Antibiotic effects on yqfA protein function in *Escherichia coli* metabolism

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Introduction

After recent sequencing and analysis of the genome of the soil bacterium *Azospirillum brasilense*, the presence of several chemotaxis signaling pathways in the organism have become evident. Chemotaxis pathways allow the organism to sense and respond to concentration gradients of chemical stimuli in the environment. One of these pathways, called the Che1 pathway, plays a role in regulating phenotypic behaviors including cell surface adhesion, cell-to-cell clumping and swimming velocity (Bible, Russell, and Alexandre, 2012). Included in this pathway is the CheA1 protein, which contains an N-terminal domain that is similar to the hemolysin III family of proteins. While hemolysin proteins are known to cause lysis of red blood cells by forming pores in the cell membrane, the function of the CheA1 protein's N-terminal domain has yet to be defined (Bible, 2012). This particular domain also manifests in the form of a single domain protein in other species of bacteria, including *Escherichia coli* (Bible, 2012). In *E. coli,* the gene coding for this specific protein has been named *yqfA*. By studying the role of the yqfA protein in the model organism *E. coli*, the function of the N-terminal domain of the CheA1 protein in *A. brasilense* may also be established.

The yqfA protein is a component of the inner layer of gram-negative bacterial cell membranes. Like the N-terminal domain of the CheA1 protein, yqfA has been discovered to be a member of the hemolysin III (Hly-III) family of bacterial inner membrane proteins. While the *yqfA* gene encoding the protein has been identified and the protein has been localized to the inner membrane, the specific function of this protein in the membrane is still largely unidentified (Feng and Cronan, 2011). It

appears to play a role in regulation of the properties of the cell membrane and the cell length phenotype. Mutations of these proteins have been shown to impair the chemotactic abilities of the cells and affect cell membrane properties such as fluidity by altering fatty acid composition of the membrane (Bible, 2012). In addition, it has been suggested that deletion of this yqfA protein through mutation leads to upregulation of metabolism in the mutant *E. coli* cells, though how this occurs is still unknown.

To examine whether the presence of the yqfA protein factors into regulation of *E. coli* metabolism, two *E. coli* strains, the wild type MG1655 strain and a *yqfA*-deletion mutant strain called *yqfA*^{o}, were treated with two classes of antibiotics. Bacteriostatic antibiotics, including tetracycline and chloramphenicol, inhibit bacterial growth by affecting protein production, suppressing the cells' metabolism and confining them to the stationary, lag phase of growth. Bactericidal antibiotics, such as gentamicin and ampicillin, do the opposite; causing cell death by accelerating the metabolism of bacterial cells or inhibiting synthesis of the cell wall or plasma membrane (Lobritz *et al.*, 2015). If the yqfA protein functions in regulating metabolism of *E. coli* cells, then treatment with antibiotics that affect bacterial metabolism would result in noticeable differences in growth between the colonies of the wild type MG1655 strain and colonies of the mutant *yqfA*^{o} strain. Lack of yqfA function in the mutant cells would affect colony growth compared to that of the wild type cells. These differences would be indicated by the minimum inhibitory concentration (MIC) for each antibiotic, the lowest concentration of antibiotic at which the growth rate of the bacterial cells was observably inhibited.

MIC values are used both to confirm antibiotic resistance by microbes and to measure the efficacy of antibiotic agents. They are one of the most commonly used measures of antibacterial activity (Turnidge, Ferraro, and Jorgensen, 2011).

Materials and Methodology

To begin the experiment, first the *yqfA*^{o} deletion mutant strain had to be constructed. This was done by the overlap extension polymerase chain reaction method. This method uses two sets of primers to generate DNA fragments whose overlapping ends contain the desired gene deletion. The two DNA fragments are combined and annealed to create hybrid duplexes. The hybrids are then extended and amplified via PCR to produce recombined PCR products, which are digested by restriction endonucleases and cloned via plasmid vectors (Lee, 2010). Following construction of the mutant strain via overlap extension PCR, two different cultures of *E. coli* cells were streaked for isolation on Luria broth (LB) nutritionally rich solid agar plates without antibiotics. One culture consisted of the wild-type MG1655 cells, while the other consisted of the *yqfA*^{o} mutant cells. After 48 hours of growth on the agar plates, cells were extracted from the plates and inoculated in 5 mL of LB liquid media without antibiotics. These liquid cultures were allowed to grow overnight at a temperature of 37°C (the optimum temperature for *E. coli* growth) with slight agitation of 120 revolutions per minute. After this period of overnight growth, the cultures were removed from their state of incubation and agitation. 100μL of each culture were then inoculated into 5mL of fresh LB liquid medium, and these reinoculated cultures were returned to the 37°C incubation with agitation to grow

for another 1-2 hours. After 1-2 hours of growth, the cultures were removed from incubation. The absorbance, or optical density, of each culture was measured at a wavelength of 600nm (OD600) using a spectrophotometer calibrated with fresh LB liquid medium. This procedure was used to determine the concentration of bacterial cells in the liquid medium. The linear relationship between absorbance and concentration of solution as stated by the Beer-Lambert Law ($A= \varepsilon bc$, with A=absorbance, ε=molar absorption coefficient, b=path length, and c=concentration), allows the concentration of the cultures to be evaluated. Cultures were grown with 37°C incubation and agitation until each culture reached an optical density of 0.1-0.2. If the cells had overgrown this OD600 range, 100μL of each overgrown culture were reinoculated in another 5mL of fresh LB liquid medium and allowed to grow in 37°C incubation with agitation until they reached OD600 values of 0.1-0.2, as measured by the spectrophotometer.

Once the cultures had grown to the desired OD600 values, measurement of cell growth with antibiotic exposure could be performed. A total volume of 200μL of cells + antibiotics were added to each well of a 96-well plate, with the exception of the LB blank control wells containing 200μL of LB liquid medium. These LB blanks served as controls for any possible substances in the LB medium that could affect the optical density readings of the cultures. Concentrations ranged from 0-10μg/mL for chloramphenicol (0.34mg/mL), 0-14μg/mL for tetracycline (1.0mg/mL), 0-14μg/mL for ampicillin (1.0mg/mL), and 0-100μg/mL for gentamicin (5.0mg/mL) [Appendix A]. The specific concentration ranges were determined after conducting outside research on the accepted MIC values for each antibiotic when applied to

MG1655 cells. These concentrations were calculated using a total volume of 200μL for each well. Appropriate volumes of cells and antibiotics were added to the wells of the plates to arrive at the desired concentration of antibiotic. Multiple biological replicates were prepared for each antibiotic-strain combination, with a minimum of 3 and a maximum of 6 runs performed for each antibiotic due to time constraints. Two technical replicates were created for each biological replicate. Use of 2 technical replicates provided a measure of variation within cultures and controlled for potential outliers caused by errors in measurement. The plates were read using a BioTek Cytation3 plate reader and Gen5 software. The experiments were conducted for 12 hours, with OD600 read every 15 minutes. The plate reader was kept at a temperature of 37°C and continuously shaken. After 12 hours of OD600 measurements, data for each run, in the form of both numerical values for OD600 and plots of OD600 vs. time, were collected and analyzed.

Data Analysis

The data obtained after the absorbance readings were in the form of a series of tables, each cell corresponding to a well of the 96-well plate. Each reading produced a table of OD600 values, for a total of 49 tables per run. These values were then compiled to create scatter plots of OD600 vs. time corresponding to each well. To determine the MIC for each combination of strain and antibiotic, the series of plots were examined and compared as the concentration of antibiotic added to the well increased. The antibiotic concentration of the first plot to show a noticeable decline in slope was noted. This concentration became the observed MIC value, as it

was the lowest concentration at which growth became affected. The MIC values for each replicate of the antibiotic-strain combinations were then averaged, producing a series of mean minimum inhibitory concentration measurements.

Results

Table 5: Cm Mean Minimum Inhibitory Concentration Measurements (μg/mL)

Cm		
	MG1655	*yqfA°*
Run3	2	3
	1.75	3
Run4	2	3
	2	3
Run5	2	3
Average	**1.95**	**3**

Table 6: Tet Mean Minimum Inhibitory Concentration Measurements (μg/mL)

Tet		
	MG1655	*yqfA°*
Run3	0.5	2
	0.5	2
Run4	0.5	1.5
	0.5	1.5
Run5	0.5	1
	0.5	1
Run6	0.5	1
	0.5	1
Average	**0.5**	**1.375**

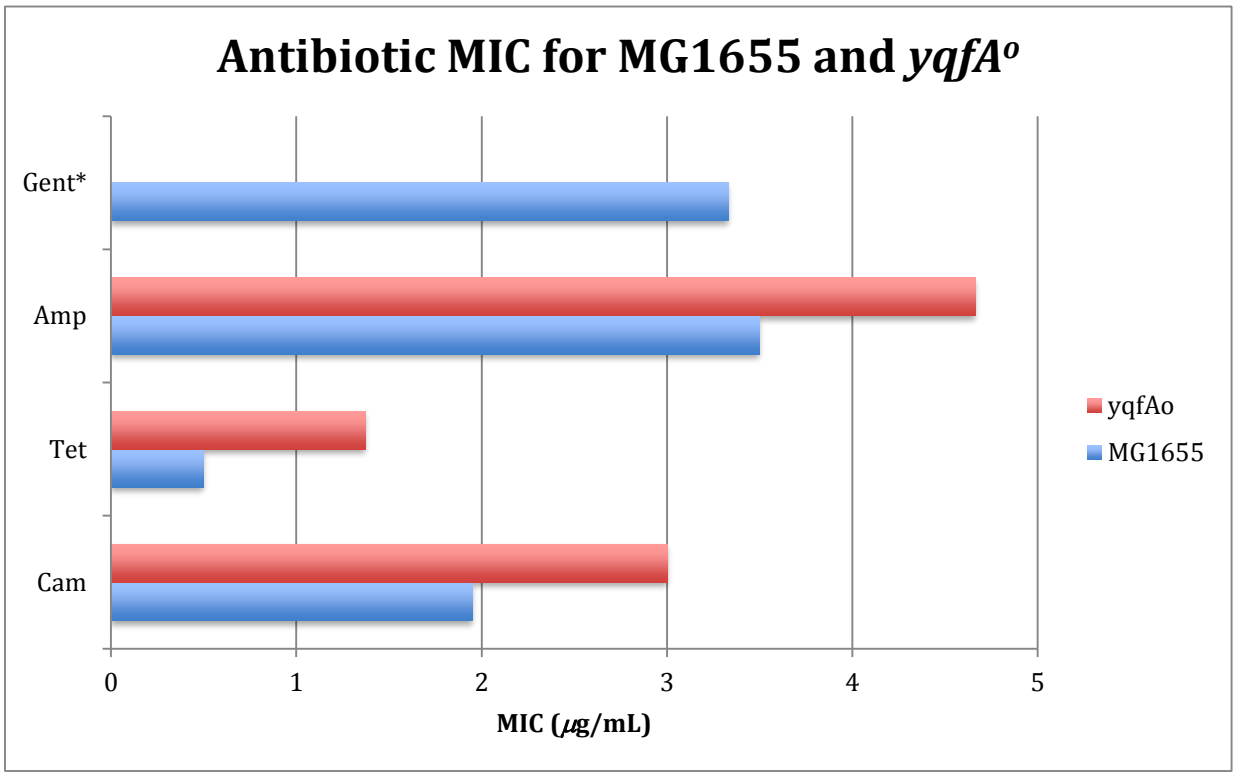
Table 7: Amp Mean Minimum Inhibitory Concentration Measurements (μg/mL)

	Amp	
	MG1655	*yqfA*^{o}
Run 1	3	4
	3	5
Run 2	3	5
	3	5
Run 3	4	4
	5	5
Average	**3.5**	**4.666666667**

Table 8: Gent Mean Minimum Inhibitory Concentration Measurements (μg/mL)

	Gent	
	MG1655	*yqfA*^{o}
Run 1	3	-
	4	-
Run 2	4	-
	2	-
Run 3	5	-
	2	-
Average	3.333333333	-

Figure 1: Mean Minimum Inhibitory Concentration Measurements



Discussion

After examining the series of plots, identifying changes in plot shapes, determining the concentrations at which these changes occurred, and averaging these minimum concentrations, consensus values for each antibiotic's MIC when applied to the 2 strains were obtained. Based on analysis and comparison of the mean MIC measurements obtained from the series of reads, there were noticeable differences between the MIC values for the MG1655 and *yqfA°* strains. For each of the 4 antibiotics, the MICs for the MG1655 cells were almost always observed at lower concentrations than the MICs for the *yqfA°* mutant cells. Chloramphenicol was observed to affect growth of MG1655 cells at concentrations as low as 1.75-2

μg/mL, while it affected *yqfA*^{o} growth closer to 3 μg/mL. Tetracycline caused decreases in MG1655 growth at minimum concentrations of 0.5 μg/mL, while causing decreases in *yqfA*^{o} growth at approximately 1.5 μg/mL. Ampicillin inhibited MG1655 growth at concentrations as low as 3 μg/mL but as high as 5 μg/mL. It inhibited *yqfA*^{o} growth at slightly higher values, more uniformly at 5 μg/mL. When applied to MG1655 cells, gentamicin caused inhibition of growth at minimum concentrations between 2 and 5 μg/mL, at an average of 3 μg/mL. However, when *yqfA*^{o} cells were treated with gentamicin, no effects on growth were observed, regardless of the concentration of antibiotic used. Cells were resistant to gentamicin at concentrations as high as 100 μg/mL. This anomaly was particularly unexpected, as the wild type MG1655 strain clearly responded to gentamicin treatment as evidenced by clear changes in growth rate. It is possible that production of the yqfA protein may be a target in the mechanism of gentamicin action, which involves binding irreversibly to the aminoacyl-tRNA site of the smaller 30S ribosomal subunit (Yoshizawa, Fourmy, and Puglisi, 1998). Thus, the lack of the yqfA protein in the inner membrane of the *yqfA*^{o} cells would minimize one of the effects of gentamicin bactericidal action. Further experimentation with [Gent]>100μg/mL would be needed to establish this possibility.

The MG1655 MICs of each antibiotic obtained from the assays deviated from the expected MICs gathered from outside literature, however. The observed MIC of gentamicin for MG1655 was slightly higher than the expected value of 1μg/mL (Lobritz *et al.*, 2015). The observed MIC of ampicillin for MG1655 was approximately equal to an accepted value of 3.4 ± 0.42 μg/mL (Maisonneuve, 2011),

but slightly higher than another accepted value of 2 μg/mL (Lobritz *et al.*, 2015). Tetracycline had a very low observed MIC for MG1655, compared to an expected value of 2μg/mL (Lobritz *et al.*, 2015) or 3μg/mL (Jaktaji, Ebadi, and Karimi, 2012). Finally, the observed MIC of chloramphenicol was much lower than the accepted value of 10μg/mL (Lobritz *et al.*, 2015).

Conclusion

The results of the series of MIC assays showed clear differences in the effects of both types of antibiotics on the wild type strain and the yqfA mutant strain of *E. coli.* The bacteriostatic antibiotics chloramphenicol and tetracycline inhibit *E. coli* growth by slowing metabolism. If deletion of the yqfA protein results in an upregulation of metabolism in the mutant cells, as has been suggested, then perhaps these mutant cells would require higher concentrations of bacteriostatic antibiotics to slow metabolism and ultimately inhibit growth, compared to the concentration of antibiotic needed to inhibit growth of wild type cells. The bactericidal antibiotics would seemingly affect the *yqfA*^{o} mutant cells at lower concentrations due to their already elevated metabolism. It would likely require a lower amount of antibiotic to overwhelm the cell's metabolic machinery and cause rapid cell death if it was already working at a high rate due to the deletion of the yqfA protein. The results of this research did not show this, however. The MICs observed from these reads were the opposite of what was expected. The MICs obtained for the *yqfA*^{o} mutant cells were noticeably higher for both ampicillin and gentamicin. It is possible that the metabolisms of the mutant cells are upregulated to the point that accelerating

metabolism would not affect the growth rate, as the cells would already be conditioned to survive and grow at a higher metabolic rate than the wild type cells. While the deviations seen between the observed and accepted values for the wild-type MICs suggest that further experimentation is needed to correct for error, it is clear that deleting the yqfA protein affects how the *E. coli* cells respond to antibiotics that bring about changes in their metabolism. This would correlate with the suggestions of previous research. It is possible that this hemolysin III-like protein's influence on the fatty acid composition and fluidity of the *E. coli* inner membrane plays a role in the ability of antibiotics to permeate the cell and then alter the metabolic machinery. Further experimentation should aim to correct for the differences in the observed and accepted MIC values, as well as to provide evidence for more definitive MIC values for each antibiotic when applied to the two different strains. Ultimately, future research would elucidate what aspect of the yqfA protein's function permits it to affect the metabolism in the *E. coli* cell, likely its effect on the inner membrane composition and fluidity.

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Appendix A – Antibiotic Concentration Range Tables

Table 1: Concentration Ranges for Chloramphenicol Trials

	1	2	3	4	5	6	7	8	9	10	11	12
[Cm]	LB	0 ug/mL	1 ug/mL	1.5 ug/mL	2 ug/mL	2.5 ug/mL	3 ug/mL	3.5 ug/mL	4 ug/mL	4.5 ug/mL	5 ug/mL	6 ug/mL
Cm to Add (uL)	0	0	0.588	0.882	1.18	1.47	1.76	2.06	2.35	2.65	2.94	3.53

	1	2	3	4	5	6	7	8	9	10	11	12
[Cm]	LB	0 ug/mL	0.25 ug/mL	0.5 ug/mL	0.75 ug/mL	1 ug/mL	2 ug/mL	3 ug/mL	4 ug/mL	6 ug/mL	8 ug/mL	10 ug/mL
Cm to Add (uL)	0	0	0.147	0.294	0.441	0.588	1.18	1.76	2.35	3.53	4.71	5.88

Table 2: Concentration Ranges for Tetracycline Trials

	1	2	3	4	5	6	7	8	9	10	11	12
[Tet]	LB	0 ug/mL	0.5 ug/mL	1 ug/mL	2 ug/mL	3 ug/mL	4 ug/mL	6 ug/mL	8 ug/mL	10 ug/mL	12 ug/mL	14 ug/mL
Tet to Add (uL)	0	0	0.1	0.2	0.4	0.6	0.8	1.2	1.6	2	2.4	2.8

	1	2	3	4	5	6	7	8	9	10	11	12
[Tet]	LB	0 ug/mL	1 ug/mL	2 ug/mL	3 ug/mL	3.5 ug/mL	4 ug/mL	4.5 ug/mL	5 ug/mL	6 ug/mL	7 ug/mL	8 ug/mL
Tet to Add (uL)	0	0	0.2	0.4	0.6	0.7	0.8	0.9	1	1.2	1.4	1.6

	1	2	3	4	5	6	7	8	9	10	11	12
[Tet]	LB	0 ug/mL	1 ug/mL	1.5 ug/mL	2 ug/mL	2.25 ug/mL	2.5 ug/mL	3 ug/mL	3.25 ug/mL	3.5 ug/mL	4 ug/mL	4.5 ug/mL
Tet to Add (uL)	0	0	0.2	0.3	0.4	0.45	0.5	0.6	0.65	0.7	0.8	0.9

	1	2	3	4	5	6	7	8	9	10	11	12
[Tet]	LB	0 ug/mL	3.5 ug/mL	3.75 ug/mL	4 ug/mL	4.25 ug/mL	4.5 ug/mL	5 ug/mL	6 ug/mL	8 ug/mL	10 ug/mL	14 ug/mL
Tet to Add (uL)	0	0	0.7	0.75	0.8	0.85	0.9	1	1.2	1.6	2	2.8

	1	2	3	4	5	6	7	8	9	10	11	12
[Tet]	LB	0 ug/mL	0.5 ug/mL	1 ug/mL	1.5 ug/mL	2 ug/mL	2.5 ug/mL	3 ug/mL	3.5 ug/mL	3.4 ug/mL	4.5 ug/mL	5 ug/mL
Tet to Add (uL)	0	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1

Table 3: Concentration Ranges for Ampicillin

	1	2	3	4	5	6	7	8	9	10	11	12
[Amp]	LB	0 ug/mL	1 ug/mL	2 ug/mL	3 ug/mL	4 ug/mL	5 ug/mL	6 ug/mL	7 ug/mL	8 ug/mL	9 ug/mL	10 ug/mL
Amp to Add (uL)	0	0	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2

	1	2	3	4	5	6	7	8	9	10	11	12
[Amp]	LB	0 ug/mL	1 ug/mL	1.5 ug/mL	2 ug/mL	2.5 ug/mL	3 ug/mL	3.5 ug/mL	4 ug/mL	4.5 ug/mL	5 ug/mL	5.5 ug/mL
Amp to Add (uL)	0	0	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.1

Table 4: Concentration Ranges for Gentamicin

	1	2	3	4	5	6	7	8	9	10	11	12
[Gent]	LB	0 ug/mL	2 ug/mL	3 ug/mL	4 ug/mL	5 ug/mL	6 ug/mL	7 ug/mL	8 ug/mL	9 ug/mL	10 ug/mL	11 ug/mL
Gent to Add (uL)	0	0	0.08	0.12	0.16	0.2	0.24	0.28	0.32	0.36	0.4	0.44

	1	2	3	4	5	6	7	8	9	10	11	12
[Gent]	LB	0 ug/mL	2 ug/mL	4 ug/mL	6 ug/mL	8 ug/mL	10 ug/mL	12 ug/mL	14 ug/mL	16 ug/mL	18 ug/mL	20 ug/mL
Gent to Add (uL)	0	0	0.08	0.16	0.24	0.32	0.4	0.48	0.56	0.64	0.72	0.8

	1	2	3	4	5	6	7	8	9	10	11	12
[Gent]	LB	0 ug/mL	2 ug/mL	4 ug/mL	6 ug/mL	8 ug/mL	10 ug/mL	20 ug/mL	40 ug/mL	60 ug/mL	80 ug/mL	100 ug/mL
Gent to Add (uL)	0	0	0.08	0.16	0.24	0.32	0.4	0.8	1.6	2.4	3.2	4

Appendix B – Tables of Mean MIC Results

Table 5: Cm Mean Minimum Inhibitory Concentration Measurements (μg/mL)

Cm		
	MG1655	*yqfA°*
Run3	2	3
	1.75	3
Run4	2	3
	2	3
Run5	2	3
Average	**1.95**	**3**

Table 6: Tet Mean Minimum Inhibitory Concentration Measurements (μg/mL)

Tet		
	MG1655	*yqfA°*
Run3	0.5	2
	0.5	2
Run4	0.5	1.5
	0.5	1.5
Run5	0.5	1
	0.5	1
Run6	0.5	1
	0.5	1
Average	**0.5**	**1.375**

Table 7: Amp Mean Minimum Inhibitory Concentration Measurements (μg/mL)

	Amp	
	MG1655	*yqfA°*
Run 1	3	4
	3	5
Run 2	3	5
	3	5
Run 3	4	4
	5	5
Average	**3.5**	**4.666666667**

Table 8: Gent Mean Minimum Inhibitory Concentration Measurements (μg/mL)

	Gent	
	MG1655	*yqfA°*
Run 1	3	-
	4	-
Run 2	4	-
	2	-
Run 3	5	-
	2	-
Average	3.333333333	-
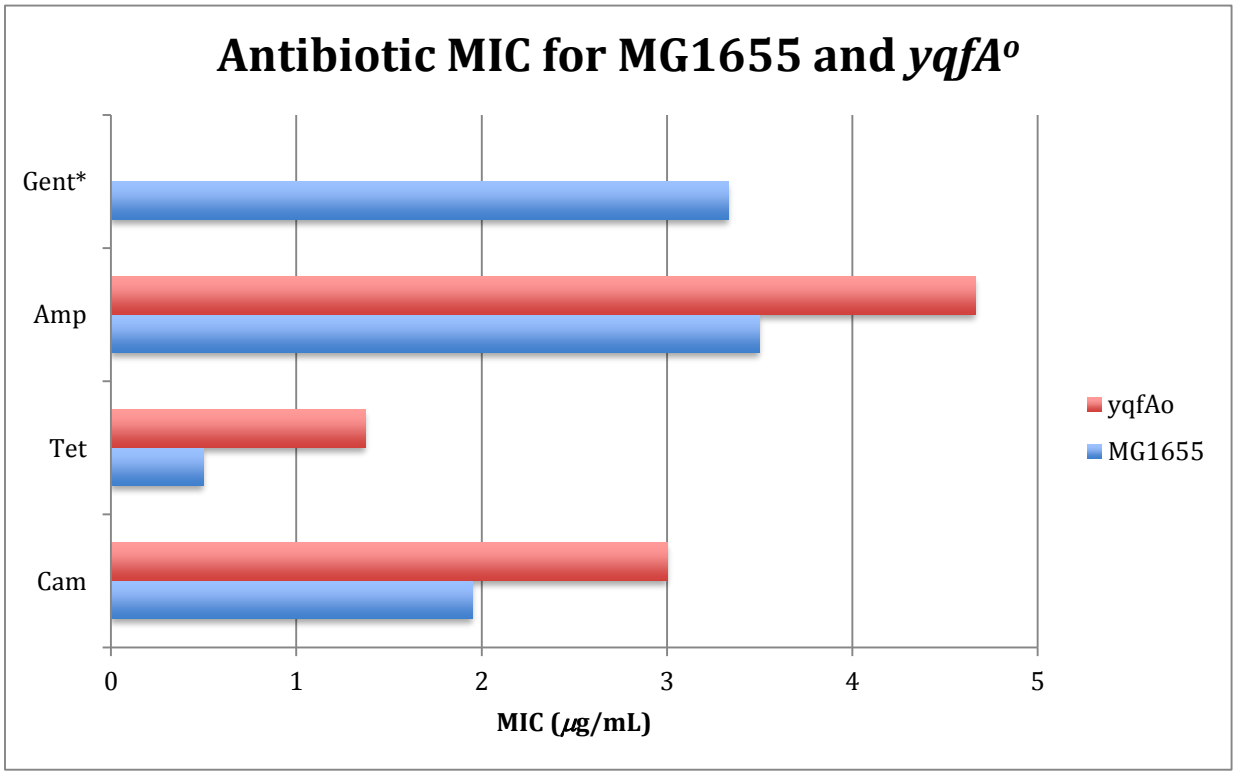
Figure 1: Mean Minimum Inhibitory Concentration Measurements