

INDOLE INHIBITION OF AHL-MEDIATED QUORUM SIGNALING IS  
WIDESPREAD IN GRAM-NEGATIVE BACILLI

by

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## ABSTRACT

The luxI/R quorum-sensing system and associated N-acylated homoserine lactone (AHL) signals is a widespread mechanism of gene regulation in gram negative bacteria. Quorum inhibition can be exploited as a strategy of bacterial competition. Although indole inhibition of *Pseudomonas aeruginosa* quorum signaling has been reported by other investigators, it has not been documented in other organisms. Co-culture with *Escherichia coli* BW25113 inhibited quorum-regulated pigmentation in *Chromobacterium violaceum*, *Serratia marcescens* and *Pseudomonas aureofaciens* (*Pseudomonas chlororaphis*). We investigated whether indole was responsible for this effect. qRT-PCR of genes under the AHL-regulated system exhibited up-regulation in untreated cells compare with cells treated with 1.0 mM indole. Loss of pigmentation did not occur during co-culture with *E. coli tnaA* (lacking tryptophanase and indole production). In the absence of *E. coli*, the presence of physiologically relevant indole concentrations (0.5-1.0mM) caused a significant reduction of *C. violaceum* biofilm formation, pigment (violacein) production, elastase and chitinase activity. Similar indole-mediated inhibition of pigmentation was also observed in pure cultures of *S. marcescens* and *P. aureofaciens*. At indole concentrations above 1.0 mM, virulence of *C. violaceum*, *S.marcescens*, and *P. aureofaciens* was reduced in a *Caenorhabditis elegans* bioassay. Overall, indole-mediated inhibition of AHL quorum signaling appears to be a general phenomenon and not exclusive to *P. aeruginosa*.

## CHAPTER I

### Introduction

Bacterial species coexist in dynamic communities in almost all environments. This continuous coexistence has led to the development of ways that allows bacteria to synchronize multicellular behavior through gene expression in order adapt and survive changing conditions and competition [1, 2]. Many Gram-negative bacteria use an array of quorum-sensing systems, such as: the luxS/Autoinducer-2, indole, and the luxI/luxR/N-acylated-homoserine lactone (AHL) systems that allow them to sense the conditions in the environment such as nutrient depletion, competition and the presence of different and their own population [2, 3].

Acyl homoserine-lactone quorum-sensing signaling system has been well-defined in many Gram-negative bacilli and it resembles the canonical quorum sensing system of *Vibrio fischeri*, which is dependent on two regulatory proteins; LuxI and LuxR [2, 4]. The LuxI-like protein synthesizes the acylated homoserine lactone signaling molecules (HSL) that diffuse out of the cell into the environment [2, 5]. The LuxR- like protein binds to the cognate HSL and forms a LuxR-like protein:HSL complex that functions as a DNA binding transcription factor [5].

Quorum sensing in *Pseudomonas aeruginosa* is intimately connected to its virulence. In *P. aeruginosa*, many of the genes responsible for virulence are under the AHL- and quinolone-signaling system [6, 7]. These include pyocyanin, rhamnolipids, elastase activity, chitinase activity and membrane vesicles [6, 7].

Competition experiments between *P. aeruginosa* and wild-type *Escherichia coli* showed down-regulation of several virulent genes under the AHL-mediated quorum

signaling in *P. aeruginosa*. Previous studies have shown that indole, a product of tryptophan biosynthesis, is able to quench the AHL-mediated quorum signaling in *P. aeruginosa* and consistently down-regulate the activity of pyocyanin, rhamnolipids, PQS and pyoverdine [4, 6, 7,].

Previous research has shown that the addition of extracellular HSLs generally up-regulates the expression of virulent genes under the AHL-mediated quorum signaling and PQS synthesis in *P. aeruginosa*. Alternatively, addition of extracellular indole induces down-regulation of these genes. Quenching of AHL-mediated quorum signaling in *P. aeruginosa* has shown to reduce the ability of *P. aeruginosa* to compete in mix-culture experiments and modulate its virulence factors [6, 7].

Although indole-inhibition of AHL-mediated quorum signaling in *P. aeruginosa* has been reported previously, its effect on other AHL-regulated systems has not been shown (7). There is an indication that quenching by indole occurs in the Gram-negative bacilli *Chromobacterium violaceum*, *Serratia marcescens* and *Pseudomonas aureofaciens*.

Even though *Chromobacterium violaceum* is a known saprophytic Gram-negative  $\beta$ -proteobacterium, it is known to be an opportunistic pathogen that can cause fatal septicemia in humans and animals [8, 9]. The most prominent characteristics of *C. violaceum* is the production of violacein and desoxyviolacein, both are indole pigments derived from tryptophan [8, 10]. Violacein is biologically relevant because it possesses antitumoral and antimicrobial properties [8, 11, 12,]. *C. violaceum*, like many other Gram-negative bacilli, relies heavily on AHL-mediated signaling system to regulate gene expression [13, 14]. CviI, a homologue of LuxI, synthesizes the autoinducer C10-homoserine lactone (C10-HSL) and CviR, a homologue of LuxR, acts as a cytoplasmic DNA binding transcription factor that

activates gene expression when density reaches its threshold. [9,13]. It is known that many phenotypic characteristics are under the regulation on the AHL signaling system and these include the production of violacein, production and degradation of cyanide, extracellular chitinase, elastase, phenazine and biofilm formation [15, 16]. It is also speculated that genes responsible for the coding of extracellular enzymes such as serine protease, collagenase and oligopeptidase are under the AHL-mediated quorum system. [9]

*Serratia marcescens* is a Gram-negative bacillus which is part of the *Enterobacteriaceae* family [17]. *S. marcescens* has become more relevant since it is known to be an opportunistic pathogen which has become common in serious nosocomial infections such as pneumonia, catheter-associated infections and urinary tract infections [17, 18]. It has been shown that *Serratia marcescens* employs AHL-mediate quorum sensing for the regulation of genes encoding for extracellular virulence [19, 20]. Many of the phenotypes regulated by the AHL-mediated quorum sensing system include swarming and sliding motility, the LipB translocation system and exo-enzymes, chitinase, prodigiosin production, antibiotic formation, biofilm formation, hemolytic activity, and butanediol fermentation [17, 21, 22,]. Previous research has shown that *Serratia* strains that are deficient in AHL signal generation were deficient in biofilm formation, prodigiosin production and exoenzyme excretion [19, 20].

*Pseudomonas aureofaciens* strain 30-84, now reclassified as *P. chlororaphis*, is a beneficial bacterium that protects wheat from the fungus *Gaeumannomyces graminis*. *P. chlororaphis*. [23, 28]. *P. aureofaciens* has two AHL-mediate quorum sensing systems. The PhzR/PhzI system regulates the production of three kinds of phenazines; phenazine-1-carboxylic acid, 2-hydroxy-phenazine-1-carboxylic acid, and 2-hydroxy-phenazine. The

other AHL-mediated quorum sensing system is still undetermined [23, 24]. Phenazine-deficient mutants exhibit deficient motility and biofilm formation. This is an indication that phenazine production and AHL-quorum sensing regulation are tightly involved in biofilm formation, motility and pathogen inhibition in *P. aureofaciens* [24, 28]

We recently found that indole production by *E. coli* helps this organism compete with *Pseudomonas aeruginosa* in co-culture, by interfering with the *P. aeruginosa* AHL-regulated pyocyanin. Other research has shown that indole decreases rhamnolipids, PQS and pyoverdine in *Pseudomonas aeruginosa* and facilitates the *Escherichia coli* growth in mixed cultures [6, 7].

Recent research in our lab seems to indicate that indole quenches the AHL-mediated quorum signaling of various Gram-negative bacilli. Initial mixed-growth competition assays with wild-type *E. coli* showed decrease in pigment production and biofilm formation in *C. violaceum*, *S. marcescens* and *P. aureofaciens* while the *tnaA* mutant, which lacks indole biosynthesis had no effect on AHL-mediated quorum sensing. Quenching of AHL-mediated quorum signaling by indole also renders *C. violaceum*, *Serratia marcescens* and *P. aureofaciens* more susceptible to bacterivore grazing. This may be an indication that virulent genes under the AHL-mediated quorum system are being quenched. Indole-mediated inhibition of AHL quorum signaling appears to be a general phenomenon and not exclusive to *P. aeruginosa*.

## CHAPTER II

### Methodology

#### Organisms and cultivation

*Chromobacterium violaceum* ATCC 12472, *Escherichia coli* BW25113, *Escherichia coli tnaA*, and *Escherichia coli* strain OP50 were routinely cultured aerobically in Luria-Bertani (LB) broth (0.5% yeast extract, 1% tryptone, 0.5% NaCl) at 37°C with 140 rev min agitation in shaking incubator prior to experiments except for *Serratia marcescens* and *Pseudomonas aureofaciens*, which were cultured in tryptic soy broth (TSB) and incubated at 25°C in similar agitation conditions. Strains were maintained in LB solidified media with 1.2% agar except for *Serratia marcescens* and *Pseudomonas aureofaciens*, which were maintained in tryptic soy agar (TSA) with 1.2% agar (w/v).

*C. elegans* nematodes were maintained on nematode growth medium (NGM) agar (L<sup>1</sup>: 2.5 g peptone, 3 g NaCl, 17 g agar, 1 ml of 1 M cholesterol, 1 ml of 1 M CaCl<sub>2</sub>, 1 ml of 1 M MgSO<sub>4</sub>, 1 ml of 1 M potassium phosphate buffer, pH 6) at 25°C and fed with *Escherichia coli* strain OP50. [32]

#### qRT-PCR of genes under the AHL-mediated quorum sensing system

Reverse transcription was performed on genes under AHL-mediated quorum sensing in *C. violaceum* (table 1). Cultures treated with 1.0mM and untreated were incubated at 37°C for 24hrs. RNA isolation was performed using RNeasy mini kit from QIAGEN. Reverse transcription was performed using ImProm II reverse transcriptase system from Promega. RT-PCR was performed using the SYBR green method.

Table 1. Primers of genes under the AHL-mediated quorum sensing in *C. violaceum* used for qRT-PCR.

Gene	Function	Primer
<i>lasA</i>	Elastase	R 5' gga gaa gaa tth cca tcc '3 F 5' cat gtg cac ttc tcg ctg at '3
<i>lasB</i>	Elastase	R 5' ggt cgc gat cag ata gaa cg '3 F 5' ggc aac gcc aag gac tac ta '3
<i>vioA</i>	violacein production	R 5' ggt tgt cga cga tca gca c '3 F 5' gct gtt caa ggc ttt cct ca '3
<i>vioB</i>	violacein production	R 5' gct gca agt gcg aga cct ac '3 F 5' ggt gct ggg cat gta gta gc '3
<i>vioC</i>	violacein production	R 5' aaa tac agc gtg tcc ttg cg '3 F 5' ttc cag cag act ttc ttc cg '3
<i>chiA</i>	Chitinase	R 5' tta caa cac cga gtg caa cg '3 F 5' cgg ccg tag tat ttc ttc ca '3
<i>hcnB</i>	cyanide	R 5' ctg gaa cag ggc gtc aac '3 F 5' aat agc cga cgc aca tct tg '3
<i>cviR</i>	AHL-mediate receptor	R 5' ggc tca gga tgt tgt caa gg '3 F 5' caa tgg aca ttg ctg gag tg '3
<i>cvil</i>	AHL synthesizer	R 5' cct cca aca tga aag cat gc '3 F 5' tcc gac aac ttg gta ttg tgc '3
<i>pykF</i>	Housekeeping- Pyruvate kinase	R 5' gca ttg cct tcc gtt tca t '3 F 5' ctg cgc atc ggc aag ttt '3

### Competition assays

#### Exogenous addition of indole

To investigate whether exogenous addition of indole inhibits AHL-quorum regulated pigment production and biofilm formation in *C. violaceum*, *S. marcescens* and *P. aureofaciens*, indole at different concentrations ranging from 0-1.5mM was added to 50ml of LB in a 125mL flask and inoculated with 100µl of overnight culture at a final concentration of 10<sup>6</sup>/mL. 500µL of planktonic culture were removed, diluted and plated at different

intervals of time and colony forming units (CFU) quantified. For biofilm silicone rubber disks, 7 mm diameter by 1 mm (Dapro Rubber Inc., Tulsa, OK) were placed in 250-ml beakers which contained 50 ml LB broth and inoculated with overnight cultures of bacteria at a final cell density of  $10^6$ /ml at 140 rpm for 2 days at 37°C. During this incubation, the biofilm-colonized disks were transferred to fresh medium after 24 h. Biofilm growth was measured using the sonication and dilution plating protocol described by Corbin et al (31).

#### Competition and exogenous addition of indole

To investigate the level by which indole produced naturally by *Escherichia coli* BW25113 and addition of extracellular indole inhibits the AHL-mediated quorum signaling in *C. violaceum*, *S. marcescens* and *P. aureofaciens*. Mixed-growth competition assays were designed to measure the amount of inhibition at different biologically relevant concentrations of indole (0-1.0 mM). For mixed-culture experiments containing no indole, *tnaA* mutant at a final concentration of  $10^6$ /mL was added to 50ml of LB in a 125mL flask. The same final concentration of *C. violaceum* was added for a 1:1 ratio. In the case of *S. marcescens* and *P. aureofaciens* the broth media used was TSB. For mixed-culture experiments containing 0.5mM of indole, *Escherichia coli* BW25113 at a final concentration of  $10^6$ /mL was added as described above. For mixed-culture experiments containing 1.0mM of indole, either *tnaA* mutant was added with 1.0mM of exogenous indole or *E. coli* BW25113 supplemented with 0.5mM of exogenous indole in the same conditions as described previously. Quantification of planktonic and biofilm cells was done as previously described.

## Pigment production

### Violacein production

*C. violaceum* was grown in 50ml of LB at 37°C 140 rev min<sup>-1</sup> agitation in shaking incubator. At regular time intervals, 500ml of planktonic cells were harvested for centrifugation and treated for violacein extraction. For extraction, bacterial cells were pelleted and treated with 1000mL 90% methanol. Cell debris was pelleted and supernatant containing violacein was collected and quantified at 585nm (12).

### Prodigiosin production

*S. marcescens* was grown in 50ml of TSB at 25°C 140 rev min<sup>-1</sup> agitation in shaking incubator. At regular time intervals, 500ml of planktonic cells were harvested for centrifugation and treated for prodigiosin extraction. For extraction, bacterial cells were pelleted and resuspended in a 1000ml of acidified ethanol (4% 1M HCl in ethanol). Cell debris was pelleted and supernatant containing prodigiosin was quantified at 534nm (19).

### Phenazine production

*P. aureofaciens* was grown in 50ml of TSB at 25°C 140 rev min agitation in shaking incubator. At regular time intervals, 500ml of planktonic cells were harvested for centrifugation and treated for phenazine extraction. For extraction, 1ml of cell-free supernatant was extracted with 1ml of acidified benzene (4% 1M HCl in benzene), the benzene phase was separated and evaporated under air. Dried phenazine was dissolved in 0.1M NaOH and quantified at 367nm (23, 25)

## Exoenzyme activity and motility

### Elastase activity

Elastin nutrient agar assay was performed as following. 1g of elastin (Sigma Chemical, St. Louis, MO) was added to 300ml nutrient top agar and overlaid on LB agar plates. Cultures exhibiting elastase activity cleared the elastin under and around the streak (16). Elastin-Congo red assay was performed as followed; 10mg of Elastin-Congo red (Sigma-Aldrich) was incubated with 100 $\mu$ L of culture supernatant overnight at 37°C in 1mL 10mM sodium phosphate buffer, pH 7.0. Unsolubilized substrate was pelleted by centrifugation. The supernatant was read at 495nm as a measure of amount of substrate solubilized (16).

### Chitinase activity

Cells were inoculated onto plates containing minimal medium supplemented with colloidal chitin (0.2% [wt/vol]) and solidified with 1.5% agar. Cultures exhibiting chitinase activity cleated the colloidal chitin under and around the streak. Colloidal chitin was prepared by partial hydrolysis with concentrated HCl followed by repeated washes with water to give a final pH of 6.5-7.0.

Chitin-azure assay (Sigma-Aldrich) was performed as followed; 10mg of chitin-azure was incubated with 100 $\mu$ L of culture supernatant overnight at 37°C in 1mL 10mM sodium phosphate buffer, pH 7.0. Unsolubilized substrate was pelleted by centrifugation. The supernatant was read at 585nm as a measure of amount of substrate solubilized (15).

### Motility

The medium used for motility assay was tryptic soy broth. Swimming assay plates contained 0.3% (wt/vol) agar. Assay plates were inoculated with bacteria from an overnight culture in TSA agar (1.5% [wt/vol] at 25°C with a sterile applicator. The plates were incubated at 25°C for 18h (23, 25).

### Toxicity

Nematode Growth Medium was supplemented with different amounts of indole (0-1.0mM). Bacteria were inoculated and allowed to grow for 48hrs. *C. elegans* eggs were harvested from large populations of gravid adults using a standard bleaching protocol (30mL 5% bleach, 15mL N KOH, 55ml DH2O). Harvested eggs were placed on lawns of fresh *E. coli* OP50 and allowed to hatch and grow to the young adult stage. 10 non-gravid young adults were transferred onto lawns of *C. violaceum*, *S. marcescens* and *P. aureofaciens*. Worms were scored for survival each day and transferred to fresh lawns every two days until all worms had expired.

### CH10-AHL complementation assay

To investigate whether indole is in direct competition with the acyl homoserine lactones, 1.0 mM of indole and different concentrations of C10-HSL were added to 25ml of LB in a 50mL flask and inoculated with 100µl of overnight culture of *C. violaceum* at a final concentration of  $10^6$ /mL. 500µL of planktonic culture were removed at different time intervals and violacein extracted as previously described.

## CHAPTER III

### Results

#### qRT-PCR of genes under the AHL-mediated quorum sensing system

qRT-PCR of untreated cultures of *C. violaceum* at 24hrs exhibited up-regulation. *VioA* was up-regulated 50-fold, *vioB* was up-regulated 33-fold, and *vioC* was up-regulated 73-fold. Elastase genes *lasA* was up-regulated 39-fold and *lasB* was up-regulated 22-fold. *HcnB* responsible in cyanide production was up-regulated 95-fold in untreated cultures and *chiA* responsible for chitinase exoenzyme was up-regulated 15270-fold in untreated cultures. *CviI* was up-regulated 1.9-fold while *cviR* did not exhibit any activity in cultures treated with indole.

#### Competition and exogenous addition of indole

*TnaA* mutant, which lacks production of indole, is outcompeted by *C. violaceum* after 24hrs in both planktonic ( $\log^{10}$  CFU for *C. violaceum* at 48hrs  $8.74 \pm 0.35$  and  $\log^{10}$  CFU for *tnaA E. coli* at 48hrs  $6.63 \pm 0.287$ ) and biofilm ( $\log^{10}$  CFU for *C. violaceum* at 48hrs  $5.54 \pm 0.40$  and  $\log^{10}$  CFU for *tnaA E. coli* at 48hrs  $4.41 \pm 0.36$ ). *TnaA* mutant was able to outcompete *C. violaceum* in the presence of 1.0mM indole after 24hrs in planktonic ( $\log^{10}$  CFU for *C. violaceum* at 48hrs  $4.66 \pm 0.49$  and  $\log^{10}$  CFU for *tnaA E. coli* at 48hrs  $8.48 \pm 0.39$ ) and biofilm ( $\log^{10}$  CFU for *C. violaceum* at 48hrs  $3.10 \pm 0.40$  and  $\log^{10}$  CFU for *tnaA E. coli* at 48hrs  $5.29 \pm 0.44$ ).

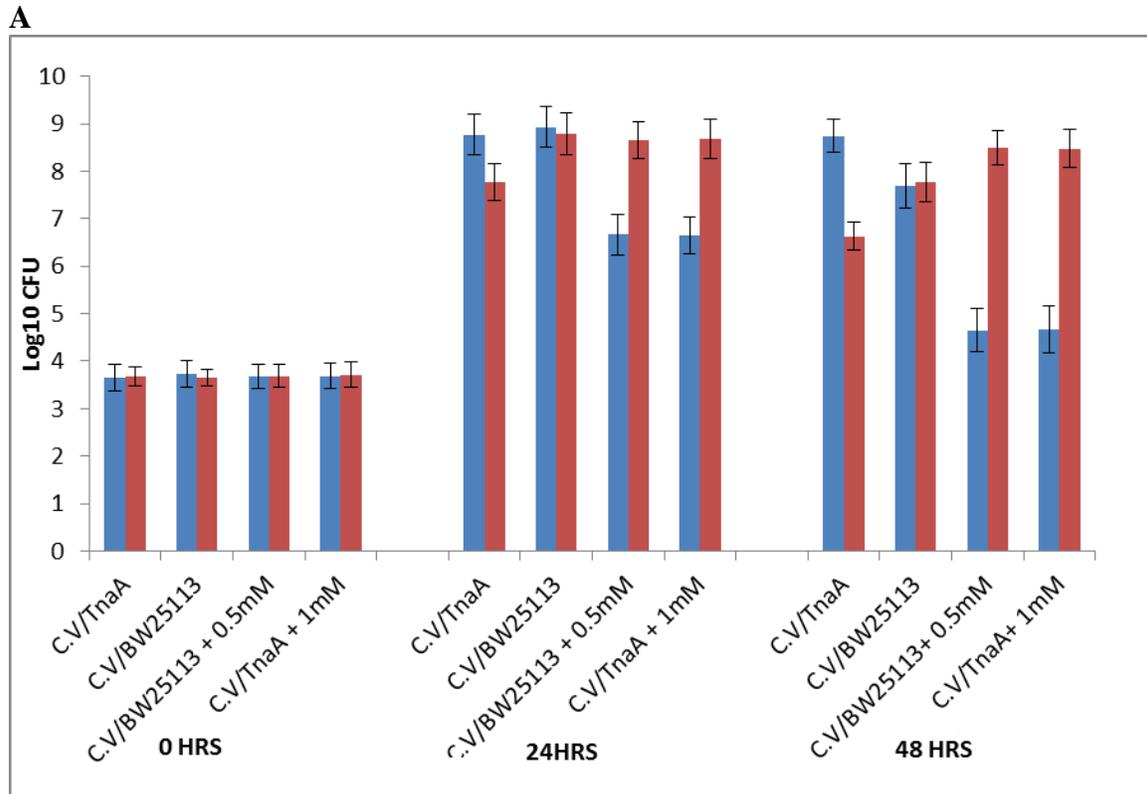
Co-cultures of *E. coli* BW25113 and *C. violaceum* did not seem to outcompete each other and maintained constant populations in both planktonic and biofilm assays after 24hrs

(log<sup>10</sup> CFU for *C. violaceum* at 48hrs 7.69±0.47 and log<sup>10</sup> CFU for *E. coli* BW25113 at 48hrs 7.78±0.41) and biofilm (log<sup>10</sup> CFU for *C. violaceum* at 48hrs 5.54±0.42 and log<sup>10</sup> CFU for *E. coli* BW25113 at 48hrs 5.26±0.43). Addition of 0.5mM of exogenous indole to co-cultures *E. coli* BW25113 and *C. violaceum* gave *E. coli* BW25113 a competitive advantage that allowed it to outcompete *C. violaceum* after 24hrs in planktonic (log<sup>10</sup> CFU for *C. violaceum* at 48hrs 4.65±0.45 and log<sup>10</sup> CFU for *E. coli* BW25113 at 48hrs 8.49±0.36) and biofilm (log<sup>10</sup> CFU for *C. violaceum* at 48hrs 3.07±0.20 and log<sup>10</sup> CFU for *E. coli* BW25113 at 48hrs 5.59±0.44) (Fig 1A and Fig 1B).

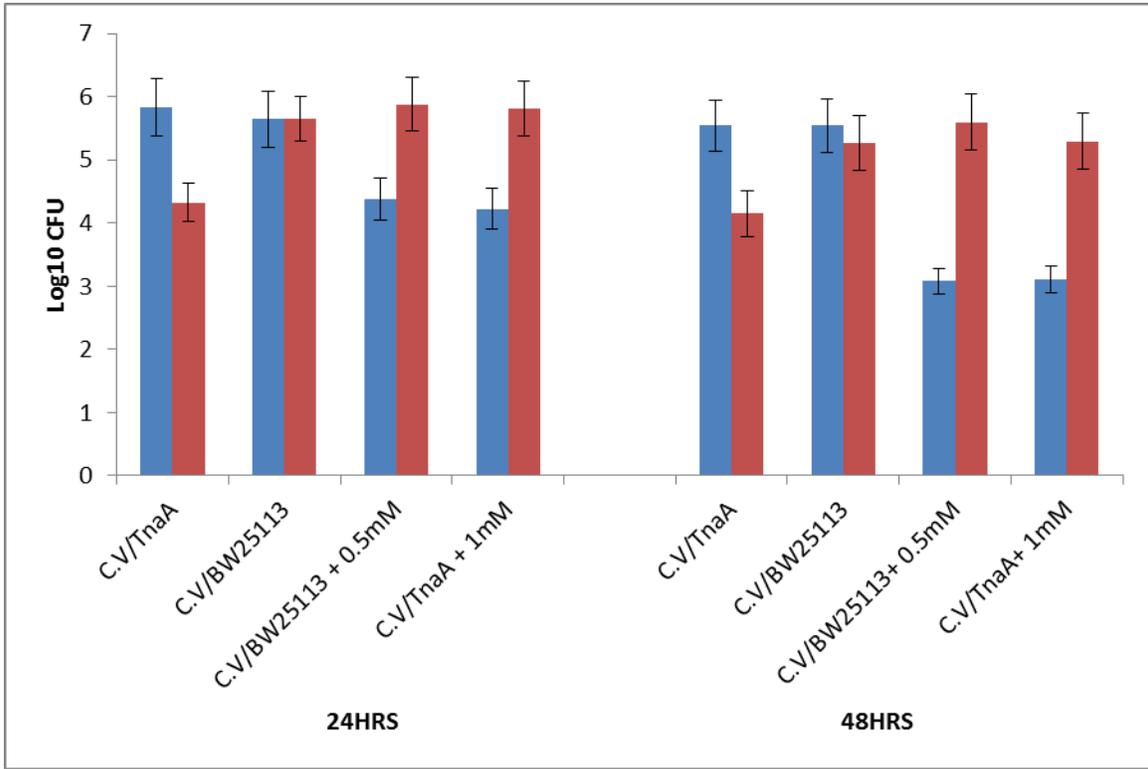
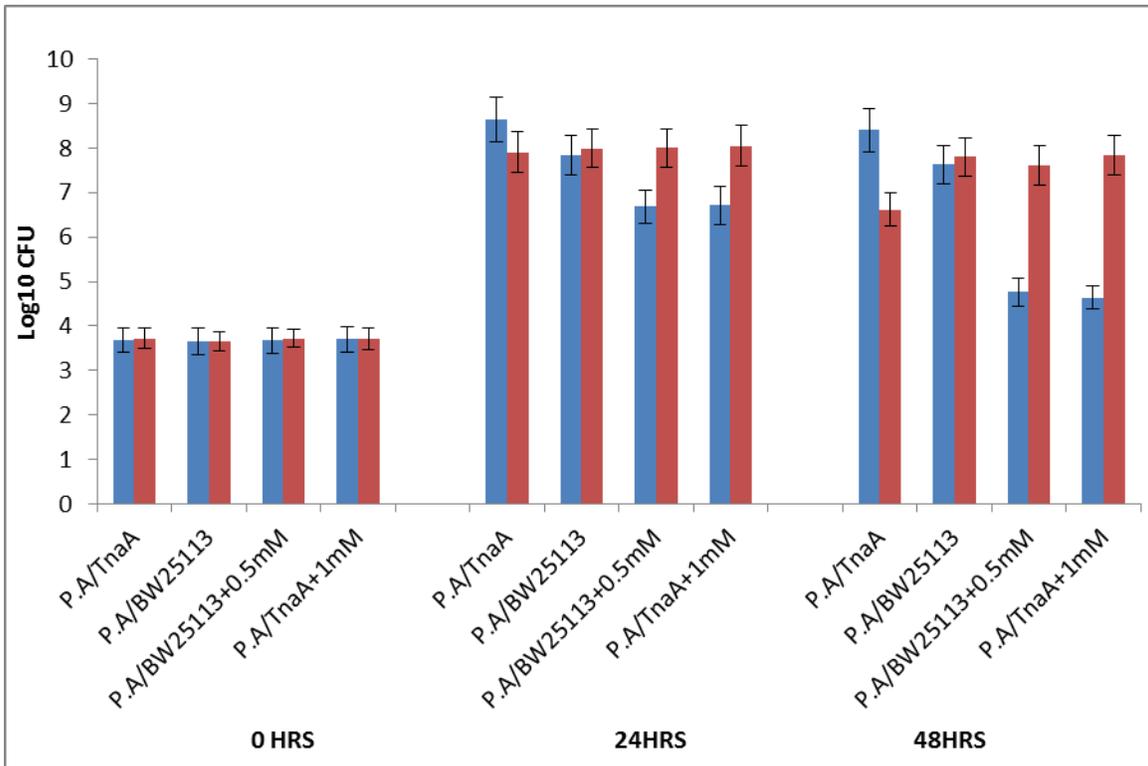
Similar results were obtained for *S. marcescens* and *P. aureofaciens*. *S. marcescens* outcompeted the *tnaA* mutant both in planktonic (log<sup>10</sup> CFU for *S. marcescens* at 48hrs 8.57±0.46 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 6.97±0.46) and biofilm (log<sup>10</sup> CFU for *S. marcescens* at 48hrs 5.87±0.48 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 4.73±0.38). Addition of 1.0mM indole reversed the advantage and *E. coli tnaA* outcompeted *S. marcescens* in planktonic (log<sup>10</sup> CFU for *S. marcescens* at 48hrs 4.75±0.31 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 7.79±0.45) and biofilm (log<sup>10</sup> CFU for *S. marcescens* at 48hrs 3.79±0.29 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 5.59±0.47) (Fig 1C and Fig 1D)

*P. aureofaciens* outcompeted the *tnaA* mutant in the absence of indole in both planktonic (log<sup>10</sup> CFU for *P. aureofaciens* at 48hrs 8.40±0.47 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 6.41±0.36) and biofilm (log<sup>10</sup> CFU for *P. aureofaciens* at 48hrs 5.74±0.43 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 4.38±0.36). *TnaA* mutant gained a competitive advantage in the presence of 1.0mM indole in planktonic (log<sup>10</sup> CFU for *P. aureofaciens* at 48hrs 4.63±0.25 and log<sup>10</sup> CFU for *E. coli tnaA* at 48hrs 7.84±0.45) and biofilm (log<sup>10</sup> CFU for

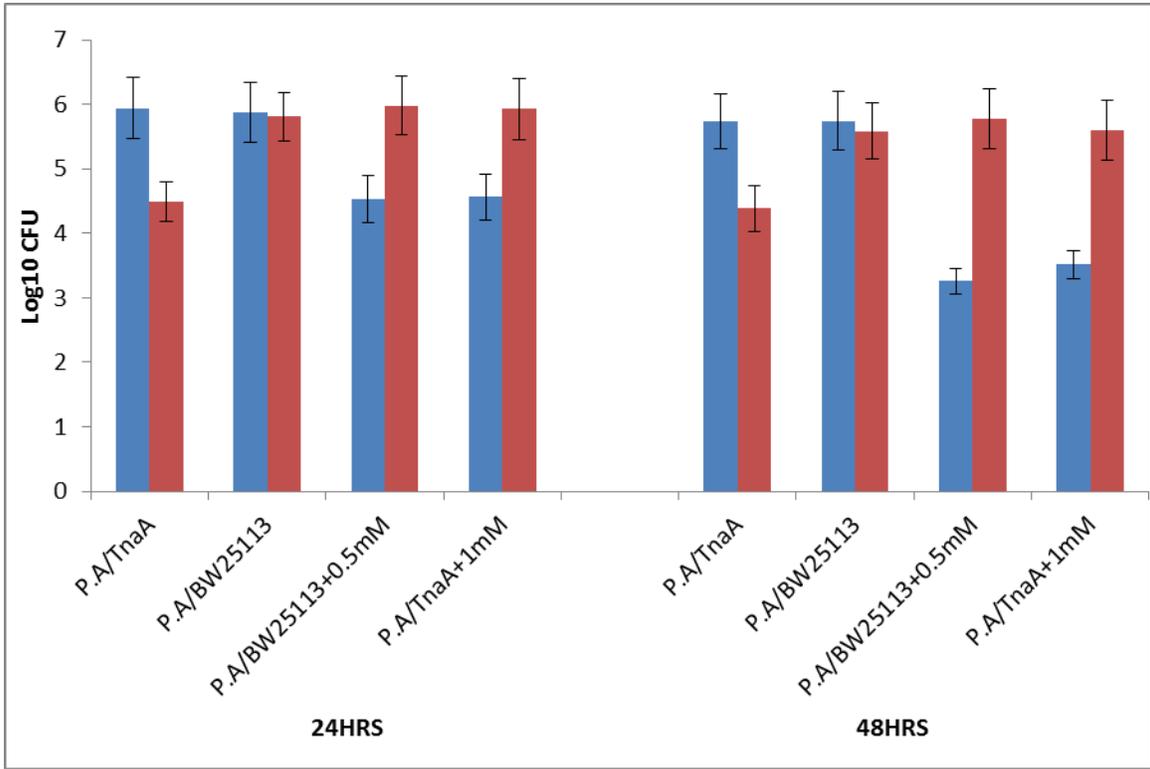
*P.aureofaciens* at 48hrs  $3.51 \pm 0.21$  and  $\log^{10}$  CFU for *E. coli tnaA* at 48hrs  $5.59 \pm 0.47$ ). (Fig 1E and Fig 1F)



**Figure 1. A) Population levels of planktonic and biofilm cultures of Gram-negative during co-culture with *E. coli*.** 100 $\mu$ l of overnight culture at a final concentration of  $10^6$ /mL are inoculated in 50ml of LB. At 24hrs *tnaA* mutant starts losing ground against *C. violaceum* and at 48hrs is outcompeted. BW25113 remains competitive and the presence of 1mM indole provides an advantage to *tnaA* mutant and *E. coli* BW251113. Both outcompeted *C. violaceum* in planktonic cultures with the addition of exogenous indole. **B)** *C. violaceum* is outcompeted by *tnaA* mutant and *E. coli* BW251113 in the presence of exogenous indole in biofilm formation. **C) and D)** The presence of 1.0 mM indole provides an advantage to *tnaA* mutant and *E. coli* BW251113. Both outcompeted *P. aureofaciens* in planktonic cultures and biofilm cultures. **E) and F)** *TnaA* mutant and BW251113 *E. coli* outcompeted *S. marcescens* in planktonic and biofilm cultures when indole was present.

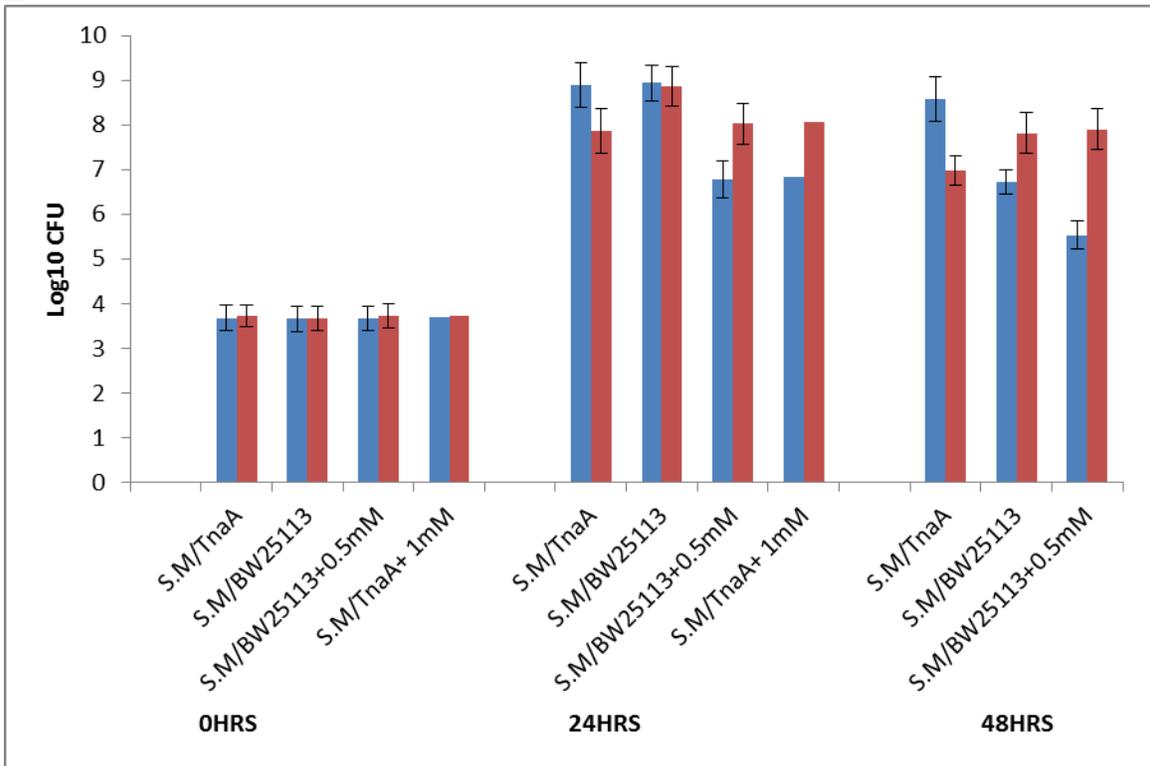
**B****Figure 1, continued****C****Figure 1, continued**

**D**

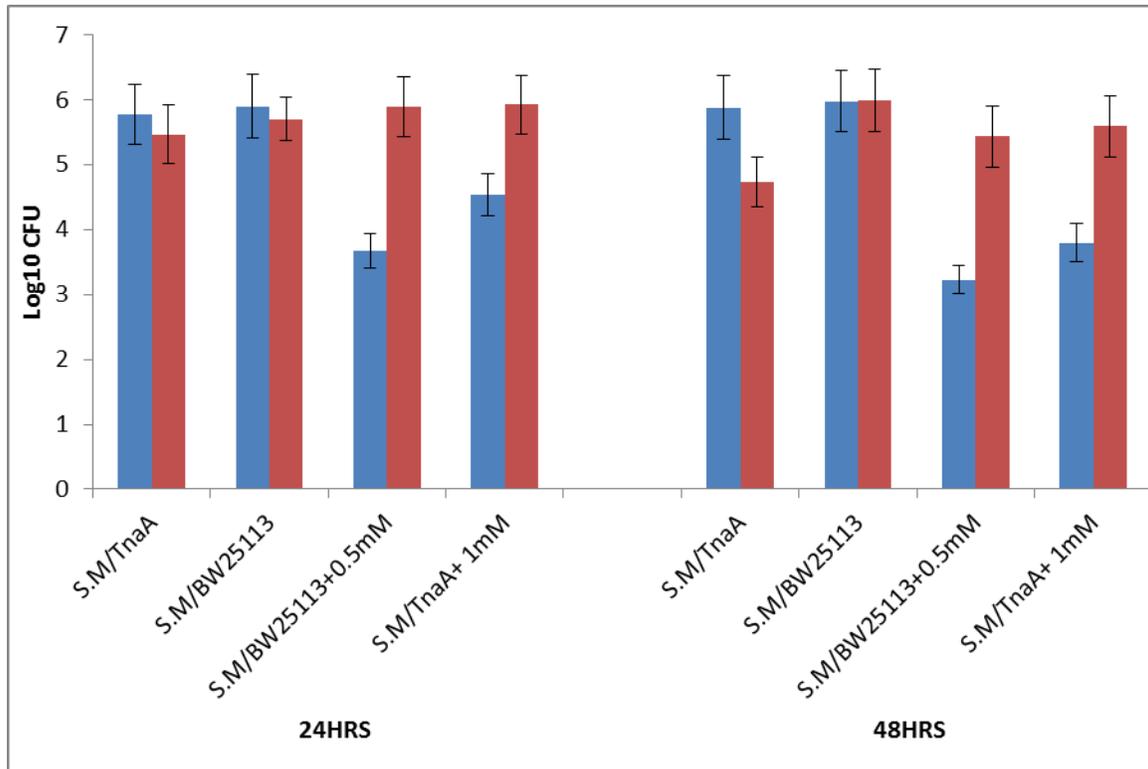


**Figure 1, continued**

**E**



**Figure 1, continued**

**F****Figure 1, continued**Pigment productionViolacein quantification

The quantitative spectrophotometric determination of violacein production showed that indole inhibited the production of violacein ( $OD_{585} = 0.054 \pm 0.02$  at 48hrs and  $0.093 \pm 0.03$  at 72hrs). Production of violacein was abundant when no indole was present in the media ( $OD_{585} = 0.745 \pm 0.066$  at 48hrs and  $1.123 \pm 0.012$  at 72hrs) (Fig 2A).

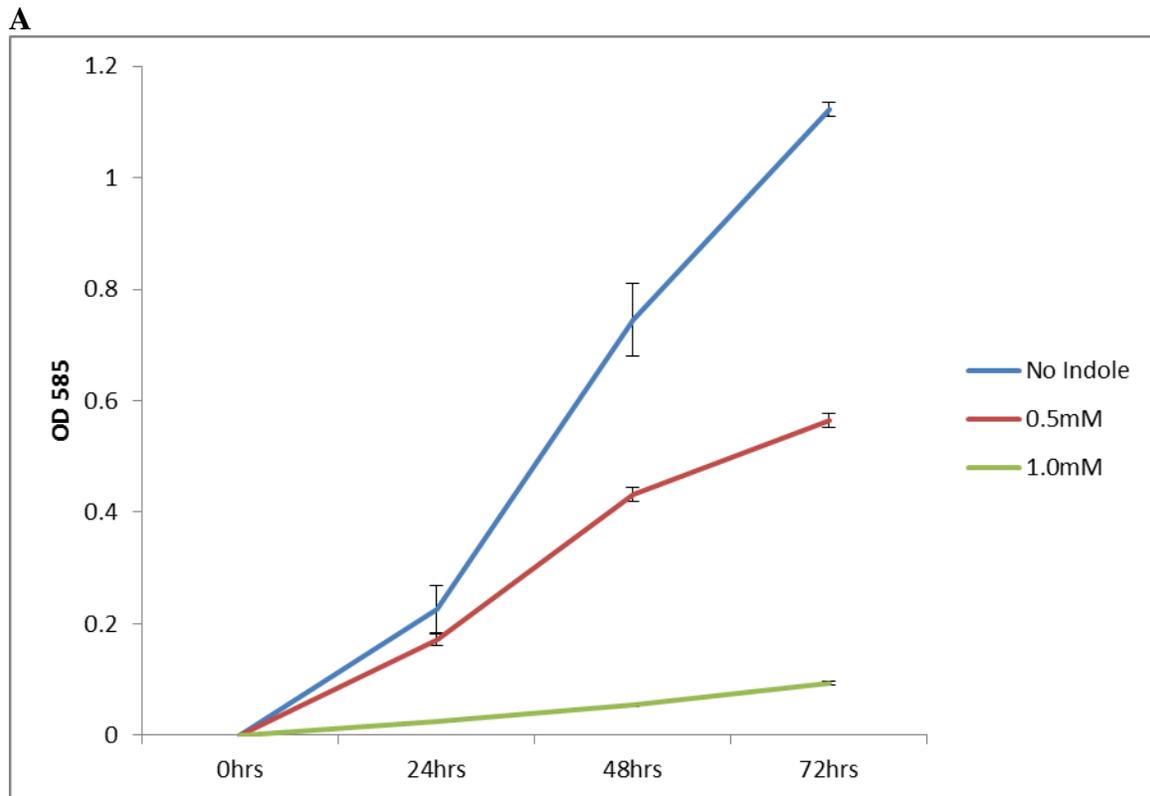
Prodigiosin quantification

Prodigiosin, the pink pigment produced by *S. marcescens*, is also regulated via quorum sensing (18). 1.0mM indole inhibited production of prodigiosin ( $OD_{534} = 0.054 \pm 0.09$  at 48hrs and  $0.067 \pm 0.010$  at 72hrs). Production of prodigiosin resumed normally when indole

was not present in the media ( $OD_{534} = 0.534 \pm 0.014$  at 48hrs and  $0.832 \pm 0.029$  at 72hrs) (Fig 2B)

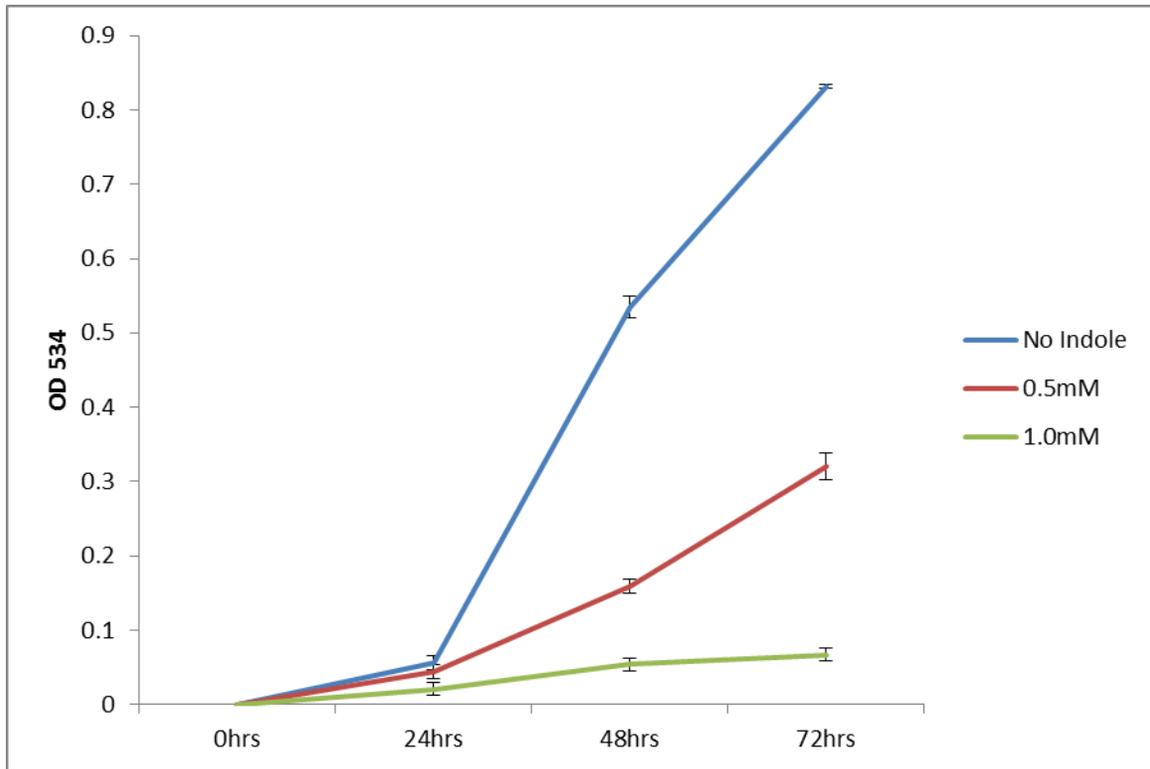
### Phenazine quantification

Phenazine production by *P. aureofaciens* also regulated by quorum sensing (25) was inhibited in the presence of 1.0mM indole ( $OD_{367} = 0.079 \pm 0.02$  at 48hrs and  $0.099 \pm 0.03$  at 72hrs). When indole was not present, phenazine production was abundant in the media ( $OD_{367} = 0.983 \pm 0.055$  at 48hrs and  $1.432 \pm 0.059$  at 72hrs) (Fig 2C).



**Figure 2. Production of violacein, prodigiosin, and phenazine in the presence and absence of exogenous indole** **A)** Violacein production peaks at 48hrs when there is no indole present. 0.5mM indole does moderately inhibits violacein production and 1.0mM indole reduced production of violacein. **B)** Prodigiosin production peaks at 48hrs when there is no indole present. In the presence of 1.0mM indole, prodigiosin production is reduced. **C)** Phenazine production starts peaking at 24hrs when indole was not present in the media. In the presence of 1.0mM indole, production of phenazine was greatly reduced.

**B**



**Figure 2, continued**

C

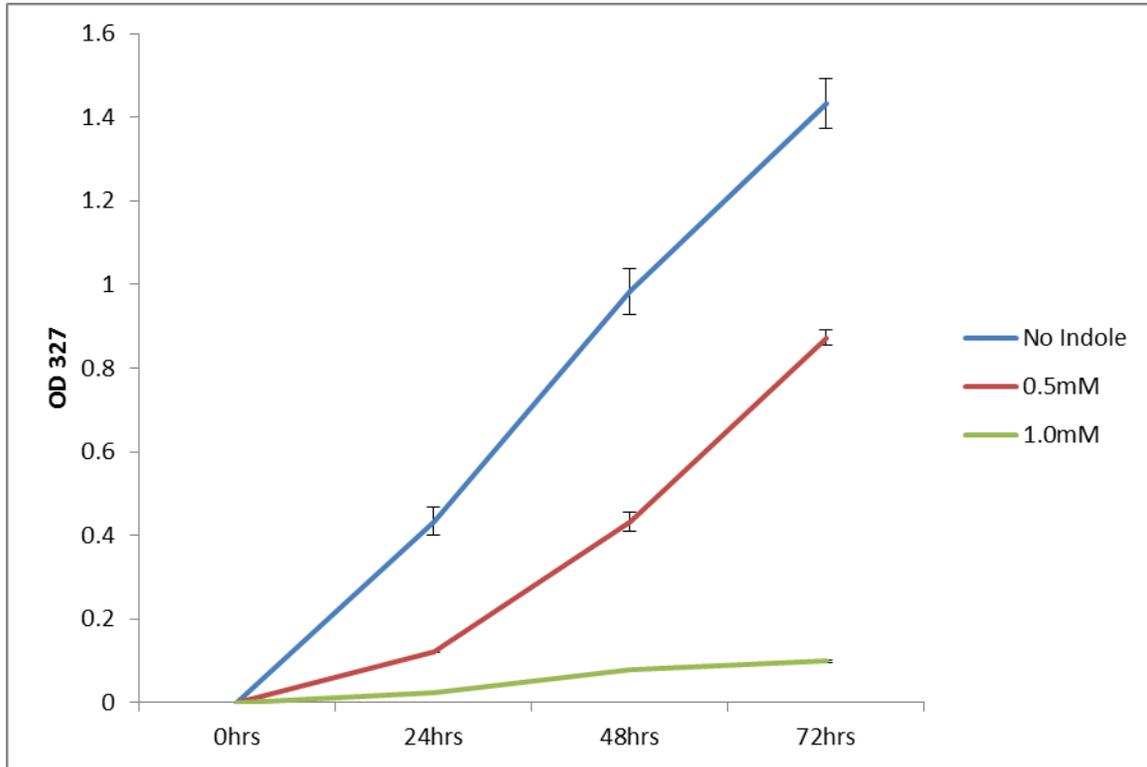


Figure 2, continued

### Exoenzyme activity and motility

#### Elastase and chitinase activity

Elastase and chitinase are regulated by quorum sensing. High elastase activity by *C. violaceum* culture supernatant was observed in the elastin-Congo red assay ( $OD_{495} = 0.534 \pm 0.0223$  at 48hrs and  $0.611 \pm 0.232$  at 72hrs). Incubation of the supernatant in 1.0mM indole inhibited elastolysis activity ( $OD_{495} = 0.089 \pm 0.0184$  at 48hrs and  $0.091 \pm 0.0343$  at 72hrs) (Fig 3A).

Elastin nutrient agar on LB exhibited elastase activity ( $2.23\text{cm} \pm 0.166$ ). Elastin nutrient in agar LB supplemented with 1.0mM indole inhibited elastase activity

(0.133cm±0.028) (Fig 3B).

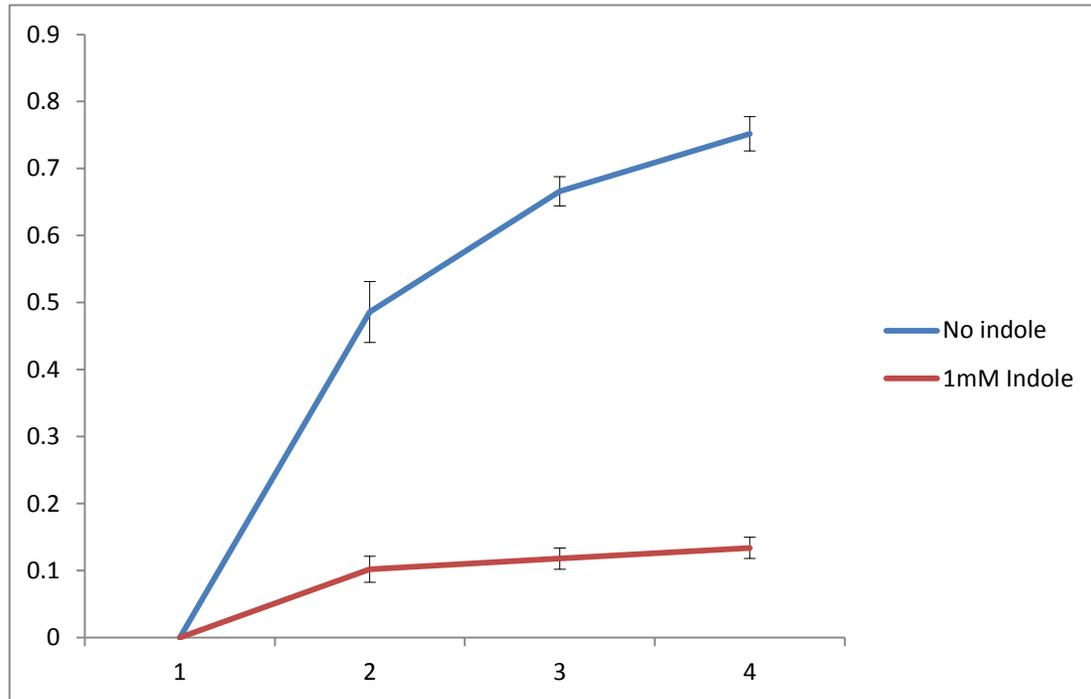
Chitinase activity by *C. violaceum* was observed in supernatant that did not contain indole. (OD<sub>585</sub>= 0.353±0.0322 at 48hrs and 0.554±0.015 at 72hrs). Supernatant extracted from samples incubated with 1.0mM indole inhibited chitinase activity. (OD<sub>585</sub>= 0.021±0.002 at 48hrs and 0.034±0.001 at 72hrs) (Fig4A).

*C.violaceum* inoculated onto plates containing minimal medium supplemented with colloidal chitin (0.2% [wt/vol]) exhibited chitinolytic activity (1.59cm±0.177). Plates containing 1.0mM indole inhibited chitanase activity (0.15cm±0.05) (Fig 4B).

### Motility

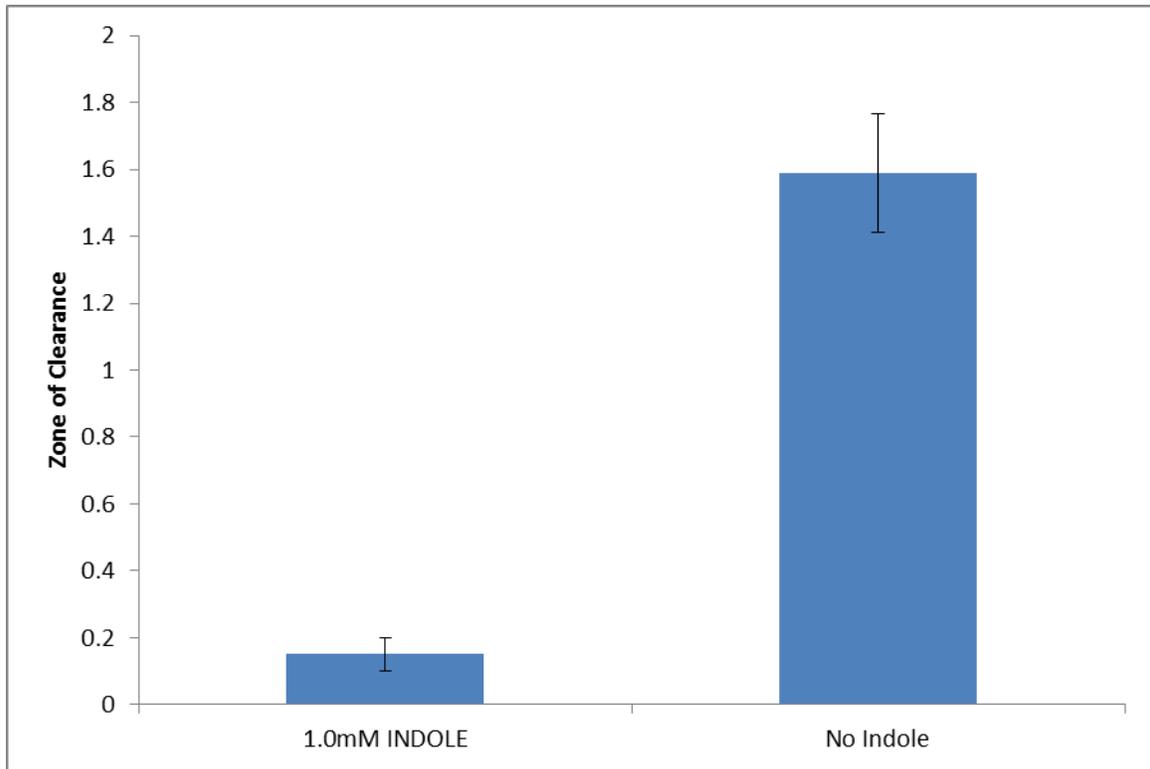
In the presence of 1.0mM indole, motility was inhibited in both *S. marcescens* (1.02 cm±0.07) and *P. aureofaciens* (0.885cm±0.130) compared to untreated media for *S. marcescens* (7.53 cm±0.15) and *P. aureofaciens* (5.43 cm±0.25) (Fig 5A and 5B).

**A**

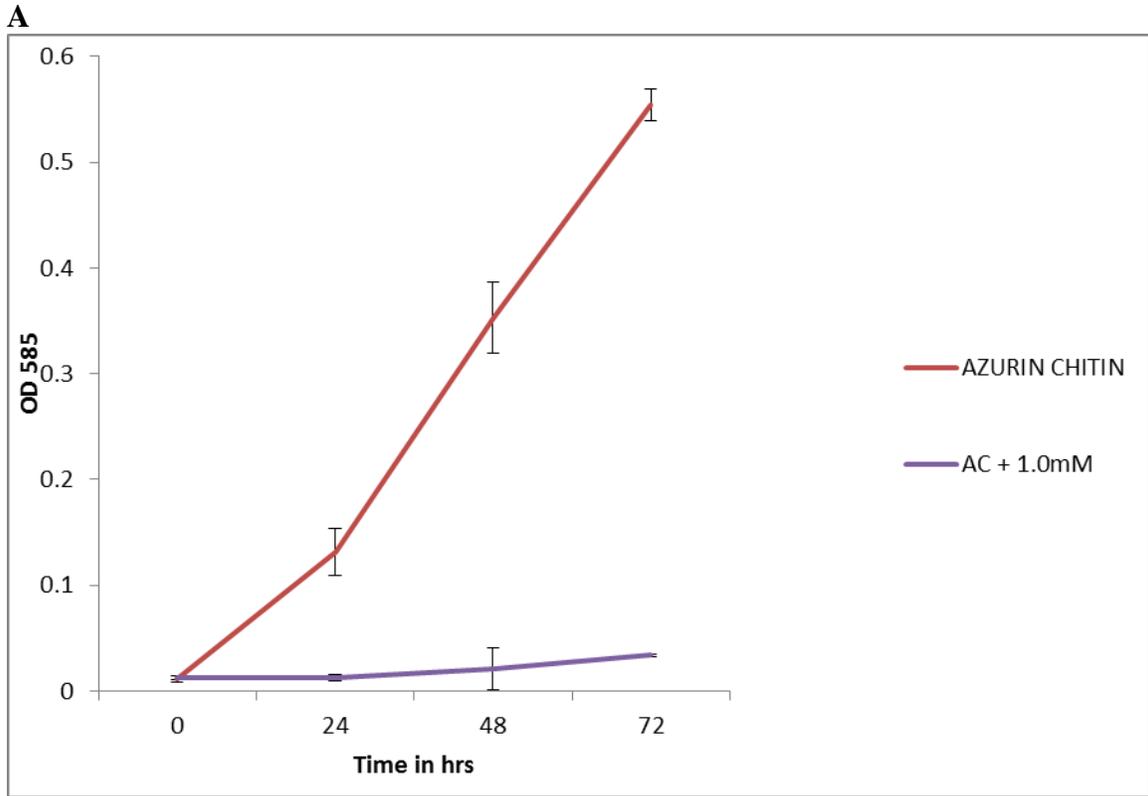


**Figure 3. A) Elastase degradation of elastin-Congo red and zone of clearance of elastin nutrient by *C. violaceum*.** Elastase activity in untreated supernatant was normal. Supernatant incubated with 1.0mM indole exhibited inhibition of the elastase enzyme and reduced optical density readings of degradation of the elastin-Congo red complex. **B) Zone of clearance** in cm. of elastin nutrient exhibited normal degradation for plates with no indole. 1.0 mM indole inhibited degradation of elastin nutrient.

**B**

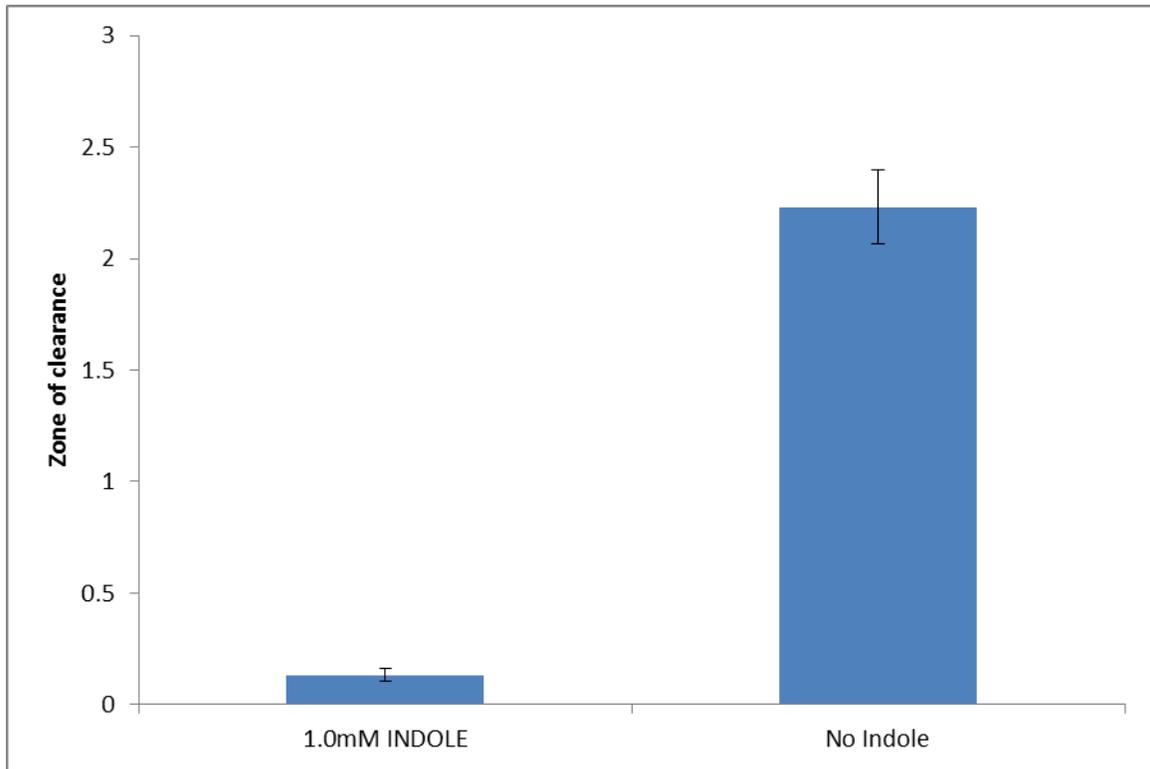


**Figure 3, continued**



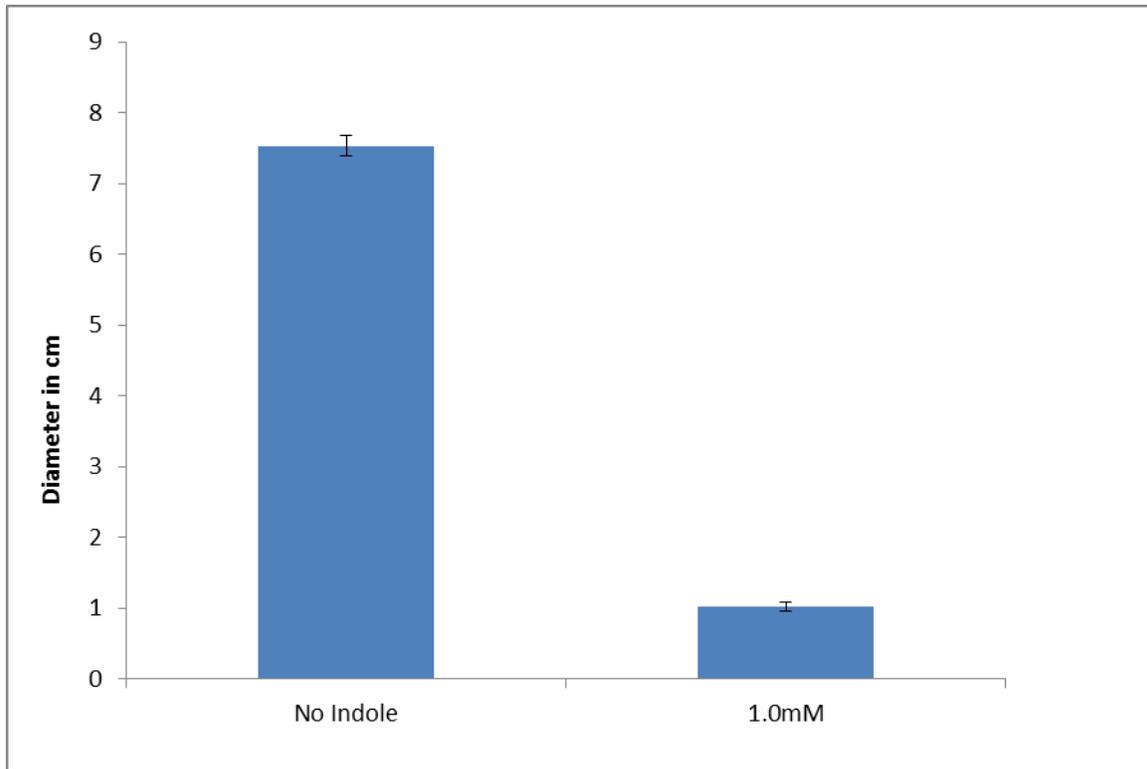
**Figure 4. A) Chitinase degradation of chitin-azure and zone of clearance of colloidal chitin by *C. violaceum*.** Chitinase activity was normal in supernatant untreated with indole for chitin-azure assays. Supernatant of cells incubated with 1.0mM indole exhibited inhibition of chitinase activity and reduced OD readings for azure. **B) Zone of clearance** in cm. of colloidal chitin exhibited normal degradation for plates with no indole. Addition of 1.0 mM indole inhibited degradation of colloidal chitin.

**B**



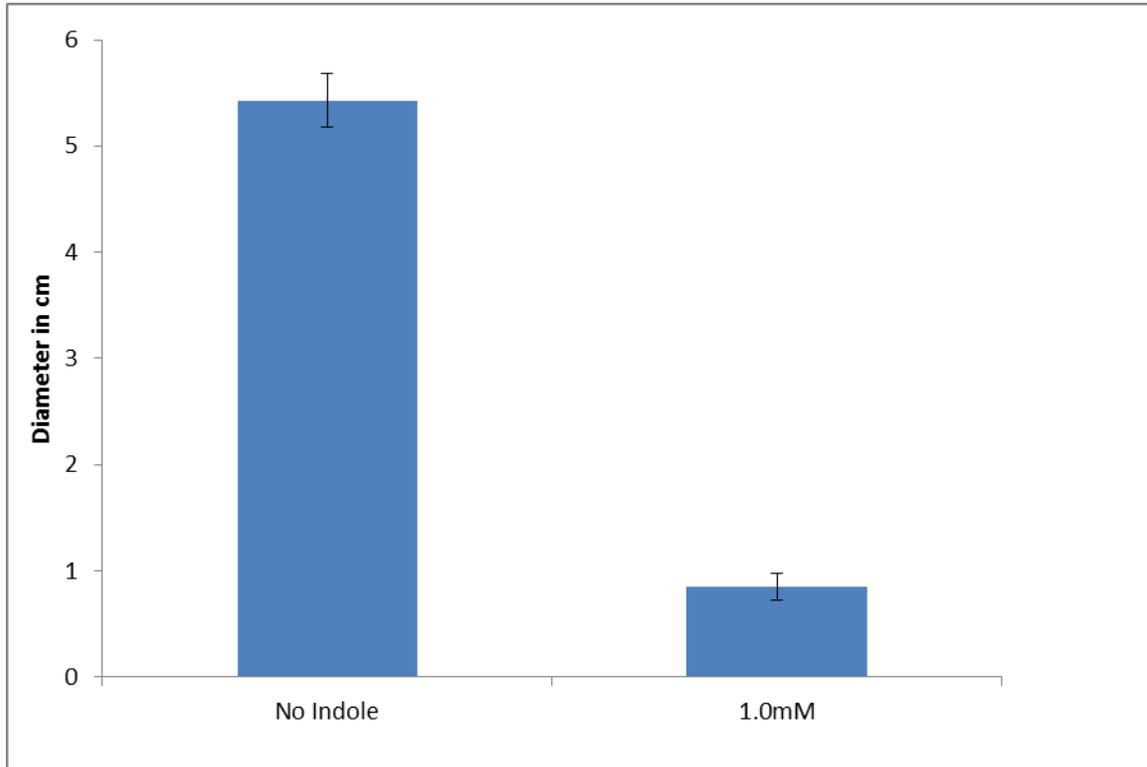
**Figure 4, continued**

**A**



**Figure 5. Motility of *S. marcescens* and *P. aureofaciens* in soft agar is inhibited in the presence of indole** **A)** Motility of *S.marcescens* in soft agar is inhibited when media is supplemented with 1.0mM indole. **B)** *P. aureofaciens* exhibited deficient motility in the presence of 1.0 mM indole. Normal motility is observed when *S. marcescens* and *P. aureofaciens* are inoculated in indole-free media.

**B**

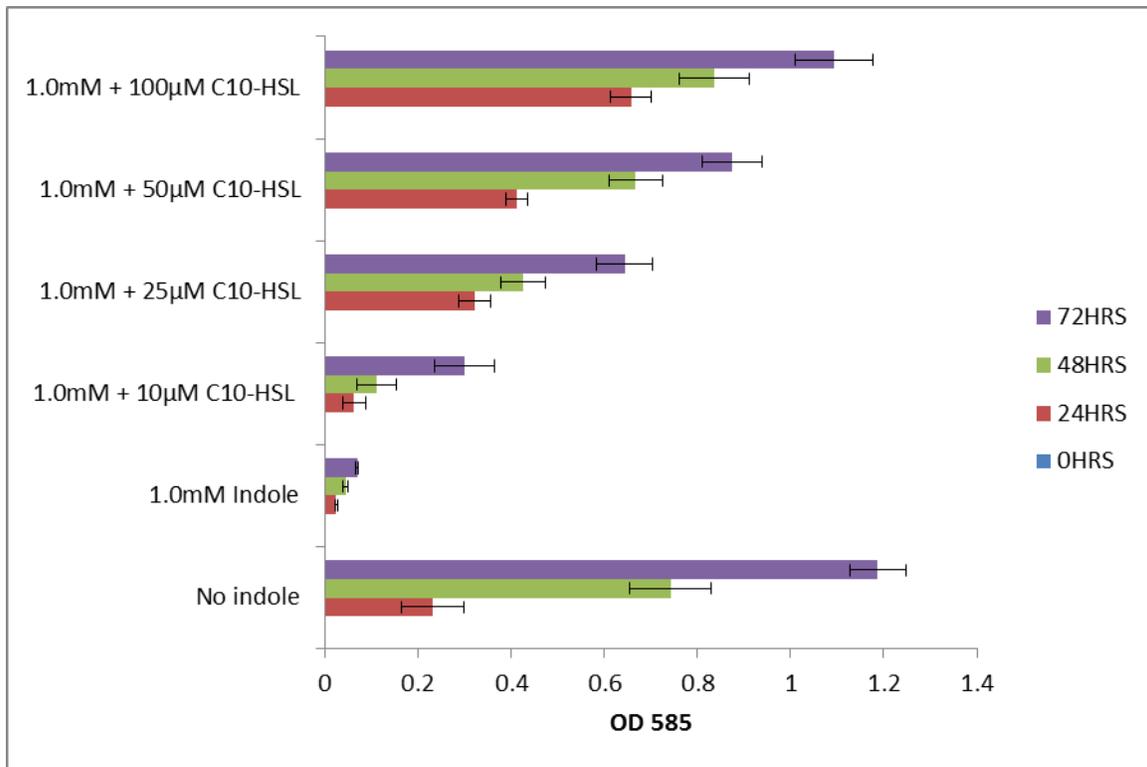


**Figure 5, continued.**

#### CH10-AHL complementation assay

*C. violaceum* was grown in 1.0mM indole and supplemented with different amounts of exogenous C10-HSL to determine if AHL- quorum-sensing could be restored. Production of violacein was restored in cultures where C10-HSL was added exogenously. Violacein recovery was proportional to the amount of C10-HSL added. Cultures with no indole had the highest violacein production as expected ( $OD_{585} = 1.187 \pm 0.06$ ). Cultures with 1.0mM indole and supplemented with 100 $\mu$ M C10-HSL exhibited higher OD readings for violacein production ( $OD_{585} = 1.093 \pm 0.0832$ ) while cultures with 1.0mM indole and supplemented with 10 $\mu$ M C10-HSL exhibited the lower OD readings for violacein production ( $OD_{585} =$

0.874±0.064). No addition of exogenous indole exhibited minimal production of violacein (OD<sub>585</sub> = 0.07±0.003). (Fig 6)



**Figure 6. Addition of C10-HSL restores production of violacein in complementation assays.** Indole inhibited violacein production. Exogenous addition of C10-HSL restored violacein production in a dose-dependent manner. Higher dosage of C10-HSL exhibited higher optical density readings for violacein.

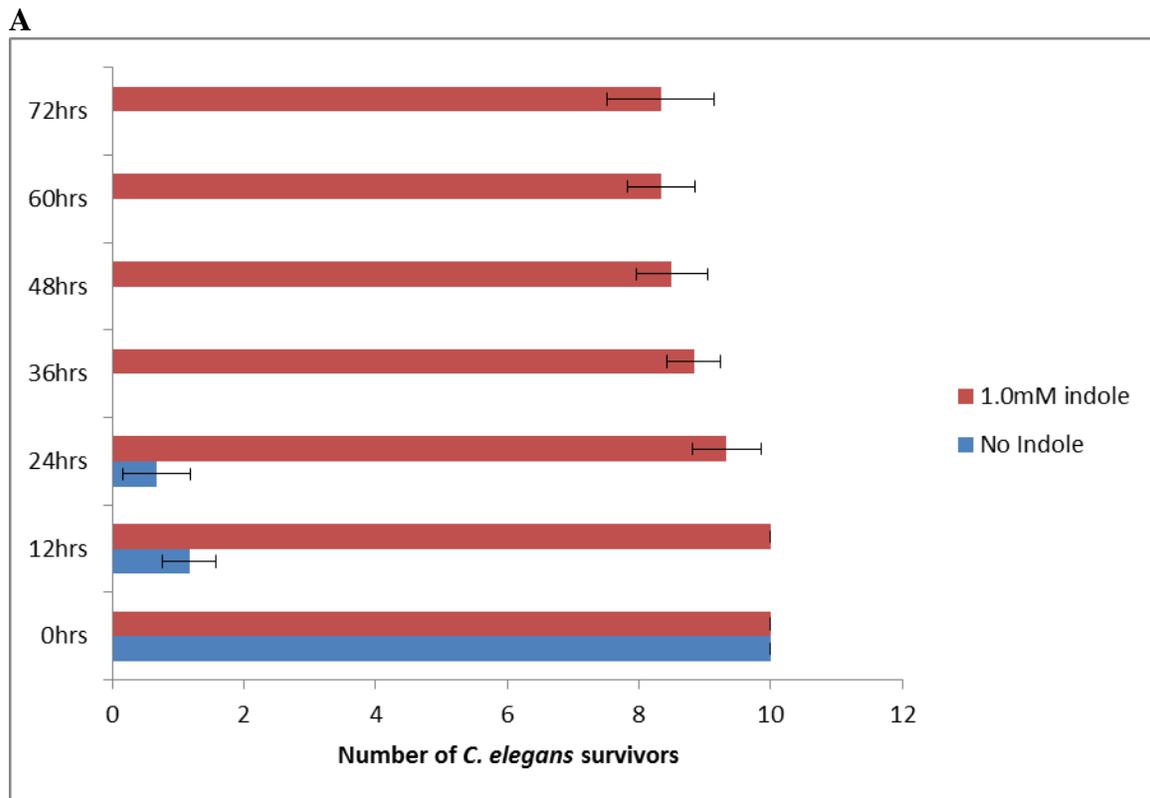
### Toxicity

*C. violaceum*, *S. marcescens* and *P. aureofaciens* exhibited different levels of toxicity toward *C. elegans* when indole was not present in the media and there was no inhibition of AHL-mediated quorum sensing. Average survival of *C. elegans* exposed to *C. violaceum* was 24hrs (0.666±0.516) (Fig 7A).

The average survival for *C. elegans* exposed to *S. marcescens* was 48hrs (2.166±1.211) (Fig 7B)

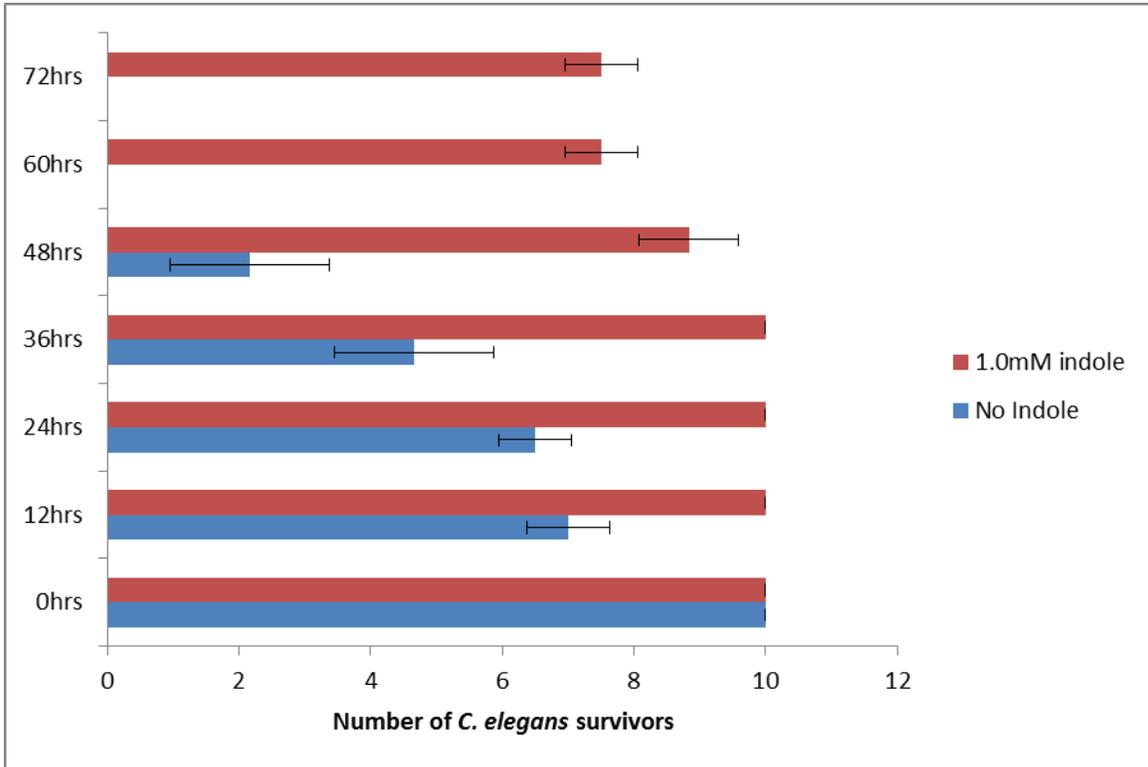
The average survival for *C. elegans* exposed to *P. aureofaciens* was 48hrs (2.166±1.211) (Fig 7C)

When 1.0mM indole was present in the media, *C. elegans* survival was 72hrs when exposed to *C. violaceum* (8.333±0.816), *S. marcescens* (7.5±0.457) and *P. aureofaciens* (4.833±1.16). Survival continued after 72 hrs but this data was not recorded.



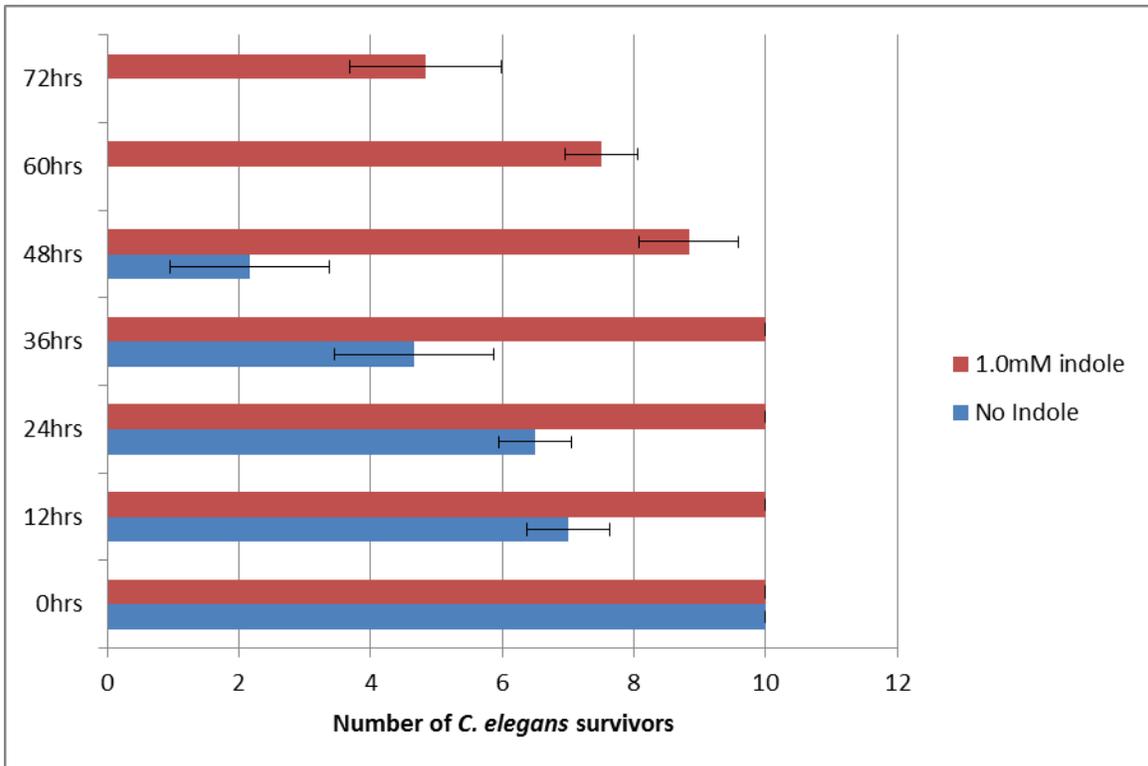
**Figure 7. *C. elegans* survival in the presence of indole** A) *C. violaceum* is more toxic when AHL-mediated quorum sensing is activated. B) and C) *S. marcescens* and *P. aureofaciens* are toxic to *C. elegans* when AHL-mediated quorum system is uninhibited. Presence of indole turned off AHL-mediated quorum sensing and increased *C. elegans* survival in the presence of Gram-negative bacilli.

**B**



**Figure 7, continued**

**C**



**Figure 7, continued**

## CHAPTER IV

### Discussion

AHL-mediate quorum sensing in Gram-negative bacteria is dependent on cell population density. When AHL autoinducers synthesized by CviI in *C. violaceum* reach a threshold, these autoinducers bind to the receptor CviR, which in turn regulates expression of violacein, chitinase, elastase, cyanide production among other genes [3, 4]. Our results indicate that genes under the AHL-mediated quorum sensing system in *C. violaceum* are up-regulated after 24hrs in untreated cells in comparison with the same genes from cells treated with 1.0mM indole. This may be an indication that indole is a general AHL-mediate quorum sensing inhibitor in Gram-negative bacteria.

Three genes in the VioABCDE cluster responsible for violacein production were up-regulated along with the genes responsible for elastase, chitinase and cyanide production in the absence of indole [14].

Genes responsible for the synthesis of the autoinducer (*cviI*) showed a 2-fold up-regulation compared with the indole-treated sample. CviR was not detectable in cultures treated with 1.0mM. Further research is needed to determine what possible inhibitory effect indole has on the AHL-receptor on *C. violaceum*.

Our results for qRT-PCR indicated an up-regulation for genes under the AHL-mediated quorum system in *C. violaceum*. Corroboration of our qRT-PCR results is important to determine if the up-regulation translated into phenotypical inhibition or expression of these genes.

Production of violacein in *C. violaceum*, prodigiosin in *S. marcescens* and phenazines in *P. aureofaciens* are under the control of AHL-mediated quorum sensing [9]. Optical density (OD) readings of pigment production are generally noticeable at 24hrs and it peaks between 48hrs-72hrs when cells are past the early lag phase. Cultures that were grown in the presence of 1.0mM indole had significantly reduced OD readings of pigment production and after 48hrs OD readings of pigment production did not increase significantly. Untreated cultures had significantly higher OD readings indicating that pigment production is being up-regulated in these Gram-negative bacteria. These results corroborate out qRT-PCR findings for *vioA*, *vioB*, and *vioC* genes.

Other research is consistent with our findings. Inhibition of AHL-regulated pyocyanin and other AHL-regulated virulence factors in *P. aeruginosa* cultures are inhibited in the presence of indole produced by *E. coli* and exogenous indole [6, 7].

Expression of different phenotypes in many Gram-negative bacteria including cell differentiation, production of secondary metabolites and exoenzymes is quorum-sensing-dependent. In *C. violaceum*, the production of exoenzymes such as chitinase and elastase are under the AHL-mediated quorum sensing system [16, 21].

Our results are consistent with RT-PCR of elastase and chitinase genes. The OD readings of elastase activity in elastin-Congo red assay and chitin azure are noticeable at 24hrs and increases after 48hrs. OD readings in supernatant treated with 1.0mM indole showed poor degradation of the elastin and chitin.

Similarly, zones of clearance were observed in plates containing elastin nutrient and colloidal chitin after 24 hrs. Media supplemented with 1.0mM and elastin nutrient or colloidal chitin inhibited zones of clearance. This indicates that indole does not only inhibit

the production of pigment but also inhibits other phenotypic characteristics under the AHL-regulated quorum sensing in *C. violaceum*.

Motility in *P. aureofaciens* and *S. marcescens* is inhibited in media supplemented with 1.0mM indole. Bacteria grown on normal media exhibited normal motility. This result is expected since research on *P. aureofaciens* has shown that phenazine production affects motility and biofilm formation [23, 25]. Phenazine structural mutant *phzR* and *phzI* are deficient in biofilm production. These data suggests that loss of phenazine production is responsible for deficient motility and biofilm formation [23, 25]. This goes in hand with our results because this would indicate that the inhibition of indole in phenazine production of *P. aureofaciens* does influence motility and ultimately biofilm formation which is supported by our data.

Previous studies have demonstrated biofilm formation and sloughing in *S. liquefaciens* and sliding motility in *S. marcescens* are under the AHL-mediated quorum sensing system [15, 23]. Other studies have shown that N-nonanoyl-cyclopentylamide (C9-CPA) was an inhibitory of quorum sensing in various *Serratia*. In *S. marcescens*, C9-CPA was able to inhibit prodigiosin production, swarming motility and biofilm formation [15]. Similarly, indole has an equal effect on *S. marcescens* as it reduces prodigiosin production, motility, and biofilm formation. These results indicate that AHL-mediated quorum-sensing in *P. aureofaciens* and *S. marcescens* are involved in bacterial motility, which in turn plays an important role in biofilm formation [15, 23]

*E. coli tnaA* mutant is deficient in the production of indole. No detectable indole was obtained in late stationary-phase cultures of *E. coli tnaA*. In competition assays with *C. violaceum*, *S. marcescens* and *P. aureofaciens*, *E. coli tnaA* was outcompeted in planktonic

and biofilm colony forming unit (CFU) counts. Planktonic and biofilm decrease of *E. coli tnaA* were more pronounced after 48hrs when AHL-mediated quorum sensing is at its highest level in these Gram-negative.

In co-cultures with to *E. coli* BW25113, that produces between 0.450mM-0.5mM indole at late stationary-phase, CFU for *E. coli* and *C. violaceum*, *S. marcescens* and *P. aureofaciens* remained at an equal footing in CFU counts, without any of them gaining an advantage over the other even after 48hrs. Indole present in amounts 0.5mM or less does have some inhibitory effect but *C. violaceum*, *S. marcescens* and *P. aureofaciens* are able to overcome it and activate AHL-mediate quorum sensing to almost normal levels.

Co-cultures with 1.0mM present (*E. coli tnaA* supplemented with 1.0mM indole and *E. coli* BW25113 supplemented with 0.5mM indole) exhibit an inhibitory effect on *C. violaceum*, *S. marcescens* and *P. aureofaciens* and affect their ability to compete in planktonic and biofilm cultures.

This may be an indication that the loss of pigment production, exoenzymes, motility and other factors under AHL-mediated quorum sensing affect the ability of *C. violaceum*, *S. marcescens* and *P. aureofaciens* to compete in mix cultures assays.

1.0mM indole is able to quench quorum sensing in *C. violaceum* and inhibit the production of violacein. Addition of exogenous C10-HSL activated violacein production in a dose-dependent manner. Higher concentrations of C10-HSL were able to increase violacein production to fairly normal levels. This could suggest that indole and C10-HSL are competing signals when both are present in the environment.

The results from the toxicity assay of *C. violaceum*, *S. marcescens* and *P. aureofaciens* indicated that pigment seems to be an important factor in the survival of these

bacteria against predators. *C. elegans* exposed to *C. violaceum*, *S. marcescens* and *P. aureofaciens* in media with no supplementation of exogenous indole survived in average 24hrs -48hrs. Violacein showed to be more toxic than prodigiosin or phenazines. Pigment production is not the only biologically active compounds produced by these bacteria. Therefore more work will be required to determine whether other factors contribute to the overall toxicity of the organism. Media with 1.0 mM indole inhibited the production of pigment and other AHL-mediated quorum sensing genes and left *C. violaceum*, *S. marcescens* and *P. aureofaciens* susceptible to predation.

Other studies had previously shown that quorum-sensing genes are necessary in the competition of *C. violaceum* with *C. elegans* and that quorum-sensing inhibitors protect *C. elegans* [5, 23]. Studies with fresh isolates of *Janthinobacterium lividum* and *C. violaceum* exhibited high toxicity to the bacterivorous nonflagellates *Ochromonas sp*, *Spumella sp* and *Bodo saltans*. The same experiment demonstrated that AHL-deficient non-pigmented mutants of *J. lividum* and *C. violaceum* allowed for high growth rates for the nonflagellates tested [26, 27, 30].

Phenazines are known secondary metabolites produced by *P. aureofaciens* and are known to exhibit antimicrobial activity against bacteria, fungi and protozoa too [25].

Our data suggests that virulent genes under the AHL-mediated quorum system are being quenched in the presence of 1.0mM indole and leaves Gram-negative bacteria more susceptible to competition and predation. Indole-mediated inhibition of AHL quorum signaling appears to be a general phenomenon and not exclusive to *P. aeruginosa*

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