Integrating the metabolic processes of *Escherichia coli* with virulence by decreasing glucose availability, inhibiting the acetyl-CoA carboxylase subunit accA with asRNA, and through the quantification of the Lux-S gene

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ABSTRACT

The study aims to demonstrate a possible link between bacterial cell metabolism and virulence by combining the metabolic mechanisms of the gram-negative bacteria, *Escherichia coli*. Glucose increases the proliferation of intestinal microflora, which augments the output of the short-chain fatty acids. Bacteria ferment glucose into short-chain fatty acids, which help regulate many biochemical processes and pathways. Each short-chain fatty acid maintains colonic pH, promotes cell differentiation, and the apoptosis of colonocytes. The long-chain fatty acids are also synthesized for plasma membrane and biofilm formation. To increase the synthesis of acetyl-CoA carboxylase, an enzyme that catabolizes glucose into short- and long-chain fatty acids, *Escherichia coli* was cultured in Luria broth enhanced with a high to a low concentration of glucose. The 15mM, a high concentration of glucose, yielded qPCR products measured for the target gene accA, which was 4,210ng/µL. The 7.5mM sample produced a concentration equal to 375 ng/µL, and the control sample measured an accA concentration of 196 ng/µL. The gene accA, one of four subunits for the acetyl-CoA carboxylase enzyme, was suppressed by asRNA, producing a qPCR product of 63 gene copies. Antisense RNA for accA reduced the amount of Lux-S, a vital gene needed for propagating quorum-sensing signal molecules. The Lux-S gene, which is responsible for releasing autoinducer 2 for cell-to-cell quorum sensing, was reduced by

the gene inhibition of accA with asRNA. The increase in Lux-S transcription augments biofilm production in support of spreading virulence. The implications of the study advocate for designing antibiotics that target bacterial cell metabolic processes to block bacterial antibiotic resistance.

INTRODUCTION

Seventy percent of the immune system resides in the gastrointestinal tract, and a healthy balance of microflora can improve insulin sensitivity, fight obesity, eradicate colon cancer cells, and reduce mental disorders such as depression. The human gut may be a conduit for treating many human diseases. For example, researchers found a tumor-inhibiting molecule released by a probiotic strain of bacteria. The *L. case* strain produces ferrochrome, which delays the metastasis of colon cancer by activating the c-Jun-terminal kinase pathway of apoptosis [1]. Increasing the production of ClpB, a chaperone protein secreted by *Escherichia coli*, reduced the food intake, limited the meal patterns, and stimulated intestinal hormones in mice as a glucagon-like peptide 1, which is an antihyperglycemic protein [1]. Another study found therapeutic opportunities for inflammatory diseases through isolating 17 clostridium strains and re-engineering mixtures of those strains, which decreased colitis and increased regulatory T cells, or the T-reg cells, in rodents. Inflammation of the airways due to allergies and inflammatory colitis are remedied by interleukin-10, a cytokine expressed by the bacteria *Lactococcus lactis* [1].

Isolating microbial organisms with the prospect of providing alternative therapies for a disease is an extremely favorable possibility for future research. The study of synbiotics is a territory that has been little explored but is gaining attention. For the extracellular process within the microflora in the gut, *Escherichia coli* ferments the extracellular glucose of the bacterial cells into short-chain fatty acid (SCFA) molecules as butyrate and into long-chain fatty acids

(LCFAs). Butyrate can be administered as a therapeutic medicine because of it acts as a histone deacetylase inhibitor, produces ATP, and as a ligand that binds G protein-coupled receptors [2]. Intestinal bacteria use enzymes to split carbohydrates with water, producing hydrogen, methane, carbon dioxide, acetate, propionate, butyrate, and lactate. The end products from bacterial fermentation engender energy for the colonic bacteria. A high fiber diet activates fermentation, which amplifies bacterial density and fecal mass, increasing the viscosity of the stool. Fermentation generates approximately 30 grams of bacteria for every 100 grams of carbohydrate [3]. *Escherichia coli* produces and secretes an enzyme called acetyl-CoA carboxylase, which metabolizes glucose into butyrate, acetate, and propionate [4].

The fermentation of glucose by *Escherichia coli* leads to SCFAs binding to GPCRs that increase or decrease cell-to-cell signaling [5]. According to Hur Vi. Lee et al. (2015), SCFAs are also subunits of signaling molecules. The GPCRs called the free fatty acid receptor 2 (FFAR2, GPR43) and FFAR3 (GPR41) are receptors for SCFAs [6]. Acetate stimulates FFAR2 in vitro; propionate presents similar traits of binding to a receptor and triggering a downward cascade of responses on FFAR2 and FFAR3; butyrate only activates FFAR3 [7]. The SCFA receptors FFAR2 and FFAR3 function in the intestine, regulating the symbiotic interaction with adjacent enteroendocrine cells of the GI tract, which express the peptide YY (PYY). An FFAR3 deficiency in mice was associated with the reduced involvement of bacteria from the microflora, which allowed an increased expression of PYY in the plasma (8).

Therefore, the metabolic processes of bacteria may provide much potential for generating and designing novel therapeutics as antibiotics. For example, the cell metabolism within *Escherichia coli* cells induces the expression of the four genes, accA, accB, accC, and accD, which code for the translation of acetyl-CoA carboxylase. The four genes for acetyl-CoA

carboxylase modify the composition of fatty acids and monitor the rate of production. The four genes also monitor the overexpression of ACCase by inserting one of the four subunits, activating antisense RNA expression [9]. The acetyl-CoA carboxylase hydrolyzes glucose into SCFAs and catalyzes the biosynthesis of LCFAs. The SCFAs include propionate, acetate, and butyrate. The SCFAs are hydrophilic, soluble, and readily absorbed into the bloodstream. Many of the body's major organ systems such as the nervous system, skeletal-muscle system, and tissues catabolize acetate. Propionate decreases the liver's production of cholesterol through the liver's ability to efficiently decay and clear propionate, which blocks the synthesis of propionate. The SCFAs initiate apoptosis [3]. The fermentation of glucose that produces SCFAs also constrains the development of disease-causing organisms by decreasing the luminal and fecal pH [3]. The SCFAs lessen the pH by decreasing the expression of unfavorable bacterial enzymes, which reduce peptide degradation through the production of ammonia, amines, and phenolic compounds [3]. The long-chain fatty acids are synthesized after the acetyl-CoA transferase, the alpha subunit of ACCase or the ACCA subunit transfers a carboxyl group to acetyl-CoA, producing malonyl-CoA, which catalyzes the elongation of the LCFAs. The LCFAs become structured into the bacterial cell-matrix and the plasma membrane. The bacteria cells grow into large populations, forcing biofilm formation, which induces a quorum-sensing response.

Bacterial cells communicate through an intercellular process called quorum sensing. Quorum sensing is a type of communication between bacterial cells that occurs through the release of small signaling molecules called autoinducers. Releasing the autoinducers regulates the aggregation of cells and monitors genetic expression. Lux-S is a gene that induces the release of autoinducer-2, which is a homoserine lactone signal for quorum sensing. The autoinducers of gram-negative bacteria consist of homoserine lactones versus gram-positive bacteria that release

oligopeptides [7]. Amplified Lux-S expression increases the release of autoinducer-2, intensifies quorum sensing, and produces more biofilm for spreading pathogenic bacteria. An increased release of quorum-sensing signals produces more biofilm, which is needed for rapid motility and the spreading of infection. The production of biofilm induces resistance to antimicrobial peptides. Biofilm maintains an intensive resistance to antibiotics as multi-drug resistant pumps utilize biofilms for excreting antibiotics from bacteria. Mutations present in genes that are a part of the metabolic pathway cause bacteria to become more susceptible to antibiotic exposure. Therefore, targeting metabolic genes such as CbrAB, which regulate cell metabolism, connects cell metabolic processes with antibiotic resistance [10]. For example, inhibiting the gene called Crc lowers the growth rate of bacteria along with providing a higher susceptibility to antibiotics [10]. A mutation in the LasR gene, which generates quorum-sensing signals, also increases antibiotic resistance in *P. aeruginosa*. Mutations of metabolic genes in *S. aureus* upregulate glycolysis and fermentation, which removes the bacterial response of antibiotic resistance.

The three phases that occur after bacterial horizontal gene transfer include: 1) the antibiotic genes received during conjugation code for the creation of efflux pumps; 2) the antibiotic-resistant genes are enclosed within the pro-antibiotic genes, which impede antibiotic drugs attaching to the antibiotic gene target sites; and 3) the antibiotic-resistant genes can direct the ubiquitous destruction of antibiotic drugs through hydrolysis [11] . In a study by Peng et al. (2015), bacteria cultured without glucose or the non-polar amino acid of alanine were less susceptible to kanamycin, an antibiotic. Only 9.5 ng/mL of the kanamycin was absorbed; however, adding alanine and glucose to the cultures increased the uptake of kanamycin to 65-123 ng/mL and then to 113-231 ng/mL [12]. Yu Bin Shu et al. (2018) performed a Michaelis-Menten kinetics experiment using multi-drug efflux pumps while adding glutamate and acetate to

growths of *Escherichia coli* [13]. They confirmed that the *Escherichia coli* cells were more sensitive to gentamicin, an antibiotic. Therefore, a link between cell metabolism and bacterial virulence may exist. We do not completely understand the exact mechanism that connects cell metabolism with the bacterial pathogenesis that causes antibiotic resistance. The author suggests that blocking the enzymatic activity of acetyl-CoA carboxylase may represent a potential aim for eliminating antibiotic resistance.

For that reason, the effects of extracellular nutrient availability on bacterial gene expression were tested in this study. The concentration of glucose in LB broth media was altered, and then RNA was extracted to test the yield of the gene expression of *Escherichia coli*. To study the intracellular process, the gene expression of accA, a subunit of the acetyl-CoA carboxylase enzyme, was quantified via real-time PCR. The ACCase complex is an important enzyme for the first step in the fatty acid synthesis. The ACCA subunit, one of the four subunits, is a carboxyl transferase that transfers a carboxyl group to an acetyl-CoA, producing the malonyl-CoA. The malonyl-CoA initiates the lipid synthesis needed for constructing cell membranes and biofilm formation. The ACCase enzyme converts pyruvate to the fatty acids called butyrate, propionate, and acetate. To quantify the activity of ACCase, a part of the intracellular metabolism, the concentration of the gene accA was measured after exposure to glucose conjoined with antisense RNA inhibition.

To measure the effects of genetic inhibition of the intracellular metabolic processes, accA was suppressed and the RNA concentrations recorded. The original bacterial protocell constrains foreign and movable DNA through the transcription of antisense RNA, which is complementary to its specific DNA target sequence [14]. The asRNAs bind to the sequences flanking the ribosome-binding site and the start codon of the target mRNAs [15]. The antisense RNA blocks

the ribosomal detection by the RBS inhibiting translation [15]. In this study, antisense RNA obtains amplification through the PCR of an antisense DNA sequence. The DNA sequence converts to an antisense sequence when the DNA is flanked with Xho1 and Nco1 restriction sites. The PCR product was implanted into the PHN1257 plasmid through ligation (Fig 1). In addition, *Escherichia coli* cells were converted into antisense expressing bacteria after transforming the competent bacteria cells with the recombinant IPTG-PT-asRNA plasmid called PHN1257. RNA extraction isolated the total RNA from the transformed cells, and the RNA was reverse transcribed into the cDNA for Real-Time PCR analysis to determine the number of gene copies for accA.

Fig. 1 Plasmid Assembly and Gene Silencing of accA

Plasmid Assembly and Gene Silencing the PCR products and the PTasRNA expression vector of the plasmid PHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI (downstream). After the restriction digestion, the antisense PCR product was ligated into the IPTG-PTasRNA inducible vector of PHN1257 at the multiple cloning site.

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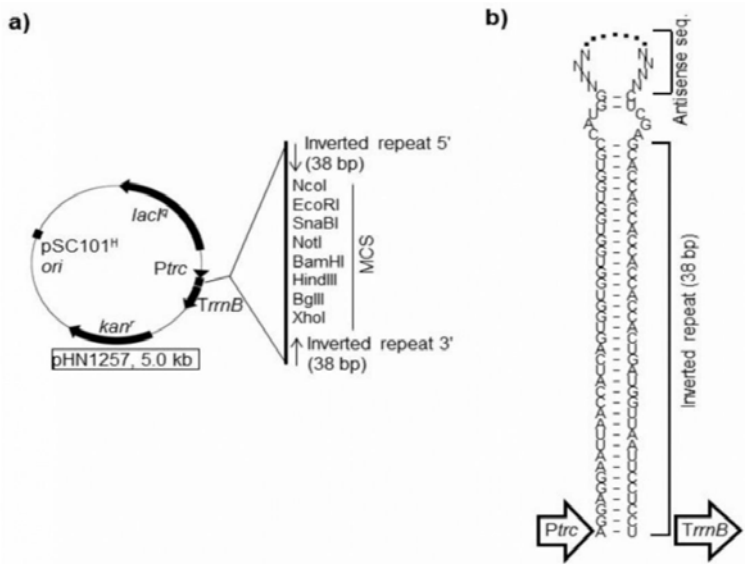


The microflora can communicate with the host cells of the gastrointestinal tract through a variety of chemical idioms [16]. A group of chemical molecules for bacterial language expression and transfer includes homoserine lactones, which are the most heavily studied. When a bacterial infection occurs, homoserine lactones form and interrelate with the immune system [17]. In addition, suppressing the accA gene can model the mechanism of the pathogenic bacteria repression of gene transcription based on the availability of nutrients and any other physiological change [18]. In order to measure the effects of extracellular and intracellular factors upon the intercellular communication between cells, the gene copy number of Lux-S will be quantified. Lux-S, an important gene for synthesizing and releasing autoinducers. Lux-S is required for quorum sensing, biofilm formation, and virulence. To test the correlation between glucose, accA, and Lux-S, the concentration of glucose was decreased while inhibiting accA. The author hypothesizes that inhibiting accA while lessening glucose availability will decrease the Lux-S gene copy number as quantified through qPCR. The author expects to determine an integration between extracellular glucose, intracellular metabolic gene expression, the effect of the two preceding stages of metabolism on intercellular cell-to-cell signaling, and pathogenesis. The purpose of the study is to demonstrate the immense potential of targeting the metabolic gene, accA, for combating antibiotic resistance.

MATERIALS AND METHODS

BACTERIAL CELL CULTURE AND RNA EXTRACTION

Escherichia coli cells grown on 25 mL Luria broth agar media plates enhanced with glucose concentrations of 200µM, 50µM, and 0µM were used for measuring the dsDNA and the RNA OD260 concentration. Varied concentrations of glucose including, 15mM, 7.5mM, 5mM, and control of glucose were added to LB liquid with *Escherichia coli*. Transformed bacterial

competent cells with (+antisense) and (-antisense) PTasRNA expression vectors were cultured with LB agar plates with the kanamycin antibiotic and incubated at 37°C for 24 hrs. Cells grown on agar plates were inoculated into 4 mL of LB liquid media with (+) and (-) PTasRNA bacterial cells expression of asRNA for accA cultured with kanamycin. Up to 3 mL of bacterial cell culture from the cells grown in the varying concentrations of glucose, the (+) asRNA, and the (-) asRNA cells were centrifuged at 4,000 x g for 10 minutes at 4°C. The medium was discarded, and the cells were resuspended in 100µL of lysozyme/TE buffer. The solution was vortexed for 30 seconds. Incubation occurred at 30°C for 10 minutes in a shaker-incubator. A lysis buffer of 350µL with 25mg of glass beads was added. The lysates were centrifuged for 5 minutes at maximum speed. RNA was extracted using HiBind RNA mini columns through RNA Wash Buffers I and II. The RNA was eluted with 50µL of DEPC water.

CDNA PREPARATION

The author used the OneScript Reverse Transcriptase cDNA Synthesis kit by Applied Biological Materials Inc. or ABM. Approximately, 1µg of RNA from each sample, high glucose, medium glucose, low glucose, and (-) glucose were added to 0.5µM oligonucleotides(dT) (10µM). For the asRNA and (-) RNA, 1 microgram of each total RNA extract was added to the initial primer/RNA and reaction mixture. The cDNA was also prepared for Lux-S quantification. The initial primer/RNA mix consisted of 1µg of total RNA, 1µL of oligo(dt), 1µL of dNTP (10mM) mix, and nuclease-free water for a 20µL reaction solution. The mixture was heated to 65°C for 5 minutes and then incubated on ice for 1 minute. The reaction mixture of 4µL 5X RT buffer, 0.5µL of RNaseOFF, and 1µL OneScript RTase was added to the initial primer/RNA mix in a 2µL microcentrifuge tube. The cDNA was synthesized by incubating the tube for 50 minutes

at 42°C. The reaction was stopped at 85°C for 5 minutes and chilled on ice. The cDNA was stored at -20°C.

PCR

A Promega PCR MasterMix volume of 25µL was added to the upstream and downstream primers each of 0.5µL specific for the accA gene target. The concentrations of the two cDNA samples added to the PCR master mix were 190ng and 230ng plus nuclease-free water. A 2X and 1X upstream primer, 10µM 0.5–5.0µl0.1–1.0µM, and a downstream primer, 10µM 0.5–5.0µl 0.1–1.0µM, DNA template 1–5µl less than 250ng, and nuclease-free water were mixed into a 50µl PCR reaction mixture. The thermocycler was set to denaturation of a 2-minute initial denaturation step at 95°C. Other subsequent denaturation steps were between 30 seconds and 1 minute. The annealing step was optimized with annealing conditions by performing the reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature in increments of 1°C to the annealing temperature. The annealing step was set at 30 seconds to 1 minute in 52°C. For the extension reaction with Taq polymerase, there was 1 minute allowed for DNA to be amplified at 72°C with a final extension of 5 minutes at 72–74°C. The PCR thermocycler completed 40 cycles of amplification instead of 35 cycles.

AGAROSE GEL ELECTROPHORESIS AND PLASMID ASSEMBLY

The condition of the PCR products, without primer dimers and contamination, for the target gene accA, were detected through agarose gel electrophoresis. The PCR products were confirmed without primer dimers and contamination, the PCR products, and the PTasRNA expression vector of the plasmid PHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI (downstream) (New England Biolabs XhoI- catalog R0146S- 1,000 units

and NcoI- catalog R0193S- 1,000 units) (Fig, 1). Each microcentrifuge tube was placed in the incubator for 30 minutes at 37°C. A heat block was heated to 90°C. The PCR products of the accA gene were ligated into the PHN1257 plasmid by mixing 1µL of the DNA insert with 2µL of the plasmids, adding 5µL of ligation mix, and then placing the tubes into a heat block of 90°C for 15 minutes (Takara Ligation Kit 6023). Competent bacterial cells were transformed with the PHN1257plasmid plus the antisense DNA insert of accA, for expressing (+) asRNA and (-) asRNA, which is the control. The competent cells were incubated with the recombinant DNA for 45 minutes at 37oC.

REAL-TIME PCR

The author enhanced each bacterial sample with the glucose concentrations of 15mM, 7.5mM, 5mM, and 0mM. The cDNA from H-glucose, M-Glucose, L-glucose, and the control (-) glucose were diluted into a 1:8 serial dilution. A 10-fold dilution of standards was prepared for bacterial samples transformed with (+) asRNA inducible plasmids and exposed to high to a low volume of glucose. The amount of gene expression for accA and the target gene Lux-S were measured through absolute quantification methods. The diluted cDNA was pipetted into a clear 96 well plate. A 0.4µl of each primer pair mixture was pipetted into a microcentrifuge tube with 10µL of SensiMix SYBR MasterMix, and with 1.2µL of DEPC water. The SYBR reaction mixture of 12µL was then pipetted into the wells with the diluted and undiluted cDNA. Optical film sealed the plate. To reposition all liquids to the bottom of the wells, the PCR plates were centrifuged for 2 minutes at 2500 rpm. The real-time PCR Amplification Protocol (Roche Lightcycler 480 machine) began with a 3 min step at 95°C for 40 cycles with a step of 10 sec at 95°C, 45 sec at 65°C, and 20 sec at 78°C with one step of 1 min at 95°C. One step of 1 min at

55°C for 10s each started at 55°C with a 0.5°C increment at each step up to 95°C. For the Roche Lightcycler 480 machine, the 96-well clear Roche plates were used for SYBR green detection.

THE qPCR ABSOLUTE QUANTIFICATION OF LUX-S

Bacterial cells were transformed with the PHN1257 plasmid expressing asRNA to inhibit the genetic expression of the accA gene. The author added 25µM and 5µM concentrations of glucose to the bacterial samples expressing antisense RNA for the inhibition of the gene accA. RNA was extracted from cells grown with 25µM glucose and 5µM of glucose. The cells grown with glucose in the medium were also transformed with antisense expressing IPTG-PT-asRNA inducible PHN1257plasmids. Cells were cultured without (-) glucose but the expression of accA was also inhibited, (+) asRNA. A control, without (-) glucose and (-) asRNA, was compared to each sample. The number of gene copies for Lux-S were measured through the qPCR amplification protocol and absolute quantification (Roche Lightcycler 480 machine).

RESULTS AND DISCUSSION

THE EFFECT OF GLUCOSE ON THE RNA CONCENTRATION AND THE EXPRESSION OF ACCA

Hydrolytic enzymes of *Escherichia coli* split one glucose molecule into two pyruvates. The acetyl-CoA carboxylase enzyme transfers a carboxyl group from the pyruvate to the acetyl-CoA, producing malonyl-CoA. The acetyl-CoA carboxylase enzyme consists of four subunits combined into the AccABCD complex. The accA gene codes for the ACCA subunit, which is the transferase domain of the ACCase enzyme. From the results, the genetic expression of accA was proportional to the increased glucose concentrations in *Escherichia coli* bacterial samples.

After culturing *Escherichia coli* with different levels of glucose, the RNA concentrations for each sample measured were 627 ng/µL for high-glucose, 452.88 ng/µL for medium-glucose, and 361.72 ng/µL for the low-glucose sample. A production of 1392, 797, 608, and 199 ng/ µL for the H-Glucose, M-Glucose, the L-Glucose, and the control, respectively (n=6, 1010±540). The control for replicate sample yielded 179 and 137ng/ µL (n=2, 158±29). The genes of accA and accD are entirely independent of the accBC lac operon.

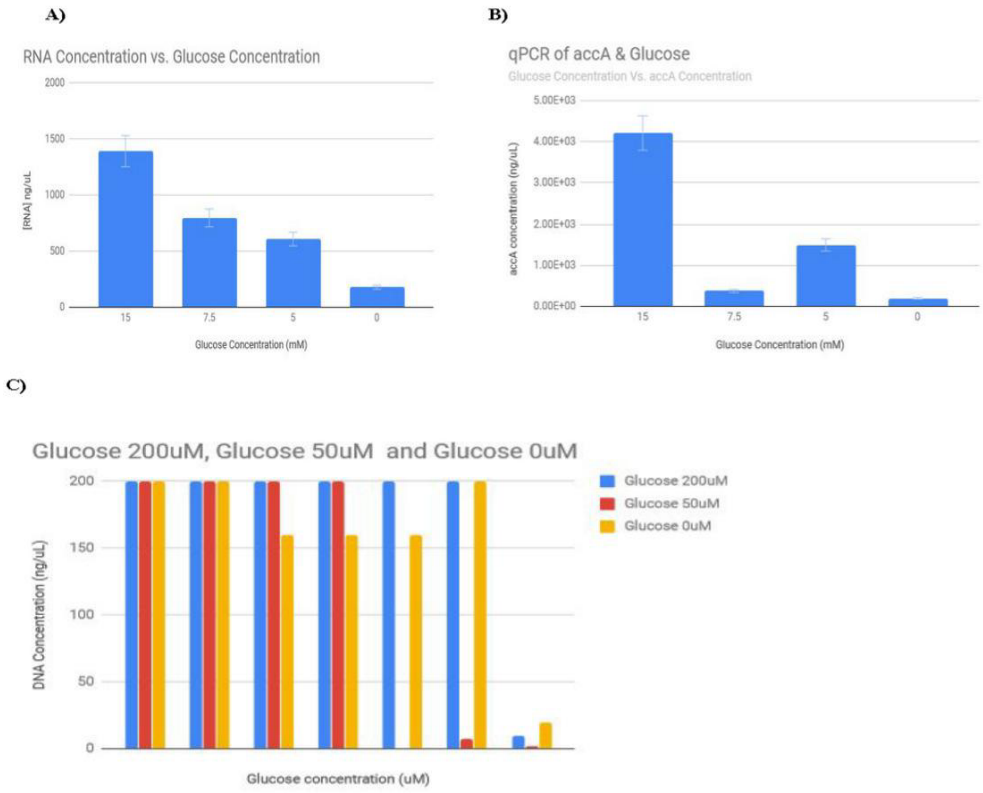
When accB is overexpressed, accBC transcription is blocked, but the overexpression of the other gene products, accA and accD, don't affect the accBC operon transcript levels [21]. A high level of glucose increases the availability of accB, and the transcription of the accBC lac operon is greatly reduced even after exposure to a small amount of accB. Although a high glucose concentration can stymie the production of accBC, the over-activated accBC lac operon did not inhibit the production of accA, the target gene of study. The subunit ACCA encoded in the accA gene may act as a more intense target for blocking bacterial cell fatty acid synthesis and metabolism. The *Escherichia coli* MG1655 bacteria cells were grown in LB broth overnight at 37°C with 15mM (High-glucose), 7.5mM (Medium-glucose), 5mM (Low-glucose), and a control of glucose concentration (Fig 2). RNA was extracted from each sample and the concentrations determined by the Implen Nanophotometer 250. The high-glucose sample produced the highest amount of RNA compared to the medium, the low, and to the control. The RNA for each sample was reverse transcribed into first-strand cDNA and absolute quantification with qPCR measured the amount of the target gene, accA, of each sample.

Fig 2 Glucose concentration versus RNA

A) Glucose concentrations of 15mM, 7.5mM, 5mM, and the control samples each yielded RNA concentrations, in ng/uL, 1392, 797, 608, 179, respectively. A) The bacterial sample with 15mM

glucose yielded the largest measure of RNA, noting a proportional relation between glucose and genetic expression of *E. coli*.

B) Includes qPCR results for 15mM, 7.5mM, 5mM, and the control. The samples with 15mM and 5mM glucose displayed the most genetic activity of accA transcription, measuring accA concentrations of 4,210 ng/uL and 1,500 ng/uL respectively. The accA qPCR concentrations for 7.5mM accA equaled 372 ng/uL and a 196 ng/uL quantity for the control. C) Displays the OD260 measure of DNA results for samples of *E. coli* grown in medium enhanced with 200uM, 50uM, 0mM of glucose.



The RNA extractions were repeated (n=20, 335.4 ± 329.2). The high-glucose sample provided a crossing point value or a Cp-value of 12.28 and the concentration of accA was 4.21E3 ng/µL. The Cp of the sample of medium-glucose equaled 16.51 with a concentration of 3.75E2

and the low-glucose Cp was 14.08 with a target gene concentration of 1.50E3 (Fig 4). The concentration of accA by qPCR analysis, in ng/µL, included: 4210, 375, 1500, and 196 for H-glucose, M-glucose, L-glucose, and for the control, respectively. The number of qPCR products ranged from 11-186, 5-18, and 0-79 gene copies for High-glucose, Medium-glucose, and the Low-glucose/control, respectively (n=12, 48±38). The control group had a Cp of 17.64 with a target gene concentration of 1.96E2. For the glucose concentrations of 200µM (n=7,173±72), 50 µM (n=7,116±105), and the control (n=7, 157±64) compared to their dsDNA concentrations showed significant variability (Fig 2). Comparing 200 µM to its control group, the difference between the samples was statistically significant (p=0.038). The cells grown in 200µM yielded a more significant and larger production of dsDNA than cells cultured as a control. After loading a 96-well plate for qPCR with 8-fold dilution standards of each sample, the standards of each sample were used to calculate a standard curve (Fig 2).

Vinals et al., (1999), found results that determined that a high glucose concentration causes the absorption of glucose to be blocked as a result of its repression of glucose phosphorylation [19]. Ferraris et al. (1990), measured the luminal glucose osmolarity and the concentrations in the small intestines. They found that the luminal glucose concentrations in a range of 50mM to 500mM exhibited errors [20]. The previous researchers measured glucose concentrations but did not recognize how osmolality is affected by Na+ and K+ salts, amino acids, and peptides [20]. Ferrais et al. (1990), discovered the average of SI luminal osmolarities were approximately 100 mosmol/kg, which were mainly hypertonic results. In the Ferrais et al. (1990) study, for an animal's diet, the SI glucose concentrations averaged 0.4-24 mM and ranged with time over a large amount of an SI region from 0.2 to 48 mM. They also found that high concentrations of glucose lessened the absorption of glucose in endothelial cells called ECV304

cell lines. There was a 60 percent reduction of 2-deoxyglucose uptake at 30 mM glucose from their study [20]. The high glucose osmolality resulted in a reduced Vmax value for 2-deoxyglucose uptake with a constant Km, calculated from the Michaelis-Menten kinetic enzyme equation [20].

THE QPCR RESULTS OF THE ANTISENSE RNA INHIBITON OF THE TARGET GENE ACCA

Ninety percent of the genome for Eukaryotes consists of non-coding regions or RNA that will not translate into proteins. Antisense transcription is the purpose of the noncoding regions of human cell DNA [22]. The non-coding transcripts are called natural antisense RNAs. Antisense activity has been recorded in mice, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Arabidopsis thaliana* [23]. Recombinant DNA was produced when the PCR product of the gene insert, accA, was ligated into the plasmid PHN1257 (Fig. 1), which was engineered to amplify antisense RNA. The PCR product and the IPTG-PT-asRNAs plasmid of PHN1257 were cut with the restriction enzymes XhoI and NcoI. The primers (Table. 1) each contained an extra 3 to 4 bps of nucleic acids to accompany the sequences for Xhol and Nco1. The primers flanked the target DNA, which totaled to 150 base pairs. The antisense sequence was constructed in a specific orientation in that the restriction sites, which NcoI normally flanks the forward primer, reversed positions. The XhoI was repositioned to flank the forward primer, and the Nco1 flanked the reverse primer, creating the antisense sequence when inserted into the plasmid for PHN1257. The PCR product was amplified (n=12, 219 ± 171), and then ligated into the PHN1257-asRNA inducible vector.

Table 1 Primers for amplifying the Lux-S gene, antisense RNA for accA, and the accA gene.

Primers	DNA Sequence
XhoI+accA Forward	GAGATGAGTCTGAATTTCCTTGATT
NcoI+accA Reverse	TGGCAGTTCATCGCTTTTTTCAC
Lux-S Forward	CATACCCTGGAACATCTGTTTGC
Lux-S Reverse	AGTTCCTGCACTTTCAGCACATC
accA Forward	TCATCACCTTTATCGACACCCC
accA Reverse	TTCACTTTATCGCCCACGCC

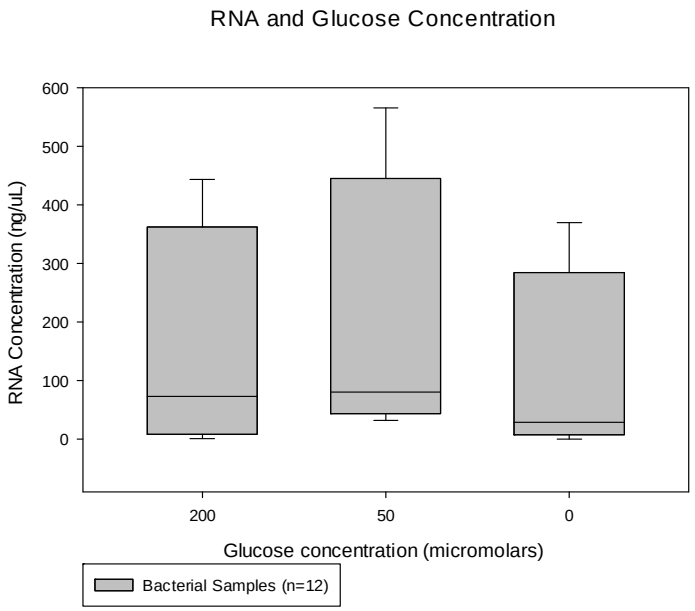
Primers for PCR Amplification and qPCR Analysis. To produce antisense RNA for accA inhibition the restriction enzyme site for XhoI was sequenced with the forward primer for accA. The restriction enzyme NcoI was added to the reverse primer for accA. Through PCR amplification, the PCR products were composed of antisense DNA for the accA gene. The PCR product would be inverted when inserted into the IPTG-PT-asRNA inducible PHN1257 plasmid, forming recombinant DNA plasmids for asRNA transcription in vitro. The plasmids with recombinant antisense DNA were transformed into bacterial competent cells for qPCR analysis of the accA gene. The primers for the Lux-S gene were applied to quantify the number of gene copies from qPCR analysis for cells grown in glucose enhanced samples. The primers for the accA gene were used for the quantification of the accA gene from the samples cultured with an increasing gradient of glucose solutions.

The total RNA concentration for the control, (-) asRNA, equaled 739.44 ng/µL. The control, (-) asRNA, yielded a miRNA concentration of 334.98 ng/µL (Fig 4). The RNA concentration for transformed bacterial cells with the asRNA of accA PHN1257plasmid was 279.28ng/µL. The miRNA concentration of the cells with asRNA measured to 240.90 ng/µL (Fig 3). The gene of accA was successfully suppressed by asRNA in vitro with 63 gene copies, qPCR

measured for bacteria cells (n=3) transformed with the recombinant antisense PHN1257 plasmid DNA (37.8 ± 35.6). The real-time PCR results for the bacterial cells without the antisense gene target and insert yielded 421.69 ng/µL for accA (Fig 4). There was a 147 % difference between cells not expressing asRNA versus cells transcribing the asRNA for accA (p=0.027). Inhibiting or lessening ACCase activity limits the synthesis of lipids, causing many physical changes. Antisense RNA expression can modify the consistency of fatty acids by upregulating the production of the ACCase enzyme [24].

Fig 3. RNA Concentrations vs. Glucose Concentration Fig 3. A) E. coli bacteria cultures (n=12) were enhanced with 200µM, 50µM, and a control of glucose (59±49). B) The 50µM of glucose in a bacterial sample highest yield was 566ng/µL, for 200uM 117ng/µL of RNA was produced, and the control measured to 76ng/µL of RNA.

A)



B)

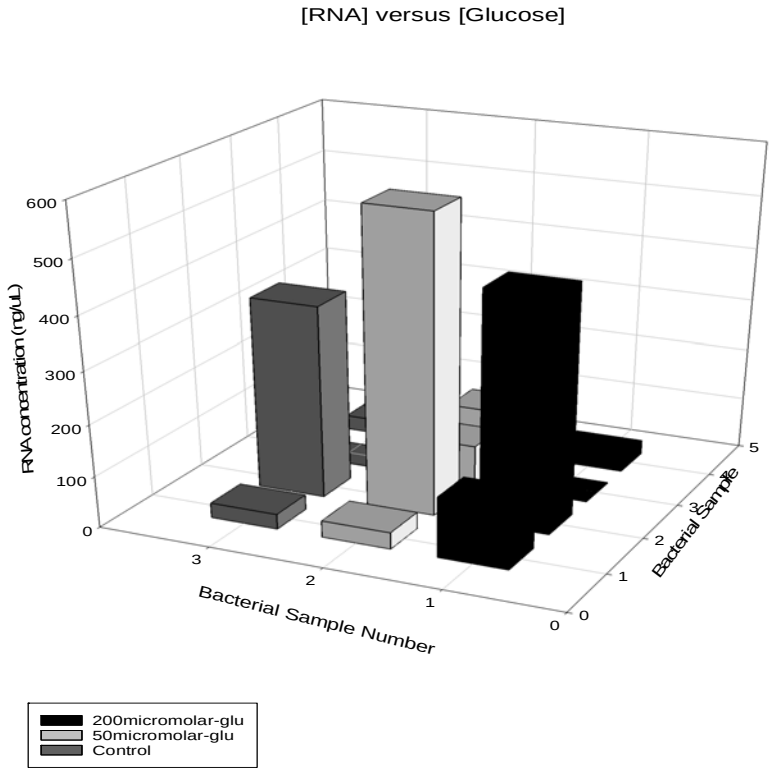
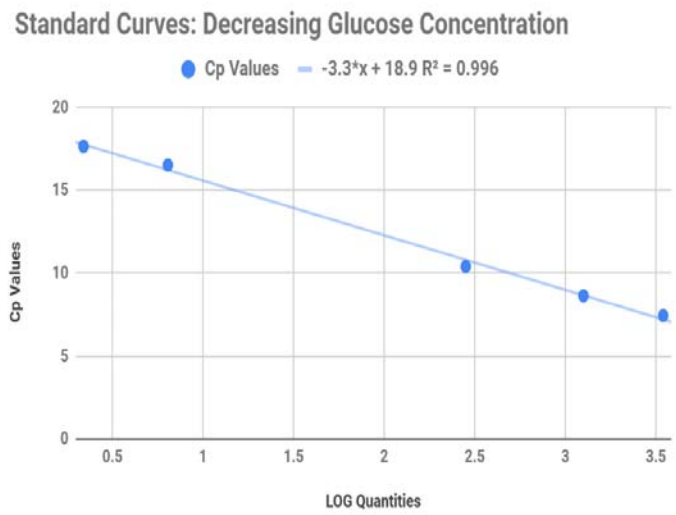


Fig 4 The qPCR results for quantifying accA concentration and the gene copy number. The RNA for each sample, 15mM, 7.5mM, 5mM, and the control, was reverse transcribed into first strand cDNA and absolute quantification with qPCR was used to measure the amount of the target gene, accA, produced by each sample. A) High glucose had a Cp of 12.28 and the concentration of accA was 4.21E3 ng/uL. The Cp of sample medium-glucose equaled 16.51 with a concentration of 3.75E2 and the low-glucose Cp was 14.08 with target gene concentration of 1.50E3. B) The gene of accA was successfully suppressed by asRNA in vitro with 63 gene copies measured for bacteria cells transformed with the recombinant antisense PHN1257 plasmid DNA. The bacterial cells with the PHN1257 plasmid but without the antisense gene target and insert produced 421.69 ng/uL for accA. There was a 147% percent di□erence between cells not expressing asRNA versus the cell transcription of the asRNA for accA (p=0.027).

A)



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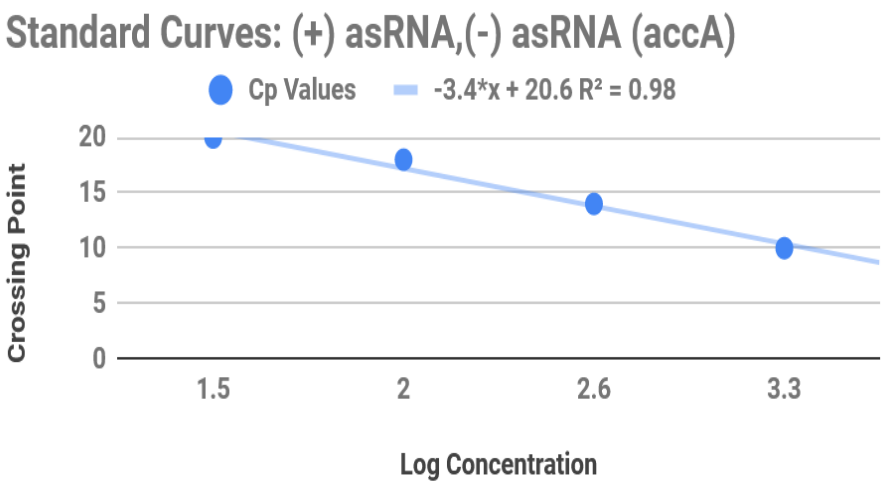


During short-chain fatty acid synthesis, malonyl-CoA is formed from acetyl-CoA, using energy from ATP and bicarbonate production [21]. Glucose is hydrolyzed into pyruvate, which is made into acetyl-CoA, forming acetate (Table.2). Through the Wood-Ljugdahl pathway, pyruvate loses two hydrogens, carbon dioxide loses oxygen, altered into carbon monoxide, and a methyl group is added to the oxidation, synthesizing acetyl-CoA into Acetate [25]. Butyrate is

produced when two molecules of acetyl-CoA combine into acetoacetyl-CoA. The acetoacetyl-CoA is transformed into butyryl-CoA, or butyrate [20]. Propionate is composed through the succinate or acrylate pathway [26]. The phosphoenolpyruvate, or PEP, is broken into pyruvate which is further metabolized with water into succinate. The succinate is reduced into propionyl-CoA, forming propionate (Table. 2). Through the acrylate pathway, lactate loses oxygen, forming propionate [25]. The LCFAs are synthesized when the ACCA subunit of ACCase transfers a carboxyl group to acetyl-CoA converting into malonyl-CoA, which then enters the fatty acid elongation cycle. The fatty acid cycle synthesizes long chains of fatty acids for the formation of the bacterial plasma membranes and a thin-layered matrix for initial bacterial attachment to a biotic or abiotic surface. After bacteria attach to a surface, more growth occurs until a microcolony of bacterial cells form. In addition, the gene accA codes for the subunit of the Acetyl-CoA Carboxylase complex. Acetyl-CoA carboxylase (ACC) initiates the first step of the fatty acid synthesis. An acyl-group or an ACP is added to the malonyl-CoA and converted into a ketoacyl-ACP. The Beta-ketoacyl-ACP is reduced, gains a hydrogen, into Beta-hydroxyacyl-ACP. The Beta-hydroxyacyl-ACP is dehydrated, losing a molecule of water, to alter into an Enoyl-ACP, which forms into an Acyl-ACP by gaining 2 hydrogen protons.

Table 2 Pathways for Biosynthesis of SCFAs from Carbohydrate Fermentation

Glucose	Glucose	Glucose
2 Pyruvates Lose a Hydrogen	2 Pyruvates Lose a Hydrogen	2 Pyruvates Lose 1 Hydrogen Gain 1 Hydrogen
Acetyl CoEnzyme A	Acetyl Coenzyme A	LACTATE Loses 1 Oxygen
B-hydroxybutyryl CoA Lose H2O	Phosphate Replaces The CoA	Creates Acryloyl-CoA

Crotonyl CoA Gains a Hydrogen	Creates Acetyl-Phosphate	Forms Propionyl-CoA
Butyryl CoA Phosphate Replaces The CoA	Phosphate added to ADP to make ATP	CoA in Propionyl-CoA Replaced with a Phosphate
Butyryl Phosphate formed The Phosphate moves to an ADP to form ATP **BUTYRATE**	**ACETATE**	Phosphate removed **PROPIONATE**

For synthesis of each SCFA, glucose is hydrolyzed into 2 molecules of pyruvate, which then lose a hydrogen, forming Acetyl Coenzyme A. For butyrate the Acetyl coenzyme A loses a water, forming B-hydroxy butyryl CoA, where it loses a hydrogen and the CoA is replaced with a Phosphate, the phosphate is removed. After the phosphate is removed, butyrate is formed. Acetate forms when the CoA in Acetyl Coenzyme A is replaced with a phosphate group, and then the phosphate is added to an ADP to make ATP. Propionate can be formed when lactate loses an oxygen, giving Acryloyl-CoA, converted into Propionyl-CoA, which the CoA is replaced with a phosphate, and then removed by a kinase, to produce propionate.

QUANTIFICATION OF LUX-S GENE EXPRESSION

The gene accA is a component of the ACCA subunit of the ACCase complex, which functions as a transferase. The ACCA subdomain of ACCase is vital for catalyzing fatty acid synthesis. Lipid biosynthesis is responsible for increased quorum sensing and biofilm formation. After glucose is hydrolyzed into two pyruvate molecules, the alpha subunit, ACCA, transfers a carboxyl group to acetyl-CoA to produce malonyl-CoA. The malonyl-CoA enters the lipid biosynthesis pathway. The fatty acids produced assist with secreting a more fluid membrane, stretching the span of the biofilm matrix. As the biofilm matrix expands the microcolonies of bacteria grow proportional to the size of the matrix. The increase in the number of colonies

induces the release of autoinducers, which accrue into a display of bioluminescence. The bioluminescence is a signal of quorum sensing. The gene Lux-S is needed to produce autoinducer-2. Lux-S monitors the amount of biofilm, flagellum movement, and regulates the factors of virulence. The ACCA subunit catalyzes the synthesis of lipids required for inducing the release of AI-2, which are signals produced during quorum sensing.

The high levels of autoinducers initiate a positive feedback loop switching all bacterial cells into producing quorum signals, which create bioluminescence [20]. As autoinducers compile in the surrounding environment of a bacterial cell, the bacterial population increases [5]. The bacteria sense the growth in the population and begin tracking the changes in the number of cells, which help to change the amount of genetic expression [28]. Quorum sensing between bacterial cells monitors the genes responsible for organizing the activities executed by different bacterial groups. Activities completed through quorum sensing include bioluminescence, spore formation, antibiotic excretion, creating a biofilm, and releasing factors for virulence [5]. Biofilm can increase the movement of pathogenic bacteria into the bloodstream [7].

Therefore, there is a link between bacterial cell metabolism and virulence. By quantifying the measurement of gene copies for Lux-S while inhibiting accA with asRNA, the connection of metabolism with pathogenesis could be demonstrated. *Escherichia coli* cells were grown in 25µM and 5µM of glucose and transformed with antisense PHN1257expressing IPTG-PT-asRNA plasmids. Cell samples with 5µM glucose- (+) asRNA produced a high measure of the Lux-S gene [11]. The gene copy number of Lux-S for (+) glucose/ (+) asRNA, and for (+) asRNA were quantified. For the results, the (-) glucose-(+) asRNA sample produced a gene copy number of 199 and 2511 Lux-S copies for (-) glucose- (-) asRNA (n=3, 277 ± 37). For the 25µM glucose-(+) asRNA and 5µM glucose (+), asRNA produced 39,810 and 316,228 gene copies of

Lux-S, respectively (Fig 4). The control samples, the (-) glucose-(-) asRNA, (n=3, 658,114 ± 483,499) and the 5µM glucose- (+) asRNA (n=3, 171571±204574) exhibited the highest amount of expression for Lux-S with a $p<0.05$ ($p=0.025$) (Fig 5-6). An ANOVA analysis of the qPCR gene copies for the Lux-S gene is displayed in Table 3.

Fig 5 Gene Copies for Lux-S determined through qPCR measurement

The qPCR Results for Lux-S The number of gene copies for Lux-S was measured through qPCR and absolute quantification A) (-) glucose-(+)asRNA sample had a gene copy number of 199. B) There were 2511 Lux-S copies for (-)glucose-(-)asRNA. The 5uM glucose-(+)asRNA yielded 316,228 gene copies of Lux-S D) For the 25µM glucose-(+)asRNA 39,810 gene copies were produced.

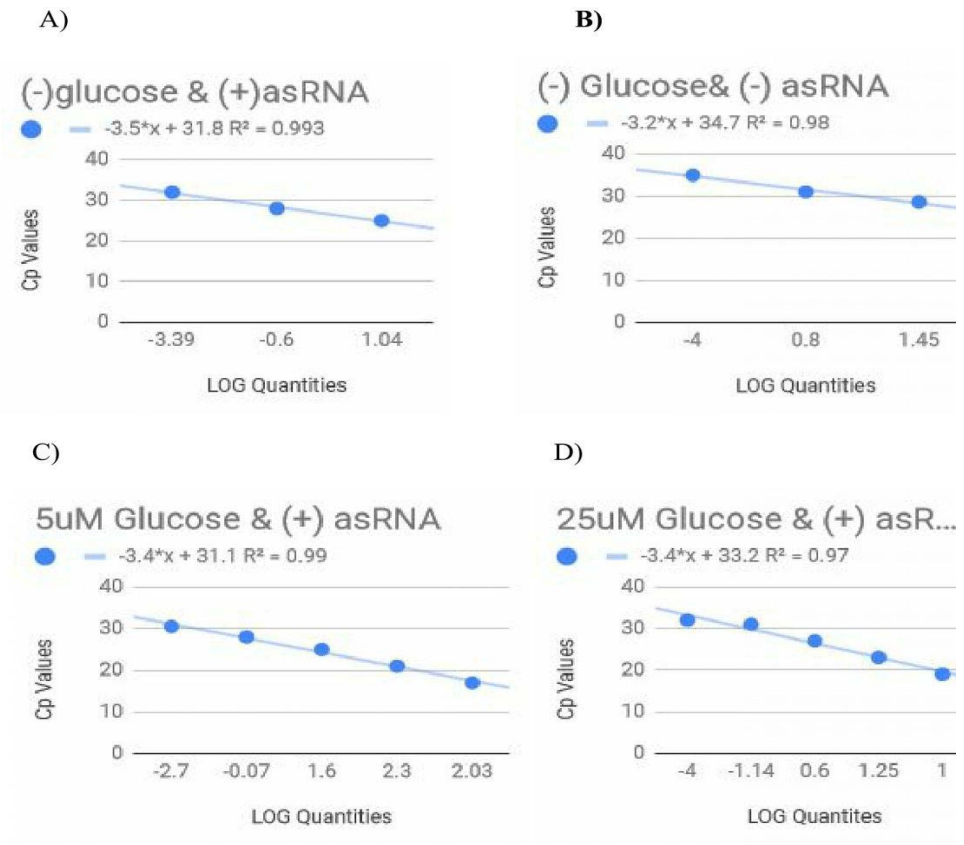
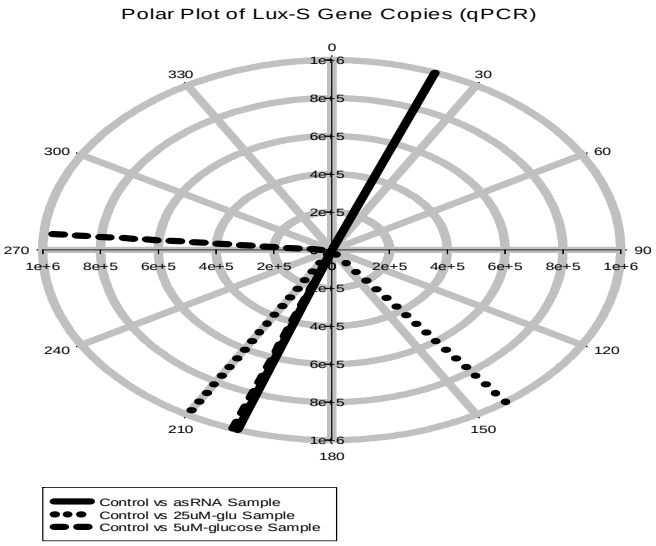


Figure 6 Total Production of Lux-S versus the Control Sample. The polar plot compares the control sample for the qPCR quantification of the gene Lux-S after exposure to glucose and antisense RNA in the form of the PHN1257-IPTG-asRNA expression plasmid. A) The control compared to the asRNA sample, to the 25micromolar-glu-asRNA sample, and versus the 5**µ**M-glu-asRNA sample equaled the following polar coordinates (radius, theta): ($1x10^6$, 180°), ($5x10^5$, 60°), and ($6x10^5$,68°), respectively. B) The control, 5, 25, and the asRNA micromolar samples production of the Lux-S gene are displayed in the 3D contour graph as an intense red, purple/blue for the 5micromolar-glu-asRNA, and purple for the asRNA including the 25micromolar-glu-asRNA sample. The control sample yielded a higher measure of Lux-S with $1x10^6$ gene copies (658,114 ± 483,499). The sample without glucose but with asRNA expression produced 199 gene copies of Lux-S (277 ± 37).

A)



B)

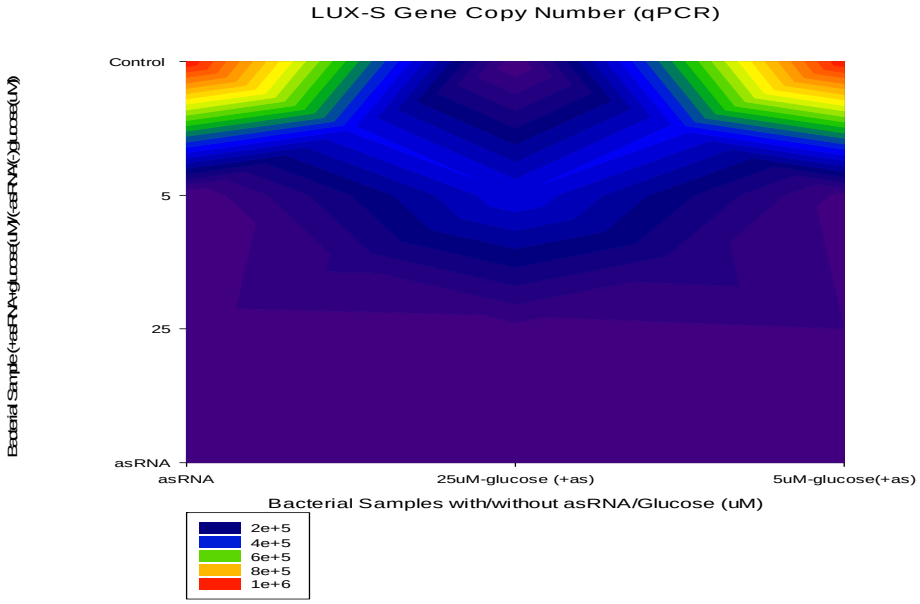


Table 3 ANOVA results for the total experimental sample for quantifying gene copies through qPCR.

ANOVA Results of qPCR Gene Copies

	Lux-S-glu/(+) asRNA	Lux-S-glu/(+) asRNA	(-)glu(-)asRNA	accA (-)asRNA (+)glu/ (-) glu	accA (+)asRNA (+)glu/ (-) glu
1	2511	316228	1000000	186	100
2	2000	26915.4	1000000	79	32
3	741	17783	39810	18	5
4	199	251	151286	7.4	3
n	4	4	4	4	4
X	1363.750	90294.350	547774.000	72.600	35.000
s	1074.010	151028.260	524165.009	81.920	45.306
X_{ave}	127907.940				
total	19	1798315304447.04			

		8			
source	**df**	**SS**	**MS**	**F**	**P-value**
treatments	**4**	**905636341810.108**	**226409085452.527**	**3.8044**	**0.0250**
error	**15**	**892678962636.940**	**59511930842.463**		
total	**19**	**1798315304447.04**			

The ANOVA results reveal an overall mean of 127907.940 for the total number of trials that yielded a total and a sum of gene copies through Real-Time PCR absolute quantification. The sample without glucose and without antisense RNA, (-)glucose-(-)asRNA, produced the highest amount of gene expression, for Lux-S and accA with 547774.000 as a mean of the total number of gene copies yielded from qPCR analysis (n=20, p=0.025).

The 5µM glucose- (+) asRNA sample expressed more of the Lux-S gene than the 25µM sample. Wang et al., (2005) found adding 0.8 percent glucose to their bacterial culture and growth medium increased the activity at the promoter site of the Lux-S gene. A glucose concentration of 5µM results in a normal exogenous osmolarity surrounding bacterial cells within the microflora in the gut of the lower digestive tract [24]. Because cells were overly saturated with a 25µM concentration of glucose, it was expected to increase the level of Lux-S gene expression. However, the excess saturation of glucose at 25µM did not result in the amplified gene expression of Lux-S. Jesudhasan et al. (2010) proved that higher levels of autoinducer-2 did not lead to the increased gene expression of Lux-S in *Salmonella typhimurium* when cultured in high concentrations of glucose [25] . Glucose affected the rate of transcription, increased the production of nucleic acids, but the inhibition of accA with asRNA significantly suppressed gene expression. From the control to the 25 µM glucose exposure, bacteria cells

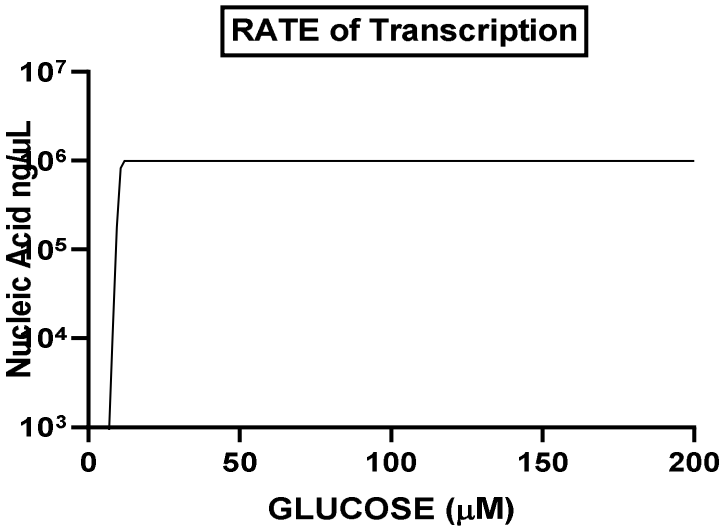
transcribed DNA at an amplified rate (Fig 7). As the glucose concentration increased to 50 µM the rate of transcription reached a sustained equilibrium with no new nucleic acid production or activity as the concentration approached 200 µM. The samples expressing asRNA inhibition of accA (n=4) produced 16% of the total yield of nucleic acid. Samples not expressing asRNA inhibition (n=20), yielded 83% of the total (n=24) quantity of nucleic acid.

Moreover, by transforming *Escherichia coli* bacterial cells with IPTG-PT-asRNA inducible vectors, which targeted the gene accA, the gene expression of Lux-S gene also decreased. Because glucose delays the movement of the Lux-S mutant strain with bacterial overpopulation but does not inhibit bacterial growth, each (+) glucose-(+) asRNA bacterial colony appeared smaller and defected. Osaki et al., (2006) showed that Lux-S mutants can be smaller in diameter with 8.0 mm versus 12.3 mm in its wildtype [29]. The bacterial colonies expressing antisense RNA (5mm) were a smaller size versus cells without antisense RNA transcription (8mm) of accA. The cell samples with (+) asRNA transcription were found by many preceding research studies to appear smaller in size [21]. Bacterial samples transformed with IPTG-PT-asRNA inducible PHN1257 plasmids and designed to inhibit the accA gene displayed the least measure of Lux-S expression. The (-) glucose- (+) asRNA sample exhibited the lowest amount of Lux-S expression of 199 gene copies (225 ± 37). As a result, when the Lux-S gene is mutated with less expression, the biofilm is thinner, looser, and displaying an appearance of bacterial cells with defects. Lux-S works to form a thicker and more viscous biofilm, which is a result of many nucleic acids released into the extracellular matrix of the biofilm.

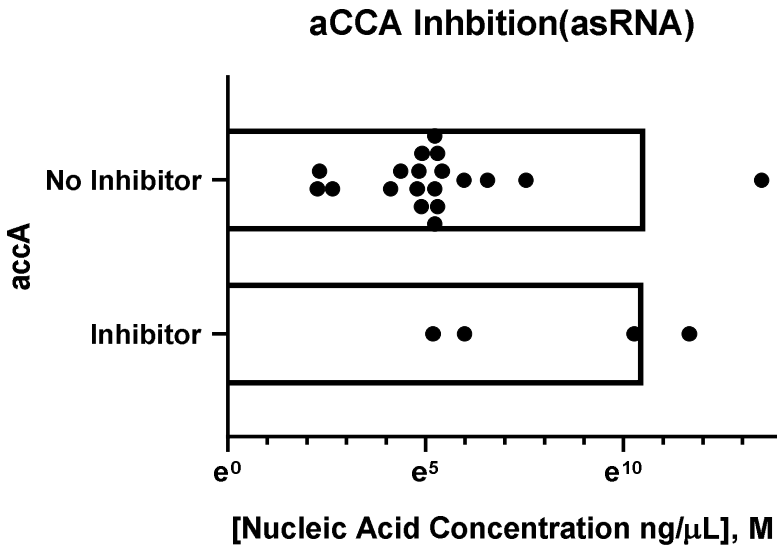
Figure 7 The effect of glucose on the rate of transcription, inhibition with asRNA, and the frequency of Nucleic Acid production. A) From the control to the 25 µM glucose exposure,

bacteria cells transcribed DNA at an amplified rate. As the glucose concentration increased to 50 the rate of transcription reached a sustained equilibrium with no new Nucleic Acid production or activity. B) The gene accA was inhibited with asRNA. The samples expressing asRNA inhibition of accA (n=4) produced 16% of the total yield of nucleic acid. Samples not expressing asRNA inhibition (n=20), yielded 83% of the total (n=24) quantity of nucleic acid.

A)



B)



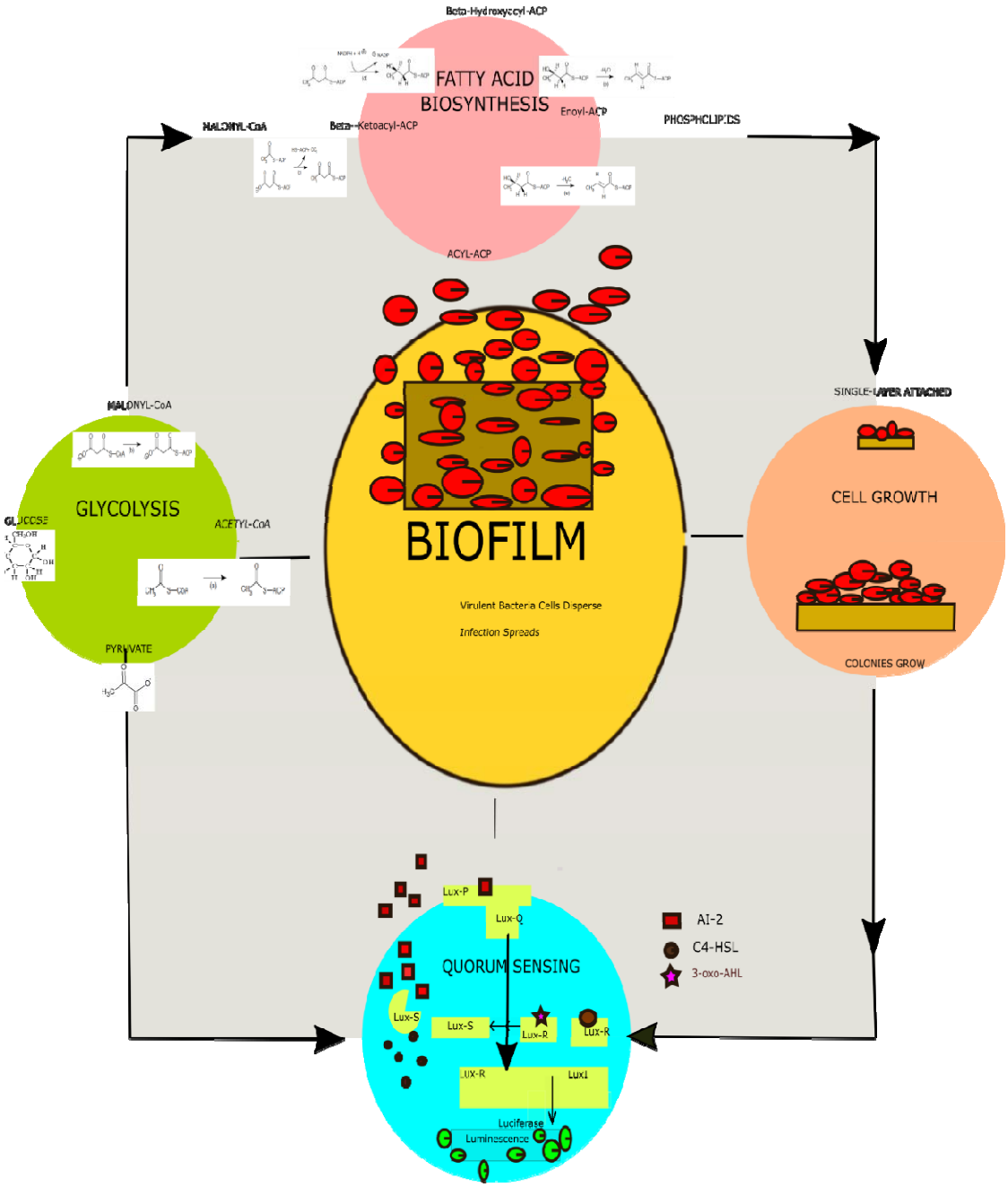
The ample amounts of DNA are released to maintain a more solid macromolecular matrix and consistency of the biofilm [30]. Lux-S is required for AI-2 production and for regulating gene expression in the early-log-growth phase. Pathogenic bacteria depend on biofilm formation to attach to epithelial cells and tissues, spreading infectious diseases. Pathogenic bacteria infect host cells by accumulating AI-2, exogenously, which then increases the amount and consistency of the formation of biofilm (31). The gene Lux-S codes for the flagella complex, biofilm creation, and controls virulent factors. The Lux-S gene, when it is more expressed, causes the spread of virulent bacteria through increasing the motility and movement of pathogenic bacteria. More Lux-S expression, as a result of mutations in the Lux-S gene, produces increased amounts of biofilm, assisting pathogenic bacteria to invade the mucosal layers and the epithelial cells of its host, developing into a systemic infection.

The autoinducer-2, which accumulates, monitors cell growth by permitting bacterial cells to sense the excess AI-2 and then terminate population growth. The excess AI-2 distributes quorum signals between bacterial cells, which notifies cells of limited nutrients, surface area, or space. An enzyme called an autoinducer synthase or Lux I produce a hormone class of acyl-homoserine lactones, AHL, which is the autoinducer 3OC6-homoserine lactone. The AHL binds to the autoinducer receptor called Lux-R for initiating a transcriptional activator of DNA [27]. After AHL is produced, it passes through the cell's plasma membrane as the number of bacterial cells increase, and then it stops diffusing after cell density reaches a threshold. When the AHL is attached to the LuxR complex, the operon is activated, transcribing the genes that code for the enzyme luciferase [27]. The LuxR-AHL bond releases, into the periphery of the cell, high levels of quorum sensing molecules that stimulate the cell to cell communication between bacteria species [24]. Autoinducer-2 requires the genetic expression and translation of Lux-S (Fig 8).

When Lux-S and autoinducer-2 are less regulated and overexpressed, infectious bacteria can propagate in stressful environments with high acidity and salinity [23]. Kendall et al., (2008) demonstrated the ability of *S. pyogenes* to adapt to acidic conditions as result of an unregulated Lux-S/AI-2 system [32].

Fig 8 Integrating the Metabolic Processes of *Escherichia coli* with Biofilm Formation a Major Virulent Factor

A glucose molecule is hydrolyzed into two pyruvate molecules. The acetyl-CoA Carboxyl transferase or the ACCA alpha subunit of the acetyl-CoA carboxylase complex, AccABCD, transfers a carboxyl group from the pyruvate to the acetyl-CoA to form malonyl-CoA. The malonyl-CoA enters the fatty acid synthesis pathway, elongating fatty acids into long-chain fatty acids of phospholipids necessary for structuring plasma membranes and for forming the initial extracellular matrix. The microcolonies attach, aggregate, and accumulate in the extracellular matrix. As the colonies accumulate into large populations a release of autoiducer-2 molecules is triggered, causing a certain bioluminescence through an exchange of cell- to-cell quorum sensing signals. The release of autoinducer-2 signals cells to increase population growth or to preempt it. The gene Lux-S is needed for releasing AI-2. The excess AI-2 distributes quorum signals between bacterial cells, which notifies cells of limited nutrients, surface area, or space. If bacterial cells continue to accrue, more biofilm is produced within the extracellular matrix, resulting in the dispersion of bacterial cells. Biofilm is a potent virulent factor. Pathogenic bacteria can disperse from and cause a systemic infection through excess biofilm production.



To form biofilm for infection, the Lux-S gene must be activated. Its excessive amplification and expression form a denser texture of biofilm [30]. Lux-S monitors virulence through regulation of the process for generating biofilm in the nasopharynx in mice with pneumonia, *Streptococcus pneumoniea*. Flagella expression increases with Lux-S mutation in the lungs and bloodstream. The Lux-S mutants can infect the lungs or the bloodstream more rapidly

than its wild-type strain [27]. Flagellin increases inflammation by activating Toll-Like Receptor Signaling or TLR5 pathways, which translates pro-inflammatory genes within the Mitogen-Activated Protein Kinase or the MAPK pathways. Flagella modulates virulence and pathogenesis by allowing more rapid motility of bacteria, infecting the colonization of host cells, and assisting infectious bacterial cells into entering the mucosal layers [33].

Pathogenic bacteria determine the level of autoinducer signals specific for their species. Pathogenic bacteria require a specific order and assortment of virulent genes to cause infection. For example, the different residues or R-groups of amino acids within autoinducers bind to the LUXR protein receptors in a conformation-specific to the amino acid side chains within the binding sites. The orientation of the AI to protein receptor binding sites produces varied types of side-chain lengths and amino acid substitutions [34]. For example, pathogenic bacteria as Enteropathogenic *Escherichia coli* EPEC release intraspecies-type of signals that help to colonize the small intestines because the small intestines are void of much commensal microflora. The Enterohemorrhagic *Escherichia coli* or EHEC pathogenic form of *Escherichia coli* conducts signals with other diverse bacterial cell types and with host cells. The EHEC communicates through quorum sensing with other colonic bacteria cells. The EHEC infect epithelial cells through activating the genes called the Locus of Enterocyte Effacement or LEE. The genes of LEE are required for A/E lesions to form when EHEC cells attach to and efface from epithelial cells, amplifying the level of pathogenicity [27].

When the production of Lux-S is blocked, the intensity of disease-causing bacteria changes. For example, the mutation of Lux-S in *Haemophilus influenzae* increases virulence that causes middle ear infections [35]. The *A. pleuropneumonia* and *M. haemolytica* bacteria have autoinducer mutations that lessen virulence in mouse models. When the Lux-S gene is inhibited,

there is a lesser amount of pustule-creation than the parental wild type strain [35]. However, autoinducer 3 is not dependent on the expression of the Lux-S gene. Lux-S genetic mutants impede the production of AI-3 (21-22). As a result, the pathogenic Escherichia coli, EHEC, collects signals from host cells in the form of hormones known as epinephrine. The AI-3 can communicate with the transmembrane protein of the host cell called Qsec [36]. The two components for quorum sensing also include Las and RhI, which are quorum systems with activator proteins that direct the synthesis of autoinducers. Las and RhI assist in turning genes on or off based on the density of bacterial populations, sensing the accumulation of autoinducers [37].

The bacteria called *P. aeruginosa* secretes two types of autoinducers called N-3-oxododecanoyl, a homoserine lactone, (3-oxo C12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL). These autoinducers released can cause virulence in people infected by *P. aeruginosa* by supplying virulent factors that help colonize and spread the infection [37]. Therefore, the results present significant data for the effect of glucose and genetic inhibition of accA on the production of the Lux-S gene, an important component of bacterial virulence. The data demonstrates that the genetic changes, as inhibiting the accA gene with antisense RNA, and the availability of nutrients such as glucose, affected the production of Lux-S. Inhibition of the Lux-S gene can reduce biofilm formation and decelerate the motility of bacteria, which the lessened motility can impede the spread of pathogenic bacteria.

CONCLUSION

The overall purpose of the study was to confirm and identify the gene accA as a possible target for blocking bacterial antibiotic resistance. Through examining the role of accA, a link between bacterial cell metabolism and virulence was attempted and demonstrated. A cumulative

perspective, integrating bacterial cell metabolism via the ACCA subunit with bacterial pathogenicity, may provide possible metabolic targets for designing new and potent antibiotics. It was previewed in this study, an ability to design antibiotics that target bacterial cell metabolic genes as accA, the binding sites of the ACCase enzyme, and the fatty acid synthesis pathway. Targeting all three areas of bacteria cell metabolism can reduce the formation of bacterial cell biofilm needed for spreading pathogenic bacteria. The strong condition of a bacterial population's metabolic state can prevent bacterial susceptibility to antibiotics [34]. The results of decreased RNA expression and cell growth in the presence of antisense RNA inhibition of accA may serve as a model for blocking microbial metabolic processes that contribute to antibiotic resistance [38].

Escherichia coli cells were cultured in high glucose to a control concentration and showed a decreasing range of gene expression for the target gene, accA. The observation of the bacterial cells, grown in an increased amount of available glucose, showed the highest measure of transcription for accA. The effect of glucose extracellular of the bacteria, on the concentration of RNA, was measured by modulating the glucose concentration gradient. The transcription of intracellular molecules as the accA gene was determined through qPCR. The accA gene was inhibited with antisense RNA, using an IPTG-inducible vector, PHN1257. There was a 138 percent difference of gene expression between cells transformed with antisense PHN1257 versus cells negative for antisense RNA during in vitro transcription. The recombinant DNA and plasmids were assembled from the IPTG-PT-asRNA of the PHN1257 (Fig 1). The constructed plasmid of PHN257 consisted of flanking inverted repeats for the target DNA, rebuilding a paired double-stranded RNA terminus that inhibited the transcription of accA. The inhibition of accA was confirmed through the qPCR results of 63 gene copies for accA (+) asRNA versus 422

gene copies for accA with (-) asRNA. The sum volume of Lux-S was determined through qPCR. By increasing the glucose concentration in increments, Wang et al. (2005) found that glucose increased the levels of autoinducer-2 of the culture as well [39]. The observations displayed the RNA concentration for bacterial cells, expressing asRNA of accA, included a concentration of 279.28 ng/µL. The miRNA concentration of the cells with asRNA measured to 240.90 ng/µL.

The results supported the hypothesis. Decreasing the glucose concentration, while suppressing the accA gene, led to an observed decrease in Lux-S expression. The author hypothesized that decreasing the sum of glucose and suppressing accA gene expression would decrease the expression of the Lux-S gene. Lux-S is an important gene for quorum sensing and releasing autoinducer-2. Increased expression of Lux-S produces more AI-2, which induces and initiates quorum sensing between bacterial cells. The increased AI-2 molecules released triggers quorum sensing for the amplified production of biofilm. Biofilm formation allows more pathogenic bacteria cells to attach, proliferate, detach, and spread into a systemic infection. The accA gene translated into the acetyl-CoA carboxyl transferase enzyme transfers a carboxyl group from pyruvate to the acetyl-CoA to form a malonyl-CoA. More glucose induces more production of the acetyl-CoA carboxyl transferase. Increased glucose also causes more AI-2 quorum-sensing signal molecules to accumulate. The malonyl-CoA enzyme is important for catalyzing the biosynthesis of long-chain fatty acids. The long-chain fatty acids are the building blocks for biofilm formation. Therefore, a decrease of biofilm production can occur due to the limited enzymatic activity of the ACCA transferase subunit via accA gene inhibition.

However, in the presence of glucose with antisense RNA transcription, *Escherichia coli* cells continued to produce large amounts of Lux-S with 316,228 copies measured through qPCR when enhanced with 5µM of glucose (n=3, 171571± 204574). The limitations of this study

include the contradictory results of the 5µM glucose-(+) asRNA sample for Lux-S. Adding a 5µM concentration of glucose to the LB broth culture increased the gene expression of Lux-S although the accA gene was inhibited. These contradictory and unexpected results may show early signs of reduced efficacy of the inhibition of the accA gene in the presence of glucose. However, it is probable that the 5uM of glucose triggered increased expression in the accD gene and of the ACCD subunit, which was not inhibited by asRNA. The gene accA can continue to act as a potent metabolic target for suppressing antibiotic resistance. This study suggests that designing antibiotics should include targeting metabolic genes and enzymes. During the past 30 years, only a few potent antibiotics have been formulated and discovered [40]. However, there is a new motivation for examining bacterial targets located in metabolic pathways. For future research, studying the gene expression, structure, and the function of the ACCase complex can increase the incentive for designing small-molecule inhibitors specific for the binding sites of bacterial-metabolic targets.

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