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Treating Resilient Medical Implant Infections with the Hidden Genius of Apple Pies and Cranberry Sauce

Abstract. Biofilms are collections of bacterial cells that create a protective protein matrix around themselves and attach to a surface such as a medical implant. Resveratrol is an extract from grapes and other plants that has been used in previous studies to inhibit biofilm formation. This research project used resveratrol to prevent biofilm growth by the bacterial strain *V. harveyi* in hopes of discovering the mechanism by which resveratrol is able to inhibit biofilm formation. Two strains of *V. harveyi*, BB120 and BB170, were used in these experiments. Our studies found that resveratrol decreased biofilm formation in both strains while decreasing cell viability in BB120 and allowing BB170 to remain completely viable. These results suggest that resveratrol inhibits the mechanisms that both strains use to form biofilms and also has an impact on some aspect of the BB120 strain that is not present in BB170. These findings present resveratrol as an effective biofilm inhibitor and offer hope that resveratrol could be harnessed into a treatment for biofilm infections.

BACKGROUND

Introduction. Bacterial biofilms can form on a medical implant anywhere in the body, from contact lenses to prosthetic implants to catheters (Samrot et al., 2021). Bacterial infections are typically treated with antibiotics. However, bacteria contained in a biofilm are less susceptible to antibiotic treatment because they can become genetically resistant and are

physically protected by the blanket they form around themselves (Di Domenico et al., 2022). Severe bacterial infections without timely treatment can progress to chronic infections, causing implant malfunction, tissue damage, and even death (Khatoon et al., 2018). Biofilm infections are not only a potentially fatal issue but also economically burdensome. A 2018 study reported that the annual cost in the US of treating patients with biofilm-related infections (BRIs) that originated in a hospital setting was $11 billion (Wi & Patel, 2018).

Biofilms. The power of a group composed of millions of individuals is always more potent than a single individual. Likewise, when bacterial cells are contained in a biofilm, the infection becomes stronger because the cells can share nutrients, carry out vital processes more efficiently, and are insulated from some antibiotic treatment (Algburi et al., 2017). The bacteria in biofilms produce a matrix (blanket) of extracellular polymeric substances (EPS), which binds the bacterial cells to one another and to the surface (Samrot et al., 2021). This matrix is difficult to penetrate, making the antibacterial treatment less effective. (Shin et al., 2021). Biofilm formation is considered a virulence factor, a behavior that is beneficial to bacterial survival but harmful to the host organism (Defoirdt, 2013).

Antibiotic Resistance. In addition to the physical barrier that biofilms create, biofilms exacerbate the issue of antibiotic resistance. Antibiotics are ineffective if bacteria are genetically resistant to this treatment (Davison et al., 2000). Antibiotics effectively eliminate bacteria that do not contain any resistance genes while allowing the antibiotic-resistant bacteria to thrive (Defoirdt, 2013). As a result, the number of antibiotic-resistant bacteria increases with the use of antibiotics. Alternatives to traditional antibiotics might overcome this problem.

Quorum Sensing. To develop preventions and treatments for BRIs, it is critical to know how biofilms form and grow. Bacterial cells “communicate” using a process called “quorum sensing” in which they synthesize and release signaling molecules that act like chemical words (Plener et al., 2015). These molecules bind to receptors in the membranes of other bacterial cells, as shown in **Figure 1**, signaling to these cells to perform a certain behavior, such as biofilm

formation (Kalia, 2012). Known as autoinducers, the signaling molecules are part of an elegant signal cascade, which exhibits a domino effect – relaying chemical messages from protein to protein within the cell, beginning with the receptor and ending with gene expression (Subramani & Jayaprakashvel, 2019). The concentration of autoinducers depends on the density of bacteria; when more bacteria are present, more autoinducers are produced. Thus, the bacteria signal to one another to carry out more self-beneficial behaviors, and more virulence will be expressed, resulting in increased biofilm formation (Zhao, Yu, Ding, 2020). Targeting the steps of quorum sensing may lead to new treatments for BRIs.

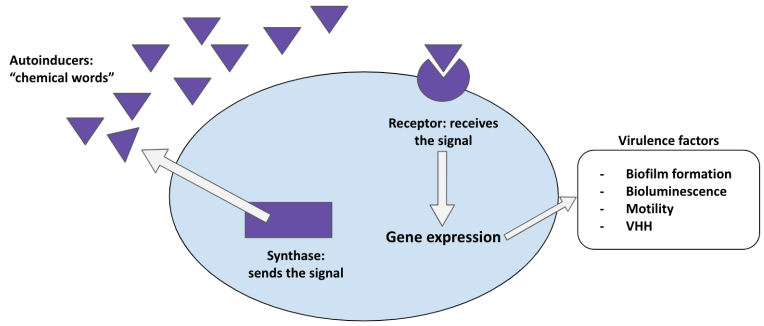

Figure 1: *Quorum sensing: autoinducers are created by the synthase protein, released outside of the bacterial cell, and received by the receptor protein. The binding of the autoinducer to the receptor then relays the signal through a cascade, resulting in the expression of genes that can produce virulence factors. (Adapted From: TED, & Bassler, B. (2009, April 8). Bonnie Bassler: The secret, social lives of bacteria [Video file]. Retrieved from https://www.youtube.com/ watch?v=TVfmUfr8VPA)*

Quorum Sensing Pathways. Most bacteria are capable of producing at least two types of autoinducers, as shown in **Figure 2**. Autoinducer 1 (AI-1) is a species-specific autoinducer; only that species of bacteria contains the pathway to synthesize AI-1 and the receptors that bind to AI-1 (Guo et al., 2013). Autoinducer 2 (AI-2) is a universal autoinducer; all bacterial cells contain the pathway that produces and receives AI-2 (Guo et al., 2013). The AI-1 and AI-2 pathways control different virulence factors.

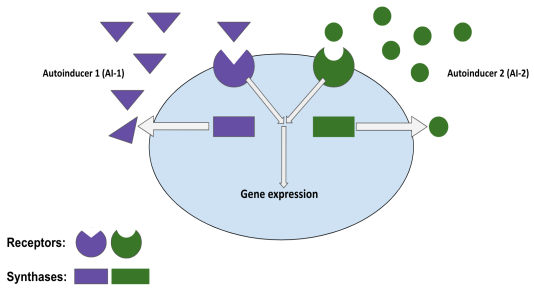

Figure 2: *The proteins responsible for making and receiving autoinducer 1 and autoinducer 2 exist in Vibrio harveyi and its mutant strains. In the wild type Vibrio harveyi depicted above, all of these proteins are active.*

Inhibiting Virulence Factors by Targeting Autoinducers. When an autoinducer is created and released, that molecule can only be received by a neighboring bacterial cell if that cell contains the receptor for the specific autoinducer. The expression of virulence factors may be prevented by targeting either the synthase, the receptor, or another step in the pathway after the autoinducer is received. Many studies that examine quorum sensing inhibition utilize the model system *Vibrio harveyi*, which contains both the AI-1 and AI-2 pathways (Zhao, Guo, Ni et al., 2020).

Resveratrol. Resveratrol is a molecule extracted from grapes and berries that plants use to defend themselves against pathogens (Augustine et al., 2014). Previous studies have concluded that resveratrol is effective at inhibiting quorum sensing and, thus, preventing further growth of biofilms at certain concentrations for certain species of bacteria (Augustine et al., 2014). However, the mechanism by which resveratrol targets quorum sensing in these bacteria is unknown. Resveratrol may inhibit the production of autoinducers, their binding to the receptor, as shown in **Figure 3**, or the communication of the signal within the receiving cell.

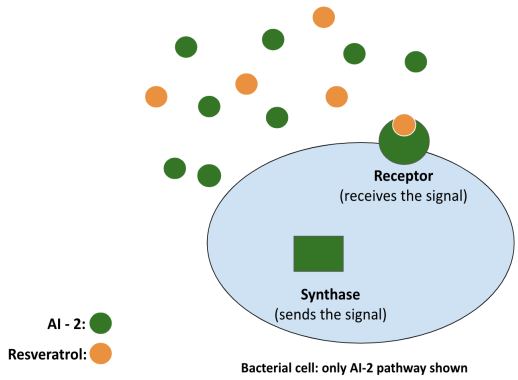

Figure 3: *Resveratrol could be a competitive inhibitor in the AI-2 quorum sensing pathway.*

Model system. *V. harveyi* was used as the model system in this study because it forms the biofilms needed for our experiment, is safe for high school students, is well studied in the primary literature, has many quorum sensing phenotypes, and has an intact quorum sensing system. It can illuminate the effect of resveratrol on various elements of quorum sensing through its mutant strains (Plener et al., 2015).

RESEARCH OBJECTIVES

In some studies, resveratrol has been suggested to decrease biofilm formation for wild type *V. harveyi* in a dose-dependent manner, but whether resveratrol targets the receptor or a step in the quorum sensing pathway remained ambiguous (Blea & Zhang, 2022). Our research aimed to learn more about how resveratrol inhibits bacterial biofilms for the purpose of developing more effective treatments for BRIs on medical implants. We studied the impact of resveratrol on *V. harveyi* strains BB120 (wild type) and BB170 (which lacks the AI-1 receptor) at varying concentrations that would inhibit biofilm formation without leading to antibiotic resistance.

Benefits of studying multiple strains. In the BB170 mutant strain of *V. harveyi*, AI-2 can be produced and received whereas AI-1 can be produced but not received (Jing et al., 2021). This research aimed to gain insights into AI-2 mediated behaviors from this strain along with the wild type because the AI-2 pathway is the only active QS pathway. Observing biofilm activity in

strains of *Vibrio harveyi* with different QS machinery active with and without resveratrol allows us to learn more about the mechanism by which resveratrol inhibits quorum sensing in *Vibrio harveyi*.

Hypothesis. Our hypothesis was as follows: If resveratrol equally inhibits biofilm formation in the wild type (BB120) and the BB170 mutant strain, then resveratrol inhibits aspects of the AI-2 pathway. We tested this hypothesis using two sets of identical experimental and control groups. On one set we conducted a crystal violet assay to quantify biofilm formation. This assay used a crystal violet stain to stain each well and the excess was washed off, allowing the groups with more biofilm to retain more stain. The amount of stain was measured using a spectrophotometer. On the other set of experimental groups, we conducted a resazurin assay (cell viability) to measure if the cells within the biofilm were dead or alive. This assay required a similar process that is discussed in the materials and methods.

Important and Impact. Researching the mechanism that resveratrol uses to inhibit biofilm formation is important because it provides insights that can aid in the development of future treatments for BRIs on medical implants. By using both strains of *V. harveyi*, we can learn which aspects of the quorum sensing pathways resveratrol targets and apply that knowledge to bacteria found in the human body. This research studies resveratrol's impact on biofilm formation and cell viability simultaneously, considering the danger of bacterial resistance to treatment. The development of a treatment for BRIs on medical implants that would not cause bacterial resistance would allow patients with implants to have safer surgeries, shortened hospital stays, and fewer complications.

METHODS

Materials. Four studies were conducted using proper aseptic techniques, including disposal of sterilized biological materials. We acknowledge previous students who optimized the methods described below. Resveratrol, *Vibrio harveyi* strain BB120 (wild type), and BB170 (mutant) were purchased for a previous group's project and were available to us at no charge. *V.*

harveyi strains were prepared by microbiologists at Oak Crest Institute of Science (Monrovia, CA). Two cultures were made for each strain by diluting 1mL of each strain of *V. harveyi* in 9 mL of Nutrient Broth (NB) media. These cultures were incubated in a shaking water bath at 30 C overnight. The incubated solutions were then diluted again by mixing 0.1 mL of *V. harveyi* with 4.9 mL of NB media in a 15 mL Falcon tube.

Stock solutions. Our resveratrol stock solution was prepared in two steps. We first made a master stock with a concentration of 65,700 uM RSV in which 0.015 g of resveratrol was dissolved in 1.0 mL of ethanol. From the master stock, we made a working stock with a concentration of 280uM RSV in which 21uL of the master stock was dissolved in 4.979mL of Nutrient Broth. We prepared a second working stock for Studies #3 and #4 with a concentration of 560 uM RSV in which 85.2 uL of our master stock was dissolved in 9.915mL of NB media. In Study #3, this working stock was mistakenly made with 9.915 mL of ethanol instead of NB media. Our working stock was sterilized using filter-sterilization supplies and kept in a 15 mL Falcon tube.

Common Materials. We used micropipettes (p20 and p200) and pipette tips (p20 and p200) to prepare our experimental groups in both a black-walled sterile 96-well microplates (Costar 3603) and a clear, round-bottom 96-well microplate (GenClone). Sterile troughs were used to transfer media and *V. harveyi* to the well plate with a multichannel micropipette. For related steps, 10 ml serological pipettes, micropipettes (p1000), and pipette tips (p1000) were used.

Biofilm Activity Assays. Resazurin (biofilm cell viability) and crystal violet (biofilm mass) assays were used to quantify biofilm formation by the bacteria in the presence and absence of added resveratrol. After plates were prepared and incubated for 16-24 hours at 30 C, the cell viability plate was inverted and liquid was shaken out. The plate was then washed in 0.1M phosphate-buffered saline (PBS). 100uL PBS and 20uL of the resazurin solution were added to each well. Next, samples were incubated at 30°C for 3-4 hours and fluorescence (excitation at

570 nm, emission read at 595 nm) was measured every half an hour using a Molecular Devices Gemini fluorescence detector. The amount of fluorescence corresponded to the amount of live biomass released from the biofilm. For the crystal violet assay, the clear plate was washed with 0.1M PBS three times and air-dried for 20 minutes. Then 200 uL of 0.3% crystal violet dye were delivered to each well and incubated for 20 minutes. The dye was then washed out with sterile water and the plate was allowed to dry overnight. 150 uL of ethanol was then added to release the dye and 20-40 uL of each well was transferred to a new plate containing 110-130 uL of ethanol. The optical density was read at 595 nm, which corresponds to the total biofilm mass (both living and dead cells).

We processed the data by averaging the five data points measured for each experimental group and subtracted the media-only blank values from the average signal. All processed data were normalized to the untreated control group (*V. harveyi* + media without RSV) for each strain of *V. harveyi*.

RESULTS

We conducted four biofilm inhibition studies in the presence of varying concentrations of resveratrol (0-140 uM RSV) in Nutrient Broth media. The results from Study #1 did not show a trend, which may be accounted for by the fact that this was our first study and our technique was likely not as sophisticated as our subsequent studies. In Study #1, the experimental group containing 54 uM resveratrol formed significantly more biofilm than the groups with either 35 uM or 70 uM RSV in both BB120 and BB170. Study #2 showed clear trend lines that will be discussed in more complete detail below.

Study #3 used a stock solution of resveratrol containing a high concentration of ethanol to aid solubility, but this led to bacterial death and those results are not shared here. In Study #4, the ethanol concentration of the resveratrol was reduced to the degree that it was not harmful to the bacteria. Both Studies #3 and #4 included experimental groups containing 28 uM, 56 uM, 70 uM, 110 uM and 140 uM RSV; the first three concentrations being similar to those used in

Studies #1 and #2 and the last two being significantly larger. The data from study #4 show that while resveratrol at concentrations of 110 uM and 140 uM inhibited biofilm formation by at least 86% in BB120 and 95% in BB170, it also inhibited cell viability by at least 96% in both BB120 and BB170.

For the purpose of illustration, data from study #2 is shown below in **Figure 4** and **Figure 5**. The experimental groups containing BB120 showed a noticeable, concentration-dependent decrease in biofilm formation relative to the control group (see purple bar data in **Figure 4**). Cell viability decreased in most of the experimental groups (see green bar data in **Figure 4**). Our preliminary analysis shows that biofilm formation at 21 uM RSV decreased by 38% relative to the control group, while cell viability actually increased by 6%. At a resveratrol concentration of 35uM, biofilm formation decreased by 60% and cell viability decreased by only 15%. The experimental groups containing BB170 also demonstrated a dose-dependent decrease in biofilm activity (see purple bar data in **Figure 5**). Biofilm formation of BB170 decreased by 29%, 27%, 42%, and 51% at resveratrol concentrations of 21 uM, 35 uM, 50 uM, and 70 uM, respectively. The cell viability of BB170 at all concentrations of resveratrol experienced no significant change (see green bar data in **Figure 5**).

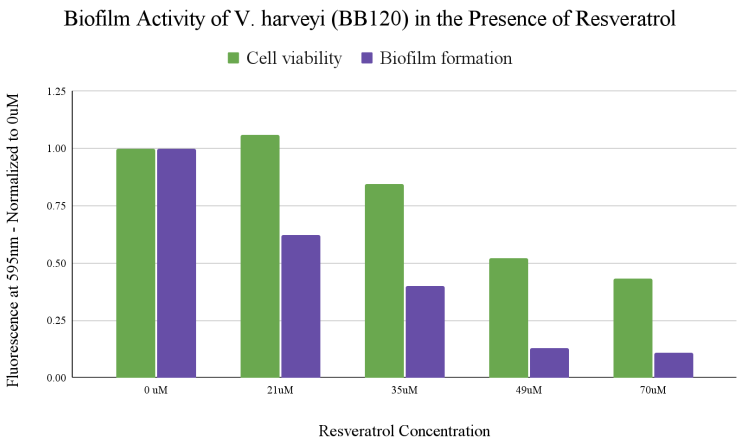

Figure 4: *V. harveyi (BB120) data from the resazurin assay (cell viability) and the crystal violet assay (mass of biofilm formation) conducted in the presence of varying concentrations of resveratrol (0-70 uM RSV) in Nutrient Broth.*

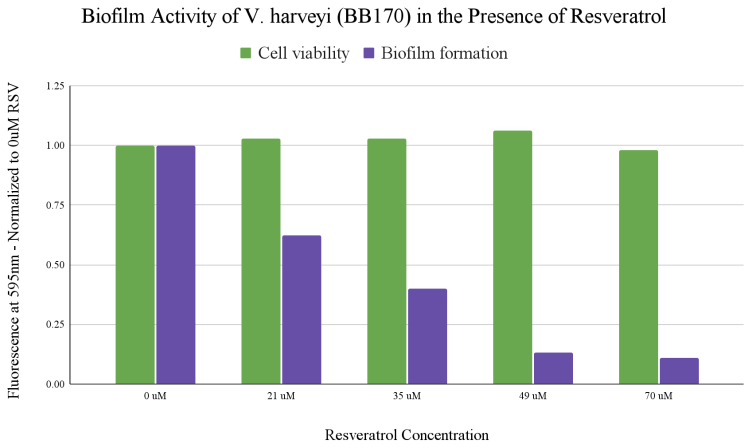

Figure 5: *V. harveyi (BB170) data from the resazurin assay (cell viability) and the crystal violet assay (mass of biofilm formation) conducted in the presence of varying concentrations of resveratrol (0-70 uM RSV) in Nutrient Broth.*

DISCUSSION & CONCLUSIONS

The results from Studies #2 and #4 support our hypothesis that resveratrol is an effective biofilm inhibitor of strains BB120 and BB170. Because the biofilm inhibition trends are so similar in the two strains, we believe that resveratrol prevents biofilm formation by inhibiting quorum sensing. The results also confirm previous results obtained in our school lab that resveratrol decreased biofilm formation in a dose-dependent manner (Blea & Zhang, 2022). In BB120, resveratrol was most effective at inhibiting biofilm formation without decreasing cell viability at a concentration of 21 uM as shown in **Figure 4**. In BB170, resveratrol effectively inhibited biofilm formation in a dose-dependent manner in all of the experimental groups without killing the bacteria as shown in **Figure 5**. This suggests that resveratrol inhibits the AI-2 pathway since it decreases biofilm formation in both strains of *V. harveyi*.

Because BB120 experienced a decrease in cell viability in the presence of resveratrol while BB170 did not, the data also suggests that resveratrol has an impact on some aspect of the BB120 strain that is not present in BB170. It may be that resveratrol also affects the AI-1

pathway, which would impact BB120 without impacting BB170 because the AI-1 pathway in BB170 is already inactive. Resveratrol also may affect another aspect of BB120 that is not relevant to BB170. In our preparations for the studies, we measured the growth of both strains without resveratrol to determine which cultures to use for our studies. In nearly all of these trials BB170 grew more successfully than BB120, suggesting that BB120 may be more fragile and susceptible to all threats to cell viability, including resveratrol. Determining the reason that resveratrol affected the cell viability of BB120 while BB170 remained alive would require further study of the survival mechanisms of the bacteria.

Interpretation. These results show resveratrol to be an effective biofilm inhibitor. These findings could lead to further research of the resveratrol's potential to be used to develop pharmaceutical treatments for BRIs on medical implants. In particular, BB170's response to resveratrol brings a lot of hope because it demonstrates that certain strains of bacteria can have biofilm formation inhibited while remaining viable in the presence of resveratrol. If the bacteria present in human biofilm infections have a similar response, then a resveratrol-based treatment could prevent harmful behavior in bacteria without killing the bacteria and causing them to become resistant.

Limitations. While the model system *V. harveyi* presented many benefits for our study, including forming biofilms and having mutant strains with different quorum sensing machinery, it is a marine bacteria and does not reside in the human body (Henke & Bassler, 2004). Because our central question revolves around biofilm infections on medical implants in the human body, using a marine bacterium as our model system may limit the relevance of our results to further research of the original problem.

We originally proposed to study three strains of *V. harveyi*: the wild type, BB120, which contains active AI-1 and AI-2 pathways; BB170, which contains only an active AI-2 pathway, and KM413, in which both the AI-1 and AI-2 pathways are inhibited (Jing et al., 2021; Meyel et al., 2015). Studying BB170 in addition to the wild type provided insight into whether or not

resveratrol targets the AI-2 pathway. KM413 has active receptors for both AI-1 and AI-2 but does not produce either signaling molecule (Meyel et al., 2015). Since KM413 is used in the literature as a "testing" strain because it doesn't make its own autoinducers, KM413 will show group behaviors only when autoinducers harvested from other strains are added to KM413. This feature allows for competitive inhibition studies. If resveratrol acts as a competitive inhibitor, then it resembles the shape of the autoinducers and binds to the receptors, preventing the autoinducers from activating the receptors. We can then compare the result of KM413 with and without autoinducers added to determine whether or not resveratrol targets the AI-2 receptor in *V. harveyi*. To learn more about resveratrol's action on the AI-2 pathway, we planned to add AI-2, isolated from the *V. harveyi* strain BB152, and resveratrol to KM413. We hypothesized that, if biofilm formation was inhibited in the group with resveratrol in comparison to the control containing only KM413 and isolated AI-2, then could conclude that resveratrol inhibits the binding of AI-2 to its receptor, rather than the production of AI-2. Due to time limitations, we did not study KM413.

Next Steps. Suggestions for further research of this topic are to study the bacteria that are commonly found in the human body and to study the impact of resveratrol on an already formed biofilm. Studying the bacteria that often form biofilms in humans, specifically the quorum sensing pathways that they use, would be beneficial because it would allow us to draw parallels between these bacteria and *V. harveyi*. These parallels would drive our thinking about how resveratrol could function as a treatment for biofilm infections. In our studies, resveratrol was added before the bacteria was incubated and biofilm was formed. Thus, our methods parallel a **preventative treatment** for biofilm infections. It would be beneficial to also research the potential of resveratrol as a **reactive treatment** by testing its ability to degrade previously formed biofilms.

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