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EDITOR'S CHOICE

Cadmium ion inhibition of quorum signalling in *Chromobacterium violaceum*

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Abstract

Single-celled bacteria are capable of acting as a community by sensing and responding to population density via quorum signalling. Quorum signalling in *Chromobacterium violaceum*, mediated by the *luxI/R* homologue, *cviI/R*, regulates a variety of phenotypes including violacein pigmentation, virulence and biofilm formation. A number of biological and organic molecules have been described as quorum signalling inhibitors but, to date, metal-based inhibitors have not been widely tested. In this study, we show that quorum sensing is inhibited in *C. violaceum* in the presence of sub-lethal concentrations of cadmium salts. Notable Cd^{2+}-inhibition was seen against pigmentation, motility, chitinase production and biofilm formation. Cd-inhibition of quorum-signalling genes occurred at the level of transcription. There was no direct inhibition of chitinase activity by Cd^{2+} at the concentrations tested. Addition of the cognate quorum signals, *N*-hexanoyl homoserine lactone or *N*-decanoyl homoserine lactone, even at concentrations in excess of physiological levels, did not reverse the inhibition, suggesting that Cd-inhibition of quorum signaling is irreversible. This study represents the first description of heavy metal-based quorum inhibition in *C. violaceum*.

INTRODUCTION

Quorum signalling (QS), first described in *Vibrio fischeri*, allows single-celled bacteria to regulate gene expression as a function of cell density [1, 2]. In QS, signalling molecules, also called autoinducers, are secreted into the environment. Many proteobacteria utilize *N*-acylated homoserine lactones (AHLs) as signalling molecules [3]. *Chromobacterium violaceum* is a Gram-negative soil β-proteobacterium that utilizes *N*-hexanoyl (C6 HSL) and *N*-decanoyl-L-homoserine lactone (C10 HSL) as quorum signals [4, 5]. When these AHLs reach a critical concentration, they re-enter the cell and bind to a DNA-binding protein, CviR transcriptional regulator, which allows activation of *C. violaceum* quorum-regulated genes [5], including biofilm formation [6], chitinase production [7] and genes involved in violacein synthesis [5, 8]. Violacein is the dark purple tryptophan derivative [8] that promotes interspecies competition in a mixed-culture environment [9]. Binding of C6 or C10 HSL to CviR also results in binding to the *cviI* promoter, increasing transcription of *cviI* and, as a result, the amount of AHL that is produced. This positive feedback loop is highly sensitive [10] and allows for synchronicity in the activation of QS [11]. *C. violaceum* ATCC 12472 is well characterized and often serves as a model for Gram-negative QS since pigmentation is a quantifiable quorum-regulated phenotype [12].

During a recent investigation of *Burkholderia multivorans,* Vega *et al.* [13] noted that low concentrations of Ni^{2+} and Cd^{2+} (0.1–0.5 mM) interfered with biofilm formation and reduced transcription of the *luxI* and *luxR* homologues, *bmuI* and *bmuR*. Here, we report the inhibition of QS in *C. violaceum* by Cd^{2+}. In contrast to a previously tested QS inhibitor, indole [14], Cd^{2+} inhibition could not be counteracted by the addition of the cognate AHLs, C6- or C10-HSL, implying that Cd^{2+} inhibition of CviR is irreversible and non-competitive. This study represents the first description of heavy metal-based QS inhibition in *C. violaceum*.

METHODS

Strains and culture conditions

C. violaceum ATCC 12472 was obtained from the American Type Culture Collection (Manassas, VA) and stored as

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Abbreviations: AHL, *N*-acyl homoserine lactone; C6 HSL, *N*-hexanoyl homoserine lactone; C10 HSL, *N*-decanoyl homoserine lactone; LB, Luria-Bertani; MH, Mueller-Hinton; OD_{600}, Optical density at 600 nm; q-RT-PCR, quantitative reverse transcription polymerase chain reaction; QS, Quorum signaling; TBE, Tris Borate EDTA; TSA, Tryptic Soy Agar.

Two supplementary tables and three supplementary figures are available with the online Supplementary Material.

frozen stock cultures at −80 °C with 12.5 % (v/v) glycerol used as a cryoprotectant. Prior to experimentation, frozen culture samples were streaked on Luria–Bertani (LB) agar and grown overnight at 30 °C to check for purity. For starter culture preparation, individual colonies were transferred to 5 ml LB broth and grown overnight in a shaking water bath (30 °C, 150 r.p.m.). For culture inoculation, the starter culture was adjusted to OD_{600} 0.2 using sterile LB broth, then 1 ml (2 % v/v inoculum) was used to inoculate 50 ml LB in a 125 ml flask. The flask was incubated in a shaking water bath (30 °C, 150 r.p.m.). Each experiment was repeated a minimum of three times. In all cases, OD_{600} readings were converted to c.f.u. ml^{-1} using experimentally derived OD_{600} calibration curves (Fig. S1, available in the online Supplementary Material) to account for differences in OD_{600} readings based on the amount of Cd^{2+} in the LB. Negative controls were grown in LB with no added cadmium. High-resistance water (18 MΩ/cm^2) was used for all media and solution preparations.

Toxicity and quorum inhibition screening

C. violaceum was screened for inhibition of QS by plating as a lawn on LB agar, Tryptic Soy Agar (TSA) and Mueller–Hinton Agar (MH). Wells of diameter 6 mm were made in the centre of each agar plate. Each well was filled with 100 µl of 10 or 40 mM metal salt solution (Table S1) or de-ionized water and incubated at 30 °C overnight. Inhibition of QS was indicated by an absence of pigment in areas with growth [4, 15].

Growth curves

C. violaceum was grown at 30 °C for 48 h in microtitre plates in 200 ul LB broth with a gradient of $CdCl_2$ (0–0.5 mM) using a plate reader as described [16], in which OD_{600} readings were taken every 20 min after a brief agitation.

Violacein quantification

Violacein pigment production was measured using the protocol described by Blosser *et al.* [12]. Briefly, cultures of *C. violaceum* were grown in 2 ml LB treated with a gradient of $CdCl_2$(0–0.5 mM) for 22–24 h at 30 °C while shaking at 150 RPM Following incubation, 1 ml culture was assayed for optical density at 600 nm (OD_{600}), and 200 µl had violacein extracted. Cells were lysed in a 5 % sodium dodecyl sulfate (SDS) solution, 500 µL water-saturated butanol was added, then vortexed vigorously for 15 s. Eppendorf tubes were centrifuged at 12 300 ***g*** for 10 min to separate the violacein-containing organic phase from the aqueous phase, then 200 µl of the organic phase was read in a plate reader at 585 nm. Absorbance values were normalized against cell number, determined from OD_{600} values using experimentally derived calibration curves to account for differences in spectrophotometric readings of cells grown with Cd^{2+}.

AHL supplementation

Cultures of *C. violaceum* were grown in 5 ml LB supplemented with 0.5 mM $CdCl_2$ (50 µl from a 0.05M stock solution) or an equal volume (50 µl) of sterile water, with the addition of C6- and C10-HSL at different concentrations for 22–24 h at 30 °C with shaking at 150 r.p.m., according to Hidalgo-Romano *et al.* [14]. Briefly, C6- or C-10 HSL in ethyl acetate was added to a test tube, then incubated at room temperature overnight to allow evaporation of the ethyl acetate. LB with or without 0.5 mM $CdCl_2$ was added and tubes were vortexed vigorously to dissolve the HSL into the media, then inoculated and grown at 30 °C for 22–24 h while shaking at 150 r.p.m. Violacein was extracted and measured as described above.

Chitinase production

Chitinase activity was quantified in *C. violaceum* cultures, using a dye-release assay as previously described [14, 17] at

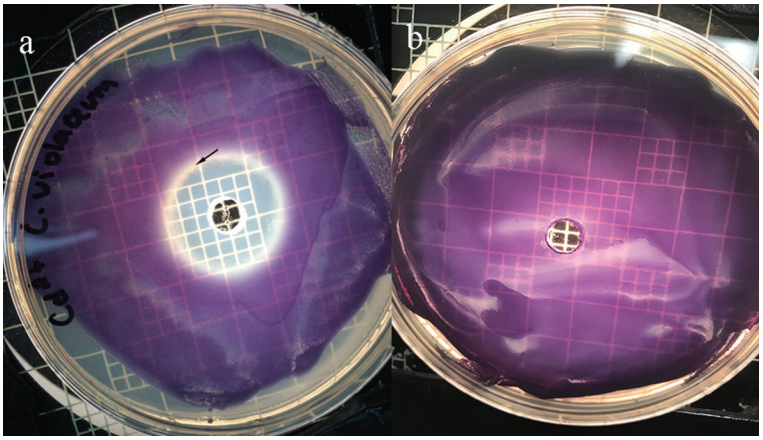

Fig. 1. Screening for *C. violaceum* QS inhibition in the presence (Fig. 1a) and absence (Fig. 1b) of Cd^{2+}. *C. violaceum* growth is purple, QS-inhibited growth is the white non-pigmented region (arrow) and the clear region is the zone of inhibition for growth. Details are in the text.

$CdCl_2$ concentrations of 0 and 0.5 mM. The assay was repeated using 0.2 U commercial chitinase from *Streptomyces griseus* (Sigma C6137) to rule out interference between cadmium ions and the chitinase enzyme. Chitinase activity was determined using a spectrophotometer (560 nm) to detect the amount of Remazol brilliant violet 5R dye released.

Biofilm assay

Biofilm assays using a crystal violet staining protocol were carried out as previously described by O'Toole [18]. Briefly, biofilms were cultured in 96-well microtitre plates by inoculation with 200 µl culture for 22–24 h at 30 °C while shaking at 150 r.p.m. Planktonic cells were washed from the cells using PBS, then biofilms were stained using 0.1 % (w/v) crystal violet solution. Unbound dye was washed from the wells and bound crystal violet was eluted using 30 % acetic acid. Eluted dye was diluted four-fold into de-ionized water, and then the absorbance (550 nm) was determined using a plate reader.

q-RT-PCR

C. violaceum cultures grown in 0.5 mM $CdCl_2$ or sterile water-treated LB media for 8 h (c.f.u.≈1.5×10^8) were treated with RNAProtect Bacteria Reagent (Qiagen 76506) to stabilize RNA. Total RNA was extracted using the RNEasy Plus Mini Kit (Qiagen 74134) according to the manufacturer's instructions. RNA quality was checked by running on 2 % agarose gel with TBE (Fig. S2). cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814). TaqMan q-RT-PCR was performed using PrimeTime Gene Expression Master Mix (IDT 1055772) and custom-designed PrimeTime qPCR probes and primers (IDT) (Table S2). q-RT-PCR was performed in the QuantStudio 3 Real-Time PCR system using a fast block. The q-RT-PCR reaction mix (20 µl) was heated for 3 min at 95 °C, then 40 cycles of denaturing at 95 °C for 5 s and annealing at 56 °C for 30 s. Transcript levels were normalized using *pykF* expression.

RESULTS AND DISCUSSION

Various organic and biological QS inhibitors have previously been described that typically act on the LuxR protein. Organic inhibitors include furanones [19], which destabilize LuxR [20], acetylsalicylic acid (aspirin),= and many autoinducer analogues that inhibit AHL binding sites [21, 22]. Biological inhibitors are frequently extracted from other organisms. Various plant extracts, for example guava leaf extract [23], have been found to inhibit QS in Gram-negative bacteria. Enzymes that inactivate AHLs have also been identified in several bacteria [24]. To date, only one metal-based QS inhibitor has ever been identified in *Burkholderia multivorans* [13], and here we report the first metal-based QS inhibitors in *C. violaceum*.

Cadmium inhibition of QS does not affect growth

When working with heavy metals in a biological system, the question of toxicity effects must be addressed. The binding of cadmium to DNA can inhibit gene replication and prevent the growth of microorganisms [25]. Cadmium can also interfere with proteins, by binding to co-factor binding sites [26] or preventing formation of tertiary structure [27].

Cadmium is known to be competitive with antagonists of calcium and magnesium in DNA binding [25], and has previously been shown to be lethal in *Escherichia coli* at 6 ppm when magnesium availability is limited [28]. Previous studies have also found that cadmium is effective in regulating gene expression in eukaryotic cells [29].

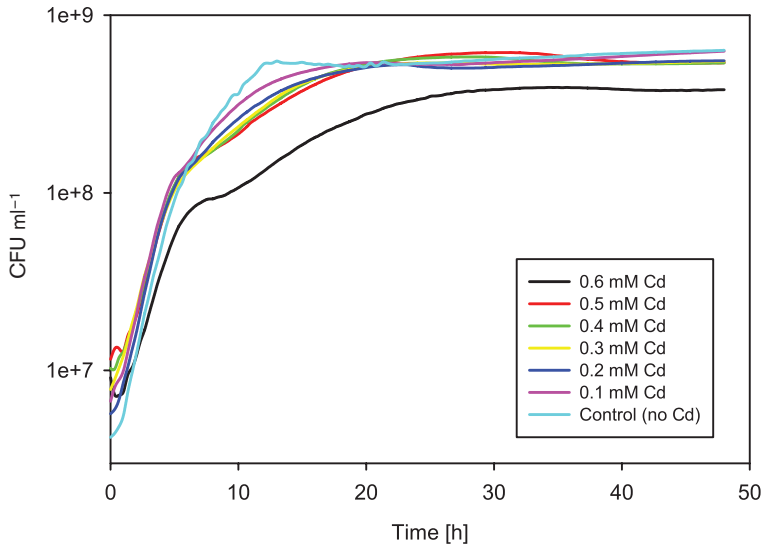

Fig. 2. Growth dynamics of *C. violaceum* with varying concentrations of Cd^{2+} (*n*=3).

Cadmium has also been shown to act as an inhibitor via competitive inhibition of enzymes that utilize manganese [30–32].

Screening of 12 metal ion salts (Table S1) revealed that cadmium was effective in inhibiting QS at sub-lethal concentrations (Fig. 1). To rule out effects by the chloride anion in $CdCl_2$, we also tested $Cd(NO_3)_2$ and saw no difference. We noted negligible amounts of inhibition by Ni^{2+} and Co^{2+}, and no inhibition by any of the other metal salts tested. Forty-eight-hour growth curves revealed that at 0.5 mM or below, $CdCl_2$ was not lethal and *C. violaceum* displayed normal growth characteristics (Fig. 2). Toxicity effects become apparent at 0.6 mM (Fig. 2).

Cadmium inhibition of QS is a global effect and is not dependent on signal availability

Cadmium inhibits QS in *C. violaceum,* as evidenced by a reduction in violacein production, chitinase production and biofilm formation. The presence of cadmium resulted in a significant decrease in violacein production (Fig. 3a), and in the absence of motility (Fig. S3). Addition of C6 and C10 HSL dids not restore the QS phenotype (Fig. 3b, c) even at concentrations well in excess of the physiological levels (approximately 1 uM) [5], suggesting that cadmium inhibition of QS in *C. violaceum* occurs through non-competitive interference rather than by competitive binding of Cd^{2+} in the AHL binding site. The apparent decrease in the amount

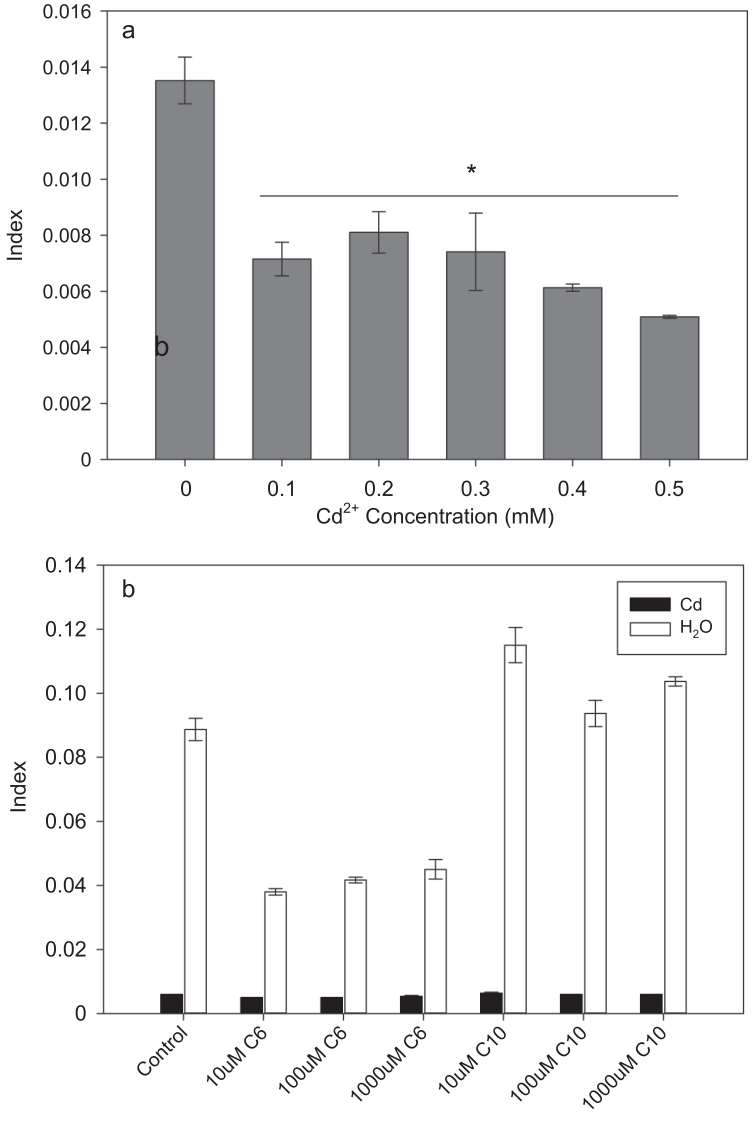

Fig. 3. Violacein production in *C. violaceum*. Fig. 3(a) Violacein production in *C. violaceum* is significantly inhibited at all concentrations of Cd^{2+} (*=P<0.05; one-way ANOVA, n=3). In Fig. 3(b), addition of increasing concentrations of C6 HSL or C10 HSL in the presence of 0.5 mM Cd^{2+} did not restore violacein production. These differences between control (H_2O) and Cd-exposure were all highly significant under each experimental condition (P<0.0001, Student's t-test, n=3). Index=A585/log(c.f.u. ml^{-1}). Error bars in all figures represent SE.

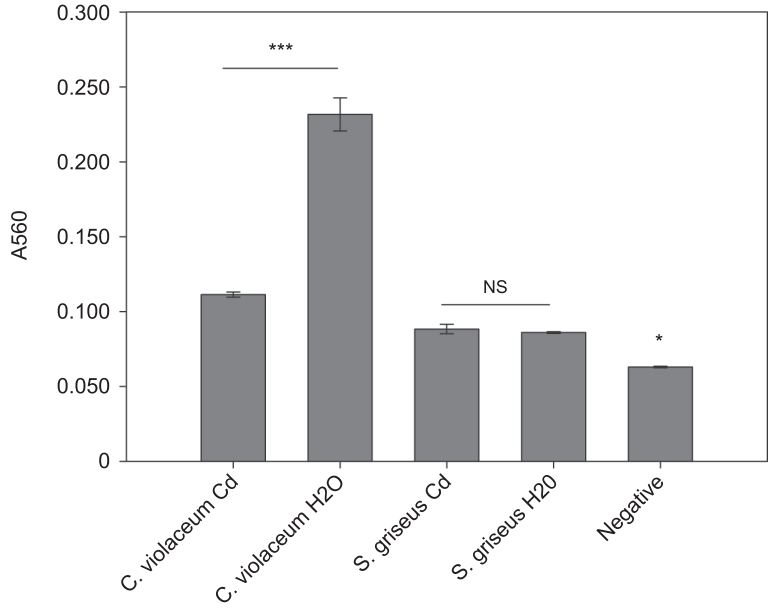

Fig. 4. Chitinase activity in *C. violaceum*. *C. violaceum* chitinase is significantly inhibited in the presence of Cd^{2+} (***P<0.001). However, the addition of Cd^{2+} to commercially available *S. griseus* chitinase enzyme had no significant effect (NS, P=0.700), whereas *chiA* transcript levels (Fig. 6) were reduced. The negative control illustrates low levels of dye release in the absence of enzyme, and these were significantly lower than in all experimental treatments (*P<0.05) (Student's *t*-test, n=3).

of violacein produced in untreated cultures when incubated with C6 HSL is likely to be an artefact of the assay, since violacein with an absorption maximum of 585 nm [12] can interfere with OD_{600} readings. Chitin digestion by *C. violaceum* is inhibited in 0.5 mM $CdCl_2$. In order to investigate possible direct Cd-inhibition of chitinase enzyme activity, we tested the activity of commercially available chitinase from *S. griseus* in the presence of 0.5 mM $CdCl_2$ and observed no inhibition (Fig. 4), so we interpret Cd-inhibition as not being caused by direct inhibition of the enzyme.

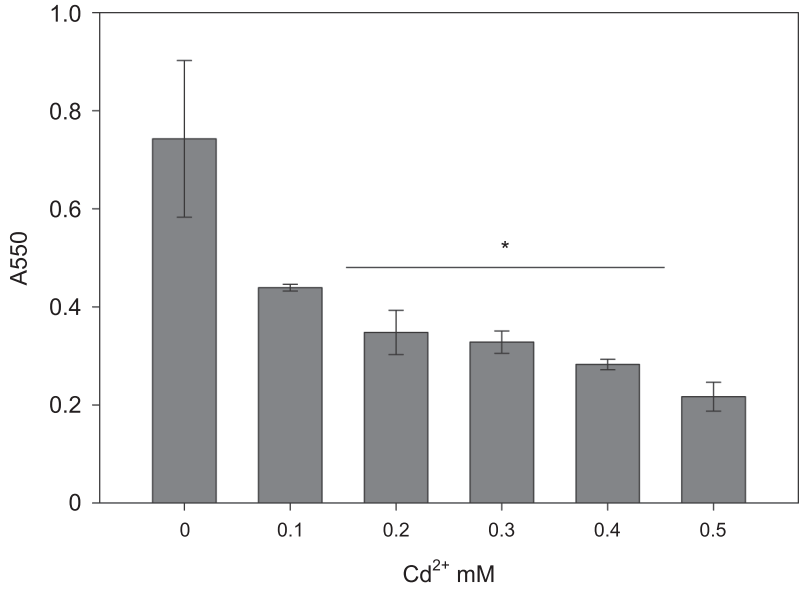

Fig. 5. Biofilm formation in *C. violaceum*. Biofilm formation is significantly inhibited in *C. violaceum* in the presence of all cadmium concentrations tested. (*P<0.05, one-way ANOVA, n=3)

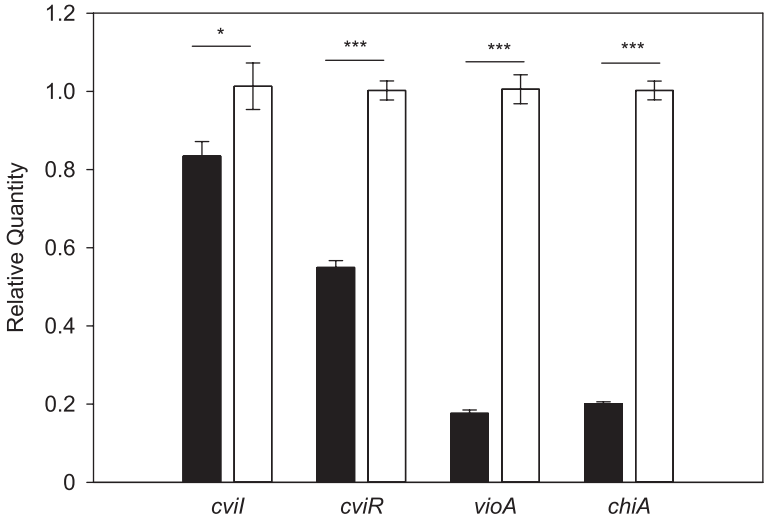

Fig. 6. Transcription levels of QS-related genes in *C. violaceum* in the presence (filled bars) or absence (open bars) of 0.5 mM Cd^{2+}. All expression levels are expressed as relative to *pykF* expression. (**P*<0.05, ****P*<0.001, Student's *t*-test, *n*=3). The difference in *pykF* expression was not significantly different (*P*=0.768, Student's *t*-test) between Cd-treated and control samples.

We also observed that biofilm formation is significantly inhibited in the presence of $CdCl_2$ (Fig. 5).

Cadmium inhibits QS in *C. violaceum* by inhibiting transcription of *cviR*

Analysis of the transcript levels of *cviI*, *cviR*, *vioA* and *chiA* indicates that inhibition of QS occurs by down-regulation of *cviR* mRNA production (Fig. 6). The down-regulation of *cviI* is also significant. Possibly *cviI* down-regulation may be due in part to the reduced CviR transcriptional regulator and its associated, positive feedback regulation, but is not likely to be biologically relevant [11].

During AHL-based QS in *C. violaceum*, the C6 HSL signal molecule binds to CviR (LuxR homologue), causing a dimerization of this protein [27] so it can then bind to several QS-controlled DNA binding sites [5]. QS inhibition can occur through a number of mechanisms, including disruption of the CviR dimerization process [27], interference with DNA recognition and, in the case of furanones, accelerating the turnover of LuxR homologues [20]. Some inhibitory actions may involve reversible binding of AHL analogues, which can be removed through competitive inhibition by the cognate signal molecule. In previous work [14], we observed that indole inhibition of QS in *C. violaceum* could be reversed by competition with C10 HSL. Other investigators have observed competitive AHL-based quorum inhibition in other Gram-negative bacteria [33]. To our knowledge, there has been only one description of non-competitive inhibition in Gram-positive *Enterococcus faecalis*, in which QS is mediated by small peptides [34]. While the study by Chen *et al.* [27] suggests that non-competitive QS inhibition and inhibition of CviR dimerization is a possible inhibitory mechanism, to our knowledge no other studies have explored this. One likely contributing factor in the present study may be the reduced *cviR* transcripts seen in the presence of Cd^{2+} (Fig. 3b). The significance of non-competitive and irreversible quorum inhibition is that the target bacterium would need to synthesize the necessary LuxR and possibly LuxI proteins *de novo*, in order to regain quorum-mediated physiology. In contrast, a bacterium could counteract competitive quorum inhibition by synthesizing excess signal, since the LuxI/R proteins would remain functional.

In summary, Cd^{2+} exhibits an inhibitory effect on *C. violaceum* AHL-based QS by non-competitive inhibition, by inhibiting the expression of the *luxR* homologue, *cviR*. While Cd^{2+} also caused inhibition of the *luxR* homologue (*bmuR*) in *B. multivorans* [13], the inhibitory effect could partially be reversed by the addition of 400 mM C8 HSL (the *B. multivorans* cognate AHL). In contrast, Cd-inhibition of *C. violaceum* QS could not be reversed by the addition of the cognate C6 or C10 HSLs at much higher (1 mM) concentrations (Fig. 3b). Finally, *B. multivorans* QS was inhibited by two other metals (Ni^{2+} and Co^{2+}) [13], whereas total QS inhibition in *C. violaceum* was limited to that by Cd^{2+}. We conclude that metal ions represent a potential mechanism for quorum inhibition.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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