Quorum sensing-controlled genes increase the survival of *Chromobacterium violaceum* during bacterial interspecies competition

By

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Abstract

Many Proteobacteria use a cell-cell communication system called quorum sensing (QS) to coordinate gene expression in a cell density-dependent manner. Cell density is detected through the production and diffusion of acyl-homoserine lactones (AHLs). The AHL concentration increases until threshold is reached, and AHLs bind the AHL receptor causing transcription of QS-controlled genes. Many QS-controlled genes include: antimicrobials, biofilm components, and other virulence factors. For this reason, we believe that QS is critical for survival during competition with other bacteria. To test our hypothesis, we have developed a competition model between two soil-saprophytes, *Burkholderia thailandensis* and *Chromobacterium violaceum.*

Our research demonstrates that QS in *C. violaceum* and *B. thailandensis* controls the production of antimicrobials that inhibit the other species' growth. *C. violaceum* using QS to increase resistance to bactobolin, a *B. thailandensis* antibiotic, through a RND efflux pump. This QS-controlled efflux pump is important for limiting QS-deficient mutants in our competition model allowing for the increase in antimicrobial producing cells and increased survival of *C. violaceum* during competition. Our results demonstrate that interspecies competition can reinforce QS-controlled behaviors by placing constraints on QS-deficient mutants.

Additionally, we demonstrate that the AHL receptor, CviR, in *C. violaceum Cv017* can bind to N-octanoyl-L-homoserine lactone (C_8-HSL) and N-3-hydroxyoctanoyl-L- (3OH-C8)-HSL produced by B. thailandensis. RNA sequencing of transcripts from C. violaceum grown in the presence of C_8-HSL or hexanoyl (C6)-HSL revealed an enrichment in the transcriptome for competition associated genes when exposed to C_8-HSL. Our transcriptomic and competition results show that the ability of C. violaceum to detect non-cognate AHLs provides an advantage during interspecies competition through the activation of competition associated genes.

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None of this would be possible without the support of my family and friends. Thank you to Dr. Saida Benomar, Dr. Patricia Martins, and Jakki Deay for our long talks on science and life. I would not have finished this dissertation without you. Thank you to everyone in the Chandler lab for all the laughs, help on weekend experiments, and awesome lab lunches! Finally, thank you to my husband, Forrest, and my mother and father, Mimi and Russ Hinshaw for your love, support, and encouragement.

Dedication

This work is dedicated to my grandparents, Robert (Bob) Hinshaw, Edgar Plapp, and Rosalie Plapp. I love you and miss you! Thank you for the example of your amazing work ethic and love of education that saw me through the tough times of graduate school.

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CHAPTER I:

Introduction

Bacteria exist in mixed microbial communities and interact with diverse species of bacteria, fungi, yeast, and protozoa. Despite bacteria existing in these mixed communities, most research focuses on bacteria in isolated cultures leading to the question of how bacteria interact with other species. There are many examples of cooperation between species such as the dental species: *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum,* and *Veillonella* species which cooperate to form biofilms on teeth [1]. However, research by Dr. Kevin Foster and Dr. Thomas Bell looked at the ability of 180 two-species mixtures to cooperate by measuring overall respiration [2]. They concluded that the majority of interactions between species was competitive and not cooperative.

In diverse environments, it is often beneficial for bacteria to function as a cooperating population instead of as an individual in order to thrive. Therefore, it is not surprising that many bacterial species have developed chemical signals to coordinate gene expression within a population. Many Gram-negative bacteria coordinate gene expression through a cell-density dependent communication system called quorum sensing (QS). Gene activation occurs when a "quorum" is reached, thus such systems are referred to as "quorum sensing" systems [3]. Gram-positive bacteria have a similar system for coordinating gene expression called pheromone signaling. While most Gram-negative bacteria have a QS system, the best-characterized QS systems can be found in Proteobacteria. The size of the QS regulon varies widely but most Proteobacteria have QS-controlled antimicrobials. Recently we and others have characterized QS-controlled antimicrobial resistance through efflux pumps [4]. We are interested in determining how interspecies bacterial competition may have influenced the collection of genes that are regulated by QS.

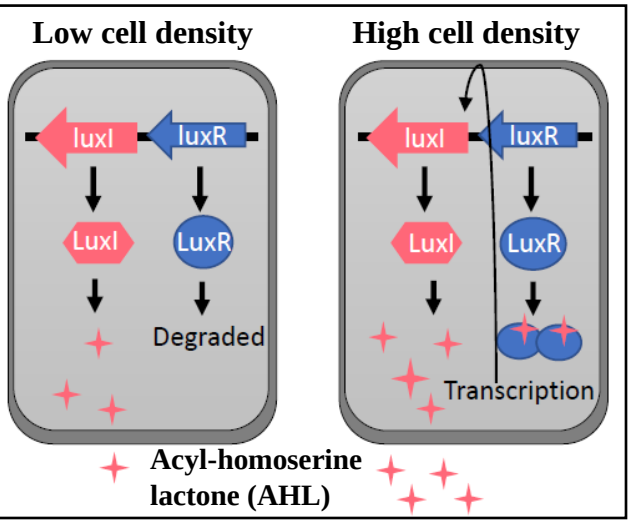
In the following sections, we will review 1) the discovery and molecular mechanism of Proteobacterial QS systems, 2) the importance of QS in the soil bacteria *Chromobacterium violaceum*, 3) a dual-species competition model, and 4) RND-mediated antibiotic resistance in Proteobacteria.

Quorum sensing in Proteobacteria

The first QS system was characterized in 1979 in the bioluminescent marine bacterium, *Vibrio fisheri* [5]. *V. fisheri* colonizes the light organ of the Hawaiian squid, *Euprymna scolopes*, forming a symbiosis where *V. fisheri* acquires nutrients and produces light that helps camouflage the squid at night. *V. fisheri* is only able to reach a high cell density, or quorum, inside the nutrient rich environment of the light organ. Light production occurs through the expression of the luciferase operon (LuxCDABEG) [6]. Activation of the luciferase operon was found to be dependent on two regulatory genes, *luxI,* which encodes the signal synthase, and *luxR*, which encodes the signal receptor and transcriptional regulator [3]. LuxI signal synthase produces the signal molecule N-3-oxohexanoyl-homoserine lactone (3-oxo-C_6-HSL). A "quorum" is reached when 3-oxo-C6-HSL accumulates inside the cell and binds LuxR activating about thirty genes, including the luciferase operon and *luxI* [7-9]. The positive regulation of *luxI* causes a rapid and significant increase in signal production ensuring uniform activation of QS genes in the population (Figure 1).

Figure 1. Mechanism of quorum sensing

LuxI-signal synthase constantly produces a low level of AHL signals that diffuse out of the cell. LuxR-signal receptor is degraded when not bound to its specific signal. Once the AHL reaches a critical concentration, the signal binds LuxR and forms a homodimer with another LuxR-AHL complex. The LuxR-AHL homodimer activates transcription of select genes, including *luxI*, creating a positive feedback loop that drastically increases the signal concentration.



LuxI homologs produce acyl-homoserine lactones (AHLs) as the signal molecule. Biosynthesis of the AHL signal generally requires the central metabolite S-adenoslmethionine (SAM) for the homoserine lactone ring and the acyl side chain from fatty acid biosynthesis [10-12]. Variation in the acyl side chain provides specificity to the individual QS systems. Acyl side chains are usually straight but can be derived from aromatic acids [13, 14]. Additionally, the acyl side chain can vary in length from 4 to 18 carbons, have varying degrees of saturation, and can have substitutions on the third carbon. This variation provides specificity to the QS system.

The AHL binds with the N-terminal of LuxR homologs at a structurally conserved AHL-binding domain [13, 14]. Without a signal, LuxR homologs are inactive and quickly degraded. Upon binding the AHL, LuxR undergoes a conformational change allowing it to bind to another LuxR-AHL complex to form a homodimer [15]. This homodimer then functions as a transcription factor and binds DNA at the C-terminal helix-turn-helix domain. LuxR-family proteins typically bind a DNA sequence called the *lux* box generally found upstream of the target gene [16]. The *lux* box is usually 14-18bp long and is a palindrome sequence following the pattern CTGNNCCNNGGNNGAC. LuxR responsive genes can be anywhere in the genome, but *luxR* and *luxI* are usually adjacent on the chromosome. LuxR-family transcriptional regulators

normally activate expression of target genes, but in a few cases, LuxR-family proteins repress gene transcription [17-19].

Most QS-controlled genes fall into several general groups: toxins (e.g., antimicrobials), exoenzymes (e.g., proteases), biofilm components, and cellular proteins (e.g., metabolism). Many of these genes encode products that are important for group behaviors that are costly to the individual cell but are beneficial at high cell density. For example, the production of biofilm proteins, proteases, or antimicrobials are less effective at low concentration and a waste of energy that is better spent on growth. Thus, QS may serve as a cost-saving strategy for metabolically expensive products that are not useful at low density [20, 21]. Additionally, it is believed that QS delays production of antimicrobials until a lethal concentration can be delivered to prevent the adaptation of competitors to a sub-inhibitory concentration [22, 23]. In this dissertation we evaluate the effect of group behaviors and competition as evolutionary forces that shaped the QS regulon in *Chromobacterium violaceum*.

Chromobacterium violaceum

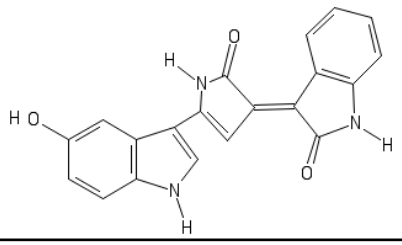
Overview of the Chromobacterium genus

In 1880 Italian scientist Bergonzini from Modena University found that his cultures were contaminated by a "fantastic" film of violet color [24]. He initially suspected *Cromococcus violaceus,* the only known bacteria to have purple coloration. However, further testing revealed this contamination to be a novel bacterium Bergonzini termed, *Cromobacterium (sic) violaceum* and published his findings in 1881. The only earlier report of a similar species was the publication of a communication by Gessard in 1867 [25]. In this publication, Gessard describes a "small organism" causing violet coloration on rice. In 1882, Boisbaudran, a contemporary of Gessard, published an article in *Compt Rendues d' Academie du Science* where he named the

"small organism" *Chromobacterium violaceum* and characterized the purple pigment, violacein, using visible absorption spectra. The spelling of *Cromobacterium (sic)* by Bergonzini was corrected later that same year cementing the nomenclature of *Chromobacterium violaceum.*

Chromobacterium violaceum is currently defined as a Gram-negative, facultative anaerobe of the β-Proteobacteria family and is found in water and soil samples of the tropics and subtropics around the world. Although *C. violaceum* is the most recognized species of the *Chromobacterium* genus, currently there are 11 species: *C. violaceum, C. subtsugae, C. haemolyticum, C aquaticum, C. piscinae*, *C. amazonense*, *C. pseudoviolaceum, C. alkanivorans*, *C. vaccinii*, *C. sphagni*, and *C. rhizoryzae.* All species are rod-shaped, motile with a single flagellum, and most species are able to ferment glucose and reduce nitrate. The most recognizable feature is the purple pigment, violacein, though it is no longer a defining feature of all species. There are four non-pigmented species: *C. aquaticum, C. haemolyticum, C. alanivorans,* and *C. rhizoryzae.* Since most species are nonpathogenic, there has been minimal research in this genus. The unique characteristics of each species are discussed in Chapter II.

Figure 2. Structure of violacein



Violacein is a bisindole (see structure in Figure 2) formed by the condensation of two tryptophan through the action of five proteins encoded by *vioABCDE* [26]. There are several bacteria genera, including: *Collimonas, Duganella, Janthinobacterium, Microbulbifer* sp., and *Pseduoalteromonas,* that produce violacein, but *C. violaceum* is the best-known species for violacein production [27]. Violacein is a broad-spectrum antimicrobial, an antifungal, and is believed to protect *Chromobacterium* from eukaryotic predators, such as *C. elegans* [28-30]. Another study demonstrated the ability of

violacein to provide protection from ultra-violet light damage [25]. Violacein has also been shown to have antimalarial properties [31].

Clinical reports of Chromobacterium infections

Most members of the *Chromobacterium* genus are nonpathogenic, but infections of *C. violaceum* and *C. haemolyticum* in humans and mammals have been reported [32, 33]. Infections commonly present with fever, abdominal pain, skin abscesses, sepsis, leukocytosis, and liver abscesses. The first infection of *C. violaceum* was documented in the 1930s and there have been over 150 documented cases to date [34]. However, 65% of those cases were reported after 1990. Whether this increase in prevalence is due to better access to healthcare, improved documentation, or due to increased pathogenicity remains to be seen. Infections of *C. violaceum* are reported every year and mortality rates range from 7% in the United States and Australia to 53% in Southeast Asia [34]. In 2017 there were 23 new cases of human infection with a mortality rate of 35% (8 deaths) [35]. The decrease in fatality rate may be attributed to improved diagnostic and treatment regimes. Antimicrobial susceptibility information for clinical specimens is limited and extremely variable. However, *C. violaceum* is known to be resistant to β-lactams and has become resistant to ticarcillin, carbenicillin, and cefoxitin [34]. The current antibiotic regiment usually involves a treatment with a fluoroquinolone, carbapenem, or ciprofloxacin for 2-3 weeks followed by 3-6 months of treatment with trimethoprim sulfamethoxazole, tetracycline, or fluoroquinolone to prevent relapse.

C. haemolyticum was first isolated from human sputum and demonstrated remarkable hemolytic activity in human and sheep erythrocytes [33]. Infections by *C. haemolyticum* are even more rare than *C. violaceum* infections and have not been associated with any mortalities.

Interestingly, *C. haemolyticum* isolated from tropical freshwater also exhibited strong β-hemolytic activity and high resistance to β-lactams as seen in the clinical isolate [36].

Until recently, there was no research into the molecular mechanisms of *Chromobacterium* virulence. *C. violaceum* ATCC 12472 has two pathogenicity islands: Cpi-1 and Cpi-2, which resemble the pathogenicity islands Spi-1 and Spi-2 in *Salmonella* [37]. Phylogeny studies revealed that Cpi-1 can be found in most species of *Chromobacterium* and deletion of this pathogenicity island resulted in a remarkable decrease in virulence in a mouse model [35, 37]. Interestingly, the genes encoding the needle complex (Cpi-1a) were located distantly from the Cpi-1 cluster and appear to be important for infection. More research is needed to identify the function of the at least 16 effector proteins associated with Cpi-1/1a. More research is also needed to determine the role of Cpi-2 in infection. In *Salmonella,* Spi-2 is important for survival in macrophages [38].

In the mouse model, *C. violaceum* is detected by macrophages through recognition of the Cpi-1a T3SS needle protein CprI [37]. Detection of CprI causes up-regulation of the inflammasome through Nod-like receptor (CARD domain-containing protein 4 (NLRC4)). The inflammasome eliminates *C. violaceum* through pyroptosis and natural killer (NK) cell cytotoxicity which eject the bacteria from macrophages and hepatocytes. The bacteria are then vulnerable to killing by neutrophils [39]. This mode of clearing the infection corresponds with what is known about human infections of *C. violaceum.* A comorbidity with chronic granulomatous disease, an autoimmune disease characterized by difficulty making reactive oxygen compounds, is often reported but is not found in all cases [34, 40]. *C. violaceum* is classified as a BSL-2 bioagent and potential emerging pathogen due to the ability of *C. violaceum* to infect humans and multiple non-primate mammals [41, 42].

Overview of Chromobacterium violaceum strain CV017

Most of what is known about *C. violaceum* comes from studies in the reference strain *C. violaceum* ATCC 12472 as this is the only complete genome [43]. However, another commonly used strain is *C. violaceum* ATCC 31532 which was isolated prior to 1982 from the New Jersey Pine Barrens [44]. Little is known about strain ATCC 31532 other than basic growth conditions and that it produces a monobactam. Compared to the reference strain *C. violaceum* ATCC 12472, strain ATCC 31532 has decreased violacein production. To increase violacein levels, a transposon was inserted into a previously unknown site (discussed in Chapter II) and this strain was named *C. violaceum* strain CV017. Research in this dissertation is conducted with *C. violaceum* strain CV017.

One of the best characterized features of *C. violaceum* strains, including CV017, are their ability to detect AHLs from other species. The LuxR homolog, CviR, can bind AHLs with saturated acyl-side chains that range from 4-12 carbons in length [45]. This feature has made *C. violaceum* a common biosensor for detecting AHLs. As a biosensor, the native AHL synthase, *cviI*, is disrupted through deletion or transposon insertion making the strain light yellow in appearance. When grown near other bacteria producing AHLs or if exogenous AHLs are added to the media, then *C. violaceum* will turn purple through the QS-controlled production of violacein. Indeed, violacein is so responsive to AHL concentration, that standard curves can be created to determine the quantity of AHLs in the media [45, 46].

In addition to regulating violacein, QS in *C. violaceum* strains has been shown to activate transcription of exoproteases, hydrogen cyanide, antibiotics (aerocyanidin, aerocavin, 3,6-Dihydroxy-indoxazene, and monobactam SB_26.180), β-lactamase, antitumoral depsipeptide (histone deacetylase), and type III secretion system proteins [25, 47, 48]. Many of the genes

responsible for these phenotypes remain unknown but are important for competition providing motivation for determining the QS regulon by RNA-sequencing in Chapter IV.

In addition to the QS regulon being unknown, the genome of strain CV017 was not sequenced prior to this dissertation (see Chapter II). At that time the only fully sequenced genome for *C. violaceum* is strain ATCC12472. Since 2015 there has been a drastic increase in the number of draft genomes for the broader *Chromobacterium* genus. Using phylogenetic analysis of these genomes, we demonstrate in Chapter II that *C. violaceum* CV017 is a member of the *C. subtsugae* species. For the sake of clarity, we will refer to *C. violaceum* CV017 as *Chromobacterium* sp. CV017 after Chapter II.

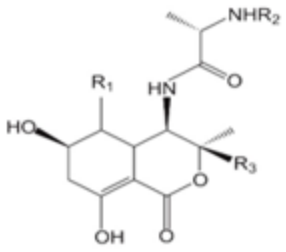
Dual-species competition model

While most QS systems control antibiotic production, there are very few studies evaluating the importance of QS during interspecies competition. The dual-species model between *C. violaceum* and *Burkholderia thailandensis* used in this dissertation was originally developed by Dr. Josephine Chandler [23]. These two bacteria were chosen for their similar laboratory growth conditions, defined QS systems, and QS-controlled antimicrobials.

B. thailandensis is also a β-Proteobacteria and soil saprophyte. While *C. violaceum* has a single QS system, *B. thailandensis* has three LuxI-LuxR pairs: BtaI-BtaR 1, BtaI-BtaR 2, and BtaI-BtaR 3 which respond to N-octanoyl-L-homoserine lactone (C_8-HSL); N-3-hydroxyoctanoyl-L-HSL (3OH-C_8-HSL) and N-3-hydroxydecanol-L-HSL (3OH-C_{10}-HSL); and 3OH-C_{10}-HSL, respectively. These QS circuits are also conserved in the human pathogens *Burkholderia mallei* and *Burkholderia pseudomallei.* Since *B. thailandensis* is nonpathogenic, it is a convenient model for studying QS and other conserved pathways for the pathogenic species.

B. thailandensis has at least 12 polyketide synthase (PKS) or non-ribosomal peptide synthase (NRPS) clusters [49]. The *bta* PKS/NRPS hybrid cluster is controlled by the BtaIR 2 QS circuit. This cluster produces the antibiotic bactobolin which is a broad-spectrum antibiotic. There are 8 classes (A-H) of bactobolins (Figure 3) with bactobolin A and bactobolin C being the most potent [50, 51]. Bactobolin is believed to disrupt ribosome activity as evolved-resistance to bactobolin in *Bacillus subtilis* occurs through the L2 protein in the 50S subunit [51]. L2 is required for association between the 50S and 30S subunits and full activity of the peptidyl-transferase center. B) Structure of each class (A-H) of bactobolin adapted from [51]. Based on the location of bactobolin-resistant mutations in *B. subtilis*, bactobolin is believed to inhibit peptidyl transfer. There is a homolog of L2, L8e, in the eukaryotic ribosome which may explain why bactobolin A and bactobolin C are also toxic to murine fibroblasts.

Figure 3. Structure of each class (A-H) of bactobolin
Figure adapted from [48].



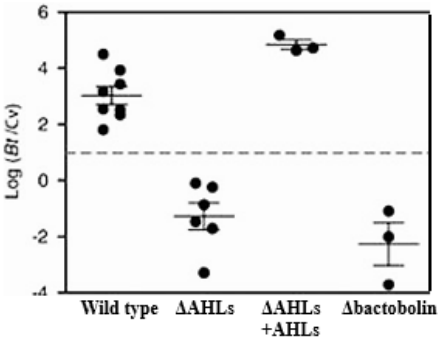
Bactobolins

A: R_1=OH,	R_2=H,	R_3=$CHCl_2$
B: R_1=OH,	R_2=L-Ala,	R_3=$CHCl_2$
C: R_1=H,	R_2=H,	R_3=$CHCl_2$
D: R_1=H,	R_2=L-Ala,	R_3=$CHCl_2$
E: R_1=OH,	R_2=L-Ala-L-Ala,	R_3=$CHCl_2$
F: R_1=H,	R_2=L-Ala-L-Ala,	R_3=$CHCl_2$
G: R_1=H,	R_2=H,	R_3=CHCl
H: R_1=H,	R_2=L-Ala,	R_3=CHCl

In competition experiments between wildtype *B. thailandensis* and wildtype *C. violaceum* (described in Chapter III), *Burkholderia* is able to increase in frequency indicating that it is able to "win" the competition (Figure 4). The ability of *B. thailandensis* to inhibit *C. violaceum* is dependent on QS. The deletion of signal synthases in *B. thailandensis* allows *C. violaceum* to increase in frequency, and the addition of *B. thailandensis* AHLs to the competition restores *B. thailandensis* to victory. Deletion of bactobolin mirrored the effect of deleting the signal synthases in *B. thailandensis* indicating that bactobolin is the QS-controlled antimicrobial that is responsible for inhibiting *C. violaceum* (Figure 4) [23, 49].

C. violaceum also has a QS-controlled antimicrobial factor that inhibits the growth of *B. thailandensis,* but the specific antimicrobial factor is unknown [23]. Additionally, the ability of *C. violaceum* to detect AHLs produced by *B. thailandensis* increases *C. violaceum* survival under certain conditions (Figure 4). Research in Chapter IV investigates which AHL from *B. thailandensis* is most important for increasing survival of *C. violaceum* and which genes respond to noncognate AHLs.

Figure 4. QS-controlled bactobolin produced by *B. thailandensis* inhibits *C. violaceum* during competition



A) Adapted from [23]. Co-culture competition of wild-type *C. violaceum (Cv)* and wild-type or mutant strains of *B. thailandensis (Bt)* indicated on the x-axis. The dashed line indicates the starting 10:1 ratio of *Bt:Cv* which offsets the slower growth of *Bt.* The ratio of *Bt:Cv* was determined after 24h by selective plating and colony counts. AHLs were extracted from *Bt* supernatant. Deletion of bactobolin (Δbactobolin) prevents killing of *Cv* demonstrating the importance of bactobolin during competition. Error bars represent the standard error of the mean.

RND efflux pumps

Upon starting my dissertation research, I demonstrated that QS in *C. violaceum* also controls an antibiotic resistance factor that increases survival during competition. Further experimentation discussed in Chapter III led to the conclusion that this resistance factor was an efflux pump. Sequencing revealed that this efflux pump most closely matched the AcrAB proteins from *Escherichia coli* and Mex pumps in *Pseudomonas aeruginosa.* Both pumps are part of the RND (resistance-nodulation division) superfamily. Importantly, at that time, very few QS-controlled efflux pumps had been identified to provide antibiotic resistance.

Efflux pumps are classified by phylogenetic similarities in structure. There are five major classes of efflux pumps: RND, MFS (major facilitator superfamily), MATE (multidrug and toxic

compound extrusion), SMR (small multidrug resistance), and ABC (ATP-binding cassette) transporters [52]. All families except ABC transporters use proton motive force to efflux their substrates while ABC transporters use ATP hydrolysis to drive exportation.

The most detailed information on RND efflux pumps comes from studies on AcrAB-TolC in *E. coli.* All RND efflux pumps consist of three proteins: an ATPase inner membrane pump (AcrB), a periplasmic adaptor(AcrA), and an outer membrane porin (TolC) [53]. In *E. coli*, *acrAB* are linked in the chromosome while *tolC* is located elsewhere. However, in most other systems all three genes are linked in the chromosome. RND systems can also be found on plasmids. Some of the efflux pumps in the MATE and ABC transporter superfamilies are also organized into tripartite complexes, but SMR and MFS superfamilies are single protein transporters located in the inner membrane [52]. Since RND and other tripartite efflux pumps can export directly into the extracellular environment, they contribute more to antibiotic resistance than SMR or MFS efflux pumps which excrete into the periplasmic region. By exporting into the extracellular environment, the effectiveness of RND efflux pumps is directly related to the ability of the compound to cross the outer membrane. In fact, permeabilizing the outer membrane has nearly the same effect as deleting the efflux pump [54]. Most RND efflux pumps have very wide specificity which may include antibiotics, detergents, dyes, free fatty acids, and quorum sensing molecules [52]. Not surprisingly most of these compounds have at least one hydrophobic domain making facilitated diffusion required. The specificity of each efflux pump seems to be linked to the size of the pore created by AcrB in the inner membrane.

RND efflux pumps are regulated by local and global transcription factors. Most RND efflux pumps are repressed at the local level by a divergently transcribed TetR repressor family protein [55]. TetR family proteins have a C-terminal ligand binding domain and a N-terminal

DNA-binding domain [56]. In the absence of antibiotics, TetR family proteins repress the RND efflux pump [57]. However, this repression is not tight allowing for constitutive expression of the efflux pump. In the AcrAB-TolC efflux pump in *E. coli*, the QS-regulator, SdiA, increases transcription of the AcrAB operon 2- to 3-fold [4].

Allocation of work

In Chapter 2, the genome sequencing was published in *Genome Announcements* (10.1128/genomeA.00080-16) [58]. The DNA libraries were created by the University of Kansas (KU) Genome Sequencing Core and the genome assembly and annotation were done by Dr. Stuart Macdonald and Xiaofei Wang. The DNA libraries were sequenced by the Kansas State University Integrated Genomics Facility. The 16S RNA phylogenetic analysis and comparison of *Chromobacterium* species is unpublished and completed by Kara Evans.

All of Chapter 3 was published in the *International Society of Microbial Ecology* (10.1038/s41396-018-0047-7) [59]. The *cdeAB-oprM* deletion mutants were created by Saida Benomar. Genome sequencing of the bactobolin-resistant, quorum sensing-deficient *C. violaceum* was done by the KU Genome Sequencing Core. The purified bactobolin A used in the casein evolution experiments was purified from *B. thailandensis* supernatant through HPLC by Ben Neuenswander. The casein evolution experiments with bactobolin A were conducted by Lennel Camuy-Velez. Ellen B Nasseri assisted Kara Evans with all other casein evolution experiments. All other experiments were conducted by Kara Evans.

The work in Chapter 4 is unpublished. To determine the amount of acyl-homoserine lactones produced by *C. violaceum*, Kara Evans completed all sample prep and Todd Williams, Director of the Mass Spectrometry Laboratory and Analytical Proteomics Laboratory at the University of Kansas, ran the time of flight-MS/MS and helped with analysis of the results. Kara

Evans conducted all the competition experiments to determine which AHL produced by *B. thailandensis* was detected by *C. violaceum*. For the RNA sequencing, Kara Evans prepared all the RNA samples while the libraries were generated by the KU Genome Sequencing Core. The bioinformatics following sequencing was done by Xiaofei Wang and Dr. Stuart Macdonald.

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CHAPTER II:

Reassessment of the taxonomic position of *Chromobacterium violaceum* CV017 and a proposal to reclassify *C. violaceum* CV017 as *C. subtsugae* CV017

Abstract

The genome of *Chromobacterium violaceum* CV017 was sequenced "*de novo*" and compared to the genome of *C. violaceum* ATCC 12472. The genome-wide comparison yielded only a 64.4% similarity to *C. violaceum* ATCC 12472 but 98% similarity to *C. subtsugae.* The genome of *C. subtsugae* has fewer nitrate reductases, a unique biosynthetic complex encoding secondary metabolites, and lacks the second pathogenicity island, Cpi-2. *C. violaceum* CV017 has these same features and also has the same quorum sensing signal, N-hexanoyl-homoserine lactone, as *C. subtsugae* leading to the conclusion that *C. violaceum* CV017 has been improperly classified. Further phylogenetic analysis using 16S RNA of all 45 sequenced genomes of *Chromobacterium* reveals an exclusive clustering of *C. subtsugae* that includes *C. violaceum* CV017.

Introduction

The soil saprophyte *Chromobacterium violaceum* is an occasional human pathogen known for its ability to produce a purple pigment, violacein [1]. The strain CV017, derived from a soil isolate (ATCC 31532), has a transposon insertion at an unknown site causing overproduction of violacein [2, 3]. Violacein is controlled by acyl-homoserine lactone-dependent quorum sensing, and derivatives of CV017 have been widely utilized as acyl-homoserine lactone biosensors [4]. CV017 is also useful to understand the role of quorum sensing in interspecies competition because of its growth characteristics and quorum-dependent antimicrobials. When we sequenced our genome in 2015, only one other *C. violaceum* strain had been sequenced, ATCC 12472 (accession NC_005085.1)[5]. To date, 17 genomes of *Chromobacterium violaceum* have been sequenced and are available on the National Center for Biotechnology Information (NCBI) website. Here we report the draft genome sequence of the genetically distinct strain CV017.

Publications on the broader genus of *Chromobacterium* have drastically increased in the last decade with research primarily focusing on *C. violaceum* and *C. haemolyticum* infections, medical and agricultural applications of secondary metabolites, and identification of novel species from soil isolates [6]. Importantly, this research has included the sequencing of numerous *Chromobacterium* strains and lead to the creation of 11 species of *Chromobacterium* (see Table 2). The rapid expansion of sequenced *Chromobacterium* species genomes allowed us to investigate the phylogenetic relationship of CV017 to all species of *Chromobacterium*. There are 45 draft or complete genomes of the genus *Chromobacterium* found in NCBI, and we used 16S RNA sequences to evaluate the relationship of each strain. We discovered that *C. violaceum* CV017 shares more sequence similarity with *C. subtsugae* than other strains of *C. violaceum* and clusters with other members of *C. subtsugae* based on 16S RNA. This homology and other

metabolic characteristics lead us to conclude that *C. violaceum* CV017 is a member of the *C. subtsugae.*

Materials and Methods

Genome sequencing and annotation

CV017 cells were grown in Luria-Bertani media, and genomic DNA was isolated using the Gentra Puregene Bacteria/Yeast kit (Qiagen). The DNA was used to make a sequencing library with 1-kb inserts and was sequenced on an Illumina MiSeq generating 300-bp paired-end reads. Raw reads were preprocessed with Scythe v0.991(https://github.com/vsbuffalo/scythe) and Sickle v1.200 (https://github.com/najoshi/sickle) to improve read quality, aligned to the phiX174 genome via Bowtie2 v2.1.0 to remove any contaminating reads, and assembled with ABySS v1.9.0 using an empirically determined optimal k-mer size of 115 [7, 8]. We annotated the assembly using NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Dendrogram construction

The phylogenetic tree is based on the 16S rRNA gene of *Chromobacterium violaceum* strain CV017 (GenBank accession no. LKIW01000119.1). The number of species was limited to the 45 strains that had genome assembly information available in NCBI. The accession number of each gene can be found in Table 1. The phylogenetic tree was constructed based on neighbor-joining and supported by maximum-likelihood and maximum-parsimony algorithms [9]. All bootstrap values were based on 1000 replications.

Table 1. GenBank accession number for 16S RNA sequence

Species name	Accession Number
Chromobacterium vaccinii strain XC0014	MF186165.1
Chromobacterium sp. ATCC 53434	CP025429.1
Chromobacterium vaccinii strain 21-1	CP017707.1
Chromobacterium vaccinii strain MWU328	JN120870.1
Chromobacterium vaccinii strain MWU205	NR_109451.1
Chromobacterium sp. MWU2920	KJ576863.1
Chromobacterium sp. MWU2387	KJ576860.1
Chromobacterium sp. MWU3525	KJ576868.1
Chromobacterium violaceum strain CV1197	CP024029.1
Chromobacterium violaceum strain CV1192	CP024028.1
Chromobacterium sp. MWU2576	KJ576867.1
Chromobacterium violaceum ATCC 12472	AE016825.1
Chromobacterium piscinae strain LMG 3947	NR_114953.1
Chromobacterium sp. strain 56AF	MG041385.1
Chromobacterium rhizoryzae strain LAM1188	NR_152068.1
Chromobacterium alkanivorans strain IITR-71	NR_153735.1
Chromobacterium haemolyticum strain H5244	MUKV01000078.1
Chromobacterium haemolyticum strain H3973	MUKT01000095.1
Chromobacterium haemolyticum strain T124	JRFR01000058.1
Chromobacterium sp. LK11	LDUR01000038.1
Chromobacterium violaceum strain ATCC 315322	PKBZ01000001.1
Chromobacterium sp. F49	LQNP01000027.1
Chromobacterium violaceum strain CV017	LKIW01000119.1
Chromobacterium subtsugae strain PRAA4-1	JYKA01000038.1
Chromobacterium subtsugae strain F49	JWJN01000034.1
Chromobacterium piscinae strain ND17	JTGE01000086.1
Chromobacterium sp. MWU14-2602	PQWB01000082.1
Chromobacterium violaceum strain GHPS1	NHOO01000041.1
Chromobacterium violaceum strain H5525	MUKX01000058.1
Chromobacterium violaceum strain H5524	MUKW01000048.1
Chromobacterium violaceum strain 16-419B	MUKR01000045.1
Chromobacterium violaceum strain 16-419A	MUKQ01000045.1
Chromobacterium violaceum strain GN5	JWPW01000031.1
Chromobacterium violaceum strain 16-454	MUKS01000046.1
Chromobacterium violaceum strain LK30	LDUX01000035.1
Chromobacterium violaceum strain LK17	LDUU01000022.1
Chromobacterium sp. LK1	LDUI01000072.1
Chromobacterium subtsugae strain MWU12-2387	MQZZ01000079.1
Chromobacterium pseudoviolaceum strain LMG 3953	MQZX01000025.1
Chromobacterium violaceum strain LK15	LDUT01000054.1

Chromobacterium violaceum strain LK6	LDUM01000020.1
Chromobacterium haemolyticum strain H4137	MUKU01000082.1
Chromobacterium violaceum strain L1B51	JYGI01000011.1
Chromobacterium sphagni strain IIBBL 14B-1	MKCT01000094.1
Chromobacterium sphagni strain IIBBL 37-2	MKCS01000001.1

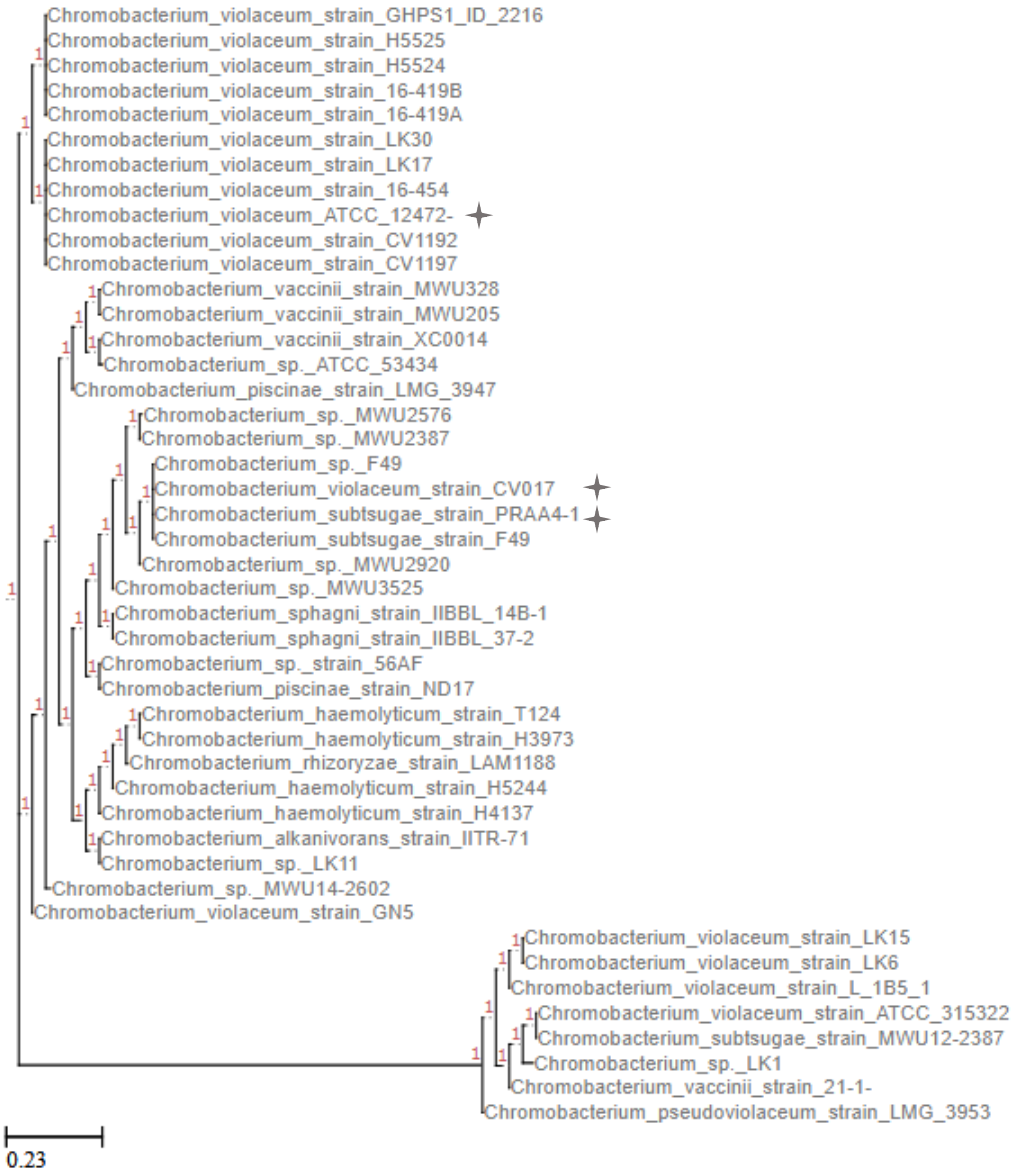
Results

The resulting assembly of *C. violaceum* CV017 contigs consists of 211 scaffolds with an *N50* scaffold size of 40,489-bp [10]. The total length of the chromosome is 4,774,638-bp and read coverage of the scaffolds is around 1,250×. The G+C content of the sequence is 64.5%. The assembly was annotated using NCBI PGAP (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), identifying 4,178 potential CDS (coding DNA sequences) on 168/211 scaffolds, and assigning a putative function to 2,559 (61%) CDS—the remainder are classified as hypothetical proteins. We additionally identified 1 noncoding RNA (ncRNA), 105 tRNA, 46 complete rRNA genes, and 3 clustered regularly interspaced short palindromic repeat (CRISPR) arrays.

One of the features that makes CV017 unique from *C. violaceum* ATCC31532 is a transposon that increases violacein expression [3]. We searched the CV017 draft genome to identify the transposon, previously described as a mercury-resistant mini-Tn5, and we found four mercury resistance genes (*merBAPT*) flanked by the conserved 19-bp Tn5 terminal ends [10]. This sequence resembles the mini-Tn5 Hg^r from plasmid pUT/Hg [11]. This putative transposon element is located 66-bp upstream of the predicted ATG start site of a CDS located between 11,559 and 11,975-bp on scaffold 184, a homolog of *vioS* in ATCC 12472 [12]. VioS is responsible for repressing violacein, protease and chitinolytic activities in *C. violaceum* ATCC31532

Based on current species delineation standards, two strains are said to be of the same species if they share greater than 70% DNA-DNA hybridization and 98% 16S rRNA similarity [13]. Using IGV to align the genomes of *C. violaceum* CV017 and *C. violaceum* ATCC 12472, we found that there is only a 65% sequence similarity. Since *C. violaceum* CV017 was not closely related to *C. violaceum* ATCC 12472, we constructed a dendrogram using 16S RNA of the 45 species with assemblies in NCBI. Based on neighbor-joining and maximum-parsimony, *C. violaceum* CV017 was grouped with other strains of *C. subtsugae* (Figure 1). Using IGV to align the genome of *C. violaceum* CV017 and *C. subtsugae* PRAA4-1, we found that there is PRAA4-1 share 98% sequence identity suggesting that *C. violaceum* CV017 is a member of the *C. subtsugae* species.

Figure 1. 16S rRNA Dendrogram of the genus *Chromobacterium*



The 16S rRNA from *C. violaceum* CV017 (GenBank accession no. LKIW01000119.1) was used to construct a phylogenetic tree of 45 draft or complete genomes. Based on neighbor-joining and supported by maximum-likelihood and maximum-parsimony, *C. violaceum* CV017 was found to group with *C. subtsugae* not *C. violaceum* ATCC 12472, .

A comparison of *C. subtsugae* strain PRAA4-1 and *C. violaceum* ATCC 12472 identified a few key genes that are unique to or absent in *C. subtsugae.* This list includes: the presence of gene cluster MY55_RS12975–13045, the lack of the *pgaABCD* operon; lack of CV_RS01430-01455, and the absence of Cpi-2 (pathogenicity island) [14]. To determine if *C. violaceum*

CV017 is really a member of the *C. subtsugae* species, we used BLAST to identify the presence or absence of the genes from this list in *C. violaceum* CV017. We found that CV017 also contains homologs for genes MY55_RS12975-13045, lacks the *pgaABCD* operon, and lacks the pathogenicity island Cpi-2, consistent with *C. subtsugae*. In pathogenicity island Cpi-1, *C. subtsugae* PRAA4-1 is also missing two hypothetical proteins when compared to ATCC 12472 [15]. Interestingly, CV017 is missing those two hypothetical proteins and two additional genes (CV_RS12855 and CV_RS12860) from Cpi-1 that encode two secretory proteins associated with virulence. In Chapter III, we identified an antibiotic efflux pump, *cdeABoprM.* We used BLAST to search for homologs of this efflux pumps in the *Chromobacterium* genus. Interestingly, *cdeABoprM* is unique to *C. subtsugae* strains further supporting our hypothesis that CV017 is a member of the *C. subtsugae* species.

The metabolic capabilities of CV017 are unknown but would provide further evidence of their membership to the *C. subtsugae* species. Unlike *C. violaceum* ATCC12472, *C. subtsugae* is unable to reduce nitrate and is oxidase positive. We performed a literature search to compare the metabolic and physical characteristics of a representative strain in each of the 11 species of *Chromobacterium* (Table 2). All species are similar in cell size and have one flagellum. Genome sizes ranges from 4.09 to 5.03Mbp with the average genome size of 4.74Mbp. All species that have been tested for hemolysis are able to lyse human red blood cells. Nitrate reduction, glucose fermentation, oxidase activity, and catalase activity all vary by species and could be used to differentiate speciation if genome sequencing is not possible.

Table 2. Summary of physical and metabolic characteristics of *Chromobacterium* species

Species/strain with published genome	Genome Size (MkB)	AHL	Violacein	Hemolysis	Glucose Fermentation	Nitrate reduction	Catalase	Oxidase	References
C.violaceum ATCC 12472	4.75	C10-HSL	Yes	Yes	Yes	Yes	Positive	Negative	(Brazilian National Genome Project, 2003)
C. violaceum CV017	4.77	C6-HSL	Yes	Yes	Yes	-	-	-	(Wang, Hinshaw, Macdonald, & Chandler, 2016) (Rekha, Young, & Arun, 2011)
C. subtsugae PRAA4-1	4.81	C6-HSL	Yes	Yes	Yes	No	Positive	Positive	(Rekha et al., 2011)
C. aquaticum CC-SEYA-1	5.00	C8-HSL	No	Yes	No	Yes	Negative	Negative	(Rekha et al., 2011; Soby, 2017a; Young et al., 2008)
C. vaccinii 21-1	5.04	-	Yes	-	No	Yes	Positive	Positive	(Soby, Gadagkar, Contreras, & Caruso, 2013)
C. piscinae ND17	4.09	-	Yes	-	No	Yes	Negative	Positive	(Kampfer, Busse, & Scholz, 2009)
C. haemolyticum DSM 19808	5.03	-	No	Yes	Yes	Yes	Positive	Positive	(Han, Han, & Segal, 2008)
C. pseudoviolaceum LMG 3953	4.66	-	Yes	-	No	Yes	Negative	Positive	(Soby, 2017b)
C. rhizoryzae LAM1188	-	-	No	-	Yes	Yes	Positive	Negative	(Zhou et al., 2016)
C. alkanivorans	-	-	No	-	Yes	No	Positive	Negative	(Bajaj et al., 2016)
C. amazonense	4.57	-	Yes	-	Yes	No	Negative	Positive	(Menezes et al., 2015)
C. sphagni IIBBL 14B-1	4.74	-	Yes	-	-	-	-	-	(Blackburn et al., 2017)

The table lists characteristics for each of the 11 species plus *C. violaceum* CV017 using the identified strain as the reference for each species. Every genome except for *C. alkanivorans* and *C. rhizoryzae* can be found in the NCBI assembly database.

Discussion

We were initially surprised by the lack of sequence identity between ATCC 12472 and CV017 since they were both classified as the same species. The overall length of the chromosome (4.8-kb) and %GC (64.5%) is consistent with most *Chromobacterium* species including *C. violaceum* and *C. subtsugae* [10]. Comparison of the CV017 scaffolds to the genome strain ATCC 12472 using MUMmer revealed that 64.4% of the bases in ATCC 12472 were covered by assembled scaffolds [16]. Outside of these regions, nucleotide conservation between the two strains appears to be modest. CV017 and *C. subtsugae* have the same AHL signaling molecule (N-hexanoyl-homoserine lactone), a subtsugae specific secondary metabolite,

and the *cdeABoprM* efflux pump. Additionally, CV017 lacks many of the genes found in ATCC 12472 including: *pga* operon, Cpi-2, and four genes found in Cpi-1. Our results based on sequence homology and phenotypic characterization strongly recommend the reclassification of *C. violaceum* CV017 as *C. subtsugae* CV017. For the sake of clarity throughout the rest of the dissertation, we will be referring to *C. violaceum* CV017 as *Chromobacterium* sp. CV017.

The transposon insert that causes an increase in violacein is located upstream of a homolog of CV_1055. Interestingly, in another study nine transposon mutants of the parental strain ATCC 31532 that had a similar phenotype as CV017 (enhanced purple pigmentation) also mapped to a homolog of CV_1055 [17]. Likely, the mini-Tn5 in CV017 is inserted into the promoter region and disrupts expression of the CV_1055 homolog. The function and structure of this protein is unknown. Homologs to CV_1055 can be found in every species that is known to produce violacein, so it is most likely a regulator of violacein.

Understanding the phylogenetic relationship and unique metabolic characteristics of each species of *Chromobacterium* is important for establishing or updating biosafety guidelines for the new species of *Chromobacterium*. Many soil isolates of *Chromobacterium* are erroneously given the name of *C. violaceum* solely for their ability to produce violacein. However, all but four species of *Chromobacterium* produce violacein leading to many strains being incorrectly identified. This also has implications for the research of *Chromobacterium* as an emerging pathogen or for industrial purposes. Currently, most species of *Chromobacterium* are biosafety level 2 despite only *C. violaceum* and *C. haemolyticum* being associated with disease. Only the secondary metabolites from *C. subtsugae* and *C. violaceum* have been studied in detail leading to the identification of novel antibiotics, antitumor medications, an insecticide, and patent for

mercury free gold purification [6]. Changing the safety level will allow for easier access to study the industrial and medical significance of new secondary metabolites found in these strains.

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CHAPTER III:

Quorum-sensing control of antibiotic resistance stabilizes cooperation in *Chromobacterium* sp. CV017

Abstract

Many Proteobacteria use quorum sensing to regulate production of public goods, such as antimicrobials and proteases, that are shared among members of a community. Public goods are vulnerable to exploitation by cheaters, such as quorum sensing-defective mutants. Quorum sensing-regulated private goods, goods that benefit only producing cells, can prevent the emergence of cheaters under certain growth conditions. Previously, we developed a laboratory co-culture model to investigate the importance of quorum-regulated antimicrobials during interspecies competition. In our model, *Burkholderia thailandensis* and *Chromobacterium* sp. CV017 each use quorum sensing-controlled antimicrobials to inhibit the other species' growth. Here, we show that *Chromobacterium* sp. CV017 uses quorum sensing to increase resistance to bactobolin, a *B. thailandensis* antibiotic, by increasing transcription of a putative antibiotic efflux pump. We demonstrate conditions where *Chromobacterium* sp. CV017 quorum-defective cheaters emerge and show that in these conditions, bactobolin restrains cheaters. We also demonstrate that bactobolin restrains quorum-defective mutants in our co-culture model, and the increase in antimicrobial-producing cooperators drives the *Chromobacterium* sp. CV017 population to become more competitive. Our results describe a mechanism of cheater restraint involving quorum-control of efflux pumps and demonstrate that interspecies competition can reinforce cooperative behaviors by placing constraints on quorum sensing-defective mutants.

Introduction

Many Proteobacteria use acyl-homoserine lactone (AHL) mediated quorum-sensing to regulate gene expression in a cell density-dependent manner [1-3]. AHLs are produced by LuxI-family synthases and when they reach a sufficient concentration, the AHLs interact with cytoplasmic LuxR-family signal receptors to affect changes in gene transcription. Many quorum-controlled genes encode factors, such as extracellular proteases, that can be shared among members of the population and constitute public goods [4]. Such public goods are cheatable by individuals that benefit without paying any costs of production [5]. In some conditions the cheaters can ultimately cause a population crash [6], presenting a significant threat to cooperation. Yet quorum-sensing mutants are relatively rare in some natural microbial populations [7, 8], suggesting there might be mechanisms to restrain cheating in certain contexts. One such mechanism relies on the quorum sensing-dependent linkage of private goods, such as cell-localized enzymes, with public goods. These private goods benefit only producing members of the population and have been shown to restrain cheating in some AHL-based quorum-sensing systems [6].

Many bacteria use quorum sensing to regulate the production of antimicrobials, a type of public good. For example, quorum sensing regulates the production of phenazine in *Pseudomonas chlororaphis* [9] and bactobolin antibiotic in *Burkholderia thailandensis* [10, 11]. Quorum-controlled antimicrobials are thought to be important for interspecies competition [12-14]. Previously, we developed a laboratory dual-species model with the soil saprophytes *Burkholderia thailandensis* and *Chromobacterium* sp. CV017 to investigate the relationship between quorum sensing and interspecies competition [12]. The *B. thailandensis* bactobolin antibiotic targets the 50S ribosome and has activity against a range of bacterial species including

Chromobacterium sp. CV017 [11, 15, 16]. Bactobolin production is regulated by one of the three *B. thailandensis* LuxR-LuxI-type quorum-sensing pairs, BtaR2-BtaI2, that senses and produces the signals 3-hydroxy-octanoyl-homoserine lactone and 3-hydroxy-decanoyl-homoserine lactone [10]. In our dual-species model, *B. thailandensis* competitiveness relies upon the production of the bactobolin antibiotic and also BtaR2 [12]. Likewise, *Chromobacterium* sp. CV017 uses quorum sensing to control production of as-yet unknown secreted antimicrobials that are important to compete with *B. thailandensis.* In *Chromobacterium* sp. CV017, there is a single LuxR-LuxI pair, the CviR-CviI quorum-sensing system. The CviR-CviI system produces and responds to hexanoyl-homoserine lactone (C_6-HSL) [17]. This system controls production of a purple pigment, violacein. Although violacein has broad-spectrum antimicrobial activity [18], the violacein biosynthetic genes were previously shown to be dispensable for competition in our dual-species model [12].

We are interested in understanding how quorum sensing benefits bacteria in natural microbial communities, and how interspecies competition might shape the evolution of quorum-sensing systems. Here we show that *Chromobacterium* sp. CV017 quorum sensing promotes resistance to bactobolin and several other antimicrobials through a CviR-dependent putative multidrug efflux pump. Because multidrug efflux pumps are cell-localized they might be considered a private good [19]. Here we show antibiotics restrain quorum sensing-defective mutants during serial passaging experiments and in direct competition with *B. thailandensis*. Such antibiotic-dependent selection might contribute to the maintenance of quorum sensing-dependent behaviors important for competition, such as antimicrobial production. Our results provide a possible mechanism controlling quorum-defective cheater variants in natural microbial populations where interspecies interactions are common.

Materials and Methods

Growth media, reagents and bacterial strains. Bacteria were grown in Luria-Bertani broth containing morpholinepropanesulfonic acid (50 mM; pH 7)(MOPS-LB), or in M9 minimal medium with 1% (wt/vol) of sodium caseinate (casein broth), or on LB with 1.5% (wt/vol) agar or skim milk agar (1/4-strength LB broth with 4% [wt/vol] skim milk and 1.5% [wt/vol] agar). All broth cultures were grown at 30°C with shaking at 230 r.p.m except *Escherichia coli*, which was at 37°C. Growth on solid medium was at 30°C for *Chromobacterium* sp. CV017 or 37°C for *B. thailandensis* and for *Chromobacterium* sp. CV017-*E. coli* matings. Growth in liquid cultures was with shaking at 30°C in 18-mm test tubes. Synthetic C_6-HSL was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in acidified ethyl acetate with glacial acetic acid (0.1 ml L^{-1}). Dissolved C_6-HSL was added to an empty sterile conical tube and dried by evaporation prior to the addition of LB agar that had been melted and cooled to 55°C. As a source of bactobolin, either filter-sterilized *B. thailandensis* culture fluid or purified bactobolin A was used (see Supplementary Information for additional information). Bactobolin A was suspended in sterile water prior to use in experiments. For casein evolution experiments we used tetracycline at 1 μg ml^{-1}, bactobolin at 2 μg ml^{-1} and gentamicin at 12 μg ml^{-1}. For strain constructions we used gentamicin at 50 μg ml^{-1} (*Chromobacterium* sp. CV017) or 15 μg ml^{-1} (*E. coli*). For selection from dual-species cultures, we used gentamicin at 100 μg ml^{-1} (*B. thailandensis*) and trimethoprim at 100 μg ml^{-1} (*Chromobacterium* sp. CV017).

Bacterial strains are listed in Table S1. *B. thailandensis* strains were E264 (wild type) or BD20, a bactobolin-deficient derivative of E264 [20]. *Chromobacterium* sp. CV017 strains are derivatives of strain ATCC31532 [21]. *Chromobacterium* sp. CV017 (referred to as wild type) has a transposon insertion in gene CV_RS05185 causing overexpression of violacein [22, 23],

CV026 is a derivative of CV017 with a second transposon insertion in the *cviI* gene [17], and CV026R is a derivative of CV026 with an in-frame deletion of the *cviR* gene [12]. The *Chromobacterium* sp. CV017 CdeAB-OprM- and CdeR H127Y mutants were constructed using homologous recombination as previously described [12] with synthetic gene fragments (IDT) cloned into the pEX18Gm-derived delivery plasmid [24]. For fragment cloning we used SphI-XbaI (for *ΔcdeAB-oprM*) or HindIII-XbaI (for CdeR H127Y). The *ΔcdeAB-oprM* construct was made with ~500-bp DNA flanking *cdeA* and *oprM* resulting in a deletion of all but the first eight codons of *cdeA* and the last thirteen codons of *oprM*. The CdeR H127Y construct was made using a ~1000-bp DNA fragment encoding *cdeR* with a missense C379T mutation and ~100-bp DNA flanking *cdeR*. All candidate mutants were screened by PCR and susceptibility testing to gentamicin, the antibiotic resistance marker on the pEX18Gm delivery plasmid. All strains and PCR-generated plasmids were verified by PCR amplification and sequencing.

Antibiotic sensitivity. To determine antibiotic sensitivity, stationary-phase broth cultures were suspended to an optical density at 600 nm (OD_{600}) of 1.0 and this suspension was serially diluted in 10-fold increments in LB broth. 10 µl aliquots of these successive dilutions were spotted onto LB agar plates containing antimicrobials at the concentration indicated or in a series of concentrations to determine the minimum inhibitory concentration (MIC). The agar plates were incubated at 30°C for 24 h and an additional 24 h at room temperature. The MIC was defined as the lowest concentration of antimicrobial in the plate that prevented visible growth of cells from the 10-3 dilution (~104 cells). *B. thailandensis* culture fluid was tested in 0.1% increments over a range from 0% to 7%, tetracycline was tested in 0.5 µg increments over a range from 0 to 2 µg per ml, ethidium bromide was tested in 10 µg increments in a range from 20 to 150 µg per ml, and gentamicin was tested in 0.1 µg increments in a range from 1 to 10 µg per ml.

Droplet digital PCR. RNA was harvested from stationary-phase *Chromobacterium* sp. CV017 cells (OD_{600} of 4) using methods described previously [25]. Droplet digital PCR was performed on Bio-Rad's QX200 Droplet Digital PCR (ddPCR) System using Eva Green Supermix. Each reaction used 1 ng μl^{-1} of cDNA template, 0.25 μM primer, 10 μl Eva Green Supermix, and 8 μl in a 20-μl volume. After generating 40 μl of oil droplets, 40 rounds of PCR were conducted using the following cycling conditions: 94°C for 20sec, 58°C for 20sec, and 72°C for 20sec. Absolute transcript levels were determined using the Bio-Rad QuantaSoft Software. In all cases a no template control was run with no detectable transcripts. The gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was used as a reference gene and the results are reported as the calculated transcript amount of a given gene per calculated *gapdh* transcript.

Casein evolution experiments. To inoculate *Chromobacterium* sp. CV017 casein evolution experiments, a starter culture was prepared by diluting stationary-phase cells from an LB broth culture to an OD_{600} of 0.05 in casein broth and growing this for an additional 24 h. To start the experiment, 40 μl from this starter culture was transferred to 4 ml fresh casein broth and at 24 h intervals 40 μl was transferred to fresh casein broth in a new tube. Tetracycline and bactobolin were added to the culture medium every day. Gentamicin was added every other day for the first seven days and every day after that for the rest of the experiment. Colony forming units (CFUs) were determined by spread-plating 10-fold dilutions of the 24 h culture onto LB agar plates. Cheater abundance was determined by the proportion of colonies identified as cheaters compared with the rest of the population. Cheaters were identified based on complete loss of purple pigmentation due to abolished production of the quorum-dependent purple pigment violacein, and their inability to form a zone of clearing on skim milk -agar plates (skim milk clearing was described previously for *P. aeruginosa* [26, 27]). Although intermediate-phenotype variants were

occasionally observed (partial pigmentation and protease production), only those variants with complete loss of each phenotype (similar to the quorum-sensing defective mutants) were counted as cheaters. As described in the text, a subset of cheaters was also verified by sequencing the *cviR* gene.

Dual-species experiments. Dual-species experiments were conducted in 1 ml MOPS-LB broth in 18 mm test tubes. The inoculum was from logarithmic-stage pure cultures of *B. thailandensis* and *Chromobacterium* sp. CV017. The initial OD_{600} in the dual-species culture was 0.05 (2-4 x 107 cells per ml) for *B. thailandensis* and 0.005 (2-4 x 106 cells per ml) for *Chromobacterium* sp. CV017. When indicated, *Chromobacterium* sp. CV017 wild type and CviIR- mutant cells were combined at a 1:1 ratio just prior to mixing with *B. thailandensis*. Colony forming units of each species were determined by using differential antibiotic selection on LB agar plates. *B. thailandensis* was selected with gentamicin and *Chromobacterium* sp. CV017 was selected with trimethoprim. Wild type and the CviIR- mutant were differentiated by color as described above.

Results

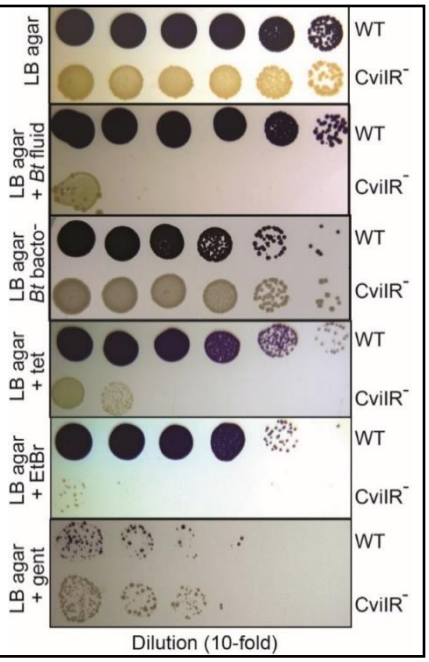
Chromobacterium sp. CV017 *quorum sensing is important for antimicrobial resistance.*

In transcriptomics studies of saprophytic Proteobacteria, quorum sensing commonly activates expression of genes predicted to be responsible for antibiotic export [28-31]. Because some efflux pumps increase antimicrobial resistance, this finding suggests there might be a connection between quorum sensing and antimicrobial resistance. Using our *B. thailandensis*-*Chromobacterium* sp. CV017 laboratory model as a starting point, we sought to test the hypothesis that *Chromobacterium* sp. CV017 quorum sensing might increase resistance to the *B. thailandensis* bactobolin antibiotic. To test this hypothesis, we supplemented Luria-Bertani (LB) agar plates with *B. thailandensis* culture fluid and compared the growth of *Chromobacterium* sp.

CV017 wild type and two quorum sensing-deficient mutants, an AHL synthase mutant (CviI-) and an AHL synthase and receptor double mutant (CviIR-) by spotting 10-fold dilutions from stationary-phase cultures onto the plates (Fig. 1 and Table 1).

Figure 1 Quorum sensing increases *Chromobacterium* sp. CV017 resistance to antimicrobials

Growth of *Chromobacterium* sp. CV017 wild type (WT) or the AHL synthase, receptor double mutant (CviIR-). The WT is purple due to production of the quorum sensing-dependent purple pigment violacein. Stationary-phase cultures were prepared in 10-fold serial dilutions in Luria-Bertani broth, and 10µl of each successive dilution was spotted from left to right onto LB agar alone (top panel), LB agar containing 0.5% *B. thailandensis* culture fluid (as a source of bactobolin, see Methods), 1% culture fluid from bactobolin-deficient *B. thailandensis* strain (BD20), 0.4µg per ml gentamicin (gent). Photos were taken after 24h growth at 30ºC and an additional 24h growth at room temperature.



We determined that wild-type *Chromobacterium* sp. CV017 was more resistant to *B. thailandensis* culture fluid than either of the quorum-sensing mutants, with a minimum inhibitory concentration (MIC) of 1.6% compared to 0.5% or 0.6% for CviIR- and CviI-, respectively. We could rescue resistance to the AHL synthase mutant by supplementing the LB agar plate with synthetic C_6-HSL, the AHL specific to CviR (Table 1). The wild type and each of the quorum-sensing mutants grew identically on LB agar with or without supplementation with 3% cell-free fluid from a *B. thailandensis* bactobolin-deficient mutant (data not shown). These results indicated *Chromobacterium* sp. CV017 quorum sensing is important for bactobolin resistance.

Table 1. Antimicrobial susceptibility of *Chromobacterium* sp. CV017 strains

Cv strain	MIC[a]			
	Bt fluid[b] (%)	Tet (μg ml^{-1})	EtBr (μg ml^{-1})	Gent (μg ml^{-1})
Wild type	1.6 (0.1)	1.2	>150	9.3 (1.2)
CviIR$^-$	0.6*	0.4 (0.1)*	60	9.3 (1.2)
CviI$^-$	0.5 (0.1)*	0.4 (0.1)*	60	7.7 (2.1)
CviI$^-$ + AHLs	1.7	1.0*	90	ND[c]
CviIR$^-$ BR[d]	3.3 (0.2)*	2.5*	>150	10
CviI$^-$ CdeR H127Y	3.4 (0.1)*	2.5*	>150	10
CdeAB-OprM$^-$	0.3*	0.1*	30	8.7 (1.2)
CviIR$^-$ CdeAB-OprM$^-$	0.3*	0.1*	ND	8.7 (1.2)

[a]The minimum inhibitory concentration (MIC) of *B. thailandensis* culture fluid (*Bt* fluid), tetracycline (Tet), ethidium bromide (EtBr) or gentamicin (Gent) was determined by spotting ~10^4 *Chromobacterium* sp. CV017 stationary-phase cells of the indicated strain onto LB agar containing a range of antimicrobial concentrations as described in Materials and Methods. Results are the average of three independent experiments and the standard deviation is in parentheses when it was not zero. Statistical analysis by *t* test compared with wild type under each treatment condition: *, $p \leq 0.0001$. For ethidium bromide, no *p*-value was given because there was no variation (standard error is 0). For gentamicin, none of the averages significantly differed from wild type.
[b]Results are from a single preparation of *B. thailandensis* fluid. Results with other preparations were similar. There were no observed growth defects in identical experiments with 3% culture fluid from a *B. thailandensis* bactobolin-deficient mutant.
[c]ND, not determined.
[d]CviIR$^-$ BR refers to spontaneous bactobolin-resistant isolates. Two isolates were tested, and results were identical.

We also evaluated the importance of quorum sensing for resistance to other antimicrobials. We tested growth of our *Chromobacterium* sp. CV017 strains on plates supplemented with the antimicrobials tetracycline, gentamicin or ethidium bromide. Our results showed that quorum sensing increases *Chromobacterium* sp. CV017 resistance to tetracycline and ethidium bromide but had no effect on gentamicin susceptibility (Fig. 1 and Table 1). Altogether our results support the conclusion that *Chromobacterium* sp. CV017 quorum sensing

regulates resistance to several antimicrobials, including bactobolin. Our results are consistent with the idea that the quorum sensing-controlled resistance factor is an efflux pump. Antibiotic efflux pumps commonly confer resistance to a subset of antibiotics with unrelated mechanisms of action, and they are frequently associated with ethidium bromide resistance [32].

Quorum sensing controls antimicrobial resistance through a putative efflux pump.

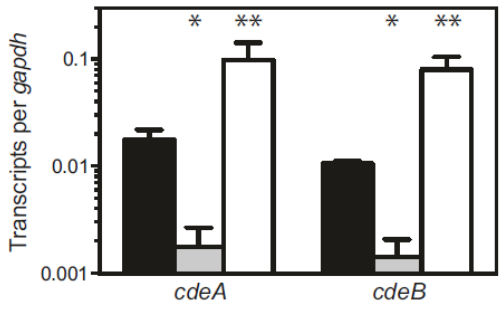
To identify the genetic factors responsible for quorum sensing-dependent antibiotic resistance, we isolated spontaneous bactobolin-resistant mutants and defined the mutations leading to resistance using a whole-genome sequencing approach. We chose this approach because antibiotic resistance mechanisms are not well understood in *Chromobacterium* sp. CV017 [23], and because initial genetic mutations that increase resistance often occur through efflux pumps, providing a relatively simple approach to identify efflux pumps with specificity for bactobolin [33, 34]. We isolated bactobolin-resistant mutants by spread-plating about 108 cells of the sensitive *Chromobacterium* sp. CV017 AHL synthase, receptor double mutant onto LB agar supplemented with ~0.6% *B. thailandensis* culture fluid, the minimal concentration needed to inhibit growth of this strain. Colonies arose after two days at 30ºC, and two mutants were isolated by streaking onto LB agar with 0.6% *B. thailandensis* culture fluid. We used a previously-described approach [15], detailed in the Supplementary Information, to isolate two bactobolin-resistant variants in the otherwise sensitive CviIR- mutant. [12] We assessed the antimicrobial susceptibility profiles of each variant relative to the parent strain. Results were identical for each variant and are described in Table 1 (strain CviIR- BR). Both variants were more resistant than the parent to bactobolin, tetracycline and ethidium bromide, but there was no difference in resistance to gentamicin, (Table 1), similar to the quorum sensing-dependent antimicrobial resistance profile described above. To define the mutations involved in bactobolin

resistance, we sequenced the genomes of our bactobolin-resistant variants using an Illumina platform (see Supplementary Information). The variants both had 11 identical single nucleotide polymorphisms (SNPs) when compared to the sequenced strain CV017 (Wang et al 2016). Two of these were in putative antibiotic resistance genes. When we PCR-amplified and sequenced the two SNP sites, only one was mutated in both variants relative to CV017. This was a C379T mutation in a predicted TetR family transcriptional regulator CV017_15440, coding for the mutation H127Y. CV017_15440 is upstream and divergent to a 3-gene cluster with homology to resistance nodulation division (RND) efflux pumps in other Proteobacteria, such as *mexAB-oprM* from *Pseudomonas aeruginosa* [35]. We named the TetR-family gene *cdeR* and the downstream genes *cdeAB-oprM* (*Chromobacterium* drug exporter).

We sought to determine whether the H127Y mutation in CdeR increased bactobolin resistance and if *cdeAB-oprM* is responsible for the quorum-dependent antibiotic resistance phenotype we had observed. We introduced the allele encoding CdeR H127Y into the genome of the *Chromobacterium* sp. CV017 AHL synthase mutant at the native *cdeR* locus. The engineered CdeR H127Y mutant demonstrated the same antimicrobial susceptibility profile as the bactobolin-resistant variants (Table 1), confirming the increased antimicrobial resistance is due to the CdeR H127Y mutation. We subsequently deleted the *cdeAB-oprM* genes in the wild type and quorum sensing-defective CviIR- mutant. Deletion of *cdeAB-oprM* resulted in a ~6-fold decrease in bactobolin resistance relative to the wild type and similar decreases in resistance for tetracycline and ethidium bromide (Table 1). Importantly, the loss of quorum sensing did not further decrease antibiotic sensitivity of the CdeAB-OprM-deficient mutant (Table 1, compare the CdeAB-OprM- mutant to the CdeAB-OprM-, CviIR- mutant), supporting the idea that quorum sensing-dependent antibiotic resistance occurs through the CdeAB-OprM putative efflux

pump. In support of this idea, both *cdeA* and *cdeB* transcripts were ~15-fold higher in wild-type cells compared with identically grown CviIR- mutant cells (Fig. 2), demonstrating that quorum sensing activates transcription of these genes. The genes were also activated in one of the spontaneous bactobolin-resistant variants, suggesting that increased resistance in this strain might have been due to increased transcription of the *cdeAB-oprM* genes.

Figure 2 Quorum sensing induction of *cdeA* and *cdeB* transcription



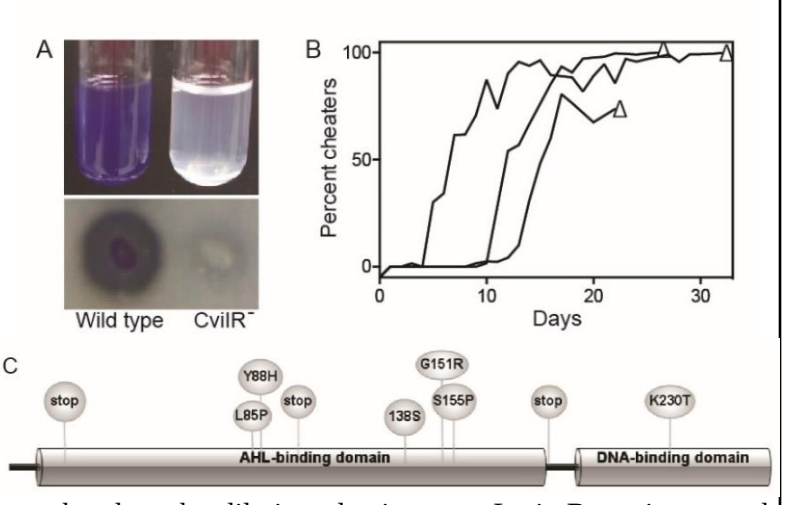
Quantitative digital PCR was used to quantify *cdeA* and *cdeB* transcripts in cells from stationary-phase cultures (optical density of 600nm [OD600] of 4). Shown are *cdeA* and *cdeB* transcripts from wild type (WT, black bars), and the AHL synthase, receptor double mutant (CviIR-, gray bars), and the bactobolin-resistant double mutant (CviIR- BR, white bars). In all cases, results were normalized to the housekeeping gene, glyceraldehyde-3-phosphatase dehydrogenase (*gapdh).* The values represent the average of three independent experiments and the error bars represent the standard deviation. Statistical analysis by *t*-test compared with wild type: *, $p \leq 0.005$, **, $p \leq 0.05$.

Antibiotics suppress the emergence of Chromobacterium sp. CV017 *cheaters during passage in casein broth*

Because CdeAB-OprM is a predicted efflux pump, and efflux pumps are membrane-localized, we hypothesized that CdeAB-OprM might be a type of quorum-controlled private good that can restrain cheating during growth with antibiotics. Previous studies of *Pseudomonas aeruginosa* and *Vibrio cholerae* demonstrated the necessity of secreted quorum sensing-dependent proteases for growth on casein as the sole carbon and energy source and, after prolonged passaging in these conditions, quorum sensing-deficient cheaters emerge [26, 27, 36]. Initially, we assessed the ability of wild type and a quorum sensing-deficient (CviIR-) *Chromobacterium* sp. CV017 mutant to grow in minimal casein broth and proteolyze skim milk protein (Fig. 3A). Wild type, but not the CviIR- mutant could grow on casein and form a zone of

clearing on skim milk agar (Fig. 3A), consistent with the conclusion that a quorum-dependent protease is required for *Chromobacterium* sp. CV017 to grow on casein.

Figure 3 Protein degradation and cheater emergence during *Chromobacterium* sp. CV017 growth on protein



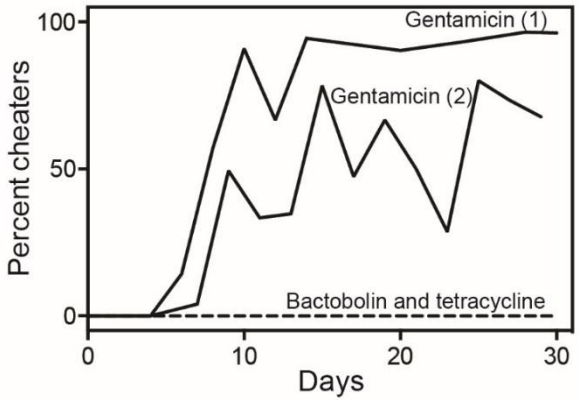
A. Growth of *Chromobacterium* sp. CV017 wild type and the AHL synthase, receptor double mutant (CviIR-) after growth in minimal casein broth or on skim milk agar. Only wild type can hydrolyze the casein in both types of media. **B.** Emergence of cheaters in *Chromobacterium* sp. CV017 populations passaged in casein broth. Cultures were transferred daily, and every 2-3 days, cheater abundance was determined just prior to sub-culture by dilution planting onto Luria-Bertani agar and enumerating the yellow colonies that also did not form a zone of proteolysis when patched onto skim milk agar plates. Each black line represents an independent experiment. In all three experiments, cheaters emerged after 2-10 days and their abundance rose of >80% of the population, followed by termination of the experiment when there was no growth after transfer. **C.** Location of nucleotide mutations in *Chromobacterium* sp. CV017 cheaters mapped to the CviR amino-acid sequence. Each unique amino-acid substitution or early termination is shown the illustration and indicated in Table S2. The AHL- and DNA-binding domains of CviR are indicated.

Having established that *Chromobacterium* sp. CV017 requires quorum sensing to grow on casein, we assessed the emergence of quorum sensing-defective cheaters by passaging a *Chromobacterium* sp. CV017 population every day into fresh casein growth media and spread-plating dilutions of the culture onto LB agar every 2-3 days to enumerate the frequency of cheaters (see Materials and Methods). Cheaters were identified as yellow colonies (due to loss of violacein production) that also did not form a zone of proteolysis when patched onto skim milk agar plates. We also sequenced the quorum-sensing receptor *cviR* in 10 of these putative mutants and in each case, there was a mutation in *cviR* (Table S2 and Fig. 3C). In three independent experiments (shown as three separate black lines in Fig. 3B), cheaters emerged between 2-10 days. At a frequency of >80% cheaters, the culture failed to grow after transferring due to a

population collapse (Fig. 3B, open triangles). The emergence of quorum sensing receptor-mutated cheaters in the population and ultimate population collapse was similar to that previously reported for *P. aeruginosa* [6, 26, 27].

In *P. aeruginosa*, quorum sensing co-regulates the expression of a secreted protease and a cellular enzyme, *nuh*, that promotes adenosine utilization, and growth on adenosine restrains the emergence of cheaters [6]. To test our hypothesis that antibiotics might similarly restrain cheater emergence in *Chromobacterium* sp. CV017, we added tetracycline or purified bactobolin A to our casein growth experiments (Fig. 4). Wild-type cultures could be passaged serially with a maximum antibiotic concentration of either 2 μg ml^{-1} bactobolin A or 0.25 μg ml^{-1} tetracycline, which caused a maximum of a 1-log reduction of the final population yield relative to cultures grown with no antibiotic (Fig. S1). In the presence of either antibiotic, cheaters remained below the limit of detection (Fig. 4, dashed line) and the population yield remained consistent throughout the experiment (Fig. S1). However, it appeared that only antibiotic substrates of CdeAB-OprM were able to restrain cheaters, because cheaters emerged in populations grown with growth-inhibitive concentrations of gentamicin, which is not targeted by quorum sensing or CdeAB-OprM (Table 1). Interestingly, during our experiments, populations grown with gentamicin never crashed as we observed in populations grown in the absence of any antibiotics (Fig. 4). These results might suggest a potentially unmeasurable quorum sensing-dependent effect on gentamicin resistance or an indirect effect of gentamicin, for example through increased selection of mutations that increase cheater tolerance [37].

Figure 4 Antibiotics suppress *Chromobacterium* sp. CV017 cheaters in casein broth.



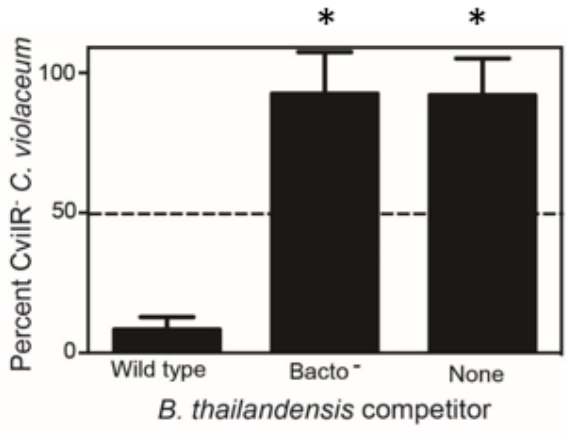
Cultures were grown as described in Fig. 3 except the culture media was supplemented with antibiotics. The solid lines show two independent experiments of populations passaged with 12 μg ml^{-1} gentamicin. The dashed line shows populations passaged with 2 μg ml^{-1} bactobolin A or 0.25 μg ml^{-1} tetracycline (two independent experiments were conducted with each antibiotic and results were identical in all cases). All antibiotic-treated populations were passaged in parallel with populations grown without antibiotics (partially represented in Fig. 3). In all cases, in populations grown without antibiotics cheaters emerged within 2-10 days and reached >80% of the population by the end of the experiment.

Bactobolin restricts growth of quorum sensing-deficient Chromobacterium sp. CV017 *mutants in dual-species cultures*

The sensitivity of *Chromobacterium* sp. CV017 quorum sensing-defective mutants to bactobolin suggests these mutants may be growth-inhibited during direct competition with *B. thailandensis*. To test this hypothesis, we used our genetically engineered *Chromobacterium* sp. CV017 AHL synthase, receptor double mutant (CviIR-). Our approach was a modification of the methods used previously to grow *Chromobacterium* sp. CV017-*B. thailandensis* cultures [12]. In our experiments, *Chromobacterium* sp. CV017 wild type and CviIR- mutant were mixed at a 1:1 ratio prior to combining with *B. thailandensis* (see Materials and Methods and [12]). As controls, we also grew the *Chromobacterium* sp. CV017 mixture without *B. thailandensis* or with a *B. thailandensis* bactobolin-deficient mutant. At the start and end of each experiment we determined the proportion of each *Chromobacterium* sp. CV017 strain by first spread-plating the entire *Chromobacterium* sp. CV017 population onto agar containing trimethoprim to select for *Chromobacterium* sp. CV017 and then enumerating the yellow (CviIR- mutant) colonies as a proportion of the total *Chromobacterium* sp. CV017 population (Fig. 5). During growth with *B.*

thailandensis, the CviIR- mutant decreased from 50% to 17% of the total *Chromobacterium* sp. CV017 population. In contrast, when grown with no *B. thailandensis* or with the bactobolin-defective *B. thailandensis*, the CviIR- mutant population increased from 50% to more than 90% of the total *Chromobacterium* sp. CV017 population. These results show *B. thailandensis*-produced bactobolin selects against CviIR- mutants, supporting the idea that bactobolin stabilizes *Chromobacterium* sp. CV017 quorum sensing during competition.

Figure 5 Competition increases the proportion of wild-type *Chromobacterium* sp. CV017 relative to quorum sensing-defective mutants

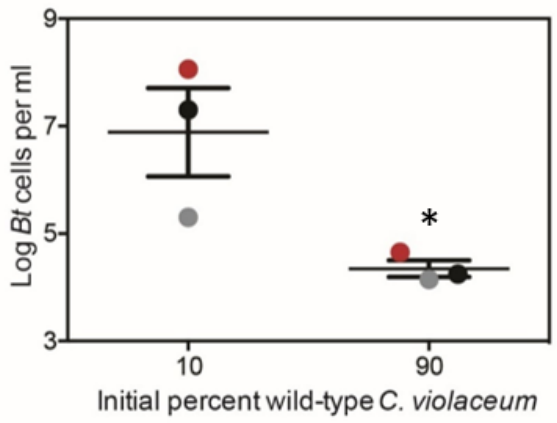


Chromobacterium sp. CV017 wild type or a quorum sensing-deficient mutant (CviIR-) were mixed at a 1:1 ratio then either combined with wild-type *B. thailandensis* or a bactobolin-deficient *B. thailandensis* mutant (Bacto-, strain BD20), or grown with no *B. thailandensis* as described in the text. The percent CviIR- mutants within the *Chromobacterium* sp. CV017 population was determined by counting the yellow colonies as a percent of the total *Chromobacterium* sp. CV017 population isolated on selective agar. The dashed line indicates the initial percent CviIR-. Shown is the mean of four independent experiments and the error represents the standard deviation. Statistical analysis by Mann-Whitney nonparametric test compared to wild type: *, $p = 0.0286$.

Because bactobolin increases the frequency of selects *Chromobacterium* sp. CV017 quorum-sensing cells (Fig. 5), and quorum sensing controls production of antimicrobials [12], we hypothesized that changes in the frequency of *Chromobacterium* sp. CV017 quorum-sensing cells would influence the outcome of competition with *B. thailandensis*. To test this hypothesis, we varied the starting ratio of wild type and CviIR- *Chromobacterium* sp. CV017 cells in our competition experiments with *B. thailandensis*. We used a *Chromobacterium* sp. CV017 starting ratio of 9:1 and 1:9 wild type to CviIR-, similar to the ratios observed at the end of the competition experiments described in Fig. 5. Our results showed that the higher frequency of

wild-type *Chromobacterium* sp. CV017 correlated with lower *B. thailandensis* growth (Fig. 6). Thus, altering the frequency of wild-type *Chromobacterium* sp. CV017 can influence the competitive ability of the *Chromobacterium* sp. CV017 population. Together, our results support the idea that antibiotic stabilization of quorum sensing can increase competitiveness.

Figure 6 *Chromobacterium* sp. CV017 competitiveness correlates with the frequency of wild type relative to quorum sensing-deficient mutants



Chromobacterium sp. CV017 wild type or a quorum sensing-deficient mutant (CviIR-) cells were mixed together at a 1:9 or 9:1 ratio and then combined with wild-type *B. thailandensis* and grown for 24h. The final *B. thailandensis* density was determined by selective plating and colony counts. The solid lines represent the means for each group. The vertical bars represent the standard deviation of each group. Statistical analysis by paired *t*-test compared with wild type: *, p = 0.0468. Paired groups are indicated by color and represent experiments performed in parallel on different days.

Discussion

In many Proteobacteria quorum sensing activates dozens to hundreds of genes, including those coding for secreted or excreted public goods, that are important for cooperation. Cooperating populations are threatened by quorum sensing-deficient cheaters, individuals utilizing the public goods without paying any cost for their production. Cheaters typically have a growth advantage over cooperators and their proliferation may ultimately cause the cooperative behavior to be lost. However, the maintenance of cooperation in natural populations suggests there are diverse mechanisms of cheater control across different bacterial groups. Indeed, several such mechanisms have been described, including kin selection through spatial structure [38, 39], mechanisms that allow cooperators to specifically recognize and interact with other cooperators (*e.g.* greenbeard traits) [40, 41], and pleiotropy, or co-regulation of genes coding for self-

benefiting private activities and genes coding for freely shared public goods [6, 42]. Results of this study demonstrate a type of pleiotropy dependent on antibiotics produced by other species, Cheater control by other species has also been shown to occur through nutrient competition [43], and through selective grazing of cheaters by protists [44, 45]. Our study expands the list of known mechanisms of cheater control and provides additional support of the idea that cheaters can be controlled through interspecies or interkingdom interactions

The results of this study demonstrate cheater control by antibiotics occurs through quorum control of antibiotic resistance through an efflux pump. What factors might contribute to maintenance of quorum-controlled resistance? The ecological or physiological factors that favor quorum-control of efflux pumps are as-yet unknown, however, it is unlikely that cheater control is sufficient for such selection [46]. Efflux pumps are known to have some fitness cost [47] thus using quorum sensing to control efflux pump production might mitigate fitness costs at low density as a means to optimize growth. Quorum sensing also commonly controls secreted toxin production, and quorum-sensing regulation of efflux pumps might serve to prevent self-toxicity. *Chromobacterium* sp. CV017 itself produces several antimicrobials, including the quorum sensing-regulated antibiotic violacein [17, 48]. However, in our experiments, CdeAB-OprM does not appear to prevent self-toxicity, as the quorum-defective variants are not growth-inhibited by the wild-type parent when the two strains are grown in co-culture, in fact, the mutants are more fit than the parent (Figs. 3 and 5).

Quorum sensing-dependent antibiotic resistance might also be important for competing with other strains or species in multispecies communities. Quorum sensing might be one of several systems involved in ‘competition sensing’ [49], providing an indirect measurement of the potential for competitors in the surrounding environment. As the population density increases,

nutrients and space become limited and there is an increased potential for conflicts with neighbors. Thus quorum-sensing systems might coordinate production of several factors, including antibiotic resistance determinants, which promote survival when competition is likely. Quorum sensing might also activate the production of efflux pumps when they directly detect competitors by sensing and responding to their signals through eavesdropping [12]. Broad signal-specificity receptors in some saprophytic species such as *Chromobacterium* sp. CV017 and *P. aeruginosa* might serve in this capacity [17, 50].

There is some evidence suggesting quorum-sensing control of antimicrobial resistance is not limited to *Chromobacterium* sp. CV017. Quorum sensing has also been reported to control resistance to tobramycin in biofilm-grown *P. aeruginosa* [51]. In *Escherichia coli,* a quorum-sensing receptor increases resistance to quinolones and several other antibiotics [52, 53]. Quorum sensing also activates expression of genes predicted to encode multidrug efflux pumps in other Proteobacteria [28-31], which may have important implications in treating infections if these antibiotics contribute to the selection or maintenance of quorum sensing or 'rewiring' of quorum-sensing systems during infections [54].

Our results indicate quorum sensing in *Chromobacterium* sp. CV017 controls antibiotic resistance through the CdeAB-OprM putative efflux pump. The genes encoding CdeAB-OprM share sequence and structural similarity to other resistance nodulation division (RND) efflux pump genes. RND efflux pumps have three proteins, an inner membrane transporter (efflux) protein (CdeB), a periplasmic accessory protein (CdeA) and an outer membrane channel (OprM) (for a review, see ref. [32]. RND efflux pump genes are frequently linked to a TetR-family regulator gene, and in the case of CdeAB-OprM, this appears to be CdeR. TetR-family members typically act as repressors by binding to a site in the promoter of the first efflux pump gene

through a N-terminal DNA-binding domain. De-repression occurs when the C-terminal ligand-binding domain interacts with ligand (*e g.* tetracycline). In many bacteria with RND efflux pumps, antibiotic resistance mutations map to the efflux pump regulator, typically the N-terminal DNA-binding domain, and cause de-repression (reviewed in [32]). The H127Y mutation in CdeR is in the N-terminal domain and might similarly affect CdeR, causing de-repression of *cdeAB-oprM*, although the role of CdeR as a repressor has not been experimentally determined. The hypothesis that CviR directly regulates *cdeR, cdeA,* or both can be tested experimentally, and such studies might provide insight into the mechanism of efflux pump regulation by quorum sensing in *Chromobacterium* sp. CV017 and other bacteria.

Supplemental Information

Materials and Methods

Preparation of bactobolin and bactobolin purification. As a source of bactobolin, we used filtered *B. thailandensis* culture fluid or purified bactobolin A, the most potent of eight bactobolins produced by *B. thailandensis* [55]. *B. thailandensis* culture fluid was prepared as follows: *B. thailandensis* was grown to stationary phase (optical density at 600 nm [OD600] of 8 to 10) in LB broth, and the culture fluid was clarified by centrifugation and filtered through a 0.22-μm pore membrane. Filtered culture fluid was stored at 4°C for up to 1 month prior to use and either used directly for experiments or used to purify bactobolin A as follows using a modified procedure similar to that reported previously [11]. The aqueous culture fluid containing bactobolin was concentrated to a residue by rotovap and then purified by reversed phase chromatography using an automated purification system capable of triggering fraction collection based on the mass signal of bactobolin. Two complementary chromatographic conditions were used. For the first condition, the bolus of dried residue was taken up in DMSO to produce a 90ml sample solution which was injected automatically in 30 preparative runs totaling 6 hours utilizing a 5 μm Waters Atlantis T3 19 x 150 mm column, an aqueous phase of 0.1% formic acid, a flow rate of 20 ml min^{-1}, and a gradient of 10% to 30% acetonitrile over 5 minutes. For the second condition it was necessary to raise the pH of the mobile phase to achieve separation of the few remaining impurities. The same column, flow rate, and fraction triggering were used but the aqueous phase was 10 mM ammonium acetate at pH 6.9 and the gradient was 5% to 50% acetonitrile over 8 minutes. A total of 5 injections of 1ml each in DMSO were performed with this condition. A final cleanup of one injection was performed using the first condition to achieve a final purity of 93.8% (auc @ 214nm) and a recovery of 16.1 mg. All preparative runs were

performed on an Agilent 1200 purification system with HTS PAL autosampler and an Agilent 6120 mass spectrometer.

Isolation and sequencing of bactobolin-resistant Chromobacterium sp. CV017 *mutants.* To isolate bactobolin-resistant *Chromobacterium* sp. CV017 mutants, we used a method similar to that described previously [15]. Briefly, ~$1x10^8$ stationary-phase *Chromobacterium* sp. CV017 strain CV026R cells were spread onto LB agar plates containing, as a source of bactobolin, filter-sterilized *B. thailandensis* culture fluid (from stationary-phase cultures, optical density [OD_{600}] 8-10, at a 0.5% final concentration). Colonies arose after two days at 30°C, and two mutants were isolated by streaking onto LB agar with 0.6% *B. thailandensis* culture fluid. DNA was isolated from bactobolin-resistant clones and used to make sequencing libraries with 300-bp inserts. The libraries were sequenced on an Illumina HiSeq 2500 System in high output mode, generating 100-bp single reads. Raw paired-end reads from the CV017 strain were preprocessed with Scythe (v0.991, https://github.com/vsbuffalo/scythe) and Sickle (v1.200, https://github.com/najoshi/sickle) to improve read quality. Initial mapping of the trimmed reads to the reference strain (ATCC 12472, accession NC_005085.1) revealed modest nucleotide conservation between strains. Thus, trimmed reads were *de novo* assembled using Velvet (v1.2.09, [56]) to build contigs for downstream variant discovery. Using an empirically determined, optimal *k*-mer size of 51, we generated 515 scaffolds with an N50 scaffold size of 18,607-bp and a summed length of 4,655,291-bp. Subsequently, we independently aligned trimmed reads from mutant strains BR1 and BR2 to the CV017 *de novo* genome build using BWA (v0.7.7, [57]), and used SAMtools (v0.1.18, [58, 59]) mpileup method to call variants. This yielded a list of both single nucleotide polymorphisms (SNPs) and insertion-deletion variants differing between each mutant strain and CV017 progenitor. We extracted all

homozygous variants, and used MUMmer (v3.23, [60]) to locate the contigs containing these variants to ATCC 12472, allowing us to annotate variants in conserved sequence regions. Using this procedure, we identified 7 single nucleotide polymorphisms in each of BR1 and BR2 that were genic and nonsynonymous. We subsequently focused on the mutation in *cdeR* because it was in a gene homologous to that identified in a similar previous screen of bactobolin resistant variants in *Burkholderia vietnamiensis* (Chandler and Greenberg, unpublished). The mutation in *cdeR* was confirmed by sequencing PCR-amplified products.

Table S1. Bacterial strains used in this study

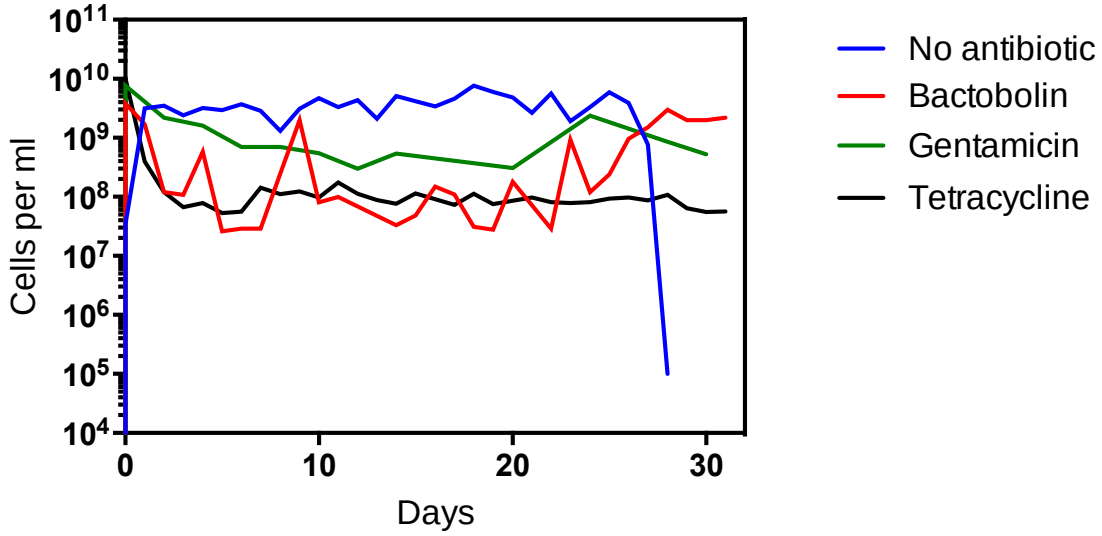
Strain or plasmid	Relevant properties	Reference or source
Strain		
B. thailandensis		
E264	Wild-type strain	[20]
BD20	*btaK* (bactobolin) mutant of E264	[10]
Chromobacterium sp. CV017		
ATCC 31532	ATCC type strain	ATCC
CV017	Uncharacterized mini-Tn5 mutant of ATCC 31532	[17]
CV026	*cviI*::Tn5 xylE Km^r insertion mutant of CV017	[17, 61]
CV026R	*cviR* mutant of Cv026	This study
CV026R BR1	Spontaneous bactobolin-resistant mutant	This study
CV026R BR2	Spontaneous bactobolin-resistant mutant	This study
CV026 H127Y CdeR	Engineered bactobolin-resistant mutant	This study
CV017 Δ*cdeAB-oprM*	*cdeAB-oprM* mutant of CV017	This study
CV026R Δ*cdeAB-oprM*	*cdeAB-oprM* mutant of CV026R	This study
Plasmid		
pEX18Gm	Suicide vector	[24]
pEX18Gm CdeR H127Y	Deletion construct used to make Cv026R CdeR H127Y	This study
pEX18Gm Δ*cdeAB-oprM*	Deletion construct used to make CV017 Δ*cdeAB-oprM* and CV026R Δ*cdeAB-oprM*	This study

Table S2. *Chromobacterium* sp. CV017 quorum-sensing cheater variants

Variant	Experiment[a]	*cviR* mutation[b]	Amino acid change[c]
cviR1	No antibiotic 1 (8)	T→C (+261)	Y88H
cviR2	No antibiotic 1 (24)	G→C (+451)	G151R
cviR3	No antibiotic 2 (13)	AGC (+411-413)	138S
cviR4	No antibiotic 2 (13)	T→C (+253)	L85P
cviR5	No antibiotic 3 (17)	Δ (+553 to +564)	Truncation (239)
cviR6	No antibiotic 3 (19)	GAG (+410-413)	138E
cviR7	No antibiotic 4 (13)	C→T (+58)	Truncation (20)
cviR8	No antibiotic 4 (15)	C→T (+301)	Truncation (101)
cviR9	Gentamicin 1 (9)	A→C (+689)	K230T
cviR10	Gentamicin 1 (10)	T→C (+510)	S155P

[a]Two cheaters were sequenced from each of four experiments where no antibiotic was added, and one experiment where gentamicin was added. Isolates were taken on the days shown in parentheses.
[b]Nucleotide changes relative to the translational start site of CV017 *cviR*.
[c]Amino acid changes relative to the CviR protein sequence of *Chromobacterium* sp. CV017.

Figure S1 Growth yield of casein-passaged cultures



One representative experiment from each of the following conditions is shown: cultures grown with no antibiotic (blue line), with 2 μg ml^{-1} bactobolin A (red line), 0.25 μg ml^{-1} tetracycline (black line) or 12 μg ml^{-1} gentamicin (green line). Cultures were transferred daily and, every 2-3 days, the growth yield was determined just prior to subculture by dilution plating onto Luria-Bertani agar and enumerating the total number of colonies.

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CHAPTER IV:

Signal promiscuity of the quorum sensing receptor, CviR, in *Chromobacterium* sp. CV017 activates a sub-set of genes that are important for competition

Abstract

C. violaceum has been widely used as a biosensor to detect short-chain acyl-homoserine lactones (AHLs) produced by other bacteria. The ability to respond to other species' AHLs, or 'eavesdropping,' may help *C. violaceum* compete with other strains or species in polymicrobial communities. Previous research in our lab and other labs has demonstrated that *Chromobacterium* sp. CV026 can detect AHLs produced by *Burkholderia thailandensis* [1]. We demonstrate that CviR in *Chromobacterium* sp. CV017 can respond to N-octanoyl-L-homoserine lactone (C_8-HSL) and N-3-hydroxyoctanoyl-L-homoserine lactone (3OH-C_8-HSL) and this response provides an increase in *Chromobacterium* sp. CV017 competitiveness during competition with *B. thailandensis*. CviR is known to have a broad range of AHL specificity, but the genes regulated by CviR in response to both cognate and noncognate AHLs are unknown. Our transcriptome analysis of genes that respond to various AHLs reveals that there is a subset of over 120 genes that respond to all signals, including many genes that are important for competition.

Introduction

Acyl-homoserine lactone (AHL) mediated quorum sensing (QS) systems have been found in many Proteobacteria and typically involve two genes coding for a LuxI-family AHL synthase and a LuxR-family AHL receptor and transcription regulator [2]. The AHL signal is diffusible, and after reaching a critical concentration, the signal binds its cognate LuxR receptor. Usually an AHL synthase produces one main AHL and several other AHLs at low levels [3]. However, the AHL receptor is specific for just the main product of the AHL synthase. There are a few notable exceptions to this dogma, including *Chromobacterium violaceum,* which has been shown to bind AHLs of varying acyl-side chains [4].

In most LuxR-homologs, noncognate signals cannot bind because the acyl-side chains do not fit in the AHL binding site. However, in *C. violaceum* noncognate AHLs have been shown to activate expression of the purple pigment, violacein [4]. Crystal structures of CviR with various signals show that CviR accommodates longer side chains by allowing the side chain to protrude from the binding site [5]. However, this does affect the overall confirmation of the CviR homodimer. When the CviR homodimer is bound to noncognate AHLs, the DNA binding domains begin to move farther apart [5]. This conformation is similar to the "locked" structure that is observed when a QS inhibitor, chlorolactone, is in the AHL binding site. In the "locked" conformation, the DNA binding domains are so far apart that no DNA binding can occur. This may explain why CviR binds cognate signal with the strongest affinity and that this affinity decreases as the acyl side chain length increases. The binding affinity of CviR for AHLs also corresponds to the level of violacein expression allowing us to measure violacein expression as a proxy for AHL binding affinity [4, 6].

The advantage of CviR binding to noncognate signals is unclear. However, it has been proposed that this ability to 'eavesdrop' on other species might be important for *Chromobacterium* sp. CV017 survival in multi-species communities [1]. We hypothesize that CviR is important for increasing competitiveness by inducing genes important for competition-associated behaviors. Further, we hypothesize that when CviR is bound to a noncognate signal, it will have a lower binding affinity for the *lux* box and only genes with high affinity *lux* boxes will be transcribed. Only certain nucleotides in the *lux* box are strictly conserved while the others can vary. Previous experiments in *C. violaceum* ATCC 12472 demonstrate that single nucleotide changes in the *lux* box can cause an increase or decrease in the binding affinity of CviR for the *lux* box [5]. To test our hypotheses, we investigated the importance of signal promiscuity (the ability of CviR to bind multiple AHLs) on survival during competition with *Burkholderia thailandensis* by adding the cognate signal, N-hexanoyl-L-homoserine lactone (C_6-HSL), and the signals produced by *B. thailandensis:* C_8-HSL, 3OH-C_8-HSL, and N-3-hydroxydecanoyl-L-homoserine lactone (3OH-C_{10}-HSL). We also carried out RNA-sequencing to identify the genes that respond to C_6-HSL, C_8-HSL, N-decanoyl-L-homoserine lactone (C_{10}-HSL), and N-butyryl-L-homoserine lactone (C_4-HSL).

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains are listed in Table 1.

Table 1. Bacterial strains

Strain	Relevant properties	Reference or source
B. thailandensis JPT125	*btaI*1-3, *btaK* (bactobolin)	[1]
Chromobacterium sp. CV017	Mini-TN5 mutant of ATCC 31531 (WT)	[4]
CV026	*cviI*::Tn5 xylE KM insertion mutant of CV017	[4, 16]
CV026R	*cviR* mutant of CV026	[1]
CviI-	*cviI* mutant of CV017	This study
CviR-	*cviR* mutant of CV017	This study

Chromobacterium sp. CV017 strains are derivatives of strain ATCC31532 [7]. *Chromobacterium* sp. CV017 (referred to as wild type) has a transposon insertion in gene CV_RS05185 causing overexpression of violacein [8, 9]. For the RNA sequencing, we used strain CV026, a derivative of CV017, with a second transposon insertion in the *cviI* gene so that it will not produce any AHLs but will respond to exogenous signals (McClean et al 1997).

In the co-culture signal addback experiments, *Chromobacterium* sp. CV017 *cviI* and *cviR* knockout mutants were constructed using homologous recombination as previously described [1] with synthetic gene fragments (IDT) cloned into the pEXG2Gm-derived delivery plasmid. The *B. thailandensis* strain, derived from strain E264, was deficient in bactobolin and all three signal synthases: BtaI, BtaII, and BtaIII [1]. To test the responsiveness of *Chromobacterium* sp. CV017 to AHLs from *B. thailandensis,* we added C_8-HSL, 3OH-C_8-HSL, and 3OH-C_{10}-HSL to the growth media. The synthetic AHLs were purchased from Cayman Chemical and dissolved in acidified ethyl acetate with glacial acetic acid (0.1 ml L^{-1}). The dissolved AHL was added to a 250mL Erylmyer flask and dried under N_2 gas. The cultures were grown in 20mL Luria-Bertani broth containing 50mM morpholnepropanesulfonic acid (MOPS). All co-cultures were grown at 30°C with shaking at 250rpm.

For quantification of AHL production by *Chromobacterium* sp. CV017, we grew a starter culture of *Chromobacterium* sp. CV017 in 15mm test tubes in 4mL of LB with 50mM MOPS to 4 OD_{600}. The flasks containing 20mL or 10mL of LB with 50mM MOPS were inoculated at 0.05 OD_{600} and incubated until they reached 2 OD_{600} or 4 OD_{600}.

Measurement of AHL in Chromobacterium sp. CV017 cultures. The cells were pelleted at 3000rpm for 20min with no break. The supernatant was removed and 0.4 nmol of D_3-C_6-HSL purchased from Cayman Chemical was added to the cultures as an internal control for

quantification. The supernatant was then filter sterilized using a 0.2μM filter. The cultures were treated with an equal volume of acidified ethyl acetate. This mixture was shaken vigorously three times, allowing the layers to separate between each agitation. The top, aqueous layer was removed. The extraction procedure was carried out two more times on the remaining organic layer. The combined ethyl acetate fractions were dried under nitrogen gas and resuspended in 1mL of acidified ethyl acetate. The samples were stored at -20°C.

The initial purification of AHL molecules was performed by solid-phase extraction. The Sep Pak Plus silica cartridges were activated by 3 successive washings of 6mL of equal volumes isooctane and ethyl ether, 6mL of acidified ethyl acetate, and 6mL of equal volume isooctane and ethyl ether. The sample was then loaded into the SPE cartridge. The cartridge was washed twice with 6mL of isooctane-ether and then eluted with 6mL of acidified ethyl acetate. The purified samples were dried and resuspended in 100μl of 1% formic acid acetonitrile and then loaded into the time-of-flight (TOF) MS/MS.

RNA isolation and RNA-seq library construction. All cultures of CV026 were grown in 10mL of LB+MOPS in 125mL baffled flasks at 30°C, 230rpm. CV026 was treated with 2μM of C_6-HSL and 2mL of cells were collected at exponential phase (2.0 OD_{600}) and the transition to stationary phase (4.0 OD_{600}). CV026 was also treated with 10μM of C_6-HSL, C_8-HSL, or C_4-HSL and 2mL of cell were collected at 4.0 OD_{600}. Two replicates of each treatment were collected. Table 2 contains a summary of each sample collected for RNA extraction. The cells were treated with Qiagen RNAprotect (catalog number 76527) following the manufacturer's protocol and frozen at -80ºC until mRNA was isolated following the protocol for Qiagen RNeasy Mini Kit (catalog number 74104). To enrich for mRNA, we used the Microb*Express* Bacterial mRNA Enrichment Kit from Invitrogen (catalog number AM1905) following the manufacture's protocol.

RNA-sequencing libraries were generated by the Genome Sequencing Core at the University of Kansas and sequencing of the single-read, 50bp fragments was performed on the Illumina HiSeq 2500 platform. The reads were analyzed by the K-INBRE Bioinformatics Core at the University of Kanas. Gene transcript abundance from each AHL treatment was compared to untreated CV026 cells to determine AHL responsive genes. We used the DESeq package with a false discovery rate (FDR) of 0.05 and only evaluated genes showing a 2-fold or greater change in transcript abundance in relation to the control CV026 group [10].

Table 2. Cell conditions for RNA collection

Strain	AHL Treatment	OD_{600}
CV026	No signal	2.1
CV026	No signal	2.1
CV026	No signal	4.2
CV026	No signal	4.1
CV026	2μM C_6-HSL	2.1
CV026	2μM C_6-HSL	1.99
CV026	2μM C_6-HSL	4.2
CV026	2μM C_6-HSL	4.1
CV026	2μM C_8-HSL	4
CV026	2μM C_8-HSL	4
CV026	10μM C_6-HSL	4
CV026	10μM C_6-HSL	4.1
CV026	10μM C_8-HSL	4.1
CV026	10μM C_8-HSL	4.1
CV026	10μM C_4-HSL	4.1
CV026	10μM C_4-HSL	4

To determine which genes respond to AHLs, we grew *Chromobacterium* sp. CV026 in the presence of the indicated amount (2μM or 10μM) of each signal (C_6-HSL, C_8-HSL, or C_4-HSL). Cultures were grown to exponential phase (~2 OD600) or transition to stationary phase (~4 OD600. As a control, we grew CV026 without any AHL. Two replicates of each condition were collected.

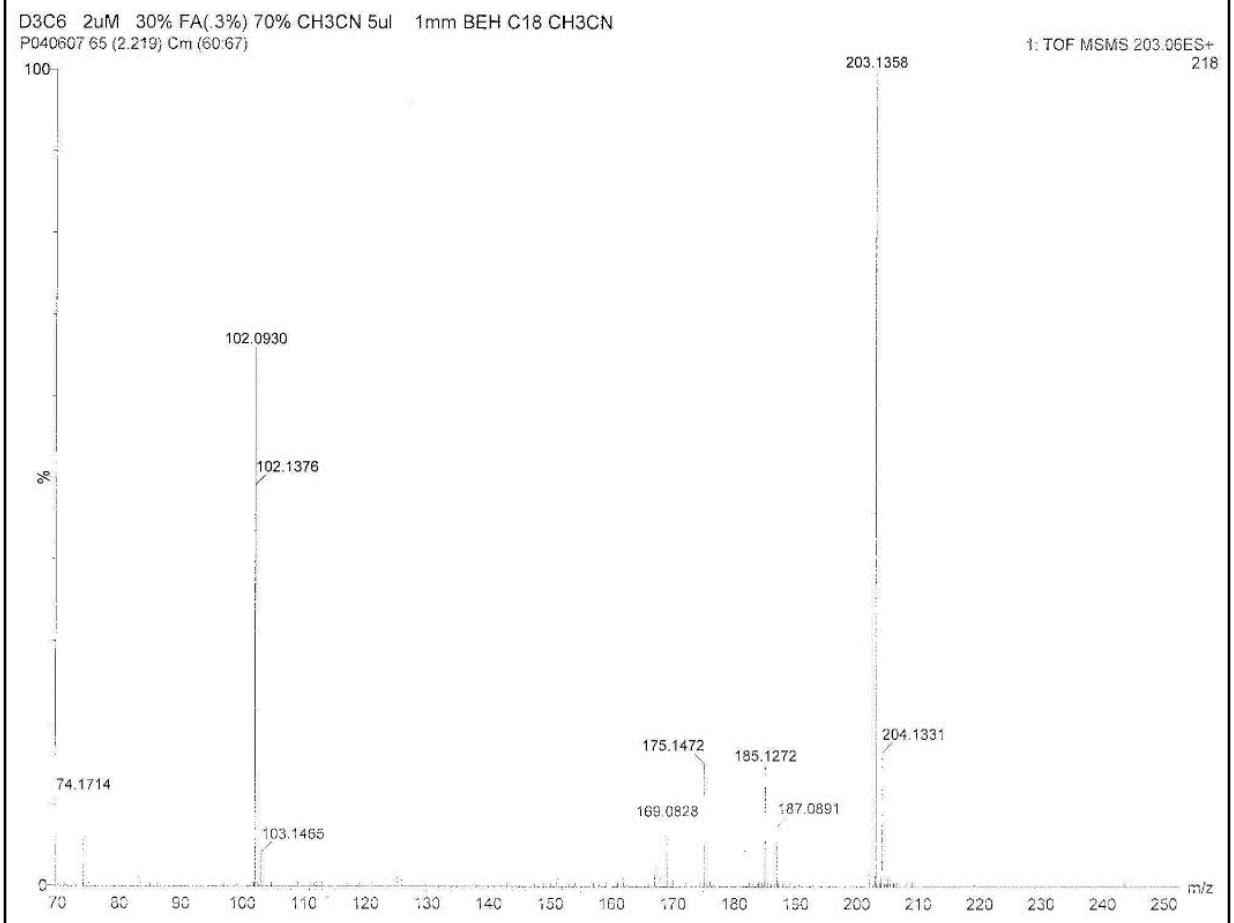
Results

Identifying the AHLs produced by CviI signal synthase

We first quantified AHLs produced by *Chromobacterium* sp. CV017 cultures to determine the concentration of AHLs to use in our experiments. To quantify the type and amount of AHL produced by *Chromobacterium* sp. CV017, we grew 20mL of culture to an OD_{600} 4.0. The culture was treated with acidified ethyl acetate in order to lysis the cells and separate the AHLs from the cell material. After separating the acidified ethyl acetate layer from the cell culture, we added deuterated C_6-HSL (D_3-C_6-HSL) as an internal control to measure the amount of signal

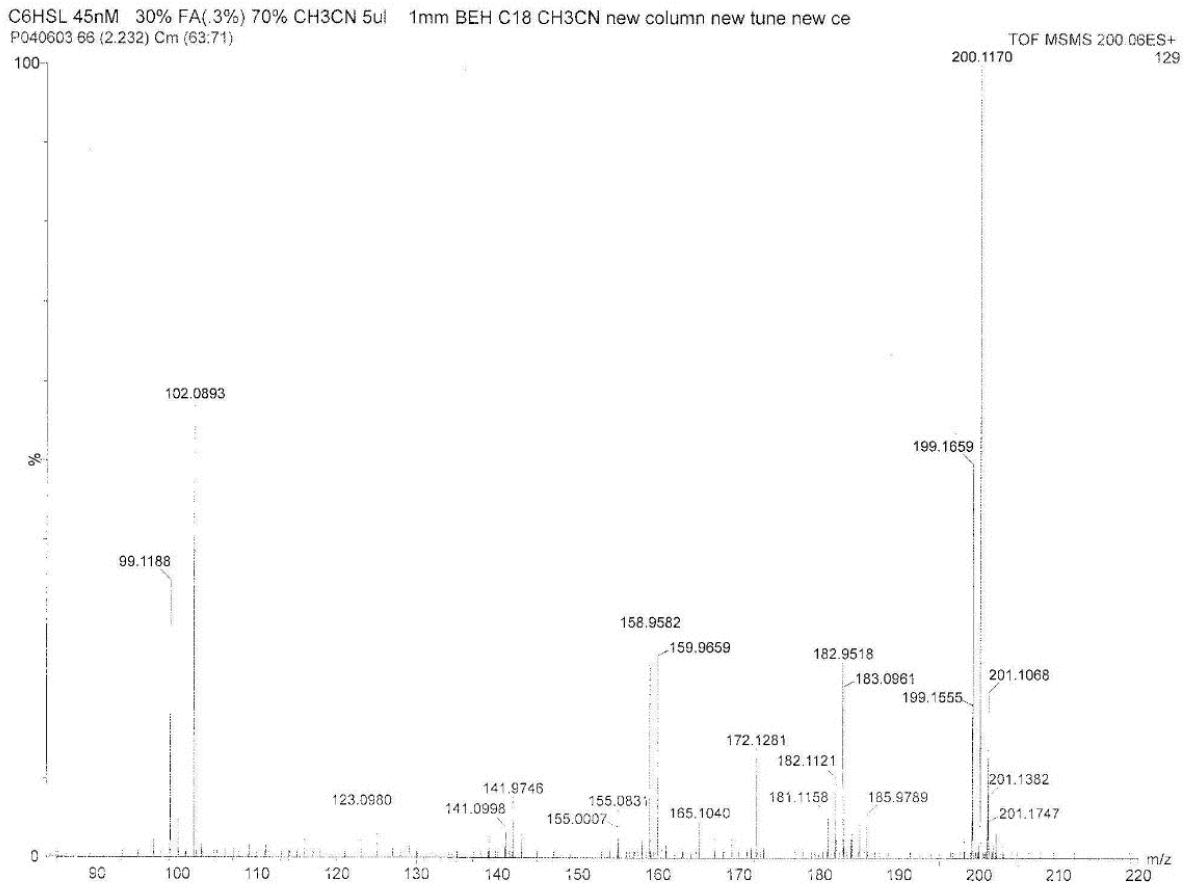
lost during further purification steps. After reverse phase SPE the sample was run through TOF MS/MS. As a control we ran D_3-C_6-HSL (Figure 1) and C_6-HSL (Figure 2) separately to make sure our mass/charge (m/z) values correspond with previously published results.

Figure 1 TOF MS/MS results for D_3-C_6-HSL



2μM of D_3-C_6-HSL was suspended in 30% formic acid and 70% acetonitrile and 5ul was loaded into the TOF MS/MS. The peak at 203.1 m/z corresponds to the full length D_3-C_6-HSL and the 102.09 m/z peak corresponds to the lactone.

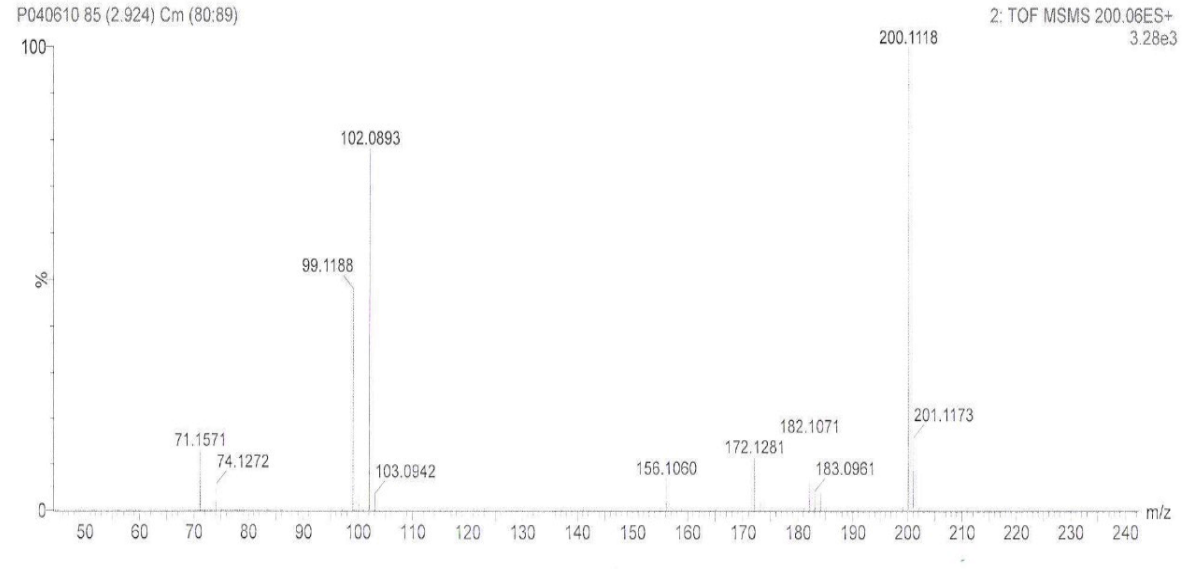
Figure 2. TOF MS/MS results for C_6-HSL



45nM of C_6-HSL was suspended in 30% formic acid and 70% acetonitrile and 5ul was loaded into the TOF MS/MS. The peak at 200.1 m/z corresponds to full length C_6-HSL, the peak at 102.09 m/z corresponds to the lactone, and the peak at 99.12 m/z corresponds to the acyl side chain.

We then ran our purified supernatant containing the D_3-C_6-HSL as an internal control. Figure 3 is a representation of the results from the 2.0 OD sample. We determined that *Chromobacterium* sp. CV017 produces around 300nM of C_6-HSL based on calculation of counts/μM of our internal standard D_3-C_6-HSL (Table 3).

Figure 3 TOF MS/MS results for purified supernatant



Purified supernatant from 2.0 OD_{600} cells was resuspended in 1% formic acid acetonitrile and loaded into the TOF MS/MS. The peak at 200.1 m/z corresponds to full length C_6-HSL, the peak at 102.09 m/z corresponds to the lactone, and the peak at 99.12 m/z corresponds to the acyl side chain.

Table 3. Calculating AHL concentration from TOF MS/MS results

Sample	Counts at 102 m/z	Sample counts/ Control counts	Dilution Factor	Concentration (nM)
2μM D_3C_6-HSL	653			
2.0 OD 10mL	6560	10	1/100	100
2.0 OD 20mL	28600	43.8	1/200	219
4.0 OD 10mL	30900	47.3	1/200	237
4.0 OD 20mL	22000	33.7	1/100	337

Chromobacterium sp. CV017 was grown in either 10mL or 20mL volumes to an optical density (OD) of 2.0 or 4.0. The supernatant was extracted and 100μl of each sample was run on TOF MS/MS. AHL concentration was calculated by determining the number of counts at the 102 m/z (lactone moiety) peak for each sample. Based on the number of counts for the internal standard (2μM D_3C_6-HSL), we could determine the amount of AHL in each sample.

There were very few peaks that did not correspond to either C_6-HSL (200.3 m/z), the lactone (102.09 m/z) or the acyl side chain (99.12 m/z). The peaks that did appear did not match any m/z values of known AHLs [3]. In many Proteobacteria the signal synthase will attach an acyl side chain of the wrong length 20%-40% of the time. However, CviI produces C_6-HSL 90%

of the time. The amount of signal produced, 300nM, also corresponds with what is seen in other Proteobacteria [3, 11]

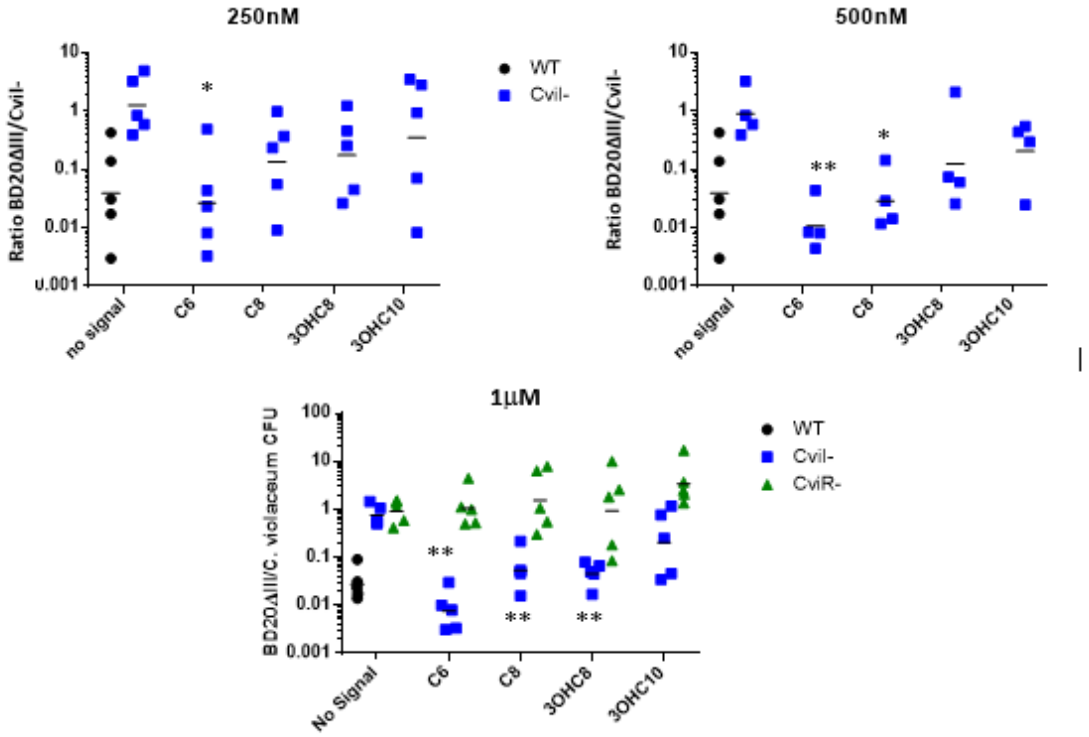
The addition of noncognate AHLs to co-cultures of C. violaceum and B. thailandensis

Previous results by Chandler et al. demonstrated that at least one of the AHLs from *B. thailandensis* supernatant activates CviR to promote survival of *Chromobacterium* sp. CV017 during competition [1]. We were interested in determining which of the three AHLs (C_8-HSL, 3OH-C_8-HSL, 3OH-C_{10}-HSL) produced by *B. thailandensis* was most effective at inducing QS in *Chromobacterium* sp. CV017. We generated a CviI knockout in CV017 (CviI-) which cannot produce AHLs but still has an intact AHL receptor, CviR, to respond to exogenously added AHLs. Since *Chromobacterium* sp. CV017 is severely inhibited by bactobolin produced by *B. thailandensis,* we used a bactobolin knockout strain that was also missing all three LuxI-homologs. This way the only AHLs present in the co-culture were those that we added to the tube.

We added C_6-HSL which is produced by *Chromobacterium* sp. CV017 as a control to demonstrate that the addition of cognate signal increased competitiveness. We then added each of the signals produced by *B. thailandensis,* C_8-HSL, 3OH-C_8-HSL, and C_{10}-HSL at 250nM, 500nM, and 1μM (Figure 3). At all concentrations, C_6-HSL was able to increase competitiveness compared to the no signal control (Mann Whitney, p-value < 0.016). We also observed increased *Chromobacterium* sp. CV026 survival as the signal concentration increased. At 1μM, all signals except 3OH-C_{10}-HSL were able to increase survival compared to no signal control (Mann Whitney, p-value = 0.008). At 250nM only C_6-HSL was able to significantly increase survival (Mann Whitney, p-value = 0.016) while C_8-HSL was approaching a significance compared to no signal control (Mann Whitney, p-value = 0.056). We created a CviR knockout in CV017 (CviR-)

and used this strain in competitions with *B. thailandensis* and 1μM of each signal. We saw no increase in CviR- survival demonstrating that the increase in survival is dependent on CviR.

Figure 3. Co-culture competition between *B. thailandensis* and *Chromobacterium* sp. CviI- in the presence of synthetic AHLs



The ability of AHLs produced by *B. thailandensis* to increase competitiveness of *Chromobacterium* sp. CviI- was determined through competition with a bactobolin and signal synthase mutant of *B. thailandensis* (BD20ΔIII). Results are shown as a ratio of BD20ΔIII:*Cv.* Synthetic AHLs were added at 1uM, 500nM, or 250nM. Statistical analysis by Mann-Whitney nonparametric test compared to CviI-: *, p-value < 0.016; **, p-value = 0.008). As a control, CviR- was also treated with 1uM of each AHL and there was no significant increase in survival.

AHL-regulated genes

Our competition experiments suggested that CviR can respond to C_8-HSL and activate expression of genes important for competition. To determine the regulon of genes controlled by C_8-HSL-bound CviR, we used an RNA-seq transcriptomic approach. We used *Chromobacterium* sp. CV026 which has a transposon in the AHL synthase, CviI. This strain allowed us to measure the effect of each noncognate AHL without interference by the cognate signal, C_6-HSL. CV026

still has a wildtype AHL receptor, CviR, so it will respond to exogenously added AHLs. First, we needed to determine the genes induced by the cognate signal compared to no AHL exposure. We compared transcripts from the exogenously added C_6-HSL cultures, at logarithmic growth phase (2 OD_{600}) and the transition from logarithmic to stationary phase (4 OD_{600}). Previous research has shown that some QS-controlled genes also require other cellular signals and may only be transcribed as the cell enters stationary phase [12]. Finally, to address which genes are responsive to noncognate AHLS, we exogenously added C_8-HSL and C_4-HSL to CV026 and collected the mRNA at 4 OD_{600}.

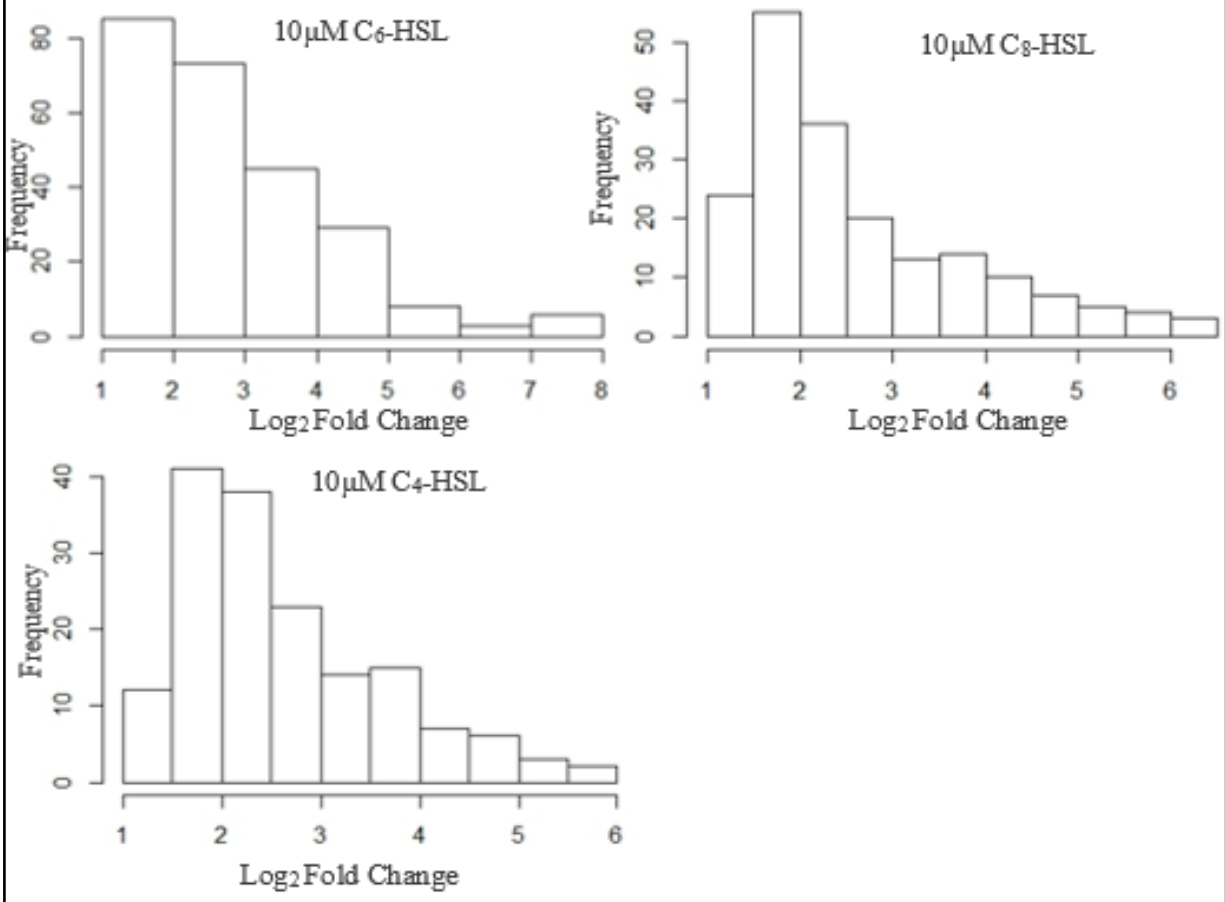
We harvested RNA after treatment with 2μM (C_6-HSL and C_8-HSL) and 10μM signal (C_6-HSL, C_8-HSL, and C_4-HSL) based on the amount of signal needed to activate production of violacein (causing cell cultures to turn visibly purple) at the same time as *Chromobacterium* sp. CV017. We sequenced the RNA libraries from two biological replicates for each signal concentration and optical density. Overall, we saw more activation than repression of genes (Figure 4A). Only 2μM C_6-HSL was tested at exponential and at the transition to stationary phase. During exponential growth, there were 244 genes that responded to 2μM of C_6-HSL with most of the genes being repressed (Figure 4A). However, during the transition to stationary phase, we saw 597 genes respond to 2μM C_6-HSL with most of the genes being activated.

Figure 4. Acyl-homoserine lactone responsive genes

A.

AHL	*OD*	*Number of genes:* *Activated*	*Repressed*
2uM C_6-HSL	2OD	90	154
2uM C_6-HSL	4OD	317	280
2uM C_8-HSL	4OD	77	56
10uM C_6-HSL	4OD	249	173
10uM C_8-HSL	4OD	191	151
10uM C_4-HSL	4OD	161	60

B.

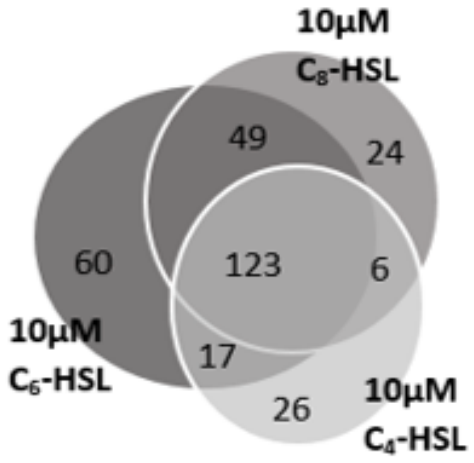


We determined AHL responsive genes based on a false discovery rate (FDR) 0.05 and compared to a no AHL control. **A)** The number of genes up- or down-regulated at least 1 $\log_2$ for each sample. **B)** Histogram of all up-regulated genes for 10μM C_6-HSL, 10μM C_8-HSL, and 10μM C_4-HSL. There is a higher frequency of 2-3-fold activated genes in response to noncognate AHLs.

All the noncognate signals were tested at the transition to stationary phase (4OD). The cognate signal C_6-HSL causes the largest change in gene transcription followed by C_8-HSL, and C_4-HSL

has the smallest effect on gene transcription (Figure 4A). However, when we look at the histogram of all up-regulated genes, there is a shift in the distribution of the degree of activation (Figure 4B). While C_6-HSL has the widest range in $\log_2$ fold-change, most genes are up-regulated 1- to 2-fold. However, both noncognate signals have less genes up-regulated 1-fold compared to C_6-HSL. The majority of non-cognate responsive genes are upregulated 1.5- to 3-fold. This lends support to our hypothesis that only genes with high affinity *lux* box sequences will respond to noncognate AHLs.

Figure 5. The number genes up-regulated for each signal.



We compared the genes up-regulated in each signal to determine which genes respond to only C_6-HSL (60 genes), all three signals (123 genes), or just the noncognate signal (6 genes).

When we compare each noncognate signal to the cognate signal, we see that most noncognate genes are also responsive to C_6-HSL (Figure 5). There is a core set of 123 genes that respond to all 3 AHLs. However, there are only six genes that respond to C_8-HSL and C_4-HSL: glutathione S-transferase and five hypothetical proteins.

Our hypothesis states that 'eavesdropping' will increase the competitiveness of *Chromobacterium* sp. CV017 by inducing genes important for competition-associated behaviors. To evaluate our hypothesis, we looked at the top and bottom 20% of differentially expressed genes for each signal (Table 4 and 5). Nearly all the genes in the top 20% are associated with competition: iron scavenging (2Fe-2S binding protein and SbnAB) [13, 14]; antimicrobials (non-ribosomal peptide and violacein); and chemotaxis [15, 16]. In the bottom 20% of differentially expressed genes most genes are associated with metabolism. However, there are a few genes that

are associated with competition including *cdeA* (RND transporter) from Chapter III and a polyketide cyclase.

While noncognate AHLs activate a smaller portion of genes than C_6-HSL, the majority of noncognate AHLs responsive genes have a higher $\log_2$ fold-change than C_6-HSL responsive genes. This may be because the 1-fold differentially expressed genes having *lux* boxes with low affinity for CviR. Since noncognate AHLs cause a decrease in the affinity of CviR for DNA, these genes may not be expressed or expressed at very low levels. Additionally, the majority of genes that are highly responsive to noncognate AHLs are associated with competition supporting our hypothesis that 'eavesdropping' improves the competitiveness of *Chromobacterium* sp. CV017.

Table 4. Top 20% of differentially expressed genes for each AHL

C6-HSL		C8-HSL		C4-HSL	
Annotation	Log2 fold change	Annotation	Log2 fold change	Annotation	Log2 fold change
(2Fe-2S)-binding protein	5.68	(2Fe-2S)-binding protein	4.34068	(2Fe-2S)-binding protein	4.74881
(2Fe-2S)-binding protein	4.69	(2Fe-2S)-binding protein	5.92664	(2Fe-2S)-binding protein	3.86678
22C3-diaminopropionate biosynthesis protein SbnA	4.76	22C3-diaminopropionate biosynthesis protein SbnA	4.74119	22C3-diaminopropionate biosynthesis protein SbnA	3.77555
22C3-diaminopropionate biosynthesis protein SbnB	5.67	22C3-diaminopropionate biosynthesis protein SbnB	5.42513	22C3-diaminopropionate biosynthesis protein SbnB	4.30374
ABC transporter ATP-binding protein	4.47	ABC transporter ATP-binding protein	4.39339	ABC transporter ATP-binding protein	3.92287
aldehyde dehydrogenase	4.57	aldehyde dehydrogenase	4.76529	aldehyde dehydrogenase	3.59651
aldehyde dehydrogenase	4.11	chemotaxis protein	3.7205	chitinase	5.61673
asparagine synthase	4.46	chitinase	5.41853	diguanylate cyclase	4.53215
chemotaxis protein	6.34	diguanylate cyclase	5.39309	iminophenyl-pyruvate dimer synthase VioB	4.93308
chitinase	5.62	iminophenyl-pyruvate dimer synthase VioB	5.90677	MarR family transcriptional regulator	4.39618
diguanylate cyclase	5.81	MarR family transcriptional regulator	4.32145	mercuric reductase	3.51453
iminophenyl-pyruvate dimer synthase VioB	7.23	MFS transporter	3.90258	monooxygenase	4.96686
MarR family transcriptional regulator	5.38	monooxygenase	6.21891	non-ribosomal peptide synthetase	3.98041
MFS transporter	4.21	O-antigen acetylase	5.13652	O-antigen acetylase	4.14542
monooxygenase	7.82	pseudogene	6.18388	pseudogene	5.42068
non-ribosomal peptide	5.53	secretion protein	4.79335	secretion protein	3.9581
O-antigen acetylase	4.68	sugar ABC transporter substrate-binding protein	4.52881	sugar ABC transporter substrate-binding protein	3.80167
peptidase M4	4.45	tryptophan hydroxylase	5.67364	tryptophan hydroxylase	4.5784
peptide synthetase	4.61	type I-E CRISPR-associated protein Cas7/Cse4/CasC	4.58554	type I-E CRISPR-associated protein Cas7/Cse4/CasC	3.54302
phasin	4.06	type I-E CRISPR-associated protein Cse1/CasA	4.33211	violacein biosynthesis enzyme VioE	4.27679
pseudogene	6.16	violacein biosynthesis enzyme	5.45507		
pseudogene	4.6				
secretion protein	4.75				
sugar ABC transporter substrate-binding protein	4.28				
sugar ABC transporter substrate-binding protein	3.97				
transketolase	4.1				
tryptophan hydroxylase	7.36				
type I-E CRISPR-associated protein Cas7/Cse4/CasC	6.1				
type I-E CRISPR-associated protein Cse1/CasA	5.92				
violacein biosynthesis enzyme	7.23				

The differentially expressed genes were sorted by $\log_2$ fold-change. The top 20% of genes for each signal (50 genes for C_6-HSL, 38 genes for C_8-HSL, 34 genes for C_4-HSL) are displayed after the hypothetical genes were removed. The majority of genes are associated with competition.

Table 5. Bottom 20% of differentially expressed genes for each AHL

C6-HSL		C8-HSL		C4-HSL	
Annotation	Log2 fold change	Annotation	Log2 fold change	Annotation	Log2 fold change
3-ketoacyl-ACP reductase	1.69	3-hydroxyalkanoate synthetase	1.62	3-hydroxybutyrate dehydrogenase	1.50
aldo/keto reductase	1.22	3-hydroxybutyrate dehydrogenase	1.53	aldo/keto reductase	1.45
aromatic amino acid transporter	1.42	acriflavine resistance protein B	1.40	aminopeptidase	1.44
carbamate kinase	1.32	aspartate-semialdehyde dehydrogenase	1.46	aminotransferase	1.75
carboxylesterase	1.30	carboxylesterase	1.34	amylase	1.57
dihydroneopterin aldolase	1.39	chitinase	1.41	aromatic amino acid transporter	1.71
dTDP-4-dehydrorhamnose 32C5-epimerase	1.45	cytochrome C biogenesis protein CcsA	1.50	chitin-binding protein	1.72
GCN5 family acetyltransferase	1.46	cytochrome C oxidase	1.63	cold-shock protein	1.79
general secretion pathway protein GspE	1.28	D-alanine/D-serine/glycine permease	1.50	cytochrome C oxidase	1.33
glutamate synthase	1.24	dihydroneopterin aldolase	1.26	DNA transposition protein	1.31
glutamate--cysteine ligase	1.35	dTDP-4-dehydrorhamnose 32C5-epimerase	1.49	glutamate synthase	1.58
glycerol-3-phosphate ABC transporter substrate-binding protein	1.51	Fis family transcriptional regulator	1.26	glutamate synthase	1.44
hemolysin	1.65	glutamine synthetase	1.44	lipopolysaccharide biosynthesis protein RfbH	1.71
histidine ammonia-lyase	1.46	heat-shock protein Hsp20	1.61	peptidase M15	1.49
isocitrate lyase	1.46	histidine ammonia-lyase	1.50	phage baseplate protein	1.78
leucyl aminopeptidase	1.65	peptidase M13	1.48	poly (3-hydroxybutyrate) depolymerase	1.66
MarR family transcriptional regulator	1.60	peptidase M3	1.26		
molecular chaperone GroEL	1.44	poly (3-hydroxybutyrate) depolymerase	1.63		
muraminidase	1.44	protein involved in meta-pathway of phenol degradation	1.56		
organic hydroperoxide resistance protein	1.53	pseudogene	1.44		
peptidase M13	1.62	purine nucleoside phosphorylase	1.26		
peptide ABC transporter substrate-binding protein	1.38	RND transporter	1.36		
peptidoglycan-binding domain-containing protein	1.43	sugar ABC transporter substrate-binding protein	1.50		
polyisoprenoid-binding protein	1.61	superoxide dismutase	1.54		
polyketide cyclase	1.55				
protease Do	1.54				
pseudogene	1.53				
PTS N-acetyl-D-glucosamine transporter	1.41				
RND transporter	1.51				
thermolabile hemolysin	1.32				
transcriptional regulator	1.44				
transcriptional regulator	1.50				

The differentially expressed genes were sorted by $\log_2$ fold-change. The bottom 20% of genes for each signal (50 genes for C_6-HSL, 38 genes for C_8-HSL, 34 genes for C_4-HSL) are displayed after the hypothetical genes were removed. There are fewer genes associated with competition and less consensus between each signal.

Discussion

The ability of *C. violaceum* strains ATCC 12472 and ATCC 31531 (parent strain of CV017) to respond to various AHLs produced by other bacteria has been widely reported [4, 17, 18]. However, few studies have addressed the potential advantages of *C. violaceum* response to noncognate AHLs, and how this relates to gene control in the cell. Only one publication examined the type of AHLs produced by *C. violaceum* ATCC 12472 by placing the *luxI*-homolog, *cviI,* in *Escherichia coli* and conducting mass spectrometry on filtered supernatant [17]. They reported that the major signal is 3OH-C_{10}-HSL with two other major products. While their m/z results (294) do match their control, it does not match other published results for known AHLs [3]. Additionally, they do not identify the other peaks observed in their mass spectrometry. Since their m/z results do not match other published results, we are unable to ascertain any other AHLs that may be produced by *C. violaceum* ATCC 12472. We show for the first time through TOF MS/MS of *Chromobacterium* sp. CV017 supernatant that the signal synthase, CviI, produces almost exclusively C_6-HSL. Over 90% of all peaks correspond with C_6-HSL and the remaining peaks do not correspond with any known AHL m/z profiles. This supports our hypothesis that *Chromobacterium* sp. CV017 'eavesdrops' on other AHLs because it gains a benefit during competition and not because CviI produces a broad-range of AHLs.

Previous research, together with our results, support the idea that *Chromobacterium* sp. CV017 detects C_8-HSL and 3OH-C_8-HSL during competition with *B. thailandensis* which results in increased survival of *Chromobacterium* sp. CV017. We were surprised to learn that *Chromobacterium* sp. CV017 also responds to 3OH-C_8-HSL since previous publication reported that *Chromobacterium* sp. CV026 did not respond to AHLs with substitutions on the 3rd carbon [4, 19]. However, 3OH-C_{10}-HSL which also had a substitution of the 3rd carbon had no effect

during competition. This may be because C_{10}-HSL already has a low binding affinity for the receptor based on violacein expression making modifications on the 3rd carbon even more detrimental [4, 19].

Our RNA transcriptomics provides the first look through mRNA sequencings at AHL responsive genes and provides some clues as to the benefits of *Chromobacterium* sp. CV017 response to noncognate AHLs. Of the noncognate signals tested, C_8-HSL most closely mimicked C_6-HSL by inducing 20.6% of C_6-HSL responsive genes at 2μM and 64.8% of C_6-HSL responsive genes at 10μM. This mimicry corresponds with what has been observed in the ability C_8-HSL to induce violacein expression in the biosensor strain CV026 [20]. Additionally, the crystal structures generated by Bassler et al. demonstrate that C_8-HSL has the smallest change to the protein conformation compared to C_6-HSL indicating that CviR is most likely exhibiting wild-type behavior [20]

Our hypothesis that noncognate AHLs will have the greatest control over competition factors was addressed by determining the genes that responded 10μm of C_6-HSL, C_8-HSL, or C_4-HSL. The majority of genes up-regulated only by C_8-HSL or C_4-HSL have no known function. However, when we evaluated the predicted function of the genes up-regulated in all three samples, we saw an enrichment in the total percent of competition-associated factors, such as: antimicrobial agents, antibiotic resistance efflux pumps, T3SS, chemotaxis proteins, and proteases. This group of genes was the only group that increased in response to noncognate AHLs lending support to our hypothesis that 'eavesdropping' is important during competition. While we have not been able to definitively answer our hypothesis, we believe we have contributed more understanding to the role of noncognate AHLs during bacterial competition.

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CHAPTER V:

Discussion

Chromobacterium sp. CV017 *reveals a need for phylogenetic comparisons during genome sequencing*

Historically, bacteria were identified by physical and metabolic characteristics. These traits, while useful in identifying and characterizing bacteria, have also lead to the improper classification of bacterial species. We believe this occurred when the parent strain, *C. violaceum* ATCC 31532, of our lab strain *Chromobacterium* sp. CV017 was identified in 1982 [1]. Based on the phylogenetic analysis of the 16S RNA, there are other *Chromobacterium* species that appear to be misidentified based on their groupings.

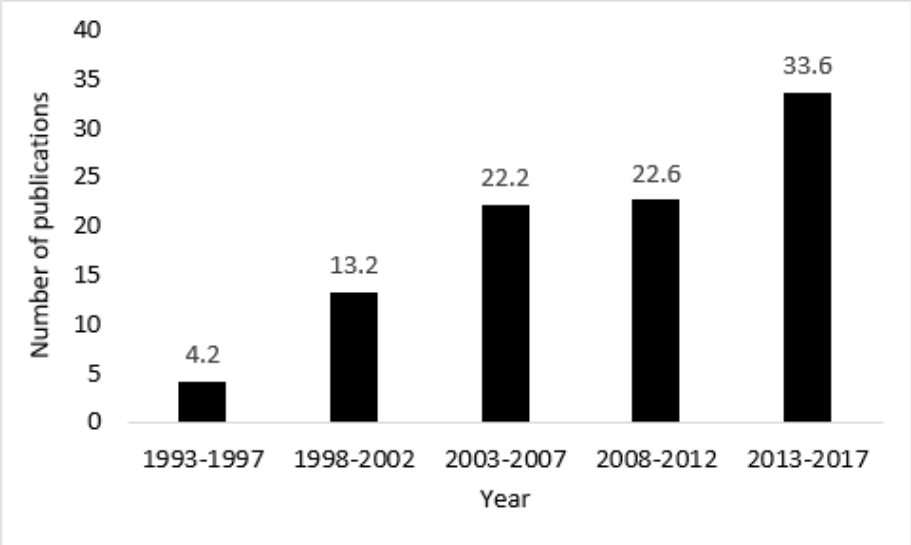
The misidentification of bacterial strains is not a new problem. The taxonomic schemes used for identifying and classifying bacteria need to be dependable, reproducible, and instructive. Historically, this means scientists focused on identifying bacterial species by phenotypes, such as: Gram staining, metabolism of compounds as energy sources, secretion of enzymes, production of toxins, and growth at various pH, temperatures, and salt concentrations [2]. However, phenotype-based taxonomy in the first half of the 1900s lead to a large multiplication of species because not enough features were used for new taxa description. The introduction of DNA-DNA hybridization in the 1970s allowed DNA homologs and molecular fingerprinting techniques (rep-PCR, amplified fragment length polymorphism [AFLP], and pulse-field gel electrophoresis [PFGE]) to be used in conjunction with physiological descriptions [3]. These methods lead to the publication of the Approved Lists of Bacterial Names that consolidation of 90% of all species described in the 1957 Bergey's Manual.

The introduction of 16S rRNA gene sequencing brought about a new system for identifying bacterial species. Current species delineation is defined as a group of strains that have greater than 70% DNA-DNA hybridization similarity, less than 5°C change in DNA melting

temperature (Tm), less than 5% difference in %GC, and greater than 98% similarity in 16S rRNA [3]. Next-generation sequencing (NGS) is changing this system again. The price of investing in and maintaining NGS systems continues to decrease making whole genome sequencing (WGS) a viable method for determining speciation [4]. WGS allows more genes to be used to delineate species than DNA-DNA hybridization or 16S rRNA sequencing alone [3]. WGS should be the standard for identifying new bacterial species. Numerous research has already demonstrated that bacterial species can be identified by unique genome characteristics [5-8] Indeed, it has been proposed that the description of new taxa should include a draft genome sequence with 20 times coverage [3].

Improper speciation is not limited to *Chromobacterium.* From 2003-2007, publications on the reclassification of bacterial strains doubled from the preceding 5 years (Figure 1). In the last 5 years, the number of publications on misidentified bacterial species has increased. The number of newly published bacterial genomes has also increased based on genome submissions

Figure 1. Average number of publications over a 5-year span



The number of publications was found using a Boolean search for articles with "reclassification" in the title and "bacteria" in the abstract on the National Center for Biotechnology Information (NCBI) pubmed.gov website. The average number of articles published in each five-year span is displayed above the bar.

to the National Center of Biotechnology Information (NCBI) genomes database. These numbers will continue to increase as genome sequencing becomes cheaper and more automated. New guidelines that require phylogenetic comparisons are needed to ensure newly sequenced genomes from bacteria that have already been named are evaluated for misclassification. The addition of a 16S or other phylogenetic comparison step in sequencing or annotation pipelines may alert researchers to the possibility that their bacterial strain has been misidentified. This check would allow researchers to publish genome announcements with recommendations for the proper nomenclature of the bacteria.

Competition provides selective advantage for quorum sensing systems

One of the leading questions in the quorum sensing (QS) field is how QS is maintained in the population when QS-deficient cheaters can be isolated from the environment, infections, and laboratory models. QS-deficient cheaters have a growth advantage over cooperating bacteria, but cheaters rarely reach a high enough frequency in the population to cause a collapse of the population. There have been a numerous examples of cheater restraint in populations through kin selection, spatial structuring (biofilm), and privatization of public goods [9]. While the coupling of public and private goods has been demonstrated to restrain cheaters, we provide the first evidence that QS-control of antibiotic efflux pumps can provide a sufficient benefit to cooperators. However, this mechanism of cheater restraint would only work if bacteria are frequently in competition with other organisms. Indeed, this may be the case as recent research indicates that competition and not cooperation dominates the interactions between microbial species [10].

It is possible that even within one species there are multiple mechanisms for maintaining QS systems. During the casein evolution experiments, we found that AHL receptor mutations

were not the only mutants to arise in the population. We observed within each culture without antibiotic treatment subpopulation of mutants with reduced violacein expression. These mutants still had intact QS genes, *cviI* and *cviR,* and produced protease but produced extremely low levels of violacein. *Chromobacterium* sp. CV017 has a transposon in the violacein repressor, VioS. This gene should be sequenced to determine if growth on casein is enough of a stress to cause a loss of this transposon and reactivation of VioS. Additionally, the ability of cheaters to emerge from these mutants during casein evolution should be tested. VioS has been shown to regulate protease and chitinolytic activity [11]. These mutants may experience less of metabolic cost from QS making it less advantageous to cheat and could be another method for limiting the burden of cheaters.

QS-regulon in Chromobacterium sp. CV026 reveals secondary metabolite clusters and hydrogen cyanide resistance

One of the problems with using *Chromobacterium* sp. CV017 in our competition experiments is that we do not know what antimicrobial factor is responsible for inhibiting *Burkholderia thailandensis* growth. Through the RNA-sequencing, we have identified the genes for a non-ribosomal peptide synthase, a polyketide synthase cluster, and hydrogen cyanide. Future work will focus on characterizing the importance of each of these genes in our dual-species competition model. Because the filtered supernatant of *Chromobacterium* sp. CV017 is not very good at inhibiting growth of *B. thailandensis,* we hypothesis that the antimicrobial agent is hydrogen cyanide.

We were also able to identify the genes that provide immunity to hydrogen cyanide based on studies in *Pseudomonas aeruginosa* and *Chromobacterium piscinae* [12]. In *Chromobacterium* sp. CV017 these genes are Cv017_17020, Cv017_17025, and Cv017_17030

which encode for cytochrome O ubiquinol oxidase I, II, and III, respectively. Interestingly these genes were only upregulated in cells at 4 OD_{600} treated with 2μM C_6-HSL. In all other treatments the genes were either down-regulated compared to no signal control or absent in the case of 10μM C_4-HSL. It is possible that there are multiple transcription factors for these hydrogen cyanide resistance genes. Future work should focus on deleting each gene separately and in-tandem in order to determine their importance for resistance in both the QS and QS-deficient background.

'Eavesdropping' in Chromobacterium sp. CV017 may provide an advantage during competition under select circumstances

In Chapter IV we demonstrate that *Chromobacterium* sp. CV017 can detect physiological levels of C8-HSL and 3OH-C8-HSL produced by *B. thailandensis.* Previous *in silico* modeling indicates that *Chromobacterium* sp. CV017 will only benefit from detecting AHLs from *B. thailandensis* when the QS threshold is high for both species [13]. This allows for the early activation of QS-controlled antibiotics and the antibiotic resistance efflux pump, *cdeAB-oprM* and increases survival of *Chromobacterium* sp. CV017.

However, our experiments assume that there is no competition between cognate and noncognate AHLs. If CviR can only form a homodimer with another subunit bound to the same signal, then there is the potential for competition between CviR:AHL homodimers. Indeed, experiments using a *vioA-gfp* reporter plasmid in *Escherichia coli* indicate that when the cognate AHL (C_6-HSL) is present, then C_8-HSL and C_{10}-HSL act as partial antagonists and decrease GFP expression [14]. To address this question, we should repeat our co-culture competition experiments with wild type *Chromobacterium* sp. CV017. Additionally, we should repeat our

transcriptomics experiments with both cognate and noncognate AHLs present during growth to determine if there is a change in the QS-regulon.

This dissertation provides evidence that *Chromobacterium* species can be classified based on WGS and unique genome characteristics instead of time-intensive physiological characteristics. There is a strong need for phylogenetic analysis when sequence any genome regardless of if it is a recent isolate. We also demonstrate QS-controlled antibiotic efflux pumps can restrain QS-deficient cheaters when antibiotics are produced during competition with other species. While this co-regulation of antibiotic production and antibiotic resistance has only been observed in a handful of species (*P. aeruginosa* and *E. coli),* there are probably many more soil bacteria that have similar systems based on the high degree of competition in soil environments [10, 15].

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