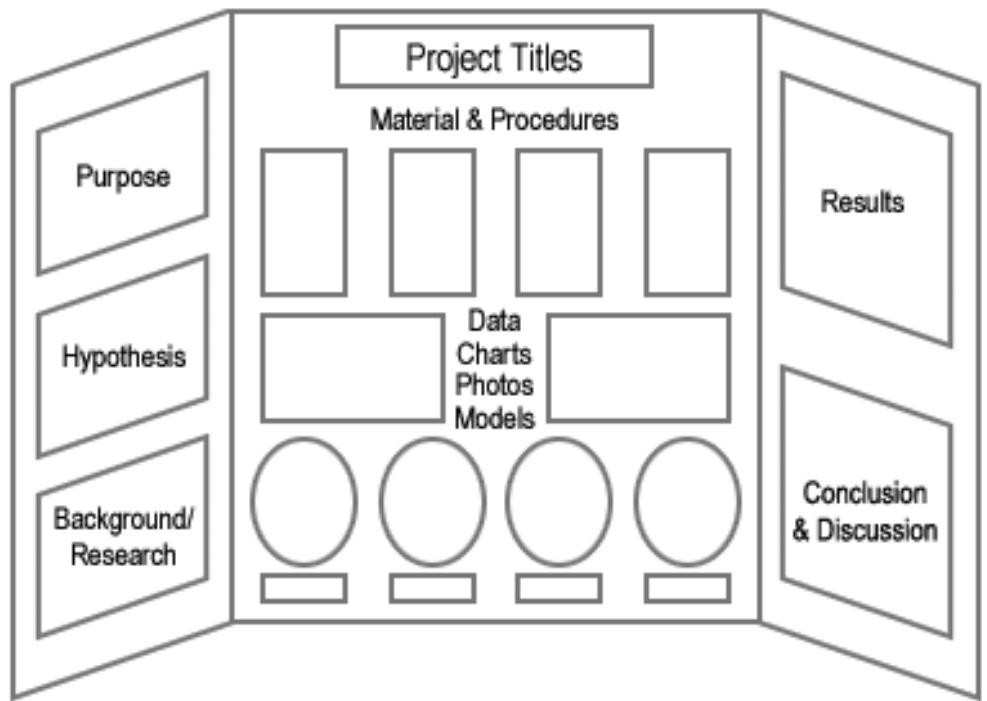


Journal of Emerging Investigators

**Science publishing is not just for Ph.D.'s.
Students can do it too!**

Sarah C. Fankhauser, Harvard Medical School
July, 2012



Environmental strains of *P. aeruginosa* are not highly resistant to rifampicin despite having sequence variability in the antibiotic target gene RNA polymerase.

John Doe¹, Jane Doe¹, members of the JEI Staff¹
¹Harvard Medical School, Local High School

Summary:
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Introduction:
 Over the last fifty years, the discovery of antibiotics has allowed humans to combat potentially devastating classes of bacterial diseases. There are several main classes of antibiotics that have many different modes of action. Some antibiotics alter bacterial cell wall stability, whereas others inhibit critical processes such as DNA transcription or translation (1-2). Over the last decade, however, there has been an emergence of increasingly resistant strains of bacteria that are becoming increasingly problematic for public health (3-4). There are several mechanisms that bacteria have developed to avoid the toxicity of antibiotics. One of the most common antibiotic resistant species is *Pseudomonas aeruginosa*. Some *P. aeruginosa* antibiotic resistant strains are able to avoid toxicity by synthesizing an efflux pump that simply pumps the antibiotic out of the bacterial cell (5). Other resistance genes have mutations in the DNA that prevent the antibiotics from working effectively. For example, many of these bacterial strains have single point mutations in the base pair sequence that alter the antibiotic codon to code for a different amino acid. These single amino acid changes can alter the antibiotic target and prevent the antibiotic from binding and working (7). One question that remains is: how prevalent are mutations in specific antibiotic targets in environmental bacterial populations? We hypothesized that all environmental *P. aeruginosa* strains isolated would be sensitive to the antibiotic rifampicin, since they have never been exposed to the drug previously and thus have not evolved resistance. Rifampicin works by binding to one subunit of the bacterial RNA polymerase (RNAP) inhibiting its essential function of transcribing DNA to RNA (8). Mutations at the DNA level, however, can still result in a mutant enzyme which is resistant to rifampicin (9). In order to test our hypothesis we examined both the sensitivity of environmental strains to rifampicin as well as the mutation frequency in the RNA polymerase gene that controls the RNA polymerase. Using a plasmid vector we used a technique called lambda Catt¹ digestion (10). Cell¹ isolated from the bacterial culture, is a restricted endonuclease that cuts double-stranded DNA at the enzyme that controls mismatched DNA. We were able to grow many strains of *P. aeruginosa* in a short period of time without the need for DNA sequencing. We found that through most strains share the same susceptibility to rifampicin polymorphisms. This suggests that mutations can occur in essential genes at a low frequency. However, most of these mutations do not result in resistance to antibiotics.

Results:
*Isolation of environmental *P. aeruginosa* strains*
 In order to examine the antibiotic-sensitivity and mutation rates of environmental strains of *P. aeruginosa* we first isolated environmental strains of *P. aeruginosa* from several unique sources. We used CaCl₂ selection agar to sample unique environmental strains (10). We grew samples from 50 locations around the Boston area including playgrounds, soil, and plants. From these 50 sample locations we were able to isolate 30 *P. aeruginosa* strains to use as controls. We grew one laboratory strain, *P. aeruginosa* PAO1, as a control. Our strain (R) is resistant to rifampicin. We found that these 30 strains were in fact *P. aeruginosa* through the use of bacterial strain identification strips. Furthermore, these strains showed similar growth patterns. All strains have a grape shape, a common feature of growing *P. aeruginosa* on agar plates (11).

*Most strains of *P. aeruginosa* are sensitive to rifampicin.*
 We next wanted to test the sensitivity of these strains to the antibiotic rifampicin. Rifamycin is an RNA polymerase inhibitor that is known to inhibit the growth of *P. aeruginosa* (12).

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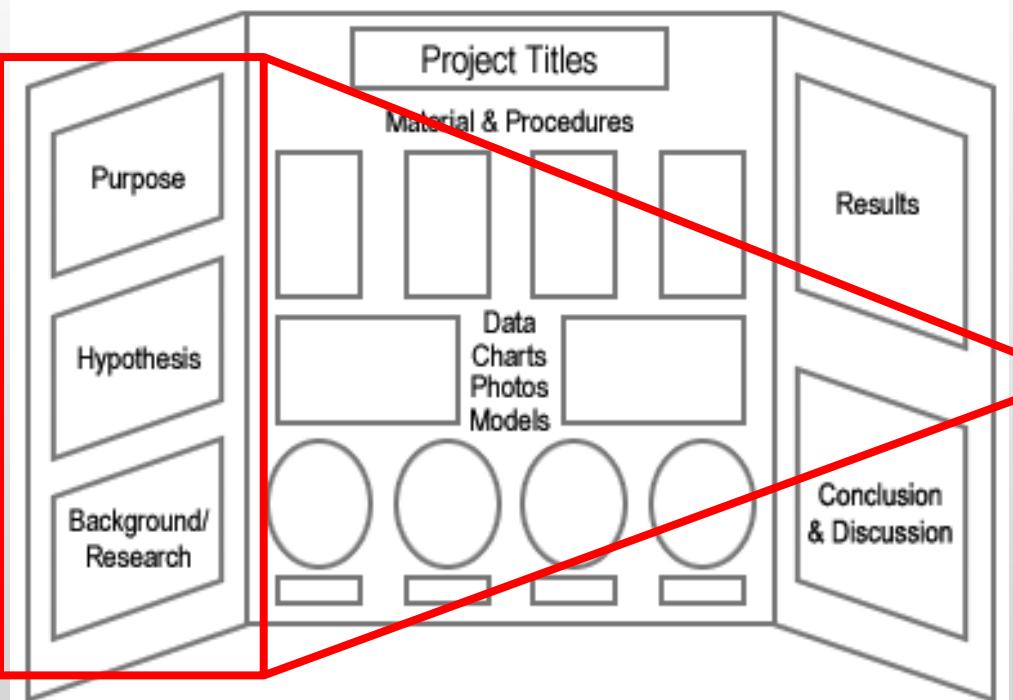
We first grew all 30 strains, along with R, in liquid culture in the presence of rifampicin at various concentrations. Optical density (or the cloudiness) is a readout of growth over time. In the 30 strains and the S strain grew in the presence of rifampicin when compared to no antibiotic present. The R strain and the R lab strain did not grow in the presence of rifampicin.

To test whether the sensitivity to rifampicin in liquid culture was also observed, we first spread a lawn of *P. aeruginosa* onto an agar plate and a negative control on one plate. Concentrations of rifampicin on our incubator we measured. After 24 hours of incubation we measured the optical density of the lawns. If all strains were inhibited in the liquid broth, the R was inhibited greatly by the plate. The two strains that did not grow in the liquid broth were R and S. These results suggest that R and S strains are in fact *P. aeruginosa*. However, since we did not have a way to measure the optical density of the lawns, we have a small percentage error. From these results it appears that R strains in liquid broth are more sensitive to rifampicin than S strains.

*Isolation of environmental *P. aeruginosa* strains*
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1

2



Sample Paper: Environmental strains of *P. aeruginosa* are not highly resistant to rifampicin despite having sequence variability in the antibiotic target gene RNA polymerase.

John Doe, Jane Doe, members of the JEI Staff
Harvard Medical School, Local High School

Summary

Antibiotic resistance of bacterial pathogens is an increasing public health concern. Patients with normally routine hospital-acquired infections are now at serious risk due to insufficient antibiotics that result in the death of thousands worldwide. One bacterial species that is partially responsible for these deaths is *P. aeruginosa*. As an opportunistic pathogen, *P. aeruginosa* normally does not cause infections, and is found in the normal environment. While many clinical *P. aeruginosa* strains are resistant to several antibiotics there have been no previous studies examining the

from the results suggest that antibiotic

Introduction

antibiotic resistant species is *Pseudomonas aeruginosa*. Some *P. aeruginosa* antibiotic resistant strains are able to avoid toxicity by synthesizing an efflux pump that simply pumps the compound out of the bacteria (5). Other resistant strains have mutations in their DNA that prevent the antibiotics from working (6-7). For example, many of these bacterial strains have single point mutations (one base pair changes) that alter the amino acids coded by the DNA. These single amino acid changes can alter the antibiotic target and thus prevent the antibiotic from binding and working (7). One question that remains is: how prevalent are mutations in specific antibiotic targets in environmental bacterial populations? We hypothesized that all environmental *P. aeruginosa* strains isolated would be sensitive to the antibiotic rifampicin, since they have never been exposed to the drug previously and thus have not evolved resistance. Rifampicin works by binding to one subunit of the bacterial RNA polymerase (RNAP) and inhibiting its essential function of transcribing DNA to RNA (8). Mutations at the DNA level, however, can result in a mutant polymerase which is resistant to

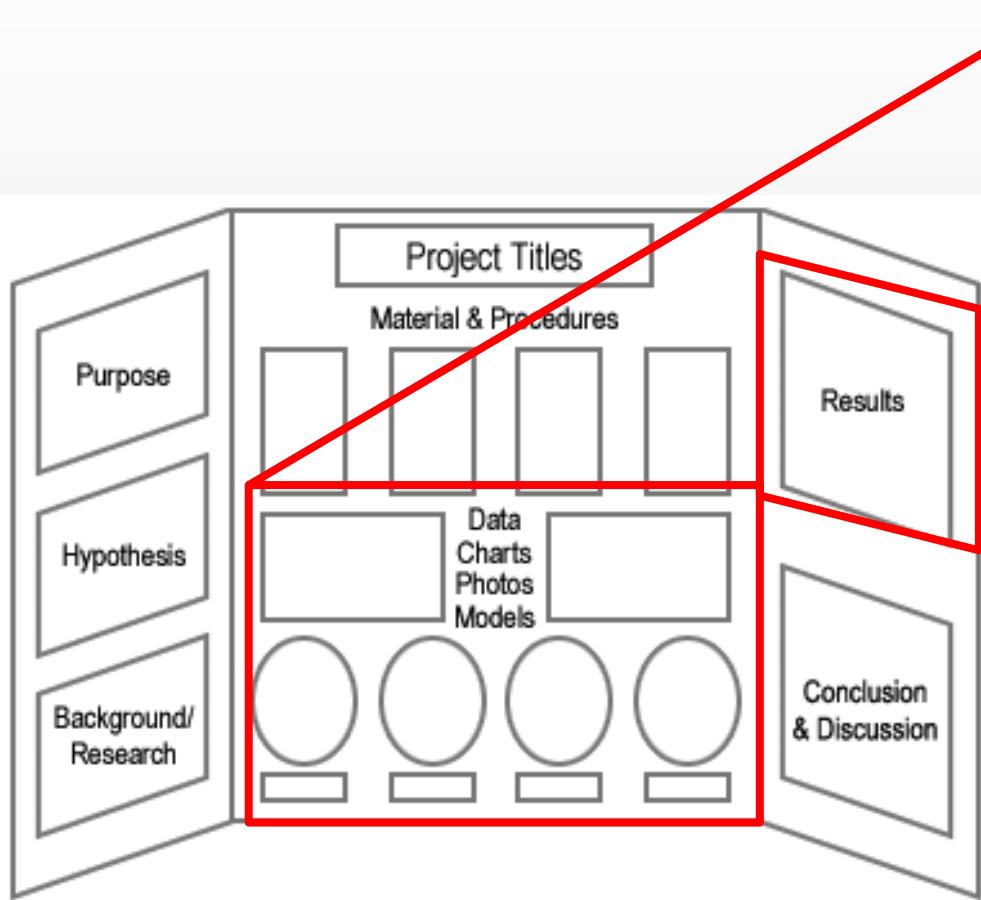
INTRODUCTION

Science Fair/ lab report

- Purpose
- Hypothesis
- Background research

Science Article

- Purpose
 - Explain the topic.
 - Why is this topic important?
- Background
 - What is already known?
 - What is unknown? What questions are you interested in?
- Hypothesis
 - What is your hypothesis and how did you come up with it?
- Brief summary
 - What are the general results that you found?



Results

characterize several strains. We used Cetrimide selective agar to isolate unique environmental strains (10). We took samples from 50 locations around the Boston area including playgrounds, soil, and plants. From these 50 sample locations we were able to grow 30 *P. aeruginosa* strains. In addition, we grew two laboratory strains in parallel as controls: one strain (R) is resistant to rifampicin and the other strain (S) is sensitive to rifampicin. We confirmed that these 30 strains were in fact *P. aeruginosa* through the use of bacterial strain identification strips. Furthermore, all strains smelled similar to the two control strains which have a grape scent, a common feature of growing *P. aeruginosa* on agar plates (11).

Most strains of *P. aeruginosa* are sensitive to rifampicin

We next wanted to test the sensitivity of these strains to the antibiotic rifampicin. Rifampicin is an RNA polymerase inhibitor that is known to inhibit the growth of *P. aeruginosa*. We first grew all 30 strains, along with our two control strains, in liquid culture in the presence and absence of varying concentrations of rifampicin. We then used the optical density (or the cloudiness) of these cultures as a readout of growth over time. In liquid culture 28 of the 30 strains and our S strain grew dramatically slower in the presence of antibiotic when compared to growth with no antibiotic present. The remaining two environmental strains and the R lab strain grew identically whether or not rifampicin was present in the culture media.

We next wanted to test whether the sensitivity to the antibiotic seen in liquid cultures was also observed when grown on agar plates. We first spread a lawn of each bacterial strain on two "blank" agar plates

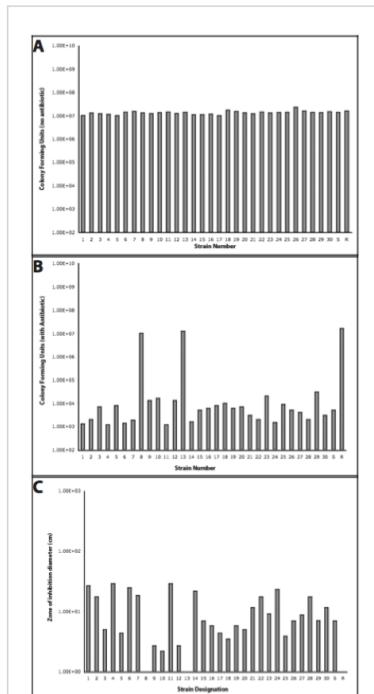


Figure 1. (A) Novel *P. aeruginosa* strains isolated from the environment grow similarly in media lacking antibiotics. All 30 isolated strains as well as two reference strains (R and S) were grown in LB broth lacking antibiotics for 24 hours. The cultures were then serially diluted onto plates lacking antibiotics and colony-forming units were calculated. Shown is a representative of two independent experiments. (B) Most novel *P. aeruginosa* strains are sensitive to rifampicin in liquid culture. All 30 isolated strains as well as two reference strains (R and S) were grown in LB broth containing rifampicin for 24 hours. The

RESULTS

Science Fair/ lab report

- Data charts/ graphs
- Variables
- Summary of data

Science Article

- Explanation of questions to test the hypothesis.
- Rationale for each experiment
 - How does each experiment address an aspect of the hypothesis? What are the variables?
- Explanation of data
 - Refer to data charts/graphs and provide summary of results.
- Data charts/graphs

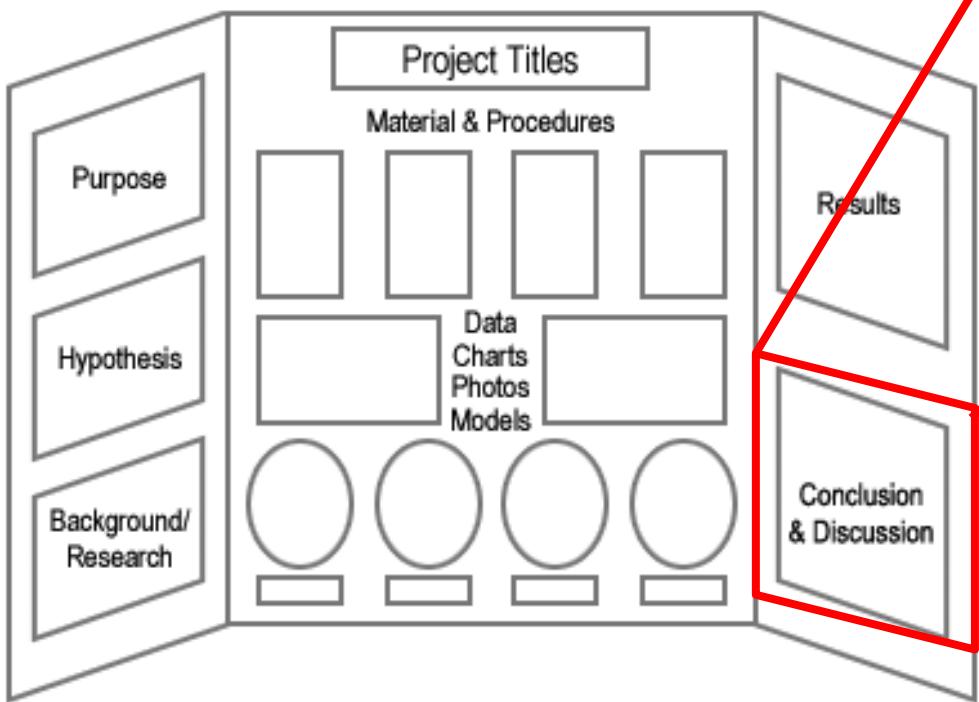
Discussion

broad range of bacterial pathogens that have emerged in recent years has recently resulted in the emergence of bacteria that are resistant to many or all forms of

current antibiotic therapy. Presently, the majority of these antibiotic resistant strains can be found in hospital settings, where the use of antibiotics is high. In this study, we wanted to determine the frequency of antibiotic resistance in environmental bacterial populations that have not been exposed to antibiotics. We chose to isolate several strains of the bacterium *Pseudomonas aeruginosa* from various locations around the Boston area. *P. aeruginosa* is an opportunistic bacterial pathogen that is notoriously antibiotic resistant. By examining environmental strains of a clinically relevant species, we hoped to gain insight into the underlying mechanisms of antibiotic resistance. From fifty unique sampling locations, we were able to isolate 30 different strains of *P. aeruginosa* using selective agar media. We re-isolated all 30 strains as clonal populations for this study individually for future use. While using these types of selective media is not always 100% effective for identification, we have the advantage that *P. aeruginosa* strains smell subtly of grapes as well as confirmation through a bacterial identification strip. We were able to use these attributes as secondary tests to confirm that the strains growing were indeed *P. aeruginosa*. While 30 strains of a single bacterial species is by no means an exhaustive examination of environmental bacteria, these experiments lay the groundwork for expanding these studies to other environmental species.

We were curious whether these unique strains would be sensitive to the antibiotic rifampicin. We hypothesized that since these strains had never been exposed to antibiotics in a clinical setting, they would all be sensitive to this drug. To our surprise, we were able to identify two different strains that were resistant to this antibiotic in both liquid culture and when grown on agar plates. This means that even in environmental settings there may be an underlying antibiotic resistant population of bacteria. This is an important finding for clinics, where giving a single antibiotic treatment may not yield the desired result. This suggests that giving multiple antibiotics as a cocktail may be a more effective strategy in order to eliminate these naturally occurring antibiotic resistant strains. However, these results confirmed our original prediction that the majority of environmental strains would be sensitive to antibiotics.

Even though the majority of the strains have the same phenotype (sensitivity to rifampicin), we wondered whether these strains had identical DNA sequences in the target of this drug, RNA polymerase. We hypothesized that there would be no differences between these strains at the DNA sequence level especially considering the essential nature of RNA polymerase. In order to test this we used Cel1 digests that allow for the sensitive detection of mismatches between DNA fragments without the need of DNA sequencing. We used PCR to amplify the RNA polymerase gene from all 30 strains and made heteroduplexes of each product with the RNAP gene from a laboratory strain. Interestingly, all 30 strains showed varying point mutations throughout this gene product, regardless of whether the strain was resistant or sensitive to rifampicin. However, a major limitation to the Cel1 digest approach is that this technique does not identify the exact location of the mutation within the gene. Even so, these results suggest that even in the environment, bacterial strains are developing new mutations in their DNA sequence. The majority of these mutations, however, are silent mutations that have no functional impact on gene products, in this case RNA polymerase. Furthermore, in two of these cases this low-level mutation frequency allowed for the development of antibiotic resistant strains even though they had not been exposed to rifampicin in a clinical setting. This important finding means that bacterial species are constantly mutating and evolving while our treatment drugs (antibiotics) have remained static for the last decade. This study paves the way for future more expansive studies across many different bacterial species that will help us gain more insight into the underlying mechanisms of acquiring antibiotic resistance.



DISCUSSION

Science Fair/ lab report

- Summary of data
- Conclusions
- Future experiments

Science Article

- Restate hypothesis
- Summary of results
- Interpretation of results
 - What are your conclusions?
- How do the conclusions agree/disagree with the hypothesis?
- Significance of conclusions?
- Future directions, remaining questions.

Methods

P. *aeruginosa* strains were collected from multiple locations in the United States. Swabs were cultured from these swabs by inoculating Cetrimide agar medium and incubating overnight at 37°C to allow single colonies. Cetrimide selectively inhibits the growth of *P. aeruginosa*, facilitating the production of a *P. aeruginosa*-specific blue-green pigment, malachite green, which can be used to identify *P. aeruginosa* strains. For rifampicin-sensitivity assays, environmental and laboratory *P. aeruginosa* strains were grown in Luria-Bertani (LB) rich medium for both liquid-culture and agar plate-based assays. When required, *P. aeruginosa* strains were grown in the presence of rifampicin.

Primers and PCR amplification of *P. aeruginosa rpoB*

We used the *P. aeruginosa* PAO1 genome sequence (GenBank accession number AE004091.2) to design forward and reverse primers to amplify the gene encoding the protein target for rifampicin, a subunit of RNA polymerase known as β (encoded by the gene *rpoB*). The sequence of the forward primer is 5' ATGGCTTACTCATACAGT 3'; that of the reverse primer is 5' TTATTCGGTTCCAGTTG 3'. We used a high-fidelity DNA polymerase (PhusionTM, Finnzymes) for the PCR amplification of the *rpoB* gene in the 30 environmental and two control *P. aeruginosa* strains to ensure that no mutations were introduced during the amplification process.

Cel1 Preparation

We prepared Cel1 enzyme directly from celery juice as described in REF. Essentially we juiced 4 lbs? of celery to produce 1.5 liters of celery juice. To each liter of celery juice we added 50ml of 2M Tris, pH 7.7, and 1ml of .1M PMSF (a protease inhibitor). The solution was centrifuged at 15,000 g for 20 min at 4 C. The supernatant was saved; we added 144 g of (NH4)2SO4 to each liter of supernatant. This solution was mixed gently for 1 hour at 4 C. We centrifuged the solution at 15,000 g for 45 minutes at 4 C. We added 390 g of (NH4)2SO4 to each liter of supernatant. This solution was mixed gently overnight at 4 C. The solution was then centrifuged at 15,000 g for 90 minutes at 4 C. The supernatant was discarded and the pellet, which contained the protein-precipitate, was saved at 4 C. We thoroughly resuspended the protein pellet in 150 mls of Buffer B: 1M Tris, pH 7.7, 0.5M KCl, 0.01% Triton X-100, and 100 μ M PMSF. We extensively dialyzed the protein solution in to 5 L of Buffer B (with 5-6 exchanges). The dialyzed protein was aliquoted and stored at -80 C.

Cel1 Digest

Heteroduplexes were formed by mixing wildtype PCR products and mutant PCR products at a 1:1 ratio; the samples were then heated at 98 C for 5 minutes to denature the strands and then slowly cooled to room temperature to re-anneal the strands. The digests were performed with 5 ul of the heteroduplex (or homoduplex control) mixed with 5 ul of the Cel1 digestion solution. The Cel1 digestion solution was made up of a 1/10 dilution of 10x Cel1 digestion buffer (100 mM HEPES (pH7.5); 100 mM MgSO4; 0.02% (w/v) Triton X-100; and 200 ng/ml BSA) in H2O with Xng of Cel1 enzyme.

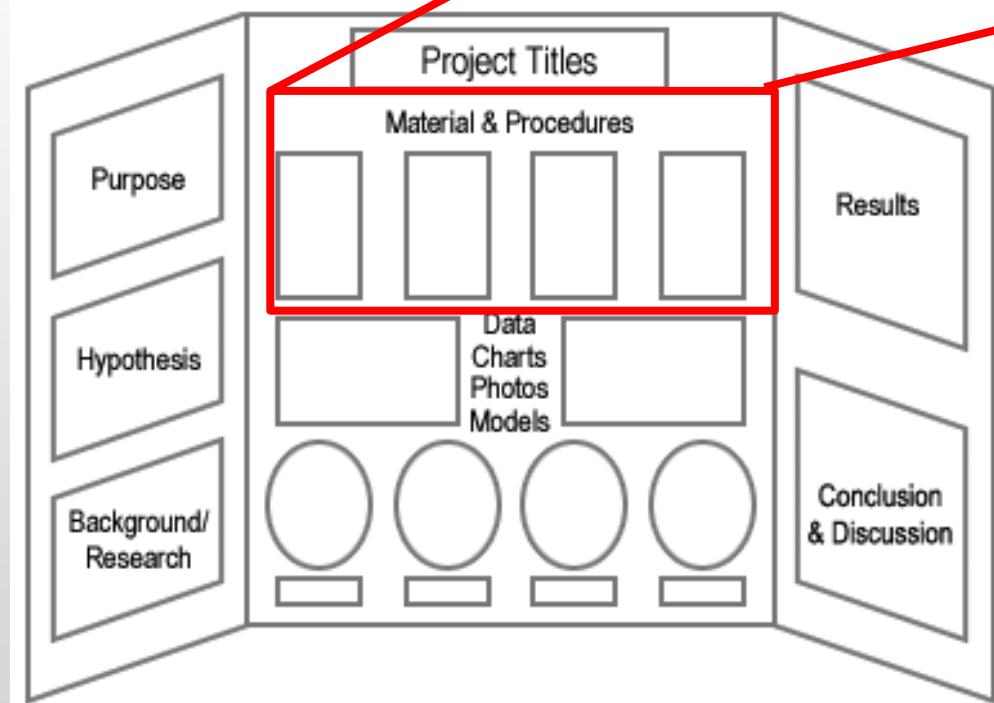
What I've been doing is making 100 μ L of a Cel1 mix that contains 20 μ L 10x buffer, 1 μ L of a 1:10 dilution of the Cel1 aliquots in the -80, and 79 μ L of H2O.

For digestions, I take 5 μ L of the heteroduplex (or homoduplex control) and mix it with 5 μ L of the Cel1 mix, and run Cel1Dig program.

Finally, I add 10 μ L of a stop solution containing 75 mM EDTA (pH 8), and 2.5 M NaCl, and run the results on a gel.

Rifampicin sensitivity assays

To assess the sensitivity of the environmentally-isolated and laboratory strains of *P. aeruginosa* in liquid culture, we inoculated culture tubes containing LB broth in the absence of rifampicin with a single colony of each strain and incubated overnight at 37°C to allow the cultures to become saturated. We then made a 1:100



Science Fair/ lab report

- List of materials
- List of procedures

Science Article

- No lists
- Methods broken up into individual sections (if many experiments were performed)
- Explanation of procedure in paragraph form
 - Description of unique materials

REFERENCES

Science Fair/ lab report

- List of references at end of document

Science Article

- References placed at end of article.
 - In order in which they are cited in the article.
- Citations are expected throughout the paper.
 - Introduction should be especially highly referenced.

Science Fair/ lab report

- Catchy title
- Abstract: shortened introduction

Science Article

- Descriptive title
- Abstract: brief summary of hypothesis, experiments and **results**. Less than 250 words.

Overview of the Scientific Review Process

Authors Submit the Manuscript

The teacher/senior author submits the completed manuscript on the JEI website.

Editor Receives the Manuscript

A JEI editor verifies that the manuscript is in accordance with our submission guidelines and distributes it to three scientific reviewers (PhD candidates with expertise in the relevant field).

Reviewers Assess the Manuscript

The JEI reviewers analyze the manuscript, including the interest of the scientific question, the quality of the scientific writing, the believability of the results, and the soundness of the conclusions.

Authors Receive Reviewer Comments and Manuscript Decision

An editor will provide the authors with the reviewer comments, which consist of suggestions to improve the manuscript (such as a topic that should be included in the introduction, an alternative interpretation of a result, or an additional experiment that could provide insight into the scientific question). These comments are designed not only to improve the manuscript but also to help the students better understand both this project and the general process of scientific inquiry. The editor will also inform the authors if the manuscript has been accepted for publication, and if so, the changes that must be made before the manuscript can be published.

JEI Publishes the Manuscript

Once the required revisions have been performed, the JEI copy-editors will prepare the manuscript for publication and the manuscript will be published on the JEI website.