TN5 MUTAGENESIS: AMELIORIZATION OF THE COST OF THE MEGAPLASMID IN *PSEUDOMONAS SYRINGAE*

By

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Abstract

An abnormally large plasmid has been discovered to naturally exist in two strains of *Pseudomonas syringae*, appropriately called the "Megaplasmid" due to the genetic content of over one million base pairs of DNA. Costs have been observed with the presence of the Megaplasmid, including growth at elevated temperatures. A technique called Tn5 mutagenesis has been utilized to ameliorate the cost to the host. Results show that initial phenotype of Tn5 mutants overcame elevated temperature growth inhibition, but presence of another mutation is likely in order for the strain to grow at elevated temperatures while harboring the Megaplasmid. Further research must be done to identify genes responsible for increased fitness.

Introduction (Statement of Purpose/ Statement of Relevance)

The study of evolution of various organisms is an ongoing and growing field as it keeps occurring. There are countless species of organisms on this earth, and possibly in other places in the universe. In order to survive, each organism holds its own niche and provides its own contributions to the ecosystem. As the environment changes over time, organisms adapt or progress with the alterations or they are naturally selected against and cease to exist. This holds true to Charles Darwin's theory of "Natural Selection", and the phrase of the survival of the fittest. There are a myriad of ways an organism is able to alter its position and possibly become more fit. Most commonly an anomaly mutation occurs by chance that thus helps that offspring cement its chances of survivor in the ecosystem. This can occur through any mutagen such as UV sun rays, carcinogenic chemicals, and so on. Beneficial mutations occur much less frequently (Russell), and much time needs to pass for species with longer life spans to evolve. The basis of my thesis and studies that occur in my Principal Investigator's (PI), David A Baltrus, lab is this entity that he discovered during his post-doctoral fellowship, which he called the "MegaPlasmid" (MP) (Baltrus). Bacteria's genomes consist of a single chromosome, along with supplemental "bonus" rings of DNA called plasmids (Smith). These plasmids are essential for different bacterial strains to transfer profound genes such as antibiotic resistance, through a phenomenon known as Horizontal Transfer (HT)(Baltrus). The exact reason and physiological process for HT is not completely concrete, but my PI's lab and several other labs around the world are furthering the knowledge that is required to know more about it. The MP is so profound and novel because it literally lives up to the Greek meaning of mega- by containing over 1 million base pairs. Commonly plasmids are in the tens-of-thousands of base pair range, as can be seen by Figure 1 below.

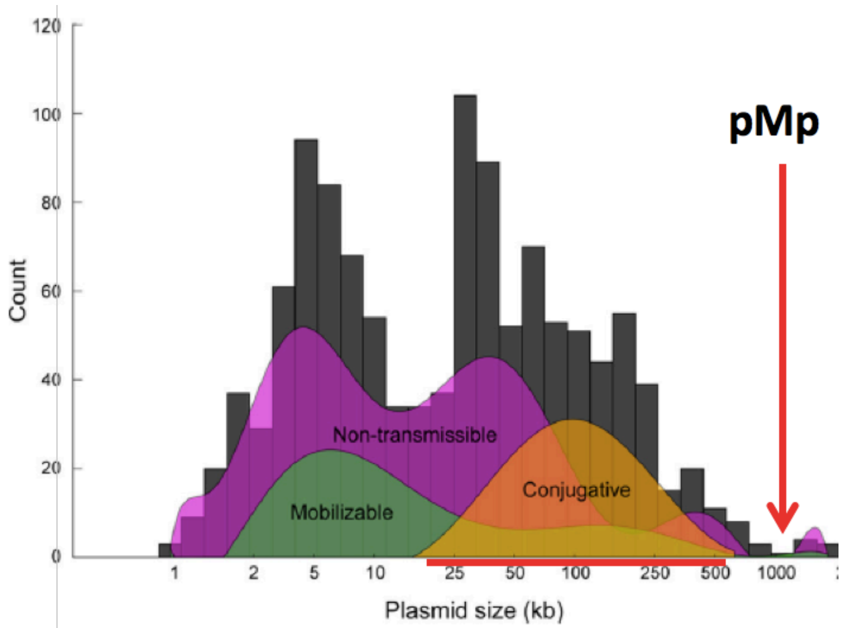

Figure 1: Size of MP compared to other plasmids

The size of the MP not only gives it the possibility of storing much more genetic material with more base pairs, but for physiology-sake it takes up much more room than a more commonly-found plasmid. According to Dunny, plasmids are highly regulated in the cell and have lots of effects on the cell transduction pathways. Namely, pathways compared between strains with highly used plasmids and those without one are drastically different in terms of what proteins interact with what genes. The MP is of interest in this regard, because it appears to have a cost on fitness and hinders the cell, rather than help it. There are many theories as to why the cell harbors it in the first place, and those will be further researched and sought after in the years to come.

According to Bennet, the general hypothesis that trade-offs occur as a result of evolutionary adaptation is certainly supported by these experimental results. This holds true to many of the experiments and theories that were performed in this thesis. Organisms adapt to a certain temperature, and when they are placed outside of their 'comfort' zone, there are adverse effects. It is true that phenotypes and pathways are environment specific, and do not necessarily exist in other environments or conditions (Bennet). In Bennet's experiments, he found that bacterial strains that were found to be fit and grow well at lower temperatures had severe difficulty growing at elevated temperatures, and vice versa but not to as great of magnitude. In Hébraud's paper, he discusses how bacteria, along with eukaryotic cells are able to respond to a dramatic drop in temperature by activating genes that then further translate for cold-shock proteins. These proteins are used in cellular transduction pathways to regulate essential functions in the cell despite the abnormally cold temperature. According to Hébraud, temperature immediately affects the interior of the cell, causing for a great spurn of interest in my own studies and overall experiments. In the grand scheme of experiments going on in my lab, temperature is one of many stress tests being performed to identify costs and fitness effects of the MP on the host bacteria cell. These experiments started when my Lab Manager placed the *P. stutzeri* strains that we now commonly work with in an elevated incubation chamber, approximately 30C, and noticed the poor growth, and especially if the strain harbored the MP. These observations ultimately lead to the basis of the experiments that make up my Senior Honors Thesis. No tests have been conducted at depressed temperatures, but will most likely in the future.

Tn5 mutagenesis is a useful technique in our experimental situation because P. stuzeri is a very easily manipulated and useful strain of bacterium. HT is used and plasmids and the MP can be exchanged during bi- or tri-parental conjugation. Genetic information are able to be passed from a donor strain to a recipient strain, with the help of a helper strain that increases the efficiency of the turnout (Baltrus). Tn5 mutagenesis comes into play as we hoped to alleviate the cost of the MP through random mutations, thereby identifying what genes on the MP that are knocked out have what effect on cell-signaling pathways and growth. Thus, by knocking out genes that possibly hinder growth at elevated temperatures, that information can tell us which genes are likely responsible for the detrimental effects of the MP in terms of growth rate and growth at elevated temperatures. Logically, the strains should get rid of the MP if there is no positive effects or reasons for harboring it, but trials of getting the ancestral strains to get rid of the MP have proven unsuccessful at all points. There is much speculation on why this occurs, and it all starts with understanding the genetic and cellular pathways that the MP is involved in and why it exists.

Materials & Methods (Methodology)

Although the MP was found in two P. syringae strains, all of the competitions and experiments used P. stutzeri strains. This was because we wanted to use an alternate model and strain of the species of bacterium, in order to see if the same effects of the MP held true and it wasn't strain-specific. Also, P. stutzeri strains are saltwater-transformable, which means when you introduce the cells to an environment with a high salt content, the osmolarity of the cell is thrown off and it opens up pores that easily and conveniently accept genetic material. This makes the strains very easy to manipulate and easy for us to move the MP around into strains of any genetic background that we wish.

The fitness competition assays were performed in order to quantitatively compare the growth rates between a MP strain and a non-MP strain. First, O/N cultures were picked to Salt-Water LB liquid media containing RIF and set in an incubated 27°C shaker. After 24 hrs of incubation and growth, a 2:2000 μL dilution is performed into plastic culture test tubes (USA Scientific, S#: 1450-0000, 5 mL Polypropylene, 12X75 cm). This conditioning step is performed so that the strains may adapt to the tubes that the competitions fitness assay will be performed in, rather than having to adjust from the glass test tube in the first step to the plastic culture tube. Also, it ensures that the cells will be in the same stage of growth when they are ready to be put into the master mix for the actual competition. After 24 hrs in the 27°C incubated shaker, the strains need to be determined how much they have grown by determining their optical density (OD 600, WPA biowave, CO8000 Cell Density Meter, S#:1829). The goal is to have each culture to contain 1-3 million CFU's, being at comparable stages of the growth cycle and concentration. From there, the master mix is divided into 8 replica plastic test tubes of 2 mL volumes each. 20μL is taken from each replica tube and serially diluted to the 10^-3 for t=0, plating and spreading 100μL of each 10^-3 dilution on separate LB plates with RIF. The replica plastic culture tubes are placed in the 27°C incubated shaker for 24-48hrs, and serially diluted to 10^-6, plating and spreading 100μL on LB-RIF plates. For strains with the LAC-Z gene, XGal sugar needs to be included in the plates so that the tester and variable strains may be differentiated. The LAC-Z gene allows the bacterium to break down XGal, which is essentially galactose attached to a blue pigment. When the pigment is separated and digested, a blue colony forms and is easily differentiated from the other white colonies. For strains that don't easily take up the LAC-Z gene, an alternate method is used. Both the t=0 and t=1 RIF plates are replica plated using filter paper and a replica plater to LB plates containing gentamyacin antibiotic, and also LB plates containing kanamyacin. The sum of both of the colonies of these replica plates should ideally equal the total colonies present on the RIF plates.

Tn5 Mutagenesis Protocol:

Tn contains an *oriR6K* origin of replication. This sequence is inactive unless the Tn is present in a cell containing the *pir* gene encoding the π replication protein. We isolated total genomic DNA the mutants and digested it with a restriction enzyme. We self-ligated the fragments and transformed the ligated mix into a special strain of *E. coli* that contains the *pir* gene encoding the replication protein. This will allow only the single chromosomal fragment that contains the Tn to replicate as a plasmid. Then, we isolated the plasmid and used DNA primers specific to the ends of the Tn to sequence into the adjacent chromosomal DNA. The sequence generated will be compared to the GenBank database to try to identify the gene disrupted by the Tn. A reaction was created that contained the isolated DNA, 10x Reaction 3 Buffer, Nano-Pure Water, and the corresponding restriction enzyme for the cutting sequences, in this case, BamH1 restriction enzyme. Then we incubated the digestion in a 37°C water bath for 2 hours. Next we column purified the digestions, then ran the completed digestions on a normal 0.5% agarose gel using electrophoresis. The ligations were incubated at 15°C overnight and stored indefinitely at 4°C.

Because of the cost of growing at elevated temperatures, other fitness costs were questioned. It was observed that MP strains had an increase in motility, but more susceptible towards Naldixic acid (Nal) antibiotic. Below is the protocol for the motility assay, but results won't be discussed because not enough reliable data was received or extrapolated in time for the final date of this thesis. The Nal protocol consists of the same competition protocol as stated above, but the mastermix is brought to a concentration of Nal 4. As you can tell by Figure 2, Nal has a large effect on the growth of MP strains. Nal 4 concentration is the "sweet spot" to study the differences in growth rates comparing the two strains, and will be heavily researched in the coming time. Data will be published in my PI's submission to an academic journal.

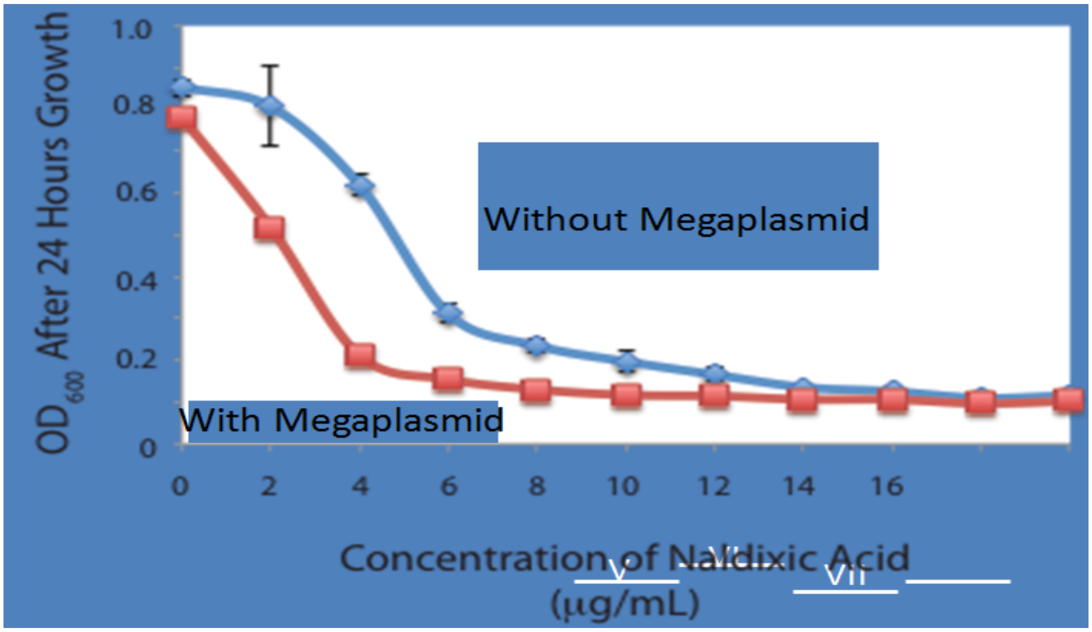

Figure 2: Effect of Antibiotic Naldixic Acid on growth of MP and non-MP strains

In order to illustrate the effect the MP had on bacterial motility, .25% agar media was used rather than .50% agar media. The complete mixture was .5% Tryptone, .5% NaCl, .25% Yeast Extract, and .25% agarose mixed and dissolved in deionionzed distilled H_2O. This mixture was pipetted into "Cyto-One" 12 well-plate w/ lid (Catalog No: CC7682-7512, polystyrene, sterile), 2.5 mL into each well. The media was allowed to cool and solidfy as much as it possibly could. Bacterial strains that were being tested were OD600ed and then normalized to an OD600 of 1. A round-bottom tooth pick was then dipped into the suspension and dipped into each well that pertains to that strain. In regards to placement of each strain on each replica 12-well plate, in order to prevent user-bias and have a "blind reading" of the results strains are rotated between wells between the replica well plates. After the plates are inoculated with bacteria and allowed to grow for 24 hours in a 27°C incubator, they are scanned under a standard Epson Perfection V500 Photo scanner, and then the program ImageJ is used to determine the area in the motility circle. The area is measured in pixels and the strains are all compared to each other, relatively.

Results

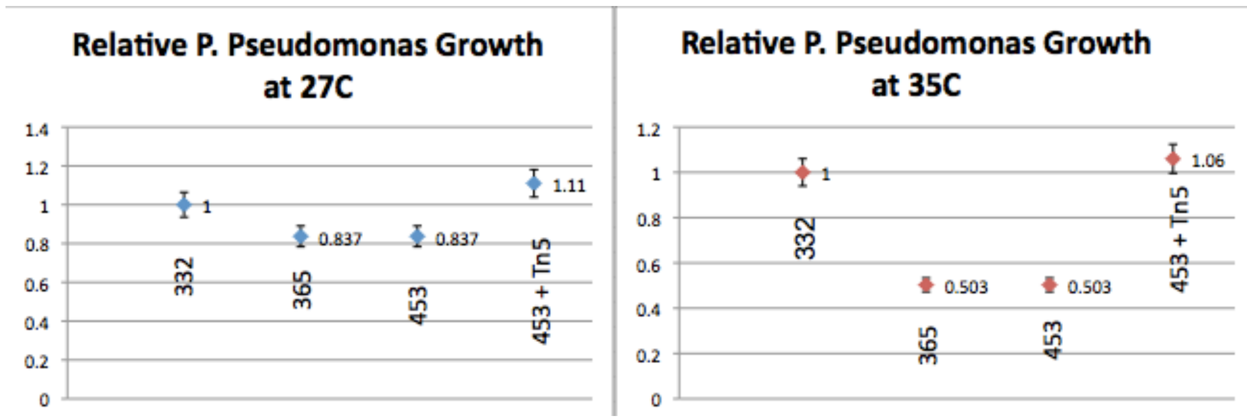

Figure 3: Comparing relative growth at 27C and 35C. All values were normalized to 332

After initial and basic observation, bacterial strains that harbor the MP have a decreased fitness at elevated temperatures. This was observed during basic growth at 27°C on LB media plates with RIF antibiotic to kill E. coli and other unwanted microbes. Bacterial strains without the MP experience thicker, denser, and much more colonies than the colonies produced by stains with the MP. At the elevated temperature of 35°C that is outside of the optimal range of P. stuzeri, MP strains were found to grow at an even lower rate than the MP-free strains. This lead to further research on the MP and why it exists in the parent strain of P. pseudomonas, even though it hinders growing ability under various environmental strains. At this point, my experiments done for this thesis is only one aspect of the overall grand scheme of things for this novel new research. My task was to achieve a Tn5 mutant strain that harbored the MP, but was able to grow at the elevated temperatures similarly to the non-MP counterpart.

As you can see from Figure 3, the Tn5 mutant outperformed all other strains at both temperatures, even the non-MP strain. As for denoting which strain is which, 332 is the non-MP strain, 365 is the rifampin, tetracyclin, and kanamyacin resistant MP strain, 453 is rifampin and sucrose resistant but kanamyacin and tetracycline susceptible, and 453 + Tn5 is 453 that was conjugated with the Tn5 ligation. Compared to other selection strategies, this one that we used is quite advantageous. This is because we were directly selecting for the phenotype in which we hoped a mutant would arise. Other selection processes require screening through thousands of samples, for example by streaking on selective media plates. Thankfully, this screening process was simple because if the bacteria could grow at an even more elevated temperature, 37C, then we suspected the Tn5 to have had knocked out a gene that was hindering the growth at elevated temperatures, or even growth altogether. Out of the the four successful Tn5 mutants that were able to grow at this highly elevated temperature, the mutant of particular interest was a Tn5 mutant that knocked out a histidine kinase enzyme. The location of the 3 chromosomal Tn5s can be seen below in

Figure 4 compared to the size of the entire genome, and what genes they effectively knocked out in the process. This was determined through sequencing the isolated ligations of the Tn5 mutations, then the genetic sequence was inserted on the BLAST website under blast-x to run through all possible reading frames. The results with the highest relevance to known genes in the *P. stutzeri* genome are believed to be the genes that were knocked out due to Tn5 mutagenesis based on the primers used at either end of the Tn5 and what matches up with the genome.

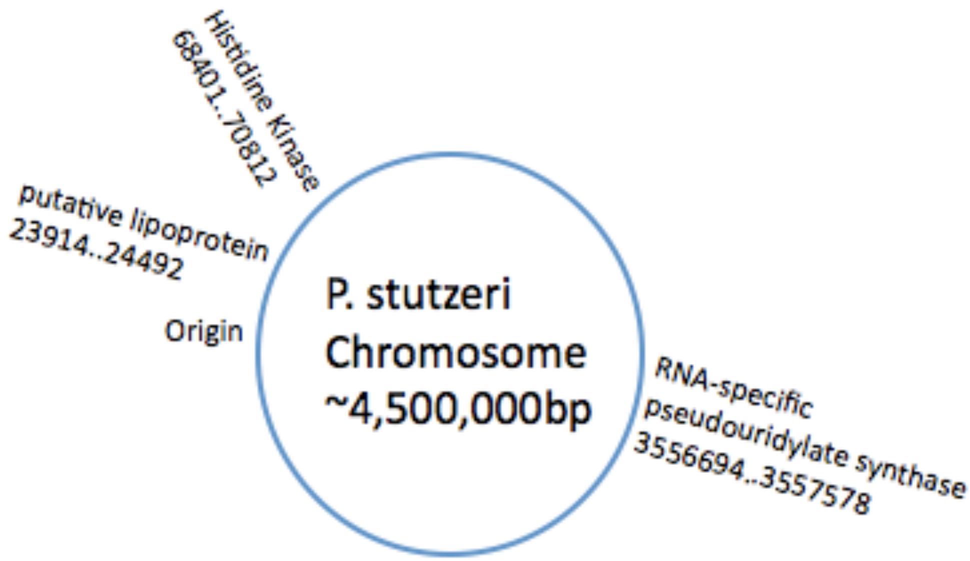

Figure 4: Location of chromosomal Tn5 mutations compared to *P. stutzeri* genome

Discussion

Although we were able to ameliorate the cost of the MP through Tn5 mutagenesis, we still were not able to fully achieve the original Tn5 mutant phenotype at 35°C with a pure background and just the Tn5 mutation. This reason can only be explained by multiple mutations in the mutant's genome that allowed it to grow better than the non-MP strain ancestor. When the Tn5 mutation was isolated using a plasmid prep mini kit and then plasmid ligation, the pure mutation DNA was sequenced using primers facing outward to identify the location of the mutation. These ligations were also conjugated back into the unmarked MP strain of bacteria. The usually used MP strain has a tetracyclin antibiotic gene located on the MP in order to differentiate it from the other MP strains and for further selection. The unmarked MP strain conjugated with the isolated Tn5 mutation was not able to grow at the same rate as the original Tn5 mutants at 35°C. This forces us to believe that because only the Tn5 mutation was conjugated on these last MP strains, that before there was another mutation that assisted with the elevated growth at elevated temperature and the alleviation of the cost of the MP. The Tn5 transposon has a kanamyacin (kan) resistance gene included in it, so the Tn5 mutants were all grown up on kan plates at 37C. This is relevant because it is possible that the Tn5 is only responsible for the strain to be able to grow on kan plates, and not that they are able to grow at elevated temperatures. It would be theoretically possible to just streak a bunch of MP bacteria on an LB rif plate and allowed to grow at 37C, and the colonies that successfully grow most likely have had a mutation arise that allows for growth at elevated temperature that would normally not occur. This technique is not favorable because it would be hard to find where the mutation is located, as we know the sequence of the primers flanking the Tn5. Although Tn5 mutagenesis is a very usefully tactic that has provided us with important information regarding the cellular transduction pathway of the MP bacterial strains at elevated temperatures, it has not ultimately lead us to the absolute reason on why our initial mutants displayed the favorable phenotype we were looking for.

More research will be done on the MP as it is still a young, novel discovery in the grand scheme of things. With that being said, the mutant that knocked out the histidine kinase enzyme is of great interest and importance because it is an important enzyme that is used in many cell-signaling transduction pathways. Thinking critically and analyzing the parameters, knocking out one step wouldn't necessarily change an entire process from occurring biologically, but knocking out one step important step is able to prevent pathways from occurring downstream, and can hinder or halt the end product protein or pathway. From the few competitions that we have performed at this point but have not included in this paper, only conjugating the histidine kinase mutant allele into the bacterial genome does not have the same phenotypic effect or results that we had hoped for, and had initially observed when we performed the Tn5 mutagenesis. Another mutation must have been present, if not several mutations, in the initially obtained mutants when Tn5 mutagenesis was first performed. Tn5 mutagenesis occurs at random locations in the genome, so it is both probable and possible for the technique to have caused multiple mutations in a single bacterium's DNA, but because the transposon wasn't found anywhere else in the mutants' genomes when they were sequenced the likely other mutations present were caused by growing at elevated temperatures. Obviously, the bacteria that had essential important genes get knocked out or thermal tolerance genes get knocked out were selected against, and were not able to survive in the elevated growth chamber.

The future path of this overall research project is to keep trying to understand the importance or reason why this plant-infecting bacterium harbors the MP. There are multitudes of answers, and with no other research being performed on the large circular DNA entity it will take great efforts from my lab to start pressing forward. At this point in time, my PI is applying for grants for further funding to study the MP. Further applications of the MP are relatively speculative and unknown, but it could possibly be very important towards the study of microbiology and can answer more questions about HT that scientists have had. It is very interesting that a bacterium would harbor something that hinders its growth rate, and especially at a temperature that it is usually known for growing well at (Baltrus). Even more curious that the ancestor strains in which the MP was found in are not able to be manipulated into shedding the MP. It is possible that there is an addictive effect, where evolution has somehow generated genes on the chromosome for toxic products, and the MP contains genes that stop them from being translated or encode for antitoxin elements. Thus, if the ancestor strain were to get rid of the MP, then it would suffer from the absence and not be able to survive. With so little information known at this point about the MP, any ideas on why the MP exists are simply supposition. In order to discover its purpose, stress test must be performed (such as this thesis) and simple characteristics must be documented to research the MP further.

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