EVALUATING PHENOTYPIC EXPRESSION OF SELECTED GENES ON PBASL58 MEGAPLASMID IN *PSEUDOMONAS*

By

MADISON ANNE MOLLICO

_____

A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Biochemistry

THE UNIVERSITY OF ARIZONA

M A Y 2 0 2 1

Approved by:

_____

Dr. David Baltrus

Abstract

Horizontal gene transfer (HGT) is the exchange of genetic material between bacterial cells. Megaplasmids, which are extrachromosomal DNA over 350 kilobases in length, can be shared between bacteria in a population through HGT. The megaplasmid pBASL58, discovered in Leaf58 *Pseudomonas* first found on *Arabidopsis* plants, contains genetic information that appears to be redundant with the bacterial chromosome (1). It is hypothesized that pBASL58 must confer fitness benefits that justify the energetic cost of plasmid retention. One such fitness benefit is a type 1F clustered, regularly interspaced, short palindromic repeat (CRISPR) system. Plasmid DNA that is transferred by HGT may be targeted by CRISPR-associated proteins, which cut DNA to prevent replication of foreign material. pBASL58 encodes the only known type 1F CRISPR system contained entirely on a megaplasmid in Gram negative bacteria. Here, we evaluate the phenotypic expression of this CRISPR system as well as a tmRNA ribosome rescue system that is also encoded on pBASL58. Such ribosome rescue systems mark and dispose of peptides that have stalled ribosomes (2). For normal cell growth and activity, the nonfunctional peptide sequence must be degraded in order to free up the ribosome for translation of more mRNA. To evaluate expression of the CRISPR system, target plasmids were created based upon the target sequences encoded in the CRISPR loci. Triparental conjugations were used to test whether CRISPR associated (Cas) proteins would cut target DNA and thus prevent replication. The tmRNA ribosome rescue system's phenotypic expression was evaluated by comparing deletion mutants of the corresponding *sspB* gene to wildtype strains in a motility assay. There is no evidence that the CRISPR system is actively being transcribed and translated in laboratory conditions. However, deletion of the transposon *sspB* gene reduces the growth of *Pseudomonas*

over a 24-hour period, possibly indicating a fitness benefit for retention of the pBASL58 megaplasmid in *Pseudomonas.*

Introduction

Megaplasmid are circular, extrachromosomal DNA found in some bacteria. By definition, they are at least 350 kilobases in length (3). Megaplasmids function as secondary replicons, whereas the bacterial chromosome functions as the primary replicon and encodes essential core genes. As such, megaplasmids are not typically essential for viability in most bacterial growth conditions. However, there must be a fitness benefit associated with the megaplasmid to justify the energy expenditure of its replication. Through horizontal gene transfer (HGT), megaplasmids can be exchanged between bacteria. HGT is therefore a means of adaptation to an environment in which a megaplasmid will confer fitness benefits.

The megaplasmid pBASL58 was found in a strain of *Pseudomonas* closely associated with the species *Pseudomonas putida*. It was discovered and characterized by Dr. Brian Smith through BlastP searches that revealed a link to the previously published megaplasmid pMPPla017 (1,4). The two megaplasmids shared functional pathways including genes for the maintenance and replication of the megaplasmid as well as some multidrug membrane efflux pumps. They also encode genes for some metabolic pathways, such as fatty acid and tRNA biosynthesis. The two megaplasmids share 57.6% of their genes but pBASL58 has 31 unique genes. Among these are a complete set of CRISPR loci and the stringent starvation protein B (*sspB*) gene, which is part of the ribosome rescue system.

CRISPR stands for clustered, regularly interspaced, short, palindromic repeats of DNA.

These unique loci contain genes for CRISPR-associated (Cas) proteins, which cleave foreign DNA, such as that of a bacteriophage, preventing replication and acting as a self-defense mechanism for the bacterium (5). The megaplasmid pBASL58 contains the only known type 1F CRISPR system contained entirely on a megaplasmid in *Pseudomonas* (1). A type 1F CRISPR system is defined by the lack of an independent small subunit of the DNA cutting complex as well as the fusion of *cas2* and *cas3* and the absence of *cas4*. Type 1F CRISPR systems can defend bacteria against DNA, but not RNA, phages (6).

The gene *sspB* is a transposable element, or transposon, meaning that it can be translocated throughout the bacterial chromosome and accompanying plasmids. It is found in bacterial genomes as well as in some chloroplast DNA (7). It encodes the stringent starvation protein B which activates ATPase activity in the ClpX-ClpP protease. This protease is responsible for degrading *ssrA*-tagged amino acids on stalled ribosomes (8). The *ssrA* gene encodes tmRNA, which functions as both a transfer RNA and a messenger RNA (9). The tmRNA serves as a tag on the truncated protein which signals for sspB to activate the ClpX-ClpP protease (10). This prevents ribosomes from permanently stalling. Ribosomes can stall when stop signals are absent, there is ribonuclease (RNAse) present, or when there is damage to the forming peptide. The tmRNA goes to the A site of a stalled ribosome and transfers the incomplete peptide to itself so that the ribosome can be recycled. The tmRNA system keeps protein production running efficiently and therefore keeps the cell growing and replicating at a normal rate. This process is termed ribosome rescue (11).

The degree to which genes of the pBASL58 megaplasmid are expressed remains unknown. Although the *Pseudomonas* strain can grow in a laboratory culture with or without the megaplasmid, some genes may be important for survival or defense in the natural world. This

study focuses on two systems encoded on pBASL58. The first is the stringent starvation protein which aids in disposal of peptide segments that have stalled during translation by a ribosome, rendering the ribosome useless and slowing the growth of the cell. The second is the type 1F CRISPR system that is completely encoded on the megaplasmid and similar to known bacterial systems of resistance against threats such as bacteriophages. The goals of this study are to determine whether these genes are active and the evolutionary advantage that they may confer to bacteria that take up the megaplasmid.

In order to evaluate the role of *sspB* in cell growth, the gene was deleted from the megaplasmid using gateway cloning and *tetA/sacB* screening. Then, a motility assay was used to compare the knockout strains to the wildtype and the positive control DBL1632, which has a similarly limited ribosome rescue system to that of the knockout strain. Growth on motility media is measured twenty-four hours after inoculation to compare growth rates of the different strains. If the growth of the transposon knockout strain is slower than that of the wildtype, this supports the hypothesis that the megaplasmid transposon functions to aid cell growth.

To evaluate the activity of the pBASL58 CRISPR system, target plasmids were created using gateway cloning. Two target plasmids exactly match the target sequences encoded within the CRISPR loci, while two others contain an altered version of the encoded target sequences. Each of the target plasmids contains the gene for resistance to the antibiotic kanamycin.
To evaluate whether the CRISPR system is translated into proteins that will cut target sequences, a triparental conjugation is performed. In this experiment, target plasmids are introduced into the megaplasmid-containing *Pseudomonas* by horizontal gene transfer. The growth of the *Pseudomonas* on selective media reveals whether the system has cut the target DNA and eliminated kanamycin resistance. If growth occurs on selective media after triparental

conjugation with CRISPR target plasmids, this indicates that the CRISPR system is not functional in preventing replications of plasmids that match the CRISPR target sequences. If there is no growth for exactly matching targets but growth for mismatched targets, this indicates functionality of the CRISPR system.

Methods and Materials

Creation of CRISPR Targets

CRISPR targets were designed to mimic spacer DNA sequences encoded on the pBASL58 CRISPR system. Two sequences that exactly match spacer regions (E1 and E3), as well as a mismatched version of the corresponding spacers (M1 and M3) were designed. These sequences were synthesized by Integrated DNA Technologies. The sequences were incorporated into *Escherichia coli* using Gateway cloning procedures. BP clonase enzyme was used to insert each sequence into the donor vector plasmid pdonr2017, which encodes gentamicin resistance. This donor vector was then transformed into *E. coli* using a 42°C heat shock. The *E. coli* were selected for gentamicin resistance and the efficacy of the BP reaction was confirmed via polymerase chain reaction (PCR) and DNA gel electrophoresis. LR clonase enzyme was used to insert the target sequence into the destination vector plasmid mtn41, which encodes kanamycin resistance. Transformation procedures were conducted as described and the *E. coli* were selected for kanamycin resistance. The success of the LR reaction was confirmed by PCR and DNA gel electrophoresis.

Triparental Conjugation

The following strains of bacteria were grown overnight: Leaf58 (the *Pseudomonas* carrying the pBASL58 megaplasmid), DBL1385 (*E. coli* conjugation helper strain), and E1, E2, M1, M3 cloned target plasmid *E. coli* strains. In a 1.65mL tube, 750µL of Leaf58, 350µL of DBL1385,

and 350μL of a target plasmid containing *E. coli* were combined and mixed by pipetting. The conjugations were plated on antibiotic-free LB agar plates and incubated at 27℃ for 24 hours. The resulting bacterial growth was scraped from the agar plate and transferred to a tube of 10mM magnesium chloride ($MgCl_2$). 10mM magnesium chloride was used to create a serial dilution of the conjugations, which was plated on LB agar with 50mM rifampicin, 30mM nitrofurantoin, and 50mM kanamycin. Rifampicin selects for pBASL58, nitrofurantoin selects against *E. coli* and kanamycin selects for the intact target plasmid.

Creation of sspB *Deletion Mutant*

Gateway cloning was used as previously described in *Creation of CRISPR Targets* to insert a DNA sequence with the *sspB* gene deletion and *tetA-sacB* to the mtn1907 destination vector plasmid. Transformation into *E. coli* was performed as previously described. Heat-shocked *E. coli* were cultured in antibiotic-free LB to allow the tetracycline resistance gene to be flipped out. The resulting *E. coli* was used as a donor strain in a triparental conjugation conducted as described in *Triparental Conjugation* to transfer the flip-out plasmid to Leaf58. The resulting *Pseudomonas* was screened for sucrose sensitivity (positive selection). The *Pseudomonas* was then screened for tetracycline sensitivity and sucrose resistance (negative selection).

Motility Assay

Motility assays were performed as previously described (12). Motility LB agar was prepared according to the following formula: 1.25g bacto tryptone, 0.625g bacto yeast extract, 1.25g sodium chloride, 0.625g bacto agar. The following strains of bacteria were grown overnight: deletion mutants, Leaf58 (negative control) and DBL1632 (positive control). Samples were adjusted to an optical density of 0.4 ±0.1 using 10mM magnesium chloride. 2 mL of molten motility media was dispensed into each well of a twelve-well plate. The media was allowed to

gelatinize but not solidify completely. The center of each well was inoculated using wooden sticks. The diameter of growth was measured 24 hours post-inoculation by hand.

Results

Triparental conjugations with exactly matching and mismatching CRISPR target plasmids produce the same amount of Pseudomonas *growth on selective media*.

The four target plasmids (E1, M1, E3, and M3) were successfully cloned to the mtn41 destination vector (Figure 1). After being introduced into the wildtype strain via triparental conjugation, growth of the resulting *Pseudomonas* was measured on kanamycin 50µg/mL LB agar plates and control LB agar plates. The target plasmids which exactly matched the pBASL58 encoded target sequences displayed the same level of growth as the mismatched target sequence plasmids (Figure 2).

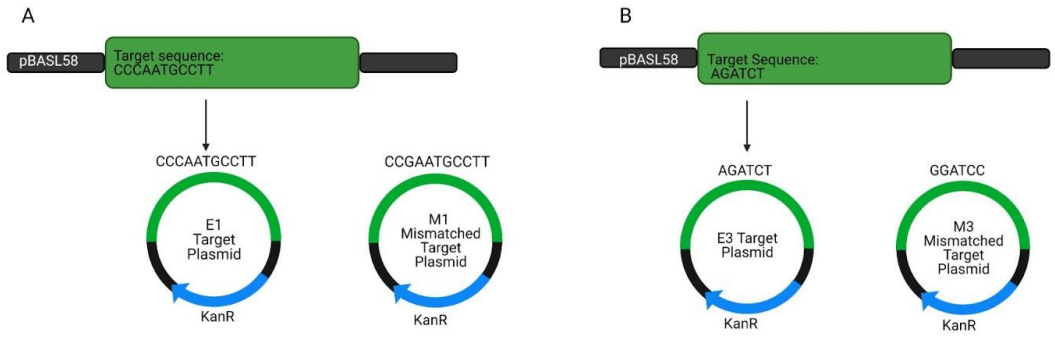

Figure 1: Target plasmid design. Sequences from target regions of pBASL58 CRISPR system were successfully cloned to the mtn41 destination vector plasmid. (**A**) Target sequence 1 and the exactly matching target plasmid E1 and mismatched target plasmid M1. (**B**) Target sequence 3 and the exactly matching target plasmid E3 and mismatched target plasmid M3. Target sequences are copied from the spacer regions of the pBASL58 sequence for E plasmids. Target sequences are slightly altered spacer regions of pBASL58 for M plasmids. Green regions represent inserted sequences. Blue regions represent Kanamycin resistance genes. Image created with BioRender.

Each conjugation resulted in significant growth on LB media which contained only rifampicin and nitrofurantoin. The wildtype Leaf58 is resistant to both of these antibiotics. When exposed to kanamycin, each conjugation grows significantly less. The E1 and E3 conjugations have the same amount of growth as the M1 and M3 conjugations on kanamycin. This shows that the CRISPR system is not targeting DNA that matches its spacer regions for cleavage.

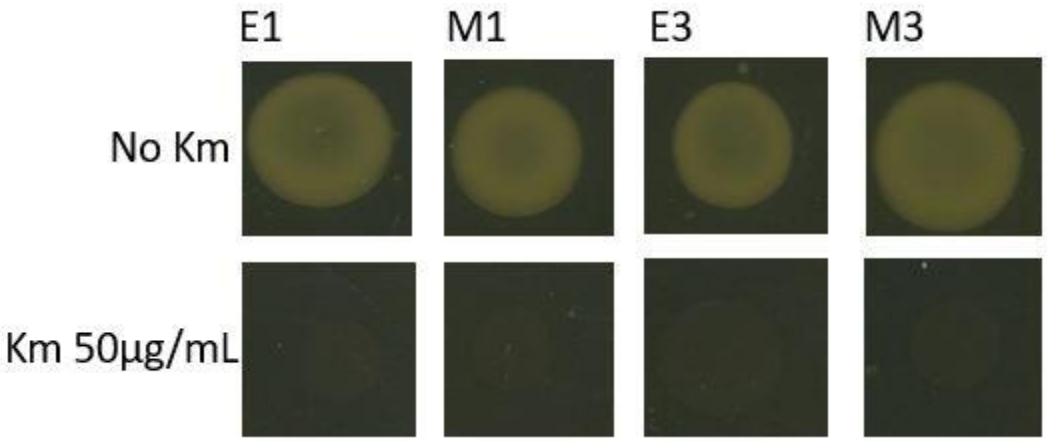

Figure 2: pBASL58 CRISPR system does not act on target plasmids. Kanamycin inhibits the growth of *Pseudomonas*.Without kanamycin, there is significant growth for all four conjugations. Under kanamycin conditions, each displays less growth, but all four conjugations have some growth on kanamycin LB. There is no difference in the inhibition of *Pseudomonas* conjugated with exactly matching targets or mismatched targets under conditions of 50µg/mL of kanamycin media. The pBASL58 CRISPR system is not acting upon its encoded target sequences.

sspB deletion reduces the growth of Pseudomonas *over a 24-hour period.*

Wildtype pBASL58-containing *Pseudomonas* grows to an average of 1.36 centimeters 24 hours after inoculation to motility LB media. In contrast, the *sspB* knockout strain grows to an average diameter of 0.96 centimeters 24 hours post-inoculation (Figure 3). The *sspB* knockout strain grows to a diameter similar to that of DBL1632, the positive control which has a ribosomal

rescue system disabled by transposon activity (p=0.891). DBL1632 and the *sspB* knockout both grow significantly less than the wildtype Leaf58 ($p<0.001$ for each). There is variation in the growth rate for both the wildtype and knockout strain that arises from slight variations in technique on the days that the assays were conducted (Table 1). The deletion of *sspB* cripples the ribosome rescue system encoded on pBASL58. Stalled ribosomes remain inactive, slowing the process of protein synthesis and ultimately slowing the growth and replication of the *Pseudomonas.*

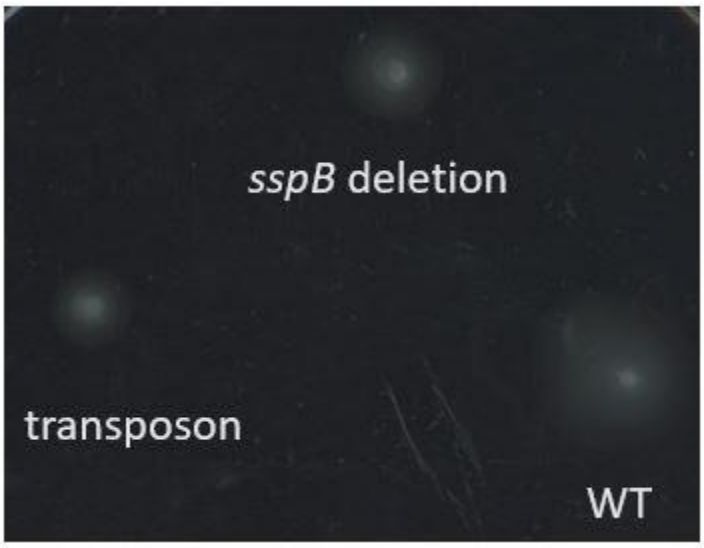

Figure 3a: *sspB* deletion inhibits growth of *Pseudomonas*. Growth of wildtype *Pseudomonas,* transposon strain, and *sspB* deletion after 24 hours on motility LB media. The diameter of *Pseudomonas* growth in motility media is reduced when the *sspB* gene is deleted. The effect is comparable to that of the positive control DBL1632, which also has a diabled ribosome rescue system.

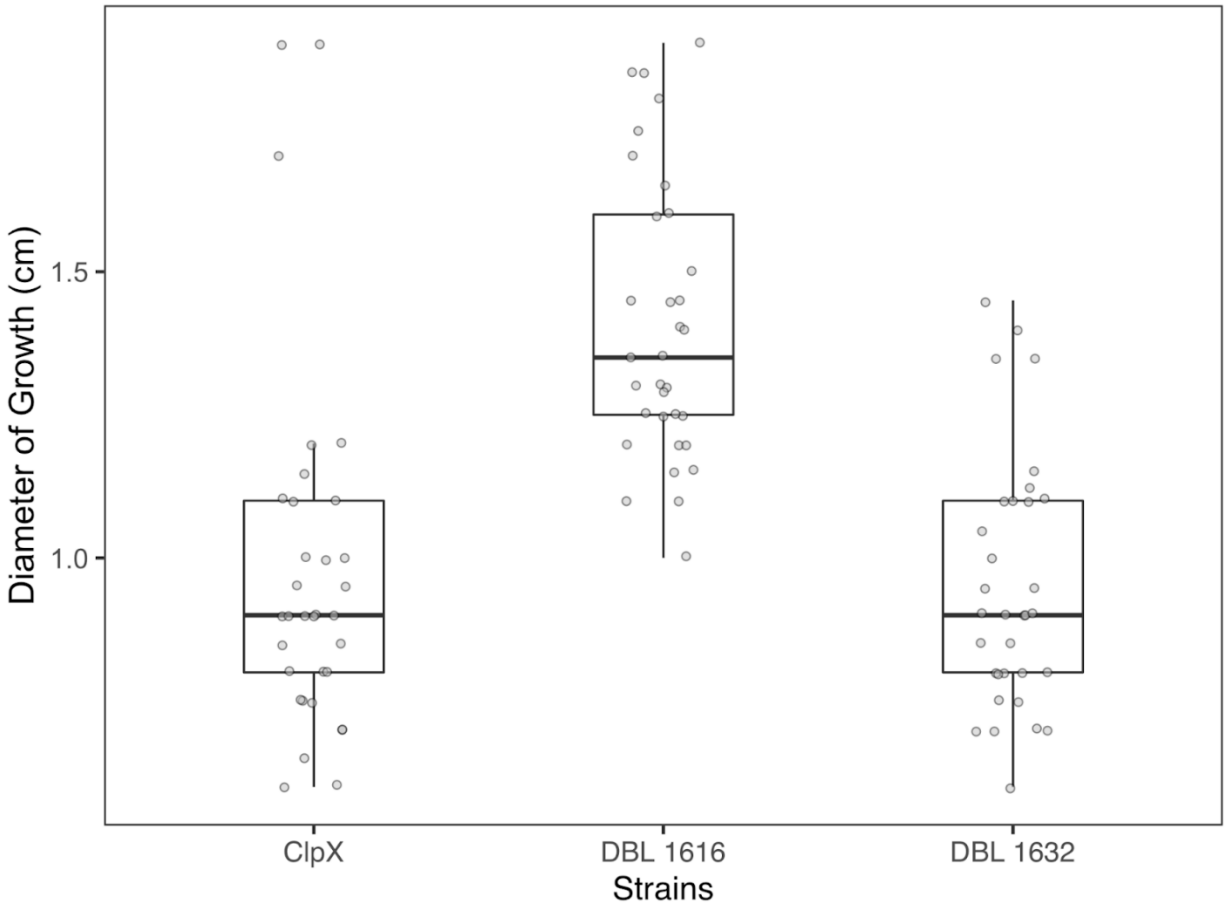

Figure 3b: Growth of *Pseudomonas* over a 24 hour period. "ClpX" refers to the *sspB* deletion mutant. DBL1616 is the wild type *Pseudomonas*, Leaf58. DBL1632 is the positive control, the *Pseudomonas* strain with a disabled ribosome rescue system. There is a statistically significant difference in the growth of the two controls, DBL1616 and DBL1632 ($p<0.0001$). The *sspB* deletion mutant grows to a similar diameter as the positive control ($p=0.891$). The *sspB* gene deletion decreases the growth of Leaf58 significantly ($p<0.001$ for ClpX to DBL1616 comparison).

Table 1: Variation in Growth between Assays

	Day 1 Average	Day 2 Average	Day 3 Average	True Average
Leaf 58	1.46	1.50	1.20	1.39
sspB knockout	1.18	0.90	0.82	0.96

Disabled ribosome rescue systems result in de novo *mutations over a 48 hour period.*

After 48 hours of growth on motility LB media, both the *sspB* knockout strain and the positive control transposon strain DBL1632 display unique growth patterns. In contrast to the crisp, circular growth of the wildtype strain, DBL 1632 and the *sspB* knockout have irregularly shaped growth that branches off from the circular growth that is closest to the inoculation site. These growths represent *de novo* mutations in *Pseudomonas* that allow it to grow nearly as rapidly as the wildtype despite the inactivation of the ribosome rescue system.

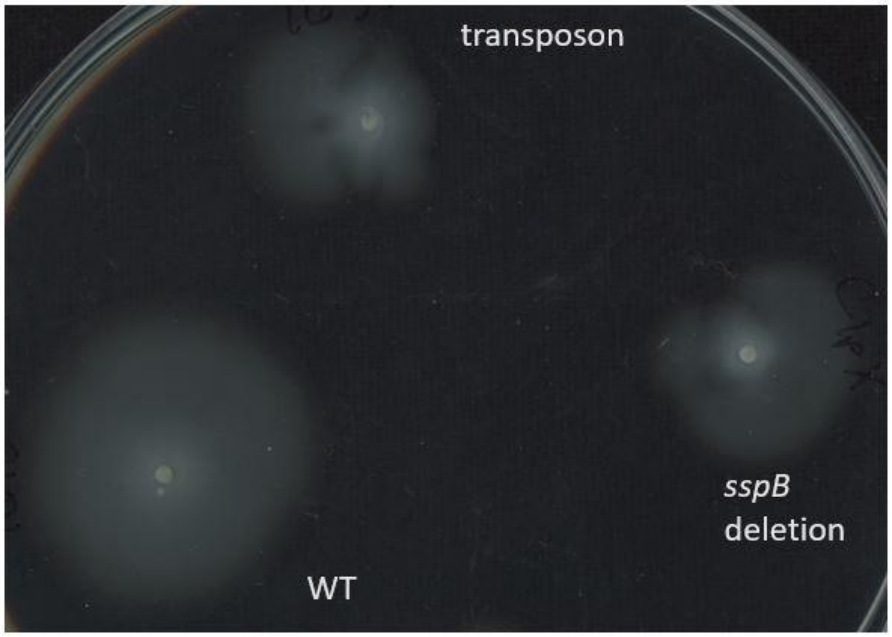

Figure 4: *De novo* mutations in *sspB* deletion and transposon strains increase motility. Growth of wildtype *Pseudomonas,* transposon strain, and *sspB* deletion after 48 hours on motility LB media. The wildtype *Pseudomonas* grows evenly, in a near-circular shape. Both the transposon strain and the *sspB* deletion strain have limited growth. They display irregularly shaped growth, indicating the presence of new mutant strains that have acquired increased motility.

Discussion

Understanding the role of megaplasmids can unlock new insights into bacterial ecology. Examination of the phenotypic expression of a megaplasmid gives insight into the reasons that megaplasmids were evolved and why they continue to be incorporated into some bacterial genomes. In this study, I sought to find evidence of phenotypic expression of the pBASL58 megaplasmid. These results do not provide evidence for functionality of the type 1F CRISPR system. However, there is clear evidence that the *sspB* gene involved in the ribosome rescue system is active and contributes to the growth and reproduction of the *Pseudomonas* which carries the pBASL58 megaplasmid. This is one of potentially many fitness benefits that are conferred by pBASL58.

This megaplasmid pBASL58 encodes 996 genes (1). Clearly, there is more information to be gathered about the functionality of these genes and their phenotypic expression. It will remain a rich source of information about bacterial ecology and potentially a standard that can be used to understand other megaplasmids that have yet to be discovered.

The type 1F CRISPR system encoded on this megaplasmid should continue to be examined for functionality. Although it is non functioning under standard laboratory conditions, it may be activated under circumstances similar to the natural environment of the Leaf58 bacteria. Leaf58, the original strain of *Pseudomonas* found to contain the pBASL58 megaplasmid, was discovered on *Arabidopsis* plants. Perhaps inoculation of Leaf58 onto *Arabidopsis* plants will create the proper conditions for its activation. Attempts to induce activity through altering the pH of growth media and the temperature of incubation have not resulted in any measurable results for CRISPR activity, but more studies should be conducted with varied environmental conditions.

It is clear from the data presented that the CRISPR system is nonfunctional. However, this does not necessarily mean that Cas proteins are not synthesized. Western blot analysis or mRNA sequencing have the potential to provide more insight into the level of phenotypic expression for this CRISPR system.

Should there be a method for manipulating activity for the pBASL58 CRISPR system, it could potentially be a useful alternative to agricultural antibiotics. The target sequences would be genetically engineered to match a part of the genome of pathogenic bacteria. All plants in a field could then be inoculated with a bacterial host for the modified CRISPR system that would be able to target and eliminate the threat. With this strategy, there is still the potential for bacterial resistance, but it would be a faster process to modify the CRISPR system targets than to design a new antibiotic drug in response to resistance. This technology could lead to higher crop yields.

The *sspB* gene will also continue to be studied. More consistent data should be gathered for the growth rates of the wildtype and knockout strains. Variations in the way each assay was conducted led to a range of growth rates for both strains. To obtain the most reliable data, it is important to inoculate the motility media at exactly the same consistency each time. Less solidified media at the time of inoculation leads to a wider diameter of bacterial growth. Additionally, it is important to use a reliable cell density meter, as any variation in the optical density of the culture will lead to inconsistent data. Despite some variation in the diameter of growth, the *sspB* knockout consistently grows less than the wildtype in a 24-hour period. Currently, there is work being done to develop a complement to the *sspB* knockout. The complement is expected to restore the growth rate to that of the wild type *Pseudomonas*, and this would be demonstrated through motility assays. This information would solidify our

understanding of the pBASL58 ribosome rescue system's contribution to the growth and replication of Leaf58 *Pseudomonas.*

Additionally, the sequence of the mutant *Pseudomonas* depicted in Figure 4 should be characterized to determine the genotypic change that has occurred to allow the bacteria to regain their motility despite the *sspB* gene knockout. The characterization of this compensatory process can reveal more information about how bacteria adapt to environmental change and continue to grow and reproduce, maximizing available resources.

Literature Cited

1. Brian A Smith, Courtney Leligdon, David A Baltrus, Just the Two of Us? A Family of *Pseudomonas* Megaplasmids Offers a Rare Glimpse into the Evolution of Large Mobile Elements, *Genome Biology and Evolution*, Volume 11, Issue 4, April 2019, Pages 1192–1206, https://doi.org/10.1093/gbe/evz066
2. Janssen BD, Hayes CS. The tmRNA ribosome-rescue system. Adv Protein Chem Struct Biol. 2012;86:151-91. doi: 10.1016/B978-0-12-386497-0.00005-0. PMID: 22243584; PMCID: PMC3358797.
3. diCenzo GC, Finan TM. The Divided Bacterial Genome: Structure, Function, and Evolution. Microbiol Mol Biol Rev. 2017 Aug 9;81(3):e00019-17. doi: 10.1128/MMBR.00019-17. PMID: 28794225; PMCID: PMC5584315.
4. Baltrus DA, Nishimura MT, Romanchuk A, Chang JH, Mukhtar MS, et al. (2011) Dynamic Evolution of Pathogenicity Revealed by Sequencing and Comparative Genomics of 19 *Pseudomonas syringae* Isolates. PLOS Pathogens 7(7): e1002132. https://doi.org/10.1371/journal.ppat.1002132
5. Makarova, K., Wolf, Y., Alkhnbashi, O. *et al.* An updated evolutionary classification of CRISPR–Cas systems. *Nat Rev Microbiol* **13,** 722–736 (2015). https://doi.org/10.1038/nrmicro3569
6. Buyukyoruk, M., Wiedenheft, B. Type I-F CRISPR–Cas provides protection from DNA, but not RNA phages. *Cell Discov* 5, 54 (2019). https://doi.org/10.1038/s41421-019-0123-9
7. Oudot-Le Secq, Marie-Pierre & Grimwood, Jane & Shapiro, Harris & Armbrust, E & Bowler, Chris & Green, Beverley. (2007). Chloroplast genomes of the diatoms

Phaeodactylum tricornutum and Thalassiosira pseudonana: Comparison with other plastid genomes of the red lineage. Molecular genetics and genomics : MGG. 277. 427-39. 10.1007/s00438-006-0199-4.

8. Wiegert T, Schuman W. SsrA-Mediated Tagging in *Bacillus subtilis.* Journal of Bacteriology Jul 2001, 183 (13) 3885-3889; DOI: 10.1128/JB.183.13.3885-3889.2001
9. Oh BK, Chauhan AK, Isono K, Apirion D. Location of a gene (ssrA) for a small, stable RNA (10Sa RNA) in the Escherichia coli chromosome. Journal of Bacteriology Aug 1990, 172 (8) 4708-4709; DOI: 10.1128/jb.172.8.4708-4709.1990
10. Lessner, F. H., Venters, B. J., & Keiler, K. C. (2007). Proteolytic adaptor for transfer-messenger RNA-tagged proteins from alpha-proteobacteria. *Journal of bacteriology*, *189*(1), 272–275. https://doi.org/10.1128/JB.01387-06
11. Keiler KC, Waller PR, Sauer RT. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science. 1996 Feb 16;271(5251):990-3. doi: 10.1126/science.271.5251.990. PMID: 8584937.
12. Baltrus DA, Dougherty K, Diaz B, Murillo R. Evolutionary Plasticity of AmrZ Regulation in *Pseudomonas*. *mSphere*. 2018;3(2):e00132-18. Published 2018 Apr 18. doi:10.1128/mSphere.00132-18