EFFECTS OF COBALT ON *CHROMOBACTERIUM VIOLACEUM* QUORUM SIGNALING IN THE ABSENCE AND PRESENCE OF OXYGEN

by

Sahar Kianarsi, B.S.

A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
May 2019

Committee Members:

Robert JC McLean, Chair

Manish Kumar

Karen Lewis

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

DEDICATION

This thesis is dedicated to my husband, Dr. Danial Faghihi, who encouraged me to achieve my dream. Thank you, Danial, for your love and support.

ACKNOWLEDGEMENTS

I would first like to thank you Dr. Robert JC McLean for his incredible support and mentorship of my work on this thesis. I would also like to thank you my lab mates, Starla Thornhill and Quentin DiPasquale for the fun and support. I would like to thank you my committee members Dr. Manish Kumar and Dr. Karen Lewis for their input and constructive advices. I would also like to thank you Drs. Rodriguez, Garcia, Carlos for use of their lab facilities.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

Abbreviation	Descriptions
AHL	N-acyl homoserine lactone
AI	Autoinducer
C6HSL	N-hexanoyl-L-homoserine lactone
C10HSL	N-decanoyl-L-homoserine lactone
EPS	Extracellular polymeric substance
LB	Luria Broth
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
OD600	Optical Density at 600 nm
OHHL	3-oxo-hexanoyl-homoserine lactone
RMP	Revolutions Per Minute
RT-PCR	Reverse-transcriptase PCR
TEM	Transmission Electron Microscopy
TTC	Tetrazolium Chloride

ABSTRACT

Bacteria exist as colonial organisms, that utilize signaling system for communication. Quorum signaling is a cell-density dependent cell-to-cell bacterial communication which regulates several phenotypes such as biofilm formation, pigmentation, and virulence. *Chromobacterium violaceum* is a gram-negative, facultative anaerobic bacterium dwelling in the soil and water of tropical environments. Quorum signaling is responsible for the *C. violaceum* biofilm formation and deep purple pigment (violacein) production. Several organic and biological molecules have been identified as quorum signaling inhibitors. As well, recent studies showed that sub-lethal concentrations of heavy metals such as cadmium and nickel also inhibited *C. violaceum* quorum signaling resulting in reduced violacein production, virulence, biofilm formation, and quorum-regulated gene expression. In the current study, I showed that cobalt inhibited suspended cell aggregation (floc biofilm formation) in both aerobic and anaerobic conditions but did not affect surface-attached biofilm. Also, cobalt inhibited expression of several genes involved in quorum signaling. This study is the first report of the effects of heavy metal on quorum signaling inhibition of *C. violaceum* in anaerobic condition.

1. INTRODUCTION

Bacteria utilize quorum signaling as a density-dependent cell-cell communication. Bacteria secrete different signaling molecules called autoinducers (AIs) to their surrounding environment which results in communication between either same genus or in mixed-culture bacteria. In gram negative bacteria AIs include diketopiperazines (DKPs), N-acyl homoserine lactones (AHLs) and AI-2 type signals [1]. Gram positive bacteria utilize a specialized transport system to secrete oligopeptide AIs [2]. Quorum signaling was first described in the bioluminescent marine bacterium *Vibrio fischeri* which utilizes Lux system, in which the LuxI protein (gene product of *luxI*) synthesizes the AHL signal, N-(3-oxohexanoyl) homoserine lactone (3-o-C6 HSL), and the LuxR protein (gene product of *luxR*), functions as a transcriptional regulator in response to a critical threshold of AHL signal. Other AHL quorum signaling systems have analogs to *luxI* and *luxR* [3] with the AHL signals having species-specific differences in the acyl moiety. Two other AHL-based quorum systems are the Las and Rhl systems in *Pseudomonas aeruginosa* in which the *luxI/R* homologs are *lasI/R* and *rhlI/R* respectively and the AHLs being *N*-3-oxo-dodecanoyl homoserine lactone (3-o-C12 HSL, for the *lasI/R* system) and *N*-butanoyl homoserine lactone (C4-HSL, for the *rhlI/R* system) [2].

Quorum signaling and expression of AIs is responsible for virulence, pigmentation, motility, and genes regulation of biofilm formation [4]. Quorum signaling is known as a positive feedback loop, because when the LuxR transcriptional protein interacts with AHL signals, it binds to the *luxI* promoter and therefore increases *luxI* expression and AHL signal production [5]. Positive feedback in *V. fischeri* happens when

exogenous increase of 3-o-C6 HSL (signal) activates the promoter of LuxI by LuxR which results in increase expression of LuxR [5]. Both gram positive and gram negative bacteria exploit quorum signaling through positive feedback loop, thus signals called "autoinducers" [2].

Chromobacterium violaceum is a gram negative bacterium commonly found in soil and water in tropical region. *C. violaceum* employs N-hexanoyl (C6 HSL) and N-decanoyl-L-homoserine lactone (C10 HSL) AHLs, as quorum signals [6] [7]. *C. violaceum* produces the purple pigment, violacein, in response to C6 HSL [8]. In this case, quorum signaling regulates by LuxI/R homologue, CviI/R, and controls biofilm formation [9], virulence, and violacein production [10], a dark purple tryptophan derivative [11]. Since the complete genome sequence of *C. violaceum* ATCC 12472 has been revealed and violacein production is a quantitative quorum-regulated phenotype [12], this bacterium is used as a model for gram negative QS characterization. Chitinase is a virulence factor in *C. violaceum* that is regulated by quorum signaling [13]. *C. violaceum* has several genes that encode chitinase [14] and utilizes chitinase to degrade chitin as a carbon source [15].

The term, biofilm, refers to a community of microorganisms in which cells stick to a surface or to each other. Cells within biofilms produce an extracellular matrix mostly composed of polysaccharides or other molecules. Proximity of organisms within biofilms results in quorum signaling, hence cells can survive biological and chemical antimicrobial agents [16]. Generally, the term biofilm is frequently related to a liquid-solid interface microbial community. Microbial flocs also exhibit similar features to biofilm groups on surfaces [17]. Bacterial flocs are suspended aggregates of EPS

encapsulated bacteria that are scattered in aquatic environments [18]. Most bacteria are motile by means of flagella, that plays an important function in biofilm formation. It has been shown that mutant *Escherichia coli* lacking flagella are unable to initiate the first few steps of biofilm formation (Figure 1)[19]. Based on the previous study, *Vibrio harveyi* QS positively regulates motility by affecting the expression of flagellar genes [20]. It has been shown that *C. violaceum* have at least 67 flagellar genes controlled by flagellar-motor supramolecular complex [21]. Thus, it is essential to investigate the link between QS and motility which affects biofilm formation. It has been shown that *C. violaceum* ATCC 12472 also is able of cellulose biosynthesis [22]. Cellulose production helps bacterial quorum signaling and adhesion in biofilm formation [23].

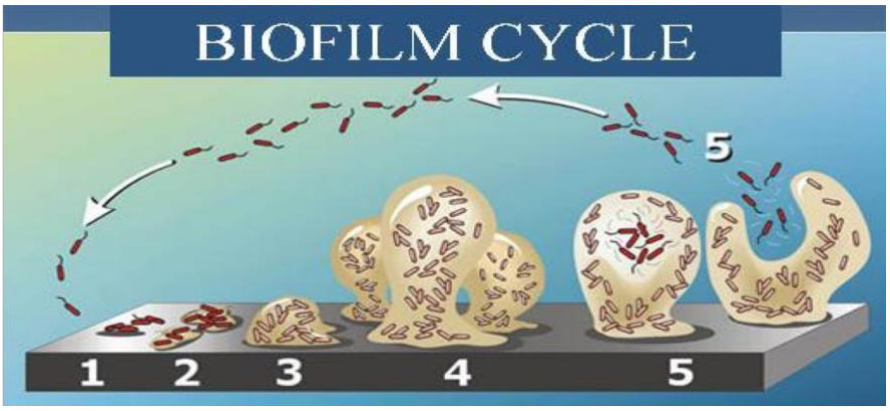

Figure 1: Biofilm formation steps. First and second steps are attachment, bacteria can adhere to each other and to a solid surface. Third and fourth steps are maturation, biofilm grow and produce extracellular polymeric matrix and become antibiotic resistant. The fifth step is release, bacteria release from the biofilm in the environment in planktonic form [24].

Several organic and biological molecules have demonstrated quorum signaling inhibition. Biological QS inhibitors have found in many plant extracts [25] [26]. Several gram positive bacteria produce lactonase and acylase enzymes that inactivate AHLs [27]. Organic inhibitors such as aspirin [28], furanones that destabilize LuxR [29] [30], and LuxI binding site inhibitors, autoinducer analogs [31] [32] have also been described.

Studies of heavy metal-based QS inhibition is more recent. In one study, low concentrations of Ni^{2+} and Cd^{2+} had inhibitory effects on biofilm formation and reduced transcription of the *luxI/R* homologues, *bmuI/R* in *Burkholderia multivorans* [10]. In another study [4], cadmium salts caused QS inhibition in *C. violaceum* resulting in a decrease of bacterial motility, biofilm formation, chitinase production, and violacein-pigmentation.

High concentrations of heavy metals are toxic for biological systems. It has been shown that binding of heavy metals such as cobalt ions to DNA terminate gene replication and inhibits growth of microorganisms [33]. Bacteria exploit different detoxification mechanisms, such as eliminating the metal ions from the cytoplasm [34], integration of metals to organic compounds [35], and hydrogen sulfide production which causes precipitation of otherwise toxic metal ions as insoluble metal sulfides [36]. Several studies have demonstrated the effects of anaerobic condition on bacterial QS. The presence of oxygen is vital for biofilm formation of laboratory strains of *Escherichia coli* [37]. Quorum signaling in *Salmonella enteritidis* was induced by AI-1 under anaerobic condition, resulting in biofilm formation [38]. To date, metal-based QS inhibitors of *C. violaceum* in anaerobic condition have not been extensively tested. This study investigates the first explanation of anaerobic heavy metal based quorum sensing inhibition in *C. violaceum*. In this regard, to attain more information on the relation between quorum sensing and molecular structure, we tested the effects of cobalt chloride susceptibility in aerobic and anaerobic conditions on quorum signaling by using RT-qPCR with *C. violaceum*.

2. MATERIALS AND METHODS

Strains and culture conditions

The bacterial strain used was *C. violaceum* ATCC 12472. Culture of *C. violaceum* was grown on Luria Bertani (LB) at 30 º on shaking incubator at 150 RMP for 24 hours. Plate culture of *C. violaceum* was grown on LB agar at 30 º. Cobalt chloride salt was added at concentrations 0.01 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM. Cobalt chloride stock solution was made by dissolving it into 1 L of high resistance "Milli Q" water (18 MΩ/cm^2) at concentration of 40 mM and filter sterilized through 0.2 µm pore-size filter.

Confocal Microscopy

Wilde type *C. violaceum* streaked on LB agar and grown overnight at 30º. Then, the individual colonies were grown in 5 ml LB and incubated at 30º in shaking incubator at 150 RPM for 24 hours. Then, *C. violaceum* culture was transferred to 5 ml LB as a control and LB plus 0.01 mM and 0.1 mM cobalt chloride. All samples were grown at 30º. Similar experiments were conducted in an anaerobic chamber. Confocal microscopy was performed using Baclite^{TM} live/dead gram stain kit. 5 µl of each sample was mixed with 0.5 µl of dye (cyto 9 and propidium) on the microscope slide.

Motility

C. violaceum was assayed for motility by using inoculating needle to stab 5 ml motility agar containing cobalt chloride [39]. *C violaceum* culture was prepared as explained before. Motility media was prepared by mixing 0.4 agar, LB, and TTC. Cobalt

chloride in different concentrations 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM were added to the tubes. Then, all inoculated media were incubated at 30º for 20-22 hours.

AHL Supplementation

Overnight *C. violaceum* colonies grown on LB agar at 30º were transferred to 5 ml LB broth and incubated at 30º in shaking incubator at 150 RPM for 24 hours. Dissolved signals in ethyl acetate were added to tubes and incubated overnight at 37º to allow the evaporation of ethyl acetate, according to Hidalgo-Romano *et al*. Motility agar was prepared as explained before. Then, tubes were filled with 5 ml motility agar, 10 mM and 100 mM of C6 and C10. In addition, different concentrations of cobalt chloride (0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM) were added to the tubes. Each tube was stabbed with overnight culture of *C. violaceum* and incubated at 30º for 20-22 hours.

Biofilm Assay

Fresh *C. violaceum* cells were streaked on LB agar and incubated overnight at 30º. Following the incubation, single colonies from the plate were inoculated in 5 mL of LB broth and incubated at 30º for 24 hours. Microtiter plates containing LB, cobalt chloride was inoculated with *C. violaceum* overnight culture. Microliter plate culture then grown in shaking incubator at 150 RPM at 30º for 24 hours. The second microliter plate culture was grown in an aerobic chamber at 30º for 20-22 hours. Planktonic cells were shaken out of the plates and the plates were washed with sterilized PBS. Then, biofilms were stained with 225 µl 1% crystal violet and incubated at room temperature for 10 minutes [40]. Unbound stain was removed using sterilized PBS three times and biofilms were eluted with 30%

acetic acid. Each well was filled with 150 µl deionized water and 50 µl cells. Then, plates were read on plate reader at 550 nm.

TEM

Overnight culture of *C. violaceum* was inoculated in 5 mL LB broth and incubated at 30º in shaking incubator at 150 RPM for 24 hours. After the initial incubation time, cultures were streaked in LB agar and different concentration of Cobalt chloride (0 and 0.3 mM) at 30º incubator for 20-22 hours. The same procedure was done for anaerobic samples in the anaerobic box. We did negative staining [41], 10 µl of 2% uranyl acetate was mixed with a loopful of each samples on a parafilm and sat for 1 minute. Then, the mixture was transferred on the TEM grid and sat for 2 minutes. Furthermore, each sample was observed using TEM.

RT-qPCR

Overnight culture of *C. violaceum* inoculated onto 5 ml LB broth and incubated at 30º in a shaking incubator at 150 RPM for overnight. Following incubation, all cultures were diluted to the fresh LB broth and 0.3 mM cobalt chloride and incubated in shaking incubator for 8 hours. The same procedure was conducted in anaerobic chamber. Total RNA was stabilized using RNAprotect Bacterial Reagent (Qiagen1018380) and extracted using RiboPure Bacterial Kit (Invitrogen AM1925). RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814) utilized lysozyme lysis and Proteinase K. qPCR was performed using PrimeTime Gene Expression

Master Mix (IDT 1055772) [39]. All primers/Probes used are shown in Table 1. Agarose Gel then performed with 5 µl samples and 2% agarose gel in TAE.

Table 1: Primers/Probes sequences used for RT-PCR.

Genes Name	Gene Function	Sequence
bscC	Cellulose synthase	Forward: 5'-GTCCAACTTCGCCTACCAG-3' Probe: 5'-CTTGCGGTTGCTGTCCACGTTC-3' Reverse: 5'-CAGAAATTGACGGTGTTGACG-3'
fliG1	Motor switch protein	Forward: 5'-ATCCAGGACAAGATGTTCGTG-3' Probe: 5'-ACCGCTCGATCCAGACCATCC-3' Reverse: 5'-TTTCCTTCAGATCGGTGCTG-3'
fliC2	Flagellin	Forward: 5'-CCAGCAACCAGATATCCGTG-3' Probe: AGGTTGGCGTTATAGCTGTTCAGGC-3' Reverse: 5'-GGTGATGTCTATCGTCTTGGTG-3'
flgM	Regulator	Forward: 5'-CTACAGCACCCAGAGCAAG-3' Probe: 5'-CTCGTGCCTCGTCCAGTTCCAG-3' Reverse: 5'-TTCACGCTGTCATCCTTGG-3'
vioA	Violacein	Forward: 5'-TTCAAGAGCCATGTCCAG-3' Probe: 5'-ACTCGTTCATCGCCCGCTTCAG-3' Reverse: 5'-TGACGAACTGGAGAAAGG-3'
chiA	Chitinase	Forward: 5'-AGTCTTGCCCCAATAACG-3' Probe: 5'-CATCGTGCAAACGCCTTACGCC-3' Reverse: 5'-CCCTTGAACACATTGCTG-3'

Table 1. Continued.

cviI	LuxI family AHL synthase	Forward: 5'-CAAGGTGGACTGGTACTG-3' Probe: 5'-ACTAAGCTGCGACAGTTGTGGGC-3' Reverse: 5'-CGGAAGATTCTGTGACGG-3'
cviR	LuxR family transcriptional regulator	Forward: 5'-ACATTGCTGGAGTGGATTC-3' Probe: 5'-CGCCCTCGGTTTCGATATGCCC--3' Reverse: 5'-ATTCTCTGGATCTGATTCTGG-3'
pykF	Pyruvate Kinase, Housekeeping	Forward: 5'-TGCTGGATGAAACGGAAG-3' Probe: 5'-ACCCGGAAGCGTTCGAGGC-3' Reverse: 5'-GTCGTTCACCAGGATCAG-3'

3. RESULTS

Confocal Microscopy

As we expected, *C. violaceum* can growth in both aerobic and anaerobic environments but prefer aerobic condition. Confocal images demonstrated that there was more biofilm formation in aerobic condition with no cobalt chloride than anaerobic condition with no cobalt chloride (Figure 2, a and d). In case of 0.01 and 0.1 mM cobalt chloride, there were more biofilm in aerobic condition (Figure 2, b, c, e, and f). Confocal images showed that cobalt chloride inhibits *C. violaceum* flocs biofilm formation in either condition. Also, Cobalt chloride has dose dependent effects on *C. violaceum*.

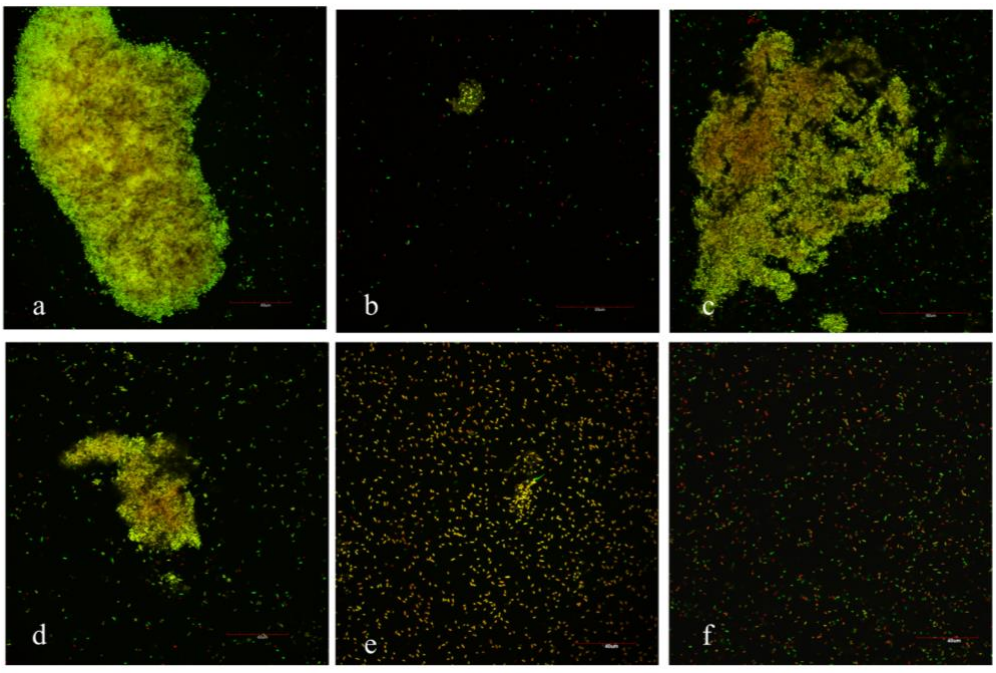

Figure 2: Confocal images. Showing Cobalt induced inhibition of biofilm formation in *C. violaceum*. a) control aerobic, b) 0.01 mM $CoCl_2$ aerobic c) 0.1 mM $CoCl_2$ aerobic, d) control anaerobic, c) 0.01 mM $CoCl_2$ anaerobic, and e) 0.1 mM $CoCl_2$ anaerobic. Red color indicates dead cells and green color indicates live cells. (60x objective) (n = 3)

Motility

Motility in *C. violaceum* was reduced in the high concentration of cobalt chloride (0.4 and 0.5 mM). Motility is indicated by red radiation away from the needle inoculation and growth is indicated by cloudy media. Organism growing in the motility media reduce colorless TTC to red-colored Formazan and indicated motile bacteria. In addition, reduced agar concentration makes the medium semi- solid, so organism can move freely. In control sample, the media was cloudy and there was an obvious red radiation away from the needle line compare to 0.5 mM sample that was less cloudy and limited red radiation was observed (Figure 3). As the cobalt chloride concentration is increased, the red radiation was limited above the needle line and media was less cloudy, this indicated that *C. violaceum* prefer oxygen.

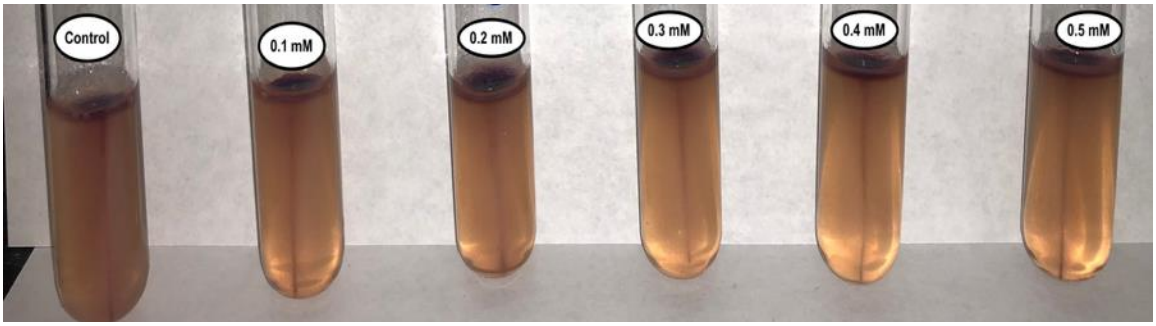

Figure 3: Motility test. From test. left to right: Motility is reduced by adding different concentration of cobalt chloride. In control, there is red radiation around the needle line and the media is cloudy due to *C. violaceum* growth. In the tube with 0.5 mM cobalt chloride, the red radiation is only observed above the needle line and the media is less cloudy compare to the control.

AHL Supplementation

As motility is a quorum-regulated phenotype in *C. violaceum*, we added signals back to the motility media to see if the inhibition is through competitive binding between AHLs and cobalt. Addition of 10 mM C10 HSL to *C. violaceum* culture with different cobalt chloride concentrations (Figure 4) had the same pattern as without AHL with cobalt (Figure 3). However, addition of 100 mM C10 HSL showed less growth and motility compare to samples without C10 HSL indicating competitive inhibition between cobalt and C10 HSL. However, addition of 10 and 100 mM C6 HSL did not restore the pattern as seen for the no AHL condition. In the sample with 100 mM C6 HSL and 0.5 mM cobalt chloride no growth was detected (Figure 4f). Thus, C6 HSL might increase toxicity of cobalt chloride.

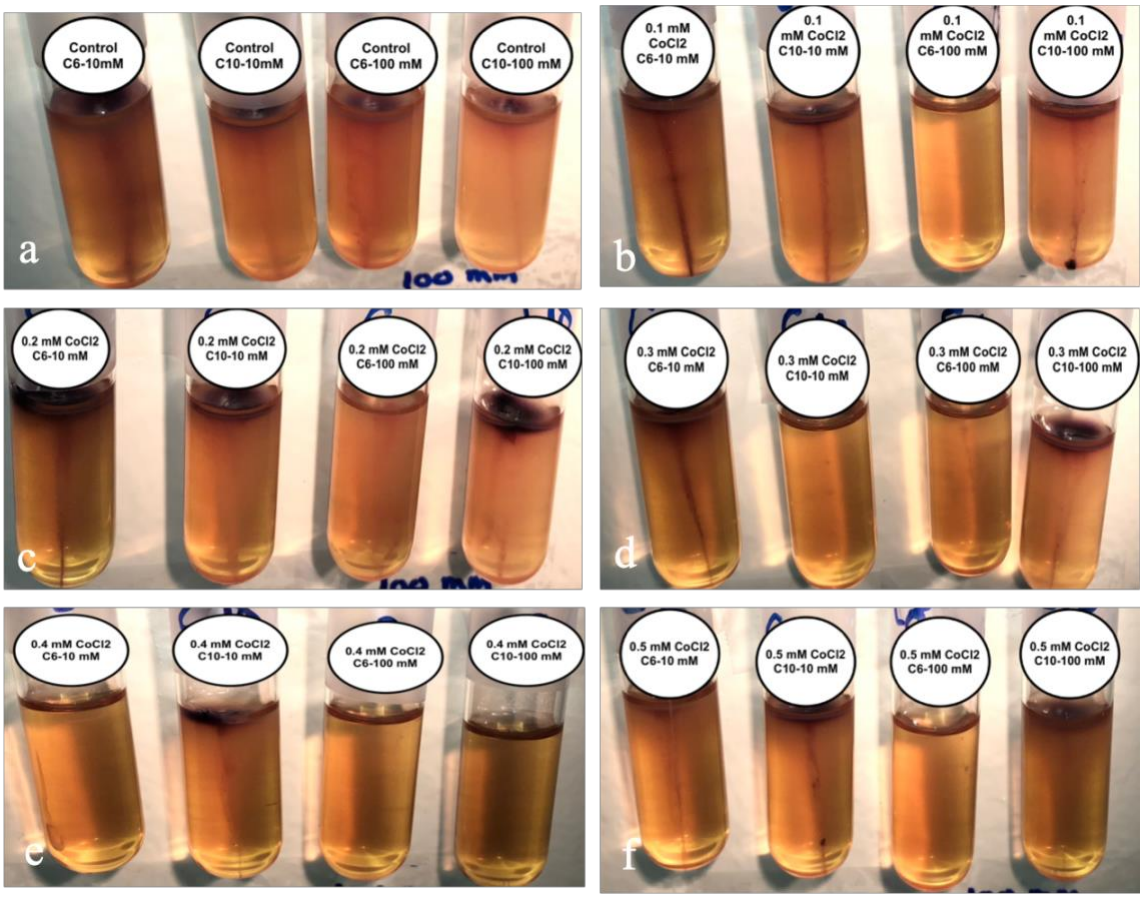

Figure 4: AHL supplementation by *C. violaceum*. Effect of AHL supplementation with two concentrations (10 mM and 100 mM) of C6 and C10 HSLs on $CoCl_2$ treatment of *C. violaceum*. Growth is shown by cloudiness of media and motility is shown by red radiation away from the stab line. a) Control no $CoCl_2$, b) 0.1 mM $CoCl_2$, c) 0.2 mM $CoCl_2$, d) 0.3 mM $CoCl_2$, e) 0.4 mM $CoCl_2$, f) 0.5 mM $CoCl_2$.

Biofilm Assay

As we expected, crystal violet staining indicated that there are notable differences in attached biofilm formation between anaerobic and aerobic conditions (Figure 5) (Table 2). *C. violaceum* can grow in the absence of oxygen but prefer aerobic condition. Also, based on the results shown in Figure 4, cobalt has no effect on attached biofilm formation of *C. violaceum* in either conditions (Table 2). However, aerobic sample with 0.2 mM $CoCl_2$ showed a difference in biofilm formation, $p = 0.058$ (Figure 5).

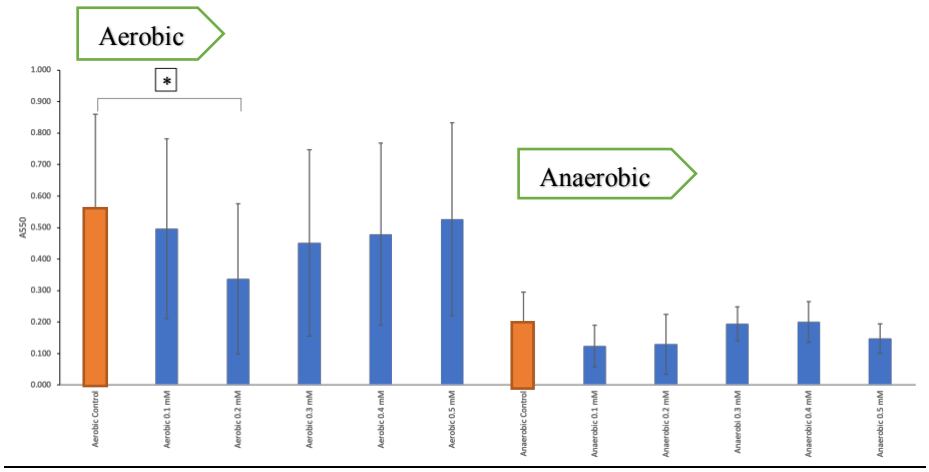

Figure 5: Quantification of biofilm formation. There is a notable difference (Table 2) in attached biofilm formation between aerobic and anaerobic conditions, while there are no significant differences (Table 2) with and without cobalt in both aerobic and anaerobic conditions. Difference marked with an asterisk (*) is statistically significant, $p = 0.058$. Error bars represent standard deviation.

Table 2: Statistical significance of biofilm formation. Under anaerobic and aerobic conditions. $p < 0.05$ (Dunnett's Test) are significantly different. This table shows the comparison between control and different concentration of cobalt chloride.

	$CoCl_2$ concentrations	**t**	**df**	**Significance**
AEROBIC	0.1 mM	-0.36479	8	0.7247
	0.2 mM	**-2.2143**	**8**	**0.05769**
	0.3 mM	-1.0086	8	0.3427
	0.4 mM	-0.7447	8	0.4778
	0.5 mM	0.20489	8	0.8428
ANAEROBIC	0.1 mM	-1.3861	8	0.2031
	0.2 mM	-1.183	8	0.2708
	0.3 mM	-0.033	8	0.9745
	0.4 mM	0.14198	8	0.8906
	0.5 mM	-1.0227	8	0.3364

Table 2. Continued.

AEROBIC vs **ANAEROBIC**	Control	2.7553	9.3609	0.02151
	0.1 mM	3.8173	8.8636	0.004224
	0.2 mM	2.429	10.491	0.03446
	0.3 mM	2.5749	8.5348	0.03121
	0.4 mM	2.8182	8.803	0.02054
	0.5 mM	3.6658	8.3721	0.00587

TEM

TEM images showed that samples treated with 0.3 mM cobalt chloride had shorter flagella than control (Figure 7, B and D). Also, aerobic samples had more flagella compare to the anaerobic ones (Figure 7, A and C). In some anaerobic treatment samples pili were observed (Figure 6).

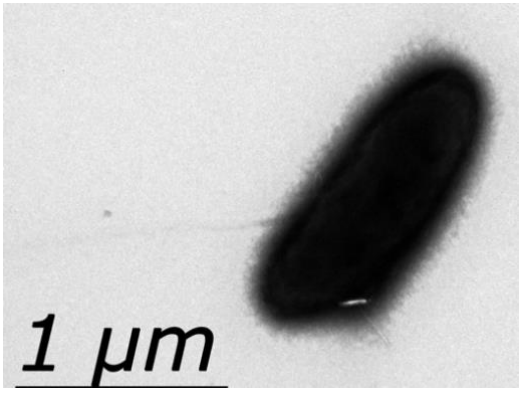

Figure 6: TEM image of *C. violaceum*, anaerobic with 0.3 mM cobalt chloride. Anaerobic samples with cobalt treatment showed shorter flagella and more pili

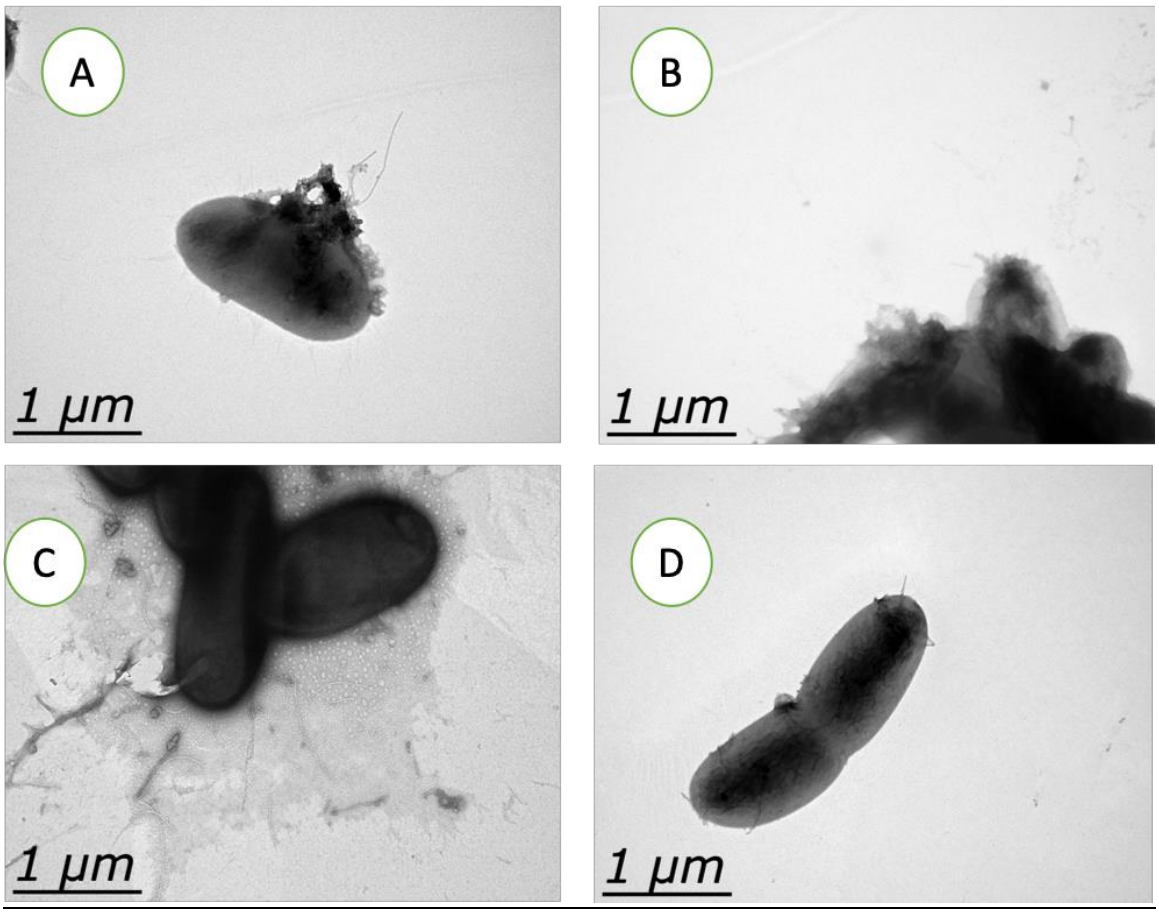

Figure 7: TEM images of *C. violaceum* in aerobic and anaerobic condition. The length of the flagella of *C. violaceum* is shorter in samples containing cobalt chloride. A) aerobic control, B) aerobic with 0.3 mM $CoCl_2$, C) anaerobic control, D) anaerobic with 0.3 mM $CoCl_2$.

RT-qPCR

We got lots of variable results, so the RT-qPCR results were inconclusive. However, we saw significant differences in gene expression with and without cobalt (Figure 8). Several genes are responsible in *C. violaceum* biofilm formation such as: *cviI/R, bscC, vioA, flgM, fliC2, fliG1*, and *chiA*. *cviI/R* are genes responsible for quorum signaling, *cviI* is signal inducer (AHLs) and *cviR* is a QS regulator. Cellulose production (*bscC*) helps bacterial quorum signaling by controlling diffusion of AHL signals as well as providing

adhesion in biofilm formation. Also, *bscC* product (cellulose) is a component of *C. violaceum* biofilm's extracellular matrix [22]. Most bacteria are motile by means of flagella, that plays an important function in initiation of biofilm formation. Previous study has shown that 67 genes are involved in chemotaxis of *C. violaceum* [21]. Some of these genes are *fliG1* is motor switch protein, *fliC2* is flagellin protein, and *flgM* is regulator. *C. violaceum* produces violacein, a dark purple pigment, which regulates by *vioA*. *C. violaceum* has several genes that encode chitinase such as *chiA*. Chitinase is a virulence factor that is regulated by quorum signaling [13]. Based on the results there was a significance difference in gene expression, *bscC* and *cviI/R*, in anaerobic samples with 0.3 mM cobalt. In case of flagella genes, *fliC2* and *flgM*, there was a significance difference in aerobic samples without cobalt. Violacein gene expression, *vioA*, showed significance differences in both aerobic and anaerobic samples.

The quantity of extracted RNA determined by the ratio of absorbance with values between 1.9-2.1 by using spectrophotometer. Additionally, the quality of extracted RNA was measured by electrophoresis. All samples had two bands representing the 16S and 23S ribosomal RNA fragments and were not degraded, which would have been indicated by a lack of bands (Figure 9).

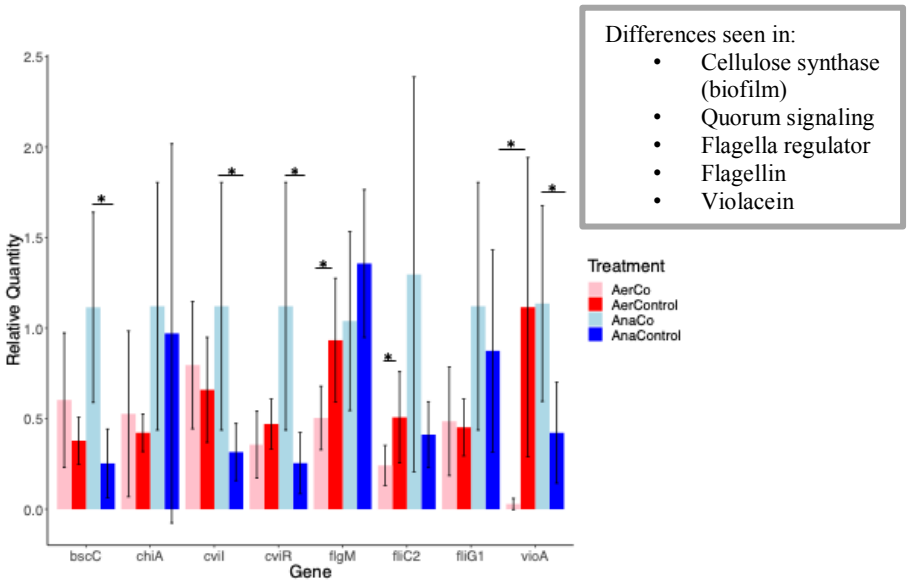

Figure 8: Transcription levels of QS-related genes in *C. violaceum*. In the presence or absence of 0.3 mM Co^{2+}. All expression levels are expressed as relative to pykF expression. Differences marked with an asterisk (*) are statistically significant from untreated samples ($p < 0.05$) (Dunnet test).

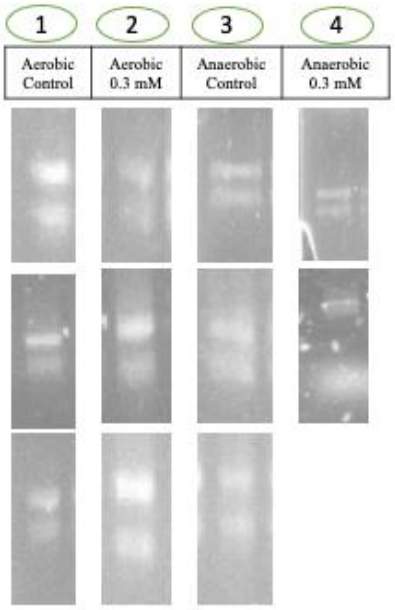

Figure 9: Image of RNA quality used for q-RT-PCR. Separate biological replicates (n = 3) of *C. violaceum* grown aerobically in LB, control (column 1), aerobically grown in LB + 0.3 mM $CoCl_2$ (column 2), anaerobically grown in LB, control (column 3), and aerobically grown in LB + 0.3 mM $CoCl_2$ (column 4). After extraction, total RNA was used for reverse transcription and qPCR tests. BioRad ™ EZ imager was used to observe gel.

4. DISCUSSION

Quorum signaling is a cell-to-cell communication that indicates the ability of bacteria to recognize and respond to chemical molecules, autoinducers. QS is a cell density dependent mechanism [42]. In Gram negative bacteria, signaling molecules are called AHLs. Here, R protein, transcriptional regulator, and I protein, autoinducer synthase, are involved in QS [43] [44]. Quorum signaling is responsible for biofilm formation, virulence, pigmentation, and motility. Biofilms are bacterial communities that are embedded in an extracellular matrix and have different phenotype compared to planktonic cells [45].

Heavy-metal based quorum signaling inhibitors have recently gained researchers' attention and required extra work to completely understand the inhibition mechanisms. Generally quorum inhibitors are non-lethal, low molecular weight, and stable molecules that cannot be metabolized [31]. Also, quorum inhibitors must be exclusive for the specific regulator, LuxR homologue. It has been shown that sub-lethal concentrations of nickel and cadmium can inhibit LuxI homologue transcription [10] [4].

Characterization of some quorum regulated phenotypes in *C. violaceum*, such as violacein production, showed that quorum signaling inhibition occurs at the transcription level [4]. In this study, we showed that cobalt inhibits floc biofilm formation in *C. violaceum*. However, cobalt did not affect attached biofilm formation in *C. violaceum*. As we expected, there was a significant difference between biofilm formation in aerobic and anaerobic conditions. *C. violaceum* prefer oxygen, so there was more biofilm in aerobic condition. We used sub-lethal concentrations of cobalt chloride (0.1, 0.2, 0.3, 0.4, and 0.5 mM) that did not have lethal effects on the cell population.

Motility in *C. violaceum* was reduced in high concentrations of cobalt chloride (0.4 and 0.5 mM). TEM images showed that *C. violaceum* flagella is shorter in samples treated with 0.3 mM cobalt chloride. Also, these images showed that there were more pili in anaerobic with 0.3 mM cobalt chloride samples compare to other samples. AHL supplementation test was done to see if the inhibition is quorum signaling related or it happens because of the competitive inhibition. Addition of C10-HSL restored the pattern of motility with cobalt, indicating competitive competition between AHL and cobalt. In both tests, red radiation was limited above the needle line which indicates that *C. violaceum* prefer oxygen. There was a significance differences between gene expression in samples with and without cobalt. *vioA*, associated with violacein production, showed cobalt-induced expression differences in both aerobic and anaerobic condition while cellulose synthesis (*bscC*), and genes involve in quorum signaling (*cviI/R*) showed differences only in aerobic samples with cobalt. Since these results were so variable, we cannot reach to a definite conclusion.

Cobalt, cadmium, and nickel are inhibitors of enzyme function [46] [47] [48] [49]. It has been shown that cobalt, cadmium, and nickel are compete with magnesium and calcium in DNA binding site [50] [33]. Cobalt and nickel are able to bind to DNA only in the presence of specific ligands [51].

Bacteria utilize several resistance mechanisms to heavy metal toxicity like metallothioneins, efflux pumps, and biofilm formation [52]. Metallothioneins are low molecular weight, cysteine-rich proteins that specifically bind to a single metal ion. Metallothioneins can be determined by the metal buffer concentrations which is required by the cells and by the Irving- Williams series [53]. Metallothioneins bind to multiple

metal ions such as cobalt [54] [55] [56], cadmium [57] [58] [59] [60], and nickel [61] [62]. Bacteria exploit efflux pumps to actively transfer toxic molecules out of the cell [63]. *C. violaceum* utilize several efflux pumps such as metal transporters like cobalt, nickel, and cadmium [64].

Biofilm formation is one of the most important resistance mechanisms exploited by bacteria. Bacteria within biofilms are more resistant to toxic metal than planktonic form by acquiring several resistance mechanisms against toxic metal compounds [65]. Aside from the previously mentioned efflux pumps, the biofilm matrix can bind and slow metal ion penetration. As well, there are a number of biofilm-specific resistance genes that have been identified [65]. Several bacteria have shown biofilm resistance mechanisms to heavy metals such as *Xylella fastitiosa* and *E. coli* [65], and *P. aeruginosa* [66].

Addition of heavy metals such as cobalt, cadmium, and nickel to drinking water systems are not advised due to toxicity to human body. However, these heavy metals can be added to non-potable water sources, to prevent biofilm formation and corrosion which results in reduction of maintenance costs of these systems.

This study is the first metal ion based report of quorum signaling inhibition in anaerobic condition in *C. violaceum*.

LITERATURE CITED

[1] N. A. Whitehead, A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond, "Quorum-sensing in Gram-negative bacteria.," *FEMS Microbiol. Rev.*, vol. 25, no. 4, pp. 365–404, 2001.

[2] W.-L. Ng and B. L. Bassler, "Bacterial Quorum-Sensing Network Architectures," *Annu. Rev. Genet.*, 2009.

[3] W. C. Fuqua, S. C. Winans, and E. P. Greenberg, "Quorum sensing in bacteria: The LuxR-LuxI family of cell density- responsive transcriptional regulators," *Journal of Bacteriology*. 1994.

[4] S. G. Thornhill, M. Kumar, L. M. Vega, and R. J. C. McLean, "Cadmium ion inhibition of quorum signalling in chromobacterium violaceum," *Microbiol. (United Kingdom)*, 2017.

[5] D. J. Sayut, Y. Niu, and L. Sun, "Construction and engineering of positive feedback loops.," *ACS chemical biology*. 2006.

[6] K. H. McClean, M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams, "Quorum sensing and Chromobacterium violaceum: Exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones," *Microbiology*, 1997.

[7] D. L. Stauff and B. L. Bassler, "Quorum sensing in chromobacterium violaceum: DNA recognition and gene regulation by the CviR receptor," *J. Bacteriol.*, 2011.

[8] H. C. Lichstein and V. F. Van De Sand, "Violacein, an antibiotic pigment produced by chromobacterium violaceum," *J. Infect. Dis.*, 1945.

[9] K. L. Visick and C. Fuqua, "Decoding microbial chatter: Cell-cell communication in bacteria," in *Journal of Bacteriology*, 2005.

[10] L. M. Vega, J. Mathieu, Y. Yang, B. H. Pyle, R. J. C. McLean, and P. J. J. Alvarez, "Nickel and cadmium ions inhibit quorum sensing and biofilm formation without affecting viability in Burkholderia multivorans," *Int. Biodeterior. Biodegrad.*, 2014.

[11] C. J. Balibar and C. T. Walsh, "In vitro biosynthesis of violacein from L-tryptophan by the enzymes VioA-E from Chromobacterium violaceum," *Biochemistry*, 2006.

[12] R. S. Blosser and K. M. Gray, "Extraction of violacein from Chromobacterium violaceum provides a new quantitative bioassay for N-acyl homoserine lactone autoinducers," *J. Microbiol. Methods*, 2000.

[13] L. S. Chernin, M. K. Winson, J. M. Thompson, S. Haran, B. W. Bycroft, I. Chet, P. Williams, and G. S. A. B. Stewart, "Chitinolytic activity in Chromobacterium violaceum: Substrate analysis and regulation by quorum sensing," *J. Bacteriol.*, 1998.

[14] M. D. P. Lobo, F. D. A. Silva, P. G. de C. Landim, P. R. da Cruz, T. L. de Brito, S. C. de Medeiros, J. T. A. Oliveira, I. M. Vasconcelos, H. D. M. Pereira, and T. B. Grangeiro, "Expression and efficient secretion of a functional chitinase from Chromobacterium violaceum in Escherichia coli," *BMC Biotechnol.*, 2013.

[15] F. Streichsbier, "Utilization of chitin as sole carbon and nitrogen source by Chromobacterium violaceum," *FEMS Microbiol. Lett.*, 1983.

[16] J W Costerton, K J Cheng, G G Geesey, T I Ladd, J C Nickel, M Dasgupta, and and T J Marrie, "Bacterial Biofilms in Nature and Disease," *Annu. Rev. Microbiol.*, 1987.

[17] S. R Robertson and R. JC McLean, "Beneficial biofilms," *AIMS Bioeng.*, 2015.

[18] M. Hassanpourfard, Z. Nikakhtari, R. Ghosh, S. Das, T. Thundat, Y. Liu, and A. Kumar, "Bacterial floc mediated rapid streamer formation in creeping flows," *Sci. Rep.*, 2015.

[19] L. A. Pratt and R. Kolter, "Genetic analysis of Escherichia coli biofilm formation: Roles of flagella, motility, chemotaxis and type I pili," *Mol. Microbiol.*, 1998.

[20] Q. Yang and T. Defoirdt, "Quorum sensing positively regulates flagellar motility in pathogenic Vibrio harveyi," *Environ. Microbiol.*, 2015.

[21] M. Pereira, J. A. Parente, L. A. M. Bataus, D. D. D. D. P. Cardoso, R. B. A. Soares, and C. M. D. A. Soares, "Chemotaxis and flagellar genes of Chromobacterium violaceum," in *Genetics and Molecular Research*, 2004.

[22] D. O. S. Recouvreux, C. A. Carminatti, A. K. Pitlovanciv, C. R. Rambo, L. M. Porto, and R. V. Antônio, "Cellulose biosynthesis by the beta-proteobacterium, Chromobacterium violaceum," *Curr. Microbiol.*, 2008.

[23] C. Solano, B. García, J. Valle, C. Berasain, J. M. Ghigo, C. Gamazo, and I. Lasa, "Genetic analysis of Salmonella enteritidis biofilm formation: Critical role of cellulose," *Mol. Microbiol.*, 2002.

[24] K. Sauer, A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies, "Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm.," *J. Bacteriol.*, 2002.

[25] R. Ghosh, B. K. Tiwary, A. Kumar, and R. Chakraborty, "Guava leaf extract inhibits quorum-sensing and chromobacterium violaceum induced lysis of human hepatoma cells: Whole transcriptome analysis reveals differential gene expression," *PLoS One*, 2014.

[26] P. Truchado, M. Larrosa, I. Castro-Ibáñez, and A. Allende, "Plant food extracts and phytochemicals: Their role as Quorum Sensing Inhibitors," *Trends in Food Science and Technology*. 2015.

[27] Y.-H. Dong, "AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora," *Proc. Natl. Acad. Sci.*, 2000.

[28] S. A. El-Mowafy, K. H. Abd El Galil, S. M. El-Messery, and M. I. Shaaban, "Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in Pseudomonas aeruginosa," *Microb. Pathog.*, 2014.

[29] M. Givskov, R. De Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, Sø. Molin, P. D. Steinberg, and S. Kjelleberg, "Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling," *J. Bacteriol.*, 1996.

[30] M. Manefield, T. B. Rasmussen, M. Henzter, J. B. Andersen, P. Steinberg, S. Kjelleberg, and M. Givskov, "Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover," *Microbiology*, 2002.

[31] T. B. Rasmussen and M. Givskov, "Quorum sensing inhibitors: A bargain of effects," *Microbiology*. 2006.

[32] A. L. Schaefer, B. L. Hanzelka, A. Eberhard, and E. P. Greenberg, "Quorum sensing in Vibrio fischeri: Probing autoinducer-LuxR interactions with autoinducer analogs," *J. Bacteriol.*, 1996.

[33] M. P. Waalkes and L. A. Poirier, "In vitro cadmium-DNA interactions: Cooperativity of cadmium binding and competitive antagonism by calcium, magnesium, and zinc," *Toxicol. Appl. Pharmacol.*, 1984.

[34] I. Chopra, "Decreased Uptake of Cadmium by a Resistant Strain of Staphylococcus aureus," *J. Gen. Microbiol.*, vol. 63, no. 2, pp. 265–267, 1970.

[35] G. M. Gadd and A. J. Griffiths, "Microorganisms and heavy metal toxicity," *Microb. Ecol.*, 1977.

[36] A. W. Lawrence and P. L. McCarty, "The role of sulfide in preventing heavy metal toxicity in anaerobic treatment," *J. (Water Pollut. Control Fed.*, vol. 37, no. 3, pp. 392–406, 1965.

[37] T. Cabellos-Avelar, V. Souza, and J. Membrillo-Hernández, "Spent media from cultures of environmental isolates of Escherichia coli can suppress the deficiency of biofilm formation under anoxic conditions of laboratory E. coli strains," *FEMS Microbiol. Ecol.*, 2006.

[38] F. A. de Almeida, N. de J. Pimentel-Filho, U. M. Pinto, H. C. Mantovani, L. L. de Oliveira, and M. C. D. Vanetti, "Acyl homoserine lactone-based quorum sensing stimulates biofilm formation by Salmonella Enteritidis in anaerobic conditions," *Arch. Microbiol.*, 2017.

[39] S. G. Thornhill, "INHIBITION OF QUORUM SIGNALLING AND BIOFILM FORMATION IN CHROMOBACTERIUM VIOLACEUM IN THE PRESENCE OF CADMIUM, COBALT AND NICKEL DIVALENT CATIONS," Texas State University, 2016.

[40] G. A. O'Toole, "Microtiter Dish Biofilm Formation Assay," *J. Vis. Exp.*, 2011.

[41] S. De Carlo and J. R. Harris, "Negative staining and cryo-negative staining of macromolecules and viruses for TEM," *Micron*. 2011.

[42] N. C. Reading and V. Sperandio, "Quorum sensing: The many languages of bacteria," *FEMS Microbiology Letters*. 2006.

[43] A. M. Lazdunski, I. Ventre, and J. N. Sturgis, “Regulatory circuits and communication in gram-negative bacteria,” *Nature Reviews Microbiology*. 2004.

[44] C. Fuqua, M. R. Parsek, and E. P. Greenberg, “Regulation of Gene Expression by Cell-to-Cell Communication: Acyl-Homoserine Lactone Quorum Sensing,” *Annu. Rev. Genet.*, 2001.

[45] G. Brackman and T. Coenye, “Quorum Sensing Inhibitors as Anti-Biofilm Agents,” *Curr. Pharm. Des.*, 2014.

[46] G. M. N. Araujo, C. B. Silva, and A. Hasson-Voloch, “Comparison of the inhibitory effects of mercury and cadmium on the creatine kinase from Electrophorus electricus (L.),” *International Journal of Biochemistry and Cell Biology*. 1996.

[47] H. Chen, N. C. Giri, R. Zhang, K. Yamane, Y. Zhang, M. Maroney, and M. Costa, “Nickel ions inhibit histone demethylase JMJD1A and DNA repair enzyme ABH2 by replacing the ferrous iron in the catalytic centers,” *J. Biol. Chem.*, 2010.

[48] C. Pan *et al.*, “Cadmium is a potent inhibitor of PPM phosphatases and targets the M1 binding site,” *Sci. Rep.*, 2013.

[49] Y.-B. Yu and S. F. Yang, “Auxin-induced Ethylene Production and Its Inhibition by Aminoethyoxyvinylglycine and Cobalt Ion,” *PLANT Physiol.*, 1979.

[50] P. H. ABELSON and E. ALDOUS, “Ion antagonisms in microorganisms; interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc, and manganese,” *J. Bacteriol.*, vol. 60, no. 4, pp. 401–413, 1950.

[51] N. Shahabadi, S. Kashanian, and M. Mahdavi, “DNA Interaction Studies of Cobalt (II) Mixed-Ligand Complexes Containing Dimethyl-1, 10-Phenanthroline and Dipyrido[3,2-a:2′,3′-c]Phenazine: The Role of Methyl Substitutions on the Mode of Binding,” *DNA Cell Biol.*, 2011.

[52] S. Silver and G. Ji, “Newer systems for bacterial resistances to toxic heavy metals,” in *Environmental Health Perspectives*, 1994.

[53] A. W. Foster and N. J. Robinson, “Promiscuity and preferences of metallothioneins: The cell rules,” *BMC Biology*. 2011.

[54] J. S. Cavet, G. P. M. Borrelly, and N. J. Robinson, “Zn, Cu and Co in cyanobacteria: Selective control of metal availability,” *FEMS Microbiology Reviews*. 2003.

[55] S. B. Ceyhun, E. Aksakal, D. Ekinci, O. Erdoğan, and Ş. Beydemir, “Influence of cobalt and zinc exposure on mRNA expression profiles of metallothionein and cytocrome P450 in rainbow trout,” *Biol. Trace Elem. Res.*, 2011.

[56] H. Falfushynska, L. Gnatyshyna, O. Fedoruk, N. Mitina, A. Zaichenko, O. Stoliar, and R. Stoika, “Hepatic metallothioneins in molecular responses to cobalt, zinc, and their nanoscale polymeric composites in frog Rana ridibunda,” *Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol.*, 2015.

[57] D. Beyersmann and S. Hechtenberg, “Cadmium, gene regulation, and cellular signalling in mammalian cells,” *Toxicology and Applied Pharmacology*. 1997.

[58] A. Gupta, A. P. Morby, J. S. Turner, B. A. Whitton, and N. J. Robinson, "Deletion within the metallothionein locus of cadmium-tolerant Synechococcus PCC 6301 involving a highly iterated palindrome (HIP1)," *Mol. Microbiol.*, 1993.

[59] N. Naz, H. K. Young, N. Ahmed, and G. M. Gadd, "Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria," *Appl. Environ. Microbiol.*, 2005.

[60] M. A. Pagani, M. Tomas, J. Carrillo, R. Bofill, M. Capdevila, S. Atrian, and C. S. Andreo, "The response of the different soybean metallothionein isoforms to cadmium intoxication," in *Journal of Inorganic Biochemistry*, 2012.

[61] J. Asselman, S. P. Glaholt, Z. Smith, G. Smagghe, C. R. Janssen, J. K. Colbourne, J. R. Shaw, and K. A. C. De Schamphelaere, "Functional characterization of four metallothionein genes in Daphnia pulex exposed to environmental stressors," *Aquat. Toxicol.*, 2012.

[62] R. M. Nezhad, A. Shahpiri, and A. Mirlohi, "Discrimination between two rice metallothionein isoforms belonging to type 1 and type 4 in metal-binding ability," *Biotechnol. Appl. Biochem.*, 2013.

[63] K. Kawamura-Sato, K. Shibayama, T. Horii, Y. Iimuma, Y. Arakawa, and M. Ohta, "Role of multiple efflux pumps in Escherichia coli in indole expulsion," *FEMS Microbiol. Lett.*, 1999.

[64] T. B. Grangeiro, D. M. D. M. Jorge, W. M. Bezerra, A. T. R. Vasconcelos, and A. J. G. Simpson, "Transport genes of Chromobacterium violaceum: An overview," in *Genetics and Molecular Research*, 2004.

[65] S. Koechler, J. Farasin, J. Cleiss-Arnold, and F. Arsène-Ploetze, "Toxic metal resistance in biofilms: Diversity of microbial responses and their evolution," *Res. Microbiol.*, 2015.

[66] C. C. Chien, B. C. Lin, and C. H. Wu, "Biofilm formation and heavy metal resistance by an environmental Pseudomonas sp.," *Biochem. Eng. J.*, 2013.