THE EFFECT OF INDOLE PRODUCTION ON THE GROWTH OF *ESCHERICHIA COLI* WHEN CO-CULTURED WITH *ENTEROCOCCUS FAECALIS*

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biology
August 2014

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Robert JC McLean. This project was really a team effort, and I am eternally grateful for his support, guidance, feedback, supervision, and encouragement. In particular, I am grateful for all of the opportunities (especially speaking at the ASM Branch meeting and having the opportunity to do curriculum writing) I would never have had without him, or knew were even possible. I am very lucky to have had the opportunity to work with not only a great scientist, but also such a friendly, supportive, and forward-looking mentor.

I would also like to thank my committee members Dr. William Coons, Dr. Dittmar Hahn, and Dr. Gary Aron for their advice and support. Each of them acted as a mentor in some capacity, and I deeply appreciate the time they took to work with me despite the busy schedule they all have. Their feedback, advice and support was indispensable, and I am forever indebted.

Lastly, I'd like to thank the others in the "slime gang" lab, specifically Ernesto Valenzuela, Logan Warren, Mark Holliday, Leigh Mayberry, Lance English, Chris Munoz, and Benjamin Hidalgo-Romano, for their help, insights, advice, and friendship.

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LIST OF ABBREVIATIONS

Abbreviation	Description
Efs	*E. faecalis* supernatant
Ecs	*E. coli* supernatant
BW	*E. coli* wild-type
Ef	*E. faecalis*

ABSTRACT

In nature, bacteria live in dynamic communities surrounded by a vast number of other bacterial species. Recent studies indicate that one mechanism by which *Escherichia coli* thrives within such a multitude is via production of the molecule indole. Evidence indicates that indole thwarts the quorum sensing system of acyl-homoserine lactone (AHL) producing bacteria such as *Pseudomonas aeruginosa, Chromobacterium violaceum,* and *Pseudomonas aureofaciens*. Impeding the signaling system of these bacteria ultimately leads to a decrease in toxic secretions such as pyocyanin and proteases. The aim of this research was to determine if the production of indole by *E. coli* is a general mechanism by which it competes in mixed culture. To do this, the effect of indole on the growth of *E. coli* in mixed culture with *Enterococcus faecalis* was studied. *E. faecalis* is a Gram-positive, non-AHL producing bacteria found alongside *E. coli* as normal flora in the human intestine. *E. faecalis* has increasingly become a concern as it is now a leading cause of hospital-acquired infection and has developed resistance to "last-line" antibiotics such as vancomycin. Colony counts and turbidity of *ΔtnaA* (the *E. coli* mutant incapable of degrading tryptophan and thus deficient in indole production) were measured in mixed culture with *E. faecalis.* Indole was then reintroduced at physiologically relevant concentrations and its effect was measured. Contrary to previous research, in competition with *E. faecalis* the population size of *E. coli* is inhibited and indole has a further inhibitory effect.

CHAPTER I

INTRODUCTION

Although major advancements in biology have been made in of the study of isolated bacteria, naturally microbes are found in species-rich, dynamic environments. As a result, bacteria have evolved to survive within a vast network of community interaction. This includes, in part, competing for nutrients, defending against antimicrobial agents, and responding to chemical signals received (Wintermute & Silver, 2010). These signals can be cell-density (quorum) regulated or be produced as metabolic byproducts, and function to help bacteria to sense the environment and respond to it in a way that confers a competitive advantage (Garg et al., 2014). There are three major classes of signals: acyl-homoserine lactone (AHL) derivatives (produced by Gram-negative bacteria), auto-inducing peptides (AIPs) (Gram-positive bacteria), and the autoinducer (AI-2) signal, produced by Gram-positive and Gram-negative organisms alike (Waters & Bassler, 2005). Depictions of various bacterial signals are illustrated below (Appendix Fig. 9).

Often bacteria emit multiple signals and have many quorum-sensing circuits (Wintermute & Silver, 2010). For example, *Pseudomonas aeruginosa* produces two acyl-homoserine lactone (AHL) autoinducers in addition to quinolone-based signals, and *Vibrio harveyi* produces two AHLs and one autoinducer-2 signal, each of which has its own cognate receptor (Jayaraman & Wood, 2008). As a result of the multiple signals/systems complex, some non-specific signaling or "cross-talk" occurs (Di Cagno et al., 2011), and some bacteria have evolved to intercept other species' signaling systems. Recent studies indicate *E. coli* competes in mixed culture in such a manner, via the molecule indole.

Indole is produced when the amino acid tryptophan is hydrolyzed by the enzyme tryptophanase (encoded by *tnaA*). It is synthesized in high concentrations by *E. coli* (~0.6mM) during stationary phase, when its population is high and its carbon resources are low (Lee & Lee, 2010). Its production is regulated by cyclic AMP (cAMP), whose synthesis and reception are encoded by *cyaA* and *crp* (respectively) (Chu et al., 2012). As an intraspecies signal in *E. coli*, indole has been shown to influence the transcription of many factors, including but not limited to biofilm development (Martino et al., 2003), drug resistance (Hirakawa et al., 2005), and plasmid stability (Field & Summers, 2012). As an *inter*species signal indole has been linked to increased drug resistance (Nikaido et al., 2008), decreased cell growth (Lee & Lee, 2010), and attenuation of virulence (Chu et al., 2012).

Studies indicate that indole produced by *E. coli* directly affects quorum regulated virulence of neighboring bacteria. For example, in co-culture with *P. aeruginosa,* indole negative *E. coli* mutants are unable to prevent the formation of toxic products such pyocyanin and elastase, molecules that are quorum regulated (Chu et al., 2012). Other AHL derivative producing bacteria (i.e., *Serratia marcescens*, *Chromobacterium violaceum*, and *Pseudomonas aureofaciens*) and non-AHL producing bacteria (*Staphylococcus aureus*) are similarly affected (Lee et al., 2013; Hidalgo-Romano et al., submitted for publication 2014). A brief summary of indole's effects as an intra- and interspecies signal is shown in Appendix Fig. 10.

The secretion of indole and the ecological success of *E. coli* are particularly important in the digestive tract, where *E. coli* is predominantly found. The digestive tract in humans not only harbors a plethora of bacteria, but also is commonly associated with

contamination-based infection such as urinary-tract infection and gastroenteritis (Falagas et al., 2008). As an inhabitant of the GI tract, studies indicate that *E. coli* can inhibit and also accentuate virulence of neighboring species. For example, though *E. coli* attenuates the virulence of *P. aeruginosa, E. coli* increases the drug resistance of *Salmonella typhimurium* (Vega et al., 2013) and has a synergistic effect when found co-infecting with *Enterococcus faecalis* (Lavigne et al., 2008).

E. faecalis is a Gram-positive, non-spore-forming facultative anaerobe found in the digestive tract of mammals and, to a lesser degree, the oral cavity (Kayser 2003). *Enterococci* are the third most prevalent genus obtained from bloodstream infection, and the most frequent cause of surgical–site infection in intensive care units (Vebo et al., 2009). *E. faecalis* is able to survive a range of stresses and environments, including those of extreme temperature and pH (Fisher & Phillips, 2009). It is ranked as one of the leading causes of nosocomial infection worldwide, with a mortality rate from infection being up to 61% (Lopes et al., 2005). *E. faecalis* infections have recently become a particularly pressing concern, as vancomycin (considered a "last-line" antibiotic) resistant strains have continued to become more and more prevalent (Howden et al., 2013). This antibiotic resistance is readily transferred via plasmid, and resistance has been correlated with other virulence determinants (Rathnayake et al., 2012).

Several *E. faecalis* virulence factors such as cytolysin, gelatinase, adhesins, and surface antigens have been identified (Lempiäinen et al., 2005), and this virulence has been shown to be partially quorum related. In *E. faecalis*, the quorum sensing system is mediated by *fsr*, which shows a ~38% similarity to the *S. aureus agr* system (Qin et al., 2000). The *fsr* system mediates an autoinducer peptide (AIP) named gelatinase

biosynthesis-activating pheromone (GBAP) that induces extracellular proteases, gelatinase, and regulation of biofilm formation (Nakayama et al., 2006).

In recent years studies have been undertaken to determine potential quorum sensing inhibitors of *E. faecalis*, with some promising results. For example, one study indicates that siamycin, an antibiotic secreted by *Streptomyces*, attenuates virulence without inhibiting *E. faecalis* growth (Nakayama et al., 2007). Another study indicates that *E. faecalis* in mixed culture with *Candida albicans* displays attenuated virulence in *Caenorhabditis elegans* infection (Cruz et al., 2013). Additionally, synthetic quorum inhibitors have been developed (Nakayama et al., 2013). Thus, virulence inhibition of this species has been shown to occur. No studies to date show the effect of indole on the *E. faecalis* quorum sensing system, however, though indole is naturally present *in vivo* and has been shown to attenuate the virulence of many bacterial species, including species with very similar signaling systems.

The main objective of this study is to determine the effect of *E. coli*'s indole production on its growth when co-cultured with *E. faecalis*. Our hypothesis is that *E. faecalis* does not significantly inhibit *E. coli*, and that the production of indole by *E. coli* is required for its competitive fitness when in mixed culture.

CHAPTER II

METHODOLOGY

2.1 Bacterial strains, media and chemicals

The strains used in this study are *Escherichia coli* BW25113 (Baba et al., 2006), *E. coli* BW25113 Δ*tnaA* (Baba et al., 2006), and *Enterococcus faecalis* OG1RF. The strains were maintained in Luria-Bertani (LB) or Brain Heart Infusion (BHI) medium supplemented with kanamycin (50μg/ml) for *E. coli* Δ*tnaA*. For long-term storage, liquid cultures were frozen at -80^{O}C using glycerol (12.5% v/v) as a cryoprotectant. Prior to experimentation, samples from cultures were thawed from frozen stock, streaked onto LB agar plates, and incubated for 24 hours at 37^{O}C to check for contamination. Subcultures were then incubated overnight in LB or BHI broth at 37^{O}C with aeration (125 rpm) and adjusted to an optical density (600nm) of 0.1 (~10^{7}CFU/ml). During mixed-culture studies, Eosin-methylene blue agar (to select for *E. coli*) or phenyl ethyl alcohol agar and LB agar with gentamycin (10μg/ml) and nalidixic acid (20μg/ml) (to select for *E. faecalis*) were used. A stock solution of indole (25mM) was prepared in dimethyl formamide (DMF), filter sterilized using a 0.2-μm-poresize sterile filter and stored at 4^{O}C.

2.2 Competition experiments, and the effect of indole

2.2.1 Direct growth measurement of *E. coli* with *E. faecalis*

E. faecalis OG1RF and *E. coli* BW25113 strains were thawed from frozen stock and streaked on LB agar plates to check for contamination. They were then grown overnight as pure cultures in LB broth, adjusted to an OD_{600} of 0.1 and inoculated in together in a

1:1 ratio in LB broth (0.5ml of bacterial culture/100ml of LB) and incubated at 37^{o}C, with aeration (125rpm). Samples were removed at 0, 4, 8, 24, 48, and 72 hours. 0.5 ml at each time point was serial diluted into 4.5 ml sterile water and plated onto Eosin-Methylene Blue agar and phenyl ethyl Alcohol agar to select for either *E. coli* or *E. faecalis*. For biofilm studies, cultures were prepared as above, with the addition of two sterile silicon discs (7mm diameter x 1mm thickness) for each time point (24, 48, and 72 hours) serving as biofilm attachment sites. At the time of sampling, the two biofilm discs were aseptically removed with forceps, rinsed with sterile water, and inserted into 4.5mL of sterile water in a scintillation vial. The discs were then sonicated for 5 minutes and vortexed for 2 minutes to release bacteria from the discs. 0.5 ml from the scintillation vial was then serial diluted and plated as above. Pure culture planktonic and biofilm samples were similarly cultured, diluted and plated on LB agar for comparison.

2.2.2 Effect of exogenous indole on growth of *E. coli* in mixed culture with *E. faecalis*

E. coli ΔtnaA (the BW25113 mutant lacking the ability to produce indole) and OG1RF were inoculated in LB broth for 24 hours, adjusted to an OD_{600} of 0.1 and mixed in a 1:1 ratio (0.5ml of each strain into 100ml of LB broth). Exogenous indole at concentrations of 0.5 mM and 1.0mM was then added. Planktonic and biofilm cultures were analyzed via dilution plating, as above. Pure culture *ΔtnaA*, BW25113, and OG1RF were grown in LB broth and supplemented with 0.5 mM and 1.0mM indole for comparison.

2.2.3 Effect of mixed culture on indole production by *E. coli*

Overnight cultures of BW25113 and OG1RF were inoculated in a 1:1 ratio as above. At 24 hours, indole concentration was measured via the following: 1ml samples from each

flask were removed and centrifuged for 10min at 15,000xg. 50µl of the supernatant was then removed and added to 1ml of Kovac's reagent, vortexed, and the absorbance at 540nm was measured. Indole concentrations were determined via standard curve (Appendix Fig. 11).

2.3 Effect of cell-free supernatant of *E. faecalis* on *E. coli* growth, with and without indole

2.3.1 Growth of *E. coli* in *E. faecalis* supernatant

Supernatant from OG1RF was prepared as follows: OG1RF was cultured in LB or BHI broth overnight and the cell density adjusted to an OD_{600} of 0.5. The culture was then centrifuged for 5 minutes at a speed of 13,000 rpm and the supernatant was removed and filter sterilized using a 0.2-µm-poresize filter and stored at $4^{O}C$. BW25113 was inoculated overnight in LB or BHI broth and then adjusted to an OD_{600} of 0.1. ~1µl of *E. coli* culture was then transferred into 200µl of the *E. faecalis* supernatant on a 96 well micro plate. The optical density at 600nm was recorded every 20 minutes for 12-24 hours using the micro plate reader. As a control, BW25113 supernatant was prepared and inoculated with BW25113 exactly as OG1RF above.

2.3.2 Effect of exogenous indole on the growth of *E. coli* in *E. faecalis* supernatant

Indole was added to the OG1RF supernatant (described above) to final concentrations of 0.1mM, 0.5mM, 1mM, 1.5mM, and 2.0mM. Overnight cultures of Δ*tnaA* and BW25113 were adjusted to an OD_{600} of 0.1. ~1µl of the cultures were then transferred into 200µl of the *E. faecalis* + indole supernatant on a 96 well micro plate and turbidity was measured as above. For comparison, a BW25113 supernatant solution was prepared and inoculated

with Δ*tnaA* or BW25113 exactly as OG1RF above.

2.4 Antimicrobial activity

2.4.1 Effect of *E. faecalis* supernatant on *E. coli* growing on solid media

OG1RF was cultured in LB or BHI broth for 24 and 48 hours and the cell density adjusted to an OD_{600} of 0.5. The culture was centrifuged for 5m at 13,000 rpm and the supernatant was removed and filter sterilized (0.2-μm). 20μl of the supernatant was then pipetted onto sterile (7mm) paper disks, which were then dispensed onto lawns of BW25113 or Δ*tnaA* growing on Mueller-Hinton agar and cultured overnight at 37^{O}C.

To test for antimicrobial production under nutrient-poor conditions, BW25113 and OG1RF were cultured for 24 hours in 50% BHI broth and the turbidity of both was adjusted to an OD_{500} of 0.5. Sterile 7mm paper discs were then saturated with 20μl of respective cultures and placed next to each other on 50% BHI agar plates. In addition, the paper discs were saturated and placed onto BHI plates as above, one culture 24 hours before the subsequent culture.

2.4.2 Initial studies to ascertain the nature of the antimicrobial substance

To determine the effect of heat on the efficacy of the supernatant, OG1RF supernatant was autoclaved and then inoculated as above. To determine the effect of protease and lipase on the efficacy of the supernatant, the supernatant was treated with protease or lipase (at final concentrations of 1mg/ml and 0.1mg/ml), incubated at 37^{O}C for 2 hours, and inoculated with BW25113 or Δ*tnaA* on the micro plate as above. The experiment was completed in LB broth, BHI medium, and Davis minimal medium (lacking dextrose) for comparison.

CHAPTER III

RESULTS

3.1 Growth of *E. coli* in mixed culture with *E. faecalis*

3.1.1 Direct growth measurement of BW25113 and Δ*tnaA* with OG1RF

The population of *E. coli* was significantly inhibited by OG1RF (Fig. 1). Specifically, at 24 hours cell counts had decreased by roughly 50%, as there was a decline from an average of 3.9×10^9 to 1.8×10^9 CFU/ml when pure and mixed cultures were compared. The Δ*tnaA* mutant (incapable of indole production) indicated a similar pattern, as growth in mixed culture led to a roughly 50% decline in CFU/ml. The inhibitory effect of *E. faecalis* on *E. coli* decreased with time, however, as at 48 and 72 hours mixed culture colony counts more closely approximated pure culture. Δ*tnaA* in particular was less affected by *E. faecalis* with time, as 72 hour pure and mixed culture sample CFU/ml counts were roughly equivalent. Biofilm and planktonic cultures were similarly affected (Appendix Figs. 12 & 13).

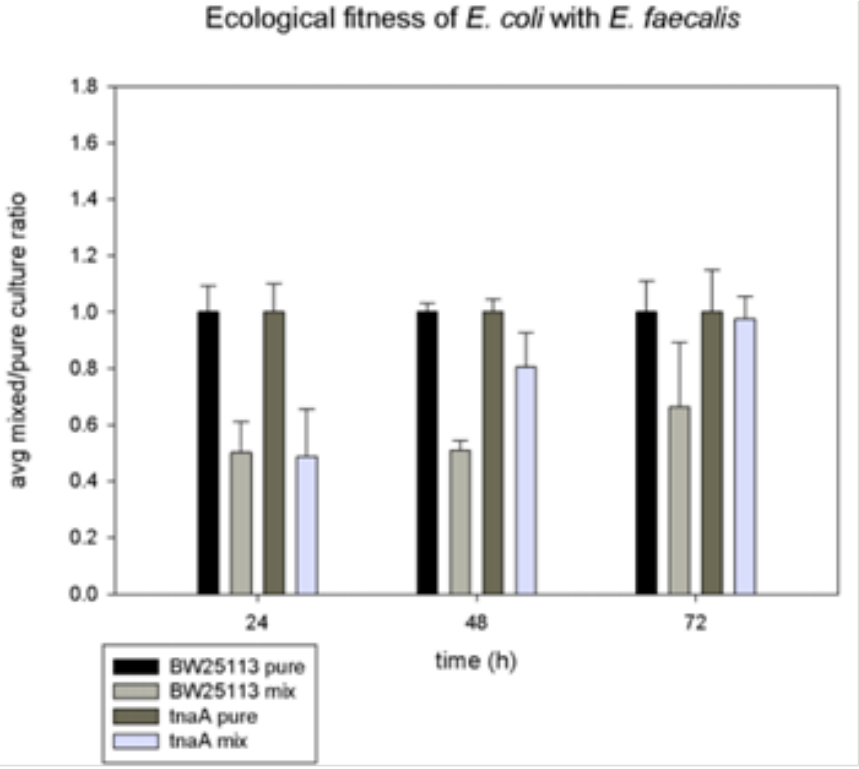


Fig. 1
The ratio of mixed culture *E. coli* BW25113 and Δ*tnaA* to pure culture.

3.1.2 Effect of indole on *E. coli* growth

The addition of exogenous indole was inhibitory, as 24 hour CFU/ml counts fell from 1.8 x 10^9 (Δ*tnaA* with 0mM indole) to 1.41 x 10^9 (0.5mM indole added) and further to 1.09 x 10^9 (1.0mM indole added) in mixed culture (Fig. 2). Pure culture Δ*tnaA* and pure and mixed culture BW25113 had similar results (Appendix Figs. 15 & 16).

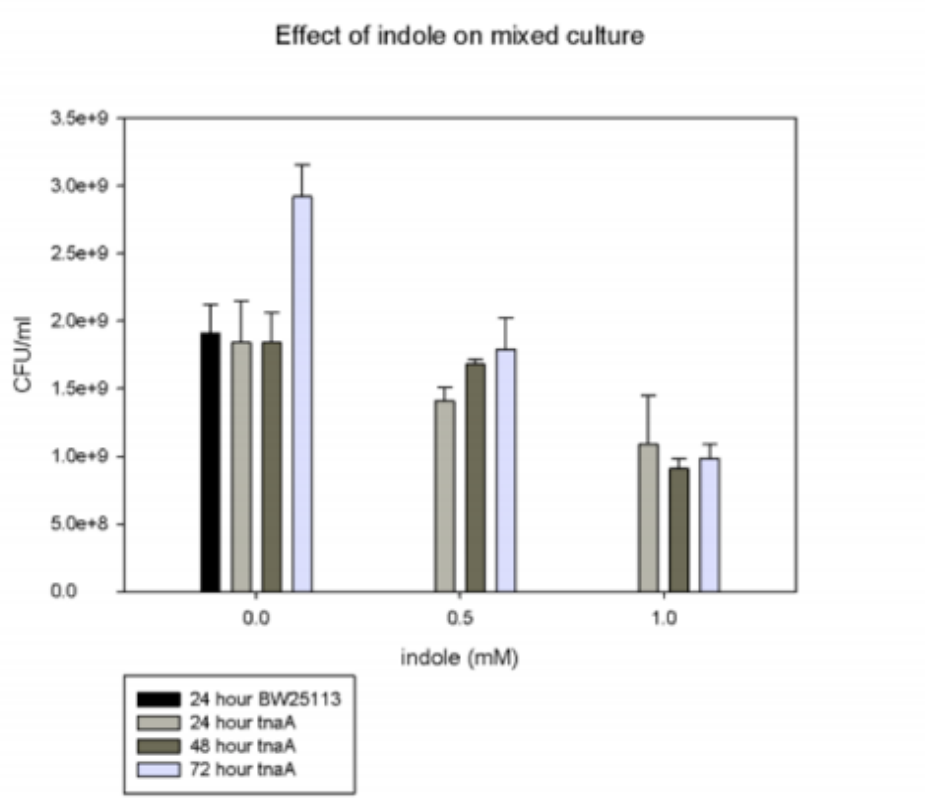


Fig. 2
The effect of increasing indole concentrations on 24, 48, and 72 hour samples of *E. coli* in mixed culture

3.1.3 Effect of indole on *E. faecalis* growth

Addition of exogenous indole to pure and mixed culture samples increased the population of *E. faecalis* when compared to control (Fig. 3). This was particularly true upon the addition of 1mM indole to an OG1RF/BW25113 mix, as the 24 hour colony count exceeded 10^9, whereas mixed culture sans indole reached 4.5 x 10^8, a 45% decrease in cell yield.

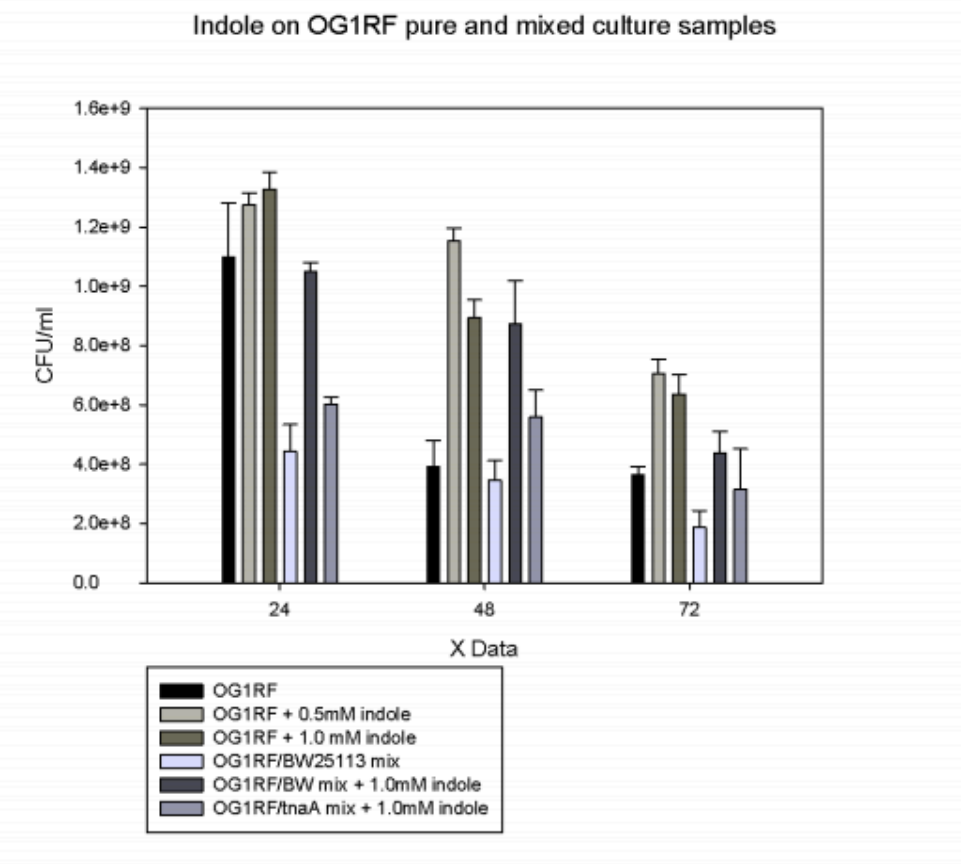


Fig. 3
The effect of indole on pure and mixed culture OG1RF

3.1.4 Effect of co-culture with *E. faecalis* on indole production by *E. coli*

Mixed culture samples of BW25113 and OG1RF indicated slightly lower concentrations of indole production when compared to pure culture (Table 1). Indole concentrations were approximated via standard curve (Appendix Fig.11).

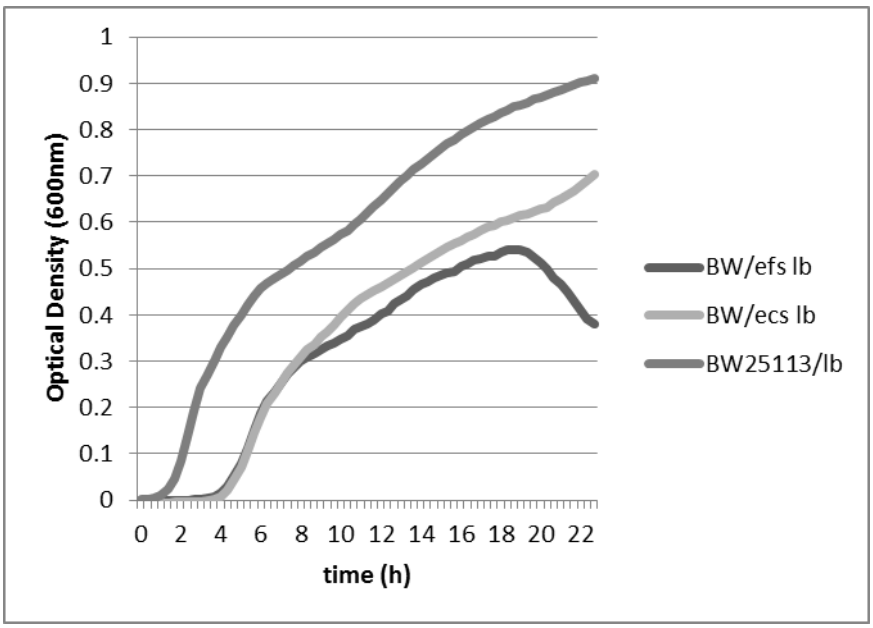
TABLE 1 – Indole concentration calculated from optical density via standard curve

Culture	OD (600nm)	Calculated Indole Concentration (mM)
BW25113	0.093	1.05
BW25113 + 0.5mM indole	0.147	1.59
BW25113 + 1mM indole	0.260	2.72
BW25113/OG1RF mix	0.073	0.85
BW25113/OG1RF mix + 0.5mM exogenous indole	0.143	1.55
BW25113/OG1RF mix + 1.0mM exogenous indole	0.231	2.43
tnaA/OG1RF mix	0.005	0.18

3.2 Effect of *E. faecalis* cell-free supernatant on *E. coli* growth, with and without indole

3.2.1 Effect of supernatant on wild-type growth

E. coli growth was inhibited by *E. faecalis* supernatant, in comparison to growth in its own supernatant (Fig. 4). This inhibition was much more prominent in stationary phase (achieved at roughly 4-6 hours post inoculation) than exponential phase, and reached a maximum inhibitory effect at around 12-16 hours (in BHI). Additionally, the magnitude of the effect was somewhat medium dependent, as when comparing the supernatant of *E. faecalis* grown in BHI to that grown in LB, a greater inhibition was indicated.



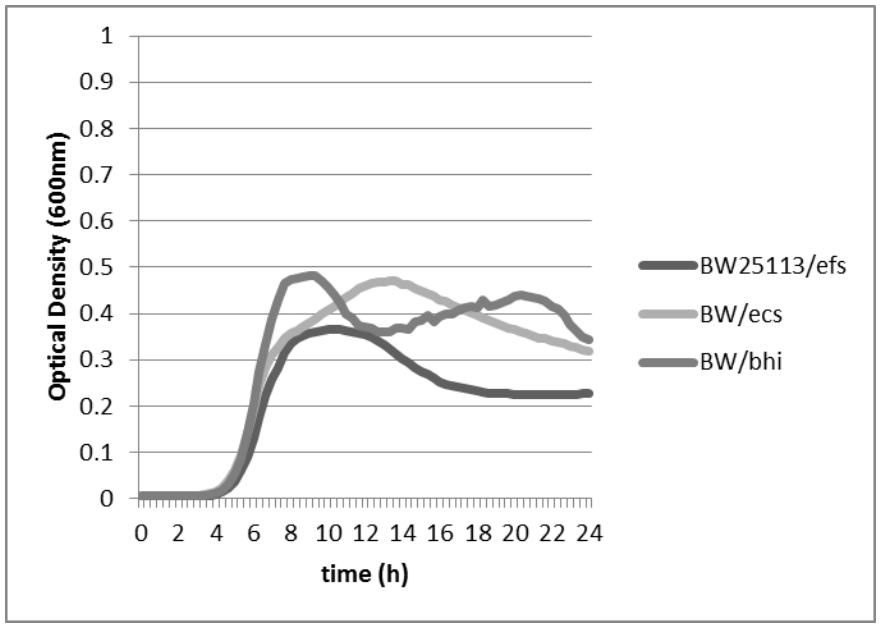


Fig. 4
OG1RF cell-free supernatant (efs) and BW25113 supernatant (ecs) on BW25113 growth. Bacteria grown in LB (top) and BHI (bottom) media.

3.2.2 Effect indole on the growth of *E. coli* in cell-free *E. faecalis* supernatant

The addition of exogenous indole to Δ*tnaA* inhibited the growth of *E. coli* in the *E. faecalis* supernatant. Increasing indole concentration from 0.1mM to 2.0mM resulted in a decrease in optical density from ~0.6 to ~0.25, corresponding to a decrease from ~$1.5x10^8$ to ~$1.75x10^7$ – a decline of approximately 12% (Fig. 5, Appendix Fig. 22). As with direct growth, similar results were seen in LB and BHI media and with the wild-type control (Appendix Figs. 18 and 19).

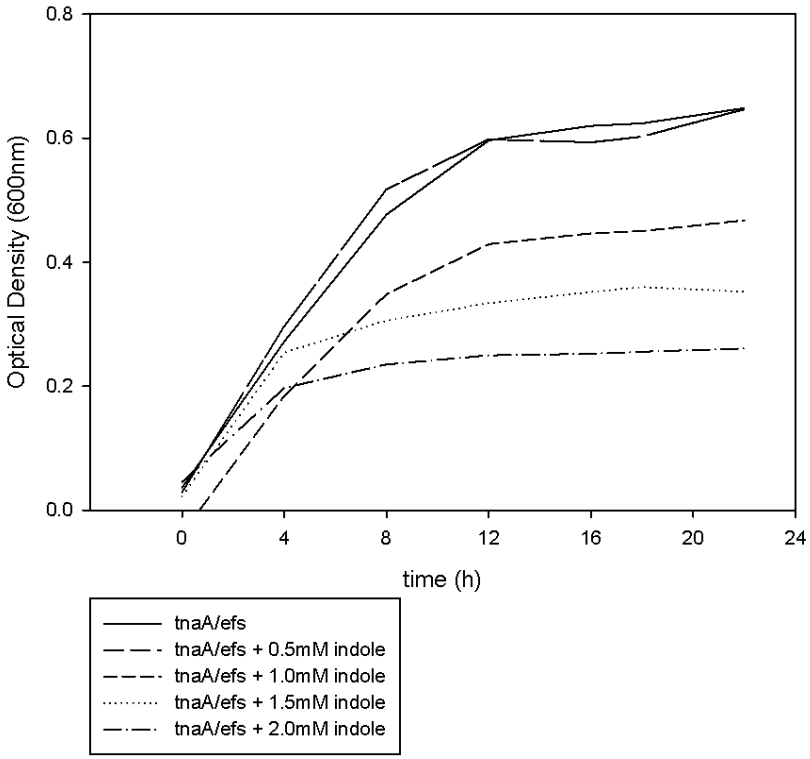


Fig. 5
Increasing indole concentration in OG1RF cell-free supernatant (efs) and BW25113 supernatant (ecs) on BW25113 growth.

3.2.3 Effect of heat, protease and lipase on *E. coli* growth in *E. faecalis* supernatant

Autoclaved OG1RF supernatant was less inhibitory than untreated supernatant (Fig. 6). The addition of 1.0mg/ml concentrations of protease and lipase also resulted in a decreased inhibition. This was observed in both BHI and LB broth (Fig. 7, Appendix Fig. 20). Bacteria grown in minimal media with protease or lipase showed a slight increase in growth as compared to minimal media alone (Appendix Fig. 22).

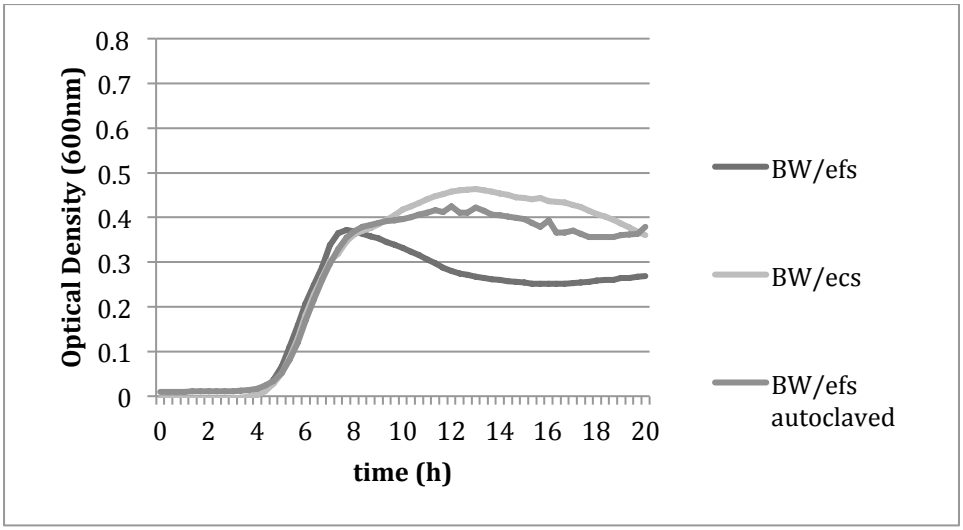


Fig. 6
E. coli growth in OG1RF supernatant (efs) and BW25113 supernatant (ecs), with and without autoclave treatment

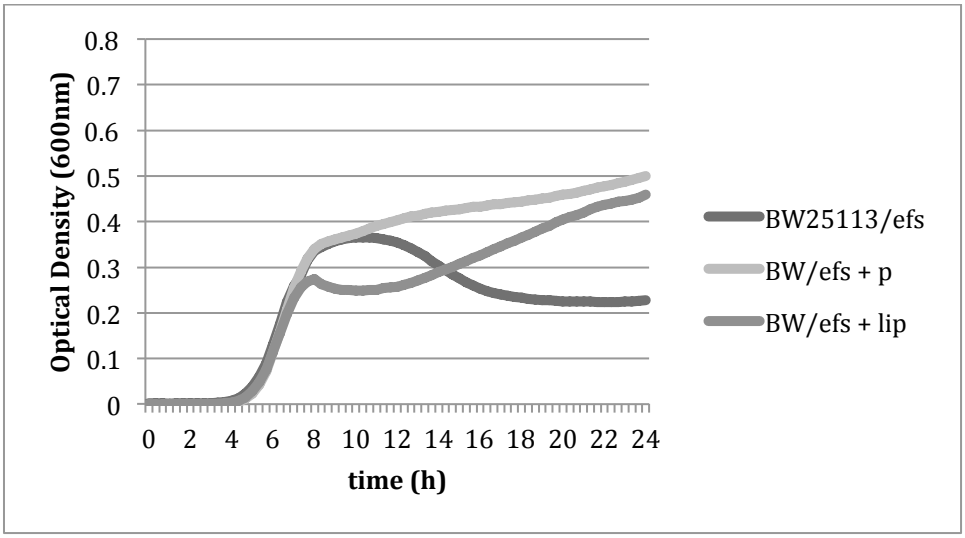


Fig. 7
The effect of 1mg/ml protease (p) and 1mg/ml lipase (lip) on *E. coli* growth in *E. faecalis* supernatant (efs)

3.2.4 Effect of *E. faecalis* on *E. coli* growth on agar

On solid medium the supernatant had no effect on the growth of BW25113 or Δ*tnaA*. Similarly, under nutrient poor agar conditions, no antimicrobial effect of live *E. faecalis* against *E. coli* was evidenced.

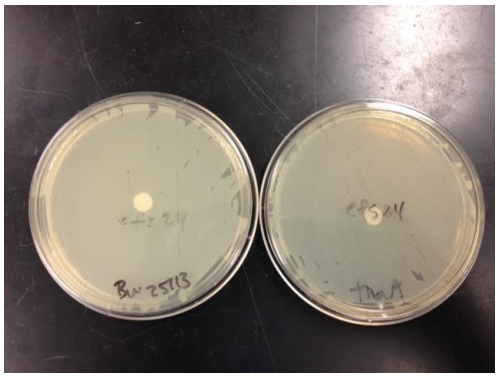


Fig. 8
The effect of OG1RF cell-free supernatant on BW25113 and Δ*tnaA* growth on Mueller-Hinton Agar

CHAPTER IV

DISCUSSION AND CONCLUSION

Indole production has been shown to increase the fitness of *E. coli* in mixed culture with *P. aeruginosa* and other AHL-producing bacteria (Chu et al., 2012, Romano-Hidalgo et al., submitted for publication 2014). This study demonstrates that this is not the case in mixed culture with *E. faecalis*. *E. coli* mutants incapable of indole production were not inhibited by the absence of indole (Figs. 1 & 2, Appendix Fig. 15). This effect was not due to the degradation or prevention of indole production by *E. faecalis*, since indole was present in a BW25113 + OG1RF culture in physiological amounts (Table 1), and the effect was observed in supernatant lacking live OG1RF cells (Appendix Fig. 19).

In this study indole was primarily inhibitory, since the supplementation of exogenous indole to Δ*tnaA* in mixed culture and to cell-free supernatant resulted in a decline in cell numbers and turbidity, with increasing indole concentrations resulting in greater inhibition (Figs. 2 & 5, Appendix Fig. 15). Similar results were seen in both pure and mixed culture, and thus cannot be attributed to the mixed-culture environment (Appendix Figs. 16 & 17). These results are most likely due to the fact that for *E. coli* indole is a molecule that functions as a signal for cells to minimize resource consumption and prepare for nutrient depletion (Gaimster et al., 2014). Thus the lack of indole production by Δ*tnaA* resulted in higher cell numbers than wild-type during stationary phase, and the addition of indole resulted in attenuated growth. This observation is corroborated in the literature. In Gaimster et al. (2014), Δ*tnaA* mutants grow more readily

than wild-type counterparts during the first few days of growth, and subsequently (after ~4 days) experience a steep decline in numbers, while wild-type cultures remain relatively steady for up to 9 days. In the Chu et al. study (2012) it was also determined that addition of indole to pure culture *E. coli* inhibits growth. The inhibitory effect of high concentrations of indole on cell division by *E. coli* in general has been thoroughly documented (Chimerel et al., 2012; Lee & Lee, 2010).

Initially it was surprising that the addition of exogenous indole to Δ*tnaA* samples at physiologically relevant concentrations did not result in wild-type growth (Fig. 2). This may be the result of several factors. One, it may be related to the inherent wild-type and Δ*tnaA* + indole difference. Indole is a molecule whose chemical nature attracts it to the interfacial region of lipid bilayers (Gaede et al., 2005), and is thus found in greater concentrations associated with cell membranes than in aqueous regions (Norman & Nymyer, 2006). The extracellular addition of indole likely leads to superficially high levels of indole outside of the cell, and may not have the physiological effect caused by indole produced within the cell. Additionally, in one study (Gaimster et al., 2014) it was purported that wild-type cells experience a brief (20 min) "pulse" at the onset of stationary phase, during which intracellular indole concentrations reach extremely high (~60mM) levels – a pulse that Δ*tnaA* + indole variants do not receive. Adding indole to Δ*tnaA* cultures, while giving some sign as to the effect of indole in general, cannot mimic naturally occurring mechanisms.

Research indicates that indole attenuates the virulence of *S. aureus* (a Gram-positive organism that has a signaling system similar to *E. faecalis*), but does not affect its growth (Lee et al., 2013). Therefore, it was unexpected that *E. faecalis* would show an

increase in cell numbers upon addition of indole (Fig. 3). The argument could be made that in mixed culture exogenous indole resulted in a decline in *E. coli* cell numbers, which led to increased resources available for *E. faecalis*. Also, virulence of *E. faecalis* may actually increase with the presence of indole. This is especially likely as studies have shown that *E. coli* and *E. faecalis* display a virulent synergism (Lavigne et al., 2008). However, neither account for indole's positive effect on the pure culture population of *E. faecalis*. One possible explanation for this phenomenon is that indole was used by *E. faecalis* to synthesize tryptophan, which was then used as an energy resource. Another is that indole had the opposite effect on *E. faecalis* than it did on *E. coli*, and signaled cells to grow and divide. Further research as to indole's effect on the growth and virulence of *E. faecalis* is clearly warranted.

This study demonstrated that *E. coli*'s growth was greatly inhibited by co-culture with *E. faecalis* (Fig. 1, Appendix Fig. 12). First, to ensure that the effect was the result of a harmful metabolite present in the media (as opposed to depleted resources), a turbidity experiment was performed using supernatant from *E. coli* (ecs) as a control (Fig. 4). To determine the effect of an alternate nutrient source, the experiment was also repeated using Brain-Heart-Infusion broth (Fig. 4). In all cases inhibition of *E. coli* was observed, indicating that a toxin was secreted and the inhibition was not likely a result of nutrient depletion. To determine the nature of the inhibiting substance we found the inhibition was reversed upon autoclaving the supernatant, indicating that a heat-liable substance was denatured or degraded (Fig. 6). Upon addition of proteinase and lipase (1.0mg/ml concentrations) we observed the inhibition similarly reversed, indicating the possible degradation of a toxic proteinaceous or lipid substance (Fig. 7).

Research indicates that *E. faecalis* produces many substances that may be the heat liable and/or proteinaceous substance causing inhibition of *E. coli*. First, *E. faecalis* produces a range of toxins, including cytolysin and several enterocins (Fisher & Phillips, 2009). *E. faecalis* also produces aggregation substance, Ace (belonging to the MSCRAMM family), and extracellular surface protein (Fisher & Phillips, 2009). None of these is likely to cause significant inhibition of *E. coli*, however, as the toxins are primarily effective against Gram-positive bacteria and the surface proteins' major function is adherence and colonization of the host. Virulence factors that are more likely causing inhibition are the degradative enzymes gelatinase and serine protease. These enzymes are quorum regulated and induced when the auto-inducing peptide GBAP stimulates transcription of the *fsr* locus (Bourgogne et al., 2006). Further research with these mutants (*fsrABC, gelE,* and *sprE*) is necessary to determine the effect of these genes on the competitive fitness of *E. faecalis* with *E. coli*. Additionally, *E. faecalis* produces substantial amounts of reactive oxygen species (Huycke et al., 2002). Though *E. coli* is catalase positive (Loewen et al., 1985), if these oxygen species are being produced at high amounts *E. coli* population growth may be inhibited. Research with *E. faecalis* mutants deficient in H_2O_2 and O^{2-} production (such as *aroC* and *menB*; Huycke et al., 2002) is additionally necessary.

Classically, inhibition of bacterial growth is identified on solid media and detected via zones of inhibition. In this study no zones of inhibition were evidenced. There are several possible reasons for this. One, the antimicrobial was produced in insufficient amounts to be detectable on solid media or was unstable (such as the light-sensitive compound hydrogen peroxide). Two, *E. coli* growing on solid media may have

been able to overcome the inhibition given a new, nutrient dense environment. Additional tests were conducted with these considerations. Supernatant from a 48-hour culture (as opposed to a 24-hour culture) was tested, as was growing *E. coli* and *E. faecalis* under nutrient starved conditions (50% BHI agar). In neither case was inhibition observed. Further tests that could be undertaken are seeding the supernatant directly into the agars, testing on more media types with various constitutions, testing under anoxic conditions, or, upon extraction, testing the purified substance directly.

The overall aim of the study was to determine the effect of indole production on the growth of *E. coli* in co-culture with *E. faecalis*. This is significant since *E. faecalis* has become a serious nosocomial pathogen, and uncovering a natural mechanism by which *E. coli* competes may be useful in microbial control of *E. faecalis*. Unfortunately, however, evidence indicates that *E. faecalis* produces substance(s) that inhibit *E. coli* growth, and indole confers it no competitive advantage. Further research is necessary to determine the nature of the substance(s) produced and the effect indole has on the growth and/or virulence of *E. faecalis*.

APPENDIX SECTION

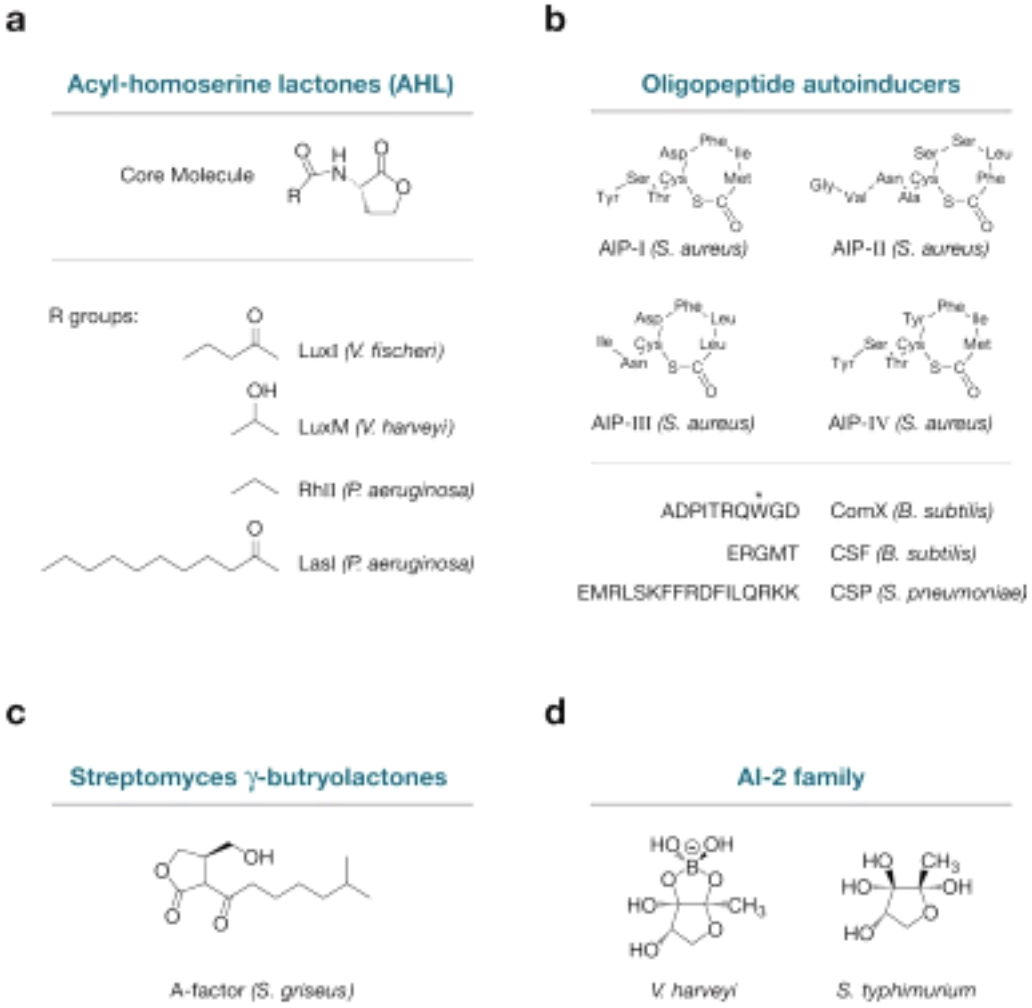


Figure 9
Some examples of quorum sensing signals. (Waters & Bassler, 2005)

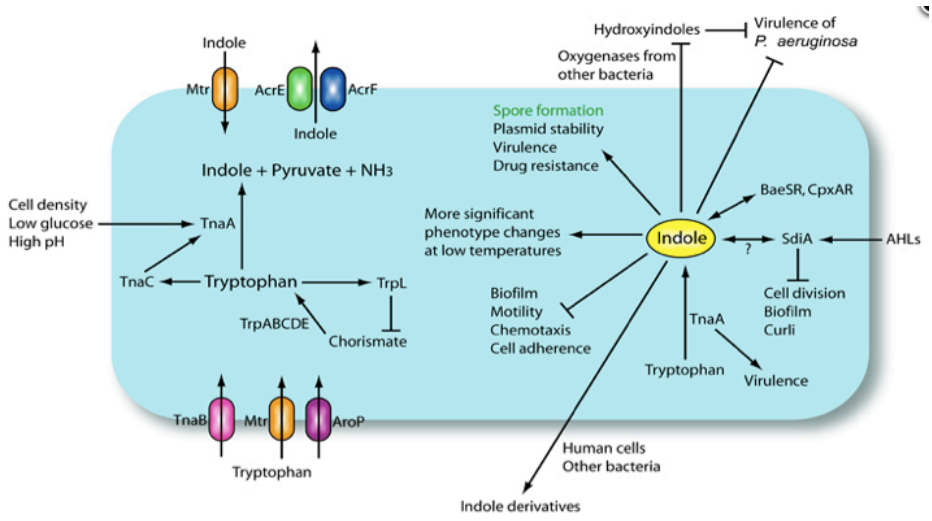


Figure 10
The role of indole as a signal molecule; enzymes associated with indole and tryptophan synthesis, metabolism, and transport.
Source: FEMS Microbiology Reviews, Jul2010, Vol 34 Issue 4, p426-444, 19p, 3 Diagrams, 3 Charts Diagram; found on p 429; as cited in Lee et al. 2010

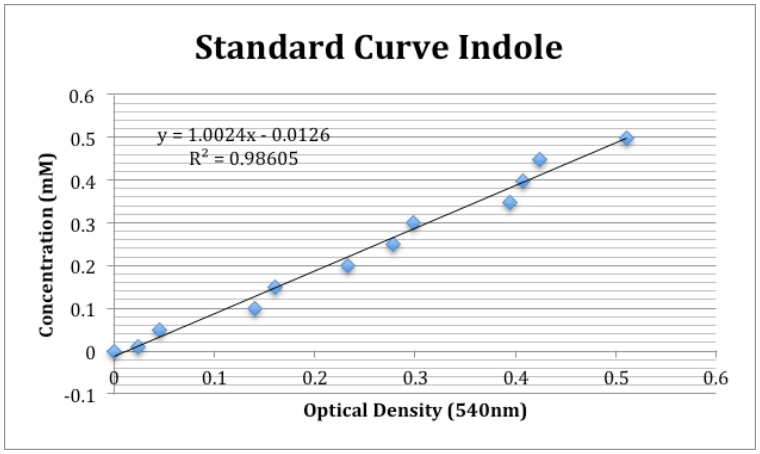


Figure 11
Standard curve of indole

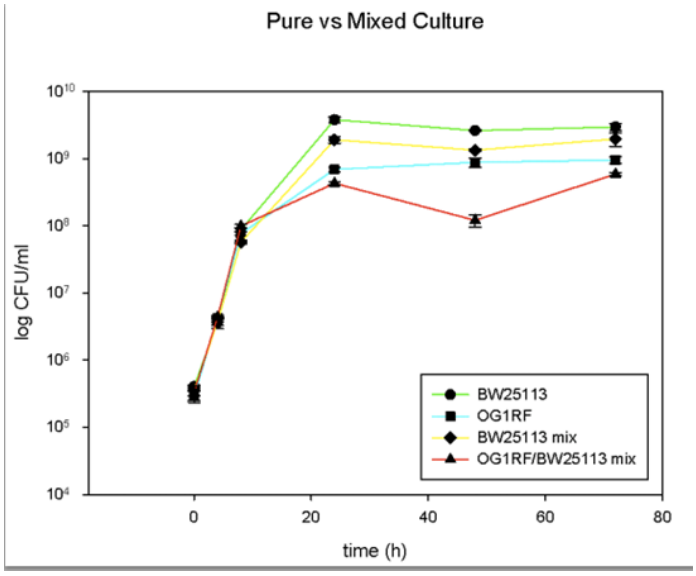


Figure 12
72 hour growth curve of *E. coli* and *E. faecalis* in pure and mixed culture

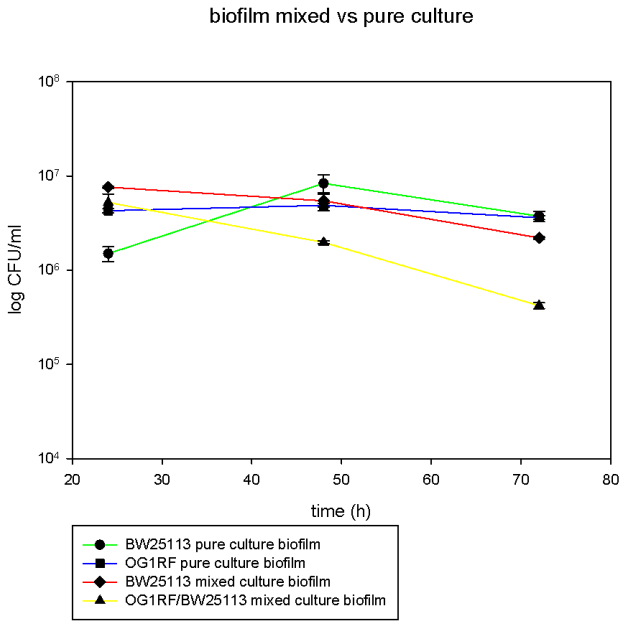


Figure 13
24, 48, and 72 hour biofilm CFU counts of *E. coli* and *E. faecalis* in pure and mixed culture

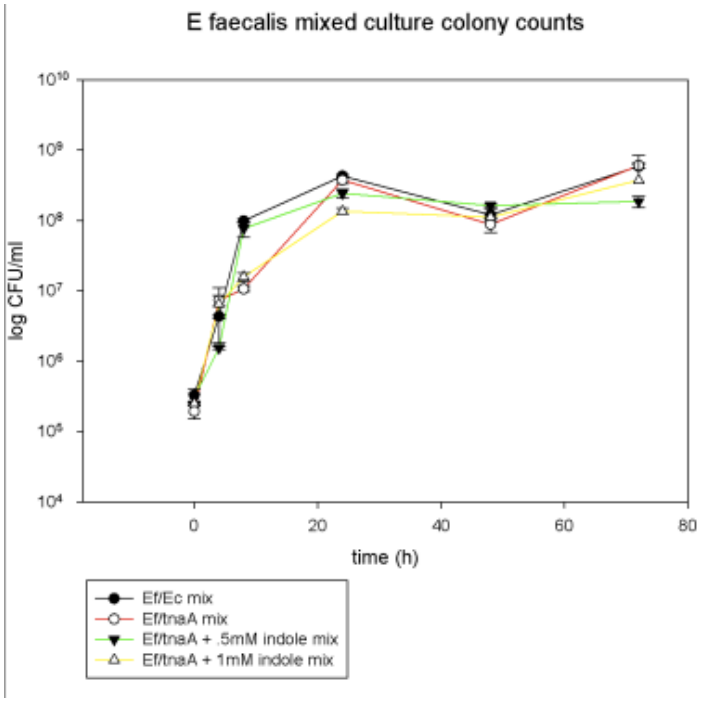


Figure 14
The effect of indole on mixed culture *E. faecalis*

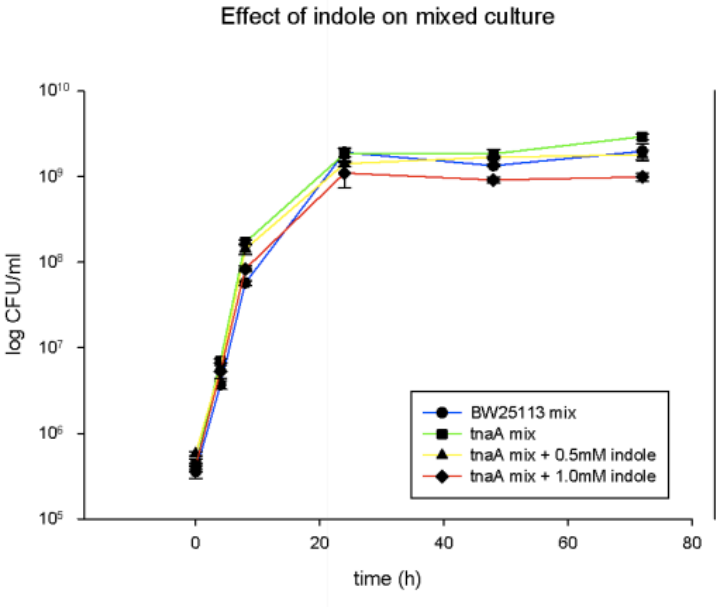


Figure 15
The effect of indole on *E. coli* in mixed culture

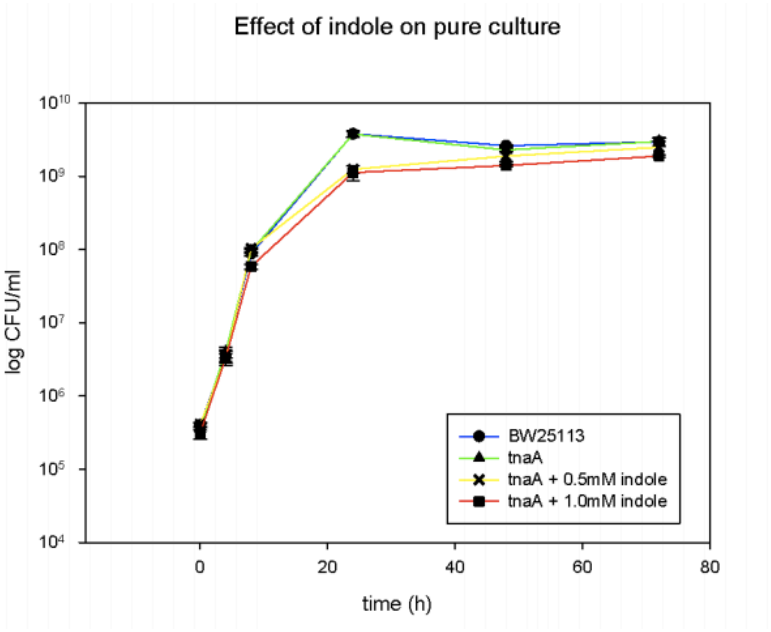


Figure 16
The effect of indole on pure culture *E. coli*

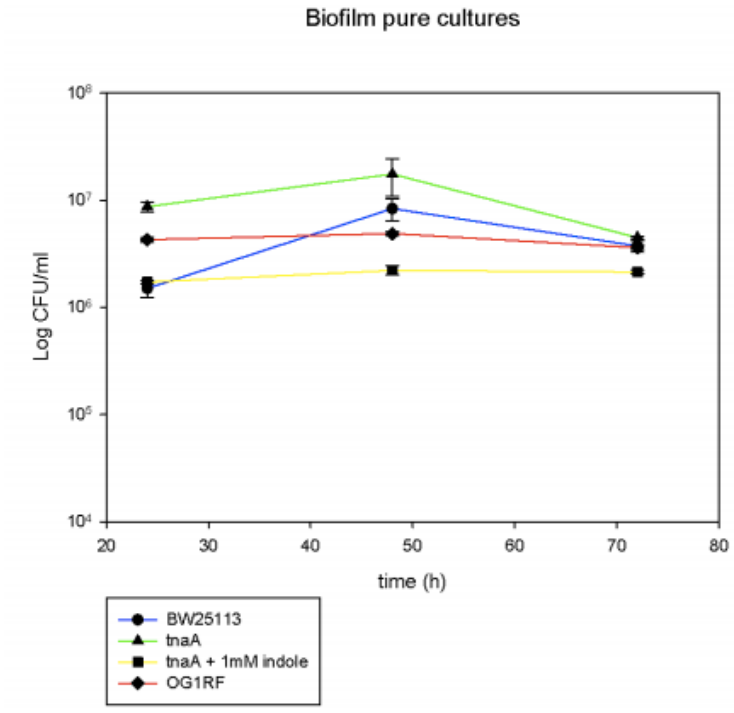


Figure 17
The effect of indole on pure culture *E. coli* in a biofilm

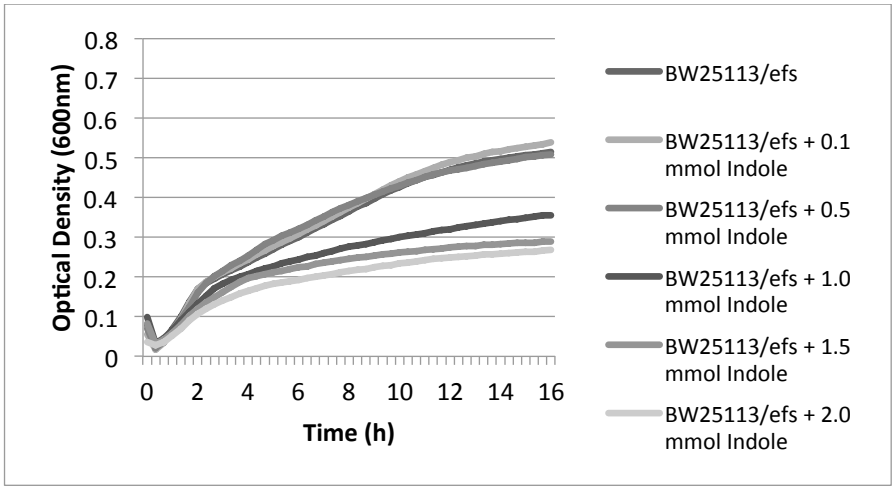


Figure 18
Increasing indole concentrations on pure culture *E. coli* in Efs

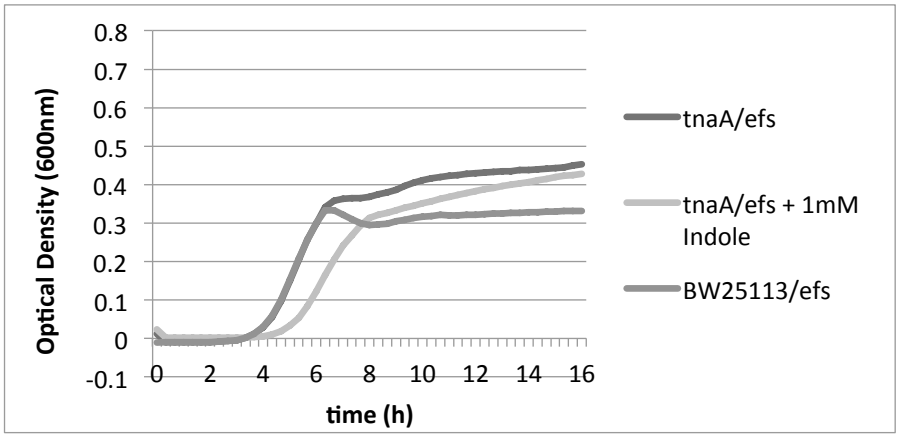


Figure 19
Indole on *E. coli* in Efs grown in BHI

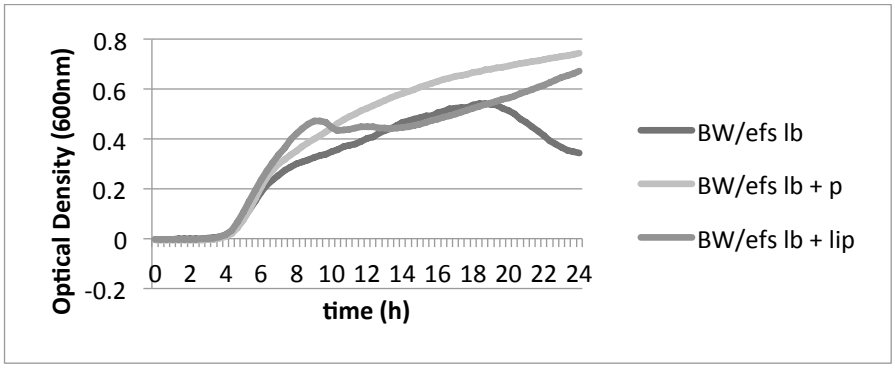


Figure 20
Protease and lipase on *E. coli* in Efs grown in LB

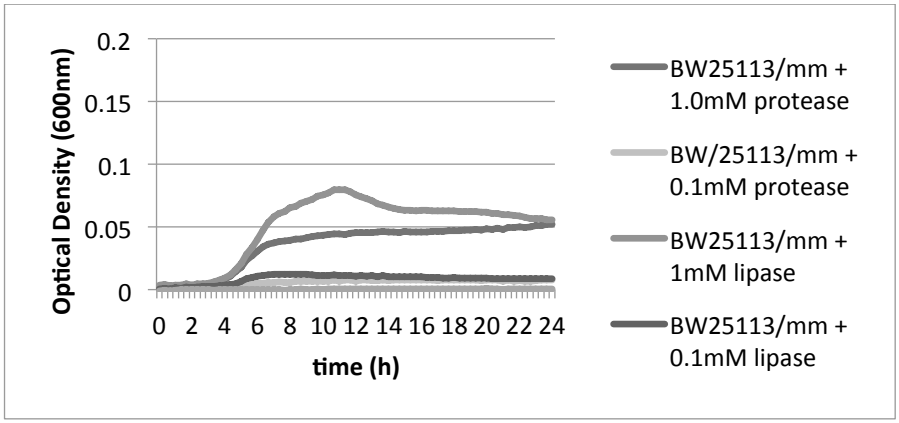


Figure 21
Protease and lipase on *E. coli* growth in minimal media

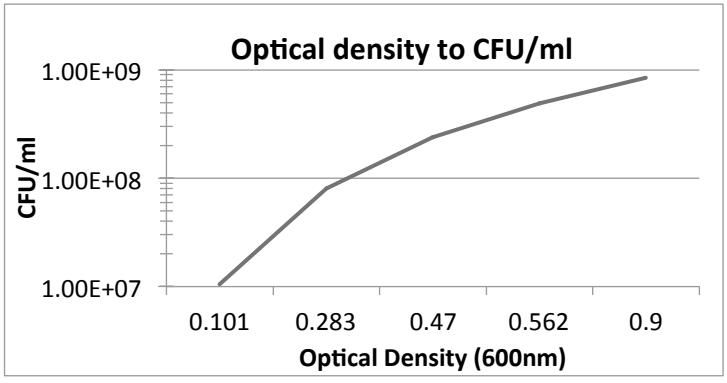


Figure 22
CFU/ml count corresponding to reading from microplate reader

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