Winter 12-10-2015

# Super-Killers: Environmental Isolates that Antagonize Pathogenic Vibrio

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# SUPER-KILLERS: ENVIRONMENTAL ISOLATES THAT ANTAGONIZE PATHOGENIC *VIBRIO*

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HONORS PROJECT

Submitted to the University Honors Program at Bowling Green State University in partial fulfillment of the requirements for graduation with

UNIVERSITY HONORS

DECEMBER 9, 2015

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**Acknowledgments**

I thank Hans Wildschutte, Andrew Torelli, Steven Norris, Payal Chatterjee, Sarah James and Elizabeth Davis for their assistance and guidance through this process and for taking the time to teach and challenge me to grow this project. All of the environmental isolates used in this experiment are due to the collection efforts of Kathryn Kauffman and members of Martin Polz's laboratory. Recognition of Hans Wildschutte again for review of this article, my parents and sisters for their support and encouragement, and the Biological Sciences Department and University Honors Program at Bowling Green State University for their support. This project was funded by the National Institutes of Health and by the Building Strength Award from Bowling Green State University.

**Abstract**

*Vibrio* are members of a bacterial group that thrive in diverse aquatic environments including on the surface of aquatic animals, free-living in the water column, and in association with suspended particles. The total *Vibrio* counts in the coastal ocean ranges from $10^3$-$10^5$ per milliliter of water depending on seasons and water temperature. Although many different species of *Vibri*o persist in the water column, pathogenic strains, such as *Vibrio cholerae* and *Vibrio parahaemolyticus* are absent or rare in marine environments. We hypothesize that the low abundance of these pathogenic species may be due to interspecific competition among environmental strains, in which the pathogens are inhibited or out-competed by distantly related non-pathogenic isolates. In order to test this hypothesis, 3,456 environmental strains isolated from size fractionated marine particles were individually tested for their ability to inhibit *Vibrio* pathogens that cause disease in humans. Because pathogenic *Vibrio* species are not observed in coastal marine environments, natural strains exhibiting antagonistic activity are expected to produce antibiotics that inhibit the growth of *Vibrio* pathogens that cause cholera around the world. In this study, we identified 131 environmental isolates that showed an antagonistic phenotype against a panel of eight different *Vibrio* pathogens. Of the 131 environmental strains, eight isolates were able to kill six or more pathogens and were defined as super-killers (SKs) because of their ability to inhibit multiple pathogens. These SKs represent a promising source of novel antibiotics.

**Introduction**

*Vibrio* are heterotrophic bacteria of the family *Vibrionaceae* that persist and thrive in the marine environment [1].These bacteria are abundant at $10^3$-$10^5$ per milliliter of ocean water and thrive in diverse marine habitats including in association with various sizes of suspended particles, on and in the surfaces of marine animals, and free-living in the water column [2]. While many *Vibrio* species are harmless, non-disease causing variants that play important roles in nutrient cycling in the marine environment, there are a few *Vibrio* species that are pathogenic and pose devastating health risks in areas with high levels of poverty, overcrowding, and poor sanitation [1, 3].

*Vibrio* pathogens, including *Vibrio cholerae* and *Vibrio parahaemolyticus*, cause disease in nearly one third of the world's population [4, 5]. These pathogens are spread by consuming contaminated food or water and subsequently colonize the intestines and cause cholera and cholera-like diseases. After colonization, the pathogens produce toxins, such as the cholera toxin, that results in the loss of large volumes of water into the intestinal lumen [5]. This then causes severe diarrhea that leads to dehydration and even death if untreated. Without treatment through rehydration or antibiotics, the mortality from severe cholera is approximately 50% of those infected [5, 6]. Unfortunately, *Vibrio* pathogens have evolved resistance to most antibiotics currently being used to treat infection. Making this problem even worse is that many large pharmaceutical companies have ceased development of treatments and antibiotics against human pathogens due to low profitability [3]. Thus, research labs at universities, including the work done in the Wildschutte lab at Bowling Green State University, now spearhead the effort of antibiotic discovery in order to discover novel antibiotics that can be used to treat human pathogens.

In the absence of cholera outbreaks, sampling studies show that pathogenic *Vibrio* are rarely detected in the coastal ocean environment, suggesting their abundance is low or absent [2]. As an explanation of this low fitness, we hypothesize that the natural strains found in the ocean environment exhibit strong competition for nutrients that inhibit the growth of pathogens. A potential trait resulting in this competitive pressure is the production of novel antibiotics that allow certain strains to out-compete other isolates, especially pathogens that have evolved to colonize the human host. Previous research by Cordero and Wildschutte demonstrated that interspecific competition does occur by natural isolates of Vibrio but their effects against pathogens remain unknown [7]. To test our hypothesis, we investigated whether non-pathogenic *Vibrio* isolates have the capability to inhibit pathogenic *V. cholerae* and *V. parahaemolyticus* through the production of inhibitory compounds. We used a collection of 3,456 environmental strains and tested their ability to inhibit pathogenic strains consisting of five *V. cholerae* and three *V. parahaemolyticus* isolates. Results showed that 131 strains exhibited strong antagonistic activity against the panel of pathogens. Environmental isolates that inhibit the *Vibrio* pathogens represent a source of inhibitory compounds that will be

further characterized for novel antibiotic activity.

**Methods**

*Environmental strain isolation and growth conditions*

Water samples were collected on Aug 10th, September 18th, and October 13th of 2010 from the marine estuary off the coast of Nahant, Massachusetts and processed for the isolation of *Vibrio*s. Water samples were size fractionated and passed through a 63 µm, 5 µm, and 1 µm filters. Suspended particles captured on these filter were homogenized, resuspended, and passed through a 0.2µm filter and then placed on Marine Tryptic Soy Broth media (MTSB) for the selection of *Vibrio* isolates. Strains were serially passaged three times on Tryptic Soy Broth (TSB) media with a final concentration of 2% NaCl (TSB2) to ensure pure colony isolation. From these sampling efforts, 3,456 environmental strains of *Vibrio* were isolated and organized in a 96 well plate format (Table 1). For storage, isolates were frozen in 140 µL of TSB2 media and 60µL of 70% glycerol. When cultured for antagonistic assays, the strains were streaked for isolation and individual colonies were used to inoculate TSB2 media. All cultures were incubated at 23°C for 48 hours with shaking at 250 rpm.

*Pathogenic strain isolation and growth conditions*

Eight pathogenic strains of *Vibrio cholerae* and *Vibrio parahaemolyticus* (Table 2) were co-grown with the environmental isolates to test for antagonistic activity. Pathogens of diverse serotypes were chosen that were well characterized and originally isolated at different times and locations. Many of the pathogens were originally collected during outbreaks of cholera in India, Thailand, and Bangladesh. Prior to experimentation, the pathogens were streaked on TSB2 agar plates for isolation and incubated at 37°C for 18-24 hours. A single colonies was picked and used to inoculate a 3 ml of liquid TSB2 media. All cultures were incubated at 250rpm at 23°C for 18-24 hours.

*Antagonistic Assays*

To test for antagonistic activity against the pathogenic strains of *Vibrio,* environmental isolates were co-grown with each of the eight pathogens. All assays were performed in a 96 well

format. Each well contained 1100 microliters of TSB2 which were inoculated with *Vibrio* environmental isolates. The strains were incubated at 23°C for 48 hours. The pathogens were incubated in 5 milliliters of liquid TSB2 at 37°C for 18-24 hours. The antagonistic assay was performed on a 150mm x 15mm petri dish with TSB2 agar media. To test for inhibitory activity, 50 µl of the pathogen were spread-plated onto the TSB2 agar plates and then 1 µl of the environmental strains were transferred to the spread-plated strain using a 96-prong stamper. All isolates were co-grown at 23°C for 18 to 24 hours and then screened for antagonistic by observing a zone of growth inhibition of the pathogen.

**Results**

*Strain isolation strategy.*

To determine if environmental strains can inhibit the growth of *Vibrio* pathogens in the marine environment, natural isolates were collected from water samples off the coast of Nahant, Massachusetts and processed for the isolation of *Vibrio* species. If natural isolates out-compete pathogens in the coastal environment through antibiotic production, we predict that strains exhibiting antagonistic activity will persist over temporal and spatial scales. To address this issue, we utilized a collection of strains that was compiled over the course of three months, on August 10$^{th}$, September 18$^{th}$, and October 13$^{th}$ of 2010, and from different sizes of suspended marine-derived particles (Table 1). This collection of strains represent isolates across temporal and spatial scales. We reasoned that if natural isolates inhibit pathogenic *V. cholerae* and *V. parahaemolyticus,* then persistent bacterial competition may contribute the low abundance of *Vibrio* pathogens, which is observed in the coastal marine environment. We competed the environmental strains against a panel of *Vibrio* pathogens that are well characterized and known to cause human cholera and cholera-like disease (Table 2).

*Antagonistic Activity*

To determine if environmental isolates could inhibit pathogenic *Vibrio*, we tested all 3,456 strains against a panel of eight *Vibrio* pathogens consisting of *V. cholerae* and *V. parahaemolyticus* using a high-throughput antagonistic assay. Briefly, the environmental

isolates and pathogenic strains were co-grown on nutrient agar media to represent competition on solid marine particles. After 18-24 hours of incubation, each assay was screened for zones of clearing, which represent the inhibition of growth by the pathogen (Figure 1). After testing all 3,456 strains, 131 environmental isolates exhibited strong antagonistic activity against *Vibrio* pathogens (Figure 2). Strains that showed antagonistic activity inhibited at least one pathogenic strain with some isolates inhibiting all eight pathogens. These strains represent sources of potentially new antibiotics.

Of the 1,152 environmental strains collected on August 10$^{th}$ 2010, 39 were observed to have an antagonistic phenotype (Table 1, strains in parentheses). Twenty-two out of the 39 inhibitory strains (56%) from this day were associated with 63µm particles with the remaining 17 isolated from 5ul and 1ul size fractionated particles and free-living in the water column. The samples collected on September 18$^{th}$ contained 62 isolates that showed antagonism against the pathogens and half of these strains (50%) were free-living. Of the remaining isolates on September 18, 14 strains (22%) were associated with 1µm particles, 4 strains (6%) were associated with 5µm particles, and 12 strains (19%) were associated with 63µm particles. The final collection date on October 13$^{th}$ yielded another 30 environmental isolates that were able to antagonize the pathogenic *Vibrio* strains; 14 out of 30 strains (47%) were free-living isolates making these the most common antagonists from this collection period. Strains associated with 63µm being the second most common with 6 (20%), and bacteria associated with 5µm and 1µm particles each had 5 strains (16%) that killed pathogens. Together, these antagonistic strains represent isolates dispersed over temporal and spatial scales that inhibit *Vibrio* pathogens.

*The identification of Super-killers*

Of those strains that showed antagonistic activity, eight were identified as Super-Killers (or SKs) meaning that they have the ability to inhibit at least six pathogens. The eight SKs can be identified in Figure 1 and are separately listed in Table 3. Of the samples taken on August 10$^{th}$, three isolates were identified as SKs. One of these SKs were isolated from 1µm size fractionated suspended particles and the other two were isolated on 5µm size fractionated particles. There were two SKs found from the samples taken on September 18$^{th}$; these were isolated on 5µm

and 63µm size fractionated suspended particles. The final three SKs were isolated from 1µm size fractionated suspended particles and two on 5µm fractionated suspended particles collected on October 13th. Interestingly, all SKs were isolated on particles and none were found as free-living in the water column. Because these antagonistic strains are found on different sized particles, isolated on separate days, and inhibit different pathogens, we predict these environmental isolates are producing diverse compounds that represent a source of novel antibiotics. Members of the Wildschutte lab are currently using genetic methods to identify and characterize the inhibitory products.

**Discussion**

Cholera outbreaks are usually observed in geographical areas where human overcrowding, poverty, and poor sanitation exist. The disease results from ingesting fecal contaminated food and water, and during outbreaks, the *Vibrio* pathogen persists at high levels in the water column. In contrast, pathogenic strains of *Vibrio* are rarely isolated from coastal marine environments where outbreaks are not observed, but instead, other species of non-pathogenic *Vibrio* that do not cause disease are abundant and range from $10^3$-$10^5$ per milliliter of water [2]. This incongruity suggested a possible antagonistic relationship between strains commonly found in the open ocean and pathogenic strains that found outbreaks around the world. Should such a competitive relationship exist, whereby environmental strains could inhibit devastating pathogens, then these non-pathogenic strains may be a source of novel antibiotics that can be used to treat cholera-infected patients.

In a previous study by Cordero and Wildschutte, the authors showed that environmental isolates exhibit interspecific-competition meaning that distantly related strains antagonize each other [7]. Based on this work, we propose that the pathogens are outcompeted in the coastal marine environment by other unrelated *Vibrio*. If such interspecific-competition occurs, we would predict that non-pathogenic environmental *Vibrio* that are able to inhibit *Vibrio* pathogens persists in the ocean across temporal and spatial scales. As predicted, our results show that natural *Vibrio* strains isolated from diverse habitats of suspended particles and over a three month period can effectively inhibit *Vibrio* pathogens. Furthermore, because the

environmental isolates were isolated from diverse habitats, on separate days, and inhibit the growth of different pathogenic stains, we expect these isolates to produce different inhibitory factors. Of the 131 total antagonistic strains identified, 53 were isolated as free-living strains in the water column. Although 40% of the isolates that were able to antagonize at least one pathogen were free-living bacteria, there were no free-living strains that were able to kill 6 or more pathogens. Thus, all SKs in this study were particle-associated. Four of the eight SKs were found in association on 1µm particles; of the other four SKs, one was associated with 5µL particles and the last three on 63µm particles. A recent study suggests that populations of environmental *Vibrio* have exhibit competitive tradeoffs whereby some strains preferentially attach to large particles while other isolates exhibit strong chemotaxis and disperse among smaller particles [8]. Accordingly, we suggest that free-living bacteria are specialized in chemotaxis and exploit small organic compounds for a food source; in contrast, bacteria associated with larger particles persist on these substrates and could be more adapted to competition. Thus, particle-associated isolates may have evolved to produce more potent and broad-range acting antibiotics that allows them to outcompete other bacteria for their food source. Based on our results, it is evident that there are multiple antagonistic relationships between the environmental and pathogenic strains.

The discovery of novel antibiotics is of vital importance for human society. The misuse of antibiotics has led to a global crisis concerning the emergence of pathogens that are resistant to all known antibiotics. For instance, cholera affects one third of the world's population, and many outbreaks involving strains of *V. cholera* that are now resistant to all antibiotic treatments [5, s3]. Making the crisis even worst is the fact that multiple major pharmaceutical companies have stopped antibiotic production due to lack of profit since bacterial resistance is observed just months after the antibiotic use by the public. Now, the discovery of antibiotic production is critical and performed mostly by research labs at universities, such as work performed by Dr. Wildschutte at Bowling Green State University [3].

*Future Implications and Work*

Now that we have identified *Vibrio* SKs that inhibit pathogens, members of the Wildschutte lab are utilizing genetic techniques in order to identify genes encoding antibiotic production. One

of these methods include transposon mutagenesis that will allows identification of the genes involved in compound production through mutagenesis and subsequent screening for loss of antagonistic activity. By identifying SKs, we have potentially identified novel antibiotics that may be used to treat cholera infections that currently have no antibiotic treatment. Therefore, this study is extremely important and significant in regards to the identification of novel antibiotics that could provide the world with the needed antibiotic resources we currently lack.

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**Table 1.** Environmental *Vibrio* strains isolated in 2010 and their ability to inhibit pathogens.

| Habitat | Date sampled and # of Strains Isolated: total (antagonistic) | | | Total Stains Isolated |
|---|---|---|---|---|
| | Aug 10th | Sept 18th | Oct 13th | |
| 63µm particle | 288 (22) | 288 (14) | 288 (6) | 864 (42) |
| 5µm particle | 288 (7) | 288 (4) | 288 (5) | 864 (16) |
| 1µm particle | 288 (3) | 288 (12) | 288 (5) | 864 (20) |
| Free-living | 288 (7) | 288 (32) | 288 (14) | 864 (53) |
| Total Strains | 1,152 (39) | 1,152 (62) | 1,152 (30) | 3,456 (131) |

**Table 2.** Vibrio pathogens used in this study.

| Pathogen | Description | Reference |
|---|---|---|
| V. cholerae N16961 | Serotype O1 El Tor, Inaba; stool from cholera patient, Bangladesh in 1971 | Dziejman et al. 2001; ATCC 39315 |
| V. cholerae MO10 | Serotype O139 El Tor; India in 1992 | Mizunoe Y et al. 1999 |
| V. cholerae O395 | Serotype O1 Classical; India in 1965 | Dziejman et al. 2001; ATCC 39541 |
| V. cholerae VO-146 | Serotype O10; Bangkok in 1993 | Dalsgaard et al. 1999 |
| V. cholerae VO-258 | Serotype O8; Bangkok in 1993 | Dalsgaard et al. 1999 |
| V. parahaemolyticus EB101 | Shirasu food-poisoning; Japan in 1965 | Fujino T et al. 1965; ATCC 17802 |
| V. parahaemolyticus BB22OP | Strain LM5312; Bangladesh in 1980s | Jensen RV et al. 2013 |
| V. parahaemolyticus 954 | Clinical isolate | ATCC 49398 |

**Table 3. Super-Killers: environmental strains that inhibit six or more pathogens.** The block and cell numbers indicate the location of the Super-Killer in the frozen stocks.

| Block Number | Cell | Pathogens Killed | # Of Pathogens Killed |
|---|---|---|---|
| 222.49 | G1 | 0395, MO10, VO-258, VO-146, N16961, MO10 | 6 |
| 222.51 | B5 | VO-146, 954, VO-258, 0395, MO10, N16961, 5316 | 7 |
| 222.53 | H2 | VO-146, N16961, VO-258, MO10, 0395, 954, BB22OP, EB101 | 8 |
| 261.53 | B8 | N16961, VO-146, VO-258, 0395, MO10, BB22OP | 6 |
| 261.54 | E7 | BB22OP, N16961, VO-258, MO10, EB101, 954 | 6 |
| 286.49 | D1 | MO10, 0395, VO-258, VO-146, N16961, 954, BB22OP, EB101 | 8 |
| 286.5 | D12 | BB22OP, MO10, VO-258, VO-146, N16961, 0395 | 6 |
| 286.52 | B3 | 0395, MO10, VO-258, VO-VO-146, N16961, BB22OP | 6 |

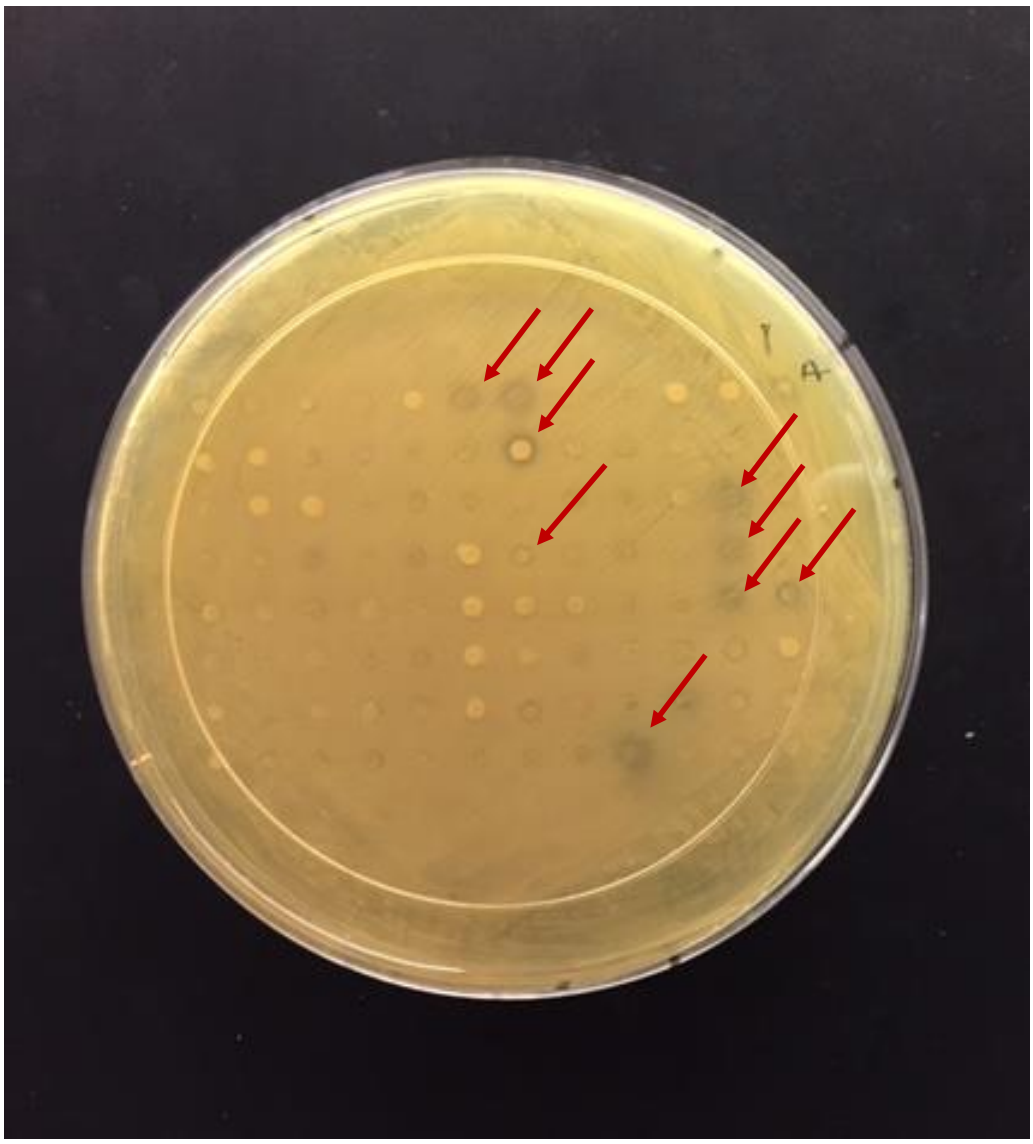

**Figure 1. Photograph of an antagonistic assay.**
Ninety-six environmental strains were co-grown with *V. cholerae* O139 pathogen. The arrows show nine strains that inhibit the growth of the *Vibrio* pathogen.

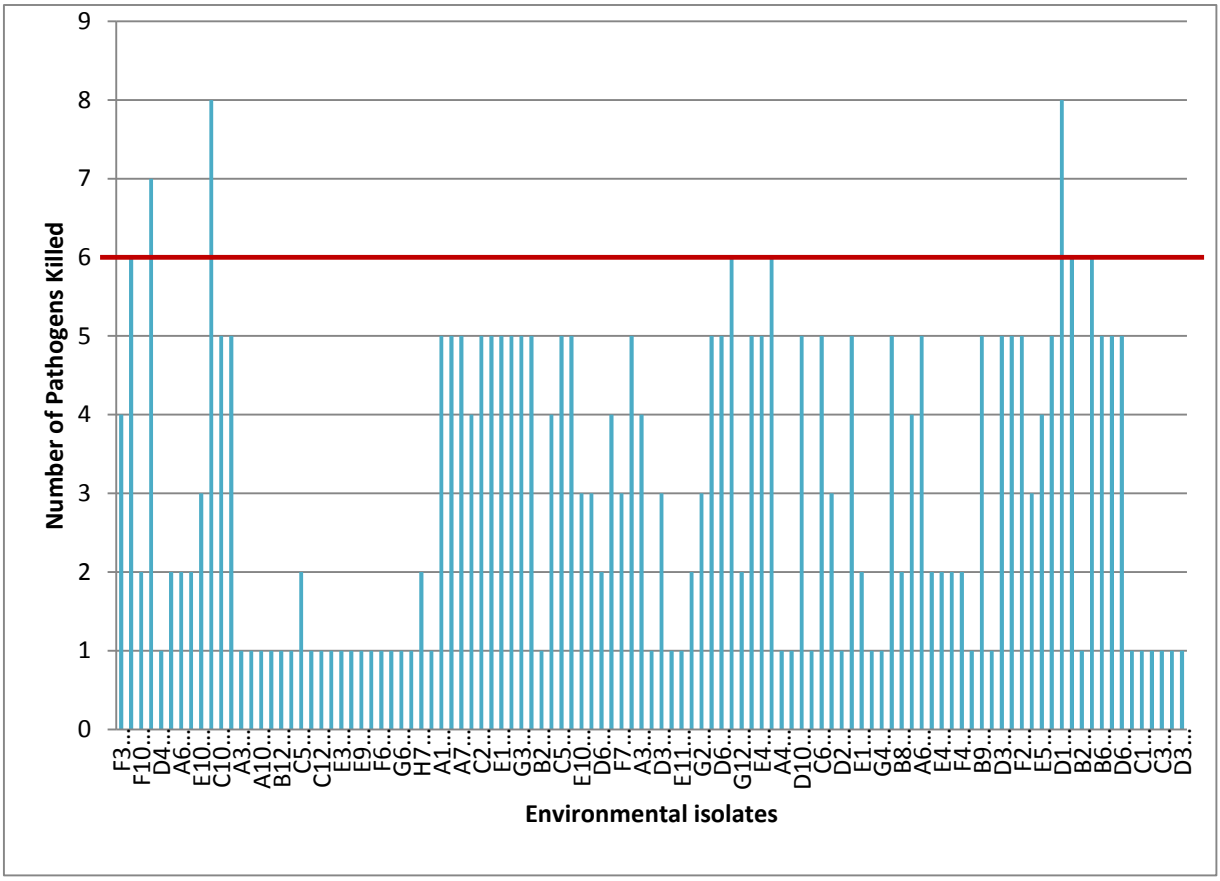


**Figure 2. Environmental isolates inhibit *Vibrio* pathogens.** One hundred thirty-one environmental strains were able to inhibit at least one pathogen. Strains inhibiting six or more pathogens are defined as Super-Killers (identified by the lines that reach or extend above the red line).