EXPLORING THE EFFECTS OF GUT-DERIVED MICROBIAL METABOLITES ON EXPRESSION OF SPI-1 GENES AND ANTIMICROBIAL RESISTANCE IN *SALMONELLA TYPHIMURIUM*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Exploring the Effects of Gut-derived Microbial Metabolites on Expression of SPI-1 Genes and Antimicrobial Resistance in *Salmonella typhimurium*. (May 2014)

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The commensal bacteria that naturally inhabit the human gut are known to be beneficial to the host in a variety of ways, including producing compounds, such as indole and tryptamine, that reduce the invasion and colonization capability of enteric pathogens, such as *Salmonella enterica* serovar Typhimurium. Here we explore the effects of other tryptophan metabolites on the expression of genes that *S. typhimurium* uses for invasion into host cells: the *Salmonella* Pathogenicity Island-1 (SPI-1) genes. We also test whether smaller dosages of one tryptophan metabolite, indole, confers antimicrobial resistance to *S. typhimurium* (thereby benefiting the pathogen rather than the host)*,* through upregulation of the SPI-2 genes and subsequent outer membrane modification.

DEDICATION

This paper is dedicated to my wonderful, lively, happy son, Daegan, who always provides me necessary comic relief during stressful times and to my very supportive partner, Marc, who is my rock. This research would not have been possible without their support.

ACKNOWLEDGEMENTS

CHAPTER I

INTRODUCTION

Salmonella enterica serovar Typhimurium, the bacterium that causes nontyphoidal salmonellosis, is a growing cause for concern. This is due to the fact that 1) it causes over a million cases of nontyphoidal salmonellosis in the U.S. per year;[1] 2) it is the leading cause of mortality in cases of food-borne illness;[2] 3) it is especially fatal in developing countries and in certain populations, including the immunocompromised;[3] and 4) the number of cases of infection with multi-drug resistant salmonella has increased in recent years.[4] Further investigation into the host-microbe interactions is warranted. This is to be accomplished by analyzing the effects of commensal microbial metabolites on the expression of certain genes in *S. typhimurium*, as well as analyzing the effects of the host's commensal gut bacteria on invading *S. typhimurium*. The expected result of these endeavors is to increase the body of knowledge related to this subject in order to develop better means of treating nontyphoidal salmonellosis.

Salmonella typhimurium causes approximately 1.4 million cases of nontyphoidal salmonellosis in the U.S. alone[1] and approximately 93.8 million cases worldwide[5]. Of these, roughly 155,000 (with a 95% Confidence Interval of 39,000 – 303,000) are fatal[5]. *S. typhimurium* is known to be especially fatal for children under five years of age (in the U.S.)[5], those who are malnourished[6], and those with compromised immune systems[6]. For example, in Sub-Saharan Africa, the pathogen has a mortality rate up to 45%, which is now suspected to be associated with the compromised immune systems of those with comorbid HIV or *Plasmodium* spp. infections[6]. Regarding this epidemic in Africa, the majority of infections with *S. typhimurium* are ones that

exhibit multidrug-resitantance[7], which is also a growing cause for concern in developed

countries due to the very nature of the mechanisms of antibiotic resistance in bacteria in general.

While infection with *S. typhimurium* is a very serious public health issue in developing countries,

it also causes 31% of mortalities associated with food-borne illness in the United States[2]. In

addition, *S. typhimurium* is also a potential bioterrorism agent: in 1984, the Rajneesh cult seeded

the salad bars of several restaurants in Oregon with the bacterium, resulting in 751 people

contracting the illness[8].

Steps have been taken by researchers to better understand host-microbe interactions in order to

help alleviate these current problems, but much more is needed. It has been discovered that *S.*

typhimurium can modulate the host immune system in a variety of ways: according to Alaniz et

al., one way *S. typhimurium* avoids the host's immune system is to regulate the production and

location of the protein that the bacterium uses to form its flagella, FliC, which makes the protein

then unavailable to dendritic cells.[9] It has also been discovered that metabolites produced by the

host commensal gut bacteria can have effects on invading pathogens: Alaniz et al. found that a

metabolite produced by the host's gut bacteria, indole, induces dendritic cell antimicrobial

activity and reduces the invasion capability of the bacterium (perhaps by reducing the expression

of SPI-1 genes) concurrent with increased intracellular replication and survival (possibly through

a small upregulation in the SPI-2 genes) (Crisp Z, Kohli N, Mueller C, Jayaraman A, Alaniz RC.,

unpublished data). While these results are very promising, more investigation is needed.

CHAPTER II

METHODS

Minimum Inhibitory Concentrations

Minimum Inhibitory Concentrations (MICs) were performed according to the Agar Dilution

Method protocol developed by the NCCLS, with the exception that no spot-inoculator was used.

A known concentration of bacteria was inoculated onto the agar using a spreader kit in the place

of the spot-inoculator.

SPI-1 Reporter Assays

The reporter strains used contain *hilA, invF, or prgH* genes from the Salmonella Pathogenicity

Island (SPI-1) joined to the lacZ gene from *Escherichia coli,* which produces ß-galactosidase.

The agar on which the particular strain of *S. typhimurium* was grown is made selective by being

provided with a substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or X-Gal) that

produces a blue color when cleaved by ß-galactosidase. The genes used are also contained on a

resistance plasmid and grown in the presence of Tetracycline to maintain selectivity for the gene.

Resorufin ß-D-galactopyranoside (Res-Gal) is also a substrate for ß-galactosidase that fluoresces;

the amount of fluorescence was measured using a plate reader.

The particular *S. typhimurium* strain used was grown for 16 hours at 37°C and 110 rpm with

aeration in 10 mLs Luria-Bertani (LB) broth with a tryptophan metabolite or negative (solvent)

control. The culture was then back-diluted to 1:32. This back-diluted culture was then

inoculated into a 96-well plate with 200 µLs per well in triplicate for each condition: wild-type

with no treatment, solvent control, and titrated doses of the tryptophan metabolite. Conditions

for fluorescence were then added: 5.0 µg/mL Res-Gal to each well or DMSO as a solvent

control. Fluorescence was then measured at 544 nm excitation and 590 nm emission and

absorbance at 600 nm for optical density of bacteria to visualize bacteria going into log phase

every half hour for three hours. The plate was kept in an incubator at 37°C in between

measurements. The measurements were then placed into graphical form using GraphPad Prism 5

software.

Antimicrobial Resistance Assays

Wild-type *S. typhimurium* +/- 100-25µM indole (+ 0.005% DMF as negative control) were

grown in 10 mLs LB broth at 37°C and 110 rpm with aeration for 22 hours, as consistent

CFU/mL are obtained at this time point. The cultures were then back-diluted 1:100 in LB broth

and grown for three hours at 37°C and 110 rpm with aeration to achieve log phase. Each culture

condition was then plated at the 10^{-5} dilution in triplicate on LB agar and incubated overnight at

37°C and 5% CO_2. The antibiotic (10X MIC or 5X MIC for Ampicillin) was then added to each

condition and incubated for 4 hours at 37°C and 110 rpm with aeration to allow killing of all

sensitive cells. Each culture condition was then plated at 10^{-3} dilution in triplicated and

incubated overnight at 37°C and 5% CO_2 . CFU/mL was then determined for both the pre-

antibiotic treatment and post-antibiotic treatment cultures. CFU/mL before Ampicillin treatment

was divided by CFU/mL after Ampicillin treatment to quantify bacterial survival; these values

were then entered into graphical form using GraphPad Prism 5 software.

CHAPTER III

RESULTS

The effects of the tryptophan metabolite tryptamine on SPI-1 genes *hilA, prgH,* and *invF* were tested using the SPI-1 reporter assay. The effects of the tryptophan metabolites 5-hydroxyindole and indole-3-carbinol were tested on SPI-1 gene *hilA*; this gene was chosen as the main gene to test since it encodes the main transcription regulator of the other SPI-1 genes. Indole was used as a positive control in each of these experiments, since it has already been established that indole suppresses the expression of SPI-1 genes[10] . Tryptamine did not appear to have any significant effect on the expression of the SPI-1 genes *hilA, prgH,* or *invF* (Figure 1)*;* 5-hydroxyindole did not appear to have any significant effect on the expression of *hilA* (Figure 2)*.* However, indole-3-carbinol did appear to suppress the expression of *hilA* (Figure 2), though not to the same extent that indole does. It should be noted that indole-3-carbinol is not a microbial metabolite but is instead found in cruciferous vegetables.
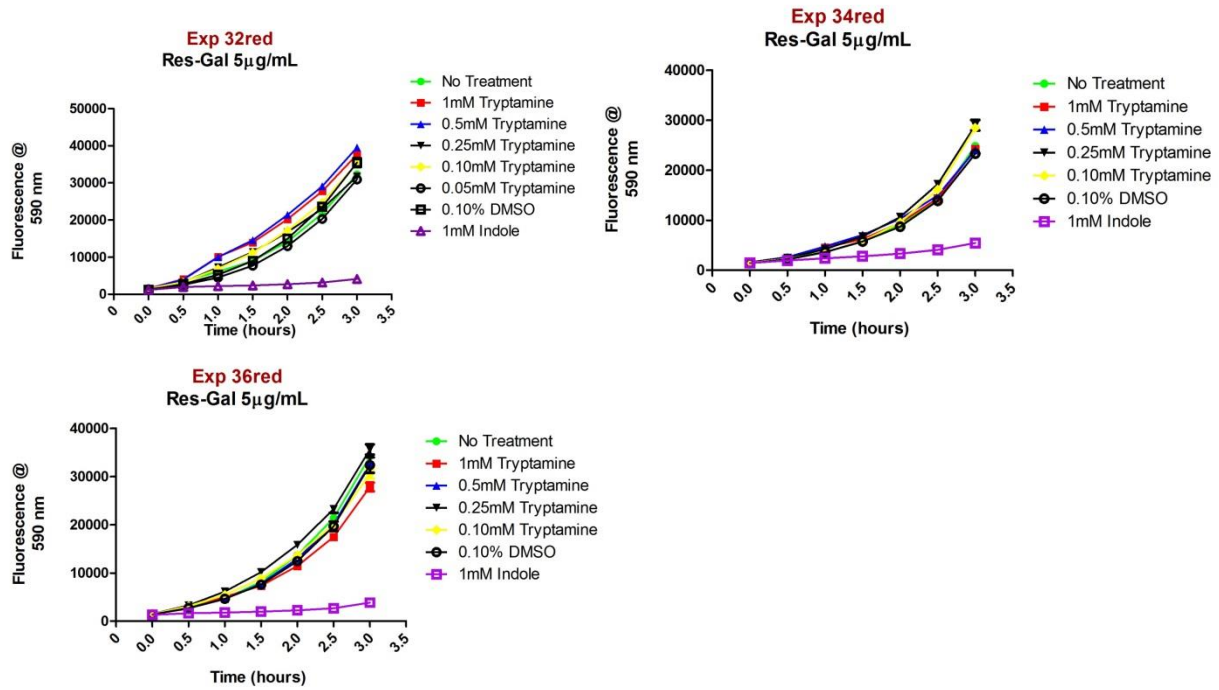
Figure 1. Effects of different doses of tryptamine on *S. typhimurium* SPI-1 genes *hilA, prgH,* and *invF*, respectively.
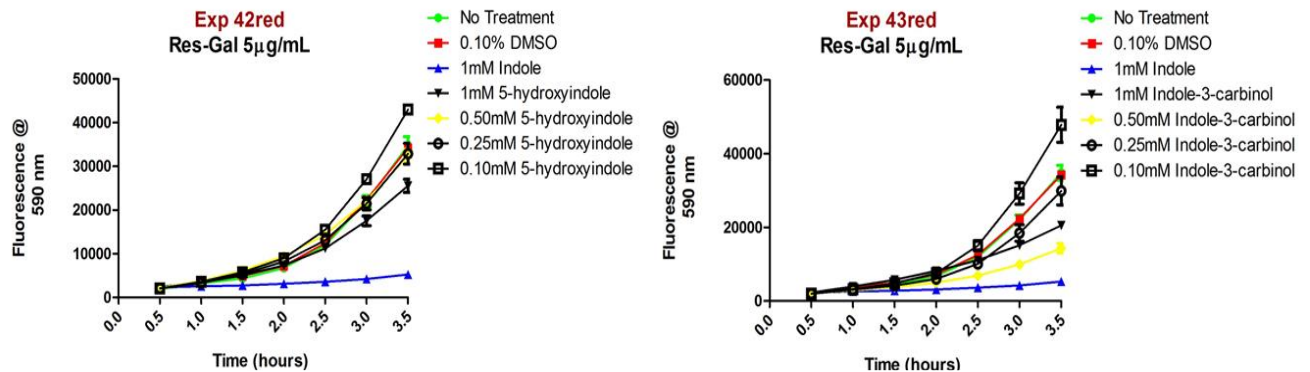

Figure 2. Effects of different doses of 5-hydroxyindole and indole-3-carbinol on *S. typhimurium* SPI-1 gene *hilA*, respectively.

The effects of indole on antimicrobial resistance in this bacterium was assesed using the

antimicrobial resistance assay described above. Micromolar amounts of indole appear to confer

antimicrobial resistance on *S. typhimurium* when treated with both Ampicillin (Figures 3 and 4)

and Polymyxin B (Figures 5 and 6). Ampicillin was chosen because 1) it is clinically relevant

and 2) it attacks the core polysaccharide of lipopolysaccharide (LPS) to gain ingress to the

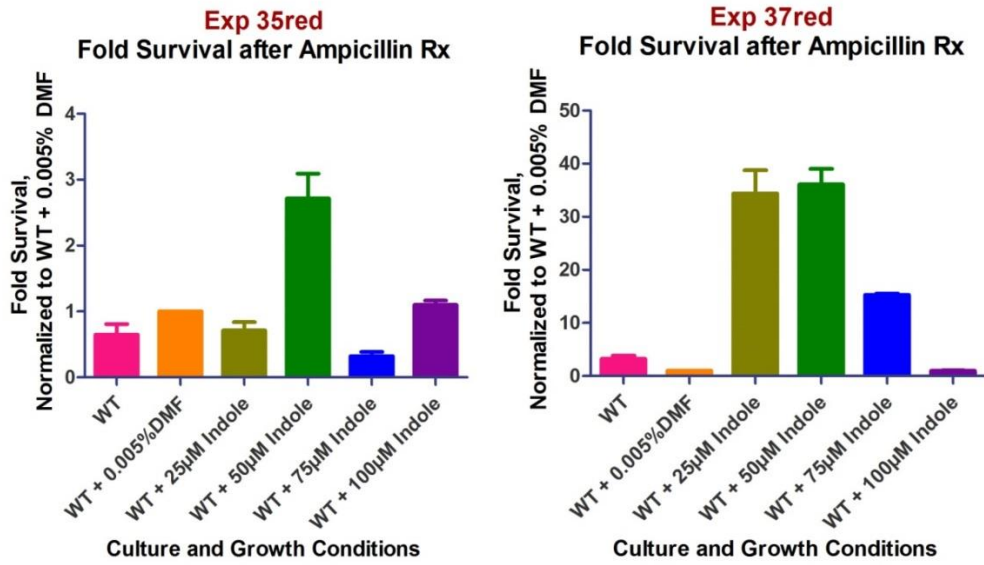bacterium to prevent the transpeptidase reaction.

Figure 3. Fold survival of WT *S. typhimurium* and different dosages of indole after 100μg and 150μg Ampicillin, respectively.
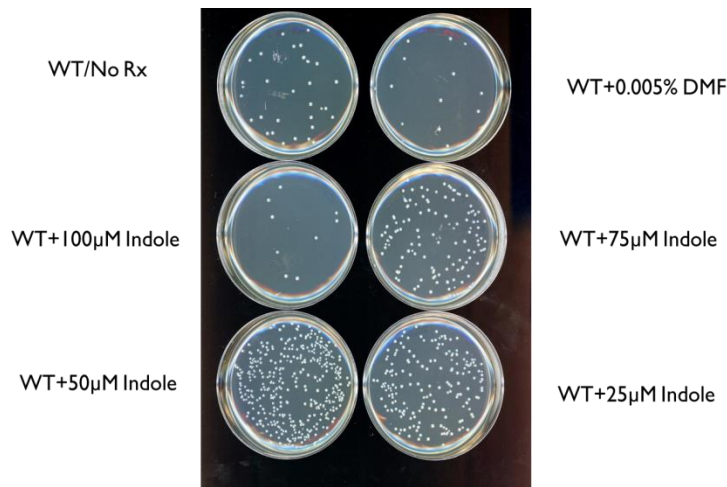

Figure 4. Representative pictures of *S. typhimurium* incubated with varying amounts of indole + DMF negative control then treated with Ampicillin.

Polymxyin B was chosen because it creates pores in the outer membrane of the bacterium by attacking the lipid A portion of LPS, thus destabilizing the outer membrane and causing lysis. Since both of these antibiotics act on the outer membran of the bacterium, the mechanism of this resistance may be through modification of the outer membrane by the bacterium.
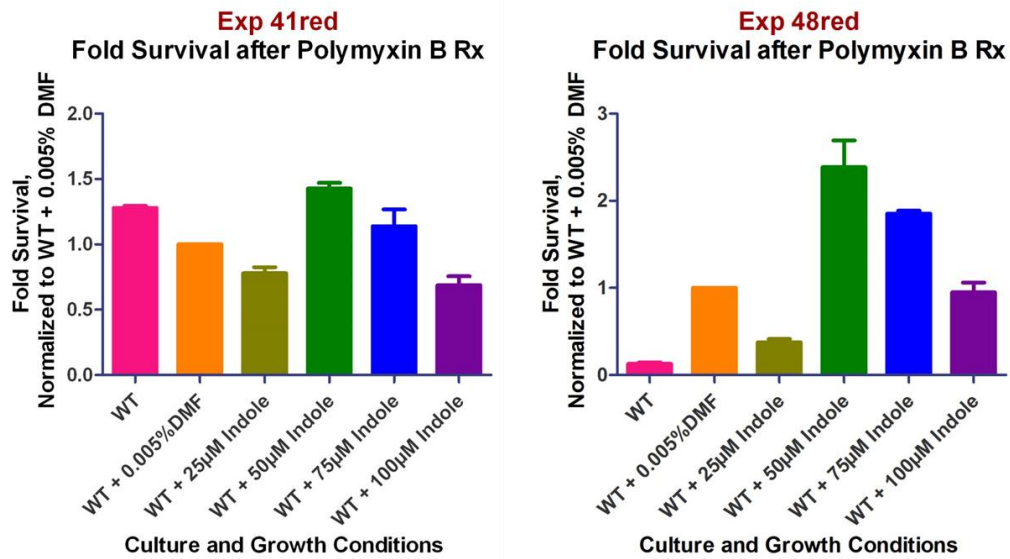

Figure 5. Fold survival of WT *S. typhimurium* and different dosages of indole after 1.563µg and 1.600µg Polymyxin B, respectively.
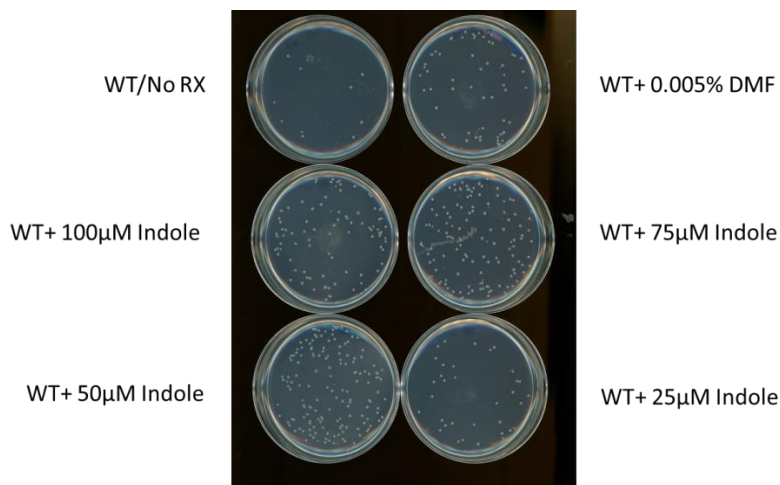

Figure 6. Representative pictures of *S. typhimurium* incubated with varying amounts of indole + DMF negative control then treated with Polymyxin B.

CHAPTER IV

DISCUSSION

These data suggest that indole, in millimolar amounts, reduces SPI-1 genes, and subsequently invasion by the bacterium, the most effectively as compared to other tryptophan metabolites. This benefits the human host. However, these data also suggest that indole, in micromolar amounts, confers antimicrobial resistance on the bacterium, benefiting the pathogen. These conclusions demonstrate the complex relationships that can evolve between the host, its commensal symbiotic bacteria, and invading pathogens. The reduction in the SPI-1 genes (which encode a type III secretion system (T3SS) that facilitates invasion into host cells, including formation of a "needle complex" to mechanically gain entry into the host cell) by indole has been supported by functional assays using HeLa and DC 2.4 cells (Crisp Z, Kohli N, Mueller C, Jayaraman A, Alaniz RC, unpublished data). This antimicrobial resistance mediated by indole is hypothesized to be caused by an upregulation in the SPI-2 genes, which is the type III secretion system (T3SS) that is used by the bacterium when located intracellularly in a host cell. These genes facilitate survival and replication of the bacterium while in this hostile environment: one of these functions is to extensively modify the outer membrane. This would be consistent with previously discovered data (Crisp Z, Kohli N, Mueller C, Jayaraman A, Alaniz RC, unpublished data). Since it is hypothesized that this antimicrobial resistance is mediated through a modification in the outer membrane of the bacterium, it would be interesting to assess the effects of micromolar amounts of indole treatment on *S. typhimurium* treated with antibiotics that target other parts of the cell.

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virulence of Salmonella enterica serovar Typhimurium revealed by genome-wide analyses. Gut Pathog. 4: 5, doi: 10.1186/1757-4749-4-5.

APPENDIX A

NEGATIVE CONTROLS AND GROWTH CURVES FOR REPORTER

ASSAYS

Negative controls were generated for each of the SPI-1 Reporter Assays by running a solvent

control for Res-Gal, DMSO, alongside the fluorescent compound in triplicate. The solvent

control is the only difference in this case. Graphs were generated for each experiment based

upon the same scale as the experimental data using Res-Gal. In addition, growth curves were

generated for each experiment to ensure that the bacteria properly entered log phase and that they

were growing as expected. As explained above, this was measured by taking the absorbance of

the sample at 600nm at regular intervals (the same intervals in which the fluorescence was

measure for the effect on the SPI-1 genes) using a plate reader. Graphs were generated for these

data as well, and can be seen alongside the negative control data in Figures 7-11 for each
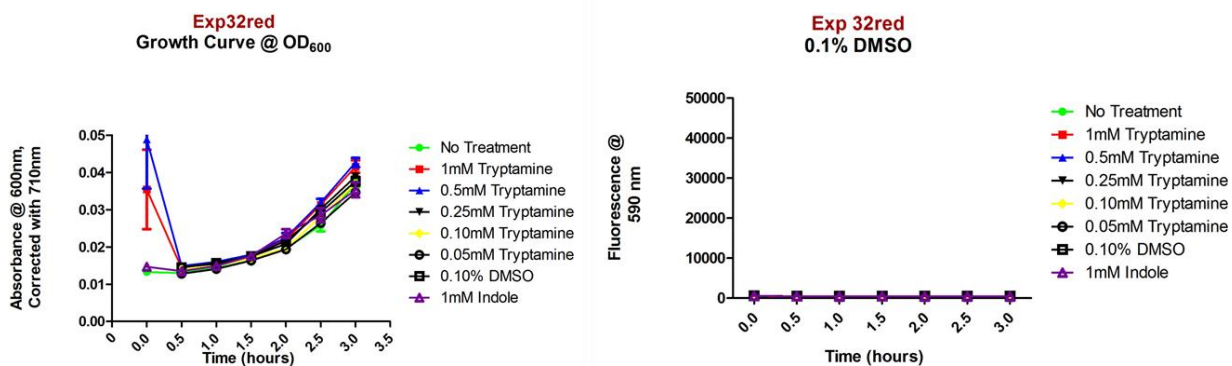
experiment in numerical order.

Figure 7. Growth curve and negative control data, respectively, for Experiment 32: effect of different doses of tryptamine on *S. typhimurium* SPI-1 gene *hilA*.
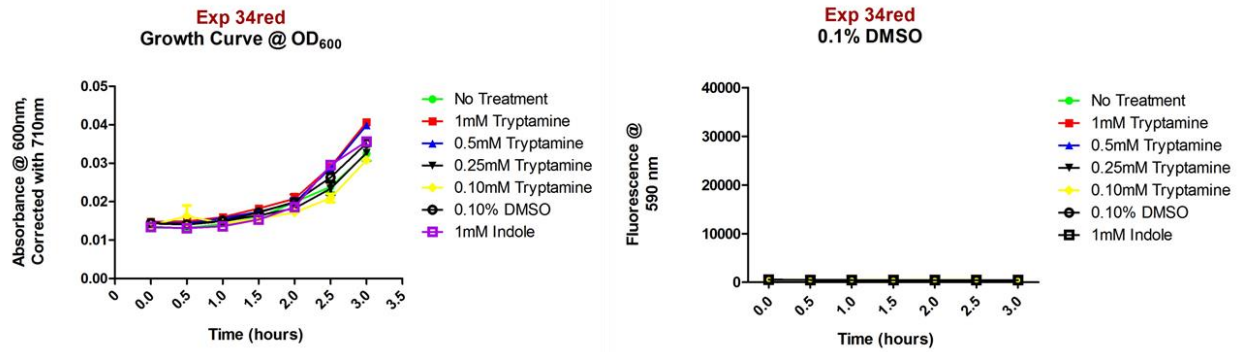
Figure 8. Growth curve and negative control data, respectively, for Experiment 34: effect of different doses of tryptamine on *S. typhimurium* SPI-1 gene *prgH*.
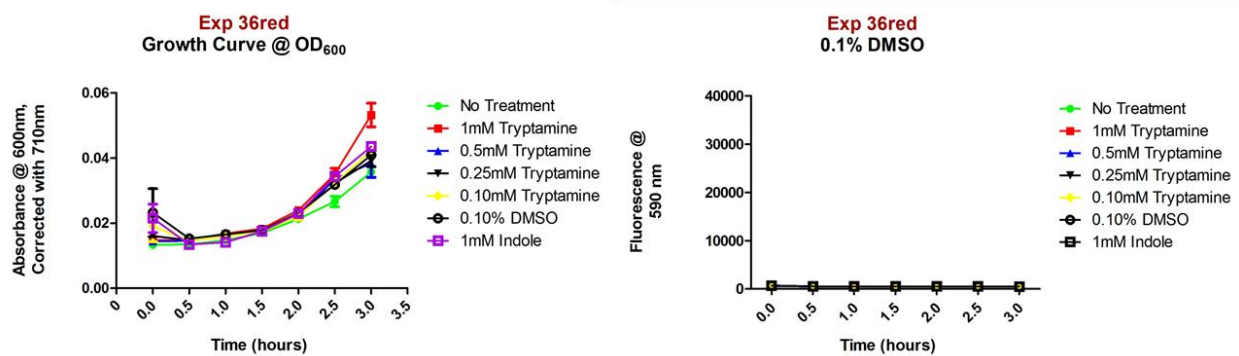

Figure 9. Growth curve and negative control data, respectively, for Experiment 36: effect of different doses of tryptamine on *S. typhimurium* SPI-1 gene *invF*.
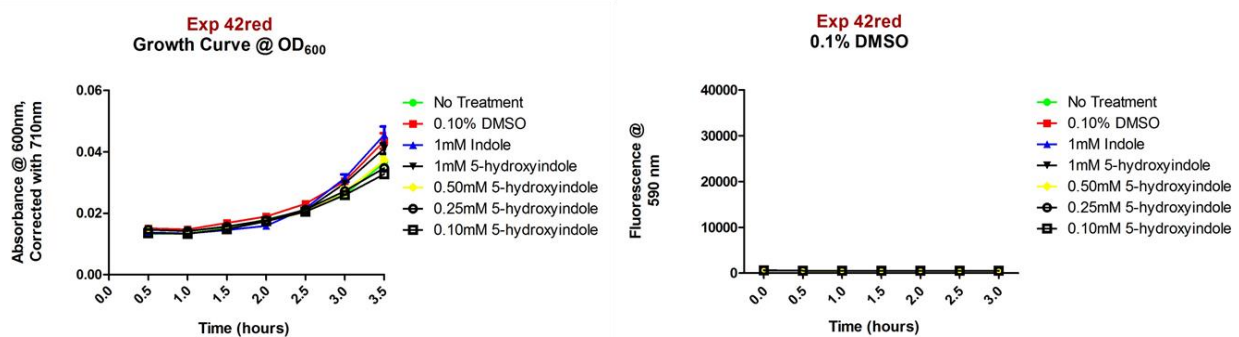

Figure 10. Growth curve and negative control data, respectively, for Experiment 42: effect of different doses of 5-hydroxyindole on *S. typhimurium* SPI-1 gene *hilA*.
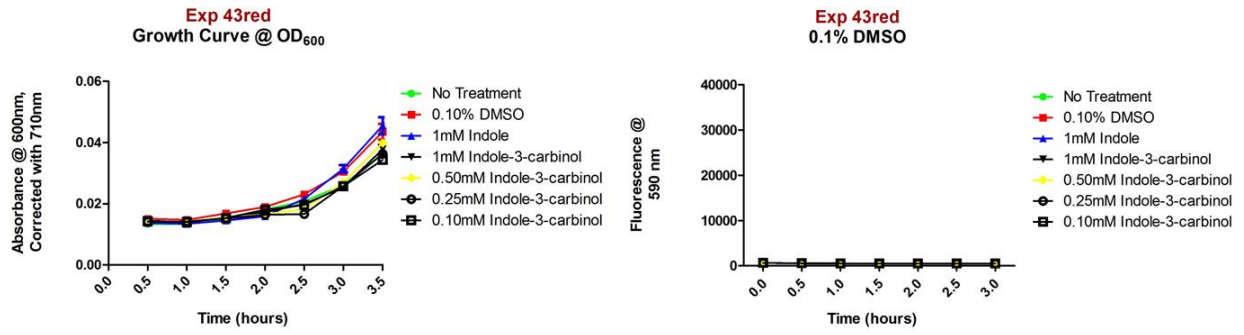
Figure 11. Growth curve and negative control data, respectively, for Experiment 43: effect of different doses of indole-3-carbinol on *S. typhimurium* SPI-1 gene *hilA*.