Integrating the extracellular, intracellular, and intercellular metabolic processes of *Escherichia coli* through glucose saturation, inhibition of the acetyl-CoA carboxylase subunit accA with asRNA, and through quantifying cell to cell quorum-sensing

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ABSTRACT

The study aims to demonstrate the link between bacterial cell metabolism and virulence through integrating the environmental, genetic, and cell to cell signaling molecular processes. Dietary fiber metabolized into glucose, increases the proliferation of intestinal microflora, which augments the outputof the Short Chain Fatty Acids. Bacteria ferment the glucose, from fiber, into Short Chain Fatty Acids, which help regulate many biochemical processes and pathways. Each SCFA maintains colonic pH, promotes cell differentiation, and the apoptosis of colonocytes. To model a high-fiber diet, increasing the synthesis of Acetyl-CoA carboxylase, an enzyme that catabolizes glucose into SCFAs, *Escherichia coli* was cultured in Luria Broth enhanced with a high to 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 equaled to 375 ng/µL, and the 0µM sample measured an accA concentration of 196 ng/µL. The gene accA, 1 of 4 subunits for the Acetyl-CoA Carboxylase enzyme, was suppressed by asRNA, producing a qPCR concentration of 63ng/µL. Antisense RNA for accA reduced the amount of Lux-S, a vital gene needed for propagating quorum-sensing signal molecules. The Lux-S gene, 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 increases biofilm production for spreading virulence. The further implications of the study propose designing antibiotics that target bacterial cell metabolic processes to block bacterial antibiotic resistance.

INTRODUCTION

The Western diet includes high amounts of fats, sugars, and simple carbohydrates. Due to the Western diet, diabetes, cancer, and many neurological disorders have proliferated and quickly increased the diagnosis of these diseases (1). Ingestion of dietary fiber in the U.S and in European diets is approximated to be more than a few grams per day. Non-digestible oligosaccharides give between 1 and 2 kcal/g of calories (1). A cause for the prevalence of diseases may be the Western diet that lacks a high source of dietary fiber. There are two main types of dietary fiber, soluble and insoluble fiber. Soluble fiber is found in fruits and vegetables while insoluble fiber includes wheat, cellulose, and inulin. Insoluble fiber is necessary because it maintains a healthy microflora in the gut of the gut by allowing waste in the colon to become bulky the facile removal of fecal matter from the colon. Insoluble fiber allows for the absorption of water to produce bowel movements more readily without blockage. Fiber regulates and promotes a healthy gut microflora in the gut. A healthy Microflora in the gut has many beneficial properties. A well-balanced microflora in the gut prevents colon cancer by the apoptosis of cancerous cells (6). Butyrate, a short chain fatty acid produced from bacterial fermentation of insoluble fiber, suppresses tumors because it obstructs cell propagation and induces apoptosis when added to different types of tumor cell lines (6). An important bacterium for maintaining homeostasis within the colon and digestive tract, includes Escherichia coli, a gram-negative bacterium that resides within the large intestines (6).

From dietary fiber, or undigested carbohydrates, SCFAS are produced and can catalyze hormonal signals within enteroendocrine cells, lining the digestive tract. Short Chain Fatty Acids can bind to G-protein coupled receptors, leading to a downward cascade of reactions within endocrine cells. For example, SCFAs bind to the G-protein coupled receptor, which then causes the secretion of the hormone Peptide Tyrosine. Peptide YY increases the rate of digestion and conserves the energy from the diet (21). Including more dietary fiber in the Western style diet is one of the best ways to restore balance and a healthy condition of the intestinal microflora in the gut. Human well-beings dependent upon a healthy gut microflora in the gut (3). Butyrate is the most significant metabolite because it provides energy for cells in the colon, prevents inflammation, controls genetic expression, differentiates, and eradicates cancerous cells (3).
Also, Bifidobacterium make up 1 to 3 percent of the population of bacteria that resides in the gut of adults (4). For infants who are breastfed, the Bifidobacterium presenting the gut is approximately 90 percent. The Bifidobacterium maintains homeostasis in the gut, regulates immune responses and blocks many cancer-causing activities (4).

Since 70 percent of the immune system resides in the gut and a healthy balance of E. Coli can improve insulin sensitivity, fight obesity, eradicate colon cancer cells, and reduce mental disorders as depression, the human gut may be a source of a conduit for treating many human diseases. For example, there has been found a tumor-inhibiting molecule released by a probiotic strain of bacteria. The L. case strain of ATCC 334 produces ferrochrome, which delays the metastasis of colon cancer by activating the c-Jun-terminal kinase pathway of apoptosis (27). Increasing production of Club, a chaperone protein secreted by *Escherichia coli*, reduced food intake, limited meal patterns, and stimulated intestinal hormones in mice as a glucagon-like peptide 1, an antihyperglycemic protein (27). 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 T-reg cells in rodents.

Inflammation of the airways due to allergies and inflammatory colitis have been shown to be remedied by interleukin-10, a cytokine expressed by the bacteria, *Lactococcus lactis* (27). Isolating microbial organisms with the possibility of providing alternative therapies for disease is extremely favorable for future research. Synbiotics is a territory minutely explored but is gaining noticeability. For the extracellular process within the gut's microflora in the gut, E. coli ferments the glucose outside of the bacterial cells into Short Chain Fatty Acid molecules of Butyrate. Butyrate is an attractive therapeutic molecule because of its wide array of biological functions, such as its ability to serve as a histone deacetylase (HDAC) inhibitor, an energy metabolite to produce ATP and a G protein-coupled receptor (GPCR) activator (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. Approximately, about 30 grams of bacteria are generated for every 100 grams of carbohydrate made through fermentation (1). *Escherichia coli* produces and secretes an enzyme, called acetyl-CoA carboxylase, metabolizing glucose into butyrate, acetate, and propionate (6). Identifying and integrating more parts of the GI tract are needed. One way to locate parts of the microflora in the gut specific for symbiotic study is through the live cell imaging of bone marrow cell lines

cultured with 3-oxo-C12-HSL. The 3-oxo-C12-HSL is a P. aeruginosa signal molecule called a homoserine lactone reported to inhibit the function of Peroxisome proliferator-activated receptor or PPARs in mammalian cells, combating lung disease (24). Bone marrow white blood cells, as macrophages can be incubated with synthetic 3-oxo-C12-HSL and C4-HSL, for 24 hours. The induced apoptotic activity of 3-oxo-C12-HSL can be demonstrated in neutrophils and monocytic cell lines U-937and P388D1, respectively (7). Cells treated with 3-oxo-C12-HSL can reveal morphological alterations indicative of apoptosis (3). A live cell image of bone marrow cells can display the real time apoptotic activity at the cell membrane level. Qualitative data of apoptosis can be assayed with live cell fluorescence microscopy. Bone marrow cells can be cultured with an assay called the passive REAL-TIME Apoptosis Fluorescent Microscopy Kit. The assay of apoptosis can give the real time data-analysis of apoptotic signaling at the cell surface (17).

Studying the intracellular factors for cell metabolism within the E. Coli cells, includes: the four genes, accA, accBaccC, and accD, which code for the translation of Acetyl-CoA carboxylase. These four genes for Acetyl-CoA carboxylase translation can modify the composition of fatty acids and monitor the rate of production. The four genes also monitor the over expression of ACCase with inserting one of the four subunits to activate antisense RNA expression (19). Acetyl-CoA carboxylase hydrolyzes glucose into Short Chain Fatty Acids including propionate, acetate, and butyrate. The SCFAs are hydrophilic, soluble, and the bloodstream readily absorbs each SCFA. Many of the body's major organ systems as the nervous system, skeletal-muscle system, and tissues catabolize acetate directly. Propionate decreases the liver's/14production of cholesterol through the liver's ability to efficiently decay and clear propionate, which blocks its synthesis. The Short Chain Fatty Acids initiate apoptosis (1). Fermentation of glucose to produce SCFAs also constrain the development of disease-causing organisms by decreasing luminal and fecal pH (1). By lessening the pH, the expression of unfavorable bacterial enzymes decreases due to reduced peptide degradation and by the production of ammonia, amines, and phenolic compounds (1).

Also, to analyze factors affecting how bacterial cells communicate through an intercellular process, the levels of quorum sensing can be measured and analyzed. Quorum sensing is communication between bacterial cells through a release of small signaling molecules called autoinducers. The autoinducers are released to regulate the aggregation of cells and 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 are oligopeptides (35). The microflora in the gut of bacterial cells can communicate with the gut, using a variety of chemical idioms, which, their host cells can detect (25). This study has considered the relevance for the link between an individual's overall health to bacterial cells' sensitivity to glucose, and the availability of metabolites. However, the author also focused on presenting evidence for the impact of effects from bacterial cells potential for detecting host signaling molecules upon the immune system (9). A group of chemical molecules for bacterial language expression and transfer includes homoserine lactones, which are the most heavily studied. When bacterial infection occurs, homoserine lactones are formed, and interrelate with the immune system (26).

When bacterial genetic expression of the genes accA, accBC, and accD are inhibited, the production of SCFAs is disrupted, delaying hormonal responses. The fermentation of glucose by Escherichia coli leads to SCFAs binding to G-coupled protein receptors that increase or decrease cell-to-cell signaling (12). Hormones affect the balance of flora within the microflora in the gut. According to Hur Vi. Linn et al. (2015), SCFAs are also subunits of signaling molecules. The G protein-coupled receptors called Free fatty acid receptor 2 (FFAR2, GPR43) and FFAR3 (GPR41) have been identified as receptors for SCFAs. Acetate stimulates FFAR2 in vitro; propionate presents similar traits of binding to a receptor and triggering downward cascade responses on FFAR2 and FFAR3; and butyrate only activates FFAR3 (26). The SCFA receptors FFAR2 and FFAR3 are both expressed in the intestine and maintain symbiosis with adjacent enteroendocrine cells in the mucosal lining of the digestive tract that express the Peptide YY (PYY). TheFFAR3 deficiency in mice was associated with a reduced involvement of bacteria from the microflora, which allowed an increased expression of PYY in the plasma (12).

To test the extracellular components a gradient of glucose concentrations was applied to monitor the *Escherichia coli* output of genetic expression through measuring RNA concentration. To study the intracellular process the gene expression of accA, a subunit of the Acetyl-CoA Carboxylase enzyme, was quantified via Real-Time PCR. ACCase is an important enzyme for the first step of fatty acid synthesis. The accA subunit, 1 of 4, is a carboxyl transferase that transfers a carbon dioxide to an acetyl-CoA, producing the malonyl-CoA. The malonyl-CoA catalyzes lipid synthesis needed for constructing cell membranes and biofilm formation. The ACCase enzyme also catalyzes the first step for converting pyruvate to the fatty acids as

butyrate, propionate, and acetate. To quantify the activity of ACCase, intracellularly, the concentration of the gene accA was measured after glucose exposure and antisense RNA inhibition.

By measuring the RNA concentration of accA after its suppression through antisense RNA, the author could better understand how genetic inhibition can affect the metabolic process within, intracellular, the bacterial cells of the microflora. For example, the original bacterial protocell could constrain foreign and movable DNA through transcription of antisense RNA, complementary to its specific DNA target sequence (18). The asRNAs bind to the sequences flanking the ribosome-binding site and the start codon of the target mRNAs (15). They block ribosomes from detecting the RBS, and therefore inhibit translation (15). In this study, antisense RNA was amplified through PCR of an antisense DNA sequence flanked with Xho1 and Nco1 restriction sites. The PCR product was ligated into the PHN1257 plasmid (Fig.1). Competent bacterial cells were transformed with the recombinant IPTG-PT-asRNA plasmid calledPHN1257. The total RNA was extracted, and reverse transcribed into cDNA for Real-Time PCR analysis to determine the number of gene copies for accA.

We wanted to study the accA gene to determine how glucose and genetic changes affect cell growth and gene expression. The genes accBC and accD cause increased amounts of regulation. If the gene accD is inhibited, the gene accA would be inhibited as well, giving obscured results. We focused on suppressing the accA gene to model how pathogenic bacteria in the gut repress genetic transcription of certain genes based on the availability of nutrients and any other physiological change (43). In order to measure the effects of extracellular and intracellular factors upon intercellular communication between cells, the amount of quorum sensing between *Escherichia coli* cells was quantified. We exposed the bacterial cells to varied concentrations of glucose and to antisense RNA suppression of the gene accA. After saturating bacterial cells in glucose media and suppressing the accA gene with asRNA, the number of gene copies of Lux-S, an important gene for synthesizing and releasing autoinducers, were quantified through real time PCR, or qPCR. The authors analyzed the effects of glucose and asRNA on the gene Lux-S to better understand the relation between environmental, genetic factors, and maintaining quorum sensing or communication between bacterial cells. Studying the effects of quorum sensing is beneficial because many bacterial cells use quorum sensing to regulate genetic expression in accord with their overall density in a location of a population of bacterial cells.

There was an observation of glucose increasing bacterial cell growth and gene expression. As a result, if the glucose concentration is increased while inhibiting the accA gene, we hypothesize that the qPCR quantification of Lux-S will increase as well. More Lux-S expression increases the release of AI-2, intensifies quorum sensing, and produces more biofilm for spreading pathogenic bacteria. More quorum sensing produces biofilm needed for increasing motility, spreading virulence. The purpose for the study is to demonstrate a link between cell metabolism and virulence. Through integrating bacterial nutrient availability, gene expression, and cell-to-cell interactions, possible methods to target and block antibiotic resistance can be adduced.

MATERIALS AND METHODS

BACTERIAL CELL CULTURE AND RNA EXTRACTION

Escherichia coli cells were grown on 25 mL Luria Broth agar media plates enhanced the glucose concentrations of 200µM, 50µM, and 0µM were used for measuring dsDNA concentration. 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 24hrs. 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. The E.Z.N.A.® Bacterial RNA Kit allowed for rapid and reliable isolation of high-quality total cellular RNA from a wide variety of bacterial species. Up to 3 mL of bacterial cell culture from the cells grown in the varied 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 cells 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. The lysis buffer of 350µL with 25mg of glass beads were added. It was 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. Up to 1 billion bacterial cells were processed. The system combined the reversible nucleic acid-binding properties of Omega Bio-Tek's HiBind® matrix with the speed and versatility of spin column technology to yield approximately 50-100 µg RNA.

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 was added to 0.5µM oligonucleotides(dT) (10µM). For the asRNA and (-) RNA, /14 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: 1µg of total RNA, 1µL of oligo(dt), 1µL of dNTP (10mM) mix, 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 2µLmicrocentrifuge 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 Master Mix volume of 25µL was added to upstream and downstream primers each of0.5µL 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 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 was mixed into a 50µl PCR reaction mixture. The thermocycler was set to a 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 was1 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 PHN1257were 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 with2µL of the plasmids, adding 5µL of ligation mix, and then placing the tubes into a heat block of 90°C for15 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. The competent cells were incubated with the recombinant DNA for 45 minutes at 37 degrees Celsius.

REAL TIME PCR

The author enhanced each bacterial sample with the glucose concentrations of 15mM, 7.5mM, 5mM, and0mM. The cDNA from H-glucose, M-Glucose, L-glucose, and (-) glucose were diluted into a 1:8 serial dilution. A 10-fold dilution of standards were prepared for bacterial samples transformed with (+) asRNA inducible plasmids and exposed to high to low volumes of glucose. The amount of genetic 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 get 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 80 cycles of

10 sec each starting at 55°Cwith 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.

QUORUM SENSING

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 inhibition of the gene accA. RNA was extracted from cells grown with25µM glucose and 5µM of glucose. As a second experimental procedure, 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. A control, without (-) glucose and (-) asRNA in vitro translation, was compared to each sample. The number of genes copies for Lux-S were measured through the qPCR amplification protocol and absolute quantification, (Roche Lightcycler 480 machine).

RESULTS AND DISCUSSION

THE RNA CONCENTRATION AND ACCA GENETIC EXPRESSION OF THE GLUCOSE SAMPLES OF BACTERIAL CELLS

The *Escherichia coli* MG1655 bacteria cells were grown in LB broth overnight at 37°C with 15mM,7.5mM, 5mM, and 0 mM of glucose concentration for high-glucose, medium-glucose, low, and zero glucose as a control, respectively (Fig. 2). RNA was extracted from each sample and the concentrations determined by the Implen Nanophotometer 250. The RNA concentrations for each sample measured were1392 ng/µL for high-glucose, 797 ng/µL for medium-glucose, 608 ng/µL for low-glucose, and 179 ng/µLfor the control. The high-glucose sample had the highest amount of RNA compared to the medium to low and to the control. The RNA for each sample 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. The RNA extractions were repeated through 20 trials with a standard deviation of 335.4 ± 329.2. High glucose had a Cp of 12.28 and the concentration of accA was 4.21E3 ng/µL. The Cp of the sample medium-glucose equaled 16.51 with a concentration of 3.75E2 and the low-glucose Cp was 14.08with a target gene concentration of 1.50E3 (Fig. 3). The control group had a Cp of 17.64 with a target gene concentration of 1.96E2.

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) For the glucose concentrations of 200µM, 50 µM, and 0µM compared to their dsDNA concentrations, the standard deviation was 148±204,190±252, and 107±176, respectively (Fig. 2). Comparing 200 µM to its control group, the difference between the samples was statistically significant. The cells grown in 200µM had a more significant and larger production of dsDNA, with a p-value of 0.038, than cells grown in 0µM of glucose. Vinals et al., (1999), found results that determined that high glucose concentration causes the absorption of glucose to be blocked as a result of its repression of glucose phosphorylation (8).

Therefore, the rate of forming SCFAs is dependent on the glucose concentration within the lumen of the intestines (16). Ferraris et al. (1990), measured the luminal glucose osmolarity and concentrations of the small intestines. They found the assumptions of luminal glucose concentrations being 50mM to500mM to exhibit some errors (16). The previous studies measured glucose concentrations but did not recognize how osmolality is affected by Na+ and K+ salts, amino acids, and peptides (16). 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 a SI region from 0.2to 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 at30 mM glucose from their study (16). The high glucose osmolities resulted in their reduced Vmax values for 2-deoxyglucose uptake with a constant Km, calculated from the Michaelis-Menten kinetic enzyme equation (16).

However, from the results, the genetic expression of accA was proportional to the increasing glucose concentrations in *Escherichia coli* bacterial samples. After culturing E. 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, 361.72 ng/µL for low-glucose, and 137.60 ng/µL for the control. The concentration of accA, in ng/µL, for each sample included: 4210, 375, 150, and 196 for H-glucose, M-

glucose, L-glucose, and No-Glucose, respectively. The author reached these specific results for genetic expression amplified in conjunction with increased glucose concentration because the four subunits for the acetyl carboxylase complex seem to be regulated only by the accBC lac operon and each gene, 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 three gene products don't affect the accBC operon transcript levels (22). When there is a high glucose concentration, the availability of accB increases, and the transcription of the accBC lac operon is greatly reduced even after an exposure to a small amount of accB. Although a high glucose concentration may have stymied the production of accBC, it did not seem to inhibit the amplification of accA, the target gene and DNA sequence of study.

THE QPCR RESULTS OF ANTISENSE RNA FOR THE TARGET GENE ACCA

Ninety percent of the genome for Eukaryotes consists of non-coding regions or RNA that does become translated into proteins. Antisense transcription is the purpose for the noncoding regions of human cell DNA (23). 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), that 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 andNco1. The primers flanked the target DNA, which totaled to 150 base pairs. The antisense sequence was constructed in a specific orientation where the restriction enzymes, which NcoI normally flanks the forward primer reversed positions. The XhoI was repositioned to flank the forward primer, and theNco1 would flank the reverse primer, creating the antisense sequence when inserted into the plasmid forPHN1257.

The PCR product was amplified 12 times with a standard deviation of 219 ± 171.

The total RNA concentration for the control, (-) asRNA, equaled 739.44 ng/µL with an A260/A280of 1.9. The control, (-) asRNA, had a miRNA concentration of 334.98 ng/µL, including an A260/A280of 2 (Fig.3). The RNA concentration for transformed bacterial cells with the asRNA of accA PHN1257plasmid was 279.28ng/µL, having an A260/A280 of 2.011. The miRNA concentration of the cells with asRNA measured to 240.90 ng/µL and an A260/280 absorbency of 2.073 (Fig.3). The gene of accA was successfully suppressed by asRNA in vitro with 63 ng/µL, qPCR measured, for bacteria cells transformed with the recombinant antisense PHN1257 plasmid DNA. The standard deviation for the real time PCR experiment was 37.8 ± 35.6 for 12 repetitions. The bacterial cells with the PHN1257 plasmid but without the antisense gene target and insert produced 421.69 ng/µL for accA (Fig.3). There was a 138 percent difference between cells not expressing asRNA versus cells transcribing the asRNA for accA. A p-value of 0.027 showed highly significant data for the accA gene target concentration ofPHN1257(+) asRNA versus PHN1257(-) asRNA, or without asRNA. 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 ACCase (19).

The four genes accA, accB, accC, and accD code for the subunits of the complex, Acetyl-CoA Carboxylase, which catabolizes dietary fiber in the form of glucose to begin many biosynthetic processes. Acetyl-CoA carboxylase (ACC) initiates the first step of fatty acid synthesis. During fatty acid synthesis, malonyl-CoA is formed from acetyl-CoA, using energy from ATP and bicarbonate production (22). 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 an oxygen, altered into carbon monoxide, and a methyl group is added to this reduction to make acetyl-CoA into Acetate (20). 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 (41). 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 an oxygen, forming propionate (20).Therefore, inhibiting accA genetic expression with asRNA can hamper the production of SCFAs, which are highly significant for initiating the downward cascades for hormonal responses, regulating metabolism, controlling hunger signals to the brain and affecting many psychological behaviors.

QUANTIFICATION OF LUX-S GENE EXPRESSION

For the mechanism of quorum sensing, the gene accA, a part of the ACCA subunit of the ACCase enzyme, functions as a transferase, catalyzing the first stage of bacterial lipid biosynthesis. Lipid biosynthesis is responsible for increased quorum sensing and biofilm formation. The gene accA codes for the alpha subunit for the Acetyl-CoA Carboxylase enzyme. After glucose is hydrolyzed into two pyruvate molecules, the alpha subunit, ACCA of the ACCase enzyme, transfers a CO2 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 biofilm matrix. The fatty acids induce the release autoinducers, which accrue into a display of bioluminescence. The bioluminescence is a tremendous sign and effect of quorum sensing. Lux-S is needed to produce autoinducer two. Lux-S monitors the amount of biofilm, flagellum movement, and monitors virulence. The high levels of autoinducers released initiates a positive feedback loop switching all bacterial cells into producing quorum signals, which create a bioluminescence (25). As autoinducers compile in the surrounding environment of a bacterial cell, the bacterial population increases (10). 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 (10). Quorum sensing between bacterial cells monitors the genes responsible for organizing the activities executed by bacterial groups. Activities completed through quorum sensing includes: bioluminescence, spore formation, antibiotic secretion, creating biofilm, and releasing factors for virulence (10) Biofilm increases the movement of bacteria into the bloodstream (35). Therefore, the results demonstrated a link between bacterial cell metabolism as lipid synthesis via acetyl-CoA carboxylase, and quorum sensing, after measuring the number of gene copies of Lux-S while inhibiting accA with asRNA.

Quantifying the gene copy number of Lux-S was replicated a total of 12 times with a standard deviation of 17449.87 ± 16640.06 for (+) glucose/ (+) asRNA, and 277 ± 37 for (+) asRNA only. Continuing for quorum sensing the standard deviation was 658,114 ± 483,499 for (-) glucose/ (-) asRNA. For the results, the (-) glucose-(+) asRNA sample had a gene copy number of 199 and 2511 Lux-S copies for (-) glucose- (-) asRNA. For the 25µM glucose-(+) asRNA and 5µM glucose (+) asRNA produced 39,810 and 316,228 gene copies of Lux-S, respectively (Fig. 4). The samples with(-) glucose-(-) asRNA and with 5µM glucose- (+) asRNA exhibited the highest amount of expression for Lux-S, which displays an increased amount of quorum sensing releasing more autoinducer-2 molecules, directly affected by the bacterial cells increased sensitivity to glucose (Fig. 5). The p-value was 0.025 for the ANOVA results of the qPCR gene copies for the Lux-S gene samples with glucose and antisense RNA expression and for the samples, (-) glucose-(asRNA), and for (-) glucose- (+) asRNA (Table. 3). The 5µM glucose- (+) asRNA sample showed the most expression of Lux-S because 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 (28). Because cells were overly saturated with a 25 µM concentration of glucose, we expected to find more release of autoinducer-2 signaling molecules with less regulation of genetic expression.

However, the over saturation of glucose at 25 µM did not result in more genetic expression of Lux-S. Jesudhasan et al. (2010) proved that higher levels of autoinducer-2 did not lead to increased genetic expression of Lux-S in *Salmonella typhimurium* when cultured in high concentrations of glucose (29). By transforming *Escherichia coli* bacterial cells with IPTG-PT-asRNA inducible vectors, which target the gene accA, the Lux-S gene was inhibited. Because glucose delays the movement of the Lux-S mutant strain, but does not inhibit bacterial growth, each (+) asRNA sample appeared smaller and defected from the LB agar plates of cultures. 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 (30). Figure 6 shows bacterial colonies expressing antisense RNA with a smaller size versus cells without antisense RNA of accA. Cell samples with (+) asRNA transcription were smaller in size (30). Bacterial samples transformed with IPTG-PT-asRNA inducible PHN1257 plasmids, designed to inhibit the accA gene had the least measure of Lux-S expression. The (-) glucose- (+) asRNA sample had the lowest amount of Lux-S expression of 199 gene copies, 277 ± 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 results from a large amount of DNA released. The ample amounts of DNA released maintains a more solid macromolecular matrix and consistency of the biofilm (39). 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 (37). The gene Lux-S codes for the flagella complex, biofilm creation, and controls virulence factors. The Lux-S gene, when it is more expressed in other bacterial types, causes the

spread of virulent bacteria through increasing the motility and movement of pathogenic bacteria. More Lux-S expression, from mutations in Lux-S, produces increased amounts of biofilm, assisting pathogenic bacteria to invade the mucosal layers and epithelial cells of the GI tract lining.

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 acyl-homoserine lactone, 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 (25). After AHL is produced, it liberally 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 (25). The LuxR-AHL bond releases into the periphery of the cells high levels of quorum sensing that allows for cell to cell communication between bacterial autoinducers. E. 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 the highest amount of the Lux-S gene (28).

Streptococcus pneumoniae, a gram-negative bacterium, results in the death of 2 million people each year. *Streptococcus pneumoniae* lives symbiotically with another microflora in the nasopharynx region of the respiratory system. After a month of commensal habitation, Streptococcus pneumoniae begins to infect other parts of the body, leading to disease. To form biofilm for infection, the Lux-S gene must be activated. Its excessive amplification and expression lead to a denser texture of biofilm (39). Lux-S monitors virulence through regulation of the process of generating biofilm in the nasopharynx in mice with pneumonia. 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 (36). 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 a more rapid motility of bacteria, infecting the colonization of host cells and assisting infectious bacterial cells with entering the mucosal layers (38). Pathogenic bacteria need to determine the autoinducer signals specific to their species. Pathogenic bacteria require a specific order and assortment of virulent genes to infect, spreading disease.

For example, the different residues or R-groups of amino acids within autoinducers bind to 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 produces varied types of side chain lengths and amino acid substitutions (40). However, *E. Coli* in the gut respond to Lux-S transcription differently than in the respiratory system. For example, pathogenic bacteria as Enteropathogenic E. coli EPEC has intraspecies signals that help to colonize the small intestines. The small intestines are void of many commensal microflora. The Enterohemorrhagic E. coli or EHEC pathogenic form of E. coli conducts signals with other bacterial cell types and with host cells. EHEC communicates through quorum sensing with another normal large intestine microflora. EHEC infects 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 (34). Autoinducer 2 requires the genetic expression and translation of Lux-S. When Lux-S and autoinducer-2 are less regulated and overexpressed, infectious bacteria can propagate in stressful environments with high acidity and salinity (32). Kendall et al., (2008) demonstrated S. pyogenes adapting to acidic conditions when the luxS/AI-2 system was unregulated.

When the production of Lux S is blocked, the levels of disease-causing bacteria changes. For example, the mutation of Lux-S in *Haemophilus influenzae* increases virulence that cause middle ear infection (33). 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 (33). However, autoinducer 3 is not dependent on expression of the Lux-S gene. Lux-S genetic mutants impede the production of AI-3 (31-32). As a result, the pathogenic Escherichia coli, EHEC, collects signals from host cells in the form of hormones as epinephrine. The AI-3 can communicate with the transmembrane protein called Qsec (31). The two components for quorum sensing also includes 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, recognizing the accumulation of

autoinducers (11). The bacteria called *P. aeruginosa* secretes two types of autoinducers, N-3-oxododecanoyl homoserine lactone (3-oxo C12-HSL) and N-butyryl-Lhomoserine lactone (C4-HSL). These autoinducers released can cause virulence in people infected by *P. aeruginosa* by supplying virulence factors that help colonize and spread the disease (11).

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 for the process of quorum sensing. The Lux-S gene is an important gene for regulating the virulence of pathogenic bacteria within the GI tract. The data reveal genetic changes, as inhibiting the accA gene with antisense RNA, and the availability of nutrients, as glucose, affect the production of Lux-S. Adding glucose to bacterial samples expressing antisense RNA, allowed for more expression of the gene Lux-S. The increased production in the presence of glucose, with asRNA, was a significant result because more glucose can increase the production autoinducer 2, causing more Lux-S transcription. Also, mutations of the gene Lux-S produce more biofilm than the wild type for spreading the infection and disease, as in the *Streptococcus pneumoniae* infection. When Lux-S is inhibited, in some bacteria, the amount of biofilm produced is greatly reduced, lessening the motility of bacteria. The lessened motility impedes the spread of pathogenic bacteria.

CONCLUSION

The overall purpose for the study was to confirm and identify the gene accA as a possible target for blocking bacterial antibiotic resistance. We attempted to provide a more integrative perspective for the effects of glucose metabolism on gene expression and the process of quorum sensing. A cumulative perspective, linking bacterial cell metabolism to the quorum sensing between bacteria cells, can provide possible molecular targets for preventing antibiotic resistance in bacteria. Antibiotics can be designed to target bacterial cell metabolic pathways, reducing bacterial cell biofilm needed for spreading pathogenic bacteria. The interaction of bacterial cell metabolism, cell growth, and gene expression within *E. coli* cells was examined. The strong condition of a bacterial population's metabolic state can prevent bacterial susceptibility to antibiotics (45). The gene inhibition of accA compiled with a high to low addition of glucose, demonstrated that glucose in excess increased production of accA even when inhibited by antisense RNA. The results of decreased RNA expression and cell growth in the presence of antisense RNA inhibition of accA, may serve as a model of intervention for blocking microbial metabolic processes that contribute to antibiotic resistance (44). *E. coli* cells were cultured in high glucose to a non-glucose concentration and showed a decreasing range of genetic 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 accA gene was inhibited with antisense RNA, using a, IPTG-inducible vector called PHN1257. There was a 138 percent difference of genetic expression between cells transformed with antisense PHN1257versus cells with no antisense RNA in vitro transcription. The high levels of glucose, from high fiber intake, increased production of accA into mRNA for translation into acetyl-Carboxylase enzyme, which produce SCFAs. 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 that flank for the target DNA, rebuilding a paired double-stranded RNA terminus that inhibited the transcription of accA (15). The effect of glucose, outside or extracellular of the bacteria, on the concentration of RNA, were measured through increasing the gradient of glucose concentrations. Transcription of intracellular molecules as theca gene were determined through qPCR. High levels of glucose increased the expression of accA.

The results supported the hypothesis. Decreasing the glucose concentration to zero mM, while suppressing the accA gene, led to an observed decrease in Lux-S expression. Lux-S is an important gene for quorum sensing, 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 pathogenic bacteria in the GIT to proliferate and cause a more systemic infection. The accA gene translated into the acetyl-CoA carboxyl transferase enzyme transfers a carbon dioxide from the glycolysis of glucose to the acetyl-CoA Carboxylase, the ACCase, 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, by inhibiting accA with asRNA there is less

release of AI-2, less Lux-S and decreased biofilm production. Inhibition of accA was confirmed through qPCR results of 63 ng/µL for accA (+) asRNA versus 422 ng/µLfor accA (-) asRNA. The amount of intercellular quorum sensing between bacterial cells was quantified through qPCR. The volume of Lux-S was determined through qPCR. The qPCR results demonstrated that the end yield of Lux-S and autoinducers, AI-2, cells are dependent on the supply of glucose. By increasing the glucose concentration in increments, Wang et al. (2005) found that it increased the levels of autoinducer 2 of the culture (28). The observations displayed the RNA concentration for bacterial cells, expressing asRNA of accA, included a concentration of 279.28 ng/µL, having anA260/A280 of 2.011. The miRNA concentration of the cells with asRNA measured to 240.90 ng/µL and an A260/280 absorbency of 2.073, with a p-value less than 0.05. The concentration for cells without antisense RNA was 422 ng/µL compared to 63ng/µL for cells expressing asRNA. However, in the presence of glucose with antisense RNA transcription, bacterial cells still produced large amounts of Lux-S with 316,228 copies measured through qPCR when enhanced with 5µM of glucose, with a p-value of 0.002. For further research, the use of antisense RNA to target and inhibit bacterial cell metabolism, providing possible methods for blocking bacterial antibiotic resistance.

For many researchers, the microflora in the gut is still a mysterious organ, and there is still a highly motivated search to fill such a huge vacuum of unknowns, concerning the gut microflora. Until 2005 with the creation of metagenomics, measuring the large community of commensals in the human gastrointestinal tract was not possible (42). Measuring the amount of microflora of the gut was impossible since many of the bacteria were anaerobic and could not be grown via culture. Therefore, researchers' understanding of the common microflora in the gut patterns and processes, combining with determining a healthy versus a diseased pattern of condition, is still new and less understood (42). Currently, there have only been a small number of findings that have been established based on human studies. For example, the Human Microflora in the gut Project, which was financed and supported by the NIH from 2007-2015 and the American Gut Project were the only two major studies in the US attempting to characterize the diverse bacteria in the human microflora in the gut, establishing a library of data of human microbial communities. Researchers are currently elucidating the many pathways and processes of the microflora in the gut of the GI tract. Beyond providing solutions to combat antibiotic resistance, researchers may find that searching through the gut could provide ample alternative treatments and therapies.

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Table 1 (on next page)

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

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.

Table 1. 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.

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

Table 2(on next page)

Pathways for Biosynthesis of SCFAs from Carbohydrate Fermentation

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-hydroxybutyryl CoA, where it loses a hydrogne 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.

Links between diet, gut E. Colicomposition and gut metabolism - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Metabolic-routes-for-butyrate-and-propionate-formation-by-representati ve-bacterial-genera_ fig1_266323963 [accessed and modified on 18 Feb 2019] Attribution: Flint [CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0)]

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**Formed	Phosphate removed to make **PROPIONATE**

Fig. 6 Short Chain Fatty Acid Biosynthesis through Bacterial Fermentation Diagram

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-hydroxybutyryl CoA, where it loses a hydrogne 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.

Links between diet, gut E. Colicomposition and gut metabolism - Scientific Figure on ResearchGate. Available from:

https://www.researchgate.net/figure/Metabolic-routes-for-butyrate-and-propionate-formation-by-representative-bacterial-genera_ fig1_266323963 [accessed and modified on 18 Feb 2019]

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Anova Results of each experimental sample for quantifying gene copies through qPCR.

Anova Results of each experimental sample for quantifying gene copies through qPCR. The ANOVA results reveal an overall average of 127907.940 for the total number of trials that yielded a total of 1798315304447 gene copies through Real-Time PCR absolute quantification. The sample without glucose and without antisense RNA produced the highest amount of gene expression, of Lux-S and accA, at 547774.000 as an average.

ANOVA Results of qPCR Gene Copies

	Lux-S-glu/ (+) asRNA	Lux-S-glu/ (+) (-) glu (-) asRNA		accA (+) glu/accA (-) glu	accA (+) glu/accA (-) glu
1	2511	316228	1000000	186	100
2	2000	26915.4	1000000	79	32
3	745	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	1798315304447.048

source	df	SS	MS	F	P-value
treatments	4	905636341810.108	226409085452.523.8044 7		0.0250
error	15	892678962636.940	59511930842.463		
total	19	1798315304447.048			

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.

doi: 10.3390/ijms15022773

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3958881/figure/f6-ijms-15-02773/

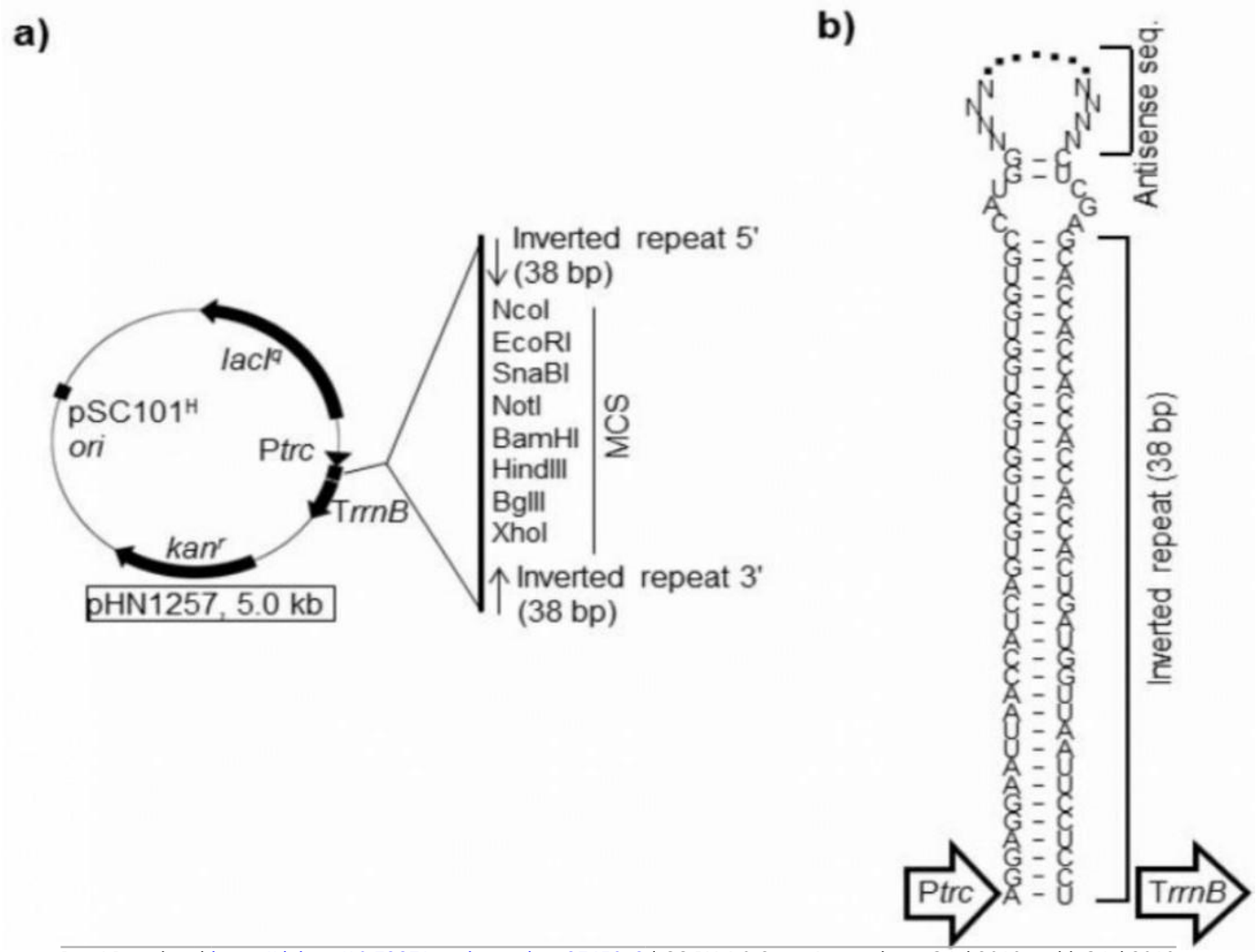


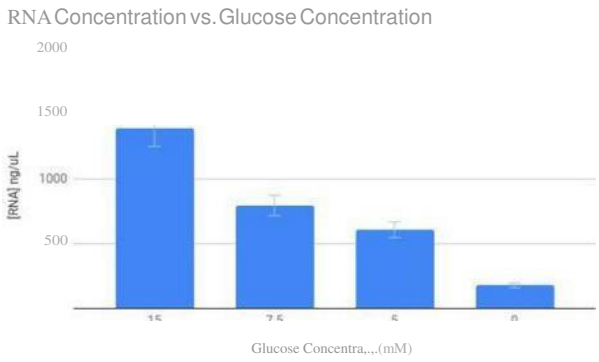
Figure 2

Glucose concentrations with RNA Concentration results

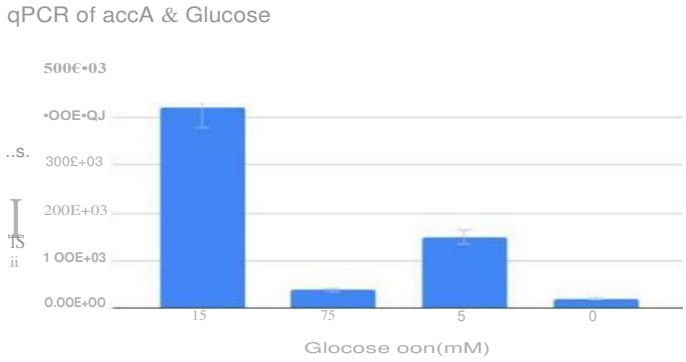
A) Glucose concentrations of 15mM, 7.5mM, 5mM, and the control samples each had RNA concentrations, in ng/uL, 1392, 797, 608, 179, respectively. A) The bacterial sample with 15mM glucose, a high concentration, had the largest measure of RNA, noting a direct proportional link between glucose and genetic expression in gram negative bacteria as E. coli.

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

A)


RNA Concentration vs. Glucose Concentration
2000
1500
1000
500
[RNA] ng/uL
Glucose Concentra,.,.(mM)

B)


qPCR of accA & Glucose
500€•03
•OOE•QJ
300£+03
200E+03
1 OOE+03
0.00E+00
15
75
5
0
Glocose oon(mM)

C)

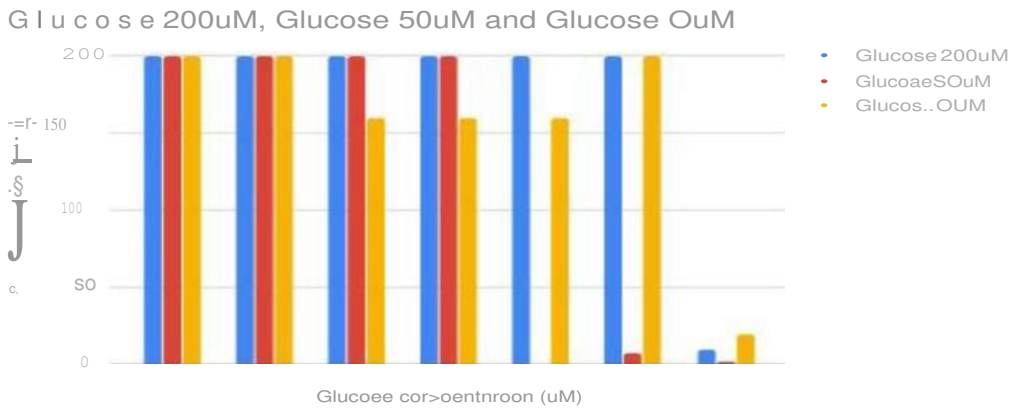

G l u c o s e 200uM, Glucose 50uM and Glucose OuM
200
150
100
SO
0
Glucose 200uM
GlucoaeSOuM
Glucos..OUM
Glucoee cor>oentnroon (uM)

Figure 3

The qPCR results for quantifying accA concentration and the gene copy number.

Standard Curves for Quantifying Gene Copies the RNA for each sample 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 ng/uL 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 138% percent di erence between cells not expressing asRNA versus cells transcribing the asRNA for accA. A p-value of 0.027 showed highly significant data for the accA gene target concentration of PHN1257(+) asRNA versus PHN1257(-) asRNA, or without asRNA.

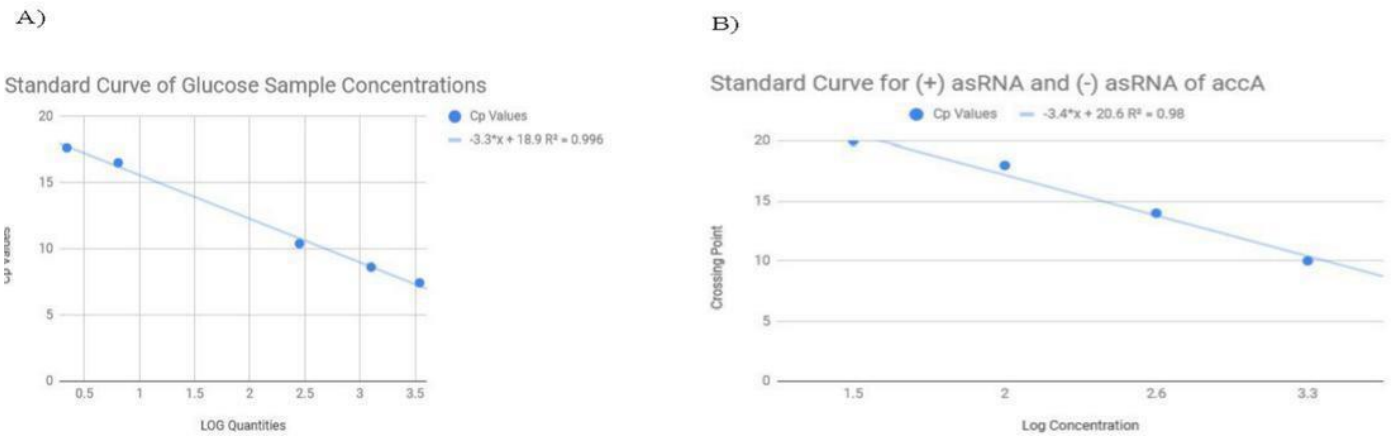
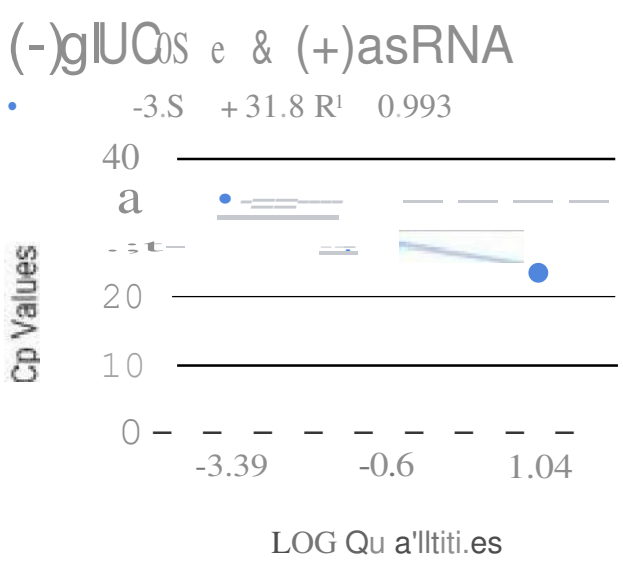


Figure 4

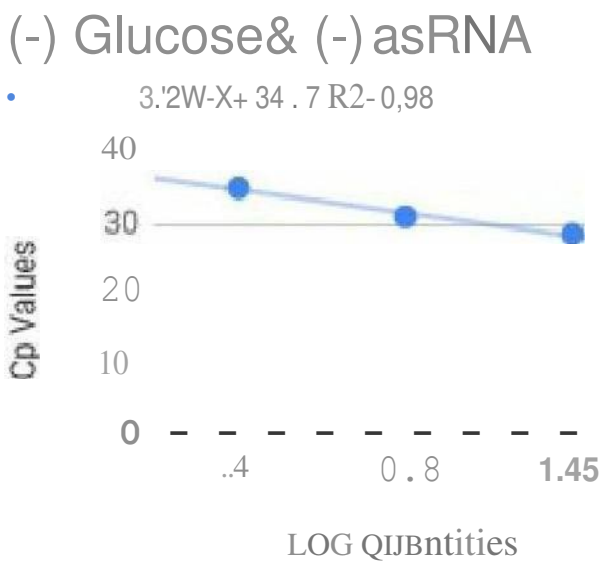
Gene Copies for Lux-S determined through qPCR measurement

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

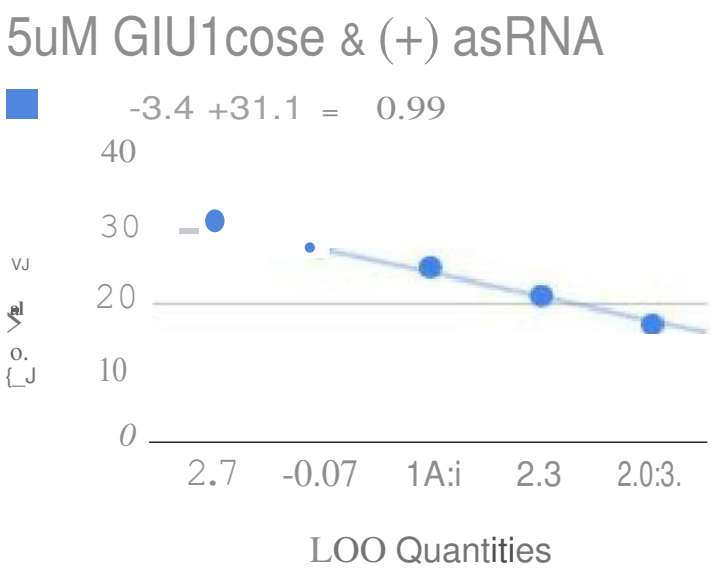
A)


(-)gIUC0S e & (+)asRNA
-3.S + 31.8 R¹ 0.993
40
a
20
10
0
Cp Values
-3.39
-0.6
1.04
LOG Qu a'lltiti.es

B)


(-) Glucose& (-) asRNA
3.'2W-X+ 34 . 7 R2- 0,98
40
30
20
10
0
Cp Values
..4
0 . 8
1.45
LOG QIJBntities

C)


5uM GIU1cose & (+) asRNA
-3.4 +31.1 = 0.99
40
30
20
10
0
2.7
-0.07
1A:i
2.3
2.0:3.
LOO Quantities

D)

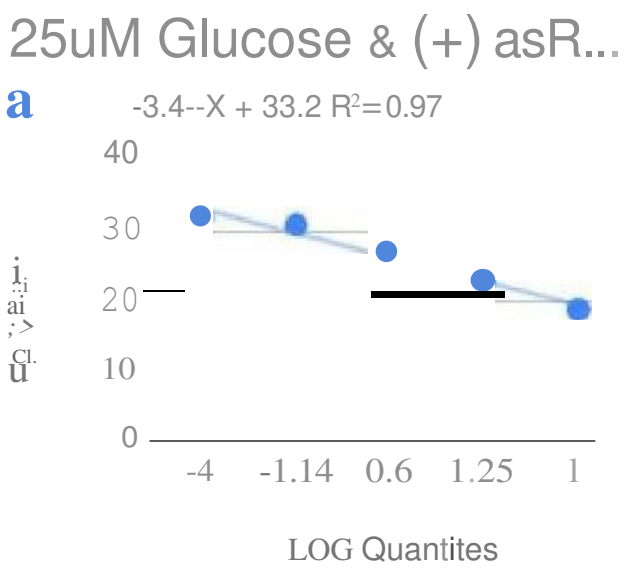

25uM Glucose & (+) asR...
a
-3.4--X + 33.2 R²=0.97
40
30
20
10
0
-4
-1.14
0.6
1.25
1
LOG Quantites

Figure 5

A graph of each sample with LuxS, expressing asRNA, enhanced with glucose, without glucose, and without asRNA.

Bacterial Samples enhanced with 25 micromolars of glucose displayed the least amount of gene expression for the gene Lux-S with 1363 copies and samples with 5 micromolars of glucose showed more Lux-S expression with 90,294 copies averaged. Bacterial samples without glucose and without asRNA transcription had the highest amount of Lux-S and accA gene expression. The (-) glu (-) asRNA sample averaged 547,774 gene copies. The samples with glucose and without glucose averaged a total of 107 gene copies for accA.

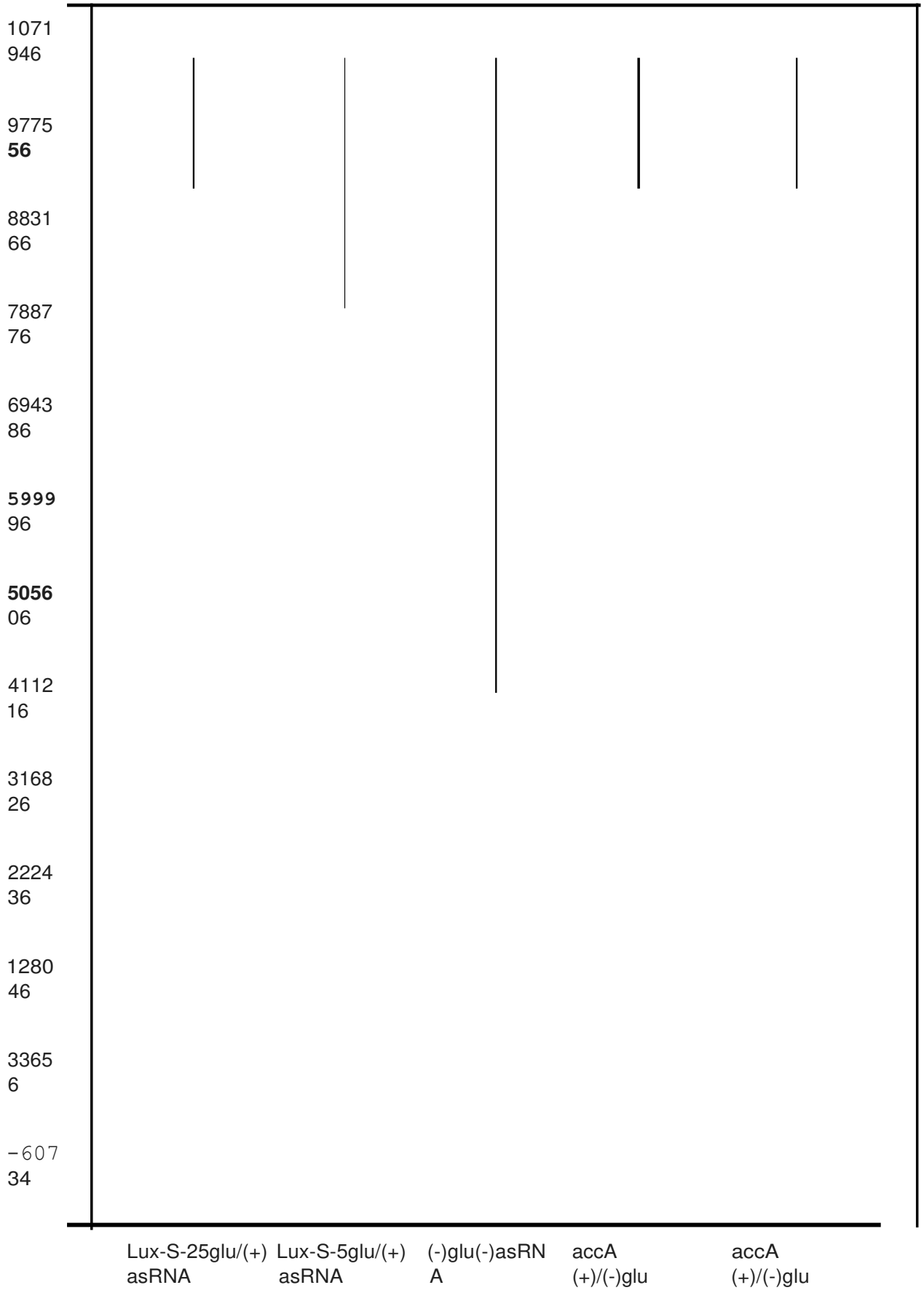


Figure 6

Comparing Bacterial Colony Sizes

Comparing Bacterial Colony Sizes Fig. 6 (+) asRNA vs. (-) asRNA Bacterial Colony Size When comparing the sizes of bacterial colonies, the. bacterial colonies with antisense RNA expression (left image) were smaller in size than the bacterial colonies without antisense expression (right image). The size of each culture was measured and determined. A) For the bacterial colony with antisense RNA transcription (+) asRNA, the size was 2 mm in diameter (left image). B) The bacterial colony without asRNA (right image) had a size of 5mm in diameter.