 µm) and a portion in 7C (1.56 µm) as they are not extensively intertwined with other pili. Interestingly, pili in 7C do not only connect the two cells together but also branch out and create their own

complex of pili. In estimating the total length of the pili in 7C, it is possible that they are 3-4 times longer than the cell body. Pili do not protrude out of every cell, there are cells that do not possess pili at all and some with a very minimal protrusion. Those without pili do not share physical contact with other cells and are seen to be existing on their own.

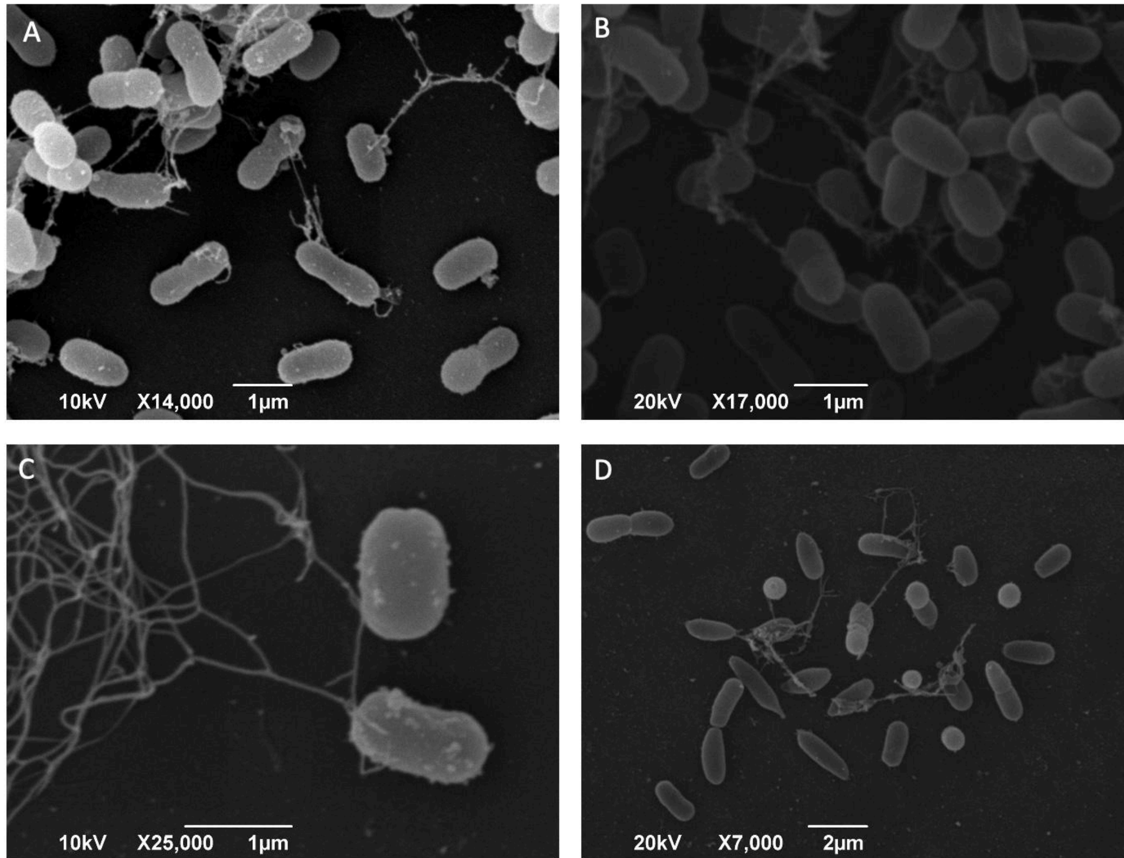

Figure 7. Pili structure observed under SEM for all mutants
Fiber-like structure adhering bacteria in clusters are observed under various magnifications from 7,000X to 25,000X. A is CCL2508, B is CCL 2511, C and D are CCL2514. The approximate length of the bacterial cell in all samples is from 1.5μm to 3.0 μm whereas the pili structure extended 3-4 times longer than the cell body. In A and B, bacteria and pili staggered on top of each other whereas C and D showed clear separation of each cell.

Discussion

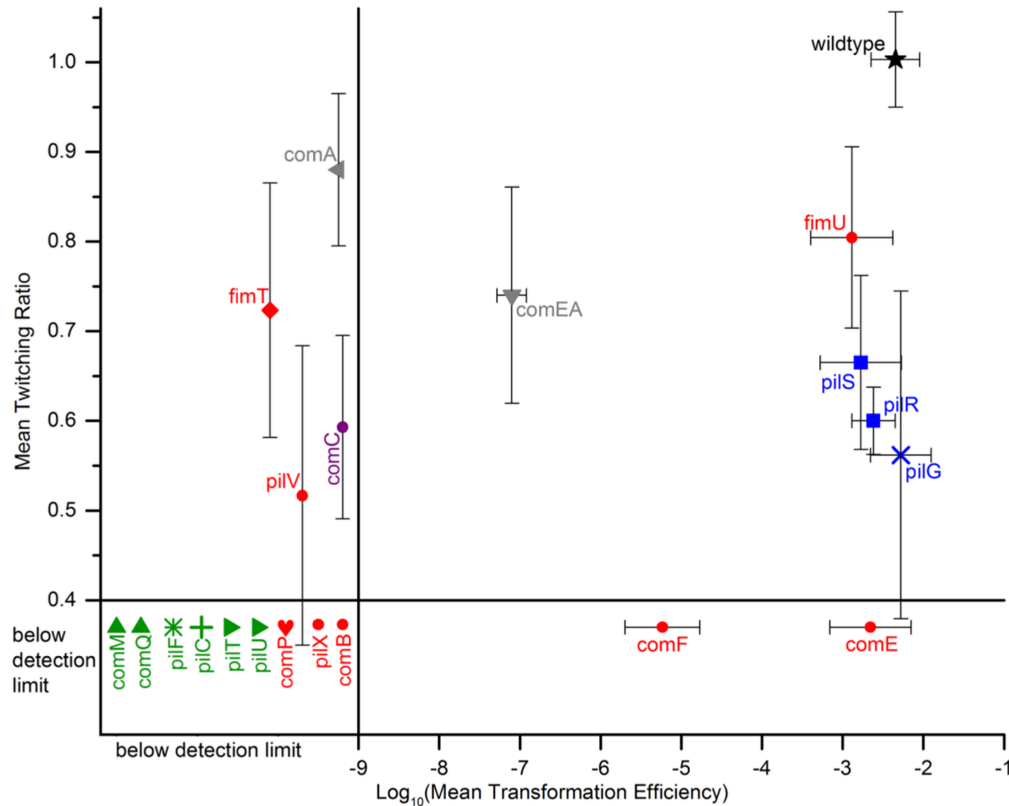

Figure 8. Competence and twitching phenotypes of ADP1 null mutations, Leong et al. 2017

Altered transformation efficiency is unlikely due to ComP gene interruption

In answering the research questions, the result has shown us that mutants with altered twitching motility result in 67% (2 out of 3) altered natural transformation efficiency. As T4P is responsible for both mechanisms, we can speculate that the transposon insertion could possibly interrupt genes related to T4P function and/or structure. However, looking closely at the altered phenotype observed in these mutants, we can notice a wide range of variation. Firstly, the twitching motility in mutants is not completely lost. CCL2508 and CCL2511 twitching ratio is 4 times more than the *comP::kan* negative control and CCL2514 ratio is twice higher. Secondly, the observed natural transformation phenotype of each mutant appears to be incongruent with their respective twitching motility alterations. For instance, CCL2508, despite exhibiting a relatively high twitching ratio, demonstrates an almost complete loss of the transformation ability, whereas CCL2514, characterized by a notably low twitching ratio, exhibits a transformation efficiency surpassing that of the wild type. It is very likely that transposon interruption occurs at different sites in each mutant. In CCL2508 where DNA integration process could potentially be affected, the mutation might cause the loss/partial loss of function for comEA which is a DNA transport protein. According to research done by Leong et al (Figure 8), a *comEA* knockout mutant greatly lost the ability to transform (~ 10^{-7} cells transformed) but possessed a twitching ratio of ~0.75. On the other hand, CCL2514 seems to have defects in pili structure and function which did not prevent DNA integration.

Candidate protein that could be affected is comE which is a pilin protein essential for twitching. Compared to Leong's result in *comE* knockout mutant which found twitching ratio to be less than 0.4 and have ~10^{-3} cells transformed, CCL2514 is somewhat similar phenotypically but more trials need to be done for a quantitative result and comparison. This could suggest that comE is partially knocked down in CCL2514 and the transposon interruption might also cause the enhancement in function of DNA processing protein which might or might not have been already discovered in *A. baylyi*. CCL2511 is the only mutant that maintains a relatively normal phenotype. In this case, we can assume that pilin protein such as FimU is interrupted since FimU is revealed to have no effect at all in twitching or transformation. Or alternatively, the transposon might not have interrupted any T4P related gene at all.

We have some good reasons to believe that in all T4P interruptions that could potentially occur, *ComP* is unlikely to be one of them. First, considering the phenotype and the measurement of both twitching motility and transformation efficiency from Leong's result, a *ComP* knockout would have a complete loss of both. We did not see this in any of the mutants. This first speculation however, is made without the knowledge of the *ComP* knockdown phenotype. Second, through ComP protein bands observed at 70kDa and 15kDa on the Western blot membrane. Even though the result is inconclusive due to the appearance of bands in the negative control, bands appearing in all other samples align with one of the expected sizes of ComP protein which is 15kDa. As ComP could run as a multimeric protein and that β-mercaptoethanol might not have completely broken down the disulfide bond and other polypeptide bonds, bands at 70kDa is also a possibility. This second speculation does not baer much weight for concrete evidence as this first but it is worth considering as a way to improve this particular procedure.

Once again, these hypotheses are formed based on the known data. Other proteins might have contributed to these particular phenotypes and transposon insertion sequencing will provide answers to many speculations formed in this research.

Morphological appearance of ADP1 mutants under SEM is similar to ADP1 wild type observed under AFM

The main purpose of the SEM investigation of ADP1 mutants morphology is to determine whether their pili structures are present or not. Note that an observable pili structure does not imply a functional pili. Here, we use "*A Nanoscopic Investigation of Pili and Pilus Production in Response to Environmental DNA in Acinetobacter baylyi"*, a thesis by Caroline Boyd as a reference to my analysis. First, it is important to address that we cannot use Boyd's observation as a baseline for a thorough comparison and analysis since our experimental methodology and condition differs in many aspects. For instance, Boyd grew ADP1 in liquid media then treated the culture with exogenous DNA incubation. Boyd's tested ADP1 cells are also 3 hours old which is the growth period where the cell is most competent (Leong, 2016). Our ADP1 mutants were taken directly from the colony that grew on the LB plate without any external DNA incubation and the colony is more than a day old. Therefore, we aim to only superficially compare basic ADP1 morphology such as the cell body (shape and size) and the pili structure (organization and length).

Using an Atomic Force Microscope (AFM) in identifying and characterizing the ADP1 pili, Boyd note some key features including (i) small diameter, (ii) the propensity to cross over other pili and (iii) the ability to bundle and/or branch from or into more than one pilus. Unlike AFM, SEM does not provide accurate measurement for both two and three dimension scans therefore we were unable to discern the diameter of the pili. However, other two features mentioned are observable under SEM. The pili are seen

to be lying on top of one another as a result of samples being air dried after suspending in the liquid. This makes it challenging to trace its path in order to quantify the pili in each cell and to accurately measure its length. Lastly, pili are seen to be connected to one another (Figure 7A, B and D) and/or branched out of one primary pilus (Figure 7C). These comparisons are drawn to the very least, confirming that these fiber-like structures are indeed pili of ADP1.

Revision in the 2 Round PCR protocol to obtain a discrete band

As seen in gel electrophoresis, smears are observed in all samples (Figure 4). In most cases the observation of a smear after gel electrophoresis of PCR amplification indicates a failed reaction due to nonspecific primer binding and / or contamination of the template DNA. As for this PCR, a smear is possible and likely to be expected due to the degenerate primers (Table 8) used and the protocol that has not been customized specifically for T26 transposon amplification (see Appendix 1: Recipe).

In an ideal situation the 3' end of the degenerate primers which have 4 or 5 unique nucleotides (**aagc** for CEKG 2T, **ataaa** for CEKG 2U and **ttct** for CEKG 2V) will bind to a location in the bacterial DNA fragment containing the specific complementary nucleotides. The following degenerate portion (**NNNNNN**) will then be able to continue binding the strand. The 5' end portion (**ggccacgcgtcgactagtac**) is not complementary to the bacterial template DNA nor the T26 portion and thus will bind to neither but still get amplified along with the degenerate and the 3' end in the following cycle. As we also use primers specific to T26 in the first round of PCR (Pgro172), the hope is that once this portion of T26 and the adjacent unknown DNA sequence got amplified multiple times, there will be enough of these specific fragments in the solution that the degenerate primers are able to bind to. The second round PCR uses another specific T26 primer (Pgro116) to further amplify the T26 portion closer to the adjacent unknown DNA sequence (that should already have one of the degenerate primers attached to it). The remaining PCR round 2 primer is CEKG 4 which contains only **ggccacgcgtcgactagtac** is used to finally exponentially amplify the portion only containing the complementary of degenerate primers which are connected to the T26 portion. This should result in a discrete band and some smear band on the background accounting for the possibility that degenerate primers can also over-amplify many other regions in the bacteria genome.

Our result showed that a revision in the PCR protocol including the mixture proportion and the thermal cycler set up is needed.

Conclusion

Transposon mutagenesis in *Acinetobacter baylyi* ADP1 generates mutants with altered twitching motility and transformation efficiency compared to the wild-type strain. The Type IV pilus is the protein complex responsible for both mechanisms, directing our attention toward different pilin units and DNA transport proteins that could be affected by the transposon insertion. As we use transposon insertion sequencing to provide precise results on affected proteins, this experiment on twitching and transformation efficiency can serve as a supplementary method to screen for mutants with interesting characteristics for further research.

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Appendix 1: Recipes

LB broth

In a 1 L graduated cylinder, dissolve in 800 mL of tap water using a stir bar: 10 g tryptone, 10 g NaCl, 5 g yeast extract. Top up to 1 L exactly. Pour the solution into 1 L bottle. Autoclave within an hour.

LB Agar

Using a graduated cylinder, pour exactly 500 mL of LB broth into a 1 L bottle. Add 7.5 g of BD BactoTM Agar, swirl to disperse the agar and autoclave within an hour. As the liquid cools down to 55 C add the required antibiotic with a 1:1000 ratio (500 µL / 500 mL broth).

Antibiotic Stock Solution (1000X)

Tetracycline (5 mg/mL): In a 50ml conical tube, dissolve 0.125 g of tetracycline powder in 25 ml 95% ethanol. Wrap the tube with tin foil and constantly shake the solution to completely dissolve the powder. Store at -20 C.

Streptomycin (20 mg/mL): In a 50 mL conical tube, dissolve 0.3 g of streptomycin powder with 15 mL of water. Store at 4 C.

Trimethoprim (20 mg/mL): In a 50 mL conical tube, dissolve 0.3 g of trimethoprim powder with 15 mL of DMSO. Store at 4 C.

LB-20% Glycerol

Using a stir bar, dissolve 2 g Tryptone, 2 g NaCl and 1 g yeast extract in 150 mL of water. Pour into a 250 mL graduate cylinder and add water to reach 180 mL. Carefully pour 20 mL of glycerol. Cover the top of the cylinder completely with parafilm. Mix by inverting at least 10 times. Use a filter flask to sterilize the LB-20% glycerol.

Phosphate Buffered Saline (PBS)

Using a stir bar, dissolve 8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1 L of H_2O. Autoclave before use and store at room temperature.

PCR Round 1

This recipe is obtained from 2023 Block 7 Leah McLeland, Casey Feringo and Zoe Zizzo Lab Notebook)
In a sterile microfuge tube, add 233 µL DDH_2O, 18.9 µL Pgro172, 7 µL CEKG-2T, 7 µL CEKG-2U, 7 µL CEKG-2V and 70 µL Go-Taq 5X Master Mix. Change pipette tips every time a new solution is added. Store at -20 C or keep on ice when in use. In a PCR tube, pipette 49 µL of the master mix and 1 µL of template DNA. PCR thermocycler is set up (Gallagher et al, 2015) as follows. Note that this protocol has been originally designed to use in <KAN-2> insertions.

PCR Round 1

Step	Temperature	Time	Notes
1	94°	12′	Colony denaturing
2	94°	30″	Denaturing
3	42°	30″	Annealing, decreased temperature 1° each cycle
4	72°	3′	Extension
5	Go to step 2		6 cycles, then to step 6
6	94°	30″	Denaturing
7	64°	30″	Annealing
8	72°	3′	Extension
9	Go to step 6		25 cycles, then step 10
10	72°	7′	Final extension
11	4°	Hold	

′, minutes; ″, seconds.

PCR Round 2

This recipe is obtained from 2023 Block 7 Leah McLeland, Casey Feringo and Zoe Zizzo Lab Notebook)
In a sterile microfuge tube, add 224 µL DDH2O, 17.5 µL Pgro116, 17.5 µL CEKG-4 and 70 µL Go-Taq 5X Master Mix. Change pipette tips every time a new solution is added. Store at -20 C or keep on ice when in use. In a PCR tube, pipette 47 µL of the master mix and 3 µL of template DNA. PCR thermocycler is set up (Gallagher et al, 2015) as follows. Note that this protocol has been originally designed to use in <KAN-2> insertions.

PCR Round 2

Step	Temperature	Time	Notes
1	94°	10′	Initial denaturing
2	94°	30″	Denaturing
3	64°	30″	Annealing
4	72°	3′	Extension
5	Go to step 2		30 cycles, then step 6
6	72°	7′	Final extension
7	4°	Hold	

′, minutes; ″, seconds.

Table 8 T26 insert primer sequences

	Primers to sequence T26 insert	Length	Annealing Temperature	Use for
Pgro-172	tgagctttttagctcgactaatccat	26	56.2	PCR round 1
Pgro-116	atttacaccttttgcgatgtttgg	24	54.7	PCR round 2
Pgro-90	cggccgcataacttcgtataatgt	24	57.3	sequencing
CEKG 2T	ggccacgcgtcgactagtacNNNNNNNNNNaagc	34	67.7	PCR round 1
CEKG 2U	ggccacgcgtcgactagtacNNNNNNNNNNataaa	35	65.6	PCR round 1
CEKG 2V	ggccacgcgtcgactagtacNNNNNNNNNNttct	34	67.2	PCR round 1
CEKG 4	ggccacgcgtcgactagtac	20	65	PCR round 2

T26 Sequence and primers binding site

> T26

CTGTCTCTTATACACATCTCAACCATCATCGATGAATTTTCTCGGGTGTTCTCGCATATTGGCTC
GAATTCTCATAACTTCGTATAGCAT**ACATTATACGAAGTTATGCGGCCGCCCCAAACATCGC**
AAAAGGTGTAAATATTATAATGCCAAAAAACTCAAATCCTATTGT**ATGGATTAGTCGAGCTA**
AAAAGCTCATATTTTTATATTCAAACTATATCCCTTCAAGCTTTGAAAAATAAACTTAATTATT
ATATATGTTATTTAGCTAGTTTTTTAATTAAAGTTAAAATCGAGAGCTTGTTTGACAAAAAAAC
AAAAAAATTTCTTGAAAATTTTTTTTTGACTCAATATCTAGACTTGCAAGAGCTTGGAACTTT
GAGATTGTTCTAAGATGCACTGCAGATTTAAGAAGGAGATATACATATGAAATCTAACAATGCG
CTCATCGTCATCCTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTAC
TGCCGGGCCTCTTGCGGGATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCT
AGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTT
GGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAGCCACTATCGACTACGCGATCATGGCG
ACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCAC
AGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTT
CGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGT
TGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTAC
TACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGA
GCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTTATG
ACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGC
GAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTG
CACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGC
CATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGG
CTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAG
GCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGC
TCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCG
AGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCG
TTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGTATGAGGCGCGCCATA

ACTTCGTATAGCATACATTATACGAAGTTATGCGAGCTCGGTACCCGGGGATCCTCTAGAGTCG
ACCTGCAGGCATGCAAGCTTGCCAACGACTACGCACTAGCCAACAAGAGCTTCAGGGTTGAG
ATGTGTATAAGAGACAG

Fast Western Blot Kit Reagent Preparation

In the Pierce™ Fast Western Blot Kit, ECL Substrate makes a working stock of the following; fast western 1X wash buffer, primary antibody working dilution, fast western optimized HRP reagent working dilution and detection reagent working solution.

Fast western 1X wash buffer: In a 250 mL Erlenmeyer flask mix 10 mL of 10X Fast western wash buffer with 90 mL of water.

Primary antibody working dilution: Shake the Antibody Diluent well before use. In a 15 mL conical tube dilute 5 µL primary antibody in 5 mL Antibody Diluent. Prepare this working dilution immediately before use.

Fast western optimized HRP reagent working dilution: In a 15 mL conical tube, mix 1ml Optimized HRP Reagent with 10 mL Antibody Diluent. Use within 1 hour.

Detection Reagent Working Solution: In a 15 mL conical tube, mix 5 mL Detection Reagent1 and 5 mL Detection Reagent2. Use within 1 hour.

Appendix 2: Laboratory Protocol

DNA Isolation using Phenol-chloroform-isoamyl alcohol

In a 1.5 mL microcentrifuge tube, suspend a cell pellet in PBS by vortexing. Put on gloves and continue the procedure in the fume hood. Add 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) into the tube, make sure to pipette the yellow organic phase and not the floating aqueous layer. Close the tube, vortex and then centrifuge for 5 minutes at maximum speed to separate organic and aqueous layers. Remove 450 µL of the top aqueous layer (containing DNA) without touching the interphase layer and place into a new tube. Add 50 µL of 3M sodium acetate into the tube. Finally add 1000 µL of 100% ethanol into the tube. Vortex hard and store at -20 C overnight. The next day centrifuge the tube at maximum speed, DNA and salt will pellet to the bottom then discard supernatant. Resuspend pellets in 100 µL of Elution Buffer from the Qiagen DNeasy Kit. Store the DNA at -20 C.

DNA Isolation using Qiagen DNeasy Kit

Centrifuge culture cells for 10 minutes at maximum speed to obtain the cell pellet at the bottom, discard supernatant. Resuspend pellet in 180 µL Buffer ATL. Add 20 µL Proteinase K, vortex and incubate at 56 C on the heat block. Vortex the sample every 15 minutes then put back on the heat block until 1 hour is completed. Add 200 µL Buffer AL to the sample and vortex. Then add 200 µL 100% ethanol and vortex again. Pipette the mixture, including any precipitate into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at maximum speed for 1 minute, discard the flow through. Place the Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW1. Centrifuge at maximum speed for 1 minute, discard the flow through. Add 500 mL Buffer AW2. Centrifuge at maximum speed for 3 minutes and discard the flow through. Place the Mini spin column in a clean 1.5 mL microfuge tube and pipette 200 µL Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute and centrifuge at maximum speed for 1 minute. Collect the flow through and store at -20 C.

Appendix 3: Calculations

Transformation Efficiency

The following formula is used to calculate how many cells are presented in 1 mL in each 10 µL puddle.
[(average colonies on LB)/(10 µL spot)] x (dilution factor) x (1000 µL/1mL)
This formula applies for both Str20 LB plate and plain LB plate. Dilution factor is the amount of dilution (such as 10^1, 10^2, …) chosen related to average colonies on LB.

The following formula is used to calculate transformation efficiency
(transformed cells/mL)/(total cells/mL)
Transformed cells are obtained from the Str20 LB plate and total cells from the plain LB plate.

Dilution Factor

Once obtained the OD600 of the 1:6 dilution, actual dilution is calculated by 1:6 dilution x 6. The desired dilution factor in this experiment is 6. Therefore, the required dilution (Fd) = actual dilution/ 1. Using the equation Fd = $V_T/V_{S,}$ where V_T is total volume (1000 µL) and V_S is stock cell volume, V_S can be calculated by V_T/Fd. The volume of PBS added will then be 1000uL - $V_{S.}$