INDOLE AND cAMP PROMOTE *Escherichia coli* SURVIVAL IN MIXED CULTURE

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Tesfalem Rezene Zere, M.S.

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INDOLE AND cAMP PROMOTE *Escherichia coli* SURVIVAL IN MIXED CULTURE

Committee Members Approved:

_____
Robert J.C. McLean, Chair

_____
Stacie A. Brown

_____
Gary M. Aron

_____
Dana M. Garcia

Approved:

_____
J. Michael Willoughby
Dean of the Graduate College

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ABSTRACT

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Tesfalem Rezene Zere, M.S.

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SUPERVISING PROFESSOR: ROBERT J.C. MCLEAN

In their natural environments, bacteria typically are found as mixed species communities and are often attached to some type of surface. There has been considerable interest in studying bacterial biofilms; however, little is known about the mechanisms that enable them to grow as mixed populations in biofilms or planktonic communities. Recent results from the McLean laboratory on transcriptional profiles of *Escherichia coli MG1655* in mixed culture with *Pseudomonas aeruginosa PAO1* showed a number of *E. coli* genes to be upregulated including *purA-F* and other genes associated with purine synthesis. In contrast, genes associated with pyrimidine synthesis were unaffected. Competition experiments in both planktonic and biofilm cultures, using three purine synthesis mutants, *purD, purH,* and *purT* showed little difference in *E. coli* survival from the parent strain.

As purines are components of the cell signals cAMP and c-di-GMP, in this study we conducted competition experiments with *E. coli* mutants lacking adenylate cyclase (*cyaA*), cAMP phosphodiesterase (*cpdA*), and the catabolite receptor protein (*crp*), as well as diguanylate cyclase (*ydeH*) associated with c-di-GMP synthesis. Survival of the *cyaA* and *crp* mutants during co-culture were significantly less than the parent strain. Supplementation of the media with 1mM cAMP could restore survival of the *cyaA* mutant but not the *crp* mutant. In contrast, survival of the *cpdA* mutant was similar to the parent strain. Survival of the *ydeH* mutant was unaffected, suggesting that cAMP has more impact on *E. coli* mixed culture growth than c-di-GMP. Addition of 1 mM indole restored the survival of both the *cyaA* and *crp* mutations. Mutants in genes for tryptophan synthesis (*trpE*) and indole production (*tnaA*) showed a loss of competition and recovery through indole supplementation, comparable to the *cyaA* and *crp* mutants. Overall, these results suggest indole and cAMP as major contributing factors to *E. coli* growth in mixed culture.

CHAPTER I

Introduction

In nature, bacteria are more likely to grow in polymicrobial communities than in monoculture. Interactions among community members are required for community development and maintenance [1] and can involve a variety of mechanisms including the generation of antimicrobial compounds, nutritional interactions and interspecies signaling [2]. The use of chemical signals for bacterial communication is a widespread phenomenon. In Gram-negative bacteria, these signals can be *N*-acyl derivatives of homoserine lactone (AHLs), cyclic dipeptides, quinolones and indole [3, 4]. Central metabolism, long regarded as reflecting a number of routine "housekeeping functions" is now being reexamined for its role in mixed culture interactions within biofilm and planktonic populations [2, 5].

One component of central metabolism in *Escherichia coli* involves purine and pyrimidine nucleic acid synthesis (reviewed in [6]. Pathways for de novo synthesis and salvage pathways exist for both nucleotides. During purine synthesis, ribose-5-phosphate, an intermediate in the pentose phosphate cycle, is converted first to 5-phosphoribosyl-1-pyrophosphate (PRPP) and then through a series of ten steps (catalyzed by the gene products of various *pur* genes) to inosine monophosphate (IMP) and then to the end products adenosine monophosphate (AMP) and guanosine monophosphate (GMP). In the

salvage pathway, guanine- and adenine-containing compounds are converted through a series of steps to IMP or else directly to AMP and GMP. De novo purine synthesis is regulated overall by allosteric regulation by AMP and GMP in the first step (PRPP amidotransferase, PurF) [7 8].

Aside from being nucleic acid components, purines serve other important functions in bacteria. These functions include energy transfer (ATP and GTP) and cell signaling (cAMP, bis - (3'- 5')-cyclic di-GMP (c-di-GMP), and guanosine tetraphosphate (ppGpp)) [6, 9, 10]. The secondary signal molecule, cAMP, is synthesized from AMP by adenylate cyclase, encoded by *cyaA*[8] and is broken down by cAMP phosphodiesterase, encoded by *cpdA* [11]. During c-di-GMP synthesis, GMP is phosphorylated to GTP and two GTP molecules are then converted by diguanylate cyclase to c-di-GMP, encoded by a variety of proteins having a common amino acid motif of GGDEF [9]. Phosphodiesterase activity, responsible for c-di-GMP breakdown is contained in proteins having either an EAL or HD-GYP domain [9]. The receptor for cAMP is the cAMP receptor protein, encoded by *crp* [8]. In contrast, receptors for c-di-GMP are quite diverse [9]. The second messengers, cAMP and c-di-GMP, have been linked to a number of cellular functions in *E. coli*. c-di-GMP has been associated with a number of functions related to biofilm formation [9]. Traditionally, cAMP has been associated with regulation of carbon catabolism [12], although recently it has been also associated with a number of stress responses [13]. One catabolic function associated with cAMP regulation is indole production from tryptophan [14]. Since the discovery of its formation from tryptophan in the early 20th century [15], indole production has been employed as a biochemical test

for distinguishing *E. coli* from other members of the *Enterobacteriaceae.* Indole is now recognized as a bacterial signal molecule [3, 4].

Previous results from our lab (contributions of MM Weber, M Whiteley, and RJC McLean, contained in W Chu, TR Zere, MM Weber, TK Wood, M Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted) showed that genes associated with de novo purine synthesis were upregulated in *E. coli* during co-culture with *Pseudomonas aeruginosa*. Pyrimidine synthesis genes were unaffected, which tended to rule out a requirement for DNA or RNA synthesis. Using deletion mutants, we then explored the role of purine-containing, secondary signaling pathways, cAMP and c-di-GMP (contributions of W Chu, MM Weber, and RJC McLean, contained in W Chu, TR Zere, MM Weber, TK Wood, M Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted). The most notable loss of *E. coli* competitiveness was observed in mutants lacking the gene for adenylate cyclase (*cyaA*, cAMP synthesis) and the cAMP receptor protein (*crp*). Chemical supplementation with cAMP could restore competitiveness in *cyaA* but not *crp* mutants. In contrast, no significant effects in either planktonic or biofilm coculture populations were seen in *ydeH* mutants, lacking diguanylate cyclase (c-di-GMP synthesis) [15]. As indole production was diminished in cAMP mutants, we then explored the role of indole using mutants lacking indole receptor protein (*sdiA*), and indole production (*tnaA*) [3, 4]. As was the case with cAMP mutants, loss of tryptophan or indole production greatly reduced *E. coli* competitiveness in mixed culture. Chemical supplementation with a physiologically relevant concentration of indole reversed the loss of competition seen in *trpE* and *tnaA* mutants as well as the cAMP mutants *cyaA* and *crp*. Although indole was not toxic to *P. aeruginosa*, its presence did reduce the levels of

several quorum-regulated compounds, notably pyocyanin [16]. Overall, the chemical signals cAMP and indole play an important role in promoting *E. coli* fitness in mixed cultures.

CHAPTER II

METHODOLOGY

2.1 Bacterial strains, media and chemicals

The strains used in this study are listed in Table 1. The strains were maintained in Luria-Bertani (LB) medium supplemented with kanamycin (50 µg/ml) for *E. coli* mutants. For long-term preservation, liquid cultures were frozen at -80 ^{o}C using glycerol (12.5 % v/v) as a cryoprotectant. Prior to experimentation, cultures were revived from frozen stocks, and cultured overnight in LB agar. The cultures were then subcultured in LB broth from LB plates and incubated overnight at 37 ^{o}C with aeration (100 rpm). Unless stated otherwise, during experimentation the turbidity of the overnight cultures was adjusted to OD_{600} of 0.1 [$1.1 \pm 0.2 \times 10^5$ CFU/ml] using sterile LB broth. During mixed culture experiments, we used LB plates with ampicillin (100 µg/ml) to select for *P. aeruginosa* or LB plates with cefsulodin (20 µg/ml) to select for *E. coli* [17]. Chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO). Stock solutions of cAMP and indole were prepared in deionized water and in dimethyl formamide (DMF), respectively. The stock solutions were filter sterilized using 0.22-µm-poresize sterile filter and stored at -4 ^{o}C until use.

Table 1: List of *E. coli* and *P. aeruginosa* strains used in this study.

Strain			Mutation	Reference
E. coli	*BW25113*	*BW25113*	Wild type (*wt*)	[18]
		BW25113 crpA::kan	cAMP receptor protein (CRP)	[13, 18]
		BW25113 cyaA::kan	Adenylate cyclase	[18, 19]
		BW25113 cpdA::kan	Phosphodiesterase	[11, 18]
		BW25113 ydeH::kan	Diguanylate cyclase	[18]
		BW25113 tnaA::kan	Tryptophanase	[14, 18]
		BW25113 trpE::kan	Anthranilate synthase component I	[18]
		BW25113 tnaC::kan	Tryptophanase leader peptide	[14, 18]
		BW25113 trpL::kan	Trp operon leader peptide	[18]
		BW25113 sdiA::kan	Indole receptor protein	[4, 18, 20]
P. aeruginosa	*PAO1*	*PAO1*	*wt*	[21]
		PDO100 ΔrhlI	Quorum-sensing-deficient mutants	[21, 22]
		PAO-MW1 ΔrhlI ΔLasI	Quorum-sensing-deficient mutants	[21, 23]

2.2 Competition experiments

2.2.1 Competitiveness of cAMP and c-di-GMP mutants

We investigated the competitiveness of several *E. coli* strains (Table 1) during growth in mixed culture with *P. aeruginosa PAO1*. Prior to experimentation, cells were revived from frozen stock cultures, subcultured, and the OD_{600} adjusted to 0.1 as described above. For mixed culture analysis, 100 µL inocula from each strain of *E. coli* were mixed with 100 µL of *PAO1* in 50 ml sterile LB broth. For biofilm analysis, eight sterile silicon discs (7 mm diameter) [24] were placed into the flasks as biofilm colonization substrata. The experiment was run for 96 h at 37 ^{o}C with a shaking speed of 100 rpm. Every 24 h, 500 µL (for the planktonic) and a pair of silicon discs (for the biofilm) were taken for bacterial and chemical analyses. The sonication and serial dilution protocol for biofilm analysis and dilution plating protocol for planktonic populations are described previously [17]. In addition to the mixed culture analysis, pure culture experiments for each strain were also conducted as controls. Chemical analysis is described below. Each experiment was replicated a minimum of three times.

2.2.2 Exogenous addition of cAMP

To investigate whether exogenous addition of cAMP can restore competitiveness of the mutant strains of *E. coli* in mixed culture growth with *PAO1 ,* cAMP from a stock solution (section 2.1) was added to the culture medium at different concentrations (0.2, 0.5 and 1 mM) and the competition experiments were conducted as described above (section 2.2.1).

2.2.3 Exogenous addition of indole

To investigate whether exogenous addition of indole can restore competitiveness of the mutant strains of *E. coli* in mixed culture growth with *P. aeruginosa*, we added indole from a stock solution (section 2.1) to the culture medium at different concentrations (ranging from 0.1 - 1 mM) and conducted monoculture and mixed culture competition experiments as described above (section 2.2.1). Samples were removed every 24 h for indole analysis as described previously. We also investigated whether indole could inhibit growth of *P. aeruginosa* or promote growth of *E. coli* (Fig 8) and thus account for any direct indole-related enhancement of *E. coli* competitiveness. Here, the media was supplemented with 1mM indole and pure and mixed culture experiments were performed as described previously, with samples taken every 24 h for bacterial and chemical analysis.

2.3 Indole assay

2.3.1 Colorimetric Protocol

Bacterial cultures were tested for intracellular and extracellular indole production in pure and mixed culture using the modified protocol by Kawamura-Sato et al. [4, 25, 26].

2.3.2 Indole production in pure vs. mix culture

In order to compare the amount of indole produced by each of the *E. coli* strains in pure vs. mix culture and to investigate the importance of indole for *E. coli* competitiveness in mixed culture, the extracellular indole produced by each *E. coli* strain (both in pure and mixed culture) was quantified as illustrated above (section 2.3.1). At the same time, samples were taken from the same culture media for colony forming units (CFU) of each strain in pure and mix culture. Then the amount of indole produced by each strain (in

pure and mixed culture) at each time point was normalized to the CFU/ml of each strain in pure and mix culture.

2.3.3 Degradation of indole in mixed culture

Whether indole is degraded (or used as a nutrient) by *E. coli* and/or *PAO1* strains was investigated both in pure and mixed culture. Here, we used two strains of *E. coli cyaA* and *crp* that are unable to produce indole. This study was done by adding indole to the culture medium (1 mM for the *crp* and *PAO1* and 0.5 mM for the *cyaA*). Cultures were inoculated as described in section 2.2.1 and incubated for 96 h with samples removed every 24 h for indole analysis (section 2.3.1).

2.4 Mechanism(s) of ecological fitness

2.4.1 Effect of indole and cAMP on *PAO1*

We investigated whether the effect of cAMP and indole on the coexistence of *E. coli* in mixed culture with *PAO1* is direct (on *E. coli*) or indirect (by affecting *PAO1*). The main strategy used in this context was the *E. coli* strain selection (Table 1) with and without chemical complementation with cAMP or indole. Strains lacking the ability to produce cAMP (*cyaA*), respond to cAMP (*crp*), synthesize the precursor of indole, namely tryptophan (*trpE*), or lacking in the enzyme responsible for indole production (tryptophanase, *tnaA*) were deficient in indole production. The *sdiA* mutant lacks the receptor for *E. coli* indole signaling [4, 18, 20]. Competition tests were done as described in section 2.2.1.

2.4.1.1 Toxicity effect

In order to see whether indole supplementation affected cell growth, we conducted growth curve experiments with pure cultures of *E. coli* and *P. aeruginosa PAO1*. Here,

pure cultures of *E. coli* and *P. aeruginosa* were grown for 48 h at 37 ^{o}C at 100 rpm in LB broth, using side-arm flasks, in the presence (1 mM) and absence of indole. By using sidearm flasks, cell growth could be monitored by turbidity (OD_{600}).

2.4.1.2 Effect on pyocyanin production

The pyocyanin extraction was conducted as described elsewhere [27, 28]. Briefly, the samples were centrifuged at 151.2 rcf for 15 min and the supernatants were transferred to 15-ml conical tubes. For pyocyanin extraction, 3 ml of chloroform was added into the tube and vigorously vortexed 30 s. The samples were then centrifuged at 2,000 rcf (Eppendorf AG 23331, Hamburg) for 5 min, and the upper layer – aqueous layer – was carefully discarded. In order to reextract pyocyanin from the organic phase into the aqueous layer, 1 ml of HCl (0.2 M, aq) was added to the samples and vigorously vortexed for 15 s, followed by centrifugation at 151.2 rcf for 5 min. After this, pyocyanin (pinkish, upper aqueous layer) was carefully recovered. The absorbance of the solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the absorbance at 520 nm (SmartSpec Plus Spectrophotometer, 273 BR 01423) by 17.072 [27, 28].

The amount of pyocyanin produced by *PAO1* in pure vs. mixed culture (with *E. coli*) was compared in order to find out if there is a difference in pyocyanin production by *PAO1* in pure vs. mixed culture. The pyocyanin produced by *PAO1* in pure and mixed culture was extracted and quantified as described above (section 2.4.1.2). Similarly, samples were taken from the same culture media for cell count and the concentration of pyocyanin was normalized to the respective cell number of *PAO1*.

2.5 Effect of cell-free culture fluid of *E. coli* on *PAO1* in mixed culture

2.5.1 Effect on pyocyanin production

The effect of cell-free culture fluid of *E. coli* strains on CFU counts and pyocyanin production of *PAO1* was investigated as described above. Briefly, overnight cultures of *E. coli* strains were pelleted by centrifugation. The supernatant was filtered with 0.22 μM filter papers. The cell-free culture fluid of the *E. coli* strains was then added to *PAO1* culture (OD_{600} of 0.1) at different dilutions (1:50 – 1:10). The *PAO1* culture was then incubated at 37 ^{o}C with aeration (100 rpm). Every 24 h, samples were taken for pyocyanin extraction and for CFU counts. The extraction and quantification of pyocyanin was done as described above. Parallel, control experiments were also conducted without *E. coli* culture fluids for comparison.

2.5.2 Antibacterial activity

In order to determine whether antimicrobial compounds were produced in pure or mixed culture by *E. coli* strains, liquid culture samples were collected, centrifuged (12,000 g for 10 min) and the supernatant was filter sterilized using a 0.2 μm-pore size sterile filter (Millipore). Drops containing 20 μl of the filter-sterilized spent culture were placed onto lawns of *P. aeruginosa* growing on either LB agar or Mueller-Hinton agar and cultured overnight at 37 ^{o}C. The diameter of any zone of inhibition due to the spent media was measured in mm with a ruler.

2.6 Indole degradation by *PAO1*

2.6.1 Mechanism(s) of indole degradation

P. aeruginosa is a non-indole producing bacterium and we observed that it can degrade indole produced by *E. coli* in mixed culture. As illustrated in section 2.3.3, we analyzed

the degradation of indole by *PAO*1. In order to answer the question how *PAO1* degrades indole, we considered two working hypotheses: The first hypothesis was that *PAO1* might use (consume) indole as a source of nutrient. However, other strains of *PAO1* (the *PAO-MW1* and *PDO100*) do not degrade indole (Fig 14). Our second hypothesis was that *PAO1* might catabolically degrade indole by secreting some metabolites. To investigate the second working hypothesis, we grew *PAO1* in LB broth for 72 h and took samples every 24 h starting from time zero. The samples were then centrifuged and filtered (as in section 2.3.1) and the cell-free supernatants were analyzed for indole degradation. That is, 0.5 mM indole was added into 25 ml LB broth, and then the cell-free supernatants were added to the indole solution (in the ratio of 1:10). The degradation of indole was analyzed (as in section 2.3.3). Parallel with this, a control experiment (LB plus 0.5 mM indole) was also conducted for comparison.

2.6.2 Indole degradation and quorum signaling

To find out if *PAO1* needs quorum signaling mechanism to degrade indole, we used two mutant strains of *PAO1*; *PAOMW1* and *PAO100*. The degradation of indole by the two mutant strains was then analyzed as in section 2.3.3.

CHAPTER III

RESULTS

3.1 Competitiveness of *E. coli* in mixed culture

3.1.1 Competitiveness of cAMP and c-di-GMP mutants

Aside from their function as nucleic acid components, purines are components of the secondary cell signaling molecules cAMP and c-di-GMP. To explore this possibility, we investigated the competitiveness of *E. coli* in mixed culture using several strains deficient in cAMP or c-di-GMP signaling. As shown in Figure 1, significant loss of *E. coli* competitiveness in both planktonic (Fig 1A) and biofilm (Fig 1B) populations was seen in mutants unable to produce cAMP (*cyaA*) or lacking the cAMP receptor gene (*crp*). There was a slight stimulation of *E. coli* competitiveness in the strain lacking the ability to degrade cAMP (*cpdA*). In contrast, no changes in competitiveness were seen in *E. coli ydeH* mutants (Figs 1A and 1B) suggesting that the loss of *ydeH*-encoded diguanylate cyclase [16] had no effect on competitiveness. We cannot rule out, however, the influence of c-di-GMP on competitiveness as *E. coli* has multiple diguanylate cyclase genes [9].

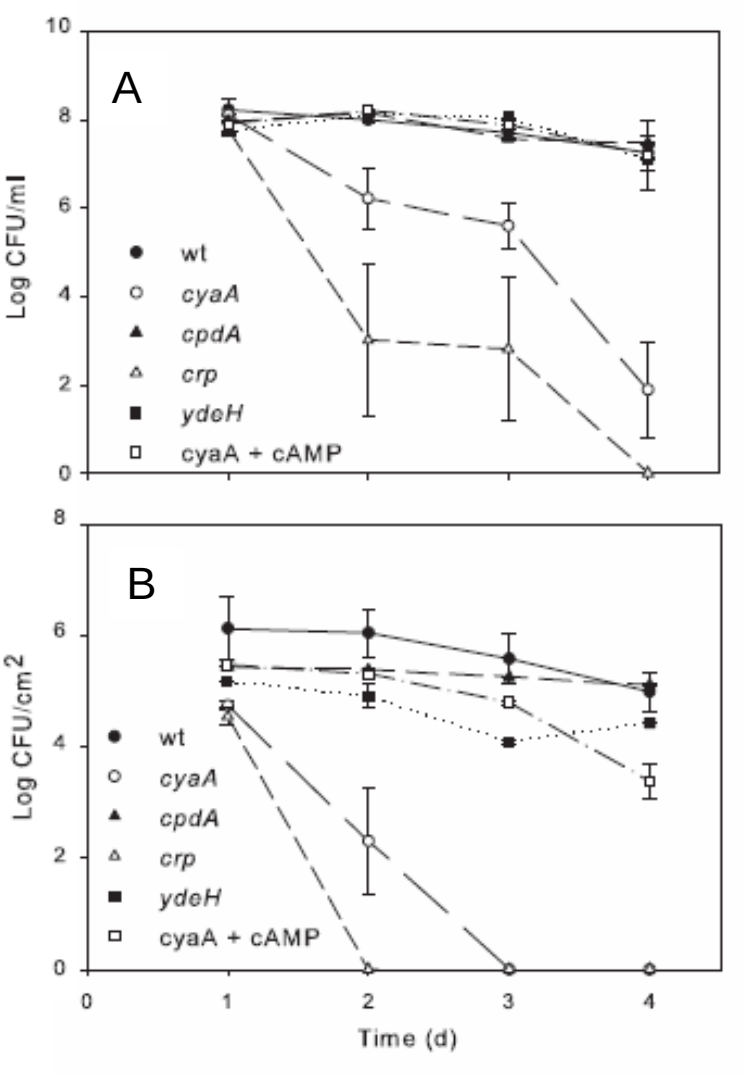

Figure 1 – Influence of second messenger-related gene mutations on *E. coli* competitiveness in mixed culture planktonic (A) and biofilm cultures (B). Similarly, the influence of exogenous addition of cAMP on *cyaA* (*cyaA* + cAMP, open squares) competitiveness in mixed culture planktonic (B) and biofilm cultures (D).

3.1.2 Competitiveness upon addition of exogenous cAMP

In order to find out whether chemical complementation could restore the competitiveness of the mutant strains, we added different concentrations of cAMP to the growth media and checked *E.coli's* competitiveness. As shown in (Figs 1A and 1B), competitiveness in the *cyaA* mutants could be restored to *wt* levels for 24 h by addition of 1 mM cAMP;

however, after 48 h these effects had disappeared, likely due to depletion of the cAMP by the culture. As anticipated, cAMP supplementation had no effect on the cAMP receptor mutant (*crp*). Surprisingly, there was no change in the *cpdA* mutant due to cAMP supplementation, suggesting that the levels of cAMP present were not inhibitory under these growth conditions. On the other hand, addition of 1mM cAMP did not show any significant effect on *cyaA* mutant in pure culture planktonic (Fig 2A) or the biofilm population (Fig 2B).

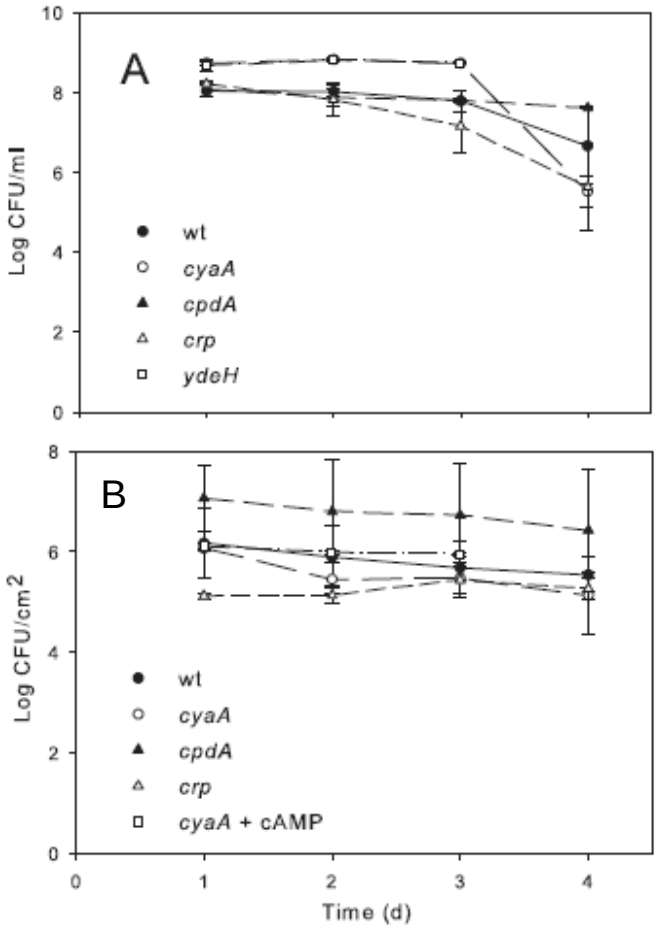

Figure 2 – Influence of second signal gene mutations on *E. coli* monoculture planktonic (A) and biofilm (B) cultures. Similarly, the influence of exogenous addition of cAMP on *cyaA* competitiveness in pure culture planktonic (A) and biofilm cultures (B).

Pure culture planktonic (Fig 2A) and biofilm (Fig 2B) experiments as well as growth curve experiments showed that cAMP and c-di-GMP disruption did not affect *E coli* growth. Therefore, the loss of competitiveness of the cAMP mutant strains observed in (Figs 1A & 1B) is not due to growth defect.

3.1.3 Competitiveness upon exogenous addition of indole

Similar to the effect of cAMP, we also investigated the influence of indole on *E. coli* competitiveness in mixed culture. This investigation was done by testing the competitiveness of *E. coli* mutants deficient in indole production (*tnaA*) and in indole receptor (*sdiA*). As shown in figure 3, *E. coli tnaA* mutant was notably less competitive than their *wt* counterparts in both planktonic (Fig 3A) and biofilm (Fig 3B) cultures. In both cases, supplementation with 1mM indole restored mutant populations to *wt* levels at 24 h (Figs 3A and 3B).

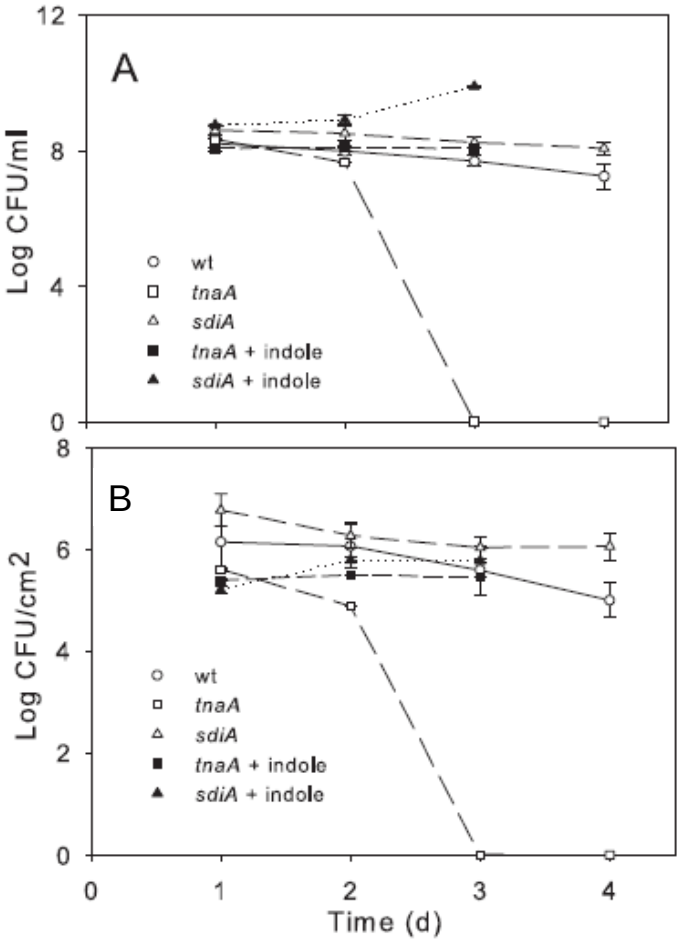

Figure 3 – Influence of indole on *E. coli* growth in mixed culture. Loss of competition in *E. coli* planktonic (A) and biofilm (B) cultures due to *tnaA* mutations could be restored to *wt* levels for 48 h by addition of 1mM indole; however deletion of the indole receptor (*sdiA*) did not affect *E. coli* competition in planktonic (A) and biofilm (B) cultures.

Surprisingly, indole supplementation could also restore the competitiveness of the cAMP mutants *cyaA* and *crp* both in planktonic (Fig 4A) and biofilm (Fig 4B) cultures. *E. coli* competiveness in the complemented mutants appeared to be correlated with indole concentrations.

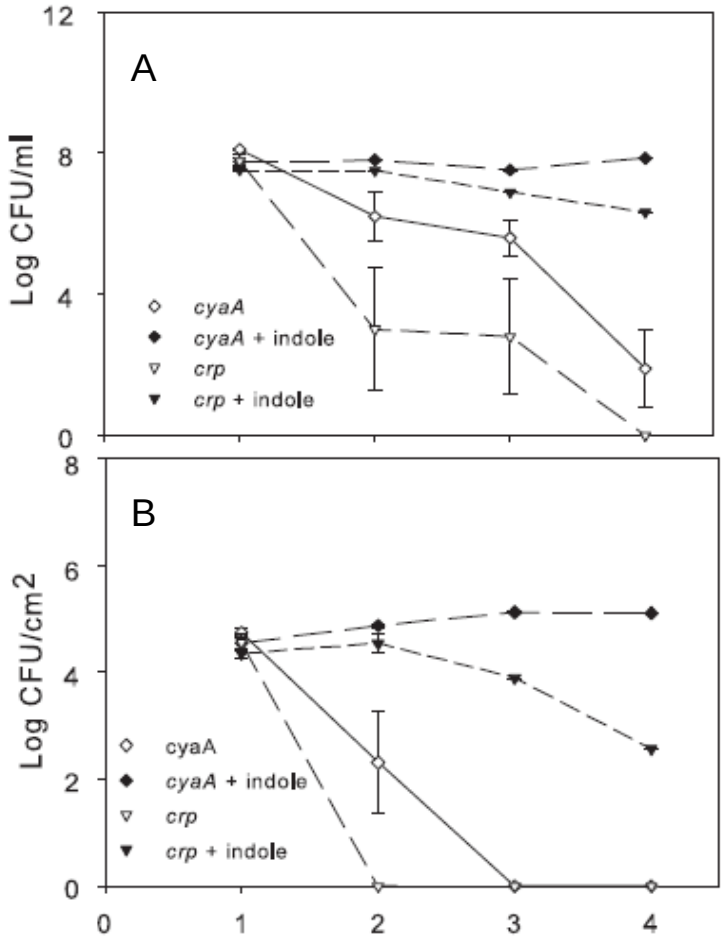

Figure 4 – Influence of exogenous addition of indole on *E. coli* growth in mixed culture. Indole restored competitiveness of *cyaA* and *crp* mutants in planktonic (A) and biofilm (B) cultures.

3.2 Indole production in *E. coli*

3.2.1 Extracellular and intracellular indole

As shown in figures 4A and B, addition of indole restored the competitiveness of cAMP mutants. To find out if the loss of competitiveness of the cAMP mutants is related to indole production and to investigate if the disruption of cAMP has any effect on indole production in *E. coli,* we measured indole production of the *E. coli* strains. In agreement

with previous work [14], we found that both extracellular and intracellular indole production was inhibited in *cyaA* mutant and absent in *crp* mutant. On the other hand, no effect in indole production was observed in the *cpdA* mutants. Results for the extracellular indole measurements of the strains are shown in figure 5A for pure and figure 5B for mixed culture, respectively.

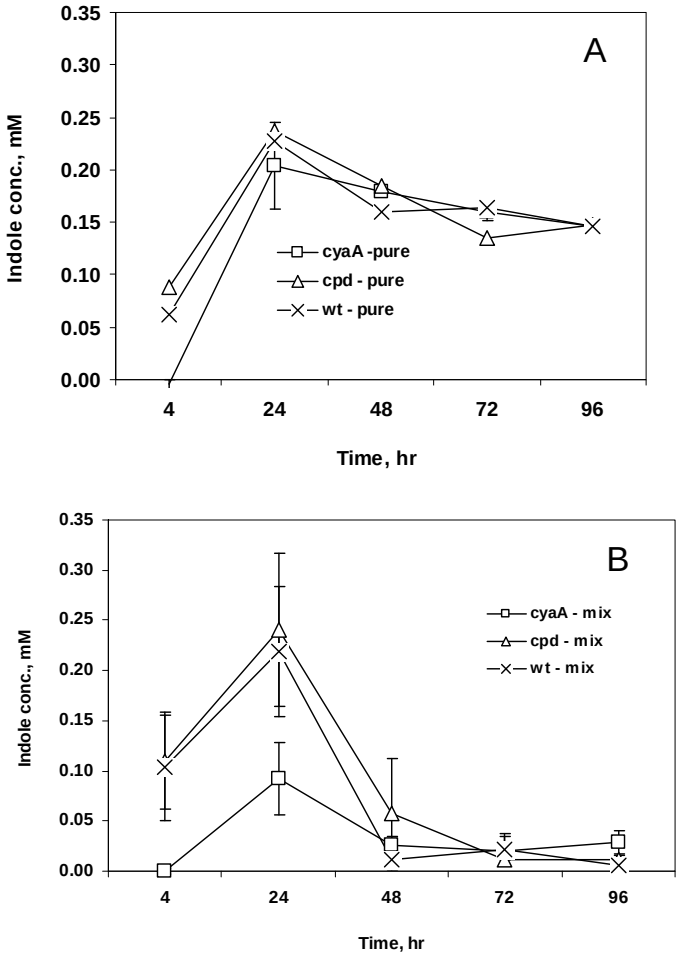

Figure 5 – Extracellular indole production by *E. coli* in pure and mixed culture. Indole concentrations were measured 4 - 96 h after inoculation. Experiments were performed in pure (A) and mixed (B) culture with *PAO*1.

Similarly, the intracellular indole measurements shown in figure 6A for pure and figure 6B for mixed cultures, showed similar effect. Detectable indole concentrations were

measured only in the *cpdA* and the *wt* strains. On the other hand, no intracellular indole was detected from the *crp* or the *cyaA* mutants.

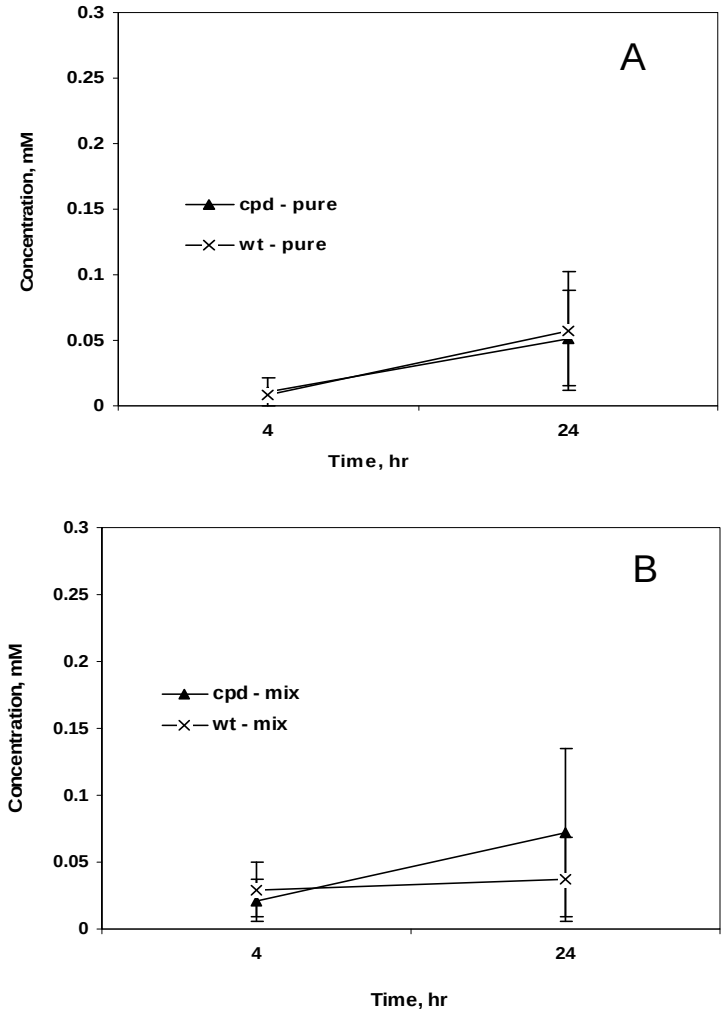

Figure 6 – Intracellular indole production by *E. coli* in pure and mixed culture. Indole concentrations were measured 4 - 24 hr after inoculation. Experiments were performed in pure (A) and mixed (B) culture with *PAO1*.

3.2.2 Indole production in pure vs. mixed culture

As indicated in the above (Figs 5A and 5B), indole production was inhibited in *cyaA* and absent in *crp* mutants and their competitiveness in mixed culture was restored by the addition of indole. Thus, to find out whether *E. coli* needs more indole to be competitive

in mixed culture, we normalized the extracellular indole produced by each strain to the cell number (CFU/ml) of each strain in pure and mixed culture, respectively. As shown in Figures 7A-C, while the indole levels were unaffected by mixed culture growth in the *cyaA* mutant strain (Fig 7B), a slight increase in indole production was observed in mixed culture with the *wt E. coli* culture (Fig 7A).With the *cpdA* mutants strain on other hand, the indole production was significantly enhanced in mixed culture as compared to the pure culture (Fig 7C).

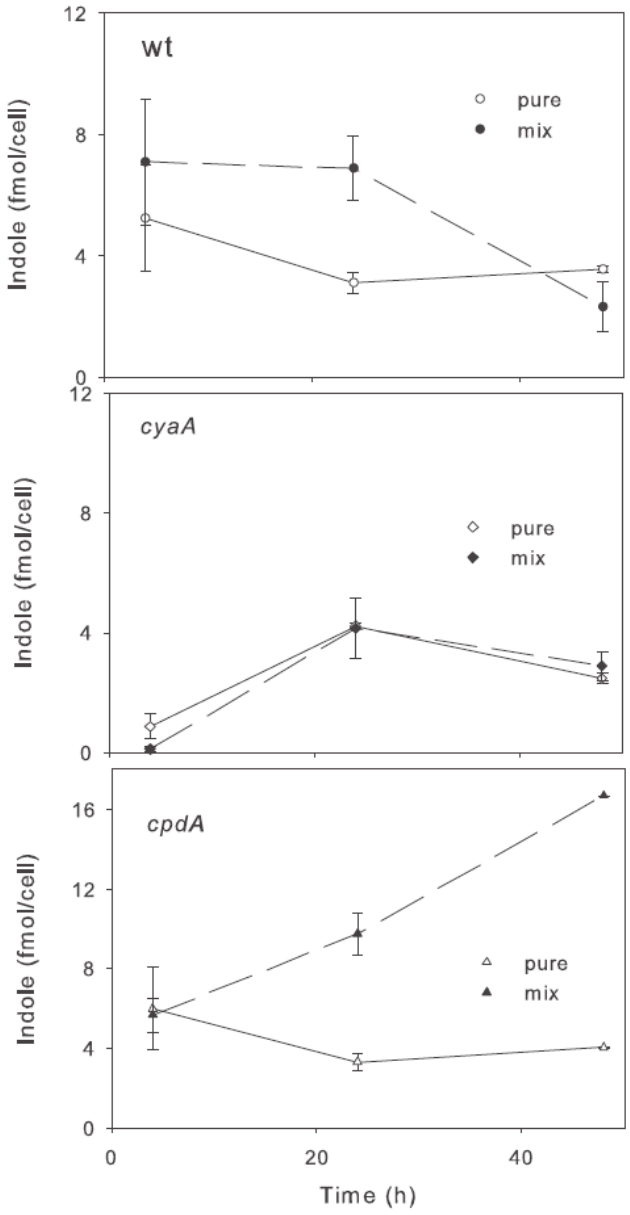

Figure 7 – Influence of pure and mixed culture growth on indole production in *wt*, *cyaA*, and *cpdA* mutants. Indole concentrations are calculated per *E. coli* cell.

3.3 Effect of indole and cAMP on *PAO1*

3.3.1 Toxicity effect

To find out whether indole makes *E. coli* competitive in mixed culture through a direct or indirect effect on *P. aeruginosa*, we checked the effect of indole on several quorum regulated virulence factors of *PAO1* (illustrated below). Before doing that, we checked the toxicity effect of indole on *PAO1*. As shown in figure 8, addition of indole did not show significant inhibitory effect on the growth of *P. aeruginosa* and this organism was able to remove indole from solution after 24 h, likely due to catabolism (Figs 12A-C).

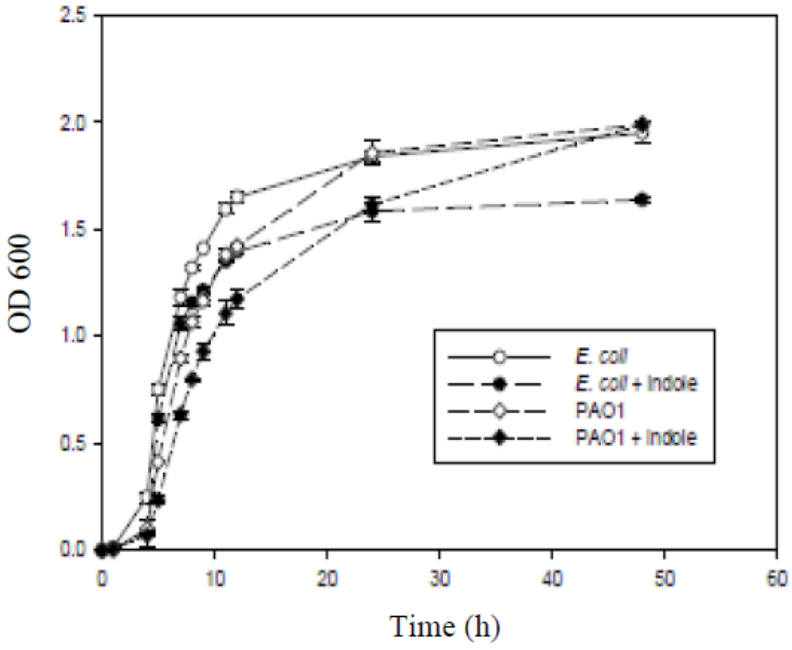

Figure 8 – Effect of indole on *E. coli* and *PAO1* growth. Addition of 1 mM indole showed short-term effect on *PAO1* growth; however, since *PAO1* metabolizes indole after 48 h (Figure 12), the effect disappeared.

3.3.2 Effect on pyocyanin production

As shown above figure 8, addition of indole and cAMP did not show significant inhibitory effect on the growth of *P. aeruginosa*. On the other hand, we observed that pyocyanin production by *PAO1* was inhibited by the addition of indole both in pure (Fig

9A) and mixed culture with *cyaA* (Fig 9B) and with *crp* (Fig 9c), respectively. On the other hand, while it is slightly enhanced in pure culture (Fig 9A), pyocyanin production was inhibited by the addition of cAMP in mixed culture with *cyaA* (Fig 9B).

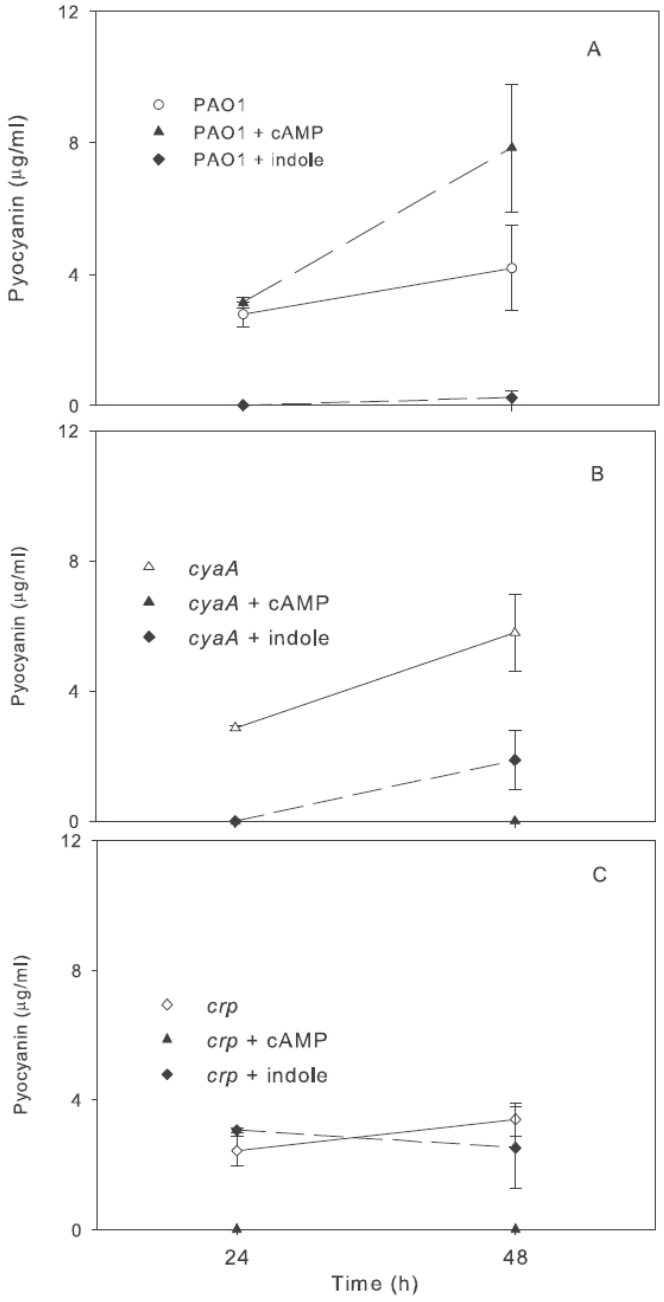

Figure 9 – Effect of 1mM cAMP and 1mM indole supplementation on pyocyanin production by *P. aeruginosa* in pure culture (A), and mixed culture with *E. coli cyaA* (B), and *E. coli crp* (C). The two *E. coli* strains are unable to produce indole.

3.4 Effect of cell-free culture fluids of *E. coli* on *PAO1*

3.4.1 Effect on pyocyanin production

Pyocyanin is one of the different virulence factors of *PAO*1 [27] and as can be seen from figure 10A, *PAO1* produce pyocyanin when co-cultured with the *crp* and *cyaA* mutants but not with the *cpdA*, *ydeH* and the *wt* strains. Therefore, to understand how *E. coli* downregulates pyocyanin production in *PAO1*, the effect of cell-free culture fluid of *E. coli* on pyocyanin production was analyzed. This was done by adding different proportions of the cell-free culture of each *E. coli* strain to *PAO1* cultures. Every 24 h, samples were taken for pyocyanin quantification. As shown in figure 10B, no effect was observed by the *crp* and *cyaA* cell-free culture fluid on the pyocyanin production by *PAO1*. On the other hand, both the *cpdA* and the *wt* strains showed a strong effect on the pyocyanin production by *PAO1* (Fig 10B).

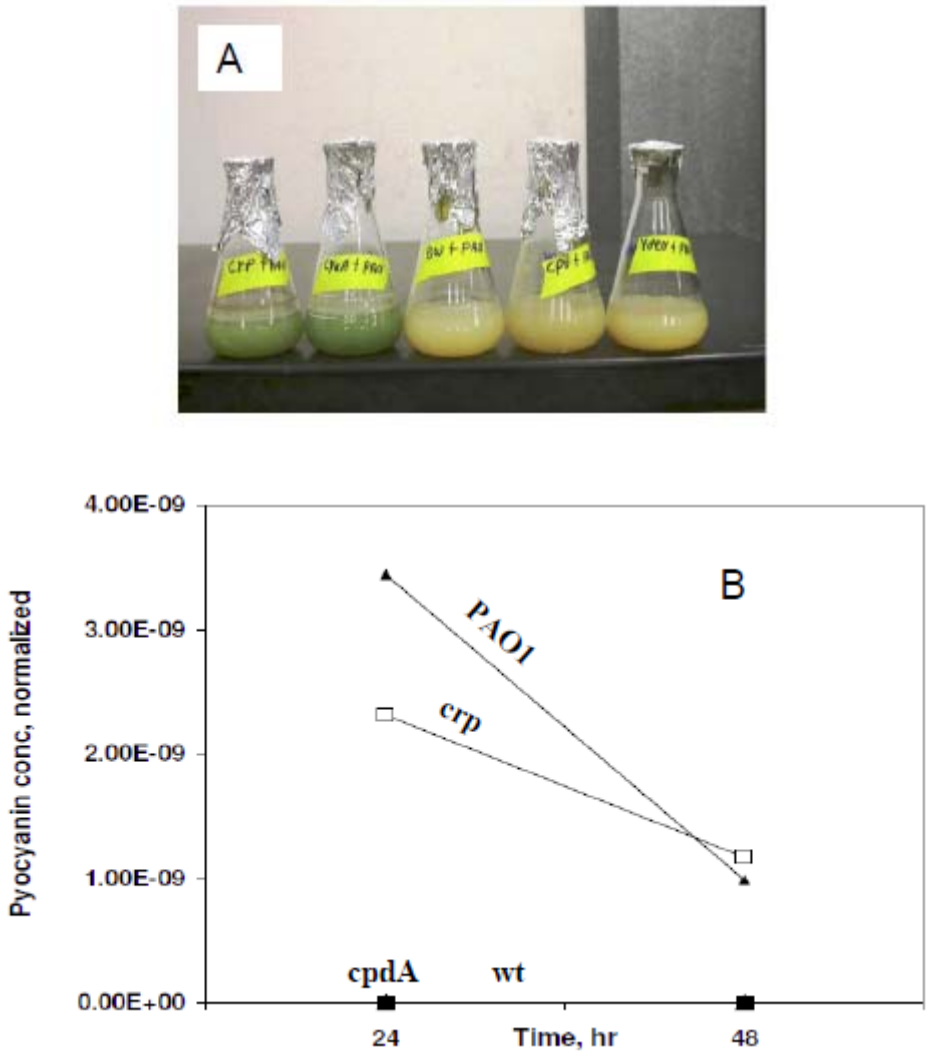

Figure 10 – Influence of *E. coli* on *PAO1*. Pyocyanin production by *PAO1* in mixed culture growth with *E. coli* (A). Effect of cell-free culture fluid of *E. coli* on pyocyanin production of *PAO1* (B). Pyocyanin concentration shown in figure B is normalized to cell number.

3.4.2 Antibacterial inhibitory effect

To see whether indole was the only factor involved in promoting *E. coli* mixed culture competitiveness, filter sterilized spent culture media from pure and mixed cultures was placed on lawns of *E. coli* and *P. aeruginosa* and incubated overnight. As seen in figure 11, mixed culture spent media inhibited *P. aeruginosa* growth. This finding was very

pronounced in *E. coli* wt (Fig 11D) and *cpdA* (Fig 11C) mixed cultures, but was reduced in *E. coli crp* mixed culture (Fig 11A) and absent in *E. coli cyaA* mixed culture (Fig 11B). There was no growth inhibition from spent culture from pure cultures of *E. coli* nor *P. aeruginosa* (top portion of Figs 11A-B &D and lower portion of Fig 11C), nor was there evidence of growth inhibition of spent culture media from pure or mixed culture on *E. coli* strains (Figs 15 and 16).

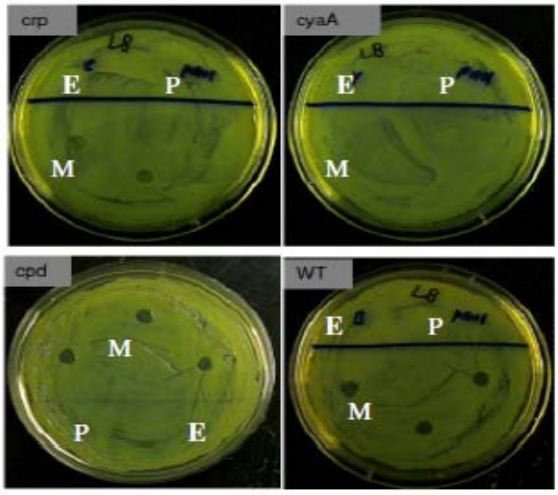

Figure 11 – Antibacterial activity of *E. coli* on *PAO1*. Spent media from mixed cultures (M) but not pure cultures of *P. aeruginosa* (P) or *E. coli* (E) caused growth inhibition of *PAO1*. This was seen during *P. aeruginosa* coculture with *wt*, however it was absent in *cyaA* cocultures and reduced in *crp* cocultures.

3.5 Indole degradation by *PAO1*

As can be seen from figures 4A & B, exogenous addition of indole restored the competitiveness of the *crp* and the *cyaA* mutant strain. However, the competitiveness declines to the levels of non-supplemented mutants after 48 h. Therefore, we thought that this loss of competitiveness of the mutant strains after 48 h might be due to the depletion of the exogenously added indole. To find out if this is the case, we added indole in to *PAO1* mixed culture (with *crp* and with *cyaA*) and monitored its concentration over time

(section 2.3.3). In mixed culture, we found that the concentration of indole starts to decline after 24 h and reaches zero after 72 h both in the *cyaA* (Fig 12 A) and in the *crp* (Fig 12 B) strains. In the pure culture, however, only slight decline of indole concentration was observed both in the *cyaA* (Fig 12 A) and *crp* (Fig 12 B) strains. Thus, to find out whether the rapid decline of indole concentration in mixed culture is due to the activity of *PAO1*, similar experiments were conducted on *PAO1* pure culture. It was found that *PAO1*, though it doesn't produce indole, depletes indole produced by the *E. coli* strains (Fig 12 C).

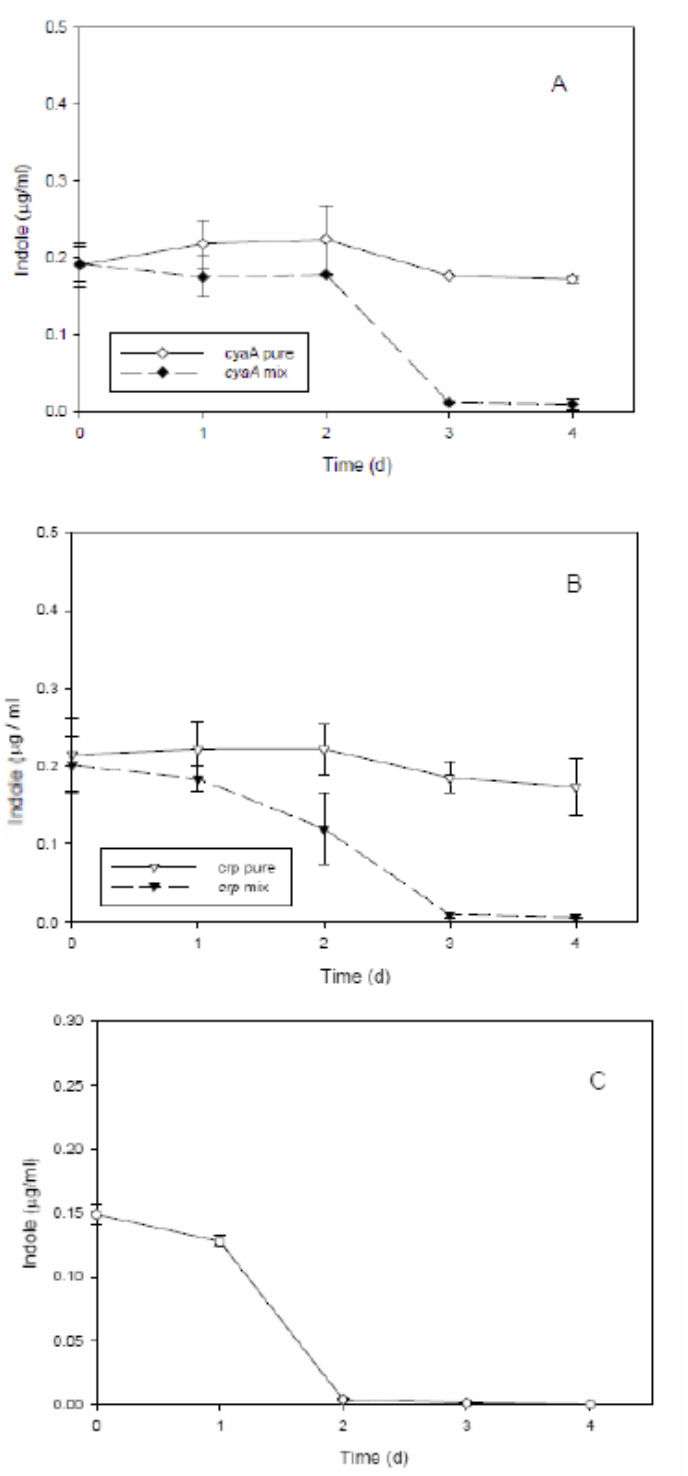

Figure 12 – Degradation of indole by *PAO1* in pure and mixed culture. Supplemented indole was metabolized by *P. aeruginosa* over 48 h whether grown in pure (C) or mixed culture with an *E. coli* strain unable to produce indole such as *E. coli crp* (B) and *cyaA* (A). On the other hand, monoculture *E. coli crp* (A) and *cyaA* (B) strains did not metabolize indole.

3.5.1 Mechanism(s) of indole degradation

In order to understand the mechanism by which *PAO1* degrades indole, I considered two working hypotheses. My working hypotheses are that *PAO1* might use (consume) indole as a source of nutrient and/or it might catabolically degrade indole by secreting some metabolites. To investigate these hypotheses, the experiments were conducted as illustrated in section 2.6.1. Briefly, we grew *PAO1* in LB broth for 72 h and took samples every 24 h starting from time zero. The samples were then centrifuged and filtered and the cell-free supernatants were analyzed for indole degradation. That is, 0.5 mM indole was added into 25 ml LB broth, and then the cell-free supernatants were added to the indole solution (in the ratio of 1:10).The degradation of indole was analyzed (as in section 2.3.3). Parallel with this, a control experiment (LB plus 0.5 mM indole) was also conducted for comparison. The results indicate that *PAO1* degrades indole by secreting some metabolites (Fig 13).

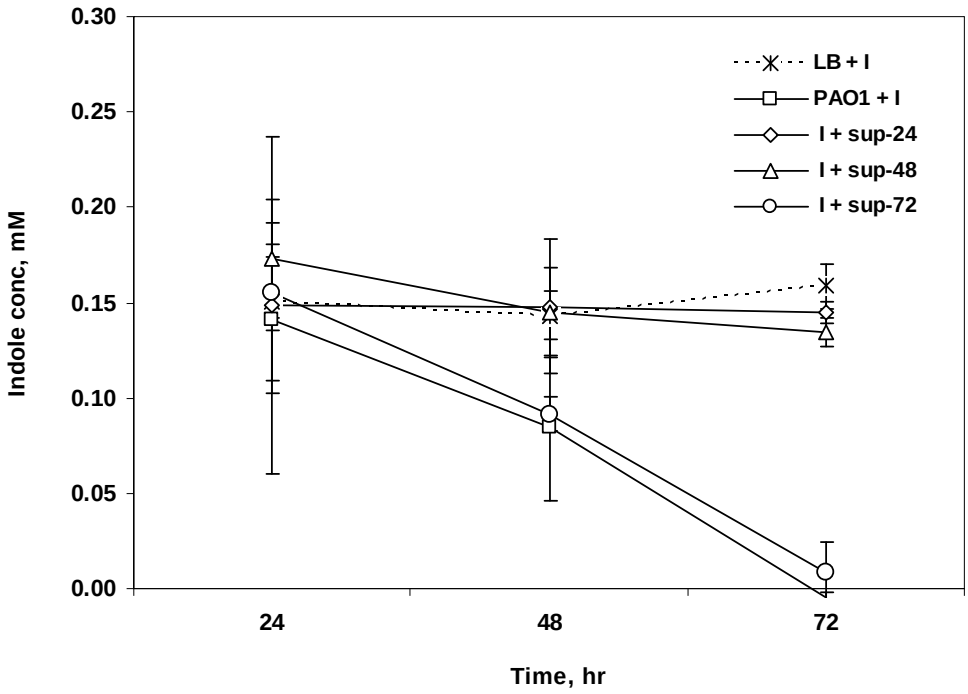

Figure 13 – Mechanism (s) of indole degradation by *PAO1*. Effect of cell-free culture fluids (from 24 – 48 h) of *PAO1* on indole degradation. The indole degradation assay was monitored over 72 h.

3.5.2 Indole degradation and quorum signaling

To find out if *PAO1* needs quorum signaling mechanism to degrade indole, I used two mutant strains of *PAO1*. The two mutant strains used are the *PAOMW1* and the *PDO100*. The indole degradation experiments for the two mutant strains were analyzed as in section 2.6.2. The results (Fig 14) showed that the two mutant strains of *PAO1* do not degrade indole.

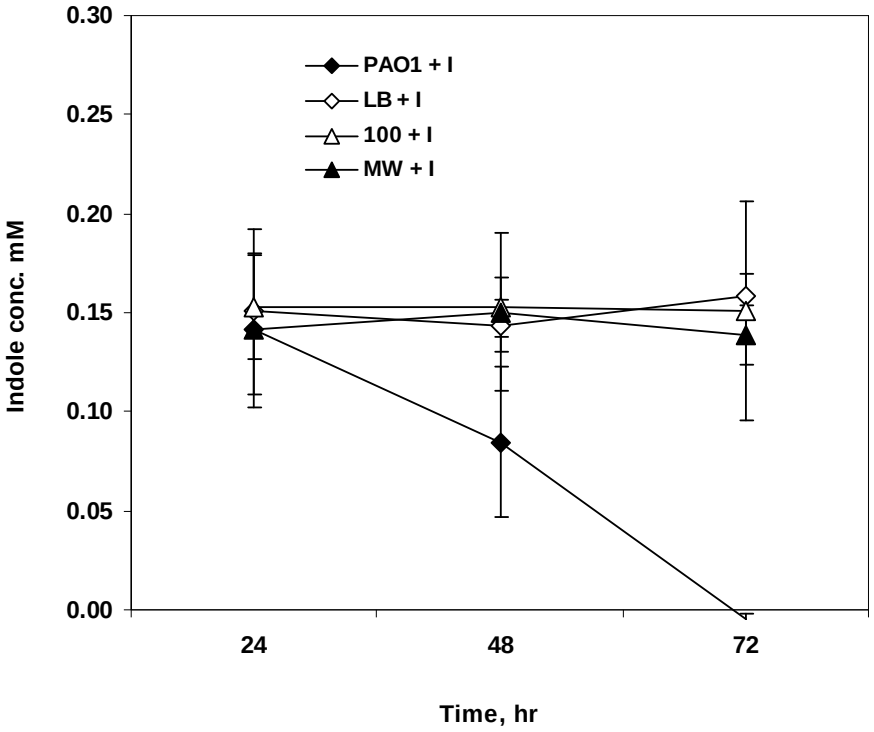

Figure 14 – Indole degradation and *PAO1* quorum signaling. *PAO1* quorum signaling mutants *PAO-MW1* and *PDO100* did not degrade indole. As shown in figure 12, supplemented indole was metabolized by the wt *P. aeruginosa* over 48 h whether grown in pure or mixed culture.

CHAPTER IV

DISCUSSION AND CONCLUSION

In nature most bacteria do not exist as pure cultures, and significant proportions of all microorganisms are associated with surfaces in complex multispecies communities called biofilms [29]. In terms of broad-based genetic studies (involving transcriptome or proteomic approaches), most mixed culture investigations have explored a gram-positive and gram-negative organism grown in co-culture. Because of the fundamental differences in the cell wall architecture between gram-positive and gram-negative bacteria [30], one can selectively lyse one member of the consortium and extract the desired cellular material for analysis. These studies have been useful in highlighting some of the novel aspects of mixed culture interactions (cf [5, 31]. When the periodontal gram-negative pathogen, *Aggregatibacter actinomycetemcomitans*, grows in mixed culture with *Streptococcus gordonii,* it preferentially uses lactate rather than carbohydrates as a carbon source [32]. Although this niche selection causes *A. actinomycetemcomitans* to use a less desirable carbon source, it removes this organism from direct competition with the faster growing *S. gordonii*. In turn, *S. gordonii* has been shown to stimulate an oxidative stress response and serum protection for *A. actinomycetemcomitans* which may explain the ability of this dental pathogen to survive passage through the blood to the heart [5]. In another example, co-culture of *Staphylococcus aureus* and *P. aeruginosa* results in down

regulation of *P. aeruginosa* iron-regulated genes as it is able to lyse *S. aureus* and use this organism as an iron source [31]. One recent study did investigate two, gram-negative organisms, *P. aeruginosa* and the plant pathogen, *Agrobacterium tumefaciens*. Using a mutagenesis approach, An et al. [33] showed several groups of genes, associated with biofilm formation (twitching motility and quorum signal regulon) as well as growth rate, influenced the success of *P. aeruginosa* and *A. tumefaciens* in this model mixed culture system. On the basis of these recent studies, microorganisms appear to be adopting several strategies for mixed culture survival, including nutritional and metabolic flexibility, mixed culture-induced gene (mcg) expression and signaling. These newer strategies must be taken in context with previously identified mechanisms, including bacteriocins, resource competition, and generation of harmful metabolites, that are used during allelopathy (cf [34, 35]).

One consistent observation from the study in our lab was the upregulation of de novo synthesis purine genes (W Chu, TR Zere, MM Weber, TK Wood, M Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted) by *E. coli* during mixed culture growth with *P. aeruginosa PAO1*. As stated earlier, this was a consistent observation with *E. coli ZK126* and *MG1655* that occurred under two different growth conditions. It is conceivable that the salvage pathway for purine synthesis [6] allowed *E. coli purD*, *purH*, and *purT* strains to compensate for these mutations. Purines serve several functions in bacteria. Along with pyrimidines, they are components of nucleic acids [6]. When phosphorylated, purines also function as energy transfer molecules (ATP and GTP) and as components of second messenger molecules cAMP and c-di-GMP [9]. Results of microarray experiments (W Chu, TR Zere, MM Weber, TK Wood, M

Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted), did not show any evidence of pyrimidine synthesis genes being affected, which we interpreted as ruling out a requirement for enhanced nucleic acid synthesis. Although ATP and GTP levels were not measured directly, no evidence was shown that *E. coli* growth was inhibited in purine synthesis mutants (*purD*, *purH*, and *purT*) either during growth in pure culture or in mixed culture (W Chu, TR Zere, MM Weber, TK Wood, M Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted). Depletion of cellular energy reserves would be anticipated to severely inhibit *E. coli* growth, which did not occur in the purine mutants tested. While we cannot rule out an ATP or GTP effect on mixed culture growth, we saw significant effects due to cAMP disruption (addressed below).

In *E. coli*, cAMP and its receptor protein, CRP, have been traditionally associated with regulation of glucose-mediated catabolite repression and other aspects of carbon catabolism [12]. Transcriptome analyses by Saier [13], Constantinidou [36], and their colleagues, have shown that approximately 200 operons are regulated directly or indirectly by cAMP. Aside from carbon catabolism, many other cell functions are now recognized as components of the CRP regulon, including some genes associated with the heat and cold shock stress responses, cell division, and amino acid metabolism including the tryptophanase gene, *tnaA [13]*. A recent study by Barth et al. [37] showed that cAMP was also indirectly involved in the oxidative stress response. In this case, a decrease in cAMP concentration, mediated by the *cpdA* gene product caused a derepression of the alternate sigma factor *rpoS*, which in turn activated the oxidative stress response. During mixed culture growth, *E. coli* would need to be flexible for nutrient utilization as

individual carbon sources are depleted and metabolites accumulate. From a nutrition perspective, a central role of cAMP in *E. coli* mixed culture growth is logical.

During competition with other species, *P. aeruginosa* produces a number of toxic compounds that are regulated by quorum signaling. These include rhamnolipids [38], pyocyanin [27] and predatory membrane vesicles [39]. The latter two agents are toxic against *Staphylococcus aureus* during pulmonary infections in cystic fibrosis [31, 39], and rhamnolipids have been shown to be toxic against eukaryotic cells [38]. As well, rhamnolipids enhance swarming motility in *P. aeruginosa* [40]. Given the roles of these compounds in *P. aeruginosa* competition, it was quite notable that co-culture with *wt E. coli* caused a decrease in elastase, rhamnolipids, pyocyanin (Fig 10A and B), and swarming motility (W Chu, TR Zere, MM Weber, TK Wood, M Whiteley, B Hidalgo-Romano and RJC McLean, manuscript submitted). This inhibition of elastase, pyocyanin, rhamnolipids, and swarming motility was not evident in the cAMP mutants *cyaA* and *cpdA*, nor in the *tnaA* mutant (indole production), but could be restored for 24h upon supplementation with 1mM indole. Our observations of indole-based inhibition of *P. aeruginosa* elastase, rhamnolipid and pyocyanin formation are in agreement with Lee et al. [41]. In order to see whether indole had any effect on *P. aeruginosa* or *E. coli*, I conducted growth experiments on these organisms as monocultures. I saw no significant difference in growth rate characteristics of these two organisms regardless of indole supplementation (Fig 8). As well, *P. aeruginosa* was able to metabolize indole after 24 h (Fig 12A-C and Fig 13), which would explain the disappearance of *E. coli* competitiveness in *cyaA, crp,* and *tnaA* mutants after 24 h. We observed that indole production was enhanced in *E. coli cpdA* strains during co-culture with *P. aeruginosa*

(Fig 7C), but not in other strains (Figs 7A&B). As *cpdA*-encoded phosphodiesterase regulates intracellular cAMP levels in *E. coli* [11], this observation shows that exposure to *P. aeruginosa* has the potential to increase indole production via enhanced cAMP levels, but that this cAMP-increase is moderated by the *cpdA* gene product [11]. These findings show an influence of *P. aeruginosa* on *E. coli* indole production and also an indole-mediated inhibition of several *P. aeruginosa* competition factors. However, they rule out indole toxicity as being a mechanism for enhanced *E. coli* competitiveness against *P. aeruginosa*.

Our data support a key role for cAMP and indole for *E. coli* competitiveness in mixed culture. Similar effects were seen in both biofilm and planktonic populations, and so we interpret this phenomenon as a global effect, rather than a planktonic- or biofilm-specific effect. Certainly, separate biofilm and planktonic differences in stress response have been seen in other studies (cf[17, 24]). During this study, cAMP mutations (*cyaA* and *crp*) and indole mutation (*tnaA*) affected both planktonic and biofilm populations of *E. coli* during mixed culture, but had no effect on monoculture populations (Figs 2A&B). However, other factors also play a role. In agar plate diffusion assays, we observed that spent culture medium from *E. coli wt* and *cpdA* mutants was inhibitory to *P. aeruginosa*, but that *cyaA* and *crp* mutants' spent culture medium did not inhibit groth of *P. aeruginosa* (Fig 11) nor did *tnaA* mutants' (Fig 16). As indole and cAMP are not inhibitory to *P. aeruginosa* growth (Fig 8), these observations would suggest that there is an additional downstream effect of indole and cAMP in the generation of one or more antimicrobial compounds. Quorum signal disruption has been described in a number of other microbial systems. Examples include the production of signal inhibiting compounds

such as furanones [42] and enzymatic degradation of signals (quorum quenching) [43]. In a related study, we found that cloning of AHL lactonase from *Bacillus thuringiensis* provided minimal competitive benefit to *E. coli* planktonic populations and no benefit to *E. coli* biofilm populations during co-culture with *P. aeruginosa* (WT Boswell, MM Weber, J Momb, W Fast, AM Duran, and RJC McLean, manuscript submitted). Based on the present study, it is likely that the indole-based inhibition of quorum-regulated phenotypes of *PAO1* masked any potential benefit of added AHL lactonase.

In summary, we conclude that cAMP and indole provide a major competitiveness to *E. coli* in mixed culture growth with *P. aeruginosa*. Certainly, some of this benefit arises from the indole-based inhibition of several *P. aeruginosa* virulence factors, notably pyocyanin, elastase, and rhamnolipids. However, there is also evidence of additional antimicrobial activity stimulated by cAMP and indole. Overall, this study provides a key mechanism to explain the natural ecological success of *E. coli* in mixed populations.

APPENDIX

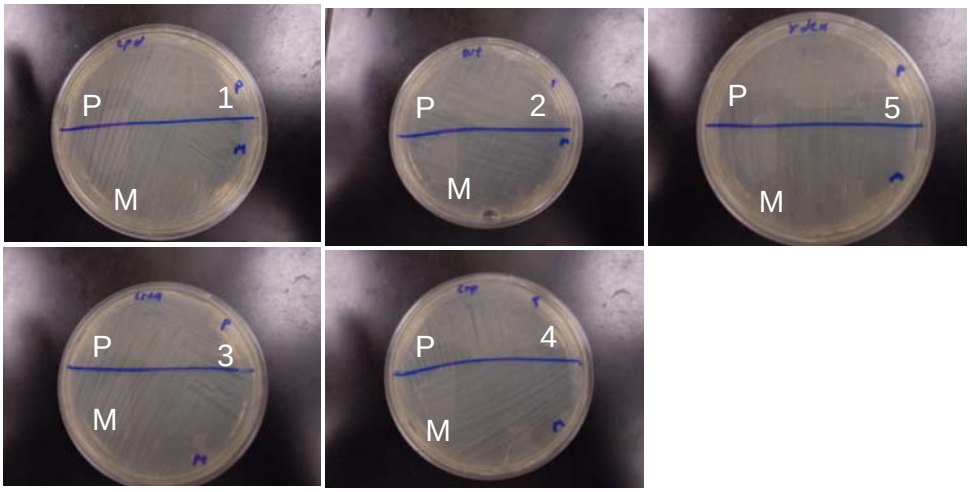

Figure 15 – Antibacterial activity of *PAO1* on *E. coli*. Spent media from mixed cultures (M) and pure cultures of *P. aeruginosa* (P) did not cause growth inhibition of *E. coli.* This was seen during *P. aeruginosa* coculture with *cpdA* (1), wt (2), *cyaA* (3), *crp* (4), and with *ydeH* coculture (5).

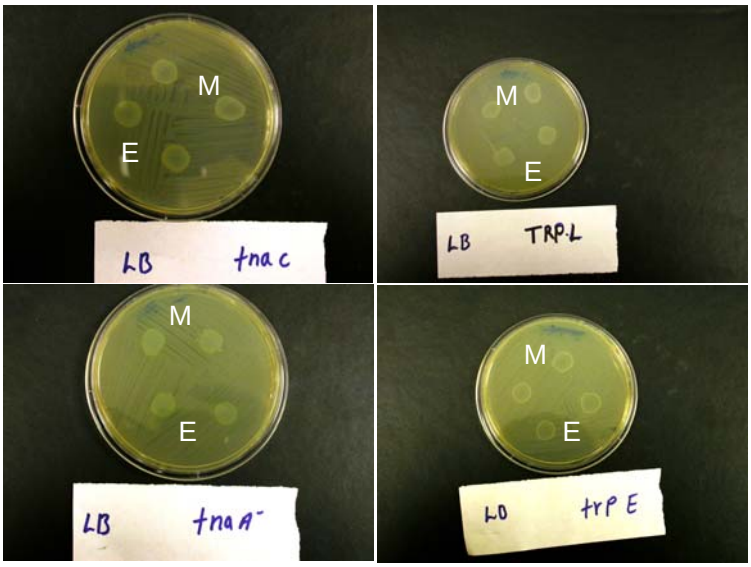

Figure 16 – Antibacterial activity of indole mutant strains of *E. coli* on *PAO1*. Spent media from mixed cultures (M) and pure cultures of *E. coli* (E) did not cause growth inhibition of *PAO1*. This was done during *P. aeruginosa* coculture with indole mutant strains of *E. coli*.

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VITA

Tesfalem Rezene Zere was born in Asmara, Eritrea. He received his Bachelor degree (in Biology) from University of Asmara in the year 2003. During the years 2003 – 2007, he was employed as a teaching assistant at Eritrea Institute of Technology and as a research assistant in the Medicinal Plants and Drug Discovery Research Center (MPDDRC), University of Asmara. He then joined Ben-Gurion University of the Negev (Israel) in the year 2008, and received his Masters degree in Water Sciences and Technology. After that, in the year 2010, he entered the Graduate College, Texas State University-San Marcos.

Tesfie98@yahoo.com

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