**Honors Thesis:**

# Finding New Signaling Pathways that Govern Biofilm Formation by *P. aeruginosa*

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Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen that is often associated with severe forms of many infections, including bronchiectasis and infections in the gut. Mortality is increased in patients who become infected with *P. aeruginosa*. *P. aeruginosa* poses a special treatment challenge due to its propensity to form biofilms, in which cells are surrounded by a self-produced extracellular matrix of proteins, DNA, and polysaccharides. Biofilms can help bacteria evade the host immune response, and the matrix represents a barrier that protects bacteria against antibiotic therapy. Because biofilms are difficult to treat once established in an infection, new strategies to prevent biofilm formation are critical to combat these infections. However, effective prevention depends on a fuller understanding of the signaling pathways that control biofilm formation. To identify new control points in the pathways governing biofilm formation by *P. aeruginosa*, we use transposon mutagenesis in conjunction with a visual assay for colony morphology, in which colony wrinkling indicates biofilm formation. Using these screens, we have been able to identify many promising candidates. For example, we have identified mutations in the genes *fdnG* and *PA14_42090* as having smoother colony morphology, suggesting that these genes are involved in biofilm formation. Conversely, we have found that mutations in the *purU2* and *trxB1* genes show increased colony wrinkling, suggesting that these genes normally suppress biofilm formation. We will continue characterizing these genes by deleting them from the genome to confirm their roles in biofilm production and then testing how their absence or presence affects other known biofilm signaling molecules such as cyclic-di-GMP. Our detailed characterization of these candidate genes will provide fundamental knowledge that can then be used to devise future treatments to prevent biofilm formation in patients infected with *P. aeruginosa*.

**Background**

*Pseudomonas aeruginosa* is a rod-shaped pathogen notorious for creating a range of infections and for making complicated infections in patients with burn wounds, cystic fibrosis, and in patients with leukemia (Bodey *et al.* 1983). *Pseudomonas aeruginosa* is a bacterium known for its intrinsic ability to resist natural host defenses as well as antibiotics (Stover *et al.* 2000). For this reason, infections can be extremely difficult to treat. Some infections occurring in hospitals are from hypermutable strains and they often account for the chronic infections found in CF patients (Oliver *et al.* 2000). In hypermutable strains of *P.* aeruginosa, the bacterium can show mutations that affect its ability to overproduce biofilms. Biofilms are considered microcolonies that are surrounded by exopolysaccharides and they are known to be prominent in virulent strains of *P. aeruginosa* by slowing cell growth, acting as a physical barrier to protect the cells, and other factors that decrease *P. aeruginosa* 's susceptibilities to antibiotics and host defenses (Van & Iglewski 1998). Biofilms cause also trouble in other areas where the bacteria can stick to surfaces such as piping for drinking water (Yu & Lee 2010). Because *P. aeruginosa* biofilms cause problems in the areas where they grow and because they are extremely difficult to get rid of or treat, it is important to increase our knowledge of biofilms and how they are formed by *P. aeruginosa*. The current understanding of the signaling pathway of biofilm formation in *P. aeruginosa* is not yet complete, and there are many signals and quorum-sensing systems at play (Lopez *et al.* 2010). In order to work against antibiotic resistance and *P. aeruginosa* infections, it is important to fully understand the signaling pathway that *P. aeruginosa* has that allows the formation of biofilms. Understanding the full pathway leading to biofilm formation will allow

for identification of potential targets to create better treatments and plans of action against biofilms.

**Methods**

Transposon Mutagenesis

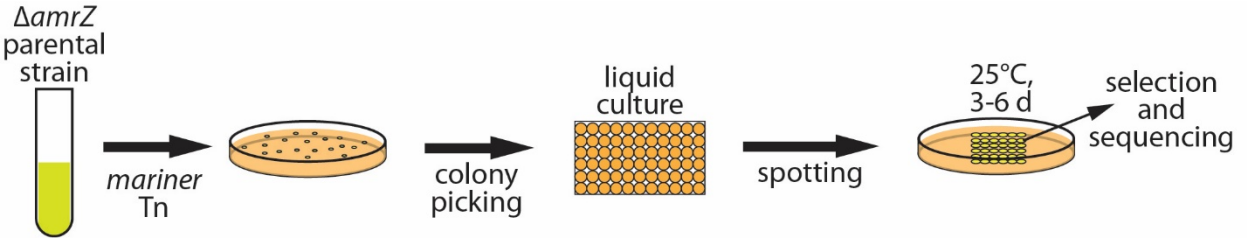


Figure 1: Parental strain, Δ*amrZ*, is mated with *E. coli* strain carrying mariner transposon and plated on antibiotic selective agar and picked for growth in 96-well plates. Well plates are them stamped on M6301 morphology agar for visual screening. Figure from Cabeen, M. T., Leiman, S. A., & Losick, R. (2016). Colony-morphology screening uncovers a role for the *Pseudomonas aeruginosa* nitrogen-related phosphotransferase system in biofilm formation. *Molecular microbiology*, *99*(3), 557-570.

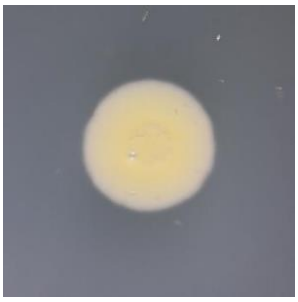

Figure 2: Non-wrinkled control strain PA14

Figure 3: Moderately wrinkled: PA14 Δ*amrZ*

In transposon mutagenesis, a moderately-wrinkled parental strain of *P. aeruginosa* is mated with an *E. coli* strain carrying a mariner transposon with a gentamycin resistance tag. The moderately wrinkled parental strain offers a dynamic range of wrinkling to include evaluation of increased wrinkling as well as decreased wrinkling (Figure 3). After the *E. coli* strain with the transposon is mated with *P. aeruginosa*, the mated culture is plated on an agar plate containing gentamycin, an antibiotic lethal to *P. aeruginosa,* and Irgasan, an antibiotic lethal to *E. coli,* to ensure that the only colonies growing are *P. aeruginosa* colonies with a transposon insertion. From this antibiotic selection plate, colonies are picked and used to inoculate a 96-well plate filled with gentamycin lysogeny broth which continues to ensure that only *P. aeruginosa* with a transposon insertion will grow. The 96-well cultures are stamped onto M6301 morphology agar (Figure 1). After an incubation period of 3-5 days, the colonies are assessed in a visual screening.

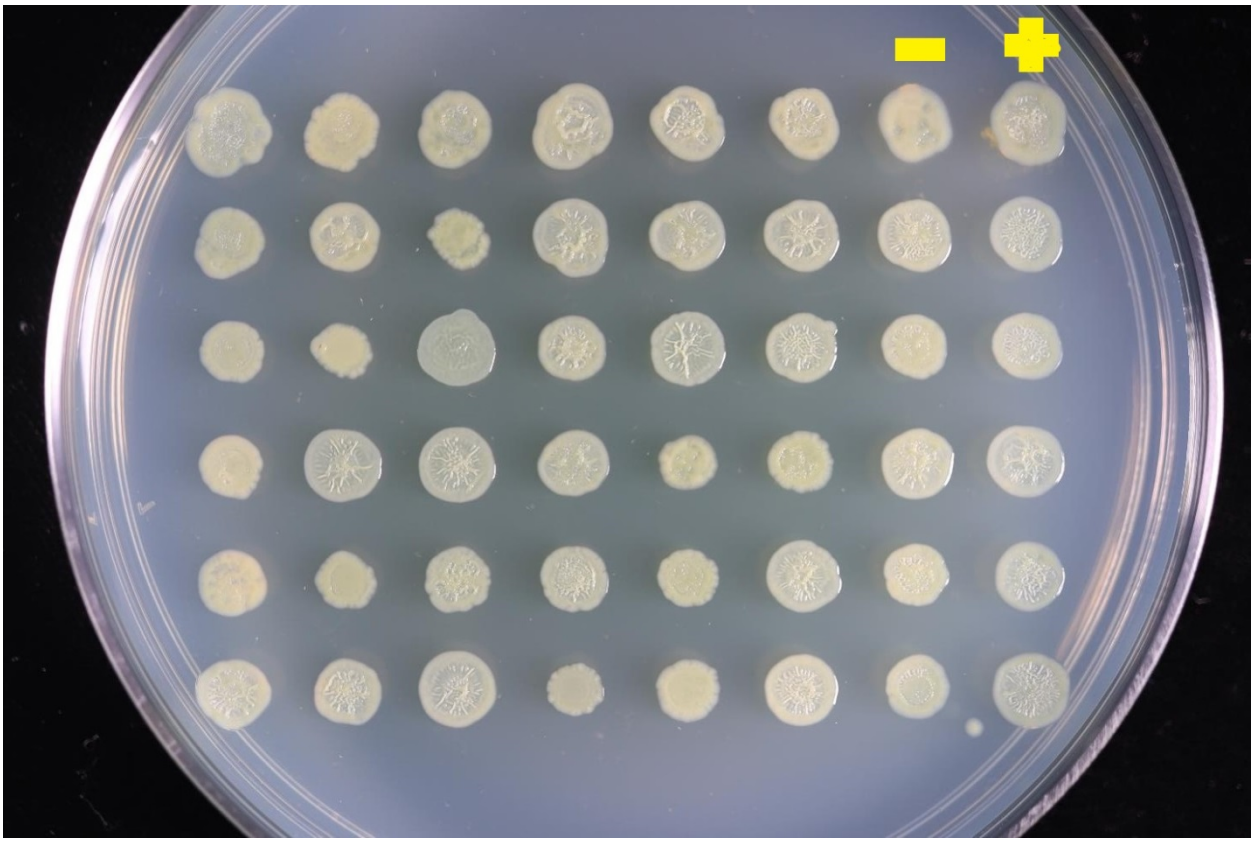

Figure 4: Visual screening of mutants against parental and control strains on M6301 agar.

– denotes smooth control, PA14

+ denotes moderately wrinkled parental colony, Δ*amrZ*

Visual Screening

*P. aeruginosa* can be visually assessed for biofilm formation by viewing wrinkling morphology of colonies (Figure 4). Increased wrinkling is directly associated with increased biofilm formation. When comparing the mutated colony with a transposon insertion to the parental strain without a transposon, an increase or decrease in wrinkling suggests that the transposon inserted into a gene that lies in the signaling pathway for biofilm formation.

Sequencing

When colonies show an increase or decrease in wrinkling after being visually assessed, those strains are sequenced to determine the location of the transposon. Colony PCR is performed with a forward primer complementary to the transposon sequence and a set of arbitrary reverse primers. The PCR product is sequenced at our on-campus sequencing facility and aligned to the *P. aeruginosa* genome using Geneious software. The alignment reveals the exact location of the transposon insertion, identifying a candidate gene that will be further characterized.

Full Gene Deletions

To test the assumption that the gene that was disrupted by the transposon has a role in the biofilm signaling pathway, the full candidate gene is deleted. This is done by amplifying a region upstream of the gene that is around six-hundred kilobases and a region downstream of the gene that is around six-hundred kilobases. Complementary bases are added to both upstream and downstream regions to anneal the two insertions together as well as anneal the ends into a

plasmid. After the amplification of the six-hundred kilobases for both the upstream and downstream regions, these two parts are then pieced together using stitch PCR, connecting the two complementary edges of the upstream and downstream region. This insert, now around 1,200 kilobases, is inserted into a plasmid using isothermal assembly and verified by sequencing. This plasmid will now carry the insert. This plasmid is electroporated into *E. coli* strain SM10, a conjugation competent strain that can transfer plasmid to *P. aeruginosa*. The SM10 strain is mated with *P. aeruginosa*, and the plasmid will integrate into the *P. aeruginosa* genome at the target locus. Integrants are selected with gentamycin and clones are screened for plasmid loss using sucrose counterselection. Plasmid loss results both in target gene deletion and reversion to the wildtype. Deletion mutants are identified using PCR at the target locus.

**Results**

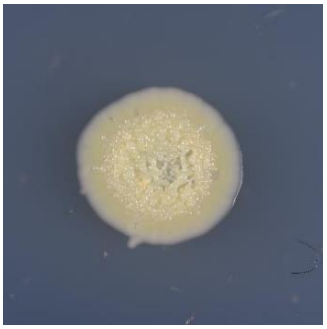

Figure 5: PA14_30280 *trxB1*

Figure 6: PA14_71530 *purU2* and *aat*

From the initial transposon mutagenesis and visual screening, fifty colonies were identified and reassessed to check for morphology changes. Of those, thirteen were seen to show consistent morphology change. From thirteen, nine genes were identified as novel genes not known for their role in biofilm formation, whereas the others had known roles in the signaling pathway.

Transposons were found in *trxB1*, *nadA*, *exoU, fdnG*, intergenic region of *purU2* and *aat*, *fdnH*, *pelG*, *pelB*, *phzF1*, *pilY1* and in hypothetical genes located at *PA14_42090*, *PA14_71150*, and *PA14_49920*. The gene *trxB1* and the location between *purU2* and *aat* both showed hyper-wrinkled mutation after transposon mutagenesis and were identified to have full deletions made for further testing (Figures 5 and 6).

**Discussion**

Of the initial 13 genes found to be disrupted by a transposon and assumed to have biofilm formation interactions, four were already characterized in the signaling pathway for biofilm formation which were *pelG*, *pelB*, *phzF1*, and *pilY1*. The mutations occurring in the *trxB1* gene and in the region between *purU2* and *aat* remained consistent and had strong phenotypic changes during the initial and secondary visual assay. Both genes had a hyper wrinkled phenotype implying that the genes play a role in suppression of the biofilm.

The gene *trxB1* is located at PA_30280 and encodes an enzyme known as thioredoxin reductase. This family of genes work by reducing thioredoxin, which donates protons to radical oxygen species and plays roles in cell growth and transformation (Mustacich & Powis 2000).

The gene purU2 is located at PA14_71530 and is known as a formyltetrahydrofolate deformylase. This gene has been shown to play a role in glycine betaine catabolism which is a precursor made into osmoprotectants for *P. aeruginosa* (Diab *et al.* 2006). Because the transposon landed in an intergenic region between *purU2* and *aat*, both genes are being deleted separately to ensure that both genes are tested to determine which gene was causing the morphology change.

**Future Direction**

Full deletions of the genes of *trxB1*, *purU2* and *aat* will be made and assembled into a plasmid to be inserted into the *P. aeruginosa* genome. They will then be used to quantify the amount of biofilm that is being made when those genes are disrupted using a Congo-red dye binding assay and testing to see if cyclic-di-GMP levels are affected by the deletion. Cyclic-di-GMP is an important signal in biofilm formation and will help to characterize the exact role that the gene and the encoded protein play in the signaling pathway.

**References**

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