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Difference in the Inhibitory Effects of Violacein on Various Yeast Isolate Strains From the Hudson Valley Region

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Public Facing Abstract

In the past years, scientists have become more and more interested in violacein, a unique substance made by some bacteria. Violacein is a natural antibiotic, the bacteria that make violacein use it to defend against other bacteria species and viral and parasitic predators. Scientists have also found that violacein has the ability to kill many kinds of fungi and cancer cells. These properties give violacein exciting promise as a drug for human use in the future. However, to find out more about violacein's effects, and to prepare it for possible use in humans, scientists need to better understand how it works. Right now, how violacein attacks tumors is not well understood, and researchers don't know why it affects some cells and not others. Yeast is often used for research on human genetics and cancer cells because it's also eukaryotic, and has the same basic structure as human cells. Because yeast is such a useful model for these interactions, for my research, I found how violacein affected different samples of yeast taken from around the Bard campus area. Violacein reduced the growth for some of the yeast, and didn't affect others. After finding how they were affected, I sequenced some of the yeast to find the identity of the species we were studying. Out of five samples, four weren't affected by violacein, and one was. I found that all the samples were the same species, called *Hanseniaspora uvarum*, however, the sample that was affected was a different strain from the ones that weren't. This is very interesting, because it opens up possibilities for future research that finds what the genetic differences between those strains are, and if the differences explain why one is affected and the rest aren't. Finding the reason behind the difference would point to how violacein works, and why it affects some cells but not others, and allow us to come up with better drugs for human use.

Abstract

Violacein is a purple pigmented compound produced by numerous bacterial species including *Janthinobacterium lividum*. Studies into violacein have found it to have a multitude of medicinal properties, from antifungal, antibiotic, to antitumor activity. Research has shown that violacein significantly inhibits both tumor and fungal growth and it has been shown to have higher cyotoxicity in pathogenic or cancerous cells than in healthy ones, giving it great potential as for use as a pharmaceutical drug in humans, alongside the fact that as a bacterial compound it's easier and faster to produce than some other drugs. Violacein has also been shown to inhibit the growth of fungi harmful to crops and amphibians throughout South America. However; not as much research has been done into the mechanics of how violacein reduces cell growth and induces cell death in eukaryotic cells, and the research which has been done is varied, and seems to suggest that different mechanisms are involved depending on cell type. It has been suggested that, as both being eukaryotic cells, the mechanisms for violacein's interaction with fungi and with cancer cells are likely similar. Yeast are also often used as study species for research on cancer and tumor growth due to being eukaryotic, with many conserved biological pathways to human cells, that replicates quickly through asexual reproduction, much like tumor cells do. I determined the effects of violacein on the growth of *S. cerevisiae* and over 20 wild yeast isolates collected from fruit in the Hudson Valley surrounding Bard college campus, and sequenced samples of both sensitive and resistant isolates. Of the 5 yeast isolates sequenced, all were found to be strains of *Hanseniaspora uvarum*. There appear to be differences in strain of resistant vs sensitive isolates, opening up the possibility of sequence alignment or genome-wide association analysis to search for a genetic linkage to a mechanism of violacein's effects against yeast and other eukaryotes.

Introduction

I. VIOLACEIN

Violacein, molecular formula $C_{20}H_{13}N_3O_3$, is an organic compound produced by multiple gram-negative bacterial species, including *Chromobacterium violaceum*, *Chitinomonas, Iodobacter*, and as used for compound production in this study, *Janthinobacterium lividium* (Venegas et al., 2019). Bacteria which produce violacein live in many different environments, and their secretion of violacein is generally thought to be for the purpose of defense against predators, and for providing competitive advantage against other, particularly gram-positive, species of bacteria (Choi et al., 2015).

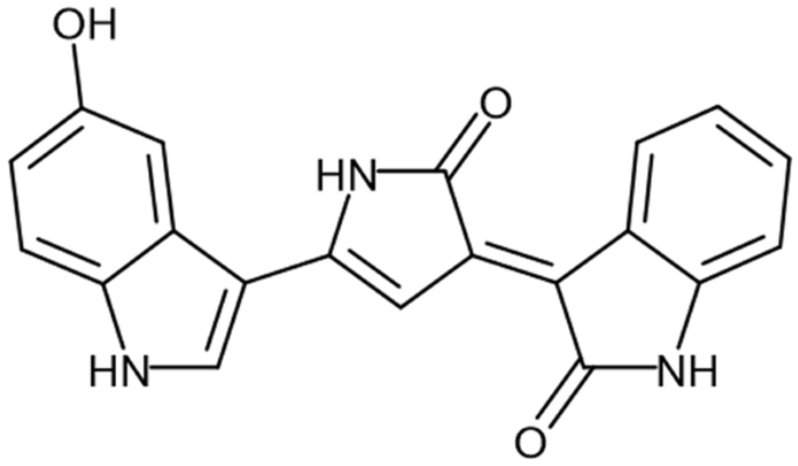

Figure 1. Molecular structure of violacein

II. PHARMACEUTICAL POTENTIAL

In the past decades research interest in violacein has rapidly expanded due to the discovery of its many antipathogenic properties. Interest has grown in the compound's potential use for helping both humans and the environment (Durán et al., 2021).

The mechanisms of violacein's antibacterial effects are generally well understood, but much less is known about the mechanics of its interactions with larger and more complex eukaryotic cells, including mammalian tumor cells, protozoa, and fungi. Despite the mechanisms for these interactions so far observed appearing to be less consistent and more unclear, there has been lots of development in research and advancement of this understanding in the past years. Some commonly observed interactions of violacein with eukaryotic cells are proposed as being major mechanisms, and many specific cell type mechanisms have been recorded.

Indications of potential for human use

Violacein's cytotoxic and antimicrobial effects against a multitude of human pathogens have piqued interest in its use as a medicinal agent for humans. Multiple other factors also point towards the possibility for the compound to be used safely by humans. A 2017 study found that violacein's toxicity results placed it in a toxicity class with LD_{50} of 500 mg/kg, suggesting it would be non-toxic for human consumption in doses under 300 mg/kg; and that violacein follows Lipinski's rules of 5, a set of standards marking a compound with potential for safe use in humans (Verma and Pandey, 2017). Another study performed micronucleus assays, tests which analyze the frequency of micronuclei (nuclei structures formed outside the nuclear envelope caused by genomic damage or instability) formation when a cell is exposed to a substance, to

examine the effects of violacein in HeLa cells. The analysis determined that the compound is not genotoxic, meaning violacein doesn't show signs of posing a risk of binding directly to or damaging DNA (Alem et al., 2020). Many studies have observed that violacein is cytotoxic to cancerous cells at much lower doses than it is to healthy cells, giving it great potential as an anticancer drug; one study found that while violacein induced apoptosis in the HL60 leukemia cell line, it had no effect on untransformed healthy human peripheral lymphocyte and monocyte blood cells (Ferreira et al., 2004). Another, *in vivo*, study on violacein's use for tumor treatment in mice found that head and neck carcinoma tumors treated with doses of 0.7 mg/kg violacein regressed, and mouse lifespan was extended during treatment. The mice also didn't appear to be negatively affected, and no difference in weight, behavior, or phenotypic features were observed between the treatment and control mice (Hashimi, Xu, and Wei, 2015).

Advantages and new opportunities

Violacein has potential in many areas for use against pathogens or cancers that are resistant to many existing treatments. The compound has been shown to be an effective bacteriostatic against certain antibiotic resistant strains of bacteria, such as MRSA (Aruldass et al., 2018). As for its anticancer effects, in one study, the cytotoxicity of violacein against many cancer cell lines was shown to be increased under hypoxia conditions. This is very useful, as an issue with many chemotherapeutic drugs is that they target proliferating cancer cells, and aren't as effective in hypoxic conditions when cancer cells are dormant. These quiescent cells are often still highly involved in tumor progression, so this can limit many chemotherapeutic drugs in fully

addressing the tumor; violacein could be used, on its own or in combination with other chemotherapeutics, to attack these chemo-resistant cells (Hashimi, Xu, and Wei, 2015).

Violacein also shows much promise as a synergistic compound which could boost or work in combination with known medications. One way in which violacein has been observed to combat cancer cells has been in melanoma cells, where violacein appeared to inhibit the cancer cell's autophagy rate, which is a strategy used by tumor cells to survive harsh conditions and evade apoptosis (Gonçalves et al., 2016). When autophagy is blocked, it makes it easier to induce apoptosis in those cells, reducing a tumor's resistance to other chemotherapeutic drugs. Violacein has also been found to work synergistically with many commercial antibiotics against microbial pathogens, without any antagonistic effects (Priya et al., 2018).

Antibacterial properties and mechanisms

Gram-negative bacteria, like the ones that produce violacein, have a thin inner cell wall made of peptidoglycan, and a tough outer membrane largely composed of lipoproteins; whereas bacteria strains negatively affected by violacein are gram-positive, having a thicker peptidoglycan cell wall but no outer membrane (Silhavy, Kahne, & Walker, 2010). Much more is known about Violacein's antibacterial mechanisms of action than its mechanisms against fungi or cancer cells. Violacein causes cytoplasmic membrane disruption of gram-positive bacteria; it has been shown to bind to and disrupt the liposomes made by bacterial phospholipids, causing increased membrane permeability, leading to leakage of intracellular content such as ATP, protons, and ions from the cell, causing osmotic imbalances which eventually induce cell death

(Aruldass et al., 2018; Cauz et al., 2019). This cytoplasmic membrane disruption has been found to be the major mechanism for violacein's antibacterial activity (Durán et al., 2021).

Antitumor properties

Broadly, multiple general kinds of antitumor activity have been displayed by violacein which give insight on its highly varied cell interactions. The compound has been shown to induce cell death, both through necrosis and apoptosis (Leal et al., 2015), to inhibit metastasis and cancer cell invasion, and to inhibit proliferative signaling which is essential to the uncontrollable and continuous replication of cancer cells (Durán et al., 2021).

A 2015 study examined the effect of violacein on the cancerous cell lines CHOK1 (Chinese hamster ovary cells), MRC-5 (fetal lung tissue fibroblasts), and HeLa (cervical cancer cells). Cell death was induced in all cell lines after violacein exposure, but interestingly, while HeLa cell death after exposure was suggested to mostly be due to apoptosis (active, programmed cell death), CHOK1 and MRC-5 cell death after exposure was indicated to mostly be due to necrosis (passive, unprogrammed cell death) (Leal et al., 2015). Another study demonstrated that violacein reduced survival of and induced cell death in a wide variety of cancer cell lines, including A549 (lung carcinoma), A431 (melanoma), MCF-7 (breast cancer), and PC3 (prostate cancer) (Hashimi, Xu, and Wei, 2015). Violacein was also demonstrated to induce apoptosis in colon cancer cell lines HT29 and Caco-2 (Carvalho et al., 2006).

Violacein has been shown to suppress proliferative signaling from cancer cells in multiple cell lines, including murine 2247 fibrosarcoma cells (Mojib et al., 2011), SKMEL-103 melanoma cells (Gonçalves et al., 2016), and colorectal cancer genes (Kodach et al., 2006).

Thirdly, violacein has displayed antitumor activity through inhibiting metastasis, the spread of cancer to other sites in the body, in multiple cancerous cell lines, including MCF-7 and SKMEL-103 (Durán et al., 2021).

Proposed antitumor mechanisms

No one mechanism for induction of cell death in cancerous cell lines by violacein has been found, but a few different major mechanisms have been contended as potential commonly held action mechanisms. The first widely observed mechanism is that violacein induces ROS generation and oxidative stress, leading to apoptosis of the cell. Violacein was found to significantly increase ROS production and levels in Caco-2 cells, causing activation of caspase-3 leading to mitochondrial membrane collapse, cytochrome *c* release, and finally culminating in apoptosis. (Carvalho et al., 2006). ROS production as the main mechanism of action in this case was further supported by the fact that incubating the cell line with both violacein and the antioxidant *N*-acetyl-cysteine resulted in almost none of the effects of violacein previously observed occurring, indicating that oxidative effects are key for violacein's cytotoxicity on this cell line. Other studies also saw an increase in caspase-3/7 activity after incubation with violacein and prior to violacein induced death for A459 cells (Hashimi, Xu, and Wei, 2015). However, this mechanism has not been nearly universally upheld. The same 2006 Caco-2 study found that violacein was incapable of increasing ROS levels in HT29, which was also less dramatically impacted by violacein than Caco-2. The 2015 study on violacein's effect on CHOK1, MRC-5, and Hela cells found that while there were signs of some oxidative stress markers, and increase of SOD levels in CHOK1 cells, oxidative stress did not seem to be the

major cause of cell death, and no dose-dependent responses of ROS production or oxidative stress due to violacein were observed (Leal et al., 2015).

Instead, this study proposed that violacein induces mitochondrial membrane hyperpolarization which triggers cell death, which is another widely regarded major mechanism proposed for violacein's cytotoxicity. There was an increase in mitochondrial membrane potential in both MRC-5 and HeLa cells when exposed to violacein, which coincided with increased percentage of cell apoptosis. This was determined by monitoring RH123 uptake, a fluorescent tracer dye used to measure mitochondrial potential, as energization of the mitochondria induces uptake, reducing fluorescence of RH123, meaning the rate of fluorescence decay is directly proportional to increasing mitochondrial membrane potential (Baracca et al., 2003). Studies have shown that violacein is associated with the structural reorganization of lipid molecules, and can induce changes of lipid positioning and ordering in the membrane, which could help explain its interaction with and change to the cell membrane (Durán et al., 2021).

Other observed likely mechanisms of violacein action include many different theorized "cell type specific mechanisms"; specific mechanisms of action only seen in how violacein affects one or two cell lines, rather than a universally applicable process. In HL60 leukemia cells, activation of NF-kB, leading to production of tumor necrosis factor activating TNF receptor 1, culminating in TNFR mediated apoptosis was observed (Ferreira et al., 2004). In HCT116 colorectal carcinoma cells, violacein was found to block cell cycle proliferation at G1 and upregulate tumor suppressor protein p53, p27, and p21 levels (Kodach et al., 2006). This same study found that violacein inhibited growth of four colon cancer lines, acting through the inhibition of Akt (protein kinase involved with cancer proliferation and survival)

phosphorylation. In murine 2237 fibrosarcoma cells, violacein treatment was observed to correlate with lower cyclin-dependent kinase (key to cell cycle progression), and higher p53 and p21 levels (Mojib et al, 2011). In MCF-7 breast cancer cells, violacein inhibited MMP-2 activity, causing a decrease in secretion of the metastasis modulator chemokine CXCL12 (Platt et al., 2014). There are a diverse multitude of other cell specific mechanisms observed in violacein in numerous different studies; one thing which is clear is that violacein can interact with and inhibit tumor growth and survival through a seemingly infinite number of particular mechanisms. Commonalities and predictability for these mechanisms, which may make the development of violacein for drug and medical use in things like human cancer treatment much easier, are still being understood, and much more research is needed.

Antifungal properties

Violacein's antifungal properties, similar to its antibacterial ones, are also a large aspect of how it benefits bacteria in nature. *Janthinobacterium* is known to form a symbiotic relationship with the amphibians it lives on, with the violacein is produces providing protection for its host against fungi such as *Batrachochytrium dendrobatidis* which grows on the skin amphibians, and can cause them to develop the deadly affliction of chytridiomycosis (Brucker et al., 2008; Harris et al., 2009). More recently, a 2015 study found violacein to be effective against the pathogenic fungal species *Cryptococcus gastricus, Trichophyton rubrum, Fusarium oxysporum, Rhizoctonia solani, Aspergillus flavus, Penicillium expansum*, and *Candida albicans*. (Sasidharan et al., 2015). Analysis of *C. violaceum* strains found that the bacteria and its metabolites (violacein being a primary one of these) were found to suppress the growth of

multiple fungi known to damage soybeans in Brazil (Barreto et al., 2008). Another study, which looked at the violacein and its derivatives deoxyviolacein and oxyviolacein, found that violacein displayed strong inhibition of *Sclerotinia sclerotiorum*, *Phytophthora capsici,* and *Fusarium oxysporum*, while oxyviolacein had the broadest range of antifungal activity, also inhibiting *Bortrytis cinera* and *Verticillium dahliae*, and deoxyviolacein only showed strong antifungal activity against *Rhizoctonia solani*. (Wang et al., 2012). This research group hypothesized that the difference in antifungal activity between the derivatives could be due to differences in the presence of hydroxyl groups (violacein having one, oxyviolacein two, and deoxyviolacein none), meaning the hydroxyl group could be essential to many of violacein's effects. One fascinating study even found that fabrics dyed with violacein from *Janthinobacterium* displayed significant antifungal activity against *Candida albicans, C. parapsilosis*, and *C. krusei*, all of which are known to cause infections in humans, especially in immunocompromised patients (Kanelli et al., 2018).

Proposed antifungal mechanisms

As with tumor cells, much is unknown about the antifungal mechanisms of violacein, and unfortunately there's not much study or theory in potential mechanisms, and more research is needed in this area. One study, looking at the effects of violacein on *S. cerevisiae,* found that at high concentrations, violacein significantly altered the membrane potential of the cell, pointing to a similar mechanism to the one seen in violacein's cytotoxic effects on some cancer cell lines (Pereira, Durán, and Volpe, 2005). Other researchers have suggested that antifungal mechanisms could be similar to certain mammalian antitumor ones, given that both cell types are eukaryotic

and have many conserved features (Durán et al., 2022). This same 2022 paper also suggested looking to the recorded mechanisms of similar methyl-indoles to violacein; structurally similar compounds have been shown to inhibit fungi by interfering in its formation of biofilm through induction of ROS production in the fungal biofilm cells (Durán et al., 2022). This again might present similarities to ROS generation mechanisms proposed in mammalian cancer cell lines (Carvalho et al., 2006).

Anti-protozoan and parasitic properties and mechanisms

Bacteria are often vulnerable to predation from bacteriovorus protozoans; in the case of violacein producing bacteria, their production of violacein appears to act as a defense mechanism, exhibiting toxicity towards predators that consume them. Bacteria containing violacein have been shown to be highly toxic to the nanoflagellates *Ochromonas sp., Spumella sp.,* and *B. saltan*, so much so that consumption of only a few bacterial cells caused fatal effects, and only small percentages of violacein producing bacteria in an otherwise non-toxic bacterial population caused significant reductions in flagellate populations (Matz et al., 2004). Violacein's toxic effects on many protozoa due to its use as a defense mechanism give it lots of potential against many parasites, as a large proportion of problematic parasites are protozoa.

One study found violacein to have anti-leishmanial activities, and that while violacein was less effective than pentamidine, a common anti-leishmanial drug, it may be less toxic to healthy mammalian cells than pentamidine, which can cause a host of other harms and side effects, making it not the most optimal treatment (Leon, De Souza, & Durán, 2001). Violacein was determined to be effective against *Plasmodium falciparum* strains (parasite associated with

malaria), including strains which are resistant to the anti-malarial drug chloroquine (Bilsland et al., 2018). This 2018 study also found that deoxyviolacein, while requiring much higher concentrations to be as toxic to the parasites as violacein, was much less toxic to mammalian cells, with the parasite being 20x more sensitive to deoxyviolacein than red blood cells were (vs the parasites being only ~5x more sensitive to violacein compared to red blood cells). In addition, no morphological change or significant mammal cell loss seen due to high deoxyviolacein concentrations, giving deoxyviolacein very interesting promise as an anti-parasitic pharmaceutical. Violacein was also shown to inhibit drug resistant *Trypanosoma cruzi* (cause of Chagas disease), in all its developmental forms at much lower concentrations than Benznidazole, the current standard treatment drug for Chagas disease. Flow cytometry indicated that the compound induced apoptosis in *T. cruzi* cells, and DCFH‑DA staining indicated an increase in ROS generation, while RH123 assay showed a decrease in mitochondrial transmembrane potential, pointing to potential mechanisms (Canuto et al., 2019). This study reveals a very important potential application, as current treatment for CD is limited to only two drugs, which both have limited effectiveness against the disease's chronic form, and very low cure rates, alongside severe negative side effects; Benznidazole has been shown to have a necrotic pathway of action against the parasite, which are generally thought to have more adverse effects on the patient than drugs which act through apoptotic ones like violacein (Canuto et al., 2019). The fact that violacein is potentially more effective against the parasite, and less harmful to the patient gives it great potential for use in treating an undertreated chronic disease, and there is high interest in seeing more research toward this development in the future.

Delivery technology improvements

One complication to violacein's use *in vivo* is its poor water solubility, which could be a detriment to its bioavailability - its ability to be absorbed into circulation and successfully reach its targets in the body (Durán et al., 2021). To deal with this, many researchers have looked into developing different delivery methods that could circumvent poor solubility, and more accurately target tumors or infections. Researchers found that they were able to transform a recombinant violacein expression vector into VPN20009 *Salmonella typhimurium*, a strain used in bacteria-mediated cancer treatment to target and colonize tumors, and that it successfully produced active violacein, providing a promising strategy for targeted violacein delivery to and production at tumors (Hashimi, Xu, and Wei, 2015). Another study used the dendrimer molecule PAMAM bound with violacein to create a delivery complex, and found that the bound violacein was twice as effective as free violacein on the Jurkat E6.1 leukemia cell line (Fakhr et al., 2012). Another study used SAIL's (ionic liquids, organic salts with low melting points that can be tailored as carriers for different molecular processes and useful for biotechnology) to dissolve violacein and act as a delivery agent, and found it gave the compound and enhanced toxicity against A549 lung carcinoma cells (Berti et al., 2020). Continued research into delivery and carrier system technology is important for improving potential for violacein's use for treatment in humans.

III. YEAST

Advantages and use as a study species

In this study, the impact of violacein on various samples of yeast will be investigated. Yeast is an interesting subject of study in this regard, because it could potentially illuminate both the relatively unknown antifungal mechanisms of violacein, and shine light on anti-tumor ones, because as previously mentioned, both being eukaryotic organisms with highly conserved genes and cell structure, they may have the same mechanisms of interaction (Durán et al., 2022).

Yeast cells, particularly those of Baker's yeast, *Saccharomyces cerevisiae*, have been used in scientific study for many years. Yeast is often used as a model species for the study of human cancer cell behaviors, as yeast being eukaryotic, many key genes and components of the cell and its functions are conserved between yeast and human, or other mammalian cells (Pray, 2008). In fact, the processes vital to our understanding of human cell function and, in terms of cancer, dysfunction, such as the cell cycle, protein synthesis, degradation of malfunctioning proteins via proteasomes, autophagy, and retrograde signaling were first characterized and studied in yeast. The process of apoptosis, programmed cell death, is seen in both yeast and mammalian cells, and the processes share many features with respect to the roles of ROS, the mitochondria, and proteases (Guaragnella et al., 2014).

Yeast is also useful as a model for cancerous cells in particular, as it shares many similarities to the physiological and metabolic shifts in cancer cells, including rapid replication and proliferation, and the use of active pathways with high synthesis of fatty acids to incorporate into lipids. Since yeast and cancer cells are both rapidly replicating, they both have high levels of

biomass synthesis which requires increased uptake of glucose and high glycolytic rates; in yeast yielding ethanol and in cancer cells yielding lactate, both of which are excreted by the cells (Natter & Kohlwein, 2013). In addition, this increased glucose uptake and resulting lactate production, known as the Warburg effect, is thought to help cancer cells evade natural immune defenses as the lactic acid accumulation results in immunosuppression (Gao et al., 2022). This phenomenon can be seen similarly to how ethanol accumulation from fermenting yeasts gives them a competitive advantage by suppressing other microorganisms (Natter & Kohlwein, 2013). Yeast being a single celled organism that rapidly replicates and spreads, in combination with the fact that many of the genes regulating its replication and cell cycle pathway are conserved in human cells make it very useful for studying and modeling cancer cells (Pray 2008).

Importance of antifungal activity

Simply gaining a better understanding of violacein's potential as an antifungal compound is very important due to the impacts of pathogenic fungi on humans, and impacts of fungi in nature. As mentioned previously, violacein, and the bacteria which produce it, play an important role in their natural ecosystems, often being involved in symbiotic relationships with other species in which the bacteria producing violacein and other similar compounds protects its host from fungal afflictions.

Although disease has not traditionally been thought of as a major cause of species extinction, more recent research has suggested that chytridiomycosis as a result of *Batrachochytrium dendrobatidis* (Bd) infection is emerging as a primary cause of amphibian extinction and loss of biodiversity in multiple regions where species and biodiversity decline had

been stumping researchers due to no typical associated causes (such as human caused habitat loss or invasive predator introduction). Earlier studies found this fungal disease to be associated with die offs in at least 93 amphibian species globally, and to be rapidly spreading and invading new communities throughout South and Central America (Lips et al., 2006). A more recent 2019 study classified the disease as a "panzootic", and found that Bd has played a role in the extinction of 90 species and decline in at least 501, classifying it as the greatest loss of biodiversity attributed to a disease to be recorded (Scheele et al., 2019). The severity of of biodiversity and species loss at the hand of this fungal disease make the role of violacein as a natural combatant to this disease, already observed *in vivo* to be part of amphibian species resistance to this disease make research into how violacein protects these species crucial, and better understanding could expand the ways scientists could use or introduce violacein with a larger number of species than it is naturally associated with, combating harmful biodiversity decline.

IV. EXPERIMENTAL GOALS

In this study, I first examined violacein's impact on liquid cultures of *S. cerevisiae* using optical density readings with the eventual goal of finding if there were changes in sensitivity for different mutant deletions. However, since in pilot studies there was no noticeable impact on the species with the concentrations of violacein I was able to obtain, the overall study shifted. I instead sought to examine the sensitivities of a variety of different samples of yeast, of different species, taken from the local area, using Kirby-Bauer disk diffusion, with the potential of identifying similarities between affected species that may help elucidate a mechanism.

Methods

Media Preparation

YPD plates were prepared by adding 2% agar, 2% Bacto™peptone, 2% dextrose, and 1% yeast extract to deionized water, autoclave sterilizing, and pouring. YPD liquid media were prepared by adding 2% peptone, 2% dextrose, and 1% yeast extract to deionized water, and autoclave sterilizing. LB plates were prepared by adding 2% agar, 1% tryptone, 0.5% NaCl, and 0.5% yeast extract to deionized water, autoclave sterilizing, and pouring.

Production and Extraction of Violacein

BJB312 strains of *Janthinobacterium lividum* were streaked and grown on LB plates. After incubating for 3 days at room temperature, and being visually observed to have produced sufficient violacein via the colonies' purple hue, the plate was scraped and contents added to methanol. This solution was vortexed to thoroughly mix. Once the cell material settled, the violacein was left suspended in the upper phase of the solvent. The upper phase was transferred to a separate container, and left to evaporate, leaving only the violacein extract as precipitate.

Determining Violacein Concentration

A stock solution of violacein was created by completely saturating DMSO with the dried violacein extract. 1:5, 1:10, and 1:100 dilutions of the stock solution in DMSO were made. Concentration was measured in absorbance using the BioSpec-1601 Shimadzu spectrophotometer set to OD_{595}. The 1:100 dilution was found to have an OD_{595} value of 0.25,

determining the absorbances for the 1:10 dilution OD_{595} to be 2.5, 1:5 OD_{595} to be 5, and stock OD_{595} to be 25.

Initial Optical Density *S cerevisiae* Trials

Liquid cultures of BY4741 *S. cerevisiae* were grown from single colonies taken from YPD plates, which were suspended in 10 mL of YPD media and incubated for 48 hours. The 10mL liquid yeast culture was added to 100 mL of YPD media in sidearm flasks. Growth curves were obtained to confirm cultures would reach stationary phase over a span of 12 hours. Growth was measured with optical density through absorbance measurements. A blank sidearm flask of 100 mL YPD media, and three 100mL 1:10 yeast cultures with different violacein dilutions were prepared: 1 mL of stock violacein solution, 1mL of 1:5 violacein solution, and 1mL DMSO with no violacein. Flasks were incubated at 30°C and rotated at 150RPM over 24hrs. Absorbance measurements were made using a Thermo Scientific™ Spectronic 20D+.

Collection of Yeast Samples

Yeast samples were collected from fruit materials at Bard College campus, Rose Hill Farm, and Tousey Winery. Original location of the sample is indicated by the letters after the dash in the labeling of the isolate, BA indicating Bard apples, RHA indicating Rose Hill Farm apples, RHGMC indicating Rose Hill grapes and mixed compost, TB indicating mixed berries from Tousey Winery, and TG indicating grapes from Tousey Winery (example: A1-BAA1 is from apples at Bard, R18-TGA3 is from grapes at Tousey Winery).

Samples of fruit and compost materials were collected, added to deionized water, and blended. The resulting solution was then plated onto YPD plates using sterile glass beads. After being left to incubate for 48 hours at room temperature, individual colonies were selected from the plates and analyzed under microscope for size to determine if they were fungi or bacteria. Bacterial colonies were discarded and fungal colonies were streaked onto separate YPD plates. Single colonies of each fungi sample were suspended in 10mL YPD media and grown overnight in a shaking incubator at 30°C and 150RPM, and 1mL of each sample suspension was added to Greiner Bio-One CRYO.S™ tubes with 1mL of sterile 40% glycerol. Glycerol stocks of each sample were placed in an Isotemp™ -80°C freezer to be saved for future use.

Kirby-Bauer Disk Diffusion

Liquid cultures of each yeast sample were incubated for 48 hrs at 30°C and 150RPM, each with a single colony suspended in 10mL of YPD media. 250µL of the liquid culture was plated onto YPD plates using sterile glass beads. Each violacein DMSO dilution was sterilized through 0.2µm VWR® sterile syringe filters, and 12µL of each violacein dilution (stock, 1:5, 1:10, and 1:100) was pipetted onto a whatman paper diffusion disk, and each disk placed onto the plate. Plates were left to grow for 24 hours, then observed and photographed (Supplementary Figure 2), using a Zeiss Stemo 2000-C microscope at 0.65 magnification with a moticam 2300 microscope camera and 60-C 1” 1.0x camera adapter, to determine if inhibition had occurred.

DNA extraction and PCR

To obtain DNA for sequencing for 20 samples, DNA was extracted and purified using the Qiagen Puregene® Yeast/Bact Kit B, according to the manufacturer's protocol (Gentra® Puregene® Handbook, 2011, pg 47). The concentration of the extracted DNA was determined using NanoDrop.

PCR were prepared with a final volume of 50µL for each sample containing 25µL of OneTaq® 2X MasterMix, 2µL of extracted DNA, 5µL of 0.5µM ITS1 Forward (5'-TCC GTA GGT GAA CCT GCG G-3') primer, 5µL of 0.5µM ITS4 Reverse (5'-TCC TCC GCT TAT TGA TAT GC-3') primer, and 13 µL of DI water. PCR were performed with a program of round 1 denaturation at 95°C for 4 min, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, then round 2 denaturation at 95°C for 30 seconds, all other steps the same, and round 2 being repeated for 28 more cycles, 30 cycles in total.

PCR cleanup was performed using IBI Scientific's Gel/PCR DNA Fragments Extraction Kit, following the PCR cleanup protocol.

Sequencing and analysis

PCR product concentrations were determined using NanoDrop, and were prepared according to Cornell's Full Service DNA sequencing Handbook. 11µL in final volume, consisting of 10µL PCR product and 1µL of 25µM ITS1 forward primer were sent to Cornell for sanger sequencing in 500ul standalone screw top vials.

Once received back from Cornell, sequences were blasted using the NCBI (Nucleotide collection nr/nt) and YeastIP databases to determine isolate species. Multiple sequence alignment of the

five successful isolate sequences was performed using Clustal Omega from the European Bioinformatics Institute (Figure 6).

Colonies of the five identified isolates were imaged using a Micro-Optics microscope at 40x magnification with a moticam 2300 microscope camera and diagnostic instruments 0.63x camera adapter.

Results

Inhibition of *S. cerevisiae* liquid cultures

To determine if violacein at any of the concentrations created would inhibit *S. cerevisiae* growth in liquid culture, optical density was measured to determine growth curves of the yeast exposed to different violacein solution concentrations. The pigment of violacein meant the culture media started off at different absorbances for each dilution, so the shape of the growth curve, and time to plateau was used as the determining measurement instead. The growth of liquid cultures of *S. cerevisiae* suspended in 100mL of YPD media with stock, 1:5, and 0 (DMSO only) concentrations of violacein were measured over the course of 25 hours with a Spectronic 20D+ spectrophotometer.

Growth curves of each flask culture were recorded. Each culture reached stationary phase in the same amount of time, growing at the same rate, with no observed difference in the shape of their growth curves (Figure 2).

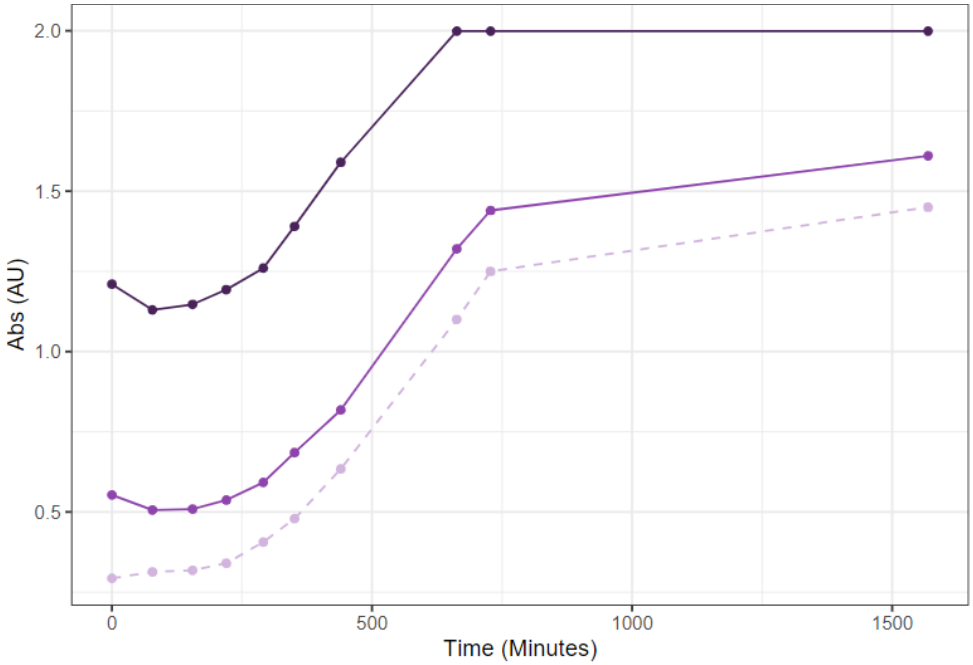

Figure 2. Growth Curves of *S. cerevisiae* exposed to different violacein concentrations: growth of *S. cerevisiae* suspended in 100mL liquid YPD culture over 24 hours of yeast incubated with 1mL of: stock violacein in DMSO (dark solid line), 1:5 dilution violacein in DMSO (purple solid line), and blank DMSO (light dashed line).

Inhibition was judged from the shape of the growth curve and time for each culture to reach stationary phase. As no difference was observed between the growth curves, it appears that violacein in liquid culture at the extracted concentrations used does not cause inhibition of growth in *S. cerevisiae*.

Inhibition of wild yeast samples

Wild yeast was collected from multiple fruit farms and wineries in the region. After colonies of these samples were isolated, I used Kirby-Bauer disk diffusion to test the effects of the extracted violacein concentrations on each sample. After being left to grow for 24 hours, plates were

examined and imaged using a dissection microscope to determine if each disk had a zone of inhibition. Isolates were labeled as sensitive to violacein inhibition if they displayed a lack of growth around any of the disks saturated with violacein DMSO dilutions, and as resistant to inhibition if they displayed consistent growth around the disks (Figure 3; Figure 4). Of the 35 isolates tested, 7 were labeled sensitive, and 28 were labeled resistant. I chose to send 20 isolates to be sequenced, 7 of which were sensitive, 13 resistant (Table 1; Supplementary Table 1).

Isolate	Inhibition	Stock	1/5	1/10	1/100
A1 - BAA1	Resistant	N	N	N	N
B2 - BAA5	Resistant	N	N	N	N
D4 - RHGMC1	Resistant	N	N	N	N
R18 - TGA3	Sensitive	Y	N	N	N
U21 - TBA2	Resistant	N	N	N	N

Table 1. Successfully sequenced isolates' Kirby-Bauer results Five isolate results are listed, with whether or not they displayed any inhibition, and if there was a zone of inhibition for each dilution. Y indicates inhibition was observed, N indicates no inhibition observed.

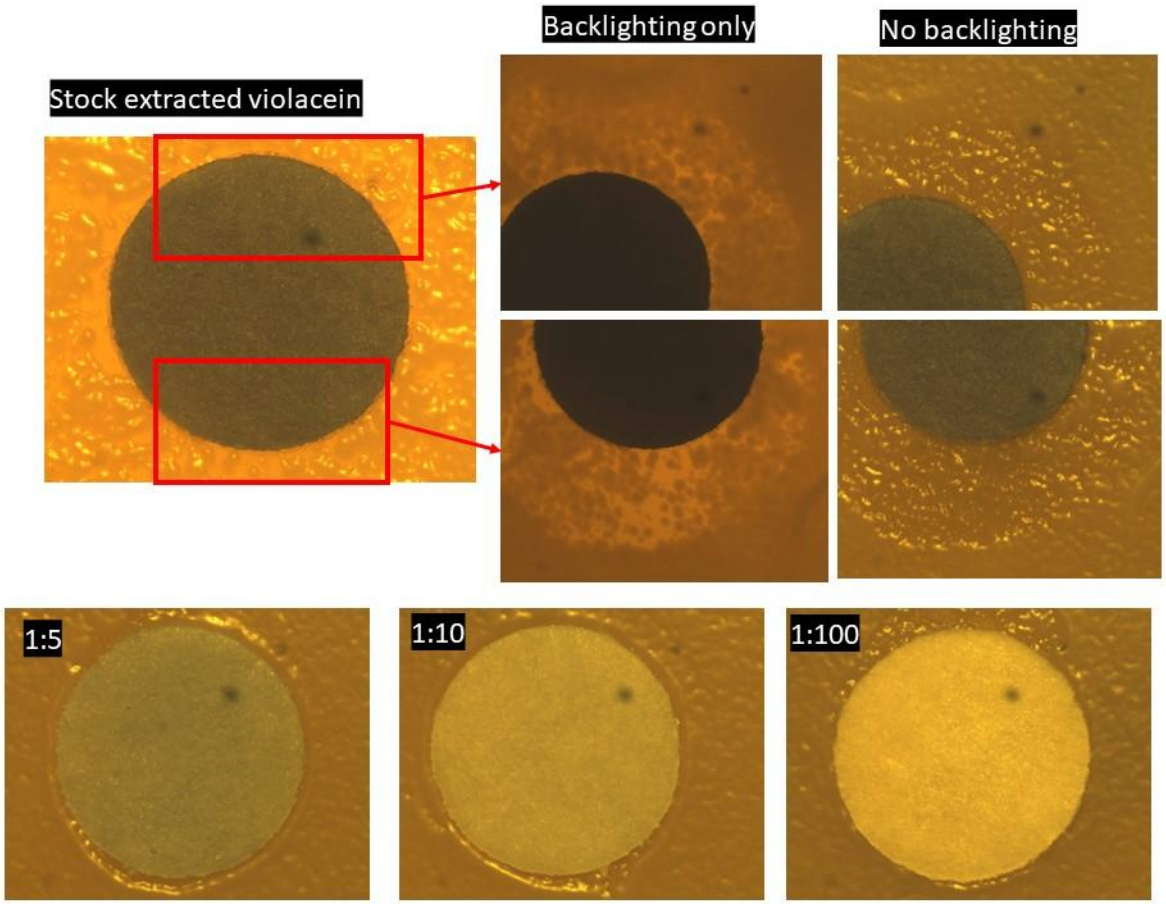

Figure 3. Kirby-Bauer results of sensitive sequenced isolate. R18-TGA3 was determined to be sensitive to violacein, demonstrating a zone of inhibition around the stock disk, as seen by disturbed and lower growth. At 1:5, 1:10, and 1:100 concentrations growth appeared relatively normal, although possibly mildly disturbed.

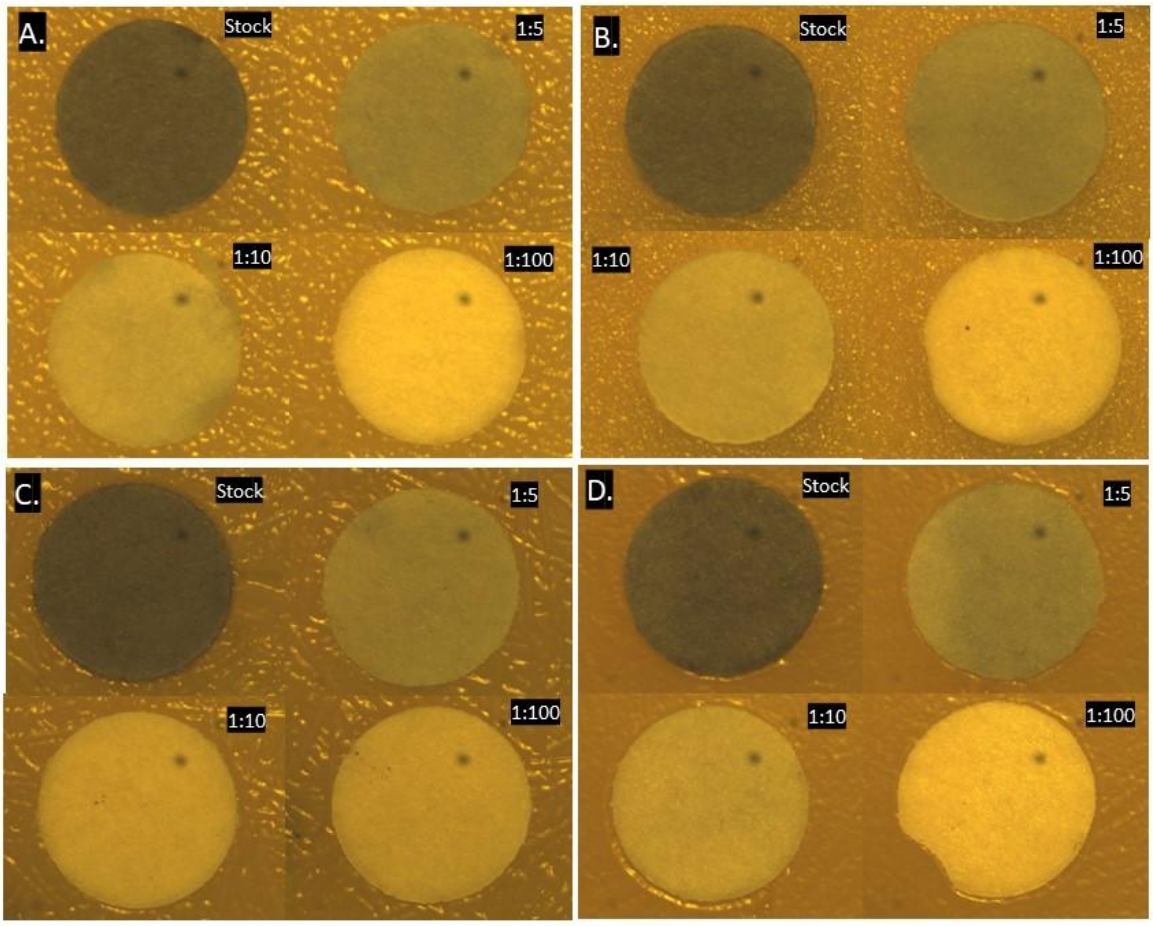

Figure 4. Kirby-Bauer results of resistant sequenced isolates. These 4 successfully sequenced samples, (A) U21-TBA2. (B) D4-RHGMC1, (C) B2-BAA5, and (D) A1-BAA1, were determined to be resistant to violacein in the tests seen above, as no zone of inhibition was present and growth was undisturbed at all concentrations.

DNA Barcoding

For 20 of the tested isolates, including the 7 sensitive samples and 14 of the resistant ones, genomic DNA was extracted from colonies, the DNA was PCR amplified using ITS1 forward and ITS4 reverse primers, and the samples were sent to the Cornell Genomics Facility for Sanger sequencing. Of the samples sent, 5 were successfully sequenced, including 4 resistant isolates and 1 sensitive one (Table 1).

The 5 successful isolate ITS sequences (named A1-BAA1, B2-BAA5, D4-RHGMC1, R18-TGA3, and U21-TBA2) were blasted, which showed that all 5 isolates likely belong to the *Hanseniaspora* genus, more specifically different strains of the species *Hanseniaspora uvarum*. Three of the resistant isolate sequences (A1-BAA1, B2-BAA5, and D4-RHGMC1) had highly similar results giving top matches to *H. sp* strain MALITTN2, *H. uvarum* isolate 3808j, and *H. uvarum* strain 20-11.YLRC3a (over 80% identity and 0% gaps for all). The sensitive isolate sequence (R18-TGA3), and 1 of the resistant isolates (U21-TBA2), had slightly different results, with R18-TGA3 top matches for *H. uvarum* strain PAT-Y271 and *H. uvarum* strain 25-16.YIRC3a (98% identity and 0% gaps for both), and U21-TBA2 top matches for *H. uvarum* strain BZL-88 and *H. uvarum* isolate 40 (94% identity and 1% gaps for both), indicating that they are possibly different strains from the others.

Multiple sequence alignment was performed on the five sequences using Clustal Omega (Figure 6), which showed that the A1-BAA1, B2-BAA5, and D4-RHGMC1 isolates are more closely related to each other than the other two isolates, R18-TGA3 and U21-TBA2 (Figure 5).

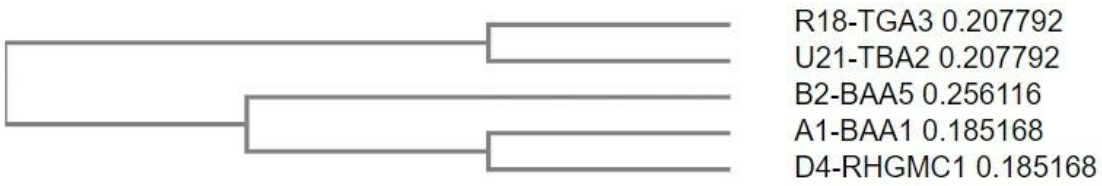

Figure 5. Phylogram of isolates from sequence alignment. Guide tree produced by Clustal Omega based on sequence alignment and similarity. B2-BAA5, A1-BAA1, and D4-RHGMC1 appear closely related, while R18-TGA3 (the sensitive isolate) and U21-TBA2 appear more distantly related to the other three, and more closely related to each other.

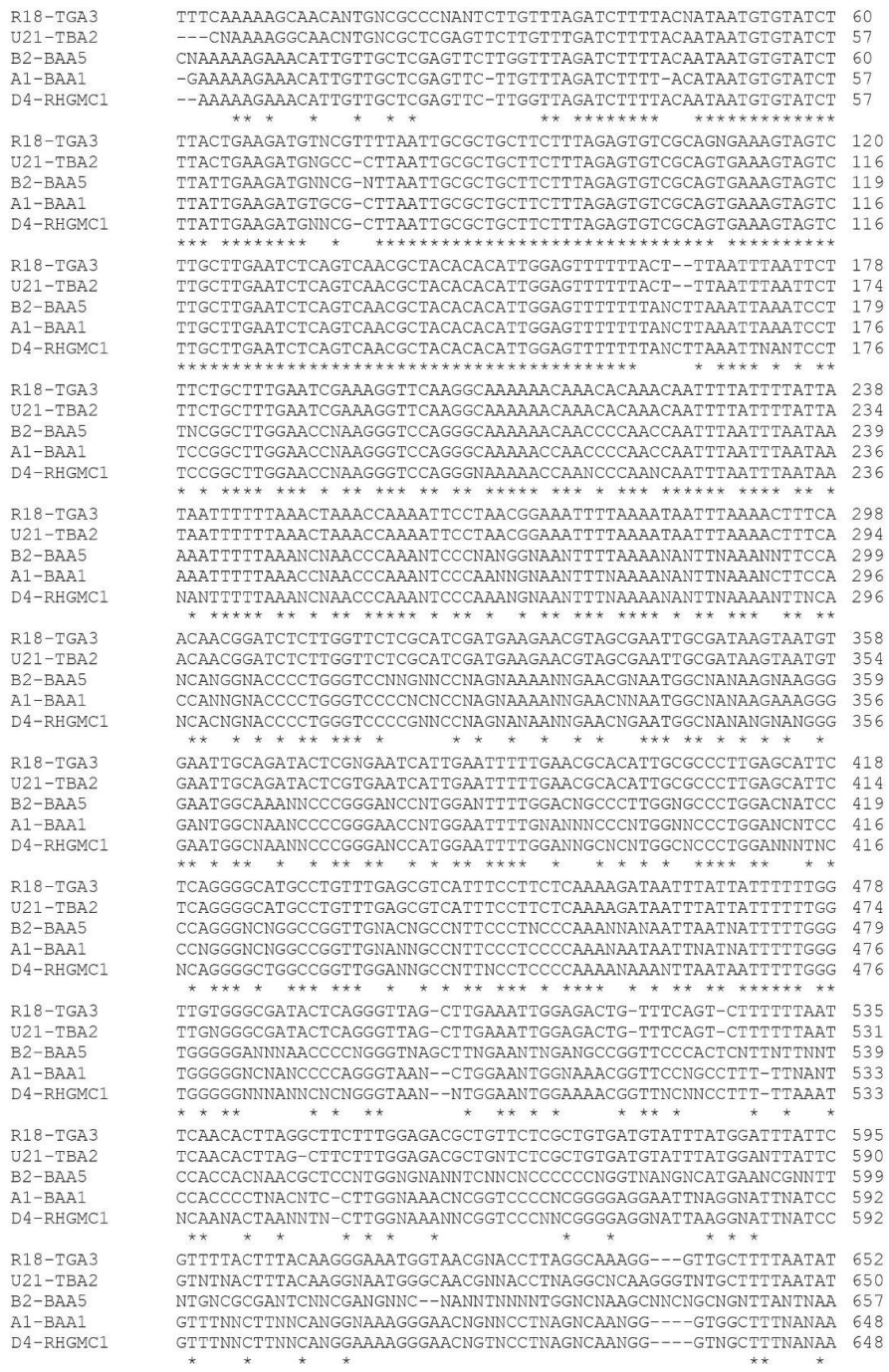

Figure 6. Multiple sequence alignment of the sequenced yeast isolates. Sequences aligned in Clustal Omega. Stars indicate positions where all bases in the sequences are identical.

Colonies of the five isolates were also examined and imaged under a microscope at 40x magnification, showing similar budding and external morphology (Figure 7).

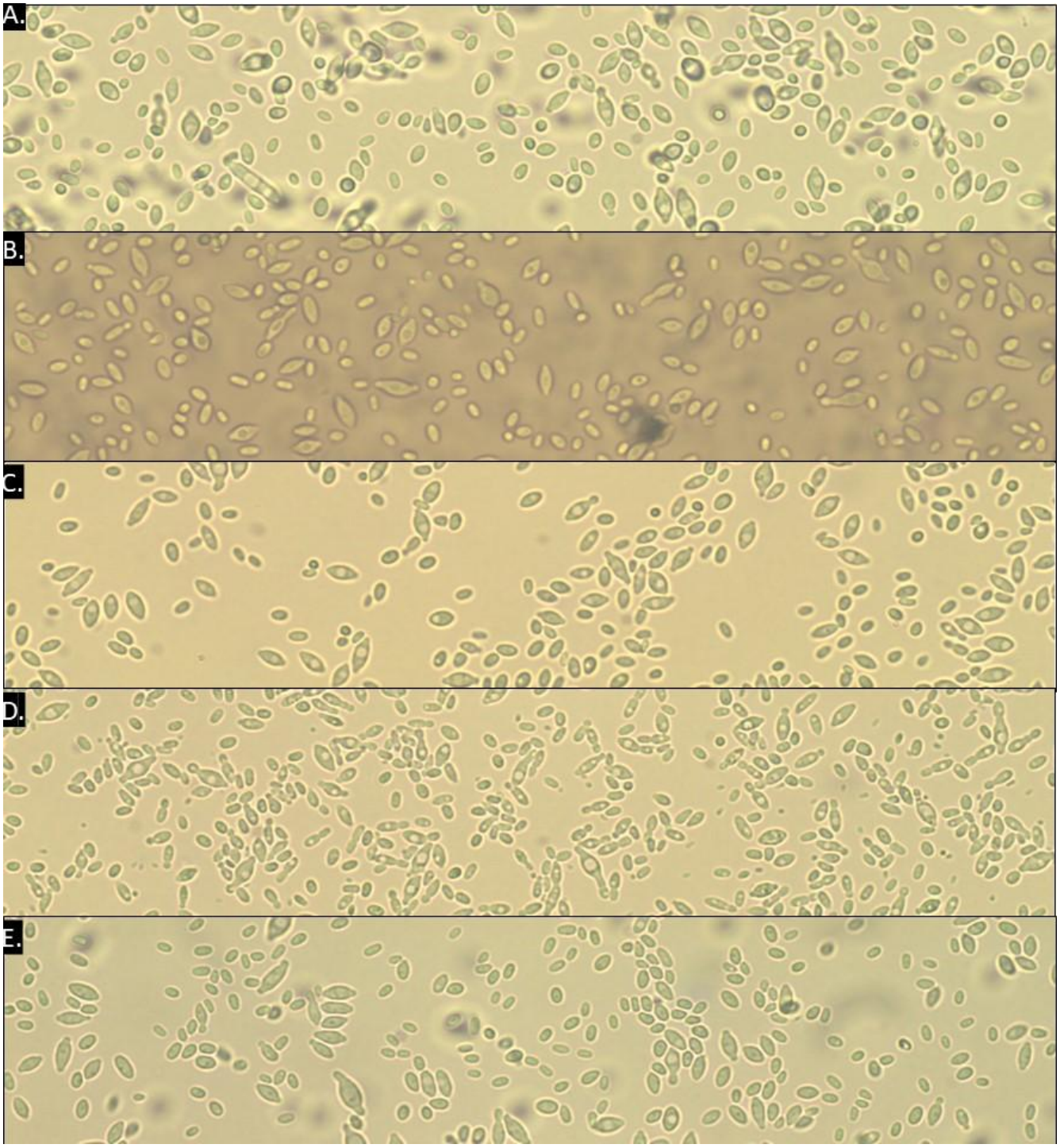

Figure 7. Images of sequenced yeast isolate cells. Cells of the 5 successfully sequenced samples (A) A1-BAA1, (B) B2-BAA5, (C) U21-TBA2, (D) D4-RHGMC1, and (E) R18-TGA3. Cells were imaged under a microscope at 40x magnification.

Discussion

In this study, I sought to determine which locally collected yeast samples would display sensitivity to violacein, to identify the species or closely related species of the tested isolates, and identify taxonomic or genetic commonalities between sensitive isolates and differences from resistant ones which might help elucidate mechanistic factors.

Of the 35 isolates which were tested, 7 displayed sensitivity to violacein through growth inhibition. Although 15 of the 20 isolates were unsuccessfully sequenced and should be re-examined in the future to continue the study's goals, the 5 which were properly sequenced were blasted and returned species results which indicated they belonged to different strains of the same species and genus, which provides very interesting implications for future study to elucidate a mechanism for violacein's inhibition.

Species determination

Through BLAST, I found all 5 isolates are all likely members of the species *Hanseniaspora uvarum*, with the one sensitive isolate, R18-TGA3, appearing to likely be a separate strain, or less genetically similar to, the majority of the sequenced resistant isolates (resistant U21-TBA2 also appeared to be a distinct strain from both the other three resistant isolates and R18-TGA3). Clustal Omega sequence alignment (Figure 6) again indicated that three of the resistant isolates, A1-BAA1, B2-BAA5, and D4-RHGMC1, were more closely related to each other, and also that R18-TGA3 and U21-TBA2 are more similar to each other

than to the other three (Figure 5). It's interesting to note that R18-TGA3 and U21-TBA2 were also collected from the same location, Tousey winery, (although from different fruit types); this could be part of why they seem genetically more similar, and more distant from the other isolates.

Hanseniaspora is a genus of yeast under the *Ascomycota* phylum. *Hanseniaspora uvarum*, a species of this genus, is the likely species of the 5 isolates sequenced and identified with BLAST, often also referred to as *Kloeckera apiculata* (its asexual, anamorph form). It is an apiculate budding yeast, commonly found on fruits, and one of the most abundant fungi species found on grapes. *H. uvarum* plays a large role in the initial stages of fruit fermentation, and is found in a high variety of environments, including soils and even in some organisms such as insects, birds, molluscs, and rarely mammals. (Albertin et al., 2015). *H. uvarum* is sometimes used in mixed cultures for wine fermentation, although the process is usually dominated by the more competitive fermentation species *S. cerevisiae* (Zohre & Erten, 2002).

Study limitations

A main issue that arose at the end of this study was issues with the sequencing of the isolates. Of the 20 that were sent for sequencing, only 5 succeeded, with the 16 other sample sequences containing too many unknown base pairs to be used for species identification. There are multiple issues that could have caused this, including low concentration of DNA product, issues during the PCR process that didn't allow for proper denaturing, annealing, or extension; or improper PCR cleanup. Ideally, I would redo the PCR amplification and cleanup, and resend the

failed samples for sequencing again, but due to time constraints that wasn't possible. In the future to continue this research, the other 16 isolates could be re-sent to analyze further species and strain differences. There were also 14 other isolates not processed or sent for sequencing due to the difficulty of processing so many samples at once, which could be sequenced for additional data on resistant isolates.

Having the species identity of the other isolates would be very useful for future study, as there are 6 other sensitive isolates with still unknown identities, along with the other unknown resistant isolates. It's very possible that there are other species overlaps like the *Hanseniaspora* ones seen in the 5 sequenced isolates which could also prove useful for identifying genetic links to violacein susceptibility.

Another limitation of this study was that inhibition was looked at on a "yes or no" basis, and among the isolates that had sensitivity, there was no comparison of degree of sensitivity. This was mainly done due to the sheer amount of samples being tested, and time limitations, which made getting consistent measurements and comparison of zones of inhibition diameter less feasible with the extent of the study. However, based on preliminary looks at the images of the different isolate disk diffusion plates, it appears that differences in degree of sensitivity to violacein do vary to a pretty wide degree (Supplemental Table 1). In the future, further trials comparing the level of sensitivity of the isolates where inhibition was seen, including measurement of the size of zones of inhibition, or optical density measurements to compare growth curves in liquid media, could allow for comparison of degree of sensitivity between isolates, which could also provide information for genomic comparative studies seeking to explain differences seen.

This study also determined concentration used by defining a "stock" concentration of fully saturated DMSO with dried violacein (OD_{595} equal to 25), with this as the highest concentration used and further dilutions made with DMSO. Other studies used known weight amounts of dried violacein to create different molarity concentrations; if this method was used in the future it could allow for more comparison of results with other studies, and the use of higher and more exact concentrations if needed. This method wasn't used in this study due to the limited amount of extracted violacein which would have been difficult to weigh and divide into different concentrations, and the OD concentration method being simpler and more efficient with the time and tools available.

Comparison to other studies

My findings differed from other research findings in regards to the inhibition of *S. cerevisiae* and *C. albicans* by violacein. While I found no effect of violacein on the growth of *S. cerevisiae* in liquid culture in my pilot study, researchers in a 2005 study reported that at concentrations of 60μM and 80μM violacein was able to significantly alter *S. cerevisiae* cell membrane potential, inducing a collapse of membrane potential leading to disruption of the membrane and cell wall and the loss of cell viability (Pereira, Durán, and Volpe, 2005). The 2005 study didn't find loss of viability at lower concentrations (20μM and 40μM), and my concentrations were measured through absorbance rather than molarity, so it's very possible that my experiments didn't use high enough concentrations to see any effect. Multiple studies found violacein to be effective against *C. albicans* (Sasidharan et al., 2015; Kanelli et al., 2018), whereas I did not. Again, it's possible that the concentrations I used were high enough to cause

inhibition of some species, while not in others like *C. albicans*, which perhaps are affected as well, but require higher concentration levels. It's also possible that differences are due to the way in which the culture was grown and exposed to violacein, as my study used disk diffusion on cultures grown on YPD-agar plate media, whereas other studies, such as the 2018 Kanelli study, examined antimicrobial activity of violacein in liquid culture.

Future studies could use higher concentrations of violacein, and could determine concentration with molarity rather than optical density in order to be more comparable to concentrations used in these other studies. More trials could also be done analyzing inhibition through other methods, such as liquid culture for all the isolates tested (*S. cerevisiae* in the pilot study was grown with violacein in liquid culture, but violacein was tested against all other isolates with disk diffusion on YPD-agar plate medium).

Future research

The sensitive and resistant strains sequenced all being species of *Hanseniaspora* opens up lots of potential for future research to determine a mechanistic reason for why violacein affects one isolate of this genus but not the others. Members of the same species will have much higher genome alignment and commonality, so differences would be easier to identify and provide a smaller amount of genetic information to analyze as potentially causing the difference in response.

One possible direction would be a genome-wide association study, using the sensitive strain and one of the resistant strains for a case-control setup. Microbial genome-wide association studies (mGWAS) are a relatively new development in research, but are emerging as

a useful method of study, similar to how human GWAS have been. Already, mGWAS have been used to identify genetic variants and genomic markers linked to microbial drug resistance (San et al., 2020). One 2018 study was able to identify genetic links to mechanisms of toxin tolerance in *S. cerevisiae* through genome-wide association analysis. The researchers then used gene knockout experiments to confirm their findings (Sardi et al., 2018). This research has similar goals and study species to a potential follow-up study to this one which might use GWA to find mechanisms determining sensitivity vs resistance to violacein, which might point to how violacein's cytotoxicity functions in fungi and other eukaryotes, and the 2018 study design could be used as a model for developing a follow-up study here.

Because of its abundance in fruit crops and fermentation, there is previous precedent for genetic analysis of *H. uvarum* strains that could prove useful in developing a followup study with these isolates. One previous study developed and used microsatellite markers, tandem repeats distributed throughout the genome which are used as markers to study genetic variation and inheritance (Vieira et al., 2016), to genotype and analyze 115 strains of *H. uvarum* from wineries across the world, exploring their genetic relationships and diversity (Albertin et al., 2015). This study and its data have great potential to provide useful information for establishing a GWAS examining *H. uvarum*.

Conclusion

In order to develop violacein's full potential for use, both in medicine and environmental efforts, it is important that scientific understanding of the extent of both the compound's effects and its mechanisms expands. As mentioned previously, gaining a better understanding of

violacein's antifungal impacts and mechanism can not only assist in utilizing the compound as an antifungal drug in humans or in nature, but would likely also shine light on it's anti-tumor mechanisms, as so much of cell biology between yeast and human cells are conserved due to both being eukaryotes, especially in the areas predicted to be central to violacein's mechanisms, such as mitochondrial homeostasis, ROS production, cell cycle replication, and membrane maintenance (Durán et al. 2021; 2022). The results of this study open up possibilities for multiple future studies to further investigate these activities and mechanisms through further sequencing and genomic analysis of the yeast studied.

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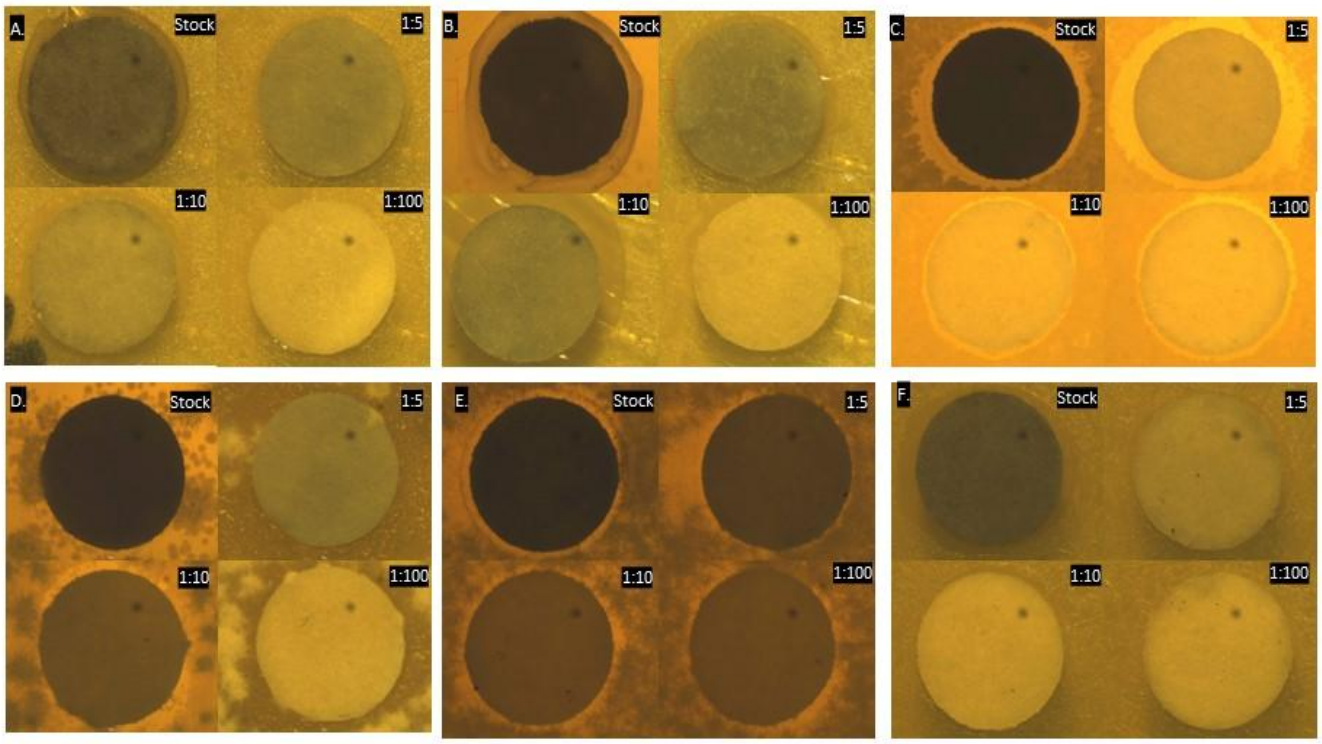
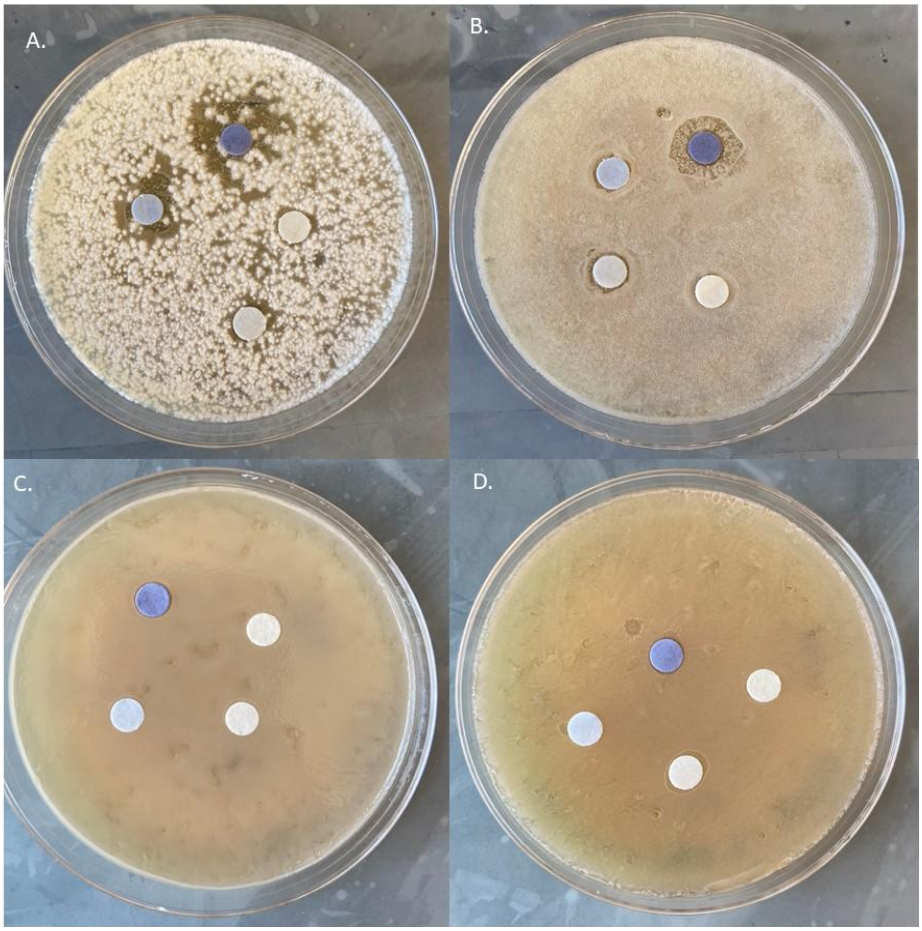
Supplementary Materials

Isolate	Inhibition	Stock	1/5	1/10	1/100
C3 - RHAA3	Resistant	N	N	N	N
E5 - RHGMCB1	Resistant	N	N	N	N
F6 - RHAA2	Sensitive	Y	Y	Y	N
G7 - RHAA1	Sensitive	Y	Y	Y	N
H8 - BAB1	Resistant	N	N	N	N
I9 - BAA7	Resistant	N	N	N	N
J10 - BAA9	Resistant	N	N	N	N
K11 - RHGMCB2	Sensitive	Y	Y	Y	N
L12 - RHAB2	Sensitive	Y	Y	Y	Y
N14 - RHAB5	Resistant	N	N	N	N
O15 - TBA3v1	Sensitive	Y	Y	Y	Y
P16 - TG1	Resistant	N	N	N	N
Q17 - BAA8	Resistant	N	N	N	N
S19 - TBA1v2	Sensitive	Y	Y	N	N
T20 - TGA2	Resistant	N	N	N	N

Supplementary Table 1. non-sequenced isolates' Kirby-Bauer results 15 isolate results are listed, with whether or not they displayed any inhibition, and if there was a zone of inhibition visible for each dilution (Y indicates visible, N indicates no inhibition visible).



Supplementary Figure 1. Kirby-Bauer images of non-sequenced sensitive isolates. These six samples were observed to be sensitive to violacein inhibition. (A) F6-RHAA2 (B) L12-RHAB2 (C) O15-TBA3v1 (D) K11-RHGMCB2 (E) G7-RHAA1 (F) S19-TBA1v2



Supplementary Figure 2. Examples of Kirby-Bauer disk diffusion plate setup: (A) a plate of sample K11-RHGMCB2, which shows inhibitory effect seen in the lack of growth surrounding disks with violacein, with degree of inhibition descending with lower concentration. (B) sample O15-TBA3v1, which also shows inhibitory effect. (C) and (D): plates of samples which show no inhibition, as displayed by consistent growth surrounding all disks.