Identification of antibiotics responsible for inhibition of bacterial growth by *Pseudomonas fluorescens* Pf-5

Edward Davis
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Introduction

Pseudomonas fluorescens Pf-5 produces many different antibiotics, and from the sequence of its genome, many more compounds that inhibit bacterial growth have been identified. In order to identify the phenotype of the newly identified genes, a set of mutants deficient in these genes has been created. We wanted to know which genes are required to inhibit the growth of *Escherichia coli* DH5α. This research project characterized which genes are essential for inhibiting growth of *E. coli* DH5α on both Nutrient Agar with Glucose and Nutrient Agar with Glycerol.

Materials and Methods

Bacterial strains and culture conditions

E. coli DH5α (L6079) was routinely cultured on Luria-Bertani (LB) medium at 37 °C and maintained on LB agar. Pf-5 and derivative strains were cultivated routinely at 27 ºC on Kings Medium B agar (KB). The Nutrient Agar with Glucose and Nutrient Agar with Glycerol were initially made as Nutrient Agar with 10% less distilled water than the recipe called for; 10% Glucose and 10% Glycerol solutions were added, respectively, to the media before pouring the plates. A 0.1M $FeCl_3$ solution was also added at a 1μL per 1mL of solution concentration ($1*10^{-4}$M final concentration).

Experimental Setup

The Pf-5 and *E. coli* cultures were taken out of the freezer and plated on KB and LB agars, respectively. After one day of incubation, Pf-5 at 27 ºC and *E. coli* at 37 ºC, a swab of each of the Pf-5 cultures was placed into 1mL of deionized water in a 1.5mL microcentrifuge tube. The cultures were spun down at 5000rpm for 5 minutes. The supernatant was removed and discarded, and the cultures were resuspended in 1mL of deionized water. The cells were then spun down again. After removing and discarding

the supernatant again, and resuspending the cells again, each culture was brought to 0.1 optical density using a spectrophotometer. Ten microliters of each culture was then spotted onto the center of the plates used in each experiment. The spotted plates were then placed in the 27 ºC incubator for two days. The plate with *E. coli* was moved into the cold room for one day. The next day, a LB broth culture of the *E. coli* was started, and the inoculated LB broth was placed into the 37 ºC incubator in a shaker. After a day in the shaker, the *E. coli* culture was taken out, and 1mL of the culture was placed in a microcentrifuge tube. The cells were spun down in the same manner as the Pf-5 cells, and the culture was brought to 0.1 OD reading as described before. The 0.1 OD *E. coli* culture was then diluted 1:10. The Pf-5 plates that were incubated for two days were removed from the incubator, and were used in the streaking of the *E. coli*. Ten microliters of the diluted *E. coli* culture were streaked four times from the outside of the plates to the inside of the plates, getting as close to the spotted Pf-5 as possible without actually touching the spot. The streaks were done in a cross fashion. The streaked plates were then incubated for one day at 37 ºC. After incubating for one day, the plates were then observed for inhibition of *E. coli* growth, and if inhibition was present, the zone of inhibition was measured, averaged, and recorded.

Positive and Negative Controls

P. fluorescens PF-5 Wild Type (JL4585) was used as a positive control, while a PF-5 *gacA* mutant (JL4577) was used as a negative control, in all of the experiments. The *gacA* mutant does not produce many of the antibiotic compounds that the wild type does, and the *gacA* mutant also does not show inhibition of *E. coli* DH5α on either Nutrient Agar with Glucose or Nutrient Agar with Glycerol when iron is added.

Determining the Effect of Medium on Inhibition

The Pf-5 Wild Type and *gacA* mutants were tested on LB, KB, NA+Gly, and NA+Glu, as well as each with iron added in order to examine the effects of the differing media on inhibition.

Experiment One

The initial experiment used Pf-5 cultures JL4859, JL4909, and JL4924. The initial experiment used KB, NA+Gly, NA+Glu, as well as each with iron added as described above.

Experiment Two

Pf-5 cultures JL4924, JL4973, JL4804, JL4805, JL4830, JL4926, JL4927, JL4928, were used as well as the WT and *gacA* mutants in experiment 2. Only NAGlu+Fe was used in this experiment.

Experiment Three

Pf-5 cultures JL4924, JL4807, JL4809, JL4855, and JL4865 were used as well as the WT and *gacA* mutants in experiment 3. Only NAGly+Fe was used in this experiment.

Results and Discussion

Testing for Differences in Media

The Pf-5 Wild Type inhibited *E. coli* growth, from best to worst, on NA+Gly, KB, NA+Glu, and LB (Table 1). The *gacA* mutant showed no inhibition once iron was added to the media (Table 2).

Table 1. Inhibition of *E. coli* growth by Pf-5 WT

Medium	Inhibition Zone (mm)
KB	15.2
KB+Fe	9.0
LB	7.9
LB+Fe	5.6
NAGly	14.8
NAGly+Fe	16.0
NAGlu	10.0
NAGlu+Fe	9.0

Table 2. Inhibition of *E. coli* growth by Pf-5 *gacA* mutant

Medium	Inhibition Zone (mm)
KB	11.0
KB+Fe	0.0
LB	8.0
LB+Fe	0.0
NAGly	13.0
NAGly+Fe	0.0
NAGlu	0.0
NAGlu+Fe	0.0

Experiment 1: Initial testing

The Pf-5 culture JL4859 inhibited *E. coli* over 5mm more on NAGlu+Fe and 4mm more on NAGly+Fe when compared to the wild type (Table 3). JL4909 showed no inhibition on either media. JL 4924 showed inhibition on NAGly+Fe and not on NAGlu+Fe (Table 3). Based on this experiment, one or more of *phlD, prnC, and pltA* were identified as important for inhibition on NAGlu+Fe, while one or both of *ofaA, and hcnB* were identified as being important for inhibition on NAGly+Fe. The genes noted are the only genes active when there is inhibition and inactive when there is no inhibition.

Table 3. Inhibition of *E. coli* on NAGlu+Fe and NAGly+Fe by JL4859, JL4909, and JL4924 when compared to the Wild Type and *gacA* mutants.

	Antibiotic genes							Inhibition (mm)	
	phlD	*prnC*	*rzxB*	*pltA*	*hcnB*	*ofaA*	*toxB*	NAGlu + Fe	NAGly + Fe
JL4859	+	+	-	+	+	+	-	13.15	19.50
JL4909	-	-	-	-	-	-	+	0.00	0.00
JL4924	-	-	-	-	+	+	-	0.00	12.13
WT	+	+	+	+	+	+	+	8.00	15.00
gacA mutant	-	-	-	-	-	-	-	0.00	0.00

Experiment 2: Testing on NAGlu+Fe

The Pf-5 cultures with a mutation in the *phlD* gene (JL4924, JL4804, JL4830, JL4927, and JL4828) all showed no inhibition of *E. coli*, while strains with mutations only in the *prnC* and *pltA* retained inhibition of *E. coli* (Table 4). Strain JL4804, which has a mutation in *phlD* and no other mutations, does not inhibit *E. coli*; the comparison between the *phlD* mutant and the controls is shown below in Figure 1. Therefore, I concluded that 2,4-diacetylphloroglucinol is responsible for inhibition of *E. coli* by Pf-5 on NAGlu+Fe.

Table 4. Inhibtion of *E. coli* on NAGlu+Fe.

	Antibiotic genes			
	phlD	*prnC*	*pltA*	Inhibition (mm)
JL4924	-	-	-	0.0
JL4793	+	-	+	8.3
JL4804	-	+	+	0.0
JL4805	+	+	-	8.1
JL4830	-	-	+	0.0
JL4926	+	-	-	7.9
JL4927	-	-	-	0.0
JL4928	-	+	-	0.0
WT	+	+	+	8.4
gacA mutant	-	-	-	0.0

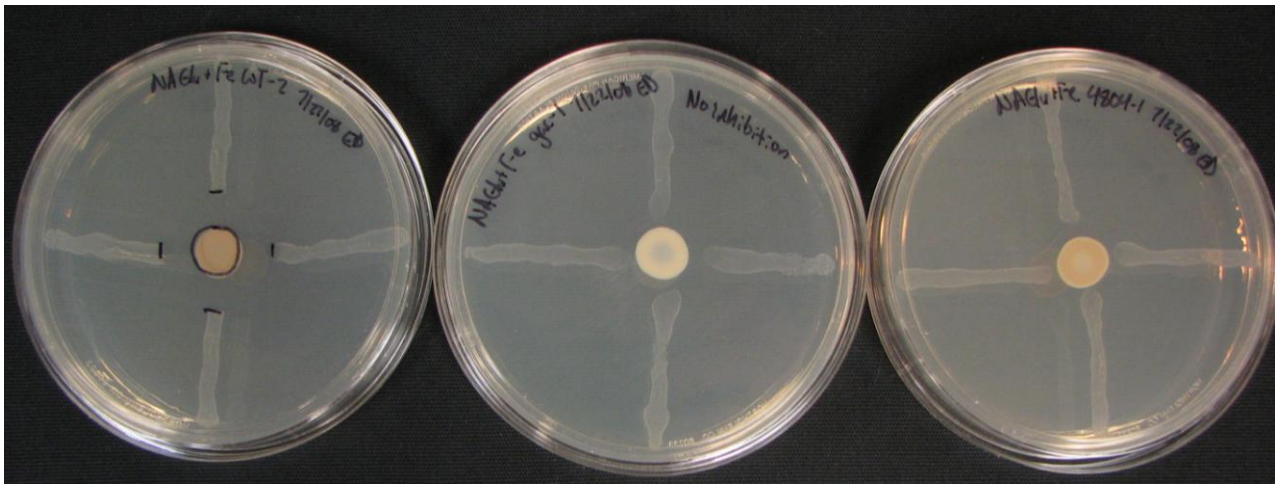

Figure 1. Inhibition by the Wild-Type (left), and no inhibition by the *gacA* mutant (middle) and *phlD* mutant (right).

Experiment 3: Testing on NAGly+Fe

Additional mutants of Pf-5 cultures were tested to identify the genes contributing to inhibition of *E. coli* on NAGly+Fe (Table 5). All of the mutants (except for the *gacA* mutant and JL4909, which was not tested in Experiment 3) showed some inhibition of *E. coli*. Because derivatives of Pf-5 with single mutations in either *hcnB* or *ofaA* inhibited *E. coli*, a strain with mutations in both the *hcnB* and *ofaA* genes was needed. Other testing also identified *phlD* as being potentially important (not shown), so a triple mutant (*hcnB*, *ofaA*, and *phlD*) was also needed. Mutants were made using procedures already established (Choi and Schweitzer 2005, T. Kadarsa, unpublished results).

Table 5. Inhibition of *E. coli* on NAGly+Fe.

	Antibiotic genes							
	phlD	*prnC*	*rzxB*	*pltA*	*hcnB*	*ofaA*	*toxB*	Inhibition (mm)
JL4924	-	-	-	-	+	+	-	12.50
JL4807	+	+	+	+	+	-	+	15.00
JL4809	+	+	+	+	-	+	+	14.00
JL4855	-	-	-	-	+	+	+	11.25
JL4865	-	-	-	-	-	+	+	6.94
WT	+	+	+	+	+	+	+	14.69
gacA mutant	-	-	-	-	-	-	-	0.00

Experiment 4: Testing New Mutants on NAGly+Fe.

The Pf-5 cultures with mutations in the *phlD* and *ofaA* genes (JL4842, JL4909, and JL4941) show no inhibition of *E. coli*, while strains with mutations in other genes retain inhibition (Table 6). The mutant JL4842 had light *E. coli* growth, indicating that the *hcnB* gene was causing some inhibition, but did not create an inhibition zone. The mutant JL4941 inhibited exactly as the *gacA* mutant, with full growth of *E. coli*. Figure 2 shows the comparison between the Wild type, the *gacA* mutant, JL4842, and JL4941. Based on these results, I concluded that both *phlD* and *ofaA* are required to inhibit growth of *E. coli* on NAGly+Fe.

Table 6. Inhibition of *E. coli* on NAGly+Fe using new mutants.

	Antibiotic genes							
	phlD	*prnC*	*rzxB*	*pltA*	*hcnB*	*ofaA*	*toxB*	Inhibition (mm)
JL4924	-	-	-	-	+	+	-	11.6
JL4842	-	+	+	+	+	-	+	0.0
JL4909	-	-	-	-	-	-	+	0.0
JL4924	-	-	-	-	+	+	-	11.6
JL4937	-	+	+	+	-	+	+	9.9
JL4939	+	+	+	+	-	-	+	15.0
JL4941	-	+	+	+	-	-	+	0.0
WT	+	+	+	+	+	+	+	15.8
gacA mutant	-	-	-	-	-	-	-	0.0

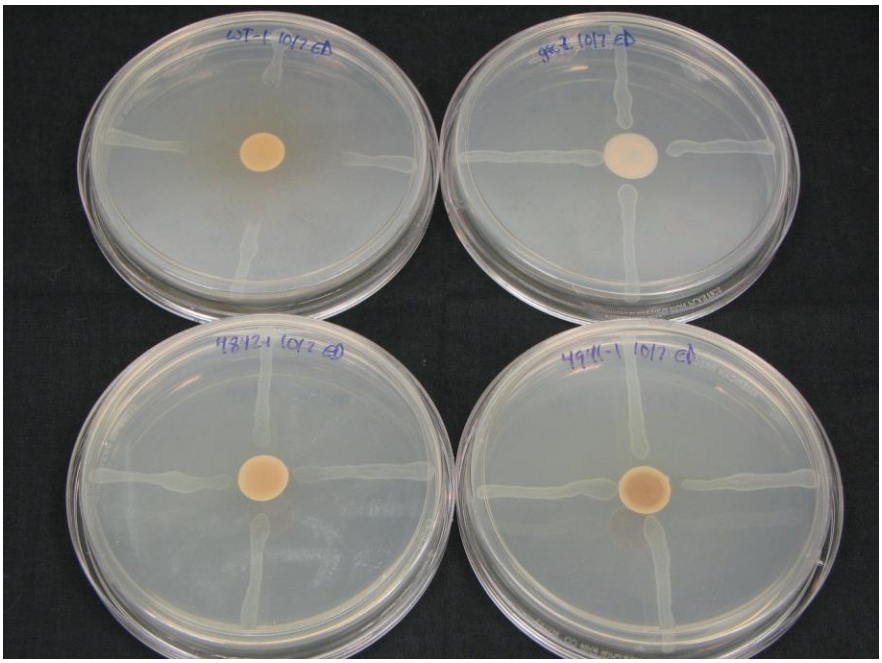

Figure 2. Inhibition by the Wild-type (upper left), and no inhibition by the *gacA* mutant (upper right), JL4842 (lower left), and JL4941 (lower right).

Conclusions

On Nutrient Agar with Glucose.
phlD is the only gene required for inhibition of *E. coli* by *P. fluorescens* Pf-5 on Nutrient Agar with Glucose. All strains with a mutation in the *phlD* gene show no inhibition of *E. coli*.

On Nutrient Agar with Glycerol.
phlD and *ofaA* are the genes required for zones of inhibition against *E. coli* by *P. fluorescens* Pf-5 on Nutrient Agar with Glycerol. Strains with mutations in both *phlD* and *ofaA* produced no inhibition zone against *E. coli*. However, growth of *E. coli* over the entire plate was less dense in the presence of Pf-5 or JL4842 (the *phlD, ofaA* double mutant) than with the *gacA* mutant or JL4941 (the *phlD, ofaA,* and *hcnB* triple mutant). Therefore, HCN production by Pf-5 also inhbits *E. coli*, and this inhibition is seen as a reduced density of growth over the entire plate rather than as a zone of inhibition.

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References

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