The antisense inhibition of *accA* suppressed *luxS* expression that is essential for quorum sensing, biofilm formation, and virulence

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ABSTRACT

Bacterial metabolism is dynamic and in constant flux. Bacteria use carbohydrate metabolism to synthesize nucleotides, proteins, and fatty acids. Because of the metabolic capabilities of microbes, bacteria can adapt to many different types of environments where specific nutrients are available, in excess, or not present. Bacteria can adapt to harsh conditions or to an overabundance of nutrients mainly through altering its genetics and changing its physiology. Microbial metabolism affects bacterial genetic activity and its structure. Bacteria convert and use ATP as energy from glucose to convert acetyl-CoA into malonyl-CoA. The conversion of malonyl-CoA into malonyl-ACP catalyzes the elongation of long-chain fatty acids. The long-chain fatty acids are produced for plasma membrane and biofilm formation. The transcription of *accA* produces the acetyl-CoA carboxylase transferase enzyme needed for long-chain fatty acid elongation. In this study, liquid cultures of *Escherichia coli* were supplemented with D-glucose. The 15 mM glucose sample yielded 4,210 ng/µL of *accA* as compared to 196 ng/µL for the control. The gene *accA* was inhibited with antisense RNA and yielded a qPCR product of 63 ng/µL. The inhibition of *accA* suppressed the expression of the *luxS* gene. The *luxS* gene is a vital virulent factor for transferring intercellular quorum-sensing signals called autoinducer-2 and to form biofilm. Bacterial cells that expressed antisense inhibition of *accA* were also more sensitive to antibiotics. These results suggest that bacterial metabolism grossly affects virulence and antibiotic sensitivity.

Keywords: Antibiotic Resistance, Antibiotics, Biofilm, Carboxylase Enzymes, Autoinducers, Quorum Sensing, Antisense RNA, and Bacterial Metabolism

INTRODUCTION

During the past 30 years, the formulation and discovery of potent antibiotics have dwindled [1]. Penicillin was discovered by Alexander Fleming in 1928. His discovery coincided with the use of antibiotics which saved many lives; however, the issue of antibiotic resistance has emerged in tandem with the overuse of antibiotics. A deep synthesis of many new and novel antibiotics was discovered between the 1960s and the 1980s [2]. Fleming's discovery came after witnessing a mold growth that reduced the staphylococci population cultivated in a petri-dish [3]. In 1940, more scientists began publishing a summary of methods to extract large quantities of penicillin for clinical trials [4]. After the first use of penicillin in 1945, the resistance to antibiotics has increased [5]. A biofilm includes tight juncture communities of bacterial cells that have an extracellular matrix outer covering [6]. The covering of the extracellular matrix allows bacteria to attach to a biotic or an abiotic surface. This extracellular matrix protects the bacterial cells from external and environmental pressures. Biofilm causes a large portion of bacterial infections in hospitals and in humans. There is not much known or fully elucidated about the metabolic fluxes that affect bacterial biofilm formation [6].

The cell envelope of gram-negative bacteria plays a pertinent role in antibiotic resistance. The cell envelope of gram-negative bacteria consists of the phospholipid inner membrane bilayer and the outer membrane. The cell envelope contains an inner membrane of a phospholipid bilayer and an outer lipopolysaccharide region termed the LPS. Due to this double membrane, gram-negative bacteria are protected from diffusible hydrophobic compounds. The LPS blocks the hydrophobic molecules, and the phospholipid bilayer blocks the hydrophilic molecules. The structure of the double membrane for Gram-negative bacteria causes antibiotic drug discovery to become an arduous task [7]. The double membrane prevents the accrual of antibiotics and of enzyme inhibitors into the Gram-negative bacterial cell.

The inner membrane (IM) for the Gram-negative bacteria is composed of a phospholipid bilayer. The phospholipid (PL) bilayer is produced by the fatty acid type II synthesis pathway [7]. The first phase of FASII includes the carboxylation of acetyl-CoA that forms into malonyl-CoA through a biotin carrier protein (BCCP) and the acetyl-CoA carboxylase transferase (CT) enzyme. To begin the process of FASII, the change from malonyl-CoA to malonyl-ACP is required. The malonyl-ACP combines with an acetyl-CoA to generate acetoacetyl-ACP by the FabH enzyme. After FASII is initiated, 2 carbons are added per every cycle of fatty acid elongation for the conversion of malonyl-ACP into acyl-ACP. Acetyl CoA combines with a carboxyl group to form malonyl-CoA in an ATP-dependent reaction [8].

Due to the structure of the gram-negative bacterial envelope, *Escherichia coli* can adapt to a variety of environments with many varied volumes of available nutrients. However, the relationship between bacterial metabolism and virulence needs further elucidation. For that reason, this study hopes to demonstrate the effects of glucose supplementation and gene expression. The *accA* codes for the AccA domain of the CT subunit. The CT catalyzes the elongation of fatty acids. The acetyl-CoA carboxylase complex is an important enzyme for the first step of fatty acid synthesis. The bacterial fatty acids produce the phospholipids required to stabilize and form the plasma membrane (inner membrane). The concentration of glucose was adjusted, and RNA was extracted to assess *Escherichia coli's* genetic activity. The AccA, a subunit of the acetyl-CoA carboxylase enzyme (CT), was quantified using Real-Time PCR. By

using antisense RNA transcripts, we suppressed the *accA* gene, recorded the subsequent RNA concentrations, and quantified the gene copies for *accA* (Fig 1). The asRNAs bind to the sequences flanking the ribosome-binding site and the start codon of the target mRNAs [9]. The antisense RNA blocks the ribosomal detection by the ribosomal binding site or the RBS that inhibits translation [9]. We measured the effects of extracellular and intracellular factors upon the intercellular communication between cells by quantifying the gene expression of *luxS*.

Lux-S is necessary for bacterial motility and the formation of a bacterial biofilm. Lux-S is an autoinducer-2-producing S-ribosylhomocysteine lyase. Autoinducer-2 is a released signaling molecule that induces quorum sensing from a cell to cell interaction. In an intercellular process called quorum sensing, bacterial cells detect AI-2 signals from other cells and interact. Amplified Lux-S expression increases autoinducer-2 production, intensifies quorum sensing, and creates more biofilm for pathogenic bacteria to spread. Gram-negative bacterial autoinducers consist of homoserine lactones and gram-positive bacteria release oligopeptides [10]. Also, the microflora of the lower gastrointestinal tract can communicate with the host cells of the gastrointestinal tract through a variety of chemical idioms [11]. A group of chemical molecules for bacterial language, expression, and transfer includes the homoserine lactones. When a bacterial infection occurs, homoserine lactones form and interrelate with the immune system [12]. Researchers treated mutant cultures with kanamycin and found that the biofilm showed a reduction in antibiotic resistance. However, after 250 generations, the resistant mutant bacteria increased in growth at the surface and the base of the biofilm [13]. In a study by Peng et al. (2015), bacteria cultured without glucose or the non-polar amino acid of alanine were less susceptible to kanamycin [14]. Bacterial metabolism can significantly affect the output of autoinducers, the production of biofilm, and antibiotic sensitivity.

The bacterial colonies within a biofilm are resistant to antibiotics. A biofilm is an organization of bacterial groups attached to a biotic or abiotic extracellular matrix. Because the extracellular biofilm matrix prevents the penetration of antibiotics, is mostly anaerobic, or lacking oxygen, bacteria within the biofilm are more tolerant of antibiotics [15]. Adding antibiotics to a bacterium's environment creates pressure that induces gene mutations called antibiotic-resistant genes. These antibiotic-resistant genes are also shared between bacteria. The natural selection process is extremely effective and essential for antibiotic resistance development. Persistent cells, for example, are the bacterial cells that survived their first antibiotic contact. The persistent cells that live after being exposed to antibiotics cause and proliferate antibiotic resistance [16]. The bacteria of *A. baumanii* inhabit hospitals, are highly contagious, and develop antibiotic resistance through horizontal gene transfer. The *A. baumanii* bacterium cause 64% of urinary tract infections during the use of catheters [17].

In order to describe the effects of bacterial fatty acid metabolism upon virulence, an integration between glucose (extracellular), *accA* (intracellular), and luxS (intercellular) was demonstrated. The integration was produced by altering the glucose concentration, inhibiting *accA*, and then quantifying the expression of *luxS*. An antibiotic resistance test was also performed that implanted tetracycline, carbenicillin, and chloramphenicol-containing disks unto bacterial cultures that expressed antisense RNA for *accA*. It is hypothesized that inhibiting *accA* while lessening glucose availability will decrease the *luxS* gene activity. It was further hypothesized that inhibiting *accA* will reduce antibiotic resistance. The objective of this study

was to demonstrate the effects of *accA* inhibition upon the *luxS* needed to synthesize QS/AI-2 signaling molecules and form biofilm.

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

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

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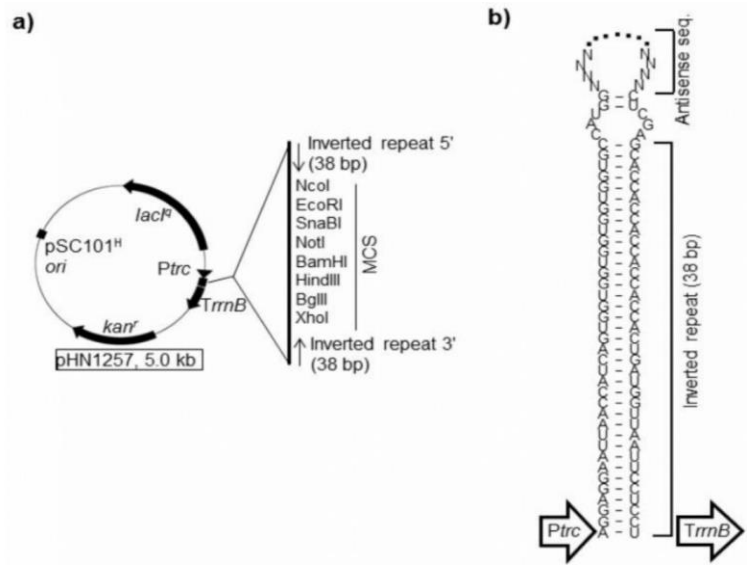


MATERIALS AND METHODS

BACTERIAL CELL CULTURE AND RNA EXTRACTION

The *Escherichia coli* TOP10 strain of cells was cultured and plated onto 25 mL agar plates. The bacterial cells were inoculated into Luria-Bertani broth, enhanced with glucose concentrations of 200µM, 50µM, and 0µM. The cultures of 200, 50, and 0 micromolar (n=7) of glucose were measured for the dsDNA and the RNA OD260 concentrations and replicated seven times. Varied concentrations of glucose, including, 15mM, 7.5mM, 5mM, and control of glucose were added to LB liquid with *Escherichia coli* cells. The plasmid pHN1257 was utilized for assembly of the antisense expression vectors. The recombinant plasmid of pHN1257 for the expression of antisense RNA was plated with the kanamycin antibiotic and incubated at 37°C for 24 hrs. Cells grown on agar plates were inoculated into 4 mL of LB liquid media with (+) and (-) PTasRNA bacterial cells expression of asRNA for *accA* cultured with kanamycin. A sum of 20 RNA extractions were completed with the Omega E.Z.N.A bacterial extraction kit.

cDNA PREPARATION

The author used the OneScript Reverse Transcriptase cDNA Synthesis kit by Applied Biological Materials Inc. or ABM. Approximately, 1µg of RNA from each sample, high glucose, medium glucose, low glucose, and (-) glucose were added to 0.5µM oligonucleotides(dT) (10µM). For the asRNA and (-) RNA, one microgram of each total RNA extract was added to the initial primer/RNA and reaction mixture. The cDNA was also prepared for *luxS* quantification. The original primer/RNA mix consisted of 1µg of total RNA, 1µL of oligo(dt), 1µL of dNTP (10mM) mix, and nuclease-free water for a 20µL reaction solution.

PCR

A Promega PCR MasterMix kit at a volume of 25µL was added to the upstream and downstream primers, each of 0.5µL specific for the *accA* gene target. PCR amplification was repeated as a triplicate. The concentrations of the two cDNA samples added to the PCR master mix were 190ng and 230ng plus nuclease-free water. A 2X and 1X upstream primer, 10µM 0.5–5.0µl0.1–1.0µM, and a downstream primer, 10µM 0.5–5.0µl 0.1–1.0µM, DNA template 1–5µl less than 250ng, and nuclease-free water were mixed into a 50µl PCR reaction mixture. The PCR thermocycler completed 40 cycles of amplification instead of 35 cycles.

AGAROSE GEL ELECTROPHORESIS AND PLASMID ASSEMBLY

The condition of the PCR products, without primer dimers and contamination, for the target gene *accA,* were detected through agarose gel electrophoresis. The PCR products were confirmed without primer dimers and contamination, the PCR products, and the PTasRNA expression vector of the plasmid pHN1257 were digested with the restriction enzymes XhoI (upstream) and NcoI (downstream) (New England Biolabs XhoI- catalog R0146S- 1,000 units and NcoI- catalog R0193S- 1,000 units) (Fig. 1). Each microcentrifuge tube was placed in the incubator for 30 minutes at 37°C. A heat block was heated to 90°C. The PCR products of the *accA* gene were ligated into the pHN1257 plasmid by mixing 1µL of the DNA insert with 2µL of the plasmids, adding 5µL of ligation mix, and then placing the tubes into a heat block of 90°C for 15 minutes (Takara Ligation Kit 6023).

REAL-TIME PCR

The author enhanced each bacterial sample with the glucose concentrations of 15mM, 7.5mM, 5mM, and 0mM (n=3). The cDNA from H-glucose, M-Glucose, L-glucose, and the control (-) glucose were diluted into a 1:8 serial dilution. A 10-fold dilution of standards was prepared for bacterial samples transformed with (+) asRNA inducible plasmids and exposed to high to a low volume of glucose. The amount of gene expression for *accA* and the target gene *luxS* were measured through absolute quantification methods of qPCR. The diluted cDNA was pipetted into a transparent 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. The optical film sealed the plate. The PCR plates were centrifuged for 2 minutes at 2500 rpm to reposition all liquids to the bottom of the wells. The real-time PCR Amplification Protocol from the Roche Lightcycler 480 machine was used.

THE qPCR ABSOLUTE QUANTIFICATION OF LUX-S

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

ANTIBIOTIC RESISTANCE TEST

Competent T7 bacteria cells were transformed with the inducible vector termed pHN1257 that expressed antisense RNA to inhibit the gene *accA.* The newly transformed bacteria cells were plated unto nutrient agar medium consisting of the antibiotic kanamycin. Three to five colonies from the pHN-1257-asRNA-*accA* bacterial transformants were selected and cultured in LB broth, enhanced with kanamycin, for 18 to 24 hours at 37 degrees Celsius in a shaking incubator. A control group that did not express asRNA for *accA* was cultured in LB broth overnight at 37 degrees Celsius. One hundred microliters of the pHN1257-asRNA-*accA* and from the control group were pipetted unto nutrient agar petri dishes (N=64) containing 50 micromolar of the kanamycin (50µg/mL) antibiotic for the sample of pHN1257-asRNA-*accA*. A swab was used to spread the 100 microliters of each experimental (with asRNA) and the control (without asRNA) unto the agar plates to form a lawn of bacterial growth. The six-millimeter sized disks contained five microliters of each the three consecutive antibiotics, including tetracycline (10µg/mL), carbenicillin (100µg/mL), and chloramphenicol (25µg/mL). Each group of eight disks were prepared with tetracycline, carbenicillin, and chloramphenicol. The antibiotic disks were placed unto the agar plates that were divided into 4 or 8 sections. The bacterial cultures grew for 18-24 hours at 37°C. The zone of inhibition for each of the disk (N=64) was measured in mm and compared to specific criteria for determining antibiotic resistance and susceptibility of bacteria.

STATISTICAL ANALYSIS

Figures were constructed with the Sigma Plot 14th edition, Microsoft excel, and the 8th edition of GraphPad Prism. The mean and standard deviations were calculated by Microsoft Excel and GraphPad Prism. The p-values and the ANOVA results were produced through GraphPad Prism. The standard curves for the absolute quantification of the genes *accA* and *luxS* were generated through Microsoft excel with linear regression coefficients added and calculated for each standard curve for the absolute quantification of *accA* and *luxS*. The antibiotic resistance test was analyzed, given descriptive statistics, and generated paired-t-test results through the OriginPro 2019B software. A t-test and a density graph for antibiotic susceptibility of pHN1257(+) asRNA for *accA* was produced through the OriginPro 2019B computer application.

RESULTS AND DISCUSSION

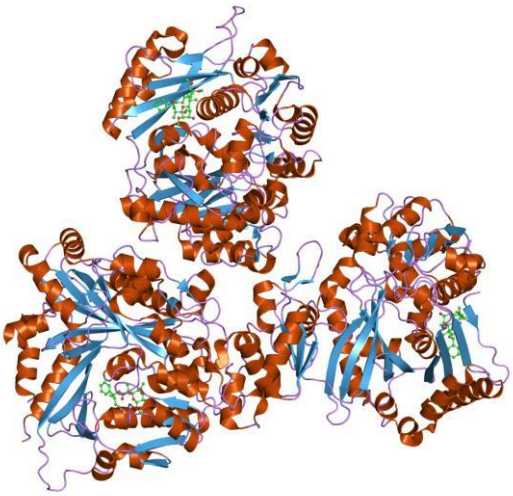
THE EFFECT OF GLUCOSE ON THE RNA CONCENTRATION AND THE EXPRESSION OF *accA*

The ACCase enzyme has three subunits that include a biotin carboxylase (BC), a biotin carboxyl carrier protein (BCCP), and a α2β2heterotetrameric carboxyltransferase (CT) [18]. The *accB* codes for the BCCP and the BC are transcribed from accC. The *accB* and the accC (*accBC*) are transcribed simultaneously by the *accBC* minioperon [19]. The genes *accA* and *accD* encode for the α-subunit and β-subunit, respectively. The α-subunit (*accA*) and β-subunit (*accD*) make-up the CT portion of ACCase. The genes of *accA* and *accD* are entirely independent of the *accBC* operon. The overexpression of *accB* is accompanied by the blocking of *accBC* expression. Conversely, when *accA* and *accD* are overexpressed, the *accBC* operon remain unaffected [20]. Glucose increases the output of *accB*, but then this overexpression reduces and regulates the transcription of the *accBC* operon. Even though excess levels of glucose regulate *accBC* expression; it did not stop the production of *accA*. The *accA* is not regulated by the BC minioperon, which only monitors the overproduction of the *accBC*.

The BC of *E. coli* is bonded into a dimer (Fig 2). Knocking out one of the dimers of BC renders it inactive and inhibited. Each dimer has an active site oriented at 25Å from the region of interaction of the dimer [21]. This dimer interface is composed of distant communications between each of the two active sites in a dimer. The two monomers of the BC dimer may share the responsibility of inducing catalysis. One of the BC monomers can bind the substrate during its initiating the reaction while the second monomer disperses the product. However, the BC dimer during the first half reaction does not exhibit cooperative behavior as confirmed through enzyme kinetic results. The biotin carboxylase has 449 amino acid side chain residues. The 449 residues are split into three domains. The three domains include a domain of the N-terminus, an ATP-grasp or a B-domain, and a C-terminal domain. The Met1-lle103 residues represent the N-terminal domain that consist of a β-sheet, five parallel stands, and four α-helices. The ATP-grasp contains the residues Val131-Tyr203, 3 β-sheets, and two α-helices [18]. The BC has residues Arg208-Lys449 in its C-terminal domain. The BC subunit is composed of 8 antiparallel β-sheet, 3 antiparallel β-sheets, and 7 α-helices [18]. Each monomeric section of the BC dimer has an active site where the residues are not shared between monomers.

Figure 2 The BC of *E. coli* is bonded into a dimer (*Retrieved from reference 22)***.** Each dimer has an active site oriented at 25Å from the region of interaction. This dimer interface is composed of distant communications between each of the two active sites in a dimer. The two monomers of the BC dimer may share the responsibility of inducing catalysis. One of the BC monomers can bind the substrate during its initiating the reaction while the second monomer disperses the product.

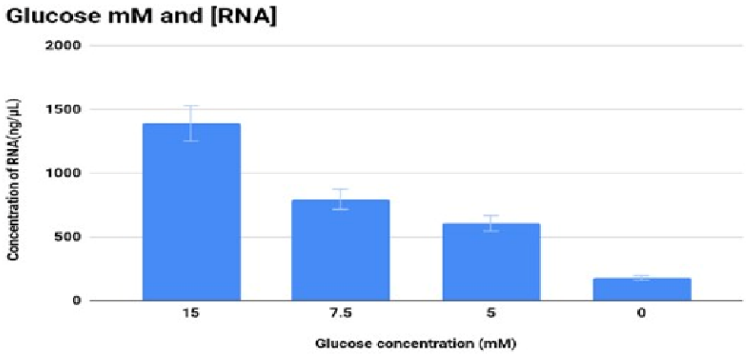
(BC)



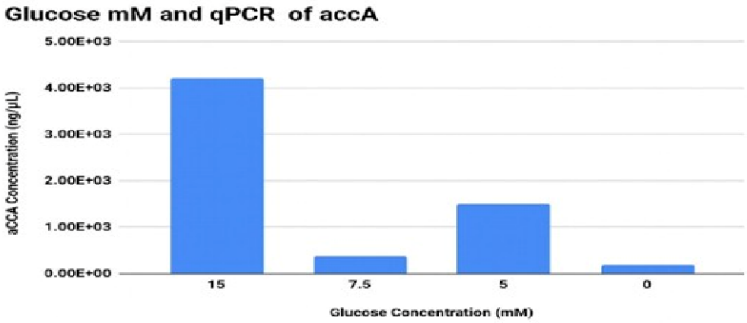
E. coli bacterial cells use enzymes that lyse glucose molecules into pyruvates. The ACCase complex subunit termed AccA transfers a carboxyl delivered by the BC-BCCP. The BC-BCCP positions a carboxyl from the pyruvate to a CT. The CT transfers the carboxyl to an acetyl-CoA to form malonyl-CoA. The gene expression of *accA* produces the AccA subunit. As we increased the glucose concentration the transcription of *accA* was amplified. After culturing *Escherichia coli* with different levels of glucose, the RNA concentrations for each sample measured were of 1392, 797, 608, and 199 ng/µL for the H-Glucose, M-Glucose, the L-Glucose, and the control, respectively (Fig 3). The RNA of each bacterial sample enhanced with glucose was extracted, and the concentrations determined (N=20, 335.4 ± 329.2). The level of *accA* by qPCR analysis, in the units ng/µL, included: 4210, 375, 1500, and 196 for H-glucose, M-glucose, L-glucose, and for the control, respectively (Fig 3).

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. The number of qPCR *accA* products ranged from 11-186, 5-18, and 0-79 gene copies for High-glucose, Medium-glucose, and the Low-glucose/control, respectively (N=12, 48±38). Comparing 200 µM to its control group, the difference between the samples was statistically significant (p=0.038) (Fig 3-4). The cells grown in 200µM yielded a more substantial and more abundant production of dsDNA than cells cultured as a control (Fig 3-4). In the Ferrais et al. (1990) study, for an animal's diet, the SI glucose concentrations averaged 0.4-24 mM and ranged with time over a large amount of an SI region from 0.2 to 48 mM. Their study confirmed a 60 percent reduction of 2-deoxyglucose uptake at 30 mM glucose [24]. The high glucose osmolality resulted in a reduced Vmax value for 2-deoxyglucose absorption with a constant Km, calculated from the Michaelis-Menten kinetic enzyme equation [24]. Increasing the glucose concentration from 50 µM to 200µM, lowered the rate of uptake of glucose into the *E. coli* cells.

Figure 3 Glucose concentration versus RNA Glucose concentrations of 15mM, 7.5mM, 5mM, and the control samples each yielded RNA concentrations, in ng/µL, 1392, 797, 608, 179, respectively. A) The bacterial sample with 15mM glucose yielded the most substantial measure of RNA, noting a proportional relation between glucose and genetic expression of *E. coli*. B) The results display the qPCR results for 15mM, 7.5mM, 5mM, and the control. The samples with 15mM and 5mM glucose showed the most genetic activity of *accA* transcription, measuring *accA* concentrations of 4,210 ng/µL and 1,500 ng/µL, respectively. The *accA* qPCR concentrations for 7.5mM *accA* equaled 372 ng/µL and a 196 ng/µL quantity for the control. C) The analysis displays the OD260 measure of DNA results for samples of *E. coli* grown in medium enhanced with 200µM, 50µM, 0µM of glucose.



A)



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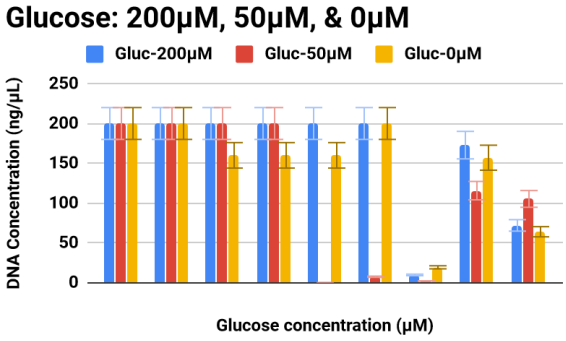
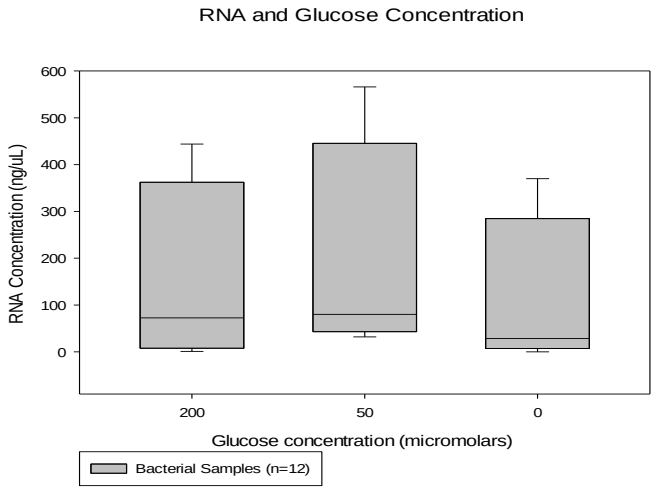


C)

Figure 4 RNA Concentrations vs. Glucose Concentration A) The enhanced *E. coli* bacteria cultures (n=3) 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 200µM 117ng/µL of RNA was produced, and the control measured to 76ng/µL of RNA.

A)



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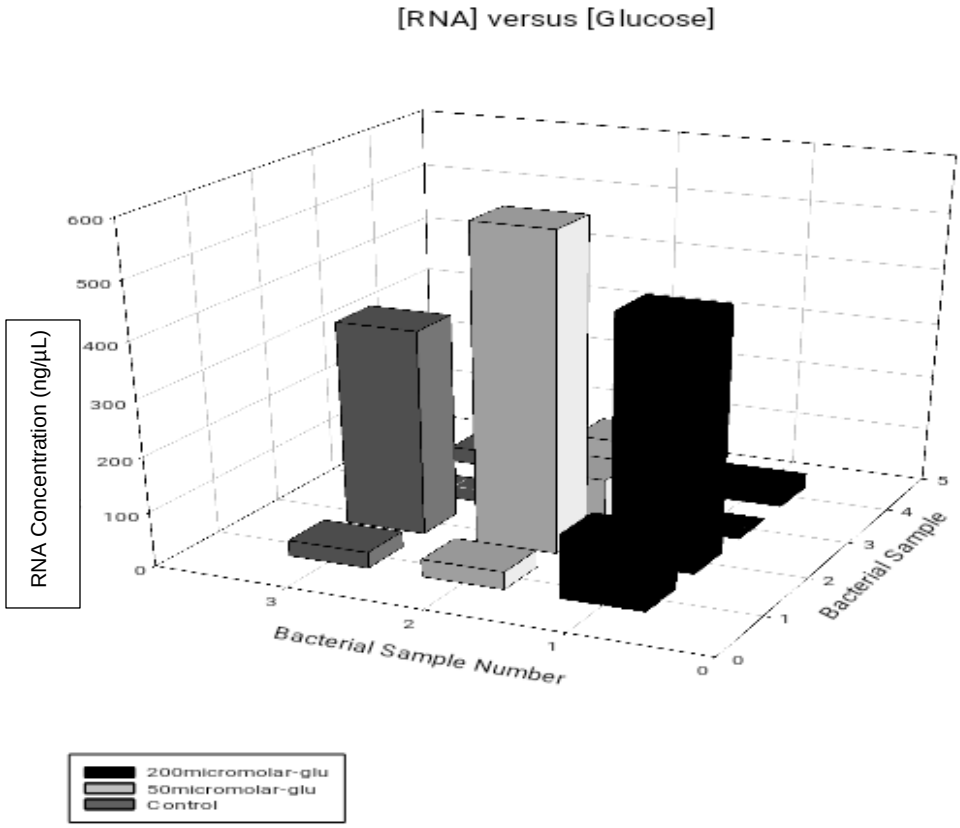


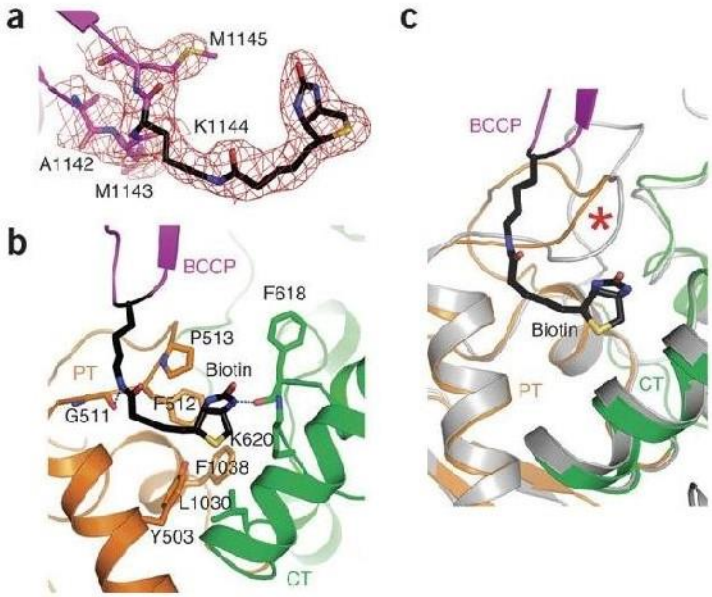
The augmentation of RNA transcription through glucose supplementation occurred because through increase carbon uptake induces more *accBC* and *accD*1 transcription and by inhibiting the flux of the Tricarboxylic acid (TCA) cycle augments the production of malonyl-CoA [25]. More available carbon nutrients from glucose addition increased the transcription of *accA* as reflected in the qPCR results for *accA*. Adding glucose to the liquid cultures, increased the production of the AccA subunit, shown in the qPCR products for *accA*. If *accA* expression was amplified, then the sum of the CT transferring a carboxyl to acetyl-CoA for conversion into malonyl-CoA also surged. Acetyl-CoA carboxylase (ACC) is a biotin-dependent carboxylase. The ACCase complex consists of a biotin carboxylase (BC), a carboxyltransferase (CT), and a biotin-carboxyl carrier protein (BCCP) subunits.

The biotin dependent ACCase enzyme carries the function for important fatty acid metabolic activity. The ACC complex completes two enzymatic reactions in two half reaction steps. The first step is catalyzed by a biotin carboxylase (BC) that carboxylates a N1' atom of a biotin vitamin cofactor, utilizing ATP, and carbon dioxide from a bicarbonate [21]. The biotin or Vitamin H has a covalent bond to a lysine side chain residue located in the biotin carboxyl carrier protein or the BCCP subunit. The second step uses a carboxyltransferase (CT) to transfer a carbon dioxide from a carboxybiotin to a carboxyl group.

The increased glucose supplementation provided excess caroboxyl groups and ATP needed for the BCCP of *E. coli* ACC to use its thumb appendage (Fig 5). As a biotin binds to the BCCP, the thumb attaches to the biotin. The thumb fortifies the BCCP, tightening the bond between biotin and the BCCP. By increasing the glucose supplementation, more energy from ATP was available for the ACCase enzyme to use the BCCP thumb to bind the biotin. The biotin is needed in all biotin-dependent carboxylase reactions. The glucose also provided the carboxyl groups needed for the BCCP to transfer the carboxyl to the CT. The BCCP of bacteria is like the ACC2 of human ACC, but human ACC2 does not contain a thumb extension [21]. The human BCCP is less restricted and can interact more with the BCCP of *E. coli*. In humans the presence of a thumb would inhibit a biotin to bind to the human holocarboxylase synthetase or HCS [21]. However, in *E. coli* the thumb does not interfere with biotin forming a bond with an enzyme called the *E. coli* BirA.

Figure 5 The BCCP of *E. coli* ACC uses a thumb appendage (*Retrieved from reference 23***).**
A) The "thumb" of a BCCP. **B)** As a biotin binds to the BCCP, the thumb attaches to the biotin. **C)** The BCCP transfers the carboxyl to the CT. The thumb fortifies the BCCP, tightening the bond between biotin and the BCCP.

(BCCP)

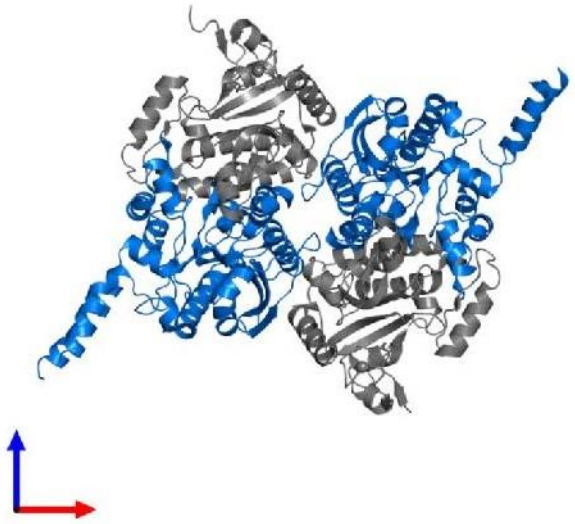


If a carboxyl is not present, then the biotin cannot attach to the CT subunit [26]. This prevents the loss of ATP as a source of energy for the BC to CT coupling reactions. The *E. coli* samples that were enhanced with glucose produced the highest concentration of *accA* and RNA because of the glucose providing more ATP for the BC to CT reactions. The glucose provided increased pyruvate molecules that yielded more carboxyl groups and ATP for the BC-CT interaction. The increased access of carboxyl induced the amplified attachment of the biotin to the CT. The CT domain has a N and a C domain. Each of the two subunits have a crotonase fold that is a β-β-α superhelix [21]. At the interface of the CT dimer of its domains there is an active site present.

The CT subunits are an $\alpha_2\beta_2$ heterotetramer [21]. The CT domains are required to bond as a dimer for the CT to be active and catalyze the second half reaction (Fig 6). An X-ray crystal structure of the CT domain shows a bond between the intermediate and the product of the reaction [27]. Every biotin-dependent enzyme requires two half reactions that have structurally isolated and separated active sites [26]. It has been confirmed that the separate active sites are physically produced in this orientation in order to regulate the interaction with biotin that stabilizes the interaction of the biotin between the BC and the CT reactions. The BC, BCCP, and the CT mimic a ping pong ball type of interaction over a distant gap of separation between the carboxylase and the transferase reaction [28].

Figure 6 The CT domain has a N and a C domain (*This figure was retrieved from the Protein Data Bank in Europe and originally published in reference 29*)**.** Each of the two subunits have a crotonase fold that is a β-β-α superhelix (Tong, 2013). At the interface of the CT dimer of its domains there is an active site present. The CT subunits are an $\alpha_2\beta_2$ heterotetramer. The CT domains are required to bond as a dimer for the CT to be active and catalyze the second half reaction. (color-grey=β/AccD, color-blue=α/AccA)

(CT)



Rather the BC, BCCP, or the CT are all bonded together as a multi-subunit protein or as separate domains relies on the type of organism and the class of the enzyme. The BC and the BCCP are conserved conformational structures. The conformation of the CT subunit, instead, depends on the behavior of the chemical substrates. Biotin is needed for producing fatty acid chain-like structures. Biotin has a C, N, and S cyclic ring with a five-carbon fatty acid chain. Biotin is a cofactor that is required for metabolism. Biotin is called Vitamin H that is bound to a lysine side chain residue of a biotin-dependent enzyme [30]. The biotin to protein bond assists with carboxylation and decarboxylation activity. To catalyze the biotin-dependent carboxylase reactions, the BC and the CT must interact with the biotin co-factor. Bacterial ACCase enzymes encase four subunits: BC at 50kD; BCCP subunit with 17kD, and two subunits called alpha and beta weighing at 33 kD for each component required for the CT reactions [21]. The ACCase enzyme has a stoichiometric ratio of 2:4:2 for BC, BCCP, and CTα with CTβ, respectively.

There was a reduction in *accA* and RNA activity because of the less availability of carboxyl groups after decreased glucose supplementation. When carboxyl groups are supplied by glucose metabolism, the ATP-grasp domain can appear as a closed conformation. When there is no carboxyl substrate bound to the biotin carboxylase, the ATP-grasp domain is in an open conformation as it extends from the main protein structure. Hence, when the glucose was decreased, less carboxyl substrates were bound, and the ATP-grasp domain remained in an

opened state. In this study, reducing the volume of glucose provided less ATP. Less ATP prevented the ATP to bind to a monomer of the ATP-grasp domain. When ATP binds to the ATP-grasp domain it rotates at an 45° angle to grab the ATP and grasp it into its active site [19]. When the BCCP domain of the human ACC2 was placed over the C-terminal alpha atoms of the image of the E. coli structure, the crystal violet image showed a new discovery. The human ACC2 did not have a "thumb." The "thumb" is a loop with 8 amino acids that extends from the BCCP-C-terminus domain [19]. The BCCP and the BC of *E. Coli* become bound together at an angle of resolution equal to 2.49 Å [18]. The BCCP and the BC are novel sites that can be targeted with antibiotics for reducing the activity of the acetyl-CoA carboxylase.

The difference between mammalian and bacterial ACCase enzymes is important for designing single-target antibiotics. An antibiotic designed for inhibiting a bacterial ACCase enzyme cannot interfere or interact with a mammalian ACCase enzyme because the mammalian ACC isoforms differ from the bacterial ACCs. Mammalian ACCase enzymes differ from bacterial ACC forms. Mammals have a different isoform of ACC called ACC2 or ACCβ. The ACCβ is conserved and matches 73% of its amino acids with the identity of isoforms, ACC1 and ACC2, from humans [21]. The ACC2 in mammals' function as an enzyme located in the outer membrane of the mitochondria. The ACC2 maintains a 140-residue oriented at the N-terminus. This 140-residue of amino acids located near N-termini is not present in the ACC [21]. The preceding 20 side chain residues of the 140 residues are almost completely hydrophobic. Citrate induces the activity of ACC1 and ACC2 in mammalian cells. In Eukaryotic cells BC has three domains, the A, B, and C domains. The side chain residue groups of A and C contain the active site. The domain of B enormously changes its conformation to cover the active site during the reaction. Eukaryotic cells have a BC domain with many partitioned and segmented residues. The side chain residues measure 550 residues between the BC and the AB connecting domains as compared to bacterial BC units that contain 450 residues [21].

THE qPCR RESULTS OF THE ANTISENSE RNA INHIBITION OF THE TARGET GENE *accA*

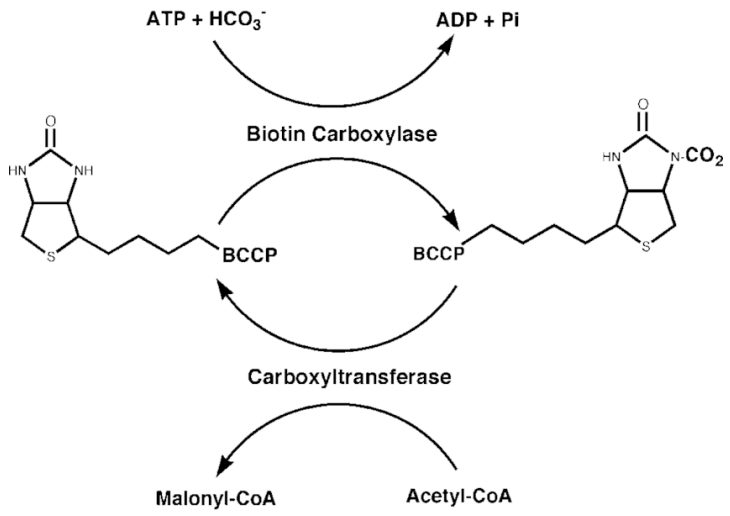
Fatty acid synthesis requires catalyzation by acetyl-CoA carboxylase that transfers a carboxyl to acetyl-CoA to produce malonyl-CoA [31]. This catalyzation requires energy from ATP, access to bicarbonate, and the vitamin cofactor of biotin. ATP from glucose is the most significant source of energy that is needed for most metabolic activities. The inner membrane (IM) for the Gram-negative bacteria is composed of a phospholipid bilayer. The phospholipid (PL) bilayer is produced by the fatty acid type II synthesis pathway [7]. The first phase of FASII includes the carboxylation of acetyl-CoA that forms into malonyl-CoA through a biotin carrier protein (BCCP) and the acetyl-CoA carboxylase transferase (CT) enzyme. To begin the process of FASII, the change from malonyl-CoA to malonyl-ACP is required. The malonyl-ACP combines with an acetyl-CoA to generate acetoacetyl-ACP by the FabH enzyme. After FASII is initiated, 2 carbons are added per every cycle of fatty acid elongation through the conversion of malonyl-ACP into acyl-ACP. Fatty Acid Synthesis II or FASII include separate subunits that interact. FASI proteins are expressed as cohesive and connected domains on one multi-polypeptide chain. *E. Coli* has an acetyl-CoA carboxylase that is a part of the FASII category.

The ACCase enzyme complex catalyzes the first step of fatty acid synthesis. The acetyl-CoA carboxylase consists of three subunits called biotin carboxylase, the biotin carboxyl carrier,

and the carboxyl transferase. The carboxyl transferase catalyzes the transfer of the carboxyl group to an acetyl-CoA, producing malonyl-CoA. The biotin carboxyl vehicle and carrier have two domains. It has a N-terminal domain, and a C- terminal region. The C-terminal domain consists of a biotin cofactor that is substrate-like. The role of the N-terminal region of the biotin carrier is still not completely elucidated. However, the N-terminal domain can attach and bind to the biotin. The utilization and function of the N-terminus seems to bind and fortify the carboxyl and biotin bond. The ACCase enzyme is also crucial for increasing cell growth. The transfer of the carboxyl from the biotin protein carrier (BCCP) through the carboxyltransferase (CT) to the acetyl-CoA is completed by two reactions [32]. The first half-reaction that uses the biotin carboxylase (BC) initiates the addition of a carboxyl to the biotin through its covalent bond to the BCCP. The second reaction is catalyzed by the CT that repositions a carboxyl to acetyl-CoA that produces malonyl-CoA.

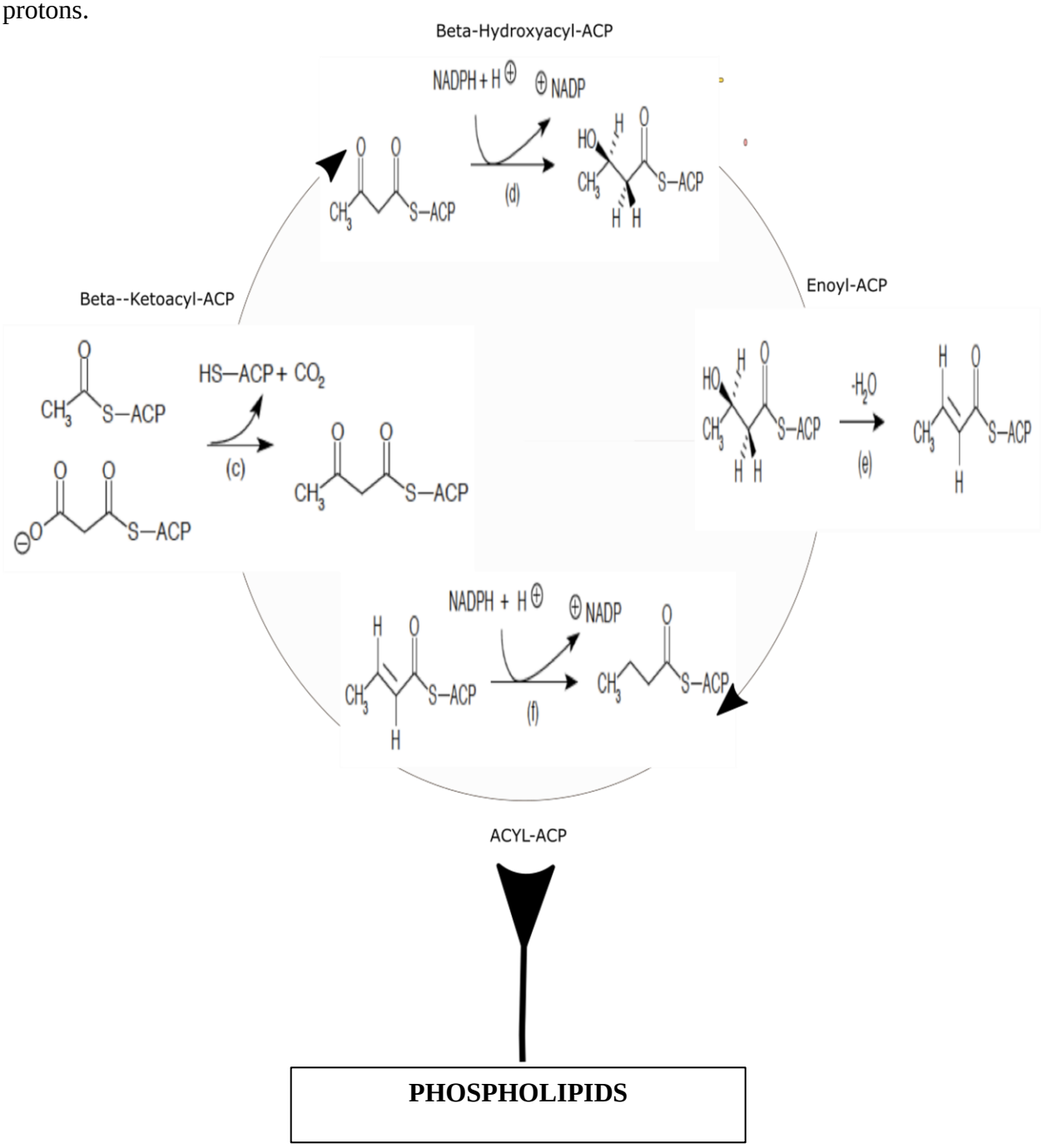
By inhibiting the *accA* gene with an IPTG-PT-asRNA vector, the CT subunit could not complete the interaction with the BCCP. Less interaction between BCCP and CT can lower the production of acyl-ACP from malonyl-ACP. The complex of acetyl CoA carboxylase catalyzes the reaction. The acetyl CoA carboxylase complex has a carboxylase and a biotin carrier subunit. The enzyme of carboxylase ignites and catalyzes the carboxyl group to be added to the biotin prosthetic group of the biotin protein carrier [8]. The carboxylase transferase initiates and transfers the carboxyl from the biotin protein carrier to an acyl group to form acyl thioesters. The gene called *accA*2 is translated into an acetyl CoA carboxylase subunit.

Figure 7 Formation of Malonyl-CoA (*This figure was retrieved from reference 33 and originally published in the Journal of Biological Chemistry)* The acetyl-CoA carboxyl transferase or the AccA and AccD, the alpha and beta subunit, of the AccABCD complex, transfers a carboxyl group from the pyruvate to the acetyl-CoA to form malonyl-CoA. The malonyl-CoA enters the fatty acid synthesis pathway, elongating fatty acids into long-chain fatty acids of phospholipids. The covalently bound biotin of BCCP carries the carboxylate moiety. A biotin carboxylase (BC) carboxylates a N1’ atom of a biotin vitamin cofactor, utilizing ATP, and carbon dioxide from a bicarbonate. The biotin or Vitamin H has a covalent bond to a lysine side chain residue located in the biotin carboxyl carrier protein or the BCCP subunit. The second step uses a carboxyltransferase (CT) to transfer a carbon dioxide from a carboxybiotin to a carboxyl group. The CT transfers the carboxyl group to an acetyl-CoA, producing malonyl-CoA.



The long chain fatty acids are produced after the ACCase complex, coded in *accA*, transfers a carboxyl group to acetyl-CoA, forming malonyl-CoA (Fig 7). The production of recombinant DNA required the ligation of the PCR product of the gene insert, *accA*, into the plasmid pHN1257 (Fig. 1). The re-engineered pHN1257 amplified the expression of antisense RNA for *accA*. The total RNA concentration for the control, (-) asRNA, equaled 739.44 ng/µL. The control, (-) asRNA, yielded a miRNA concentration of 334.98 ng/µL. 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 ng/µL of the 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.* There was a 147 % difference between cells not expressing asRNA versus cells transcribing the asRNA for *accA* (p=0.027).

Figure 8 Long-Chain Fatty Acid Synthesis An acyl-group or an ACP is added to the malonyl-CoA and converted into a ketoacyl-ACP. The Beta-ketoacyl-ACP is reduced, gains hydrogen, into Beta-hydroxyacyl-ACP. The Beta-hydroxyacyl-ACP is dehydrated, losing a molecule of water, to alter into an Enoyl-ACP, which forms into an Acyl-ACP by gaining two hydrogen protons.



Inhibiting *accA*, lowered the expanse of fatty acid production because lessening ACCase activity shortens the length of the acyl chain that can increase or decrease in elongation. Bacterial cells, in response to low temperatures or toxicity, can change the ratios of their acyl chains through saturation to unsaturation, from cis to trans double-bonded carbons, and from branched

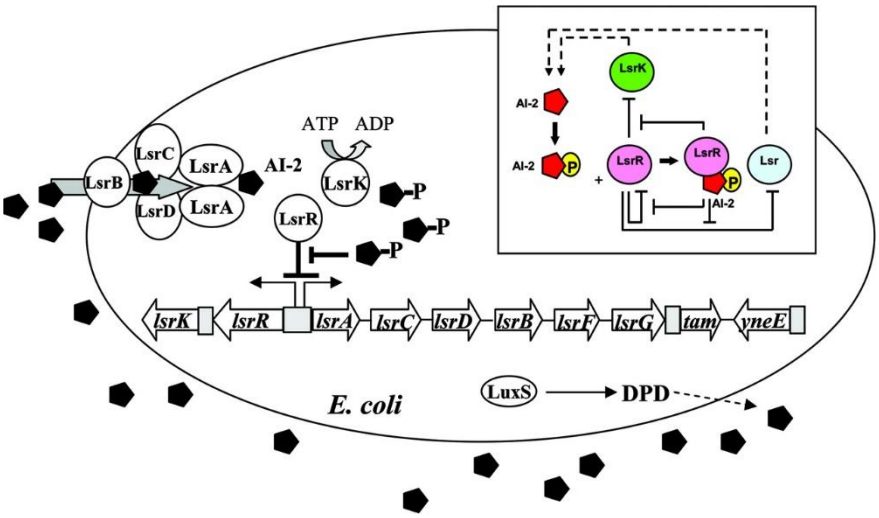
to unbranched stacks of acyl chains [34]. Lipids are the central building blocks of the inner cytoplasmic membrane. Lipids maintain the structure of bacterial cells, increase survival and increase growth by augmenting the passage of hydrophobic units via increased permeability. The lipid acyl chains that are produced during fatty acid elongation manufacture a more fluid and permeable bacterial cell (Fig 8). Environmental fatty acids can also affect the fatty acids of the bacterial plasma membrane. *S. aureus* can absorb fatty acids from its environment and increase its rate of survival and growth also after antibiotic treatment of a fatty acid pathway of synthesis. Biofilm of bacterial cells consists of larger volumes of fatty acids that increase fluidity, isolating the bacterial community from a tumultuous environmental condition [34].

qPCR ANALYSIS OF *luxS* GENE EXPRESSION

Escherichia Coli produces quorum sensing signals for regulating gene activity and growth. *Escherichia Coli* and many gram-negative bacteria regulate the influx and output of autoinducer-2. In *Escherichia Coli*, the transporter complex called LsrABCD regulates the uptake of autoinducer-2. The repressor of LsrABCD and the LsR is a kinase that is a part of the quorum-sensing system of AI-2. The cyclic AMP/cyclicAMP receptor proteins regulate the lsr operon. The expression of cyclic AMP/cyclic AMP receptor proteins occurs with the positioning of *lsrK* and *lsrR* upstream of the lsr [35]. Lsrk that is a kinase located in the cytoplasm phosphorylates AI-2, and then the Lsrk attaches to the *lsr* repressor. The activated and attached AI-2 suppresses the *lsr* repressor termed the LsrR. After the activity of the *lsr* repressor is inhibited, the release of AI-2 increases via the autoinducer-activated expression of the Lux-S protein. The autoinducer-2 that 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. Autoinducer-2 requires the genetic expression and translation of *luxS* (Fig 9).

A qPCR analysis quantified the number of l*uxS* gene copies after inhibiting *accA* with asRNA while decreasing the glucose gradient. Glucose increased the rate of transcription (Fig 10). *Escherichia coli* cells were grown in 25µM and 5µM of glucose and transformed with antisense pHN1257expressing IPTG-PT-asRNA plasmids. Cell samples with 5µM glucose- (+) asRNA produced a high measure of the *luxS* gene. The gene copy number of *luxS* for (+) glucose/ (+) asRNA, and (+) asRNA were quantified. For the results, the (-) glucose-(+) asRNA sample produced a gene copy number of 199 and 2511 *luxS* copies for (-) glucose- (-) asRNA (n=3, 277 ± 37). For the 25µM glucose-(+) asRNA and 5µM glucose (+), asRNA produced 39,810 and 316,228 gene copies of *luxS*, respectively. The control samples, the (-) glucose-(-) asRNA, (n=3, 658,114 ± 483,499) and the 5µM glucose- (+) asRNA (n=3, 171571±204574) exhibited the highest amount of expression for *luxS* with a $p<0.05$ ($p=0.025$). Wang et al., (2005) found that adding 0.8 percent glucose to the bacterial culture and growth medium increased the activity at the promoter site of the *luxS* gene [36]. Jesudhasan et al. (2010) proved that higher levels of autoinducer-2 did not lead to the increased gene expression of *luxS* in *Salmonella typhimurium* when cultured in overly saturated concentrations of glucose [37].

Figure 9 Regulatory mechanisms of the LsrR/phospho-AI-2 system in *E. coli (*Retrieved from reference 35). The AI-2 uptake repressor LsrR represses the *lsr* operon, containing *lsrACDBFG* and the operon of *lsrRK*. AI-2 can re-enter the cell through expression of LsrACDB. When the LsrK kinase phosphorylates an imported AI-2, the phosphorylated AI-2 binds to LsrR and terminates its repression of *lsr* transporter genes, inducing their expression. These regulatory steps trigger increased AI-2 uptake. (DPD=4,5-dihydroxy-2,3-pentanedione)**



The inhibition of *accA* and decreasing the uptake of glucose lowered the activity at the *luxS* promoter site because Lux-S is an AI-2-adjunct. The escalating output of AI-2 amplifies *luxS* transcription. Widmer et al., (2012) found that the *luxS* mutant strain PJ002 increased the bacterial cell growth rate after adding 25 micromolar of AI-2, which was not present in their phosphate-*buffered* saline or the PBS buffer control [38]. Adding glucose supplementation amplified the aggregates of AI-2, and then *luxS* responded with increased amplification. The heightened genetic activity of *luxS* after glucose supplementation without the inhibition of *accA* can confirm the metabolic dependency of quorum sensing and biofilm formation. Inhibiting *accA* and decreasing glucose, lowered the expression of *luxS*. Less activity of the QS LuxS/AI-2 system can reduce biofilm consistency and formation.

The reduced function of the CT can lessen the output of fatty acids that affect a bacterium's expression of virulent factors, early adhesion to a surface, and its mobility [39]. When ablating the *accA*, less *luxS* was produced, fatty acid synthesis could have decreased, and the Lux-S expression was reduced. The declining activity of Lux-S can lower the release of AI-2 molecules needed for quorum sensing. Many bacteria form fatty acyl-homoserine lactones or acyl-HSLs operate as quorum-sensing signal molecules, which bind to signal receptors to monitor gene expression. The fatty acyl group is developed from fatty acid biosynthesis and elongation [40]. For example, researchers found that the glucose present allowed *S. epidermidis* to downregulate transcription specific for biofilm production during the stationary growth phase [41]. The metabolism of carbohydrates controls biofilm generation in many bacteria species [42]. Studies have shown that AI-2 is a signaling molecule that regulates cellular processes in

Salmonella. The AI-2 signaling molecule monitors metabolism, forms biofilm, allows transport of molecules, and transduces signals (Widmer, 2012-38).

Quorum-sensing activity monitors and regulates biofilm formation. A positive feedback loop is triggered by the ample amounts of autoinducers that switches all bacterial cells to begin sharing quorum signals [43]. The autoinducers fill the external bacterial environment when the population proliferates [44]. The bacterial cells can detect the population growth, assess the number of cells, and then transmit a response to control gene expression. Quorum sensing between bacterial cells monitors the genes responsible for organizing the activities executed by different bacterial groups. Activities completed through quorum sensing include bioluminescence, spore formation, antibiotic excretion, creating a biofilm, and releasing factors for virulence [44]. For example, the malfunctioning of *fsr* that resides in the quorum sensing gene loci for *E. faecalis* decreases biofilm formation [42].

However, other bacteria from the Enterobacteriaceae species suppress its creation of biofilm as glucose availability increases. Many other gram-positive bacteria also repress biofilm formation in the presence of glucose. In bacterial species named *E. faecalis* augment its genesis of biofilm when glucose is in excess, but many different strains of *E. faecalis* can also repress biofilm construction while glucose is present [42]. The biofilm contains the total biomass of a bacterial community. The availability of nutrients determines the amount of biomass that accumulates within a biofilm. Adding glucose supplementation to the culturing medium increases the bacterial growth and total biomass [42].

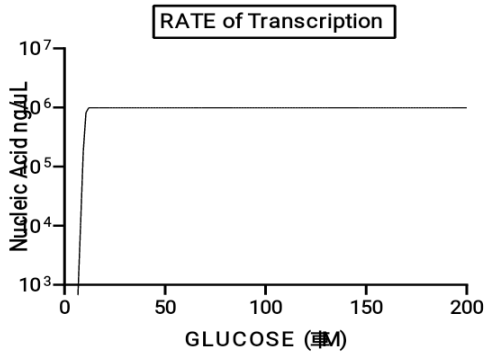
Fatty acid synthesis requires large quantities of energy and more access to phospholipids provided by the added glucose that increased the activity of the ACCase enzyme. Therefore, less production of fatty acids due to the asRNA inhibition of *accA* can negatively affect the biofilm yield and its composition. Fatty acids stabilize the plasma membrane lipid bilayer for developing the early stages of biofilm. Yao and Rock (2017) confirmed that there is a major connection between biofilm formation and fatty acid synthesis enzymes for structures in *S. aureus* [45]. Biofilm has an intricate role in the genesis of infection that cause many hospital or nosocomial infectious diseases. Biofilm allows the movement of pathogenic bacteria to become more rapid and spread an infection into the blood circulation [46]. Bacterial cells in biofilm have a greater access to nutrients and oxygen than in a culturing medium. Higher levels of fatty acids are present during the early growth phase.

Bacterial cells located in biofilm versus planktonic or free-living bacterial cells possess higher concentrations of fatty acids. The higher production and measure of fatty acids increased tolerances to various temperatures, more stacking of bacterial cells, and a more stable lipid bilayer. A heightened presence of long chain fatty acids leads to increased orientation of fatty acids in the lipid bilayer, amplifies the links between acyl chains, and stabilizes the lipid bilayer [34]. The fatty acids produced create a more fluid membrane and the fluid membrane causes the biofilm to expand. As the biofilm expands the number of microcolonies of bacteria settle and grow proportionally to the size of the matrix. The microcolonies proliferate and induce a substantial release of autoinducers, displaying much bioluminescence. The bioluminescence is a sign of quorum sensing. The gene *luxS* assist with the production of AI-2. The quality of biofilm, flagellum mobility, and the release of virulent factors are regulated by *luxS*. In addition, The

large concentrations of DNA present in biofilm serve to maintain a more cohesive matrix and a high quality of the biofilm [47].

Figure 10 The effect of glucose on the rate of transcription, inhibition with asRNA, and the frequency of Nucleic Acid production. A) From the control to the 25 µM glucose exposure, bacteria cells transcribed DNA at an amplified rate. As the glucose concentration increased to 50 micromolar, the rate of transcription reached a sustained equilibrium with no new nucleic acid production or activity. B) The asRNA caused the gene inhibition of *accA.* The samples expressing asRNA inhibition of *accA* produced 16% of the total yield of nucleic acid. Samples not expressing asRNA inhibition yielded 83% of the total quantity of nucleic acid.

A)



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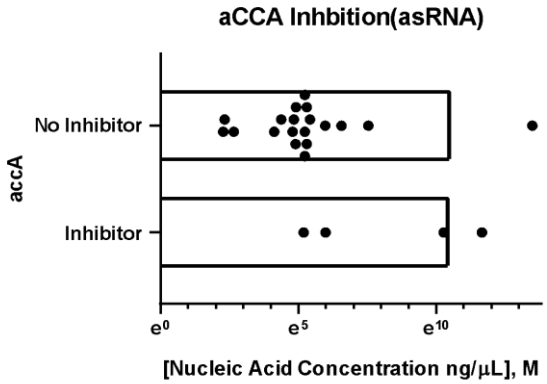


More research is needed to further elucidate the link between the phospholipid membrane and the condition of a biofilm [34]. The findings present significant results for the effect of glucose and the gene inhibition of *accA* upon *luxS,* an essential component for bacterial virulence. Inhibiting the *accA* gene with antisense RNA and altering the availability of nutrients such as glucose, decreased the production of *luxS*. There is a significant link between bacterial cell metabolism and virulence. Understanding the metabolic dependency of virulence can become fundamental for designing novel antibiotics. This understanding can also assist with re-wiring the bacterial metabolic pathways to overly produce plastic materials needed in the manufacturing and food industries.

ANTIBIOTIC RESISTANCE TEST

An antibiotic resistance test was performed to determine the susceptibility of antibiotics from bacterial samples expressing antisense RNA inhibition of the gene *accA.* The bacterial samples expressing the pHN1257-asRNA-*accA* gene suppression were predominantly susceptible to the antibiotic's tetracycline (10µg/mL), carbenicillin (100µg/mL), and chloramphenicol (25µg/mL) (Fig 11). Disks (N=64) consisting of each of the three antibiotics were placed onto agar plates divided into eight sections. The zone of inhibition was measured in millimeters. For the experimental group (with asRNA), the median was 20 mm for the measurement of the zone of inhibition. Control groups (without asRNA) were mainly resistant to antibiotics with an average of 6 mm.

Bacteria transformed with pHN1257-(+)asRNA-*accA* were more susceptible to the antibiotic's tetracycline, carbenicillin, and chloramphenicol. Fifty-nine percent of the experimental (+)asRNA-*accA* samples were sensitive to tetracycline, chloramphenicol, and carbenicillin (21±4, SEM=0.7). The (+)asRNA-*accA* treatment group displayed an intermediate sensitivity of 28% as 3% were resistant to antibiotics (13.75±10, SEM=1.8). The control exhibited more resistance with 25%, 12.5%, and 53% of susceptibility, intermediate, and resistance, respectively. The (+) asRNA-*accA* treatment group had an 82% difference in sensitivity compared to the (-)asRNA-*accA* control group. The control group showed a 77% difference in resistance when compared to the (+)asRNA-*accA* experimental group (p=0.000554). A paired t-test was performed, yielding a t-score of 3.6 and the degrees of freedom equaling 31. The t-score and the DF were used to calculate the p-value.

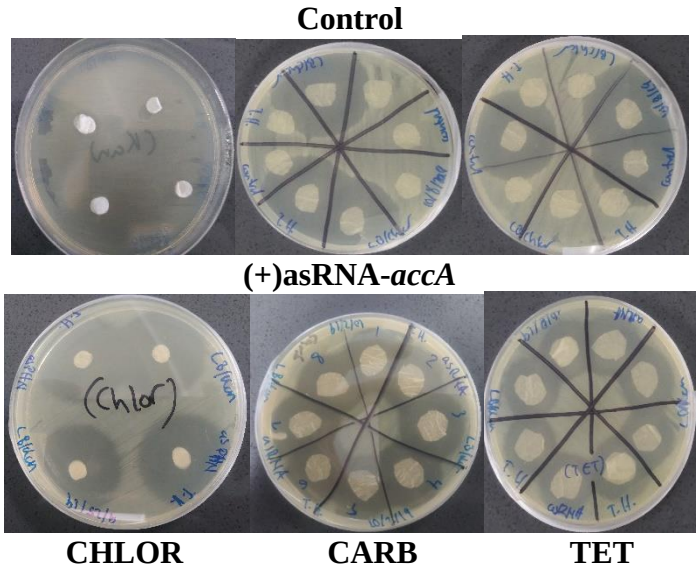
The bacteria of *S. Aureus* translate a lysine at its 164th genomic loci, forming an *S. Aureus* that is resistant to the antibiotic called thailandamide. The thaillandamide also denatures and deactivates the acetyl-CoA carboxylase enzyme with the AccA transferase subunit. The bacterium of *Salmonellae* also produces a mutation of the gene for *accA*. Other antibiotics as capistruin and malleilactone are novel synthetic antibiotics that inhibit the transformation process between bacterial cells. This blockage of bacterial transformation causes more production of antibiotics [48]. However, wild-type *S. aureus* incorporates dissimilar fatty acids in both phospholipid positions without the need for FASII antibiotic selection and rules out the previously assumed fatty acid selectivity of phospholipid-synthesizing enzymes [49].

In contrast, FASII antibiotic adaptation involves the robust bacterial proliferation and incorporation of host fatty acids. Bacterial metabolism and environmental stress play concrete roles in the outcome of antimicrobial treatments [49]. Such metabolic fortitude allows bacteria to salvage metabolites from their environments. This consideration may limit the potential of selecting targets for antimicrobial drug development. Exogenous fatty acids are converted into acyl-ACP in some gram-negative bacteria. The resulting acyl-ACP undergoes the same fates as endogenously synthesized acyl-ACP. Exogenous fatty acids are converted into acyl-phosphates in Gram-positive bacteria and can be used for phospholipid synthesis or become acyl-ACP. Only the Lactobacillales can use exogenous fatty acids to bypass FASII inhibition [45]. FASII shuts down completely in presence of exogenous fatty acids in Lactobacillales, allowing Lactobacillales to synthesize phospholipids entirely from exogenous fatty acids. Inhibition of FASII cannot be bypassed in other bacteria because FASII is only partially down-regulated in

presence of exogenous fatty acid or FASII is required to synthesize essential metabolites such as β-hydroxyacyl-ACP [45].

FIGURE 11 Zone of Inhibition for Antibiotic Susceptibility Test Bacteria that were transformed with pHN1257-(+)asRNA-*accA* showed more susceptibility to the antibiotic disk, containing tetracycline, chloramphenicol, and carbenicillin. Twenty-seven of the total 64 samples were susceptible to antibiotics. Eighteen of the samples were resistant as 13 were only intermediate. The ratio of (+)asRNA-*accA* to (-)asRNA-*accA* equaled 3:2. B) Within the range of 20 to 22 mm of the ZOI for the (+) asRNA-*accA* expressing bacteria, the antibiotic susceptibility showed a density of 0.01515. The control displayed less susceptibility at a 20 to 30 mm ZOI with a density of 0.007.

A)



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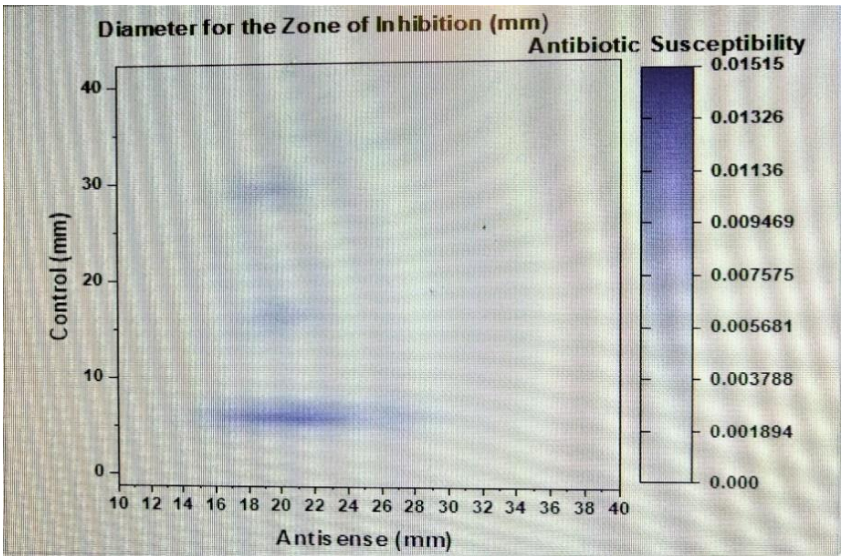

CONCLUSION

Bacterial metabolism is dynamic and complex. Bacteria cells acquire nutrients to create the energy needed for driving bacterial replication and determines host to pathogen interactions. However, a higher awareness is needed for elucidating the interaction between bacterial metabolic capabilities and virulence [50]. According to Stokes et al., (2019) we are currently at the beginning stages of understanding the link between bacterial metabolism and antibiotic efficacy [51]. To address this gap, the author attempted to describe and demonstrate an integrated metabolic process for bacterial fatty acid synthesis relative to *luxS* expression. The expression of *luxS* is an important factor for virulence. When decreasing the glucose concentration, while suppressing the *accA* gene, a decrease in *luxS* expression was observed. Lux-S is vital for quorum sensing, releasing autoinducer-2, and for biofilm formation. The increased AI-2 molecules released triggers quorum sensing for the amplified production of biofilm. Biofilm formation allows more pathogenic bacteria cells to attach, proliferate, detach, and spread into a systemic infection. To confirm and identify the gene *accA* as a possible target for lessening bacterial antibiotic resistance, an antibiotic disk diffusion test was applied to the *accA*-knockout-mutant. The bacteria transformed with (+)asRNA-*accA*, for inhibiting *accA*, displayed antibiotic susceptibility of tetracycline, carbenicillin, and to chloramphenicol. Targeting the *accA* gene, through its inhibition, can increase the antibiotic sensitivity of resistant bacteria.

The results of decreased RNA expression and cell growth in the presence of antisense RNA inhibition of *accA* may serve as a model for blocking microbial metabolic processes that contribute to antibiotic resistance [52]. The qPCR results yielded 63 ng/µL for *accA* (+)asRNA versus 422 ng/µL for *accA* with (-)asRNA. The qPCR absolute quantification results determined the total sum of gene copies for *luxS* decreased during the inhibition of *accA*. The *accA* gene, translated into the acetyl-CoA carboxyl transferase enzyme, uses BC-BCCP to transfer a carboxyl group from pyruvate to the acetyl-CoA, forming a malonyl-CoA. More glucose increases the production of the acetyl-CoA carboxyl transferase. Increased glucose also causes AI-2 quorum-sensing signal molecules to accumulate. By increasing the glucose concentration in

increments, Wang et al. (2005) found that glucose increased the levels of autoinducer-2 of the culture [36]. The proliferation of AI-2 augments the production of Lux-S.

The malonyl-CoA enzyme is vital for catalyzing the biosynthesis of long-chain fatty acids. The long-chain fatty acids are the building blocks for the function of plasma membrane translocation and initiating biofilm formation. Therefore, a decrease of biofilm production, a major virulent factor, can occur due to the limited enzymatic activity of the AccA transferase subunit via *accA* gene inhibition. The potent condition of a bacterial population's metabolic state can inhibit bacterial susceptibility to antibiotics [53]. Therefore, inhibiting a highly conserved ACCase DNA sequence, can decrease quorum sensing signaling, reduce biofilm formation, and increase antibiotic sensitivity. Because the DNA of the ACCase enzyme is conserved across many different species, targeting it with novel antibiotic-genetic inhibition, can eradicate the function and presence of the ACCase enzyme. Conserved DNA sequences do not mutate or change. If the DNA of the conserved sequence for the ACCase enzyme is inhibited or altered, then the enzyme will be missing or completely malfunction. A knockout of the conserved sequence for the ACCase enzyme can provide much potential for designing novel antibiotics as ACCase inhibitors.

Bacteria that cause diseases need biofilm to bind to epithelial cells that then spread the infection. The accumulation of AI-2 is used by bacteria to infect more host cells and the AI-2 creates a more solid biofilm [54]. The activity of the flagella is amplified with higher levels of *luxS* mutations present in the lungs and bloodstream. The mutant *luxS* allows bacteria to more rapidly infect the lungs and the blood than the wild-type strain [55]. The *luxS* gene, when it is more expressed, causes the spread of virulent bacteria through increasing the motility and movement of pathogenic bacteria. *S. Aureus* protects itself through forming biofilm. Biofilm creation is initiated with bacterial attachment to an abiotic or to a biotic surface.

When nutrients are sparse within the biofilm, the auto inducing peptides or AIP allow the detachment of bacteria from each other and from the surface [56].The bacteria begin to accumulate by binding to one another, and the synthesis of extracellular polymeric substances (EPS) is increased [56]. Platelets from the host accrue in the biofilm, forming a mature biofilm. The locus called the accessory gene regulator or agr codes for quorum sensing activity that also mediates this release of bacteria from its community and the surface. More Lux-S expression, as a result of mutations in the *luxS* gene, produces increased amounts of biofilm, assisting pathogenic bacteria to invade the mucosal layers and the epithelial cells of its host, developing into a systemic infection. When Lux-S and autoinducer-2 are less regulated and overexpressed, infectious bacteria can propagate in stressful environments with high acidity and salinity [57]. Kendall et al. (2008) demonstrated the ability of *S. pyogenes* to adapt to acidic conditions as a result of an unregulated Lux-S/AI-2 system [58]. The mutation of *luxS* in *the Haemophilus influenzae* increases virulence that causes middle ear infections [59]. The many facets of biofilm to tolerate antimicrobial therapies include many different types and subtypes of metabolic actions and the bacterial response to those metabolic changes. The subtypes of metabolic changes also include responses to pH, osmotic pressure, and the availability of nutrients [34].

There is a gap of literature, concerning bacterial metabolism and virulence. The volume of studies has increased to unlock the enigma surrounding bacterial metabolism and biofilm creation. For example, a study demonstrated the growth of biofilm temporally was suppressed

during the absence of viable nutrients within the interior of the *B. Subtilis* cells [6]. Zuberi et al. applied CRISPRi technology to inhibit the *luxS* gene in *E. coli* cells, and they found that biofilm formation depended upon the expression of *luxS*. They used a crystal violet test to assess the effect of *luxS* upon biofilm. Biofilm formation decreased as the expression of *luxS* was inhibited [60]. Hall et al. concluded that the cause of antibiotic resistance or the antibiotic sensitivity of biofilm originates from a genetic source [61]. The activity of *luxS* is augmented and regulated through fatty acid synthesis and carbohydrate metabolism. Therefore, there is an immense possibility to design antibiotics that target bacterial-metabolic enzymes and genes. For future research, studying the gene expression, structure, and the connection between bacterial metabolism and virulence could be investigated. Perhaps, this study's explanation for the interaction between *E. coli* fatty acid synthesis in the context of *luxS expression*, a major virulent factor, *contributed to our enhanced* understanding of metabolic-dependent bacterial virulence.

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REFERENCES

1. Polyak SW, Abell AD, Wilce MC, Zhang L, Booker GW. Structure, function, and selective inhibition of bacterial acetyl-CoA carboxylase. Applied microbiology and biotechnology. 2012 Feb 1;93(3):983-92.
2. Calero-Cáceres, W., Ye, M., & Balcázar, J. L. (2019). Bacteriophages as environmental reservoirs of antibiotic resistance. *Trends in microbiology*.
3. Lobanovska, M., and Pilla, G., 2017. Focus: Drug Development: Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future?. *The Yale journal of biology and medicine*, *90*(1), p.135.
4. Aminov, R., 2017. History of antimicrobial drug discovery: Major classes and health impact. *Biochemical pharmacology*, *133*, pp.4-19.
5. Landecker, H., 2016. Antibiotic resistance and the biology of history. *Body & Society*, *22*(4), pp.19-52.
6. Pisithkul, T., Schroeder, J. W., Trujillo, E. A., Yeesin, P., Stevenson, D. M., Chaiamarit, T., ... & Amador-Noguez, D. (2019). Metabolic remodeling during biofilm development of Bacillus subtilis. *MBio*, *10*(3), e00623-19.
7. Richie, D. L., Wang, L., Chan, H., De Pascale, G., Six, D. A., Wei, J. R., & Dean, C. R. (2018). A pathway-directed growth restoration assay to facilitate the discovery of lipid A and fatty acid biosynthesis inhibitors in Acinetobacter baumannii. *PloS one*, *13*(3).
8. Sweeney, P., Murphy, C. D., & Caffrey, P. (2016). Exploiting the genome sequence of Streptomyces nodosus for enhanced antibiotic production. *Applied microbiology and biotechnology*, *100*(3), 1285-1295.

9. N. Nakashima, T. Tamura. Conditional gene silencing of multiple genes with anti-senseRNAs and generation of a mutator strain of Escherichia coli. Nucleic Acids Res. 2009;103 (37).
10. Xu, L., Li, H., Vuong, C., Vadyvaloo, V., Wang, J., Yao, Y., Otto, M. and Gao, Q. Roleof the *luxS* quorum-sensing system in biofilm formation and virulence of Staphylococcus epidermidis. /14PeerJ reviewing PDF | (2018:12:33750: 1:2: NEW 8 Mar 2019) Manuscript to be reviewed Infection and immunity. 2006;74(1):488-496.
11. Telford, G., Wheeler, D., Williams, P., Tomkins, P.T., Appleby, P., Sewell, H., Stewart, G.S., Bycroft, B.W. and Pritchard, D.I. The Pseudomonas aeruginosaquorum-sensing signal moleculen(3-oxododecanoyl)-l-homoserine lactone has immunomodulatory activity. Infection and immunity. 1998; 66(1):36-42.
12. Wilson, J. W., Schurr, M. J., LeBlanc, C. L., Ramamurthy, R., Buchanan, K. L., and Nickerson, C. A. Mechanisms of bacterial pathogenicity. Postgraduate medical journal. 2002;78(918):216-224.
13. France, M. T., Cornea, A., Kehlet-Delgado, H., & Forney, L. J. (2019). Spatial structure facilitates the accumulation and persistence of antibiotic-resistant mutants in biofilms. *Evolutionary Applications*, *12*(3), 498-507.
14. Peng B, Su YB, Li H, Han Y, Guo C, Tian YM, Peng XX. Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. Cell metabolism. 2015 Feb 3;21(2):249-62.
15. Rojo-Molinero, E., Macià, M. D., & Oliver, A. (2019). Social behavior of antibiotic-resistant mutants within Pseudomonas aeruginosa biofilm communities. *Frontiers in Microbiology*, *10*, 570.
16. Verstraeten, N., Knapen, W., Fauvart, M. and Michiels, J., 2016. A historical perspective on bacterial persistence. In *Bacterial Persistence* (pp. 3-13). Humana Press, New York, NY.
17. Eichenberger EM, Thaden JT. Epidemiology and Mechanisms of Resistance of Extensively Drug Resistant Gram-Negative Bacteria. Antibiotics. 2019 Jun;8(2):37.
18. Broussard, T. C., Kobe, M. J., Pakhomova, S., Neau, D. B., Price, A. E., Champion, T. S., & Waldrop, G. L. (2013). The three-dimensional structure of the biotin carboxylase-biotin carboxyl carrier protein complex of E. coli acetyl-CoA carboxylase. *Structure*, *21*(4), 650-657.
19. Broussard, T. C., Price, A. E., Laborde, S. M., & Waldrop, G. L. (2013). Complex formation and regulation of Escherichia coli acetyl-CoA carboxylase. *Biochemistry*, *52*(19), 3346-3357.
20. E.S. James, J.E. Cronan. Expression of two *Escherichia coli acetyl-CoA* carboxylase subunits is autoregulated. Journal of Biological Chemistry. 2004;279(4): 2520-2527.
21. Tong, L. (2013). Structure and function of biotin-dependent carboxylases. *Cellular and Molecular Life Sciences*, *70*(5), 863-891.
22. Waldrop, G. L.; Rayment, I.; Holden, H. M. (1994). "Three-dimensional structure of the biotin carboxylase subunit of acetyl-CoA carboxylase". Biochemistry. **33** (34): 10249–10256. *doi*:*10.1021/bi00200a004*. *PMID* *7915138*.
23. Seti, Gita. (n.d). Discover Ideas about Protein. Retrieved from https://www.pinterest.com/pin/531002612291400564/

24. R.P. Ferraris, S. Yasharpour, K.C.K. Lloid, R. Mirzayan, J.M. Diamond, (1990) Luminal glucose concentration in the gut under normal conditions. Am. J. Physiol. 1990;259, 820–837.
25. Milke, L., Kallscheuer, N., Kappelmann, J., & Marienhagen, J. (2019). Tailoring Corynebacterium glutamicum towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin. *Microbial cell factories*, *18*(1), 71.
26. Lietzan, A. D., & Maurice, M. S. (2013). A substrate-induced biotin binding pocket in the carboxyltransferase domain of pyruvate carboxylase. *Journal of Biological Chemistry*, *288*(27), 19915-19925.
27. Lietzan, A. D., & Maurice, M. S. (2013). A substrate-induced biotin binding pocket in the carboxyltransferase domain of pyruvate carboxylase. *Journal of Biological Chemistry*, *288*(27), 19915-19925.
28. Chandrakar, B., Jain, A., Roy, S., Gutlapalli, V. R., Saraf, S., Suppahia, A., ... & Nayarisseri, A. (2013). Molecular modeling of Acetyl-CoA carboxylase (ACC) from Jatropha curcas and virtual screening for identification of inhibitors. *journal of pharmacy research*, *6*(9), 913-918.
29. Bilder P, Lightle S, Bainbridge G, Ohren J, Finzel B, Sun F, Holley S, Al-Kassim L, Spessard C, Melnick M, Newcomer M, Waldrop GL. The structure of the carboxyltransferase component of acetyl-coA carboxylase reveals a zinc-binding motif unique to the bacterial enzyme. *Biochemistry* **45** 1712-22 (2006) doi:10.1021/bi0520479
30. Ikeda, M., Nagashima, T., Nakamura, E., Kato, R., Ohshita, M., Hayashi, M., & Takeno, S. (2017). In vivo roles of fatty acid biosynthesis enzymes in biosynthesis of biotin and α-lipoic acid in Corynebacterium glutamicum. *Appl. Environ. Microbiol.*, *83*(19), e01322-17.
31. Salie, M. J., Zhang, N., Lancikova, V., Xu, D., & Thelen, J. J. (2016). A family of negative regulators targets the committed step of de novo fatty acid biosynthesis. *The Plant Cell*, *28*(9), 2312-2325.
32. Evans, A. L. (2018). The Distinctive Regulatory Mechanisms of Bacterial Acetyl-CoA Carboxylase.
33. Janiyani, K., Bordelon, T., Waldrop, G. L., & Cronan, J. E. (2001). Function of Escherichia coli biotin carboxylase requires catalytic activity of both subunits of the homodimer. Journal of Biological Chemistry, 276(32), 29864-29870.
34. Dubois-Brissonnet, F., Trotier, E., & Briandet, R. (2016). The biofilm lifestyle involves an increase in bacterial membrane saturated fatty acids. *Frontiers in microbiology*, *7*, 1673.
35. Li, J., Attila, C., Wang, L., Wood, T. K., Valdes, J. J., & Bentley, W. E. (2007). Quorum sensing in Escherichia coli is signaled by AI-2/LsrR: effects on small RNA and biofilm architecture. *Journal of bacteriology*, *189*(16), 6011-6020.
36. Wang, L., Hashimoto, Y., Tsao, C.Y., Valdes, J.J. and Bentley, W.E. Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in Escherichia coli. Journal of bacteriology. 2005;187(6):2066-2076.
37. Jesudhasan, P.R., Cepeda, M.L., Widmer, K., Dowd, S.E., Soni, K.A., Hume, M.E., Zhu, J. and Pillai, S.D. Transcriptome analysis of genes controlled by *luxS*/autoinducer-2 in Salmonellaenterica serovar Typhimurium. Foodborne pathogens and disease.2010;7(4):399-410.

38. Widmer, K. W., Jesudhasan, P., & Pillai, S. D. (2012). Fatty acid modulation of autoinducer (AI-2) influenced growth and macrophage invasion by Salmonella Typhimurium. *Foodborne pathogens and disease*, *9*(3), 211-217.
39. Nicol, M., Alexandre, S., Luizet, J. B., Skogman, M., Jouenne, T., Salcedo, S. P., & Dé, E. (2018). Unsaturated Fatty Acids Affect Quorum Sensing Communication System and Inhibit Motility and Biofilm Formation of Acinetobacter baumannii. *International journal of molecular sciences*, *19*(1), 214. https://doi.org/10.3390/ijms19010214
40. Schaefer, A., Greenberg, E., Oliver, C. *et al.* A new class of homoserine lactone quorum-sensing signals. *Nature* **454,** 595–599 (2008). https://doi.org/10.1038/nature07088
41. Dobinsky, S., Kiel, K., Rohde, H., Bartscht, K., Knobloch, J. K. M., Horstkotte, M. A., & Mack, D. (2003). Glucose-related dissociation between icaADBC transcription and biofilm expression by Staphylococcus epidermidis: evidence for an additional factor required for polysaccharide intercellular adhesin synthesis. *Journal of bacteriology*, *185*(9), 2879-2886.
42. Pillai, S. K., Sakoulas, G., Eliopoulos, G. M., Moellering Jr, R. C., Murray, B. E., & Inouye, R. T. (2004). Effects of glucose on fsr-mediated biofilm formation in Enterococcus faecalis. *Journal of Infectious Diseases*, *190*(5), 967-970.
43. C.M. Waters, B.L. Bassler. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. 2005;21: 319-346.
44. S.T. Freestone, B.L. Bassler. Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. Cold Spring Harbor Perspectives in Medicine. 2012;2(11): a012427. doi:10.1101/cshperspect. a012427.
45. Yao, J., & Rock, C. O. (2017). Exogenous fatty acid metabolism in bacteria. Biochimie, 141, 30-39.
46. Soares, A., Caron, F., & Etienne, M. (2019). Commentary: tolerance and resistance of pseudomonas aeruginosa biofilms to antimicrobial agents–how p. Aeruginosa can escape antibiotics. *Frontiers in Microbiology*, *10*, 2164.
47. Trappetti, C., Potter, A.J., Paton, A.W., Oggioni, M.R. and Paton, J.C. LuxS mediatesiron-dependent biofilm formation, competence, and fratricide in Streptococcus pneumoniae. Infection and Immunity. 2011;79(11):4550-4558.
48. Wozniak CE, Lin Z, Schmidt EW, Hughes KT, Liou TG. Thailandamide, a fatty acid synthesis antibiotic that is coexpressed with a resistant target gene. Antimicrobial agents and chemotherapy. 2018 Sep 1;62(9):e00463-18.
49. Kénanian, G., Morvan, C., Weckel, A., Pathania, A., Anba-Mondoloni, J., Halpern, D., ... & Poyart, C. (2019). Permissive Fatty Acid Incorporation Promotes Staphylococcal Adaptation to FASII Antibiotics in Host Environments. Cell Reports, 29(12), 3974-3982.
50. Passalacqua, K. D., Charbonneau, M. E., & O'riordan, M. X. (2016). Bacterial metabolism shapes the host–pathogen interface. *Virulence Mechanisms of Bacterial Pathogens*, 15-41.
51. Stokes, J. M., Lopatkin, A. J., Lobritz, M. A., & Collins, J. J. (2019). Bacterial metabolism and antibiotic efficacy. *Cell metabolism*.
52. Baquero F, Martínez JL. Interventions on metabolism: making antibiotic-susceptible bacteria. mBio. 2017 Dec 29;8(6):e01950-17.
53. Jayaraman, A., Thomas, K.W. Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. Annu. Rev. Biomed. Eng. 2008; 10:145-167.

54. Vidal, J.E., Howery, K.E., Ludewick, H.P., Nava, P. and Klugman, K.P., 2013. Quorum-sensing systems LuxS/autoinducer 2 and Com regulate Streptococcus pneumoniae biofilms in a bioreactor with living cultures of human respiratory cells. Infection and immunity. 2013;81(4): 1341-1353.
55. Vendeville, A., Winzer, K., Heurlier, K., Tang, C.M. and Hardie, K.R., Making' sense of metabolism: autoinducer-2, LuxS, and pathogenic bacteria. Nature Reviews Microbiology. 2005;3(5):383.
56. Siller, M., Janapatla, R.P., Pirzada, Z.A., Hassler, C., Zinkl, D. and Charpentier, E., 2008. Functional analysis of the group A streptococcal *luxS*/AI-2 system in metabolism, adaptation to stress, and interaction with host cells. BMC microbiology. 2008;8(1), p.188.
57. M.M. Kendall, D.A. Rasko, V. Sperandio. Global effects of the cell-to-cell signaling molecule autoinducer-2, autoinducer-3, and epinephrine in a *luxS* mutant of enterohemorrhagic Escherichia coli." Infection and Immunity. 2007;75(10):4875-4884.
58. Vidal, J.E., Ludewick, H.P., Kunkel, R.M., Zahner, ¨ D. and Klugman, K.P. The LuxS dependent quorum-sensing system regulates early biofilm formation by Streptococcus pneumoniae strainD39. Infection and immunity. 2011;79(10):4050-4060.
59. Zuberi A, Misba L, Khan AU. CRISPR interference (CRISPRi) inhibition of *luxS* gene expression in E. coli: an approach to inhibit biofilm. Frontiers in cellular and infection microbiology. 2017 May 26;7:214.
60. Hall CW, Mah TF. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS microbiology reviews. 2017 Mar 25;41(3):276-301.