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ARTICLE

Quorum-sensing control of antibiotic resistance stabilizes cooperation in *Chromobacterium violaceum*

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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 violaceum* each use quorum sensing-controlled antimicrobials to inhibit the other species' growth. Here, we show that *C. violaceum* 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 *C. violaceum* 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 *C. violaceum* 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) quorum-sensing signals 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

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interspecies competition [12–14]. Previously, we developed a laboratory dual-species model with the soil saprophytes *Burkholderia thailandensis* and *Chromobacterium violaceum* to investigate the relationship between quorum sensing and interspecies competition [14]. The *B. thailandensis* bactobolin antibiotic targets the 50 S ribosome and has activity against a range of bacterial species including *C. violaceum* [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 [14]. Likewise, *C. violaceum* uses quorum sensing to control production of as-yet unknown secreted antimicrobials that are important to compete with *B. thailandensis*. In *C. violaceum*, there is a single LuxR-LuxI pair, the CviR-CviI quorum-sensing system. The CviR-CviI system produces and responds to hexanoyl-homoserine lactone (C6-HSL) [17]. This system controls production of a purple pigment, violacein [17]. 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 [14].

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 *C. violaceum* 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 (LB) 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 *C. violaceum* or 37°C for *B. thailandensis* and for *C. violaceum–E. coli* matings. Growth in liquid cultures was with shaking at 30°C in 18-mm test tubes. Synthetic C6-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 C6-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} (*C. violaceum*) 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} (*C. violaceum*).

Bacterial strains are listed in Table S1. *B. thailandensis* strains were E264 (wild type) or BD20, a bactobolin-deficient derivative of E264 [20]. *C. violaceum* strains are derivatives of strain ATCC31532 [21]. *C. violaceum* 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 [14]. The *C. violaceum* CdeAB-OprM^{-} and CdeR H127Y mutants were constructed using homologous recombination as previously described [14] with synthetic gene fragments (IDT) cloned into the pEX18Gm-derived delivery plasmid [24]. For fragment cloning, we used *Sph*I–XbaI (for Δ*cdeAB-oprM*) or *Hin*dIII–*Xba*I (for CdeR H127Y). The Δ*cdeAB-oprM* construct was made with ~500-bp DNA flanking *cdeA* and *oprM*, resulting in a deletion of all but the first 8 codons of *cdeA* and the last 13 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. A total of 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 (~10^4 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 ml^{-1}, ethidium bromide (EtBr) 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 *C. violaceum* cells (OD_{600} of 4) using methods described previously [25]. Droplet digital PCR was performed on Bio-Rad's QX200 Droplet Digital PCR 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 H_2O 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 20 sec, 58°C for 20 sec, and 72°C for 20 sec. 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 *C. violaceum* 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 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. When indicated, antibiotics were added to the culture medium every day except for gentamicin, which was added every other day for the first week and every day after that. 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 *C. violaceum*. The initial OD_{600} in the dual-species culture was 0.05 (2–4 × 10^7 cells per ml) for *B. thailandensis* and 0.005 (2–4 × 10^6 cells per ml) for *C. violaceum*. When indicated, *C. violaceum* 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 *C. violaceum* was selected with trimethoprim. Wild type and the $CviIR^-$ mutant were differentiated by color as described above.

Results

C. violaceum quorum sensing is important for antimicrobial resistance

In transcriptomics studies of saprophytic Proteobacteria, quorum sensing commonly activates expression of genes predicted to be 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*–*C. violaceum* laboratory model as a starting point, we sought to test the hypothesis that *C. violaceum* quorum sensing might increase resistance to the *B. thailandensis* bactobolin antibiotic. To test this hypothesis, we supplemented LB agar plates with *B. thailandensis* culture fluid, and compared the growth of *C. violaceum* 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). We determined that wild-type *C. violaceum* was more resistant to *B. thailandensis* culture fluid than either of

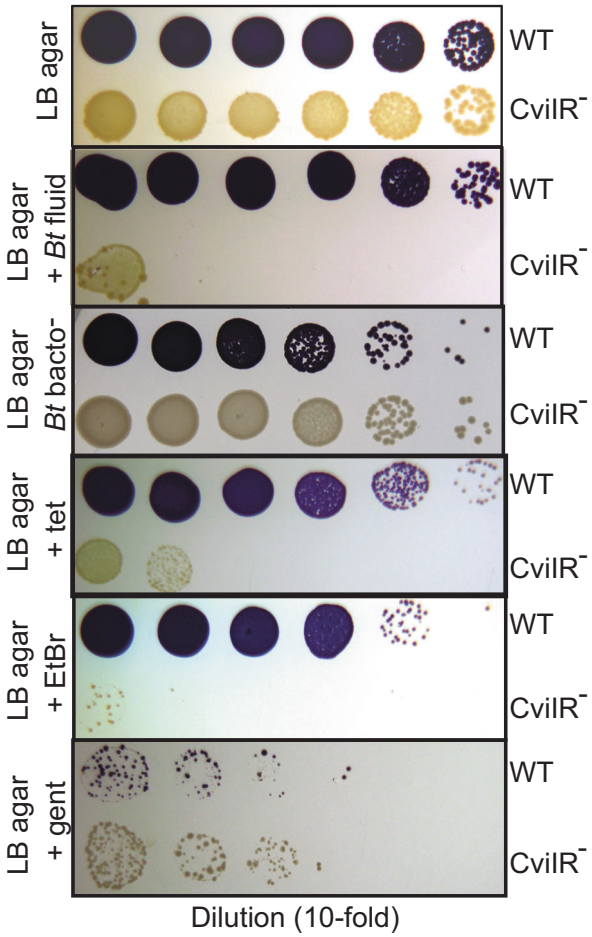

Fig. 1 Quorum sensing increases *C. violaceum* resistance to antimicrobials. Growth of *C. violaceum* wild type (WT) or the AHL synthase, receptor double mutant (CviIR$^-$). The wild type is purple due to production of the quorum sensing-dependent purple pigment violacein. Stationary-phase cultures were prepared in 10-fold serial dilutions in Luria-Bertani broth, and 10 µl 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 Materials and Methods), 1% culture fluid from the bactobolin-deficient *B. thailandensis* strain (BD20), 0.4 µg per ml tetracycline (tet), 60 µg per ml ethidium bromide (EtBr), or 8 µg per ml gentamicin (gent). Photos were taken after 24 h growth at 30°C and an additional 24 h growth at room temperature

Table 1 Antimicrobial susceptibility of *C. violaceum* 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*	0.3*	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 ~104° C. violaceum 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

the quorum-sensing mutants, with an MIC of 1.7% compared with 0.6% for both mutants. We could rescue resistance to the AHL synthase mutant by supplementing the LB agar plate with synthetic C6-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 1 or 3% cell-free fluid from a *B. thailandensis* bactobolin-deficient mutant (Fig. 1 and Table 1). These results indicated *C. violaceum* quorum sensing is important for bactobolin resistance.

We also evaluated the importance of quorum sensing for resistance to other antimicrobials. We tested growth of our *C. violaceum* strains on plates supplemented with the antimicrobials tetracycline, gentamicin, or ethidium bromide (EtBr). Our results showed that quorum sensing increases *C. violaceum* resistance to tetracycline and EtBr but had no effect on gentamicin susceptibility (Fig. 1 and Table 1). Altogether our results support the conclusion that *C. violaceum* 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 EtBr 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 *C. violaceum* [23], and because genetic mutations that increase resistance often occur through efflux pumps, providing a relatively simple approach to identify efflux pumps with specificity for bactobolin. We used a previously described approach [16], detailed in the Supplementary Information, to isolate two bactobolin-resistant variants in the otherwise sensitive CviIR$^-$ mutant. 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 EtBr, but there was no difference in resistance to gentamicin, 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 [23]. 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 CV026R. 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 *P. aeruginosa* [33]. We named the TetR-family gene *cdeR* and the downstream genes *cdeAB-oprM* (***C****hromo-bacterium* **d**rug **e**xporter).

We sought to determine whether the H127Y mutation in CdeR increased bactobolin resistance and whether *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 *C. violaceum* 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 an approximately sixfold decrease in bactobolin resistance relative to the wild type and similar decreases in resistance for tetracycline and EtBr (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.

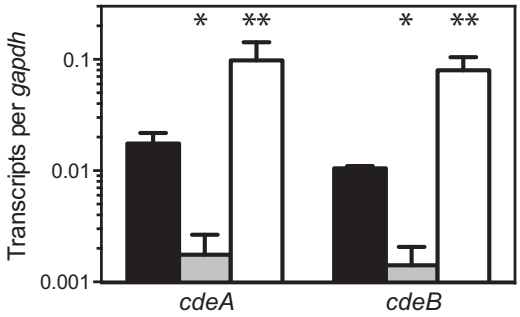

Fig. 2 Quorum-sensing induction of *cdeA* and *cdeB* transcription. Quantitative digital PCR was used to quantify *cdeA* and *cdeB* transcripts in cells from stationary-phase cultures (optical density of 600 nm [OD_{600}] of 4). Shown are *cdeA* and *cdeB* transcripts from wild type (WT, black bars), 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 encoding 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: *, $p \le 0.005$; **, $p \le 0.05$

Antibiotics suppress the emergence of *C. violaceum* 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 *P. 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, 34]. Initially, we assessed the ability of wild type and a quorum sensing-deficient (CviIR$^-$) *C. violaceum* 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 *C. violaceum* to grow on casein.

Having established that *C. violaceum* requires quorum sensing to grow on casein, we assessed the emergence of

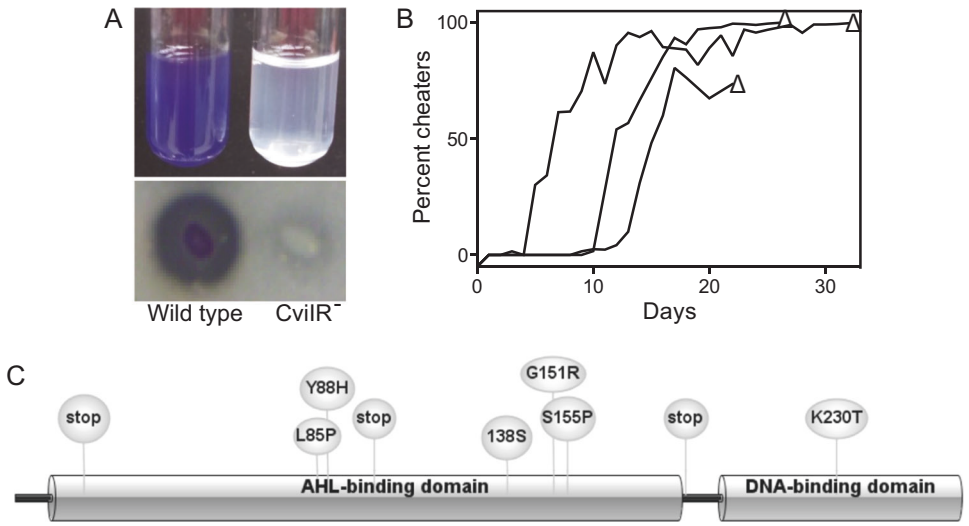

Fig. 3 Protein degradation and cheater emergence during *C. violaceum* growth on protein. **a** Growth of *C. violaceum* wild type (WT) and the AHL synthase, receptor double mutant (CviIR$^-$) after growth in minimal casein broth or on skim milk agar. Photos were taken after 24 h growth at 30°C. b Emergence of cheaters in *C. violaceum* populations passaged in casein broth. Cultures were transferred daily, and every 2–3 days, cheater abundance was determined just prior to subculture by dilution plating 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 to > 80% of the population, followed by termination of the experiment when there was no growth after transfer. **c** Location of nucleotide substitutions, deletions and insertions in *C. violaceum* cheaters mapped to the CviR amino-acid sequence. Each unique amino-acid substitution or early termination is shown in the illustration and indicated in Table S2. The AHL- and DNA-binding domains of CviR are indicated

quorum sensing-defective cheaters by passaging a *C. violaceum* 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 (owing 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 and 10 days. At a frequency of > 80% cheaters, which occurred between 22 and 33 days, the culture failed to grow after transfer 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* (nucleoside hydrolase), 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 *C. violaceum*, 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

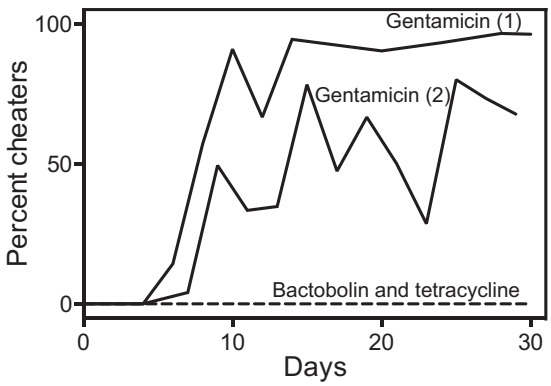

Fig. 4 Antibiotics suppress *C. violaceum* cheaters in casein broth. Cultures were grown as described in Fig. 3 except the culture media was supplemented with antibiotics. The black lines show two independent experiments of populations passaged with 12 μg ml^{-1} gentamicin. The dashed gray line shows 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

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 gray

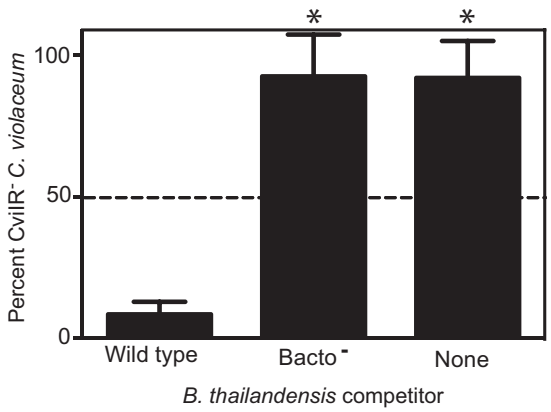

Fig. 5 Competition increases the proportion of wild-type *C. violaceum* relative to quorum sensing-defective mutants. *C. violaceum* wild type or a quorum sensing-deficient mutant (CviIR$^-$) were mixed at a 1:1 ratio then either combined with wild-type *B. thailandensis* or a bactobolin-deficient *B. thailandensis* mutant (bacto$^-$, strain BD20), or grown with no *B. thailandensis* as described in the text. The percent CviIR$^-$ mutants within the *C. violaceum* population was determined by counting the yellow colonies as a percent of the total *C. violaceum* 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 *t*-test compared with wild type: *, $p \leq 0.0001$

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, in the course of 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 [35].

Bactobolin restricts growth of quorum sensing-deficient *C. violaceum* mutants in dual-species cultures

The sensitivity of *C. violaceum* 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 *C. violaceum* AHL synthase, receptor double mutant (CviIR$^-$). Our approach was a modification of the methods used previously to grow *C. violaceum-B. thailandensis* cultures [14]. In our experiments, *C. violaceum* wild type and CviIR$^-$ mutant were mixed at a 1:1 ratio prior to combining with *B. thailandensis* (see Materials and Methods and [14]). As controls, we also grew the *C. violaceum* 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 *C. violaceum* strain by first spread-plating the entire *C. violaceum* population onto agar containing trimethoprim to select for *C. violaceum* and then enumerating the yellow (CviIR$^-$ mutant) colonies as a proportion of the total *C. violaceum* population (Fig. 5). During growth with *B. thailandensis*, the CviIR$^-$ mutant decreased from 50 to 17% of the total *C. violaceum* 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 *C. violaceum* population. These results show *B. thailandensis*-produced bactobolin selects against CviIR$^-$ mutants, supporting the idea that bactobolin stabilizes *C. violaceum* quorum sensing during competition.

Because bactobolin increases the frequency of *C. violaceum* quorum-sensing cells (Fig. 5), and *C. violaceum* quorum sensing controls production of antimicrobials [14], we hypothesized that changes in the frequency of *C. violaceum* 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$^-$ *C. violaceum* cells in our competition experiments with *B. thailandensis*. We used a *C. violaceum* 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 *C. violaceum* correlated with lower *B. thailandensis* growth (Fig. 6). Thus altering the frequency of wild-type *C. violaceum* can influence the competitive ability of the *C. violaceum* population. Together, our results support the idea that antibiotic stabilization of quorum sensing can increase competitiveness.

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

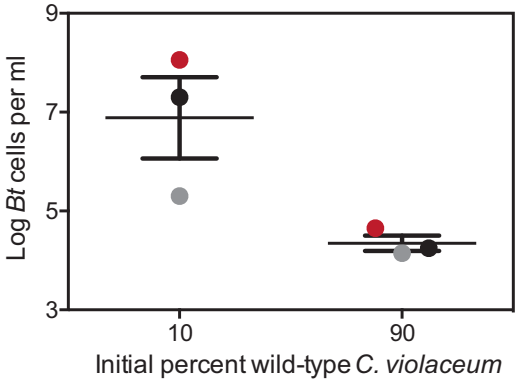

Fig. 6 *C. violaceum* competitiveness correlates with the frequency of wild type relative to quorum sensing-deficient mutants. *C. violaceum* wild type or a quorum sensing-deficient mutant (CviIR$^-$) cells were mixed together at a 1:9 or 9:1 ratio and then combined with wild-type *B. thailandensis* and grown for 24 h. 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.

[36, 37], mechanisms that allow cooperators to specifically recognize and interact with other cooperators (e.g., green-beard traits) [38, 39], and pleiotropy, or co-regulation of genes coding for self-benefiting private activities and genes coding for freely shared public goods [6, 40]. 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 [41], and through selective grazing of cheaters by protists [42, 43]. 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 a quorum-controlled antibiotic 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 [44]. Efflux pumps are known to have some fitness cost [45] 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. *C. violaceum* itself produces several antimicrobials, including the quorum sensing-regulated antibiotic violacein [17, 46]. 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' [47], 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 [14]. Broad signal-specificity receptors in some saprophytic species such as *C. violaceum* and *P. aeruginosa* might serve in this capacity [17, 48].

There is some evidence suggesting quorum-sensing control of antimicrobial resistance is not limited to *C. violaceum*. Quorum sensing has also been reported to control resistance to tobramycin in biofilm-grown *P. aeruginosa* [49]. In *Escherichia coli*, a quorum-sensing receptor increases resistance to quinolones and several other antibiotics [50, 51]. 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 [52].

Our results indicate quorum sensing in *C. violaceum* controls antibiotic resistance through the CdeAB-OprM putative efflux pump. The genes encoding CdeAB-OprM share sequence and structural similarity to other 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 *C. violaceum* and other bacteria.

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Conflict of interest The authors declare that they have no conflict of interest.

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