5-4-2010

Secondary Addiction: A Catch 22 Hypothesis

Katharina Van Santen

SECONDARY ADDICTION: A CATCH 22 HYPOTHESIS

KATHARINA VAN SANTEN

MAY 10, 2010

IN COMPLETION OF THE REQUIREMENTS FOR THE HONORS SENIOR PROJECT FOR THE HONORS PROGRAM AT THE UNIVERSITY OF ALABAMA IN HUNTSVILLE.

University Honors Program Research Project

APPROVAL PAGE

Student Name: Katharina van Santen

Department: Department of Biological Sciences

College: College of Science

Degree: Bachelor of Science

Project Advisor: Dr. Roy David Magnuson

Full title of project as it should appear in Graduation Program and transcript:

Secondary Addiction: A Catch 22 Hypothesis

Abstract:

For my Senior Research Project I worked with toxin-antitoxin systems in the *Escherichia coli* bacterium. Toxin-antitoxin systems are self-regulating protein-producing systems (genes) that invade a bacterium. The system produces a toxin and a neutralizing antitoxin. Toxin synthesis and degradation happens at a slower rate than antitoxin synthesis and degradation. Therefore, under normal conditions, there is always enough antitoxin to neutralize the toxin. If the toxin-antitoxin genes are lost, the continuing degradation of the antitoxin will eventually give rise to excess toxin—killing the cell in a processed dubbed "Plasmid Addiction".

We have recently found new evidence that the antitoxin itself could also be harmful to the cell. If the toxin producing part of the system is somehow lost, the antitoxin becomes over-expressed, causing the bacterium to die. My research was centered on finding out more information on this effect, which we have called "Secondary Addiction" (Sad). This study focused on finding out which parts of the antitoxin were necessary for secondary addiction, as well as what other activities are related to secondary addiction.

Repression and DNA binding were found to be necessary for secondary addiction. Binding specificity, however, does not seem to play a role in secondary addiction.

Approved by:

Project Advisor: _____ (signature) Date: 2010.05.04

Department Chair: _____ (signature) Date: 5/4/10

University Honors Program Director: _____ (signature) Date: 5-11-10

ACKNOWLEDGEMENTS

I have several people to thank for making this thesis possible. First, I'd like to thank my parents for showing me the joys of science, and supporting me in my endeavors. I'd like to thank Dr. Roy Magnuson, and all the recent members of Magnuson lab: Ram, Nikki, and Bobbi. Without them teaching me the ropes of biology research, I wouldn't be here today. I'd like to thank all the professors at UAH who influenced me in my life as a student and beyond, especially Dr. Bob Lawton and Dr. Maria Davis. I'm able to go on to grad school next year because of these amazing professors. And lastly, I'd like to thank all my geeky science friends. I expect PhD's from every one of you in the coming years. We must continue to push each other to achieve extraordinary things.

TABLE OF CONTENTS

List of Figures iv

List of Tables v

List of Abbreviations vii

Single Letter Amino Acid Code viii

ABSTRACT 1

INTRODUCTION 2

METHODS 8

RESULTS 12

DISCUSSION 24

REFERENCES: 30

LIST OF FIGURES

Figure 1: Normal growth of plasmid-free and plasmid-containing cells showing vertical transmission of the plasmid. Plasmid Addiction: killing of plasmid-free segregants. Larger ovals represent bacteria, smaller ovals represent plasmids, and arrows represent cell division..... 3

Figure 2: Our toxin-antitoxin system: phd/doc. This figure shows the regulatory processes of the system and the neutralization of the toxin under normal conditions. Free toxin leads to growth inhibition..... 5

Figure 3: Catch-22 Hypothesis. Once the toxin-antitoxin system has been acquired it cannot be lost. If part or the entire system is lost, cell death will occur in one of three ways: death by toxin, death by addiction, or death by secondary addiction. Here, A indicates the antitoxin, phd. T indicates the toxin, doc..... 7

Figure 4: Serial Dilutions: showing the steps in doing a serial dilution and example resultant plates from each tube. Turbid overnight broth is serially diluted with 0.85% saline solution. The number on the tube indicates the tube dilution which is used, with individual colonies counted from plates, and the amount plated, to calculate the colony forming units..... 10

Figure 5: Efficiency of plating of the wild type Phd and the vector plasmid at low induction and high induction. Wild type Phd was positive for secondary addiction, while the vector was negative for secondary addiction. Error bars indicate the standard deviation of the efficiency of plating results..... 13

Figure 6: Efficiency of Plating of selected mutants. Error bars indicate the standard deviation..... 22

Figure 7: Repression data for selected (with Phd shown for the positive control for secondary addiction). Percent unrepressed is shown on the x-axis..... 26

LIST OF TABLES

Table 1: The average log efficiency of plating and standard deviation at medium induction for mutants A36H-K49A. Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, ++ for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test. 17

Table 2: The average log efficiency of plating and standard deviation at medium induction for mutants A50D-Δ(50-73). Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, + for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test. 18

Table 3: The average log efficiency of plating and standard deviation at high induction for mutants A36H-K49A. Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, ++ for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. 19

Table 4: The average log efficiency of plating and standard deviation at high induction for mutants A50D-Δ(50-73). Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, * for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test. 20

Table 5: The average log efficiency of plating and standard deviation at medium and high induction of vector plasmid and all mutants that were SAD- at either medium or high induction 21

LIST OF TABLES

Table 6: Repression data for Ram's C-terminal mutants 23

Table 7: James McKinley's Repression activity data showing the construct, percent unrepressed, standard deviation, and repression activity. 25

Table 8: Repression activity (not induced) and Secondary Addiction for all mutants at medium induction(50 μM IPTG). 27

Table 9: Repression activity (not induced) and Secondary Addiction for all mutants at high induction (500 μM IPTG) 28

LIST OF ABBREVIATIONS

aa	amino acids
A	Antitoxin
AK	Ampicillin and Kanamycin
E. coli	*Escherichia coli*
ClpXP	ClpXP Protease
DNA	Deoxyribonucleic acid
EOP	Efficiency of Plating
phd	*Prevents Host Death*
doc	*Death on Curing*
LB	Luria Broth
IPTG	Isopropyl-β-D-thiogalactopyranoside
℃	Degrees Celsius
Sad	Secondary Addiction
SD	Serial Dilution
T	Toxin
Wt	Wild Type
mm	Milliliters
mL	Millimeters
μM	Micro Molar (10^{-6} mols)
μL	Micro Liter (10^{-6} L)

SINGLE LETTER AMINO ACID CODE

A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glysine
H	Histamine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

ABSTRACT

For my Senior Research Project I worked with toxin-antitoxin systems in the *Escherichia coli* bacterium. Toxin-antitoxin systems are self-regulating protein-producing systems (genes) that invade a bacterium. The system produces a toxin and a neutralizing antitoxin. Toxin synthesis and degradation happens at a slower rate than antitoxin synthesis and degradation. Under normal conditions, there is always enough antitoxin to neutralize the toxin. If the toxin-antitoxin genes are lost, the continuing degradation of the antitoxin will eventually give rise to excess toxin—killing the cell in a processed dubbed "Plasmid Addiction".

We have recently found new evidence that the antitoxin itself could also be harmful to the cell. If the toxin producing part of the system is somehow lost, the antitoxin becomes over-expressed, causing the bacterium to die. My research was centered on finding out more information on this effect, which we have called "Secondary Addiction" (Sad). This study focused on finding out which parts of the antitoxin were necessary for secondary addiction, as well as what other activities are related to secondary addiction.

Repression and DNA binding were found to be necessary for secondary addiction. Binding specificity, however, does not seem to play a role in secondary addiction.

INTRODUCTION

Toxin-antitoxin systems are self-regulating protein-producing systems (genes) that invade a bacterium in the form of a plasmid. Before acquiring the toxin-antitoxin system, the bacterium has its normal fitness. Once the bacterium has acquired the toxin-antitoxin system, it is not really harmed by the system, except for the cost of maintaining the system. The plasmid can be transferred vertically to daughter cells and horizontally through gene transfer (Jensen 1995).

There are many toxin-antitoxin systems with similar behaviors. Our system is the Phd/Doc system located on the P1 operon in *Escherichia coli*. The low copy number plasmid is easily acquired through horizontal gene transfer or vertical transmission from a parent cell. The plasmid is not so easily lost, however, due to its addictive properties *(Figure 1)*. Plasmid free segregants are killed or severely inhibited, a phenomenon known as plasmid addiction (Lehnherr and Yarmolinsky 1995).

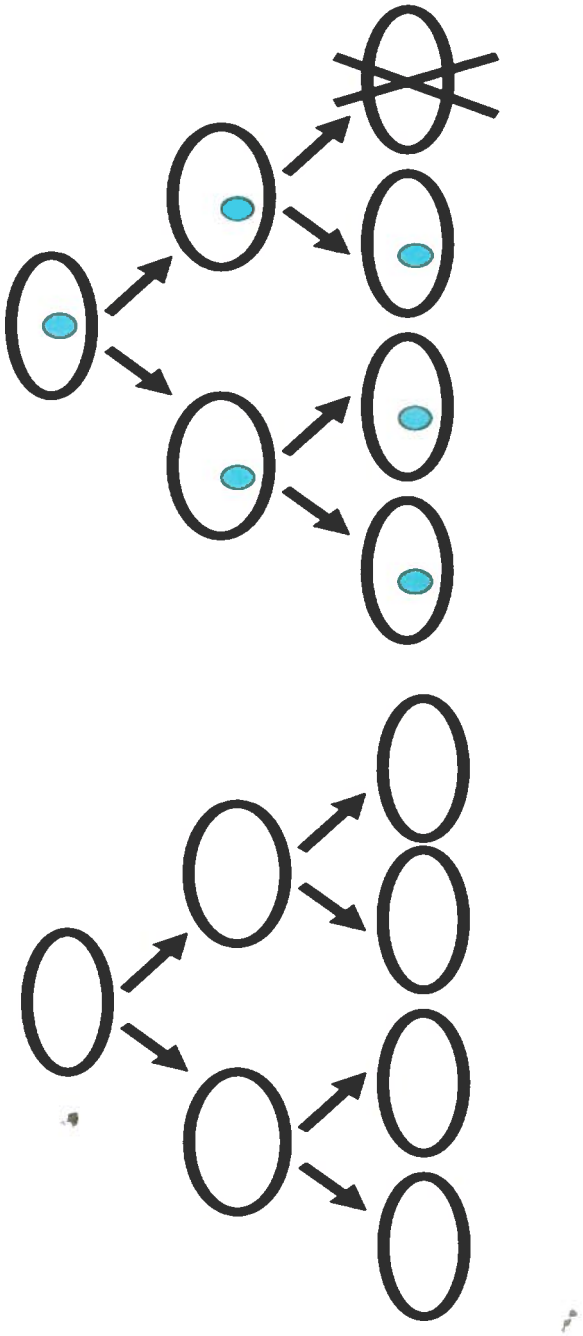

Figure 1: Normal growth of plasmid-free and plasmid-containing cells showing vertical transmission of the plasmid. Plasmid Addiction: killing of plasmid-free segregants. Larger ovals represent bacteria, smaller ovals represent plasmids, and arrows represent cell division.

Our system is a self-regulating system consisting of two parts, Phd and Doc. Doc, which stands for 'Death on Curing', is our toxin. Phd stands for 'prevents host death', and acts as our antitoxin. Its responsibility is to neutralize the toxin that is being produced by Doc. There are several known functions of Phd: binding and neutralization of Doc, dimerization, DNA binding, and regulation of transcription. While the entire plasmid is in place, the antitoxin and toxin are regulating their own production. The antitoxin is able to neutralize the toxin, and the cell remains functional. However, if the plasmid is lost, the slower degradation rate of the toxin will allow the excess toxin to kill the cell *(Figure 2)*. The addiction can be lost in one of two ways: loss of the plasmid (segregation), or by a mutation.

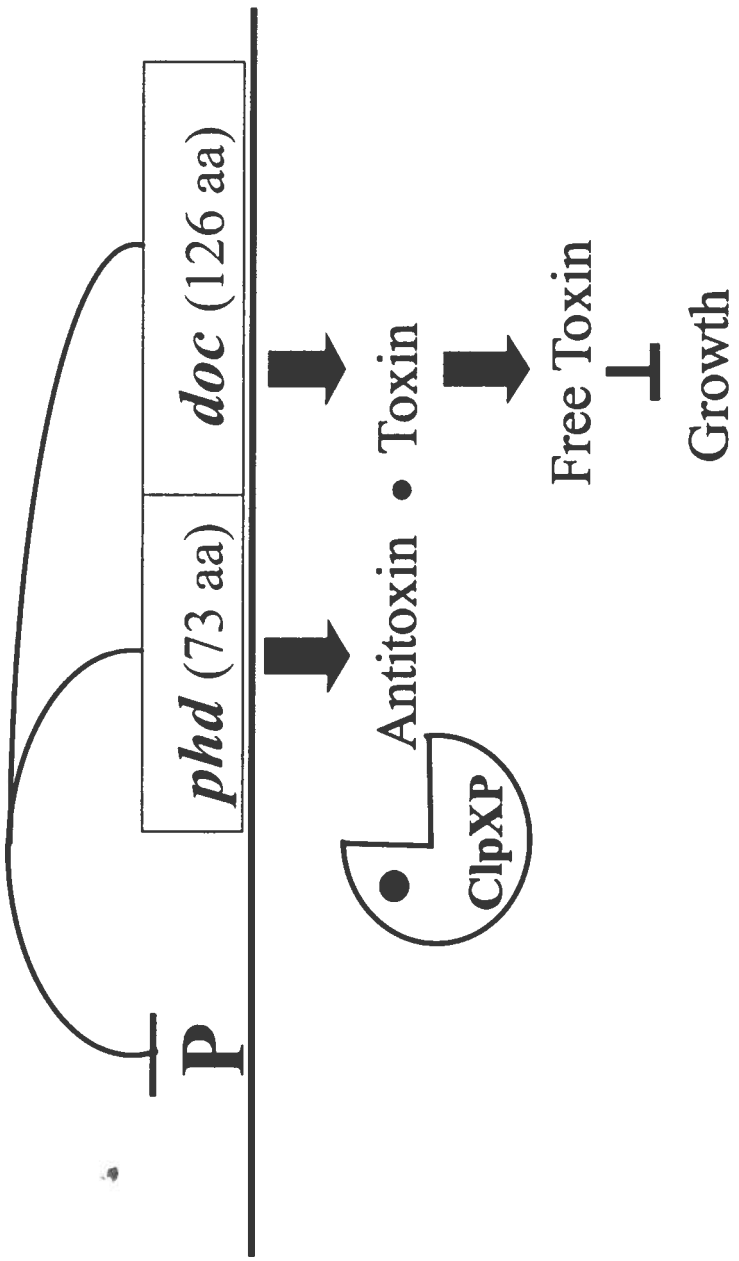

Figure 2: Our toxin-antitoxin system: phd/doc. This figure shows the regulatory processes of the system and the neutralization of the toxin under normal conditions. Free toxin leads to growth inhibition.

There are three ways in which cell death due to plasmid addiction can occur.

1. The bacterium can have death by addiction. Here, the entire toxin-antitoxin system is lost, by mutation or segregational loss. The continuing degradation of the antitoxin will leave an excess of toxin, arresting the cell.
2. The bacterium could lose just the antitoxin part of the system. This would lead to continued production of toxin, and cell death.
3. The toxin part of the system could be lost. Loss of just the toxin would interrupt the regulatory aspects of the system, causing the antitoxin to become over-expressed. We call this "secondary addiction."

We are interested in the third case. We hypothesize that the over-expression of the antitoxin could be toxic to the cell. We have dubbed this phenomenon "Secondary Addiction." Secondary Addiction is the final puzzle piece in our Catch-22 Hypothesis. That is, once a bacterium has acquired the toxin-antitoxin system it will not be able to lose it. If part of all of the system is lost, the bacterium will die either from the toxin, from addiction, or from secondary addiction *(Figure 3)*.

This "Secondary Addiction" is the focus of my research. Specifically: *Is over-expression of the antitoxin toxic to the cell?* My follow up question was: *How does the antitoxin kill the cell?* This thesis addresses these two focus questions.

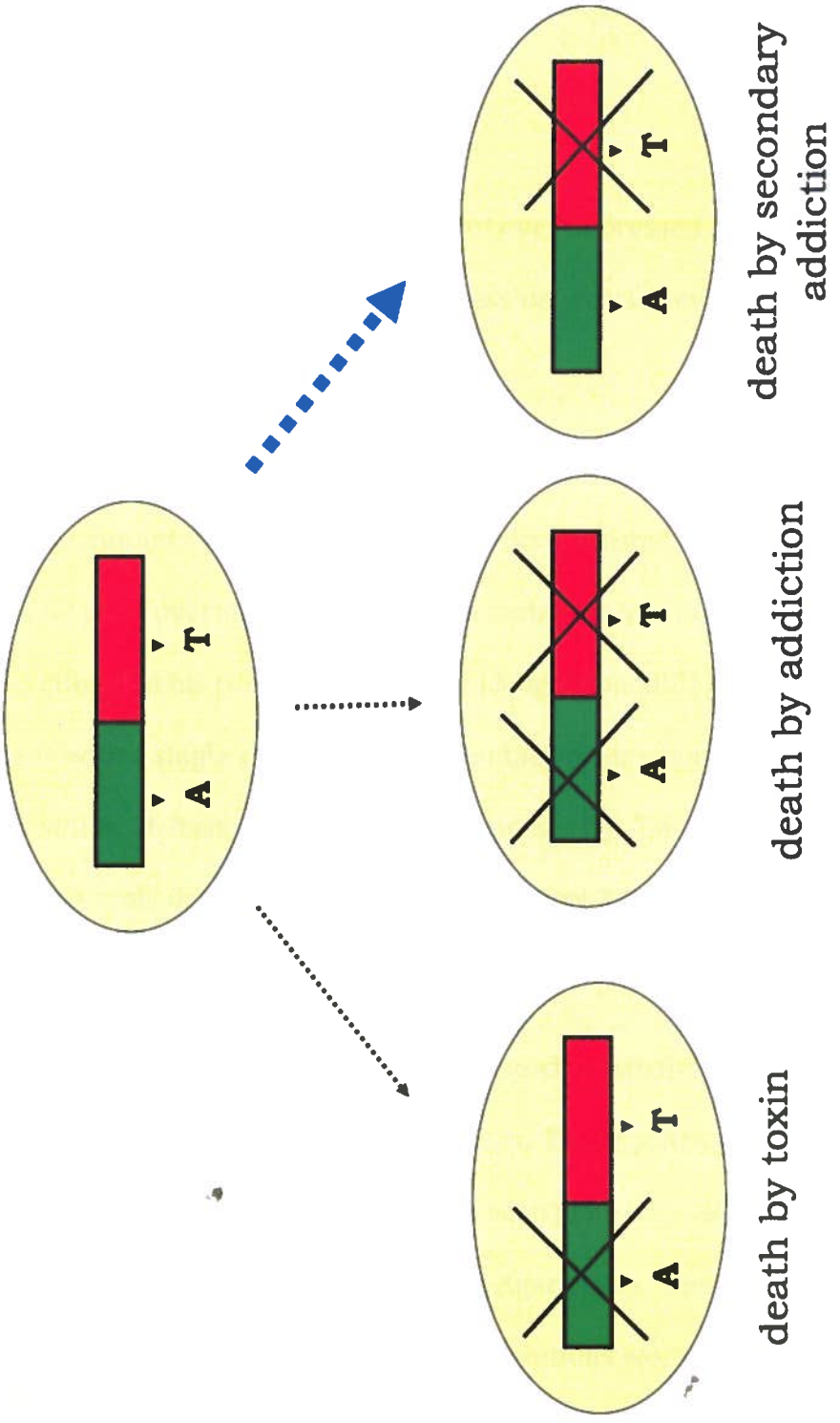

Figure 3: Catch-22 Hypothesis. Once the toxin-antitoxin system has been acquired it cannot be lost. If part or the entire system is lost, cell death will occur in one of three ways: death by toxin, death by addiction, or death by secondary addiction. Here, A indicates the antitoxin, phd. T indicates the toxin, doc.

METHODS

Preparation

This experiment required comparisons of over-expressed antitoxin with normal expression of antitoxin. This over-expression was achieved using Isopropyl β-D-1-thiogalactopyranoside (IPTG).

To obtain the *Escherichia coli* necessary for this experiment, freezer stocks of previously made mutants were used. Freezer stocks consisted of 250 μL 50% glycerol and 750 μL of overnight culture. These mutants were made by James McKinley as outlined in his paper (McKinley and Magnuson 2005). Mutants were generally made with a single amino acid point mutation, for example A36H is a point mutation at position 36 from Alanine (A) to Histamine (H). Two of the mutants consisted of large-scale deletions, a deletion of positions 2 through 29 is denoted phd Δ(2-49).

Isolation streaks of strains from a freezer stock consisting of were streaked out onto LB Ampicillin Kanamycin (AK) Agar plates. These plates were allowed to grow for 24 hours at 30°C. A single colony from each plate was selected and used to make an isolation streak on another set of LB AK Agar plates. These plates were allowed to grow for 24 hours at 30°C. Overnight solutions were made from the second set of plates. Overnight solutions consisted of 5 mL of LB AK broth with one colony of each strain inoculated into the corresponding test tube. Control tubes containing just LB AK broth were also made to ensure there was no contamination

of the broth. Overnight solutions were allowed to grow for 16 hours at 30°C in a test tube roller drum. Serial dilutions were performed on each strain.

Serial Dilutions

To prepare the serial dilutions, nine 13 x 100 mm test tubes were used for each overnight culture. Tubes were labeled 1 through 9. In tubes 2 through 9, 900 µL of 0.85% saline solution was added. The overnight culture was vortexed and 1.0 mL was added to tube 1. Tube 1 was then vortexed, and 100 µL was pipetted from tube 1 to tube 2. Tube 2 was then vortexed, and 100 µL was pipetted from tube 2 to tube 3. This process was repeated until 100 µL had been pipetted from tube 8 to tube 9 *(Figure 4)*.

Serial dilution solutions were then plated on the appropriate LB plates. Three types of plates were used. LB AK plates, LB AK and 50 µM IPTG plates, and LB AK and 500 µM IPTG plates. Each dilution of each strain had 100 µL plated on all three types of plates in order to determine the plating efficiency. Plates were allowed to grow at 30° C for approximately 48 hours. Colonies were then counted on plates containing 20 to 200 colonies. The colony forming units (CFUs) were calculated by multiplying the number of colonies by the tube dilution, and then dividing by the volume plated.

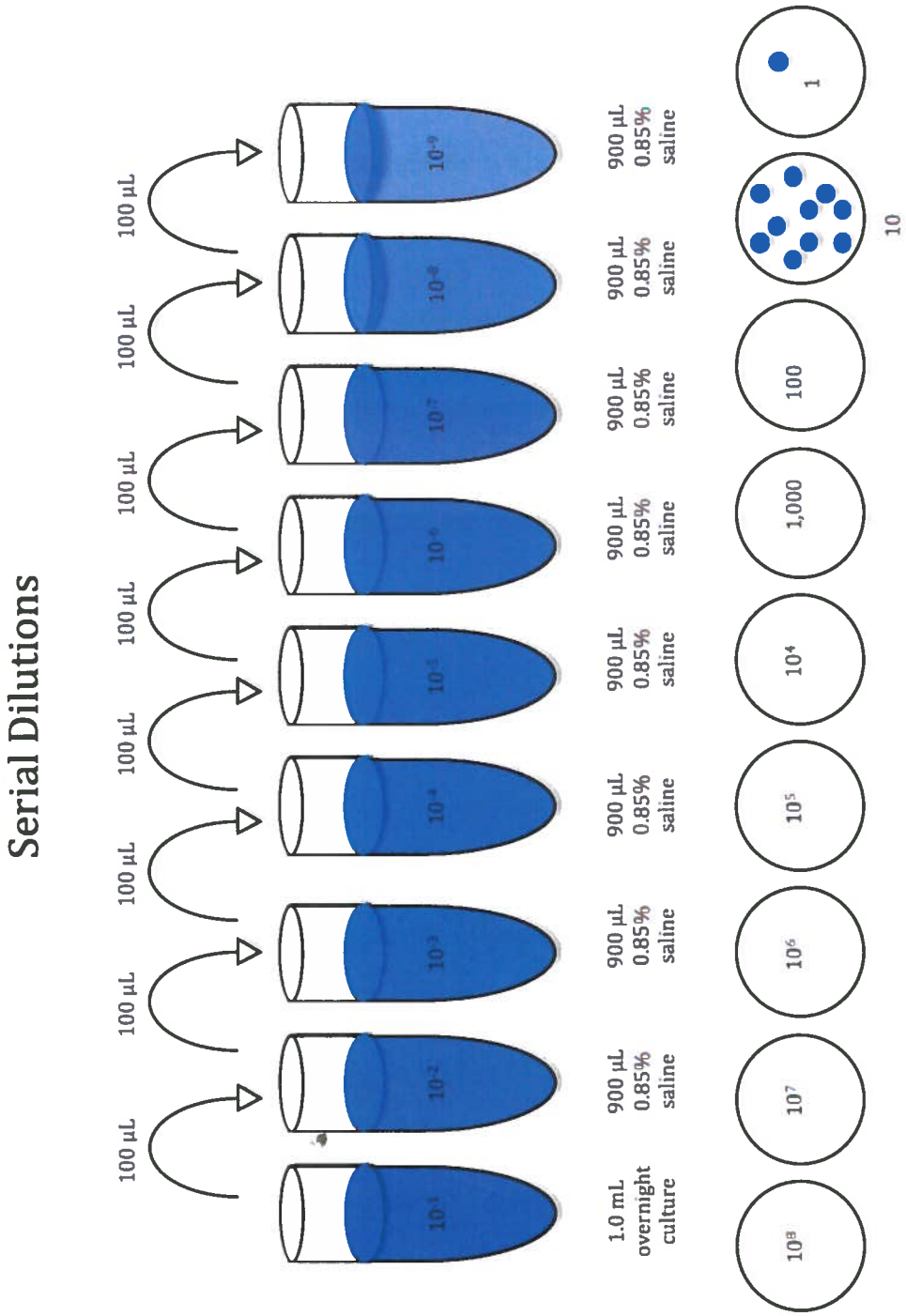

Figure 4: Serial Dilutions: showing the steps in doing a serial dilution and example resultant plates from each tube. Turbid overnight broth is serially diluted with 0.85% saline solution. The number on the tube indicates the tube dilution which is used, with individual colonies counted from plates, and the amount plated, to calculate the colony forming units.

Efficiency of Plating

In order to calculate the efficiency of plating, the CFUs of the 50 μM IPTG and 500 μM IPTG LB plates were compared with the CFUs of the plates lacking IPTG using the below equation:

$$\text{Efficiency of Plating} = \frac{\text{CFUs of over-expressed antitoxin (50 or 500 μM IPTG)}}{\text{CFUs of normal expression (no IPTG)}}$$

Efficiencies of plating were used, in the form of the log (efficiency of plating), to determine whether the strain showed the secondary toxicity effect.

RESULTS

Thirty-three mutants were tested for secondary addiction with two controls, for a total of thirty-five strains tested. The two controls were wild type Phd and pKK 223-3 (the vector plasmid). The wild type Phd acted as our positive control for secondary addiction: that is it was positive for killing. Wt Phd gave us an efficiency of plating of approximately 1.22×10^{-4} at medium induction (50 μM IPTG) and 8.36×10^{-5} at high induction (500 μM IPTG). In wt Phd, for every 10,000 cells plated, only about 1 survived, thus giving you a plating efficiency of approximately 1×10^{-4}. This shows that wt Phd is giving us secondary addiction. The vector plasmid acted as our negative control for secondary addiction. The vector gave us an efficiency of plating of approximately 9.15×10^{-1} at medium induction and 9.42×10^{-1} at high induction (Fig 5). Since the vector's efficiency of plating is close to 1, this means that IPTG itself has little to no effect on the viability of the cell. To compare the vector to the wt Phd, in the vector for every 10,000 cells plated approximately 10,000 were viable. In wt Phd, however, for every 10,000 cells plated, only about 1 cell was viable.

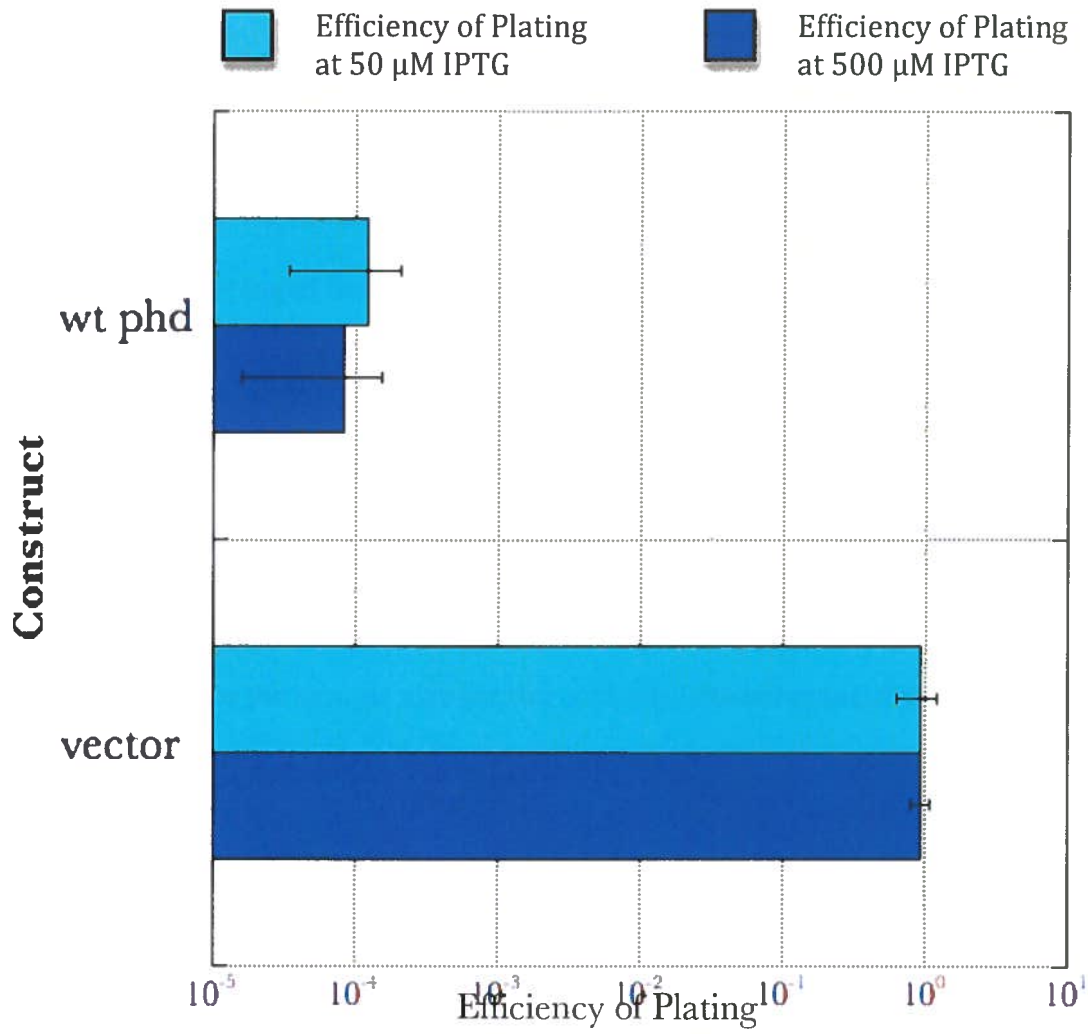

Figure 5: Efficiency of plating of the wild type Phd and the vector plasmid at low induction and high induction. Wild type Phd was positive for secondary addiction, while the vector was negative for secondary addiction. Error bars indicate the standard deviation of the efficiency of plating results.

In order to normalize the distribution of mutants, wt Phd, and vector plasmid, the log of the efficiency of plating was taken. This made the range of the data smaller, and easier to deal with. In order to compare mutants to wt Phd and vector plasmid, we used the average log of the efficiency of plating. The standard deviation of the log of the efficiency of plating was also used, as detailed below.

Mutants were statistically compared to the wt Phd and vector plasmid using the Student's two-tailed t-test. This, in very basic terms, averages out the standard deviation (σ) of the log efficiency of plating of both the control and the mutant, and takes into account the sample size. This sample size, n, as an element, is also important since the sample size for the controls was substantially large than the sample size of the mutants. The equation for the Student's two-tailed t-test is shown below.

$$t = \frac{\bar{x}_{control} + \bar{x}_{mutant}}{\sqrt{\frac{\sigma^2_{control}}{n_{control}} + \frac{\sigma^2_{mutant}}{n_{mutant}}}}$$

The t-test was done with comparison to both the wt Phd and the vector plasmid using the above formula. If a t value was negative, the absolute value was taken. In order to consider significance of t values, the total sample size must be calculated to obtain the degrees of freedom. This can be done by $n_{control} + n_{mutant} - 2 =$ degrees of freedom. When comparing mutants to wt Phd, most mutants had 12

degrees of freedom. This corresponded to significance if the t-value was above 2.18. When comparing mutants to the vector plasmid, most mutants had 14 degrees of freedom. This corresponded to significance if the t-value was above 2.14. When this was done with the SAD positive control, wt Phd, and the SAD negative control, the vector plasmid, mutants could be categorized in simple forms as SAD+ or SAD-. Not all mutants fell nicely into categories though, leaving mutants whose efficiency of plating averages were in between SAD+ and SAD- and thus were classified as SAD+/-. Those that fell outside the range were classified as SAD++, meaning that they are possibly even better than wild type at segregational killing and secondary addiction. Those that didn't fit any of these categories, due to large differences between the various log efficiency of plating values, were considered variable.

A few of our tested mutants had a sample size (n) of 1. The t-test data for these mutants did not accurately reflect their SAD classification. This was because the t-test takes into account both standard deviations, and for our sample size of 1, the standard deviation was 0. In order to classify mutants with a sample size of 1, the z-test was done. The formula for the z test only takes into account the average efficiency of plating for the control $x_{control}$ and the mutant x_{mutant}, as well as the standard deviation of the efficiency of plating of the control $\sigma_{control}$. It does not take into account the standard deviation of the efficiency of plating of the mutant strain. The equation for the z test is shown below.

$$z = \frac{\bar{x}_{control} - \bar{x}_{mutant}}{\sigma_{control}}$$

Z values of 2 or above were considered significant. If the z value was negative, the absolute value was first taken before considering significance. To be significant is to be significantly different than the control to which a mutant is being compared. So, Z values of 2 or above were significantly different than the control to which they were being compared, while Z values of less than 2 were considered to be like the control to which they were being compared. Mutants were once again categorized as SAD+, SAD-, SAD+/-, and SAD++. Mutants on which the Z-test were preformed are also included in tables 1 and 2 for medium induction, and tables 3 and 4 for high induction. They are indicated by an n value of 1, and are italicized.

Table 1: The average log efficiency of plating and standard deviation at medium induction for mutants A36H-K49A. Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, ++ for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test.

Construct	**Sample size (n)**	**Average Log Efficiency of Plating +/- Standard Deviation 50 µM IPTG**	**Secondary Addiction 50 µM IPTG**
wt phd	11	-4.06 +/- 0.43	+
pKK 223-3	13	-0.85 +/- 2.68 x 10^{-1}	-
A36H	3	-8.04 +/- 0.93 x 10^{-3}	-
V37A	3	-1.19 +/- 1.67 x 10^{-1}	-
I38A	3	-4.58 +/- 5.41 x 10^{-2}	-
V39A	3	-9.79 +/- 5.00 x 10^{-1}	+/-
S40A	2	-2.61 +/- 0.54	+/-
K41A	2	-5.34 +/- 2.33 x 10^{-1}	+/-
A42D	3	-3.99 +/- 1.14	+
T43A	3	-2.29 +/- 0.56	+/-
F44A	3	6.37 +/- 5.12 x 10^{-2}	-
E45A	2	-3.81 +/- 0.12	+
A46D	3	-2.74 +/- 0.51	+/-
Y47A	3	-3.00 +/- 0.51 x 10^{-1}	+/-
K48A	3	-1.18 +/- 1.28 x 10^{-1}	-
K49A	2	-3.67 +/- 0.18	+/-

Table 2: The average log efficiency of plating and standard deviation at medium induction for mutants A50D-Δ(50-73). Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, + for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test.

Construct	**Sample size (n)**	**Average Log Efficiency of Plating +/- Standard Deviation 50 μM IPTG**	**Secondary Addiction 50 μM IPTG**
wt phd	11	-4.06 +/- 0.43	+
pkk 223-3	13	-0.85 +/- 2.68 x 10^{-1}	-
A50D	3	-2.27 +/- 2.29	variable
L52D	3	-3.99 +/- 0.47	+
D53A	3	-4.52 +/- 0.18	++
D53R	2	-4.47 +/- 0.16	++
A54D	3	-1.15 +/- 0.62	+/-
E55A	2	-4.97 +/- 1.38	+
E55R	2	-4.72 +/- 0.43	+/-
F56A	3	-3.77 +/- 0.14	+
A57D	*1*	*-3.10*	+/-
L59A	2	-4.22 +/- 1.97	+
F60A	*1*	*-6.19*	+
D61R	2	-4.74 +/- 0.16	++
D64R	3	-4.38 +/- 1.25	+
K68E	2	-2.08 +/- 3.35 x 10^{-1}	-
E69R	2	-4.09 +/- 0.88	+
L70A	*1*	*-4.62*	+
R73E	*1*	*-4.36*	+
phd Δ(2-49)	*1*	*-2.92 x 10^{-2}*	-
phd Δ(50-73)	*1*	*1.07 x 10^{-1}*	-

Table 3: The average log efficiency of plating and standard deviation at high induction for mutants A36H-K49A. Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, ++ for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-.

Construct	**Sample size (n)**	**Average Log Efficiency of Plating +/- Standard Deviation** **500 µM IPTG**	**Secondary Addiction** **500 µM IPTG**
wt phd	10	-4.25 +/- 0.44	+
pKK 223-3	13	-3.21 +/- 7.59 x 10^{-2}	-
A36H	3	-9.42 +/- 0.89 x 10^{-3}	-
V37A	3	-1.34 +/- 1.53	-
I38A	3	-2.62 +/- 0.81 x 10^{-1}	+/-
V39A	3	-2.95 +/- 2.38	-
S40A	3	-4.40 +/- 0.95	+
K41A	3	-5.15 +/- 0.63	++
A42D	3	-5.04 +/- 0.26	++
T43A	3	-4.22 +/- 0.51	+
F44A	3	-8.16 +/- 3.92 x 10^{-2}	-
E45A	3	-3.77 +/- 0.17	+/-
A46D	3	-4.15 +/- 0.33	+
Y47A	2	-4.69 +/- 1.04	+
K48A	3	-4.46 +/- 0.08	+
K49A	3	-3.95 +/- 0.84	+

*Table 4: The average log efficiency of plating and standard deviation at high induction for mutants A50D-Δ(50-73). Secondary addiction was determined here by the Student's two-tailed t-test. For secondary addiction the symbols are as follows: + for killing, – for no killing, +/- for partial killing, * for greater than wt killing (superkiller). Variable indicates, that while the mean may fall between wt Phd and vector plasmid, the standard deviation of the mutant was too high to warrant a +/-. Mutants with n = 1 are indicated in italics, as they were analyzed using the z-test rather than the t-test.*

Construct	**Sample size (n)**	**Average Log Efficiency of Plating +/- Standard Deviation 500 μM IPTG**	**Secondary Addiction 500 μM IPTG**
wt phd	10	-4.25 +/- 0.44	+
pkk 223-3	13	-3.21 +/- 7.59 x 10^{-2}	-
A50D	3	-4.23 +/- 0.11	+
L52D	3	-3.76 +/- 0.07	+/-
D53A	3	-3.95 +/- 0.76	+
D53R	2	-5.13 +/- 0.20	++
A54D	3	-3.63 +/- 0.75	+
E55A	2	-5.68 +/- 0.80	++
E55R	2	-4.85 +/- 0.25	++
F56A	3	-4.09 +/- 1.11	+
A57D	*1*	*-4.65*	+
L59A	2	-5.50 +/- 0.73	++
F60A	2	-4.72 +/- 0.28	+
D61R	2	-5.98 +/- 0.15	++
D64R	2	-2.28 +/- 1.82	variable
K68E	3	-3.51 +/- 0.58	+
E69R	3	-4.58 +/- 1.03	+
L70A	2	-5.14 +/- 0.17	++
R73E	3	-4.60 +/- 1.36	+
phd Δ(2-49)	*1*	*-2.12 x 10^{-2}*	-
phd Δ(50-73)	*1*	*-8.67 x 10^{-2}*	-

We are concerned with what parts of the antitoxin are necessary for secondary addiction. Selected mutations are shown below in table 5 and figure 6. The efficiency of plating and standard deviation are shown below in Figure 6, note this data is not logged, but simply graphed on a logged scale.

Table 5: The average log efficiency of plating and standard deviation at medium and high induction of vector plasmid and all mutants that were SAD- at either medium or high induction.

Construct	Average Efficiency of Plating +/- Standard Deviation 50 µM IPTG	Secondary Addiction 50 µM IPTG	Average Efficiency of Plating +/- Standard Deviation 500 µM IPTG	Secondary Addiction 500 µM IPTG
pKK 223-3	$-0.85 +/- 2.68 \times 10^{-1}$	-	$-3.21 +/- 7.59 \times 10^{-2}$	-
A36H	$-8.04 +/- 0.93 \times 10^{-3}$	-	$-9.42 +/- 0.89 \times 10^{-3}$	-
V37A	$-1.19 +/- 1.67 \times 10^{-1}$	-	-1.34 +/- 1.53	-
I38A	$-4.58 +/- 5.41 \times 10^{-2}$	-	$-2.62 +/- 0.81 \times 10^{-1}$	+/-
F44A	$6.37 +/- 5.12 \times 10^{-2}$	-	$-8.16 +/- 3.92 \times 10^{-2}$	-
Y47A	$-3.00 +/- 0.51 \times 10^{-1}$	+/-	-4.69 +/- 1.04	+
K48E	$-1.18 +/- 1.28 \times 10^{-1}$	-	-4.46 +/- 0.08	+
K68E	$-2.08 +/- 3.35 \times 10^{-1}$	-	-3.51 +/- 0.58	+
phd Δ(2-49)	-2.92×10^{-2}	-	-2.12×10^{-2}	-
phd Δ(50-73)	1.07×10^{-1}	-	-8.67×10^{-2}	-

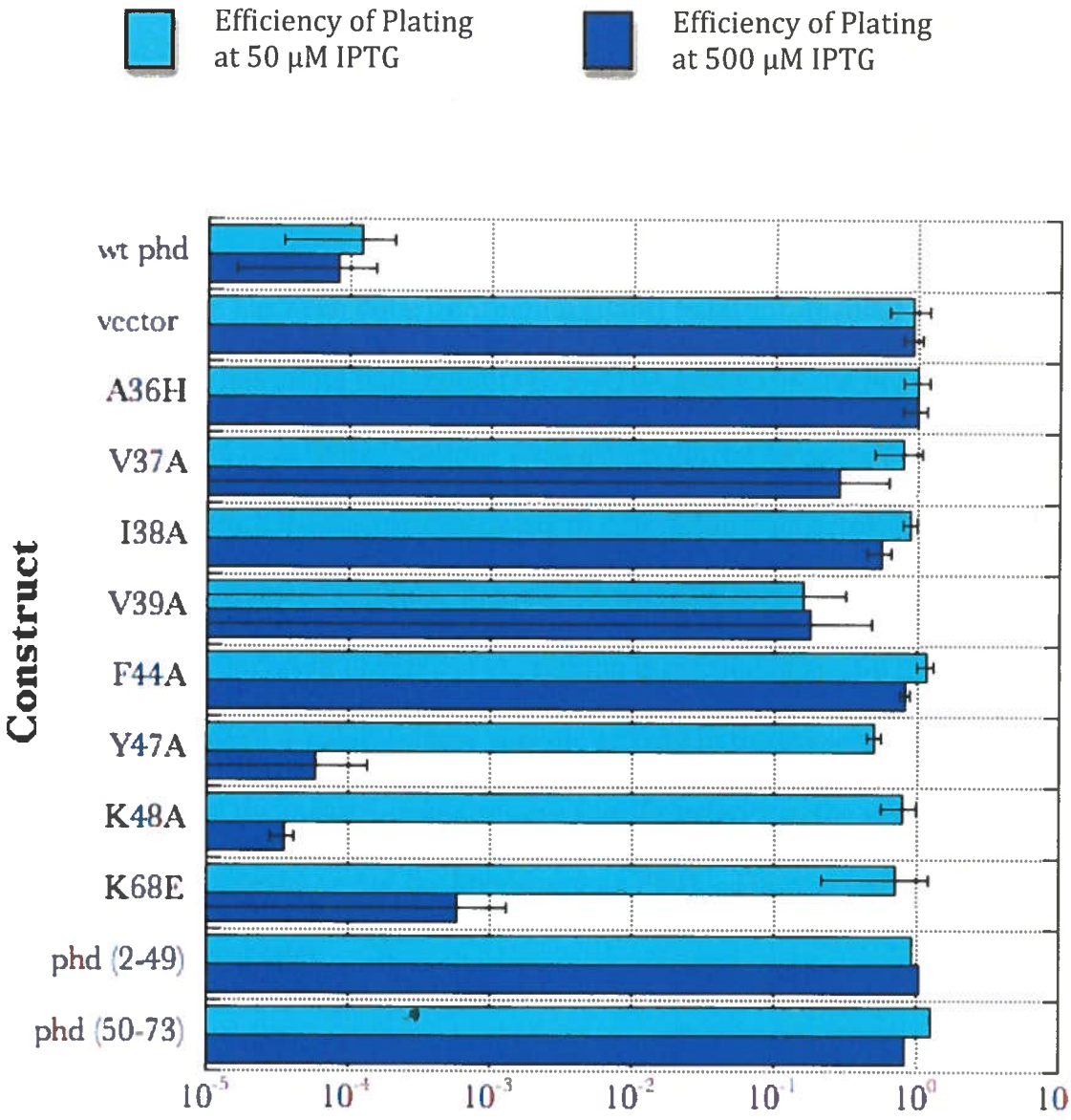

Figure 6: Efficiency of Plating of selected mutants. Error bars indicate the standard deviation.

We are not interested in the constructs involving large deletions: phd Δ (2-49), phd Δ (50-73). They simply tell us that by deleting large parts of the antitoxin, secondary addiction is removed. However, we are interested in the other mutants listed in Tables 1-4 and Figure 6. We found that the mutants A36H, V37A, I38A, V39A, F44A, K48A and K68E did not have secondary addiction, and that these locations are required for secondary addiction.

Further work has been done by Sreeram (Ram) Balasubramanian with C-terminal mutants. He has found that mutants R7S, R10A, and R10K did not exhibit secondary addiction, and thus these locations were also required for secondary addiction. This was done using a ß-galactosidase assay to determine ß-galactosidase specific repression activity of mutants. Repression was calculated as percent unrepressed, where % unrepressed or relative expression is calculated as 100 multiplied by the ratio of the mean ß-galactosidase specific activity in the presence of the Phd construct and the mean ß-galactosidase specific activity of the vector control (containing only the vector backbone and no Phd). These are shown below in table 6 and discussed later.

Table 6: Repression data for Ram's C-terminal mutants

Strain	Construct	% Unrepressed
JEM087	vector pkk223-3	100.0
JEM086	wt	11.6
SS062	**R10A**	**96.5**
SS088	**R10K**	**100.0**
XY137	R7S	76.4

DISCUSSION

This experiment was designed to build on the work previously done by James (Jamie) McKinley (McKinley and Magnuson 2005). I would also like to acknowledge the preliminary work on Phd done by Sophia Hightower. Other work that made my experiments possible Xueyan (Snow) Zhao (Zhao and Magnuson 2005) and Sarah (Sairey) Siegel (Siegel 2008). Additional repression data was provided by Sreeram (Ram) Balasubramanian.

I have already shown that secondary addiction does exist, and that it is defective in the constructs A36H, V37A, I38A, V39A, F44A, K48A and K68E. These constructs were compared with Jamie's repression data. It was found that our mutants that lacked secondary addiction were also those mutants whose repression activity had been reduced. Jamie's data can be seen in Table 7. Only those mutants that we found important are listed here. These are represented graphically in Figure 8.

Table 7: James McKinley's Repression activity data showing the construct, percent unrepressed, standard deviation, and repression activity.

Repressor Activity of Phd mutations[a]

Strain	Construct[b]	% Expression[c]	SD	Repressor Activity?
JEM086	w.t. *phd*	17.01	1.06	Yes
JEM087	pKK223-3	100 (by definition)		No
JEM165	***phd* A36H**	**76.27**	**2.38**	**Major defect**
JEM127	***phd* V37A**	**65.03**	**4.44**	**Major defect**
JEM128	***phd* I38A**	**73.37**	**2.46**	**Major defect**
JEM130	*phd* V39A	28.75	0.37	Minor defect
JEM166	*phd* K41A	25.74	2.62	Minor defect
JEM134	***phd* F44A**	**82.59**	**2.01**	**Major defect**
JEM137	*phd* Y47A	32.42	6.53	Minor defect
JEM139	*phd* K48A	31.34	2.01	Minor defect
JEM088	*phd* A50D	26.50	0.82	Minor defect
JEM144	*phd* K68E	19.44	2.53	Yes
JEM098	*phd*Δ(2-49)	113.59	15.55	**No**
JEM093	*phd*Δ(50-73)	105.64	13.17	**No**

[a]Repressor activity was indicated by the ability of the test construct to repress transcription of a *lacZ* reporter fused the promoter of the P1 addiction operon.

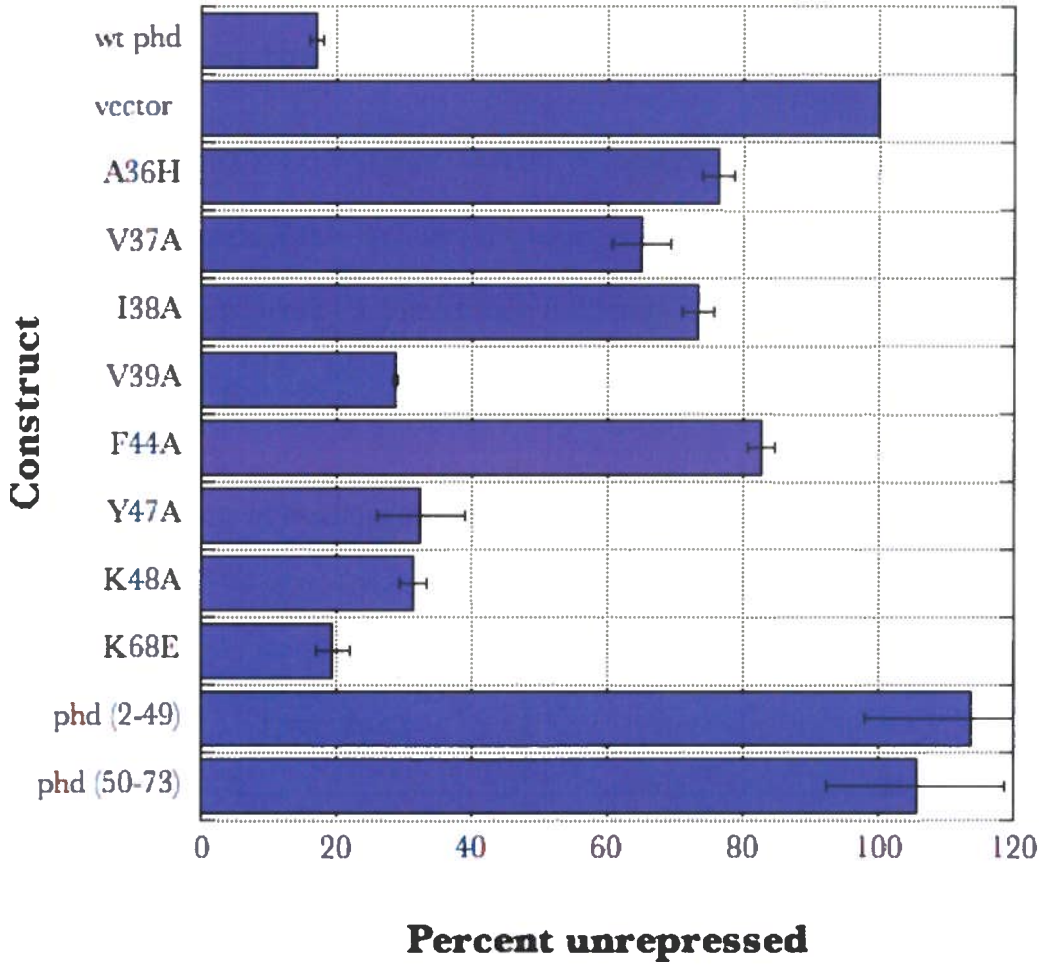

Figure 7: Repression data for selected (with Phd shown for the positive control for secondary addiction). Percent unrepressed is shown on the x-axis.

Jamie's repression data along with the new data on secondary addiction, leads us to believe that secondary addiction is really just the repressor run amuck. We believe that secondary addiction works by nonspecifically shutting off gene expression. DNA binding is required for secondary addiction, but not binding specificity. This is evidenced by three of the mutants that Ram worked with: R7S,

R10A, and R10K. These mutants are believed to have altered specificity. DNA binding is occurring, however it is not occurring on the P1 promoter.

Repression data correlated with the secondary addiction data is shown in the following two tables. Table 8 shows the repression data at medium induction, while table 9 shows the repression data at high induction.

Table 8: Repression activity (not induced) and Secondary Addiction for all mutants at medium induction(50 μM IPTG).

SECONDARY ADDICTION	**Low**	**Medium**	**High**
SAD-	vector, A36H, V37A, I38A, F44A,		K68E
SAD+/-		Y47A, V39A, A50D	*listed below
SAD+	R7S, R10K	K48A	**listed below

REPRESSOR ACTIVITY

*Other SAD +/-, high repressor mutants: S40A, K41A, T43A, A46D, K49A, A54D, A57D, D61R

**Other SAD+, high repressor activity mutants: wt phd, A42D, E45A, L52D, D53A, D53R, E55A, E55R, F56A, L59A, F60A, D64R, E69R, L70A, R73E

Table 9: Repression activity (not induced) and Secondary Addiction for all mutants at high induction (500 μM IPTG).

SECONDARY ADDICTION	Low	Medium	High
SAD-	Vector, A36H, V37A, F44A	V39A	
SAD +/-	I38A		*listed below
SAD+	R7S, R10K	Y47A, K48A, A50D	**listed below

REPRESSOR ACTIVITY

*Other SAD +/-, high repressor mutants: E45A. L52D, D64R

**Other SAD+, high repressor activity mutants: wt phd, S40A, K41A, A42D, T43A, A46D, K49A, D53A, D53R, A54D, E55A, E55R, F56A, A57D, L59A, F60A, D61R, K68E, E69R, L70A, R73E

Secondary addiction has been shown to be a factor in our toxin-antitoxin system. Over-expression of the antitoxin is toxic to the cell, as evidenced by the large difference in the efficiency of plating of the wild type Phd and some of the mutants tested. Secondary addiction kills the cell by severely reducing repressor activity, and hampering DNA binding.

The Catch-22 hypothesis of our toxin-antitoxin system now holds true, with secondary addiction shown to be valid. Our toxin-antitoxin system, once acquired, cannot be lost without causing death to the bacterium. The bacterium is in a Catch-22 scenario. If the entire system is lost, the bacterium will die from the excess toxin that is present in the cell. If the antitoxin is lost, the bacterium will die from the toxin that is still being produced. Now the final puzzle piece for the Catch-22 hypothesis is in place. If the toxin is lost, the cell will die from the over-expression of the antitoxin. Killing by antitoxin largely correlates with the ability to bind DNA/repress transcription.

REFERENCES:

Jensen, RB and Gerdes, K. "Programmed cell death in bacteria: proteic plasmid stabilization systems". *Molecular Microbiology* (July 1995): 205-210.

Lehnherr H, and Yarmolinsky MB. Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of Escherichia coli." *The Proceedings of the National Academy of Sciences* (11 April 1995); 92(8): 3274-3277.

Magnuson, R and Yarmolinsky, MB. "Corepression of the P1 addiction operon by Phd and Doc". *Journal of Bacteriology* (December 1998): 6342-6351.

McKinley, J.E. and Magnuson, RD. "Characterization of the Phd Repressor-Antitoxin Boundary". *Journal of Bacteriology* (January 2005): 765-770.

Siegel, S. "Genetic and Bioinformatic Approaches Towards Engineering a Repressor-Operator Interphase". Master's Thesis (March 2008).

Yarmolinsky, MB. "Programmed cell death in bacterial populations". *Science* (10 February 1995): 836-838.

Zhao, X and Magnuson, RD. "Percolation of the Phd Repressor-Operator Interface". *Journal of Bacteriology* (March 2005): 1901-1912.