Integrating the metabolic processes of *Escherichia coli* with virulence by decreasing glucose availability, inhibiting the acetyl-CoA carboxylase gene *accA* with asRNA, and through the quantification of the *luxs* 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 *luxs*, a vital gene needed for propagating quorum-sensing signal molecules. The *luxs* 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 *luxs* transcription augments biofilm production in support of spreading virulence. The implications of the study advocate for

28 designing antibiotics that target bacterial cell metabolic processes to block bacterial antibiotic
29 resistance.

30 **INTRODUCTION**

31 Alexander Fleming discovered penicillin in 1928. His discovery catalyzed the use of
32 antibiotics that saved many lives, however, with the overuse of antibiotics the issue of antibiotic
33 resistance has occurred in parallel. Between the 1960s and the 1980s a profound formulation of
34 many new and novel antibiotics were discovered [1]. The new and novel antibiotics were
35 superseded by a vigorous evolution of bacterial antibiotic resistance. The three phases that occur
36 after bacterial horizontal gene transfer for proliferating antibiotic resistance include: 1) the
37 antibiotic genes received during conjugation code for the creation of efflux pumps; 2) the
38 antibiotic-resistant genes are enclosed within the pro-antibiotic genes, which can impede
39 antibiotic drugs attaching to the antibiotic gene target sites; and 3) the antibiotic-resistant genes
40 can direct the ubiquitous destruction of antibiotic drugs through hydrolysis [11] .

41 Only a few of the novel antibiotics were available to treat many of the antibiotic resistant
42 bacteria infections. Therefore, there has been a reduction in exertion and effort within the field of
43 pharmaceutics to design more new and novel antibiotics. Therefore, future research can
44 investigate the processes, mechanisms, and the dynamics of antibiotic resistance to increase the
45 development of novel antibiotics that can target those specific resistant processes. Antimicrobial
46 resistance or AMR has led to the deaths of 700,000 annually including an additional 10 million
47 people who is approximated to die by 2050 [1]. Generating novel antibiotics has not solved the
48 problem of AMR since bacterial resistance has reduced the efficacy of every antibiotic
49 administered in the clinical setting. There is a demand for more creative and advanced antibiotics
50 to deal with this tremendous public health issue.

The bacterial colonies within biofilms are profusely resistant to antibiotics and the biofilm induces the continuance of chronic infections. Because the biofilm extracellular matrix prevents penetration of antibiotics, is largely anaerobic or lacking oxygen, bacteria within the biofilm are more tolerant of antibiotics [2]. Biofilms are an organization of bacterial groups attached to a biotic or abiotic extracellular matrix. However, there is insufficient knowledge about the effect of antibiotic resistant processes linked to bacterial biofilms. Bacteria use the formation of biofilm as an advantageous survival strategy.

Biofilm allows bacterial cells to develop a high tolerance for antibiotics through resistance. The biofilm formed consists of proteins, extracellular DNA, sugars, and is strongly resistant to proration by antibiotics. When antibiotics are given, the antibiotics eradicate most of the bacteria cells on the surface of the biofilm, but the bacteria embedded closer to the base of the matrix remain [3]. Biofilm can endure and persist after antibiotic exposure due to many physical, anatomical, and innate genetic factors. The pathogenic bacteria create many of their virulent actions and biofilm production through quorum sensing or cell to cell communication. Due to the cell-cell communication for biofilm formation, designing small molecules for disturbing the quorum sensing signals can inhibit biofilm development.

After researchers exposed bacterial cells within biofilm to the antibiotics to rifampicin- and kanamycin-resistant mutants within immature biofilm communities, the results displayed that each mutant amplified in growth without the presence of antibiotics. The mutant cultures of biofilm that were treated with kanamycin showed less antibiotic resistance, however, after 250 generations the resistant mutant bacteria increased in growth at the surface and at the base of the biofilm matrix [4]. *Pseudomonas aeruginosa* can produce redox reactive pigments. These pigments are called phenazines, which affect the expression of genes, cause metabolic changes,

74 and can alter bacterial cells tolerance of antibiotics [5]. Phenazines induce the survival of *P.*
75 *aeruginosa* through its ability to modify biofilm physiology.
76 Phenazines increase the formation of the initial matrix, composed of exopolysaccharide,
77 which is needed for bacteria to attach and form a mature biofilm. The Phenazines cause the
78 production of efflux pumps and affect the downward cascade of the central metabolic processes
79 of *P. aeruginosa*. Researchers measured the bacterial mutant-survivors of biofilm after exposure
80 to antibiotics and a carbon compound. They found that after biofilms of *P. aeruginosa* were
81 cultured with succinate, a carbon component that enters the mid-central metabolism of glucose,
82 the phenazine could no longer protect bacterial cells from antibiotic penetration and eradication.
83 A bacteria's increased tendency to develop biofilm bacterial populations can be a
84 significant factor of virulence. For example, most patients suffering with a skin infection known
85 as *S. epidermidis* produce a strong biofilm in vitro [6]. The exopolysaccharide matrix can lessen
86 the penetration of the deeper strata of the biofilm by antibiotics. When bacteria are subjected to
87 antibiotic exposure at deeper layers of the biofilm, resistant mutants of those bacteria can form
88 [**7**]. Although biofilm communities of bacteria can be exposed to large amounts of antibiotics for
89 longer periods of time, active and live bacterial cells can persist and survive.
90 Lux-S is a S-ribosylhomocysteine lyase that produces autoinducer-2. Lux-S is necessary
91 for the motility of bacteria and for forming bacterial biofilm. Autoinducer-2 is a signaling
92 molecule released that induces the cell to cell communication called quorum sensing. Bacterial
93 cells communicate through an intercellular process called quorum sensing. Amplified Lux-S
94 expression increases the release of autoinducer-2, intensifies quorum sensing, and produces more
95 biofilm for spreading pathogenic bacteria. The expression of the gene *luxs* induces the release of
96 autoinducer-2. Quorum sensing is a type of communication between bacterial cells that occurs

97 through the release of small signaling molecules called autoinducers. Releasing the autoinducers
98 regulates the aggregation of cells and monitors genetic expression. The autoinducers of gram-
99 negative bacteria consist of homoserine lactones versus gram-positive bacteria that release
100 oligopeptides [7].

101 In addition, the microflora can communicate with the host cells of the gastrointestinal
102 tract through a variety of chemical idioms [16]. A group of chemical molecules for bacterial
103 language expression and transfer includes homoserine lactones that are the most heavily studied.
104 When a bacterial infection occurs, homoserine lactones form and interrelate with the immune
105 system [17]. An increased release of quorum-sensing signals produces more biofilm, which is
106 needed for rapid motility and the spreading of infection. The production of biofilm induces
107 resistance to antimicrobial peptides. Biofilm maintains an intensive resistance to antibiotics as
108 multi-drug resistant pumps utilize biofilms for excreting antibiotics from bacteria. Mutations
109 present in genes that are a part of the metabolic pathway cause bacteria to become more
110 susceptible to antibiotic exposure.

111 However, the metabolism of glucose by bacteria significantly affects the output of
112 autoinducers and the production of biofilm. The acetyl-CoA carboxylase enzyme catalyzes the
113 biosynthesis of long-chain fatty acids and hydrolyzes glucose into short-chain fatty acids. The
114 LCFAs are synthesized after the acetyl-CoA transferase, the alpha subunit of ACCase or the
115 ACCA subunit transfers a carboxyl group from pyruvate to an acetyl-CoA, producing malonyl-
116 CoA, which catalyzes the elongation of the LCFAs. The LCFAs become structured into the
117 bacterial cell-matrix and the plasma membrane. The bacteria cells grow into large populations,
118 forcing biofilm formation and induces a quorum-sensing response. The *luxs* gene is essential for
119 synthesizing and releasing autoinducer-2.

120 Lux-S is required for quorum sensing, biofilm formation, and virulence. Inhibiting the
121 fatty-acid synthesis of bacterial cell metabolism can reduce Lux-S expression and biofilm
122 production. Increased metabolic fatty-acid synthesis can amplify Lux-S expression as a virulent
123 producer of biofilm, which can cause the persistence of antibiotic resistant bacteria. We do not
124 completely understand the exact mechanism that connects cell metabolism with the bacterial
125 pathogenesis of antibiotic resistance. The author suggests that blocking the enzymatic activity of
126 acetyl-CoA carboxylase may represent a potential aim for overcoming antibiotic resistance.
127 The metabolic processes of bacteria may provide much potential for generating and
128 designing novel therapeutics as antibiotics that can reduce biofilm formation. For example,
129 CbrAB is a histidine kinase that is membrane-bound, and a part of the metabolic regulatory
130 system called the CbrAB/Crc system [41]. The CbrAB/Crc system shows a connection of
131 bacterial cell metabolic processes with antibiotic resistance [10]. A process called catabolite
132 repression control or Crc inhibits translation of catabolite activities by targeting specific mRNAs.
133 The Crc protein targets mRNAs adjacent to ribosome binding sites [42]. When sources of carbon
134 availability are limited the CbrAB/Crc system is actuated. The CbrAB protein initiates the
135 expression of a small RNA termed *crcz*. The crcz alleviates the repression of the Crc-targeted
136 mRNAs. Inhibiting the *crc* gene lowers the growth rate of bacteria along with providing a higher
137 susceptibility to antibiotics [10].
138 Nutrient availability can determine the expression of metabolic genes and remove the
139 bacterial response of antibiotic resistance. In a study by Peng et al. (2015), bacteria cultured
140 without glucose or the non-polar amino acid of alanine were less susceptible to kanamycin, an
141 antibiotic. Only 9.5 ng/mL of the kanamycin was absorbed; however, adding alanine and glucose
142 to the cultures increased the uptake of kanamycin to 65-123 ng/mL and then to 113-231 ng/mL

143 [12]. Yu Bin Shu et al. (2018) performed a Michaelis-Menten kinetics experiment using multi-
144 drug efflux pumps while adding glutamate and acetate to growths of *Escherichia coli* [13]. They
145 confirmed that the *Escherichia coli* cells were more sensitive to gentamicin, which is an
146 antibiotic. Each of the previously mentioned studies displays a significant link between bacterial
147 metabolism and antibiotic resistance.

148 For that reason, the effects of extracellular nutrient availability on bacterial gene
149 expression were tested in this study. The concentration of glucose in Luria-Bertani broth media
150 was altered. RNA was extracted to test the yield of the gene expression of *Escherichia coli*. To
151 study the intracellular process, the gene expression of *accA*, a subunit of the acetyl-CoA
152 carboxylase enzyme, was quantified via real-time PCR. The ACCase complex is an important
153 enzyme for the first step of bacterial fatty-acid synthesis. The cell metabolism within *Escherichia*
154 *coli* cells induces the expression of the four genes, *accA*, *accB*, *accC*, and *accD*, which code for
155 the translation of acetyl-CoA carboxylase. The four genes for acetyl-CoA carboxylase modify
156 the composition of fatty acids and monitor the rate of production. The four genes also monitor
157 the overexpression of ACCase by inserting one of the four subunits, activating antisense RNA
158 expression [9]. To quantify the activity of ACCase, a part of the intracellular metabolism, the
159 concentration of the gene *accA* was measured after exposure to glucose conjoined with antisense
160 RNA inhibition.

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

166 the ribosomal detection by the RBS inhibiting translation [15]. In this study, antisense RNA
167 obtained amplification through the PCR amplification of an antisense DNA sequence. The DNA
168 sequence converts to an antisense sequence when the DNA is flanked with Xho1 and Nco1
169 restriction sites. The PCR product was implanted into the pHN1257 plasmid through ligation
170 (Fig 1). In addition, *Escherichia coli* cells were converted into antisense expressing bacteria after
171 transforming the competent bacteria cells with the recombinant IPTG-PT-asRNA plasmid called
172 pHN1257. Real-Time PCR analysis was performed to determine the number of gene copies for
173 *accA*.

174 **Figure 1 Plasmid Assembly and the Gene Silencing of *accA***

175 Plasmid Assembly and Gene Silencing the PCR products and the PTasRNA expression vector
176 of the plasmid pHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI
177 (downstream). After the restriction digestion, the antisense PCR product was ligated into the
178 IPTG-PTasRNA inducible vector of pHN1257 at the multiple cloning site.

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

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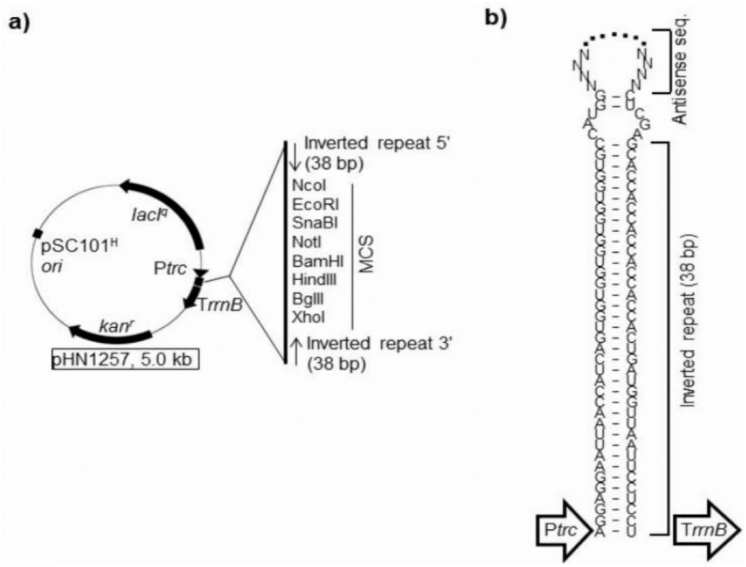

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190

191 In addition, suppressing the *accA* gene can model the mechanism of the pathogenic
192 bacteria repression of gene transcription based on the availability of nutrients and any other
193 physiological change [18]. In order to measure the effects of extracellular and intracellular
194 factors upon the intercellular communication between cells, the gene copy number of *luxs* will be
195 quantified. To test the correlation between glucose, *accA*, and *luxs*, the concentration of glucose
196 was decreased while inhibiting *accA*. The author hypothesizes that inhibiting *accA* while
197 lessening glucose availability will decrease the *luxs* gene copy number as quantified through
198 qPCR. The author expects to determine an integration between extracellular glucose,
199 intracellular metabolic gene expression, the effect of the two preceding stages of metabolism on
200 intercellular cell-to-cell signaling, and pathogenesis. The ultimate purpose of the study is to
201 demonstrate the immense potential of targeting the metabolic gene, *accA*, for overcoming
202 antibiotic resistance.

203 **MATERIALS AND METHODS**

204 **BACTERIAL CELL CULTURE AND RNA EXTRACTION**

205 The *Escherichia coli* TOP10 strain of cells were cultured and plated onto 25 mL agar
206 plates. The bacterial cells were inoculated into Luria-Bertani broth enhanced with glucose
207 concentrations of 200µM, 50µM, and 0µM. The cultures of 200, 50, and 0 micromolar (n=7) of
208 glucose were measured for the dsDNA and the RNA OD260 concentrations and repeated as
209 seven replicates. Varied concentrations of glucose including, 15mM, 7.5mM, 5mM, and control
210 of glucose were added to LB liquid with *Escherichia coli* cells. The plasmid pHN1257 was
211 utilized for assembly of the antisense expression vectors. The recombinant plasmid of pHN1257

212 for the expression of antisense RNA were plated with the kanamycin antibiotic and incubated at
213 37°C for 24 hrs. Cells grown on agar plates were inoculated into 4 mL of LB liquid media with
214 (+) and (-) PTasRNA bacterial cells expression of asRNA for *accA* cultured with kanamycin. A
215 sum of 20 RNA extractions were completed. A total of 3 mL of bacterial cell culture from the
216 cells grown in the varying concentrations of glucose, the (+) asRNA, and the (-) asRNA cells
217 were centrifuged at 4,000 x g for 10 minutes at 4°C. The medium was discarded, and the cells
218 were resuspended in 100µL of lysozyme/TE buffer. The solution was vortexed for 30 seconds.
219 Incubation occurred at 30°C for 10 minutes in a shaker-incubator. A lysis buffer of 350µL with
220 25mg of glass beads were added. The lysates were centrifuged for 5 minutes at maximum speed.
221 RNA was extracted using HiBind RNA mini columns through RNA Wash Buffers I and II. The
222 RNA was eluted with 50µL of DEPC water.

223 **CDNA PREPARATION**

224 The author used the OneScript Reverse Transcriptase cDNA Synthesis kit by Applied
225 Biological Materials Inc. or ABM. Approximately, 1µg of RNA from each sample, high glucose,
226 medium glucose, low glucose, and (-) glucose were added to 0.5µM oligonucleotides(dT)
227 (10µM). For the asRNA and (-) RNA, 1 microgram of each total RNA extract was added to the
228 initial primer/RNA and reaction mixture. The cDNA was also prepared for *luxs* quantification.
229 The initial primer/RNA mix consisted of 1µg of total RNA, 1µL of oligo(dt), 1µL of dNTP
230 (10mM) mix, and nuclease-free water for a 20µL reaction solution. The mixture was heated to
231 65°C for 5 minutes and then incubated on ice for 1 minute. The reaction mixture of 4µL 5X RT
232 buffer, 0.5µL of RNaseOFF, and 1µL OneScript RTase was added to the initial primer/RNA mix
233 in a 2µL microcentrifuge tube. The cDNA was synthesized by incubating the tube for 50 minutes

234 at 42°C. The reaction was stopped at 85°C for 5 minutes and chilled on ice. The cDNA was
235 stored at -20°C.
236
237 **PCR**
238 A Promega PCR MasterMix volume of 25µL was added to the upstream and downstream
239 primers each of 0.5µL specific for the *accA* gene target. PCR amplification was repeated as a
240 triplicate. The concentrations of the two cDNA samples added to the PCR master mix were
241 190ng and 230ng plus nuclease-free water. A 2X and 1X upstream primer, 10µM 0.5–5.0µl0.1–
242 1.0µM, and a downstream primer, 10µM 0.5–5.0µl 0.1–1.0µM, DNA template 1–5µl less than
243 250ng, and nuclease-free water were mixed into a 50µl PCR reaction mixture. The thermocycler
244 was set to denaturation of a 2-minute initial denaturation step at 95°C. Other subsequent
245 denaturation steps were between 30 seconds and 1 minute. The annealing step was optimized
246 with annealing conditions by performing the reaction starting approximately 5°C below the
247 calculated melting temperature of the primers and increasing the temperature in increments of
248 1°C to the annealing temperature. The annealing step was set at 30 seconds to 1 minute in 52°C.
249 For the extension reaction with Taq polymerase, there was 1 minute allowed for DNA to be
250 amplified at 72°C with a final extension of 5 minutes at 72–74°C. The PCR thermocycler
251 completed 40 cycles of amplification instead of 35 cycles.
252 **AGAROSE GEL ELECTROPHORESIS AND PLASMID ASSEMBLY**
253 The condition of the PCR products, without primer dimers and contamination, for the
254 target gene *accA*, were detected through agarose gel electrophoresis. The PCR products were
255 confirmed without primer dimers and contamination, the PCR products, and the PTasRNA
256 expression vector of the plasmid pHN1257 were digested with the restriction enzymes XhoI

257 (upstream) and NcoI (downstream) (New England Biolabs XhoI- catalog R0146S- 1,000 units
258 and NcoI- catalog R0193S- 1,000 units) (Fig. 1). Each microcentrifuge tube was placed in the
259 incubator for 30 minutes at 37°C. A heat block was heated to 90°C. The PCR products of the
260 *accA* gene were ligated into the pHN1257 plasmid by mixing 1µL of the DNA insert with 2µL of
261 the plasmids, adding 5µL of ligation mix, and then placing the tubes into a heat block of 90°C
262 for 15 minutes (Takara Ligation Kit 6023). Competent bacterial cells were transformed with the
263 pHN1257plasmid plus the antisense DNA insert of *accA*, for expressing (+) asRNA and (-)
264 asRNA, which is the control. The competent cells were incubated with the recombinant DNA for
265 45 minutes at 37oC.

266 **REAL-TIME PCR**

267 The author enhanced each bacterial sample with the glucose concentrations of 15mM,
268 7.5mM, 5mM, and 0mM (n=3). A triplicate of real-time PCR experiments was completed. The
269 cDNA from H-glucose, M-Glucose, L-glucose, and the control (-) glucose were diluted into a 1:8
270 serial dilution. A 10-fold dilution of standards was prepared for bacterial samples transformed
271 with (+) asRNA inducible plasmids and exposed to high to a low volume of glucose. The amount
272 of gene expression for *accA* and the target gene *luxs* were measured through absolute
273 quantification methods of qPCR. The diluted cDNA was pipetted into a clear 96 well plate. A
274 0.4µl of each primer pair mixture was pipetted into a microcentrifuge tube with 10µL of
275 SensiMix SYBR MasterMix, and with 1.2µL of DEPC water. The SYBR reaction mixture of
276 12µL was then pipetted into the wells with the diluted and undiluted cDNA. Optical film sealed
277 the plate. To reposition all liquids to the bottom of the wells, the PCR plates were centrifuged for
278 2 minutes at 2500 rpm. The real-time PCR Amplification Protocol (Roche Lightcycler 480
279 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

280 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
281 started at 55°C with a 0.5°C increment at each step up to 95°C. For the Roche Lightcycler 480
282 machine, the 96-well clear Roche plates were used for SYBR green detection.
283

284 **THE qPCR ABSOLUTE QUANTIFICATION OF LUX-S**

285 Bacterial cells were transformed with the pHN1257 plasmid expressing asRNA to inhibit
286 the genetic expression of the *accA* gene. Three replications of qPCR were performed for
287 quantifying the gene copies of *luxs*. The author added 25µM and 5µM concentrations of glucose
288 to the bacterial samples expressing antisense RNA for the inhibition of the gene *accA*. RNA was
289 extracted from cells grown with 25µM glucose and 5µM of glucose. The cells grown with
290 glucose in the medium were also transformed with antisense expressing IPTG-PT-asRNA
291 inducible pHN1257plasmids. Cells were cultured without (-) glucose but the expression of *accA*
292 was also inhibited, (+) asRNA. A control, without (-) glucose and (-) asRNA, was compared to
293 each sample. The number of gene copies for *luxs* were measured through the qPCR amplification
294 protocol and absolute quantification (Roche Lightcycler 480 machine).

295 **STATISTICAL ANALYSIS**

296 Figures were constructed with The Sigma Plot 14th edition, Microsoft excel, and with
297 Graph Prism Edition 8. The mean and standard deviations were calculated by Microsoft excel
298 and GraphPad Prism. The p-values and the ANOVA results were produced through GraphPad
299 Prism. Standard Error bars for each figure were added through Sigma plot. The standard curves
300 for the absolute of quantification of the genes *accA* and *luxs* were generated through Microsoft
301 excel with linear regression coefficients added and calculated for each standard curve for the
302 absolute quantification of *accA* and *luxs*.

303 **RESULTS AND DISCUSSION**

304 **THE EFFECT OF GLUCOSE ON THE RNA CONCENTRATION AND THE**

305 **EXPRESSION OF ACCA**

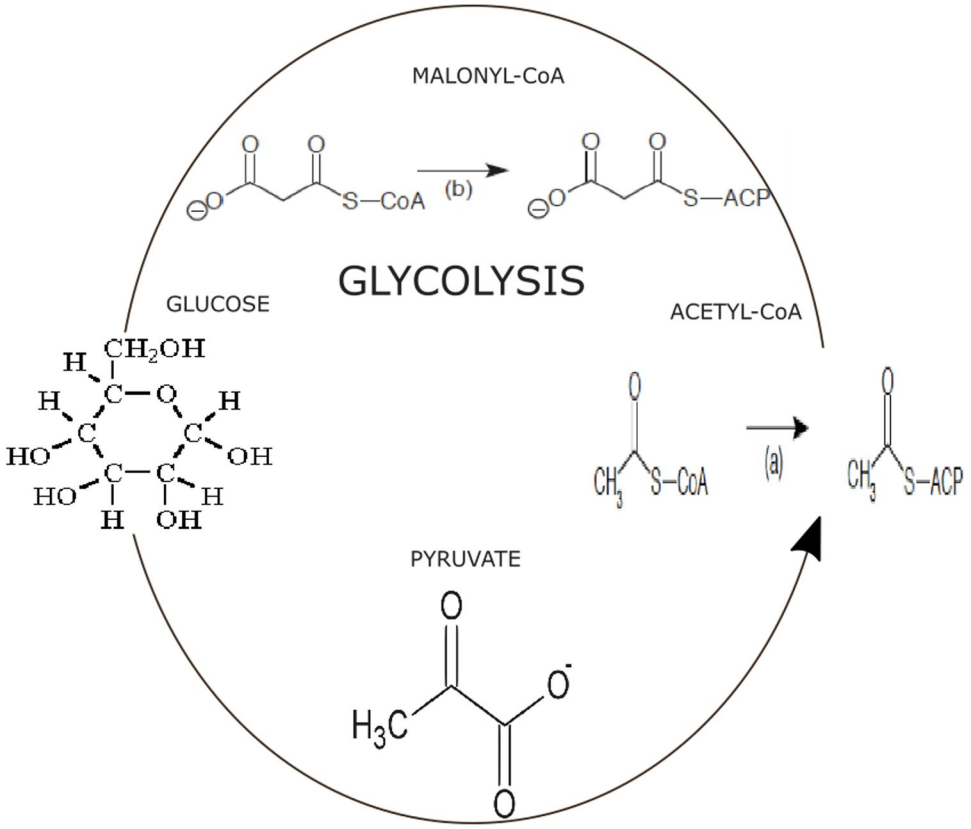
306 Hydrolytic enzymes of *Escherichia coli* split one glucose molecule into two pyruvates.
307 The acetyl-CoA carboxylase enzyme transfers a carboxyl group from the pyruvate to the acetyl-
308 CoA, producing malonyl-CoA (Fig 2). The acetyl-CoA carboxylase enzyme consists of four
309 subunits combined into the AccABCD complex. The *accA* gene codes for the ACCA subunit,
310 which is the transferase domain of the ACCase enzyme. From the results, the genetic expression
311 of *accA* was proportional to the increased glucose concentrations in *Escherichia coli* bacterial
312 samples. After culturing *Escherichia coli* with different levels of glucose, the RNA
313 concentrations for each sample measured were of 1392, 797, 608, and 199 ng/ µL for the H-
314 Glucose, M-Glucose, the L-Glucose, and the control, respectively. The genes of *accA* and *accD*
315 are entirely independent of the *accBC* operon.

316 When *accB* is overexpressed, *accBC* transcription is blocked, but the overexpression of
317 the other gene products, *accA* and *accD*, don't affect the *accBC* operon transcript levels [21]. A
318 high level of glucose increases the availability of *accB*, and the transcription of the *accBC*
319 operon is greatly reduced even after exposure to a small amount of *accB*. Although a high
320 glucose concentration can stymie the production of *accBC*, the over-activated *accBC* operon did
321 not inhibit the production of *accA*, the target gene of study. The subunit ACCA encoded in the
322 *accA* gene may act as a more intense target for blocking bacterial cell fatty acid synthesis and
323 metabolism. The *Escherichia coli* TOP10 bacteria cells were grown in LB broth overnight at
324 37°C with 15mM (High-glucose), 7.5mM (Medium-glucose), 5mM (Low-glucose), and a control
325 of glucose concentration (Fig 3). RNA was extracted from each sample and the concentrations

326 determined by the Implen Nanophotometer 250. The high-glucose sample produced the highest
327 amount of RNA compared to the medium, the low, and to the control. The RNA for each sample
328 was reverse transcribed into first-strand cDNA and absolute quantification with qPCR measured
329 the amount of the target gene, *accA*, of each sample.

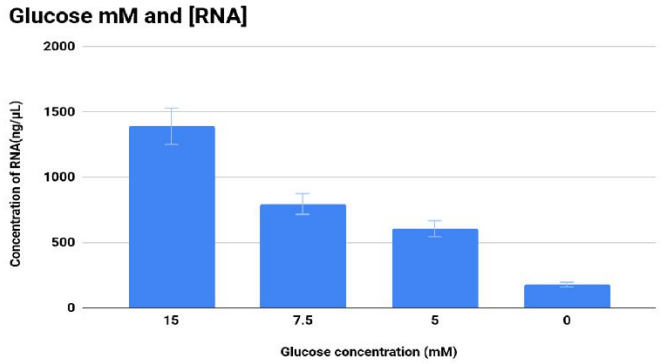
330 **Figure 2 Formation of Malonyl-CoA** A glucose molecule is hydrolyzed into two pyruvate
331 molecules. The acetyl-CoA Carboxyl transferase or the ACCA alpha subunit of the acetyl-CoA
332 carboxylase complex, AccABCD, transfers a carboxyl group from the pyruvate to the acetyl-
333 CoA to form malonyl-CoA. The malonyl-CoA enters the fatty acid synthesis pathway,
334 elongating fatty acids into long-chain fatty acids of phospholipids necessary for structuring
335 plasma membranes and for forming the initial extracellular matrix.

336

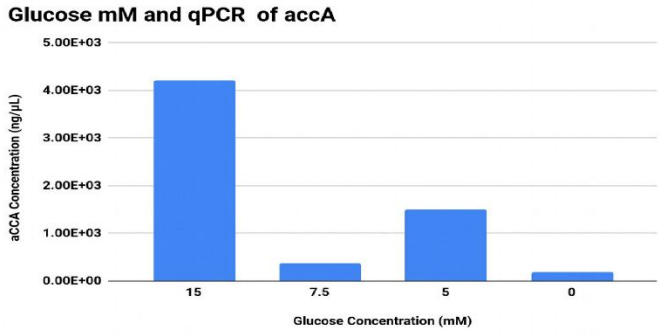


337

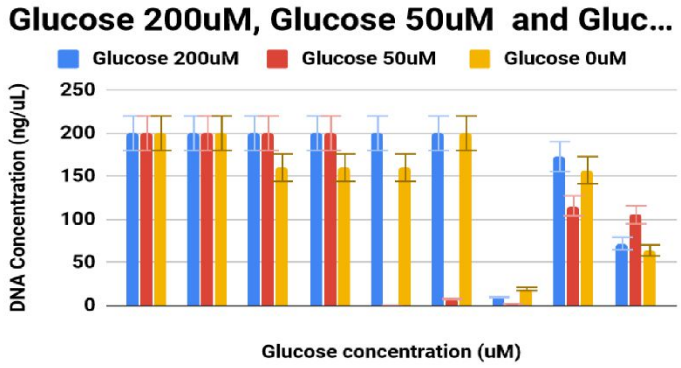
338 A) **Figure 3 Glucose concentration versus RNA** Glucose concentrations of 15mM, 7.5mM,
339 5mM, and the control samples each yielded RNA concentrations, in ng/uL, 1392, 797, 608, 179,
340 respectively. A) The bacterial sample with 15mM glucose yielded the largest measure of RNA,
341 noting a proportional relation between glucose and genetic expression of *E. coli*. B) Includes
342 qPCR results for 15mM, 7.5mM, 5mM, and the control. The samples with 15mM and 5mM
343 glucose displayed the most genetic activity of *accA* transcription, measuring *accA* concentrations
344 of 4,210 ng/uL and 1,500 ng/uL respectively. The *accA* qPCR concentrations for 7.5mM *accA*
345 equaled 372 ng/uL and a 196 ng/uL quantity for the control. C) Displays the OD260 measure of
346 DNA results for samples of *E. coli* grown in medium enhanced with 200uM, 50uM, 0mM of
347 glucose.


Glucose mM and [RNA]
2000
1500
1000
500
0
Concentration of RNA(ng/µL)
15
7.5
5
0
Glucose concentration (mM)

A)


Glucose mM and qPCR of accA
5.00E+03
4.00E+03
3.00E+03
2.00E+03
1.00E+03
0.00E+00
aCCA Concentration (ng/µL)
15
7.5
5
0
Glucose Concentration (mM)

B)


Glucose 200uM, Glucose 50uM and Gluc...
Glucose 200uM
Glucose 50uM
Glucose 0uM
250
200
150
100
50
0
DNA Concentration (ng/uL)
Glucose concentration (uM)

C)

349 The RNA of each bacterial sample enhanced with glucose was extracted and the
350 concentrations determined (N=20, 335.4 ± 329.2). The high-glucose sample provided a crossing
351 point value or a Cp-value of 12.28 and the concentration of *accA* was 4.21E3 ng/µL. The Cp of
352 the sample of medium-glucose equaled 16.51 with a concentration of 3.75E2 and the low-
353 glucose Cp was 14.08 with a target gene concentration of 1.50E3 (Fig 3). The concentration of
354 *accA* by qPCR analysis, in ng/µL, included: 4210, 375, 1500, and 196 for H-glucose, M-glucose,
355 L-glucose, and for the control, respectively. The number of qPCR products ranged from 11-186,
356 5-18, and 0-79 gene copies for High-glucose, Medium-glucose, and the Low-glucose/control,
357 respectively (N=12, 48±38).
358 The control group produced a Cp value of 17.64 with a target gene concentration of
359 1.96E2. For the glucose concentrations of 200µM (n=7,173±72), 50 µM (n=7,116±105), and the
360 control (n=7, 157±64) compared to their dsDNA concentrations showed significant variability
361 (Fig 3).Comparing 200 µM to its control group, the difference between the samples was
362 statistically significant (p=0.038). The cells grown in 200µM yielded a more significant and
363 larger production of dsDNA than cells cultured as a control. After loading a 96-well plate for
364 qPCR with 8-fold dilution standards of each sample, the standards of each sample were used to
365 calculate a standard curve (Fig 3).
366 Vinals et al., (1999), found results that determined that a high glucose concentration
367 causes the absorption of glucose to be blocked as a result of its repression of glucose
368 phosphorylation [19]. Ferraris et al. (1990), measured the luminal glucose osmolarity and the
369 concentrations in the small intestines. They found that the luminal glucose concentrations in a
370 range of 50mM to 500mM exhibited errors [20]. The previous researchers measured glucose
371 concentrations but did not recognize how osmolality is affected by Na+ and K+ salts, amino

372 acids, and peptides [20]. Ferrais et al. (1990), discovered the average of SI luminal osmolarities
373 were approximately 100 mosmol/kg, which were mainly hypertonic results. In the Ferrais et al.
374 (1990) study, for an animal's diet, the SI glucose concentrations averaged 0.4-24 mM and ranged
375 with time over a large amount of an SI region from 0.2 to 48 mM. They also found that high
376 concentrations of glucose lessened the absorption of glucose in endothelial cells called ECV304
377 cell lines. There was a 60 percent reduction of 2-deoxyglucose uptake at 30 mM glucose from
378 their study [20]. The high glucose osmolality resulted in a reduced Vmax value for 2-
379 deoxyglucose uptake with a constant Km, calculated from the Michaelis-Menten kinetic enzyme
380 equation [20].

381 **THE QPCR RESULTS OF THE ANTISENSE RNA INHIBITON OF THE TARGET**
382 **GENE ACCA**

383 Ninety percent of the genome for Eukaryotes consists of non-coding regions or RNA that
384 will not translate into proteins. Antisense transcription is the purpose of the noncoding regions of
385 human cell DNA [22]. The non-coding transcripts are called natural antisense RNAs. Antisense
386 activity has been recorded in mice, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and
387 *Arabidopsis thaliana* [23]. Recombinant DNA was produced when the PCR product of the gene
388 insert, *accA*, was ligated into the plasmid pHN1257 (Fig. 1), which was engineered to amplify
389 antisense RNA. The PCR product and the IPTG-PT-asRNAs plasmid of pHN1257 were cut with
390 the restriction enzymes XhoI and NcoI. The primers (Table. 1) each contained an extra 3 to 4 bps
391 of nucleic acids to accompany the sequences for Xhol and Nco1. The primers flanked the target
392 DNA, which totaled to 150 base pairs. The antisense sequence was constructed in a specific
393 orientation in that the restriction sites, which NcoI normally flanks the forward primer, reversed
394 positions. The XhoI was repositioned to flank the forward primer, and the Nco1 flanked the

395 reverse primer, creating the antisense sequence when inserted into the plasmid for pHN1257.

396 The PCR product was amplified (n=3, 219 ± 171), and then ligated into the pHN1257-asRNA

397 inducible vector.

398 **Table 1 Primers for amplifying the *luxs* gene, antisense RNA for *accA*, and the *accA* gene.**

399

Primers	DNA Sequence
XhoI+*accA* Forward	GAGATGAGTCTGAATTTCCTTGATT
NcoI+*accA* Reverse	TGGCAGTTCATCGCTTTTTTCAC
luxs Forward	CATACCCTGGAACATCTGTTTGC
luxs Reverse	AGTTCCTGCACTTTCAGCACATC
accA Forward	TCATCACCTTTATCGACACCCC
accA Reverse	TTCACTTTATCGCCCACGCC

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401

402 Primers for PCR Amplification and qPCR Analysis. To produce antisense RNA for *accA*

403 inhibition the restriction enzyme site for XhoI was sequenced with the forward primer for *accA*.

404 The restriction enzyme NcoI was added to the reverse primer for *accA*. Through PCR

405 amplification, the PCR products were composed of antisense DNA for the *accA* gene. The PCR

406 product would be inverted when inserted into the IPTG-PT-asRNA inducible pHN1257 plasmid,

407 forming recombinant DNA plasmids for asRNA transcription in vitro. The plasmids with

408 recombinant antisense DNA were transformed into bacterial competent cells for qPCR analysis

409 of the *accA* gene. The primers for the *luxs* gene were applied to quantify the number of gene

410 copies from qPCR analysis for cells grown in glucose enhanced samples. The primers for the

411 *accA* gene were used for the quantification of the *accA* gene from the samples cultured with an

412 increasing gradient of glucose solutions.

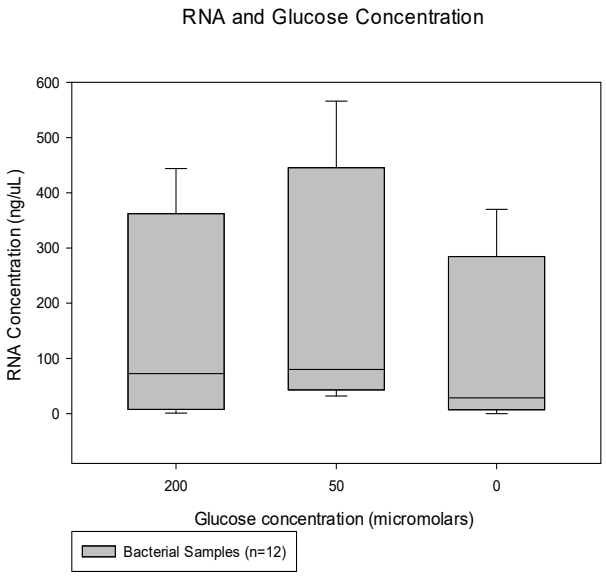
413 The total RNA concentration for the control, (-) asRNA, equaled 739.44 ng/µL. The

414 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. 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 5). 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].

Figure 4 RNA Concentrations vs. Glucose Concentration A) *E. coli* bacteria cultures (n=3) 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)



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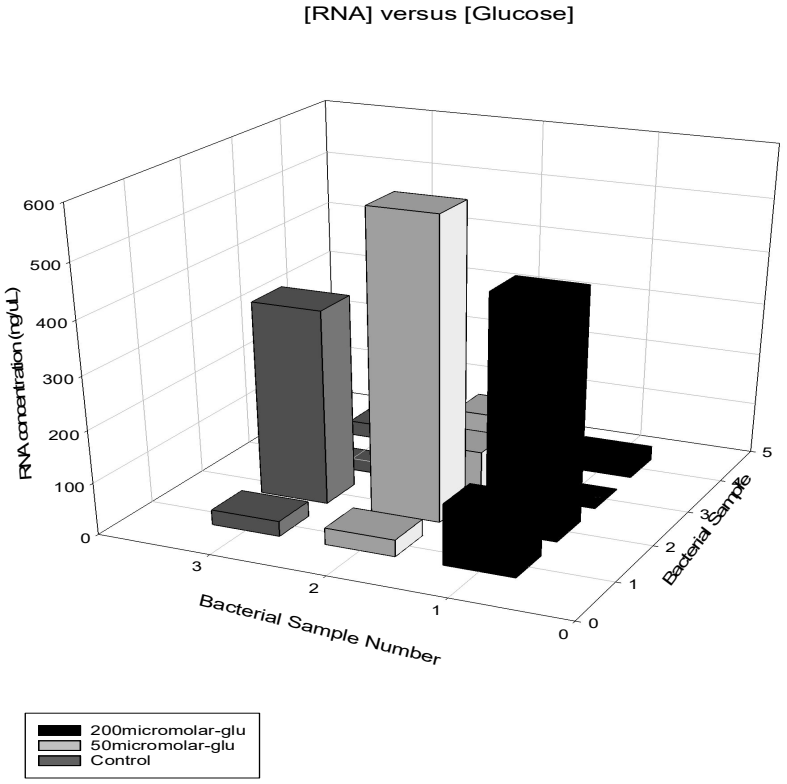
432 B)



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435 **Figure 5 The qPCR results for quantifying *accA* concentration and the gene copy number.**

436 The RNA for each sample, 15mM, 7.5mM, 5mM, and the control, was reverse transcribed into

437 first strand cDNA and absolute quantification with qPCR was used to measure the amount of the

438 target gene, *accA*, produced by each sample. A) High glucose had a Cp of 12.28 and the

439 concentration of *accA* was 4.21E3 ng/uL. The Cp of sample medium-glucose equaled 16.51 with

440 a concentration of 3.75E2 and the low-glucose Cp was 14.08 with target gene concentration of

441 1.50E3. B) The gene of *accA* was successfully suppressed by asRNA in vitro with 63 gene

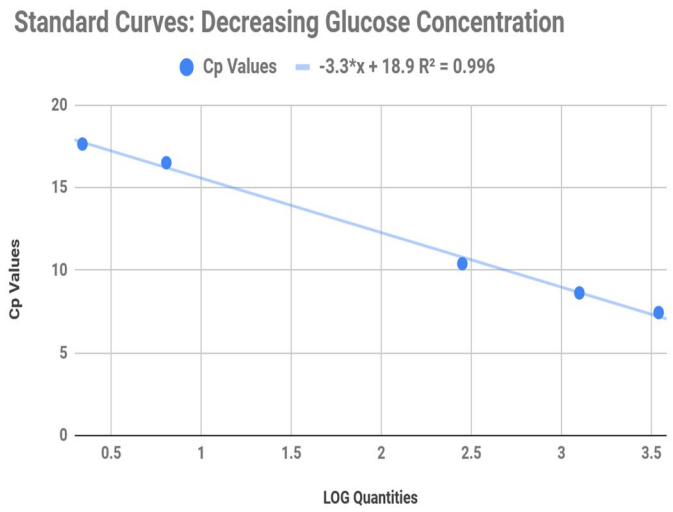
442 copies measured for bacteria cells transformed with the recombinant antisense pHN1257 plasmid

443 DNA. The bacterial cells with the pHN1257 plasmid but without the antisense gene target and

444 insert produced 421.69 ng/uL for *accA*. There was a 147% percent difference between cells not

445 expressing asRNA versus the cell transcription of the asRNA for *accA* (p=0.027).

446 A)



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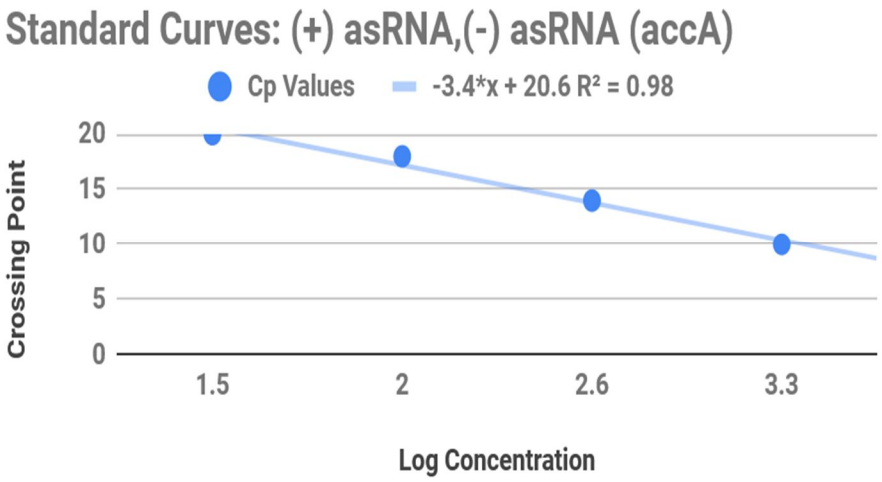
448 B)



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450 During short-chain fatty acid synthesis, malonyl-CoA is formed from acetyl-CoA, using

451 energy from ATP and bicarbonate production [21]. Glucose is hydrolyzed into pyruvate, which

452 is made into acetyl-CoA, forming acetate (Table.2). Through the Wood-Ljugdahl pathway,

453 pyruvate loses two hydrogens, carbon dioxide loses oxygen, altered into carbon monoxide, and a
454 methyl group is added to the oxidation, synthesizing acetyl-CoA into Acetate [25]. Butyrate is
455 produced when two molecules of acetyl-CoA combine into acetoacetyl-CoA. The acetoacetyl-
456 CoA is transformed into butyryl-CoA, or butyrate [20]. Propionate is composed through the
457 succinate or acrylate pathway [26]. The phosphoenolpyruvate, or PEP, is broken into pyruvate
458 which is further metabolized with water into succinate. The succinate is reduced into propionyl-
459 CoA, forming propionate (Table. 2). Through the acrylate pathway, lactate loses oxygen,
460 forming propionate [25].

461 **Table 2 Pathways for Biosynthesis of SCFAs from Carbohydrate Fermentation** For
462 synthesis of each SCFA, glucose is hydrolyzed into 2 molecules of pyruvate, which then lose a
463 hydrogen, forming Acetyl Coenzyme A. For butyrate the Acetyl coenzyme A loses a water,
464 forming B-hydroxy butyryl CoA, where it loses a hydrogen and the CoA is replaced with a
465 Phosphate, the phosphate is removed. After the phosphate is removed, butyrate is formed.
466 Acetate forms when the CoA in Acetyl Coenzyme A is replaced with a phosphate group, and
467 then the phosphate is added to an ADP to make ATP. Propionate can be formed when lactate
468 loses an oxygen, giving Acryloyl-CoA, converted into Propionyl-CoA, which the CoA is
469 replaced with a phosphate, and then removed by a kinase, to produce propionate.

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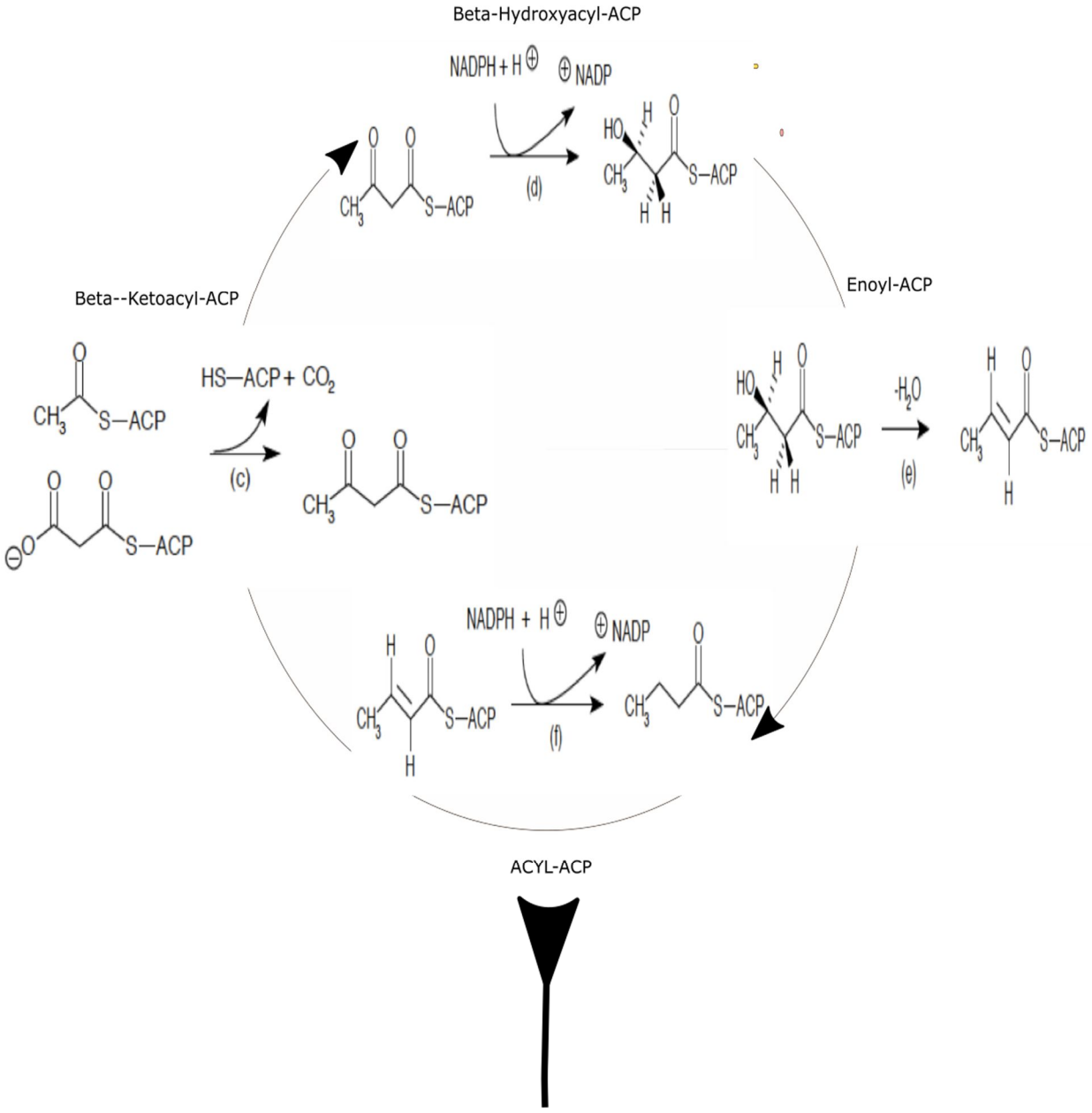
Glucose	Glucose	Glucose
2 Pyruvates Lose a Hydrogen	2 Pyruvates Lose a Hydrogen	2 Pyruvates Lose 1 Hydrogen Gain 1 Hydrogen
Acetyl CoEnzyme A	Acetyl Coenzyme A	LACTATE Loses 1 Oxygen
B-hydroxybutyryl CoA Lose H2O	Phosphate Replaces The CoA	Creates Acryloyl-CoA
Crotonyl CoA Gains a Hydrogen	Creates Acetyl-Phosphate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

481

482 The LCFAs are synthesized when the ACCA subunit of ACCase transfers a carboxyl
483 group to acetyl-CoA converting into malonyl-CoA, which then enters the fatty acid elongation
484 cycle (Fig 6). The fatty acid cycle synthesizes long chains of fatty acids for the formation of the
485 bacterial plasma membranes and a thin-layered matrix for initial bacterial attachment to a biotic
486 or abiotic surface. After bacteria attach to a surface, more growth occurs until a microcolony of
487 bacterial cells form. In addition, the gene *accA* codes for the subunit of the Acetyl-CoA
488 Carboxylase complex. Acetyl-CoA carboxylase (ACC) initiates the first step of the fatty acid
489 synthesis. An acyl-group or an ACP is added to the malonyl-CoA and converted into a ketoacyl-
490 ACP. The Beta-ketoacyl-ACP is reduced, gains a hydrogen, into Beta-hydroxyacyl-ACP. The

491 Beta-hydroxyacyl-ACP is dehydrated, losing a molecule of water, to alter into an Enoyl-ACP,
492 which forms into an Acyl-ACP by gaining 2 hydrogen protons.

493 **Figure 6 Long-Chain Fatty Acid Synthesis** An acyl-group or an ACP is added to the
494 malonyl-CoA and converted into a ketoacyl-ACP. The Beta-ketoacyl-ACP is reduced, gains a
495 hydrogen, into Beta-hydroxyacyl-ACP. The Beta-hydroxyacyl-ACP is dehydrated, losing a
496 molecule of water, to alter into an Enoyl-ACP, which forms into an Acyl-ACP by gaining 2
497 hydrogen protons.



PHOSPHOLIPIDS

503 **QUANTIFICATION OF LUX-S GENE EXPRESSION**

504 The gene *accA* is a component of the ACCA subunit of the ACCase complex, which
505 functions as a transferase. The ACCA subdomain of ACCase is vital for catalyzing fatty acid
506 synthesis. Lipid biosynthesis is responsible for increased quorum sensing and biofilm formation.
507 After glucose is hydrolyzed into two pyruvate molecules, the alpha subunit, ACCA, transfers a
508 carboxyl group to acetyl-CoA to produce malonyl-CoA. The malonyl-CoA enters the lipid
509 biosynthesis pathway. The fatty acids produced assist with secreting a more fluid membrane,
510 stretching the span of the biofilm matrix. As the biofilm matrix expands the microcolonies of
511 bacteria grow proportional to the size of the matrix. The increase in the number of colonies
512 induces the release of autoinducers, which accrue into a display of bioluminescence. The
513 bioluminescence is a signal of quorum sensing. The gene *luxs* is needed to produce autoinducer-
514 2. *luxs* monitors the amount of biofilm, flagellum movement, and regulates the factors of
515 virulence. The ACCA subunit catalyzes the synthesis of lipids required for inducing the release
516 of AI-2, which are signals produced during quorum sensing.

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

526 Therefore, there can exist a significant link between bacterial cell metabolism and
527 virulence. By quantifying the measurement of gene copies for *luxs* while inhibiting *accA* with
528 asRNA, the connection of metabolism with pathogenesis could be demonstrated (Fig 7).
529 *Escherichia coli* cells were grown in 25µM and 5µM of glucose and transformed with antisense
530 pHN1257expressing IPTG-PT-asRNA plasmids. Cell samples with 5µM glucose- (+) asRNA
531 produced a high measure of the *luxs* gene [11]. The gene copy number of *luxs* for (+) glucose/
532 (+) asRNA, and for (+) asRNA were quantified. For the results, the (-) glucose-(+) asRNA
533 sample produced a gene copy number of 199 and 2511 *luxs* copies for (-) glucose- (-) asRNA
534 (n=3, 277 ± 37). For the 25µM glucose-(+) asRNA and 5µM glucose (+), asRNA produced
535 39,810 and 316,228 gene copies of *luxs*, respectively (Fig 8). The control samples, the (-)
536 glucose-(-) asRNA, (n=3, 658,114 ± 483,499) and the 5µM glucose- (+) asRNA (n=3,
537 171571±204574) exhibited the highest amount of expression for *luxs* with a $p<0.05$ ($p=0.025$)
538 (Fig 8). An ANOVA analysis of the qPCR gene copies for the *luxs* gene is displayed in Table 3.

539 **Figure 7 Gene Copies for *luxs* determined through qPCR measurement**

540 The qPCR Results for *luxs* the number of gene copies for *luxs* was measured through qPCR and
541 absolute quantification A) (-) glucose-(+)asRNA sample had a gene copy number of 199. B)
542 There were 2511 *luxs* copies for (-)glucose-(-)asRNA. The 5uM glucose-(+)asRNA yielded
543 316,228 gene copies of *luxs* D) For the 25µM glucose-(+)asRNA 39,810 gene copies were
544 produced.

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549 **A)** **B)**

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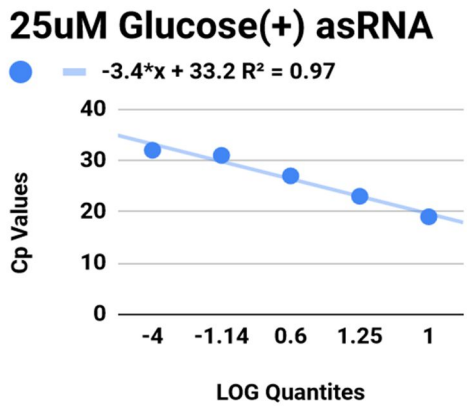

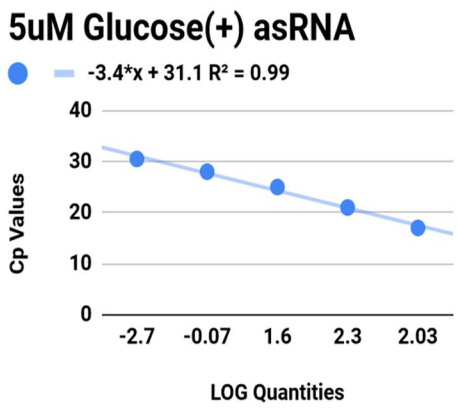

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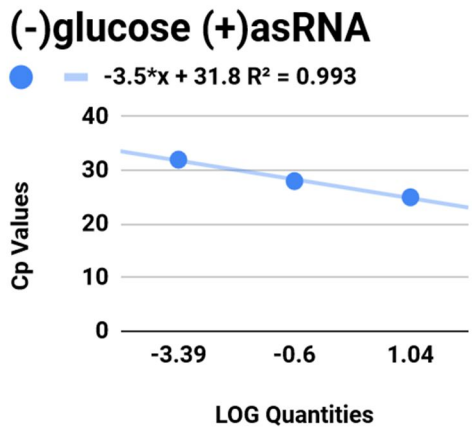
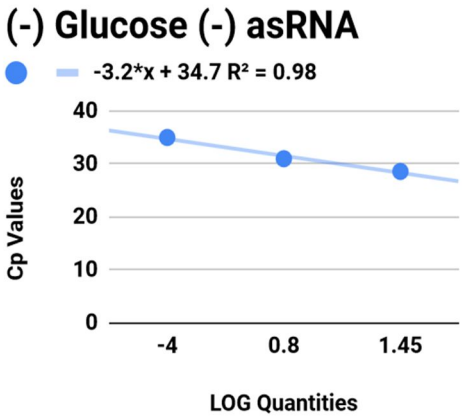
554 **C)** **D)**





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558 **Figure 8 Total Production of *luxs* versus the Control Sample.** The polar plot compares the

559 control sample for the qPCR quantification of the gene *luxs* after exposure to glucose and

560 antisense RNA in the form of the pHN1257-IPTG-asRNA expression plasmid. A) The control

561 compared to the asRNA sample, to the 25micromolar-glu-asRNA sample, and versus the 5**µ**M-

562 glu-asRNA sample equaled the following polar coordinates (radius, theta): ($1x10^6$, 180°), ($5x10^5$,

563 60º), and (6x10^5,68º), respectively. B) The control, 5, 25, and the asRNA micromolar samples

564 production of the *luxs* gene are displayed in the 3D contour graph as an intense red, purple/blue

565 for the 5micromolar-glu-asRNA, and purple for the asRNA including the 25micromolar-glu-

566 asRNA sample. The control sample yielded a higher measure of *luxs* with 1x10^6 gene copies

567 (658,114 ± 483,499). The sample without glucose but with asRNA expression produced 199

568 gene copies of *luxs* (n=3, 277 ± 37).

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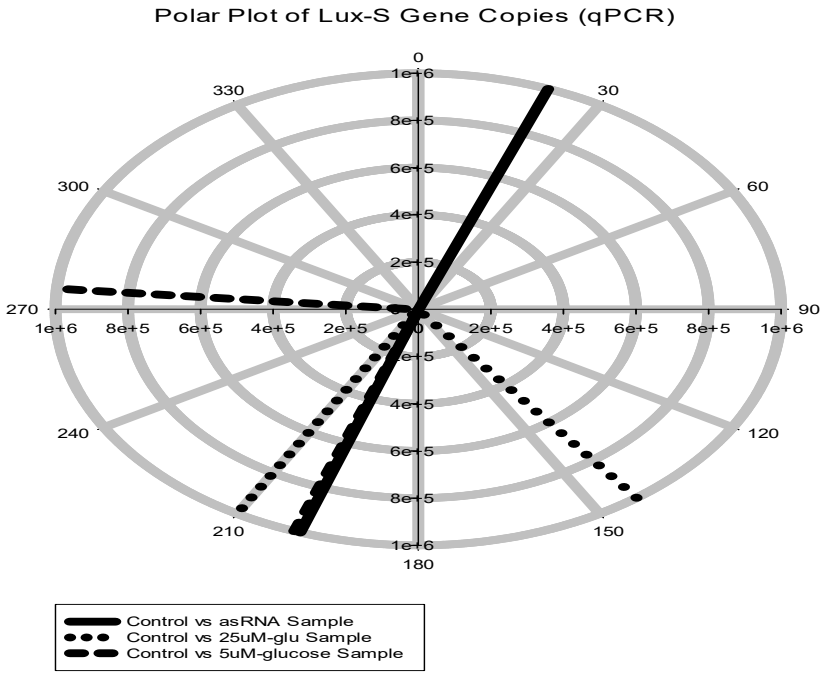
572 **A)**



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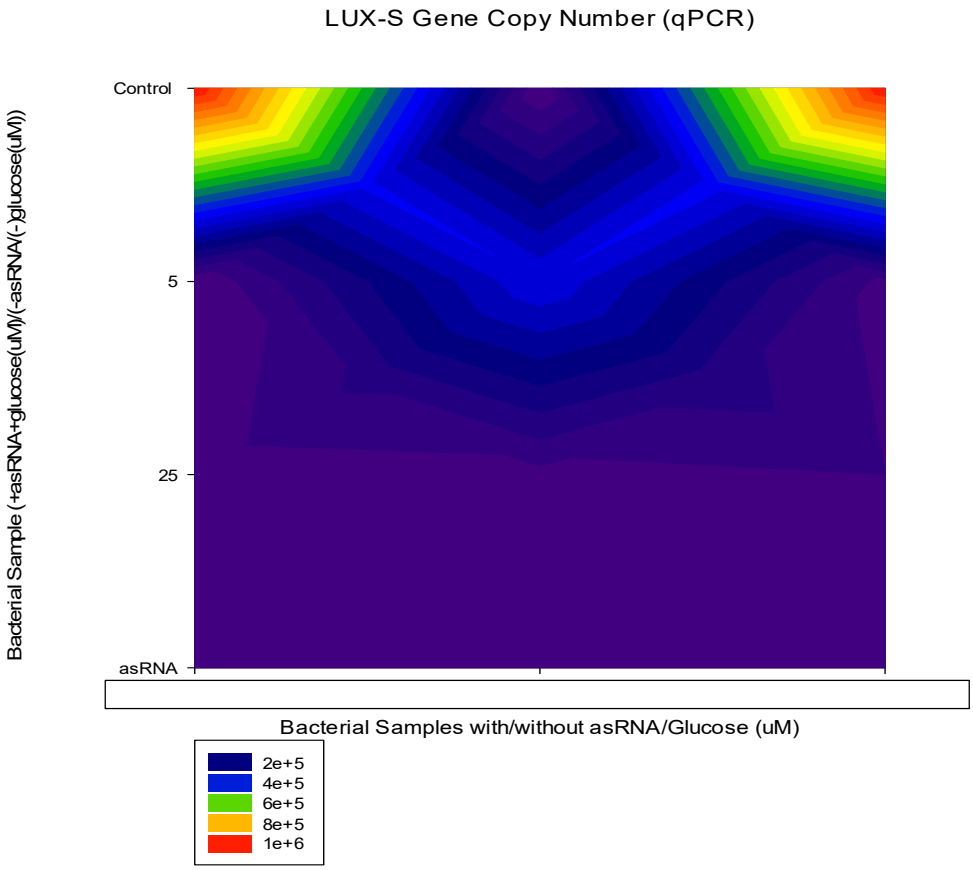
588 **B)**



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591 **Table 3 ANOVA results for the total experimental sample for quantifying gene copies**
592 **through qPCR.**
593

ANOVA Results of qPCR Gene Copies

	luxs-glu/(+) asRNA	*luxs*-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**	**1798315304447.048**			

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 *luxs* and *accA* with 547774.000 as a mean of the total number of gene copies yielded from qPCR analysis p=0.025.

The 5µM glucose- (+) asRNA sample expressed more of the *luxs* 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 *luxs* 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 *luxs* gene expression. However, the excess saturation of glucose at 25µM did not result in the amplified gene expression of *luxs*. Jesudhasan et al. (2010) proved that higher levels of

607 autoinducer-2 did not lead to the increased gene expression of *luxs* in *Salmonella typhimurium*
608 when cultured in high concentrations of glucose [25].

609 Glucose affected the rate of transcription, increased the production of nucleic acids, but
610 the inhibition of *accA* with asRNA significantly suppressed gene expression. From the control to
611 the 25 μM glucose exposure, bacteria cells transcribed DNA at an amplified rate (Fig 9). As the
612 glucose concentration increased to 50 μM the rate of transcription reached a sustained
613 equilibrium with no new nucleic acid production or activity as the concentration approached 200
614 μM. The samples expressing asRNA inhibition of *accA* produced 16% of the total yield of
615 nucleic acid. Samples not expressing asRNA inhibition, yielded 83% of the total quantity of
616 nucleic acid.

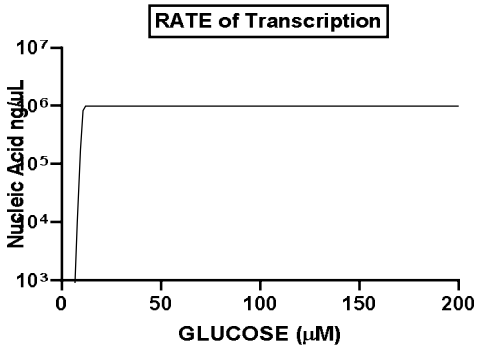
617 Moreover, by transforming *Escherichia coli* bacterial cells with IPTG-PT-asRNA
618 inducible vectors, which targeted the gene *accA*, the gene expression of *luxs* gene also decreased.
619 Because glucose delays the movement of the *luxs* mutant strain with bacterial overpopulation but
620 does not inhibit bacterial growth, each (+) glucose-(+) asRNA bacterial colony appeared smaller
621 and defected. Osaki et al., (2006) showed that *luxs* mutants can be smaller in diameter with 8.0
622 mm versus 12.3 mm in its wildtype [29]. The bacterial colonies expressing antisense RNA
623 (5mm) were a smaller size versus cells without antisense RNA transcription (8mm) of *accA*. The
624 cell samples with (+) asRNA transcription were found by many preceding research studies to
625 appear smaller in size [21].

626 Bacterial samples transformed with IPTG-PT-asRNA inducible pHN1257 plasmids and
627 designed to inhibit the *accA* gene displayed the least measure of *luxs* expression. The (-) glucose-
628 (+) asRNA sample exhibited the lowest amount of *luxs* expression of 199 gene copies (225 ±
629 37). As a result, when the *luxs* gene is mutated with less expression, the biofilm is thinner,

630 looser, and displaying an appearance of bacterial cells with defects. Lux-S forms a thicker and
631 more viscous biofilm, which is a result of many nucleic acids released into the extracellular
632 matrix of the biofilm.

633 **Figure 9 The effect of glucose on the rate of transcription, inhibition with asRNA, and the**
634 **frequency of Nucleic Acid production.** A) From the control to the 25 μM glucose exposure,
635 bacteria cells transcribed DNA at an amplified rate. As the glucose concentration increased to 50
636 the rate of transcription reached a sustained equilibrium with no new Nucleic Acid production or
637 activity. B) The gene *accA* was inhibited with asRNA. The samples expressing asRNA inhibition
638 of *accA* produced 16% of the total yield of nucleic acid. Samples not expressing asRNA
639 inhibition, yielded 83% of the total quantity of nucleic acid.

640 **A)**



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643 **B)**
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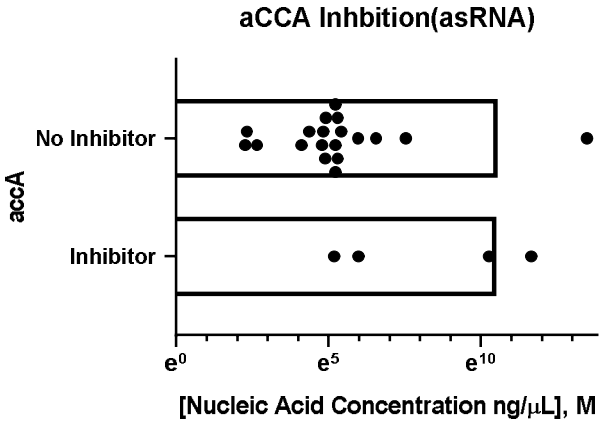

645

646 The ample amounts of DNA are released to maintain a more solid macromolecular matrix
647 and consistency of the biofilm [30]. Lux-S is required for AI-2 production and for regulating
648 gene expression in the early-log-growth phase. Pathogenic bacteria depend on biofilm formation
649 to attach to epithelial cells and tissues, spreading infectious diseases. Pathogenic bacteria infect
650 host cells by accumulating AI-2, exogenously, which then increases the amount and consistency
651 of the formation of biofilm (31). The gene *luxs* codes for the flagella complex, biofilm creation,
652 and controls virulent factors. The *luxs* gene, when it is more expressed, causes the spread of
653 virulent bacteria through increasing the motility and movement of pathogenic bacteria. More
654 Lux-S expression, as a result of mutations in the *luxs* gene, produces increased amounts of
655 biofilm, assisting pathogenic bacteria to invade the mucosal layers and the epithelial cells of its
656 host, developing into a systemic infection.
657 The autoinducer-2 that accumulates, monitors cell growth by permitting bacterial cells to
658 sense the excess AI-2 and then terminate population growth. The excess AI-2 distributes quorum
659 signals between bacterial cells, which notifies cells of limited nutrients, surface area, or space.
660 Autoinducer-2 requires the genetic expression and translation of *luxs* (Fig 8). When Lux-S and
661 autoinducer-2 are less regulated and overexpressed, infectious bacteria can propagate in stressful
662 environments with high acidity and salinity [23]. Kendall et al., (2008) demonstrated the ability
663 of *S. pyogenes* to adapt to acidic conditions as a result of an unregulated Lux-S/AI-2 system [32].
664 The *Vibrio fischeri* process of quorum sensing differs drastically compared to the
665 *Escherichia Coli*. *Vibrio fischeri* synthesizes AI-2 through an enzyme called an autoinducer
666 synthase or the Lux I that produces a hormone class of acyl-homoserine lactones, AHL, which is
667 the autoinducer 3OC6-homoserine lactone. In *Vibrio fischeri* the AHL binds to the autoinducer
668 receptor called Lux-R for initiating a transcriptional activator of DNA [27]. After AHL is

669 produced, it passes through the cell's plasma membrane as the number of bacterial cells increase,
670 and then it stops diffusing after cell density reaches a threshold. When the AHL is attached to the
671 LuxR complex, the operon is activated, transcribing the genes that code for the enzyme
672 luciferase [27]. The LuxR-AHL bond releases, into the periphery of the cell, high levels of
673 quorum sensing molecules that stimulate the cell to cell communication between bacteria species
674 [24].

675 The *Vibrio fischeri* process of quorum sensing immensely differs in comparison to
676 *Escherichia Coli.* In addition*, Escherichia Coli* and many gram-negative bacteria also regulate
677 the influx and output of autoinducer-2. In *Escherichia Coli* the transporter complex called
678 LsrABCD regulates the uptake of autoinducer-2. The repressor of LsrABCD and the LsR is a
679 kinase that is a part of the quorum sensing system of AI-2. The cyclic AMP/cyclic AMP receptor
680 proteins regulates the lsr operon. The cyclic AMP/cyclic AMP receptor proteins are expressed
681 from *lsrk* and *lsrR* that is positioned upstream of lsr [8]. Lsrk that is a kinase located in the
682 cytoplasm phosphorylates AI-2 that then attaches to the *lsr* repressor. The activated and attached
683 AI-2 suppresses the *lsr* repressor LsrR. After the activity of the *lsr* repressor is inhibited, the
684 release of AI-2 increases via the autoinducer-activated expression of the Lux-S protein.

685 A glucose molecule is hydrolyzed into two pyruvate molecules. The acetyl-CoA
686 Carboxyl transferase or the ACCA alpha subunit of the acetyl-CoA carboxylase complex,
687 AccABCD, transfers a carboxyl group from the pyruvate to the acetyl-CoA to form malonyl-
688 CoA. The malonyl-CoA enters the fatty acid synthesis pathway, elongating fatty acids into long-
689 chain fatty acids of phospholipids necessary for structuring plasma membranes and for forming
690 the initial extracellular matrix. The microcolonies attach, aggregate, and accumulate in the
691 extracellular matrix. As the colonies accumulate into large populations a release of autoiducer-2

692 molecules is triggered, causing a certain bioluminescence through an exchange of cell- to-cell
693 quorum sensing signals. The release of autoinducer-2 signals cells to increase population growth
694 or to preempt it. The gene *luxs* is needed for releasing AI-2. The excess AI-2 distributes quorum
695 signals between bacterial cells, which notifies cells of limited nutrients, surface area, or space. If
696 bacterial cells continue to accrue, more biofilm is produced within the extracellular matrix,
697 resulting in the dispersion of bacterial cells. Biofilm is a potent virulent factor. Pathogenic
698 bacteria can disperse from and cause a systemic infection through excess biofilm production.
699 To form biofilm for infection, the *luxs* gene must be activated. Its excessive amplification
700 and expression form a denser texture of biofilm [30]. Lux-S monitors virulence through
701 regulation of the process for generating biofilm in the nasopharynx in mice with pneumonia,
702 *Streptococcus pneumoniae*. Flagella expression increases with *luxs* mutation in the lungs and
703 bloodstream. The *luxs* mutants can infect the lungs or the bloodstream more rapidly than its wild-
704 type strain [27]. Flagellin increases inflammation by activating Toll-Like Receptor Signaling or
705 TLR5 pathways, which translates pro-inflammatory genes within the Mitogen-Activated Protein
706 Kinase or the MAPK pathways. Flagella modulates virulence and pathogenesis by allowing more
707 rapid motility of bacteria, infecting the colonization of host cells, and assisting infectious
708 bacterial cells into entering the mucosal layers [33].
709 Pathogenic bacteria determine the level of autoinducer signals specific for their species.
710 Pathogenic bacteria require a specific order and assortment of virulent genes to cause infection.
711 For example, the different residues or R-groups of amino acids within autoinducers bind to the
712 LUXR protein receptors in a conformation-specific to the amino acid side chains within the
713 binding sites. The orientation of the AI to protein receptor binding sites produces varied types of
714 side-chain lengths and amino acid substitutions [34]. For example, pathogenic bacteria as

715 Enteropathogenic *Escherichia coli* EPEC release intraspecies-type of signals that help to
716 colonize the small intestines because the small intestines are void of much commensal
717 microflora. The Enterohemorrhagic *Escherichia coli* or EHEC pathogenic form of *Escherichia*
718 *coli* conducts signals with other diverse bacterial cell types and with host cells. The EHEC
719 communicates through quorum sensing with other colonic bacteria cells. The EHEC infect
720 epithelial cells through activating the genes called the Locus of Enterocyte Effacement or LEE.
721 The genes of LEE are required for A/E lesions to form when EHEC cells attach to and efface
722 from epithelial cells, amplifying the level of pathogenicity [27].

723 When the production of Lux-S is blocked, the intensity of disease-causing bacteria
724 changes. For example, the mutation of *luxs* in *Haemophilus influenzae* increases virulence that
725 causes middle ear infections [35]. The *A. pleuropneumoniae* and *M. haemolytica* bacteria have
726 autoinducer mutations that lessen virulence in mouse models. When the *luxs* gene is inhibited,
727 there is a lesser amount of pustule-creation than the parental wild type strain [35]. However,
728 autoinducer 3 is not dependent on the expression of the *luxs* gene. *luxs* genetic mutants impede
729 the production of AI-3 (21-22). As a result, the pathogenic Escherichia coli, EHEC, collects
730 signals from host cells in the form of hormones known as epinephrine. The AI-3 can
731 communicate with the transmembrane protein of the host cell called Qsec [36]. The two
732 components for quorum sensing also include Las and RhI that are quorum systems consisting of
733 activator proteins. The activator proteins direct the synthesis of the autoinducers. The proteins
734 called Las and RhI turn genes on or off based on the density of bacterial populations, sensing the
735 accumulation of autoinducers [37].

736 The bacteria called *P. aeruginosa* secretes two types of autoinducers called N-3-
737 oxododecanoyl, a homoserine lactone, (3-oxo C12-HSL) and N-butyryl-L-homoserine lactone

738 (C4-HSL). These autoinducers released can cause virulence in people infected by *P. aeruginosa*
739 by supplying virulent factors that help colonize and spread the infection [37]. Therefore, the
740 results present significant data for the effect of glucose and genetic inhibition of *accA* on the
741 production of the *luxs* gene, an important component of bacterial virulence. The data
742 demonstrates that the genetic changes, as inhibiting the *accA* gene with antisense RNA, and the
743 availability of nutrients such as glucose, affected the production of *luxs*. Inhibition of the *luxs*
744 gene can reduce biofilm formation and decelerate the motility of bacteria, which the lessened
745 motility can impede the spread of pathogenic bacteria.

746 **CONCLUSION**

747 The overall purpose of the study was to confirm and identify the gene *accA* as a possible
748 target for blocking bacterial antibiotic resistance. Through examining the role of *accA*, a link
749 between bacterial cell metabolism and virulence was attempted and demonstrated. A cumulative
750 perspective, integrating bacterial cell metabolism via the ACCA subunit with bacterial
751 pathogenicity, may provide possible metabolic targets for designing new and potent antibiotics.
752 It was previewed in this study, an ability to design antibiotics that target bacterial cell metabolic
753 genes as *accA*, the binding sites of the ACCase enzyme, and the fatty acid synthesis pathway.
754 Targeting all three areas of bacteria cell metabolism can reduce the formation of bacterial cell
755 biofilm needed for spreading pathogenic bacteria. The strong condition of a bacterial
756 population's metabolic state can prevent bacterial susceptibility to antibiotics [34]. The results of
757 decreased RNA expression and cell growth in the presence of antisense RNA inhibition of *accA*
758 may serve as a model for blocking microbial metabolic processes that contribute to antibiotic
759 resistance [38].

760 *Escherichia coli* cells were cultured in high glucose to a control concentration and
761 showed a decreasing range of gene expression for the target gene, *accA*. The observation of the
762 bacterial cells, grown in an increased amount of available glucose, showed the highest measure
763 of transcription for *accA*. The effect of glucose extracellular of the bacteria, on the concentration
764 of RNA, was measured by modulating the glucose concentration gradient. The transcription of
765 intracellular molecules as the *accA* gene was determined through qPCR. The *accA* gene was
766 inhibited with antisense RNA, using an IPTG-inducible vector, pHN1257. There was a 138
767 percent difference of gene expression between cells transformed with antisense pHN1257 versus
768 cells negative for antisense RNA during in vitro transcription. The recombinant DNA and
769 plasmids were assembled from the IPTG-PT-asRNA of the pHN1257 (Fig 1).
770 The constructed plasmid of PHN257 consisted of flanking inverted repeats for the target
771 DNA, rebuilding a paired double-stranded RNA terminus that inhibited the transcription of *accA*.
772 The inhibition of *accA* was confirmed through the qPCR results of 63 gene copies for *accA* (+)
773 asRNA versus 422 gene copies for *accA* with (-) asRNA. The sum volume of *luxs* was
774 determined through qPCR. By increasing the glucose concentration in increments, Wang et al.
775 (2005) found that glucose increased the levels of autoinducer-2 of the culture as well [39]. The
776 observations displayed the RNA concentration for bacterial cells, expressing asRNA of *accA*,
777 included a concentration of 279.28 ng/µL. The miRNA concentration of the cells with asRNA
778 measured to 240.90 ng/µL.
779 The results supported the hypothesis. Decreasing the glucose concentration, while
780 suppressing the *accA* gene, led to an observed decrease in *luxs* expression. The author
781 hypothesized that decreasing the sum of glucose and suppressing *accA* gene expression would
782 decrease the expression of the *luxs* gene. The *luxs* gene is vital for quorum sensing and releasing

783 autoinducer-2. Increased expression of Lux-S produces more AI-2, which induces and initiates
784 quorum sensing between bacterial cells. The increased AI-2 molecules released triggers quorum
785 sensing for the amplified production of biofilm. Biofilm formation allows more pathogenic
786 bacteria cells to attach, proliferate, detach, and spread into a systemic infection.

787 The *accA* gene translated into the acetyl-CoA carboxyl transferase enzyme transfers a
788 carboxyl group from pyruvate to the acetyl-CoA to form a malonyl-CoA. More glucose induces
789 more production of the acetyl-CoA carboxyl transferase. Increased glucose also causes more AI-
790 2 quorum-sensing signal molecules to accumulate. The malonyl-CoA enzyme is important for
791 catalyzing the biosynthesis of long-chain fatty acids. The long-chain fatty acids are the building
792 blocks for biofilm formation. Therefore, a decrease of biofilm production can occur due to the
793 limited enzymatic activity of the ACCA transferase subunit via *accA* gene inhibition.

794 However, in the presence of glucose with antisense RNA transcription, *Escherichia coli*
795 cells continued to produce amplified amounts of *luxs* with 316,228 copies measured through
796 qPCR when enhanced with 5µM of glucose (n=3, 171571± 204574). The limitations of this
797 study include the contradictory results of the 5µM glucose-(+) asRNA sample for *luxs*. Adding a
798 5µM concentration of glucose to the LB broth culture increased the gene expression of *luxs*
799 although the *accA* gene was inhibited. These contradictory and unexpected results may show
800 early signs of reduced efficacy of the inhibition of the *accA* gene in the presence of glucose.

801 However, it is probable that the 5uM of glucose triggered increased expression in the
802 *accD* gene and of the ACCD subunit, which was not inhibited by asRNA. The gene *accA* can
803 continue to act as a potent metabolic target for suppressing antibiotic resistance. This study
804 suggests that designing antibiotics could include targeting metabolic genes and enzymes. During
805 the past 30 years, only a few potent antibiotics have been formulated and discovered [40].

806 However, there is a new motivation for examining bacterial targets located in metabolic
807 pathways. For future research, studying the gene expression, structure, and the function of the
808 ACCase complex can increase the incentive for designing small-molecule inhibitors specific for
809 the binding sites of bacterial-metabolic enzymes.

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