THE EFFECTS OF ACETOACETIC ACID ON BACTERIAL GROWTH AND BIOFILM FORMATION OF *CRONOBACTER SAKAZAKII*, *SERRATIA MARCESCENS*, AND *YERSINIA ENTEROCOLITICA*

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ABSTRACT

Prevention of bacterial biofilms is an area of research currently being investigated by many research teams. The ability of a chemical to be incorporated into a material that could be used in a medical or food production setting could be of a major value. In this study, we explored the ability of acetoacetic acid (AAA) to reduce biofilm amounts and bacterial growth. We also looked at the transcription of early and late expressed virulence genes in the presence of AAA. We conclude that AAA is a plausible candidate for preventing biofilm formation as we saw a reduction in of biofilm amounts and growth in *C. sakazakii*, *S. marcescens* and *Y. enterocolitca*. We also concluded that AAA was effecting the transcription of virulence genes.

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LIST OF ABBREVIATIONS

EPS.....extracellular polymeric substance

PEA .....β-phenylethylamine

AAA.....acetoacetic acid

DMSO .....dimethyl sulfoxide

LB.....luria burtani

TSB.....tryptic soy broth

PBS .....phosphate buffered saline

CRA.....congo red agar

CRAMP .....congo red morpholine propane sulfonic acid pigmentation agar

PCR .....polymerase chain reaction

CV .....crystal violet

ATP .....adenosine triphophate

qPCR.....quantitative real-time polymerase chain reaction

EDTA.....ethylenediaminetetraacetic acid

SDS .....sodium dodecyl sulfate

LITERATURE REVIEW

Background and significance

Bacterial biofilms are sessile communities that form on biotic or abiotic surfaces and are surrounded and protected by an extracellular polymeric substance (EPS) [1-3]. Biofilm formation from the free swimming planktonic bacteria comprises of defined stages in development; attachment, maturation, and dispersal. Each of these stages develops via a change in gene transcription producing distinct phenotypes within the biofilm [4]. These adherent cells are particularly difficult to remove by mechanical means, and this is coupled with an increased resistance to antimicrobial treatment [5, 6].

Medical community and biofilms

The biofilm characteristics, while beneficial for the bacteria, can cause a host of problems for human populations in a number of settings. Bacterial biofilms have been shown to contribute to illness in an assortment of healthcare environments, especially in the immunocompromised populations and those patients requiring indwelling medical devices, such as catheters or artificial prosthetics[7-11]. Infections involving biofilm can be problematic to treat with antibiotics, which can lead to indwelling medical devices replacement, increasing the discomfort and medical costs for the patient and the health care industry[12-14]. Patients requiring central venous line catheters are at an increased risk of catheter infection and subsequent bloodstream infections, which is consequential because 78% of all critically ill receive a central venous line catheter[15]. It's estimated that controlling infections in artificial

joints alone account for 4,000-5,000 cases a year and cost in the vicinity of $200-250 million dollars[16] .

Biofilms in the food processing industry

In addition to the impacts seen in the medical community, biofilms are also seen as a contributor to illness via food supply. Biofilms that form on food processing equipment surfaces, or the food itself, can have disastrous consequences because of the wide distribution of many of today's products. Stainless steel is a conventional material for food processing equipment, and while it may seem that it is an impervious material and easy to clean, it is still prone to bacterial attachment [11, 17]. Conveyor belts are used in the processing of many fruits and vegetables, and provide another vulnerable point of contamination[18]. As food products are being processed, fragmenting of a mature biofilm may lead to the adulteration of the product on a large scale, and a larger dose delivery in these fragments than would be expected if the product was only exposed to planktonic bacteria[19]. While there are regulations in place to reduce the risks of microbial contamination in food products, biofilms can be concealed in hard to reach and clean spaces of processing equipment [18]. Contamination, especially in ready-to-eat foods, can result in human illness and exceedingly high cost to the processing company [20]. Not only may the company in question need to make reparations to anyone personally effected by the tainted product, but must also bear the cost of lost product and reputation.

Clinical significance of the bacteria used for this study

Cronobacter sakazakii

C. sakazakii, formerly known as *Enterobacter sakazakii*, is a ubiquitous bacterium that has been isolated from a variety of sources, most notably powdered infant formula [21, 22]. *C. sakazakii* can cause illness in individuals of all ages but is most detrimental to those whose health and immune system may be compromised. This is especially hazardous because neonates, specifically premature neonates in intensive care units, are at an increased risk of fatal infections due to invasive disease [23]. Invasive diseases caused by *C. sakazakii* include sepsis, necrotizing enterocolitis and meningitis. The most severe cases, which generally occur in neonates, have a substantially increased mortality rate [24-26]. Such mortality rates can be as high as 40-80% in premature neonates, which is considerably larger than what is seen in older populations or those who are not immune compromised [26]. In addition to being susceptible to infections, premature neonates may have a nasogastric enteral feeding tube in place to aid in receiving needed nutrition. In a study done to investigate bacterial isolates in these feeding tubes was done by Edward Hurrell et. al., results confirmed the presence of *C. sakazakii* within a number of tubes [27]. Cases of infection in infants with the bacterium have also been linked to equipment used to prepare the formula in a hospital setting. It has been recommended that extreme care be taken by support staff in hospitals when preparing and administering powdered infant formula to neonates, and proper disinfection of all equipment be done appropriately [28-31].

Serratia marcescens

S. marcescens is an opportunistic pathogen that can cause significant illness in the immunocompromised population [32]. The bacterium can be isolated from virtually any environment and is a common organism on human skin, but can cause complications in postoperative patients, especially as many strains are antibiotic resistant [32, 33]. Nosocomial infections in a clinical setting due to *S. marcescens* can cause respiratory, bloodstream and urinary tract infections. Disease outbreaks in neonatal intensive care units have been attributed to *S. marcescens* with disastrous results. In one such outbreak, five premature neonates were confirmed infected with the pathogen, two of them developed septicemia and sadly passed away. This bacterium is prevalent enough to pose a considerable risk to compromised neonates that usually require invasive procedures [34]. The bacterium can also cause ocular infections and is known to form biofilms on contact lenses. The microbes use quorum-sensing to control the attachment, maturation and dispersal of the filamentous biofilms it forms [35]. The quorum-sensing also controls the sloughing off of the biofilms, which could lead to spreading of the infection given the right circumstances [36].

Yersinia enterocolitica

Y. enterocolitica is a bacterium most commonly associated with food borne illness, and can cause gastroenteritis and septicemia in susceptible populations. The very young, under one year of age, are the most prone to illness and in severe cases can develop bacteremia [37]. Infection by the bacteria can result through exposure to raw or undercooked pork products and has been associated with chitterlings (boiled porcine intestines) [38-40]. *Y. enterocolitica* is psychotropic and grows at refrigerated temperatures and forms biofilms [41]. These abilities

could lead to an increase in the number of bacteria present and the potential to cross-contaminate other food products. Pork product contamination with virulent strains of *Y. enterocolitca* have been placed at 60% or higher, revealing the potential for illness associated with those products when not handled properly [42]. Porcine are thought to be the reservoir of the pathogen, but it is unconfirmed. After infection with *Y. enterocolitica* a genetically predisposed proportion of those who contracted the disease will also develop reactive arthritis [43]. The bacterium, after invading the M cells and Peyer's patches of the host, can also invade other organs such as the spleen and liver[44]. The possibility of a life-long sequela because of a previous exposure to the pathogen increases the need to reduce the chances of infection from the beginning of the food production chain.

Research Rationale

Previous Research

Previous work was done in our lab examining prevention measures for *Escherichia coli* O:157 H:7 on beef meat pieces [45]. Signal transduction systems have been a target for novel treatment of bacteria for some time and have been reviewed by members of our research laboratory [46]. The concept of using the bacteria's own metabolic pathways to control their behaviors was taken a step further by screening 95 carbon and 95 nitrogen sources in a liquid beef broth growth medium, using the BioLog Phenotype MicroArray^{TM} (PM) technology (Biolog, Hayward, CA) to investigate each compounds ability to reduce cell counts and biofilm amounts. After screening, one carbon and one nitrogen source stood out in their ability to reduce microbial counts. These two substances were then used to perform a beef meat piece experiment, where treating the meat with β-phenylethylamine (PEA) or acetoacidic acid (AAA)

prior to inoculating with *E. coli*. After seven days of incubation at refrigerator temperature, the nitrogen source PEA was able to reduce cell counts by 90% at the highest concentration. The carbon source AAA reduced cell counts by 50% at the highest concentration [45].

Acetoacetic acid

AAA is a short-chain keto-acid produced in the liver during fatty acid oxidation. Our bodies combine AAA with acyl-CoA to produce acetoacetate-CoA, and convert it to acetyl-CoA before it enters the TCA cycle to produce energy [47]. Previous studies investigated the activity of three enzymes on ketone bodies depending on cell conditions: 3-oxo acid CoAtransferase (EC 2.8.3.5), acetoacetyl-CoA thiolase (EC 2.3.1.9), and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30).

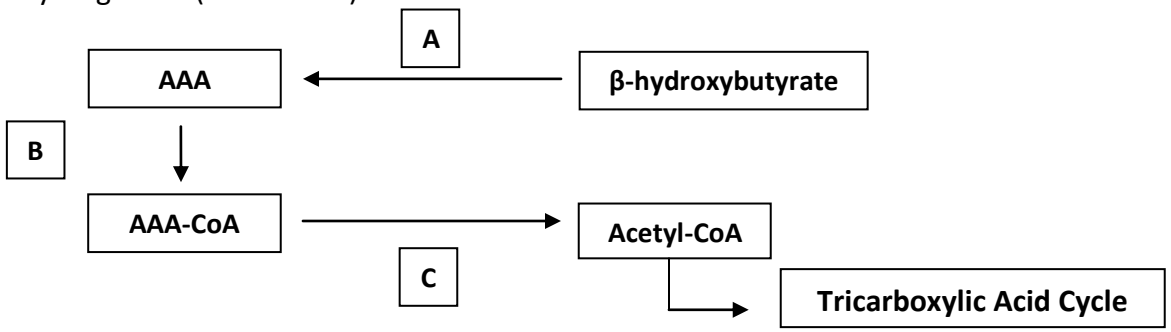

Figure 1: Eukaryotic acetoacetic acid metabolism. Box **A** is the reaction catalyzed by 3-hydroxybutyrate dehydrogenase, **B** by 3-oxo acid CoA transferase, and **C** by acetoacetyl CoA thiolase. The acetyl-CoA enters the tricarboxylic acid cycle to produce energy for the cell.

What those results elucidated was that it is not the amount of enzyme present that controls the rate of these reactions, but instead it is the concentration of available AAA that drives the reaction rate [48]. This gives us confidence that the molecule in low amounts should not be a significant hurdle for the mammalian system to process, although further research would need to be done to verify safety. At this point in time, the LC_{50} in mice has not been

established according to the MSDS for acetoacetic acid lithium salt (Sigma, St. Louis, MO) that was used for this study.

Y. enterocolitica virulence genes

One aspect that was investigated with this study was the potential of AAA to increase the transcription of virulence genes in *Y. enterocolitica*. Virulence genes of Y. *enterocolitica* fall into two separate categories, virulence genes expressed early in infection [49, 50], and genes expressed late in infection[51]. Intriguingly, the distinction between early and late virulence genes coincides with the physiological switch that *Y. enterocolitica* performs, depending on what temperature it finds itself in. At cooler temperatures, 25^{o}C, the bacteria are motile and express early virulence genes while at warmer temperatures, 37^{o}C, the bacteria become non-motile and start increasing the transcription of late virulence genes[52]. This way, temperature serves as the environmental signal that helps the bacteria switch from early to late virulence genes when they transit from the colder environment to the 37^{o}C host. It is also thought that the bacterium's DNA has a particular topography at different temperatures, and that the change to a higher temperature may allow that shape to be transformed and virulence gene transcription to occur[53].

A requirement for motility is the expression of flagellin genes , *Y. enterocolitica* contains three such genes, *fleA*, *fleB,* and *fleC*. These genes are expressed at the lower temperature of 25^{o}C and no longer expressed at higher temperatures such as 37^{o}C[49]. The correlates with the loss of motility at 37^{o}C. Intriguingly, the flagellar master regulator operon *flhD* does not show such temperature regulation [54]. In addition to the flagellar genes, the *invF* gene is another of the early virulence genes that is reduced at 37^{o}C relative to 25^{o}C [55]. The *invF* gene encodes

for an invasion factor called invasin that plays an integral role in the infiltration of host intestinal cells.

The *yadA* gene is an example of a late virulence gene with an increase in transcription at host temperature, 37^{o}C. The protein product of the gene is an adhesion that has been observed on the outside of bacterial cells almost immediately after an increase in temperature to 37^{o}C[56]. This gene may also play a role in evading the immune system, possibly by binding host factor H and reducing opsonization[57]. The *ymoA* gene is proposed to encode a histone-like protein and may play a role in the regulation of virulence genes[58]. Previous experiments examining the promoter region of *invF* found negative regulatory regions and it is thought to be regulated through YmoA, although the exact mechanism of this regulation is not completely understood[59]. The *yopQ* gene is important for the injection of bacterial toxins into host cells, and are up-regulated by the increased temperatures encountered in mammalian host. V*irF* is a transcriptional activator that is required for the expression of most plasmid-encoded late virulence genes. This gene, too, shows increased transcription at 37^{o}C [60, 61]. The virulence genes and their characteristics can be found below in Table 1.

Table 1: Characteristics of selected virulence genes

Gene	Gene Product	Early/Late Virulence	25/37^{o}C	Plasmid/Chromosome
flhD	Master regulator	Early	25^{o}C	Chromosome
invF	Invasin	Early	25^{o}C	Chromosome
fleB	Flagellin	Early	25^{o}C	Chromosome
yadA	Adhesin	Late	37^{o}C	Plasmid
ymoA	Histone-like protein	Late	37^{o}C	Chromosome
yopQ	Secreted outer protein	Late	37^{o}C	Plasmid
virF	Transcriptional regulator	Late	37^{o}C	Plasmid

Objectives

Objective I. To determine the feasibility of using AAA as an anti-microbial, Objective I tests AAA in a liquid growth medium to determine its effectiveness at reducing planktonic growth and biofilm amounts in *C. sakazakii*, *S. marcescens*, and *Y. enterocolitica*. The concentrations that will be needed to cause a 50% reduction (IC_{50}) will be determined and presented.

Objective II. To increase our understanding of the effect of AAA on *Y. enterocolitica*'s virulence gene transcription, we performed quantitative real-time PCR to determine differences in gene transcription in response to a variety of changes in the environmental conditions (including absence/presence of AAA and changes in growth temperature) for a selection of early and late virulence genes. An increase in virulence gene transcription could have major implications for the use of AAA in either medical or food production settings.

Future Perspective

The ultimate goal of the experiments currently being done is to integrate AAA into materials that can be used in relevant settings to reduce the risk of biofilm and the spread of disease. Medical devices and food processing equipment with a coating of material that can reduce the ability of pathogens to adhere to these surfaces could ultimately reduce human suffering and associated monetary losses. Currently, PEA has been integrated into polyurethane and is being tested for efficacy at reducing bacterial growth and biofilm amounts with a variety of pathogens, and is showing promising results (Schroeder et. al., manuscript in preparation). With good fortune, AAA will show the same potential when integrated into a material, and can be used either alone, or in conjunction with PEA, to reduce biofilm formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

C. sakazakii BAA-894 was isolated from powdered infant formula after a fatal neonatal case of *C. sakazakii* infection in 2001 [21]. *S. marcescens* BAA-632 was isolated from the urine of an asymptomatic adult [62]. *Y. enterocolitica* 8081v is a pathogenic wild-type BALB/c virulent O:8 strain, provided by Scott A. Minnich (University of Idaho, Moscow, ID) [63, 64]. *Y. enterocolitica* 8081c is the non-virulent, plasmid cured version of the wild-type strain [64]. pPM61 is a plasmid that was used to produce a standard curve for the qPCR. This plasmid contains the *flhD* operon [65].

All bacterial isolate stock cultures were kept at -80^{o}C in the cryoprotective agent Dimethyl Sulfoxide (DMSO). For use of the isolates, the freezer stock was struck out on to Luria Burtani (LB, tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, pH 7.0) agar plates. Plates were incubated at a combination of temperature and time that varied depending of the bacterial strain and physiological state desired (Table 2). For each isolate, single colony was picked from the plate and inoculated into 10 ml of Tryptic Soy Broth (TSB, pancreatic digest of casein 37.0 g/l, papaic digest of soybean 3.0 g/l, dextrose 2.5 g/l, sodium chloride 5.0 g/l, dipotassium phosphate 2.5 g/l, pH 7.3). Cultures were incubated aerobically while shaking at 150 rpm at times and temperatures listed in Table 2. Bacteria were pelleted from the cultures by centrifugation at 4,700 rpm at 4^{o}C for 10 min. Bacterial pellets were re-suspended in Phosphate Buffered Saline (PBS) to an optical density at 600 nm (OD_{600}) of 1.00 ± 0.02. The PBS cultures were diluted in 2 x TSB at a 1:100 ratio for use in their respective experiments.

Table 2: Bacterial isolate growth temperatures (^{o}C) and times (hrs).

Bacterial Isolate	Temperature	Time (hrs)
Cronobacter sakazakii	37^{o}C	16
Serratia marcescens	30^{o}C	24
Yersinia enterocolitca 8081c	37^{o}C	16
Yersinia enterocolitca 8081c	25^{o}C	24
Yersinia enterocolitca 8081v	37^{o}C	16
Yersinia enterocolitca 8081v	25^{o}C	24

Conformation of Plasmid Presence in Yersinia enterocolitica 8081v

Virulent strains of *Y. enterocolitica* 8081v contain a plasmid that is prone to being lost when grown in the absence of selective pressure. Therefore, all experiments using *Y. enterocolitica* 8081v contained an additional step to confirm the presence of the plasmid, both before and after the experiment. A variety of methods were tested for efficacy.

Congo Red Agar (CRA)

CRA was prepared using LB agar, 10 mM ethylene glycol tetraacetic acid (EGTA) pH 8.0, and 5 µg/ml Congo Red. Bacteria were struck out on these plates and incubated at 25 and 37^{o}C for 72 hours. Dark red pin point colonies are indicative of plasmid presence, while colorless or pale pink colonies indicate no plasmid[66, 67].

Congo Red Acid Morpholine propane sulfonic acid Pigmentation (CRAMP) Agar

CRAMP agar base (HIMEDIA Laboratories, Mumbai, India) was prepared (galactose 2 g/l, casein acid hydrolysate 2 g/l, congo red 0.005 g/l, sodium chloride 2.9 g/l, morpholine propane sulfonic acid 8.4 g/l, ammonium chloride 0.5 g/l, sodium thiosulfate 0.6 g/l, dipotassium phosphate 0.24 g/l, magnesium sulphate 0.0986 g/l, tricine 1.8 g/l, agarose 14 g/l). Bacteria were struck out on the plates, incubated at 37^{o}C for 48 h. Bacteria containing the plasmid are

supposed to yield brightly pigmented colonies versus colorless or lightly pigmented colonies[68].

Polymerase chain reaction (PCR)

Two types of PCR reactions were performed to i) identify a plasmid encoded gene that could be used to detect the presence of the plasmid and ii) detect plasmid presence in each of the experiments. To identify a gene that could be used to detect plasmid presence, three plasmid encoded genes were used: *yadA*, *yopQ*, and *virF*. PCR was performed with GoTaq Flexi DNA Polymerase (Promega, Madison, WI), 2 mM $MgCl_2$, 10 mM forward and reverse primers, and 10 mM dNTP's. PCR amplicons were electrophoresed on a 2% agarose gel with loading buffer,and bands visualized with UV light. Of the three genes, *virF* showed the greatest amount of amplification. For this reason, this gene was tested for in all subsequent experiments with the *Y. enterocolitica* 8081v isolate.

Biofilm amounts in the presence of AAA

A solution of AAA was prepared by dissolving 800 mg of AAA powder into 10 ml of double distilled sterile water (ddH_2O), then filter sterilized. The 80 mg/ml stock solution was used to make the following range of solution concentrations in mg/ml ddH_2O: 80, 70, 65, 60, 55, 50, 45, 40, 30, 20, 10, 0.

Bacterial growth and biofilm amounts

To determine bacterial growth and biofilm amounts, 0.5 ml of the 2 x TSB culture (see bacterial growth conditions) was added to each well of a 24-well polystyrene plate. AAA solution was added to each well, 0.5 ml, and each concentration was done in duplicate on triplicate plates. The final concentrations of AAA were as follows in mg/ml: 40, 35, 32.5, 30,

27.5, 25, 22.5, 20, 15, 10, 5, 0 and molarity in mM: 370, 324, 301, 278, 255, 231, 208, 185, 139, 93, 46, 0. The plates were sealed with sterile film and incubated statically in the plate reader under the conditions of Table 2. To determine bacterial growth, an OD_{600} reading was taken every 2 hours for the duration of the incubation. At the end of the incubation period, the 24-well plates were removed and biofilm amounts were determined with the crystal violet (CV, [69-71]) and ATP assays[71].

For both assays, the majority of the planktonic cells were removed by pipetting the supernatant from the wells. Wells were rinsed three times with 1 ml of PBS. To remove excess liquid between rinses, the plates were inverted and gently tapped on absorbent paper. The plates were then allowed to dry for 1 hr at room temperature.

For the CV assay, 1 ml of 0.1% CV was added to each well and allowed to incubate at room temperature for 15 minutes. The CV was pipetted off and each well was rinsed three times with PBS, again with inverted tapping between each rinse to remove excess liquid. The plates were then allowed to dry for 1 hour at room temperature. CV was resolubilized using 0.5 ml of a 80% Ethanol / 20% Acetone mixture. 150 µl from each of the 24 wells was pipetted onto a 96-well polystyrene plate and the OD_{600} was read with a plate reader.

For the ATP assay, washing of the biofilm was done with ddH_2O in place of PBS. The bacteria left in the wells were re-suspended with 1 ml of ddH_2O, and 100 µl from each well transferred to a white 96-well polystyrene plate. 100 µl of BacTiter-Glo™ (Promega, Madison, WI)) was added to each well and the plate reader was used to determine relative luminosity (RLU).

Data analysis

Averages and standard deviations across the six replicates were calculated for each bacterial isolate and growth condition. The 50% inhibitory concentrations (IC_{50}) were calculated for each bacterial isolate and experimental condition using Masterplex software. (Hitachi Solutions, San Bruno, CA) Masterplex's best fit feature was used for the calculations, which selects the best equation and algorithm for the data (4-parameter or 5-parameter). In addition to the IC_{50} values, the software also calculates a corresponding R^2 value to evaluate the goodness of fit for the calculated curve.

DNA Preparation for quanitative real-time PCR (qPCR)

Cell Preparation

To grow the cells for the qPCR experiment, 4 ml of 1:100 dilution of *Y. enterocolitica* 8081v culture in 2x TSB was added to each of the wells of 6-well polystyrene plates, and 4 ml of either ddH2O or 10 mg/ml AAA solution for a total of 8 ml/well. Plates were sealed with sterile film and statically incubated at 25/37^{o}C for 24 hours before harvesting. Supernatant was removed from the wells using serological pipettes, transferred to 50 ml conical tubes with 10% stop solution (%5 PheOH in EtOH) and placed on ice prior to centrifugation. 4 ml of 1x PBS was added to the wells and adherent cells re-suspended with sterile swabs. After re-suspension liquid was placed in 50 ml conical tubes with 10% Stop Solution. Cells were pelleted by centrifugation at 4^{o}C, 3500 rpm for 5 minutes. Supernatant from each tube was removed and discarded, and pellets were frozen with acetone and dry ice before being stored at -80^{o}C. Each experimental condition was done in triplicate for a total of 24 samples.

RNA Extraction

Pellets were defrosted on ice and re-suspended in 480 µl hot lysis buffer (2% SDS, 200 mM NaOAc, 20 mM EDTA) before adding 480 µl hot phenol solution (Stock solution as follows, mixed 4 parts to 1 part equilibrated phenol: 0.4 M sodium chloride, 1% β-mercaptoethanol, 1% SDS, 20 mM Tris (pH 7.5), 40 mM EDTA). Three phenol:chloroform extractions were performed with an increasing ratio of chloroform, drawing off the aqueous phase with each round of extraction. The resultant RNA was precipitated with isopropanol centrifugation at 10,000 g, at 4^{o}C, for 90 min. The RNA pellets were washed in 70% ethanol before a brief drying. RNeasy mini columns (Qiagen, Valencia, CA) were used to purify the RNA and two DNaseI digests done to reduce contaminating genomic DNA.

cDNA Synthesis From RNA

To synthesize cDNA, the RNA samples previously collected were incubated with random hexamers (Invitrogen™) at 70^{o}C for 10 min. The Superscript® II Reverse Transcription kit (Invitrogen™) was used to perform the reverse transcription following the manufacturer's protocol. The mixture was incubated at 42^{o}C for 2.5 hr. After incubation period, 1 N NaOH and 0.5 M EDTA was added before incubating at 65^{o}C for 15 min. The solution was neutralized with 1 M Tris, pH 7.4. Cleanup and sample concentration was done with 30 kDa centrifugal filter units. The resultant cDNA was stored at -20^{o}C until needed for qPCR.

qPCR

The qPCR was performed using the iCycler iQ Real Time PCR Detection System (BIO RAD, Hercules, CA) and iQ SYBR Green Supermix (Bio Rad, Hercules, CA) for detection. See Table 3 for a list of all primer sets used in the qPCR experiments. All 24 samples were run in triplicate

on each 96-well polystyrene plate with the primer set changing for each run. For all experimental samples, the cDNA concentration was 2ng/µl in all qPCR tests. In addition to running all experimental samples on each plate, the *Escherichia coli* pPM61 plasmid was used to generate a standard curve. Each plasmid concentration, 10^{-3}-10^{-8}, was run in duplicate on each plate. Relative transcription was calculated using $2^{-\Delta Ct}$, any transcription change greater than 2-fold was considered regulated and analyzed further[72-75]. A student t-test and ranked sign test was performed on the comparisons above the 2-fold mark to investigate if the change in gene regulation was significant, significance was determined by a p-value of 0.05 or less.

Table 3: *Y. enterocolitica* 8081v primer sets for qPCR

Primers	Forward	Reverse
Ye-*invF*	5'-CATCATCTGGTGCATCAAGG-3’	5'-TTACACAGCATCACGTTAGC-3'
Ye-*fleB*	5'-CGCAGCAGAGACAATACAGTT-3'	5'-TGAACAATACCGTGAACAACCT-3'
Ye-*ymoA*	5'-ACGAACTTTCTGACGATGAGCTGGA-3'	5'-TGTGAGTTCAGCTAAGCGGTGGTCT-3'
Ye-*yopQ*	5'-AGTTGGTGTCAATGTCGCTG-3'	5'-ACTGCGCTACTGCTCATTTAC-3'
Ye-*yadA*	5'-GCCGAATCTCCCAATGCCTTAC-3'	5'-CGGTTGGTGCTAGTGCTGAAG-3'
Ye-*flhD*	5'-CGTCTTTTAACGATAGCTCGTG-3'	5'-AACGATGAGAAAGCCTCAGC-3'
Ye-*virF*	5'-CTACAAGGGTGGAAACTAAGC-3'	5'-ATTGGTGAGCATAGAGAATACG-3'

Motility assay

An assay was performed to determine the motility of *Y. enterocolitica* 8081v at 25^{o}C and 37^{o}C, both with and without AAA. For each temperature, 6 swarm plates were poured, 3 with 5 mg/ml of AAA in the media. Each plate was inoculated by picking a single colony off a streak plate with a sterile toothpick and stabbing the center of the a swarm plate. The plates were

incubated for 24 hrs, with swarm ring measurements taken in mm every 2 hrs for the first 10 hrs, and at the 24 hr mark.

RESULTS

Bacterial growth and determination of biofilm amounts

In order to determine if AAA had an effect on growth and biofilm formation we performed biofilm and growth assays on *C. sakazakii* (Fig. 1), *S. marcescens* (Fig. 2), and non-virulent (Fig. 3) and virulent (Fig. 4) isolates of *Y. enterocolitica*. These assays were conducted to investigate bacterium response to increasing concentrations of AAA. IC_{50} values were calculated, are presented at the beginning of this chapter, and discussed throughout (Table 4).

Table 4: IC_{50} values for growth and biofilm reduction by AAA

Bacterial Isolate	Growth Temperature	Growth IC_{50}	R^2	Biofilm IC_{50}	R^2
CV Assay					
C. sakazakii	37°C	19.71	0.9629	22.08	0.9566
S. marcescens	30°C	29.26	0.9484	34.57	0.7337
Y. enterocolitica 8081c	37°C	28.76	0.834	35.20	0.8142
Y. enterocolitica 8081c	25°C	16.75	0.6944	N/A	N/A
ATP Assay					
Y. enterocolitica 8081c	25°C	19.87	0.583	12.38	0.979
Y. enterocolitica 8081v	37°C	10.65	0.645	13.76	0.834
Y. enterocolitica 8081v	25°C	N/A	N/A	19.14	0.9721

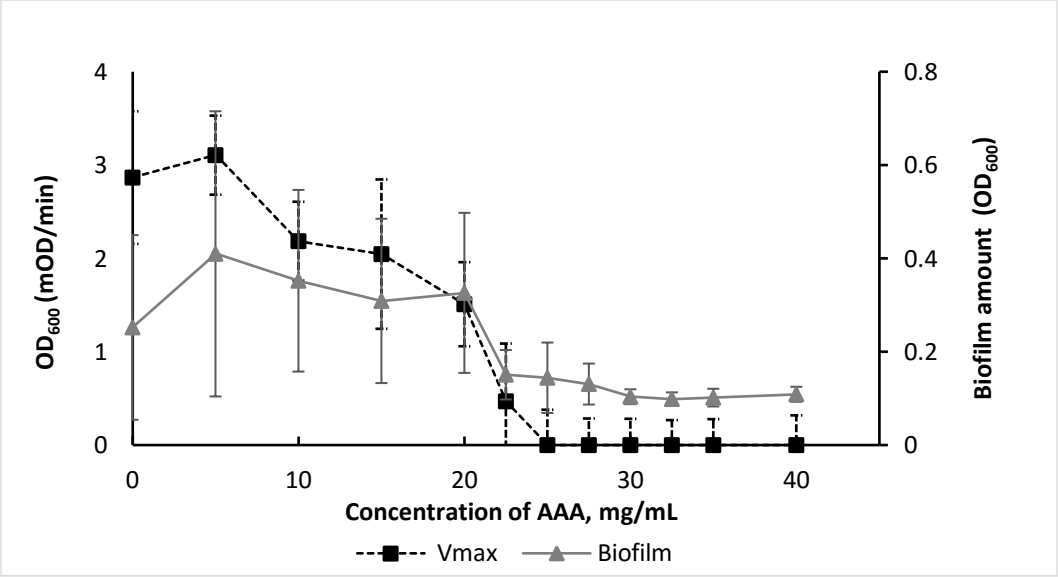

Figure 2: C*. sakazakii* growth and biofilm amounts at 37°C in TSB. The X-axis of this figure denotes the concentration in mg/ml of AAA present in a specific well of a 24-well polystyrene plate. The primary Y-axis measures the growth of the bacterial isolate (black squares and dotted lines), and the secondary Y-axis measures the biofilm amounts (grey triangles and solid lines).

The growth of *C. sakazakii* showed a slight increase from 0 to 5 mg/ml, or 0 to 46mM, of AAA and a reduction with increasing concentrations of AAA (Fig. 2) with an IC_{50} 19.71 mg/ml or 183 mM (Table 4) . The growth of the bacterium was abolished at a concentration of 25 mg/ml (231 mM). The biofilm amounts quantified using the CV assay also showed a slight increase towards 5 mg/ml and decreased as concentrations increased. A steep decrease in biofilm formation was seen between 20 and 22.5 mg/ml or 185-208 mM. The IC_{50} value for biofilm was 22.08 mg/ml, 204 mM.

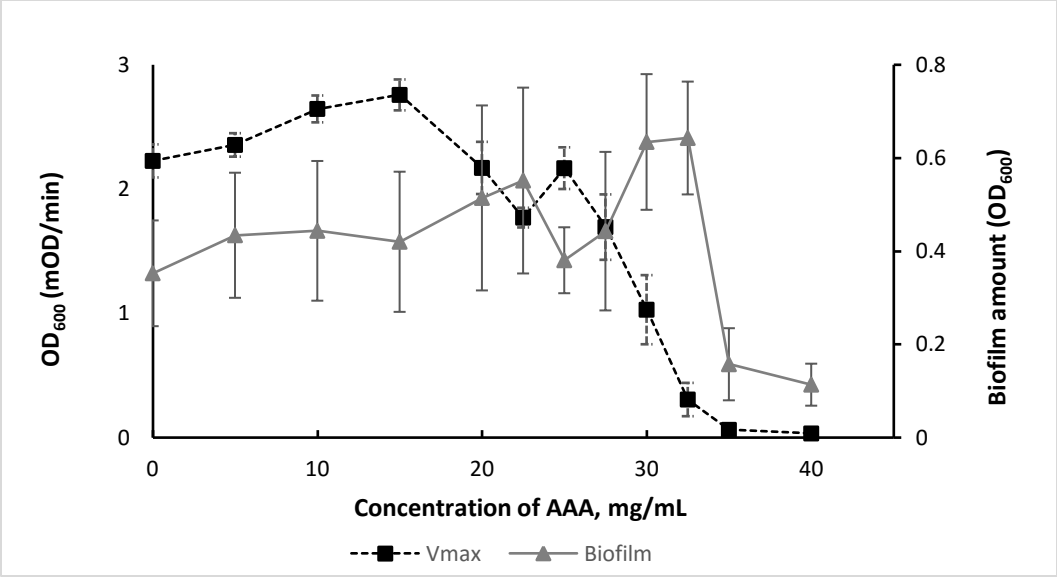

Figure 3: S*. marcescens* growth and biofilm amounts at 30°C in TSB. The X-axis of this figure denotes the concentration in mg/ml of AAA present in a specific well of a 24-well polystyrene plate. The primary Y-axis shows the growth of the bacterial isolate (black squares and dotted lines), and the secondary Y- axis resembles the biofilm amounts (grey triangles and solid lines).

The growth of *S. marcescens* increased slightly across the lower concentrations up to 15 mg/ml, 139 mM, of AAA before starting to drastically decrease with an IC_{50} of 29.26 mg/ml, 271 mM. Interestingly, there was another uptick in growth at 25 mg/ml, 231 mM, before a substantial reduction at 32.5 mg/ml, 301 mM, of AAA. The quantification of the biofilm amounts with the CV assay also showed a possible increase in biofilm formation at the lower concentrations before a sizeable decrease at 35 mg/ml, 324 mM, of AAA. The increase in biofilm formation may be a true increase but it cannot be stated with any certainty as there was a fair amount of variability between the replicates in the experiment. The *S. marcescens* IC_{50} value for biofilm was 4.57 mg/ml, 42 mM.

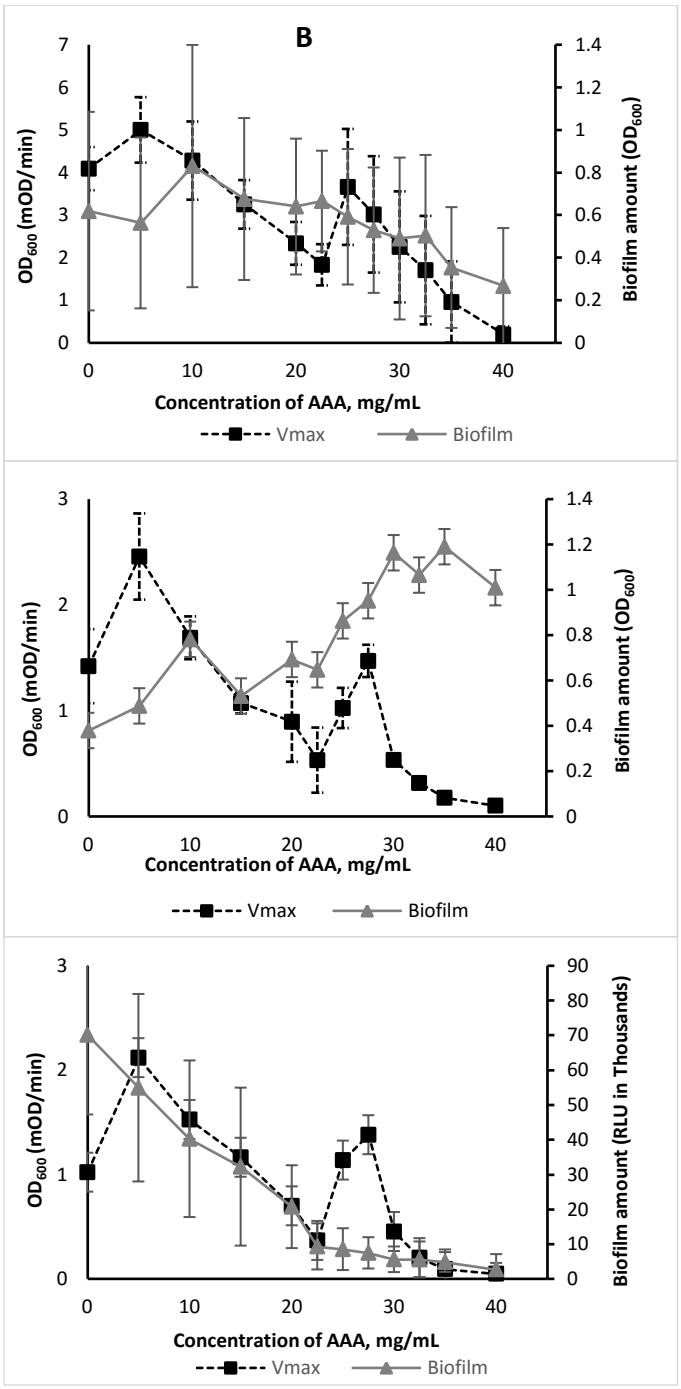

Figure 4: *Y. enterocolitica* 8081c growth and biofilm amounts at 37°C and 25°C in TSB. Bacteria were grown at 37°C (Fig. 4A) and 25°C (Figs. 4B and C). For the 37°C experiment, biofilm was quantified with the CV assay (Fig. 4A), for the 25°C experiment, biofilm was quantified with the CV assay (Fig. 4B) and the ATP assay (Fig. 4C). For each panel, the primary Y-axis shows the growth of the bacterial isolate (black squares and dotted lines), and the secondary Y- axis resembles the biofilm amounts (grey triangles and solid lines).

At 37°C (Fig. 4A), *Y. enterocolitica* 8081c growth showed a small increase towards 5 mg/ml, 46 mM before decreasing steadily until 22.5 mg/ml, 208 mM. At 25 mg/ml, 231 mM, there was a spike in growth before the data showed a continued decrease to a final low at 40 mg/ml, 370 mM, with an IC_{50} of 28.76 mg/ml, 266 mM. The biofilm amounts at 37°C decreased as the concentration of AAA was increased, but there was a large amount of variation in the data, making significant conclusions difficult to obtain. The IC_{50} value for biofilm amounts is 35.20 mg/ml, 326 mM.

Y. enterocolitica 8081c was also grown at 25°C and the CV assay was done to quantify the biofilm amounts across the AAA concentrations (Fig. 4B). As was seen in the experiments with previous bacterial isolates, there was an increase in growth between 0 and 5 mg/ml, 46 mM. In *Y. enterocolitica* 8081c, there was also another sharp increase in growth at the 27.5 mg/ml, 255 mM concentration. The growth did show a significant decrease once the 35 mg/ml, 324 mM, concentration was reached, and the IC_{50} value is 16.75 mg/ml, 155 mM. Biofilm amounts showed an increase towards 40 mg/ml, 370 mM, of AAA and therefore an IC_{50} value could not be calculated and is listed as N/A in Table 2.

The bacterium was again grown at 25°C and an ATP assay was done to quantify the biofilm amounts across the AAA concentrations (Fig. 4C). There was an increase in growth between 0 and 5 mg/ml, 46 mM, and there was also another increase in growth at the 27.5 mg/ml, 255 mM, concentration. The growth did show a significant decrease once the 35 mg/ml, 324 mM, concentration was reached, with an IC_{50} value of 19.87 mg/ml, 184 mM. Biofilm amounts show a steady decrease towards 40 mg/ml, 370 mM, and major reduction is seen at 22.5 mg/ml, 208 mM, with an IC_{50} value of 12.38 mg/ml, 115 mM.

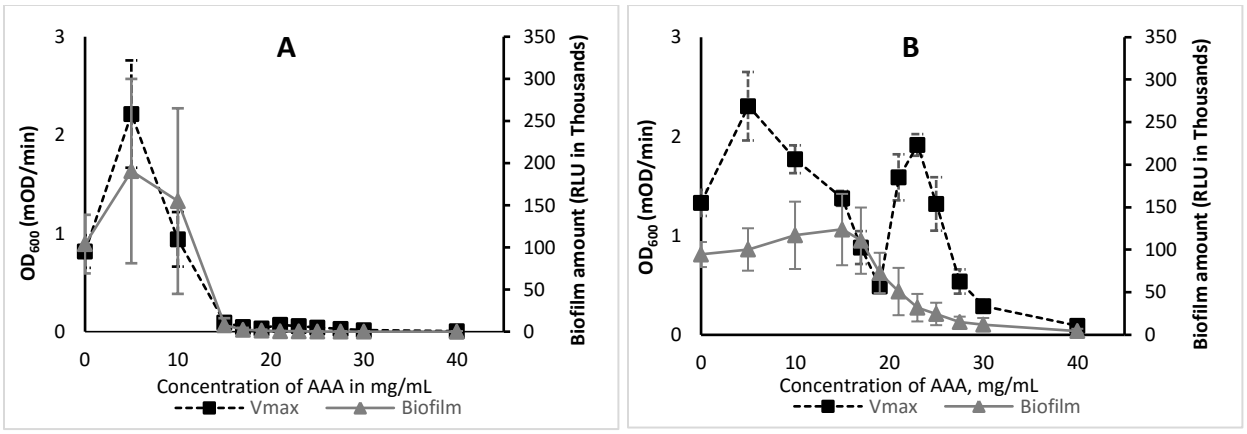

Figure 5: Y*. enterocolitica* 8081v growth and biofilm amounts at 37°C and 25°C in TSB. Bacteria were grown at 37°C (Fig. 5A) and at 25°C (Fig. 5B), biofilm was quantified with the ATP assay. For each panel, the primary Y-axis shows the growth of the bacterial isolate (black squares and dotted lines), and the secondary Y- axis resembles the biofilm amounts (grey triangles and solid lines).

At 37°C (Fig. 5A), *Y. enterocolitica* 8081v growth and biofilm showed an increase towards 5 mg/ml, 46 mM, before showing a pronounced decrease. Abolishment of both growth and biofilm occured at an AAA concentration of 15 mg/ml, 139 mM. The IC_{50} values for growth and biofilm are 10.65 mg/ml, 99mM, and 13.76 mg/ml, 127 mM, respectively.

At 25°C (Fig. 5B), growth increased towards 5 mg/ml, 46 mM, before declining towards 20 mg/ml, 185 mM. The bacteria showed another increase in growth towards 25 mg/ml, 231 mM, before again steadily decreasing. The IC_{50} value for growth could not be calculated and is listed as N/A in Fig. 2. Biofilm amounts showed a slow increase towards 15 mg/ml, 139 mM, of AAA and a distinct decrease as the concentration of AAA increased with an IC_{50} value of 19.14 mg/ml, 177 mM.

Relative gene transciption in the presence of AAA

Relative gene transription of *Y. enterocolitica* 8081v was investigated in the absence and presence of AAA. The bacterial isolates were grown at both 25 and 37°C, with 5 mg/ml, 46 mM

of AAA and without AAA. Seven genes significant to virulence and biofilm formation were selected and qPCR was performed to assess the relative transcriptions of each of these genes. For each gene, the $2^{-\Delta Ct}$ method was used to make eight comparisons between environmental conditions. Biofilm with and without AAA at each temperature was compared (25B0/25B5 and 37B0/37B5) and planktonic bacteria with and without AAA at each temperature were compared (25P0/25P5 and 37P0/37P5). In addition, the 25^{o}C biofilm and planktonic bacteria were compared to the 37^{o}C biofilm and planktonic bacteria, both with and without AAA (25B0/37B0, 25P0/37P0, 25B5/37B5, 25P5/37P5). Relative transcription differences greater than two-fold seen in the first four comparisions, 25B0/25B5, 37B0/37B5,25P0/25P5 and 37P0/37P5 were evaluated further for statistical significance ($p<0.05$).

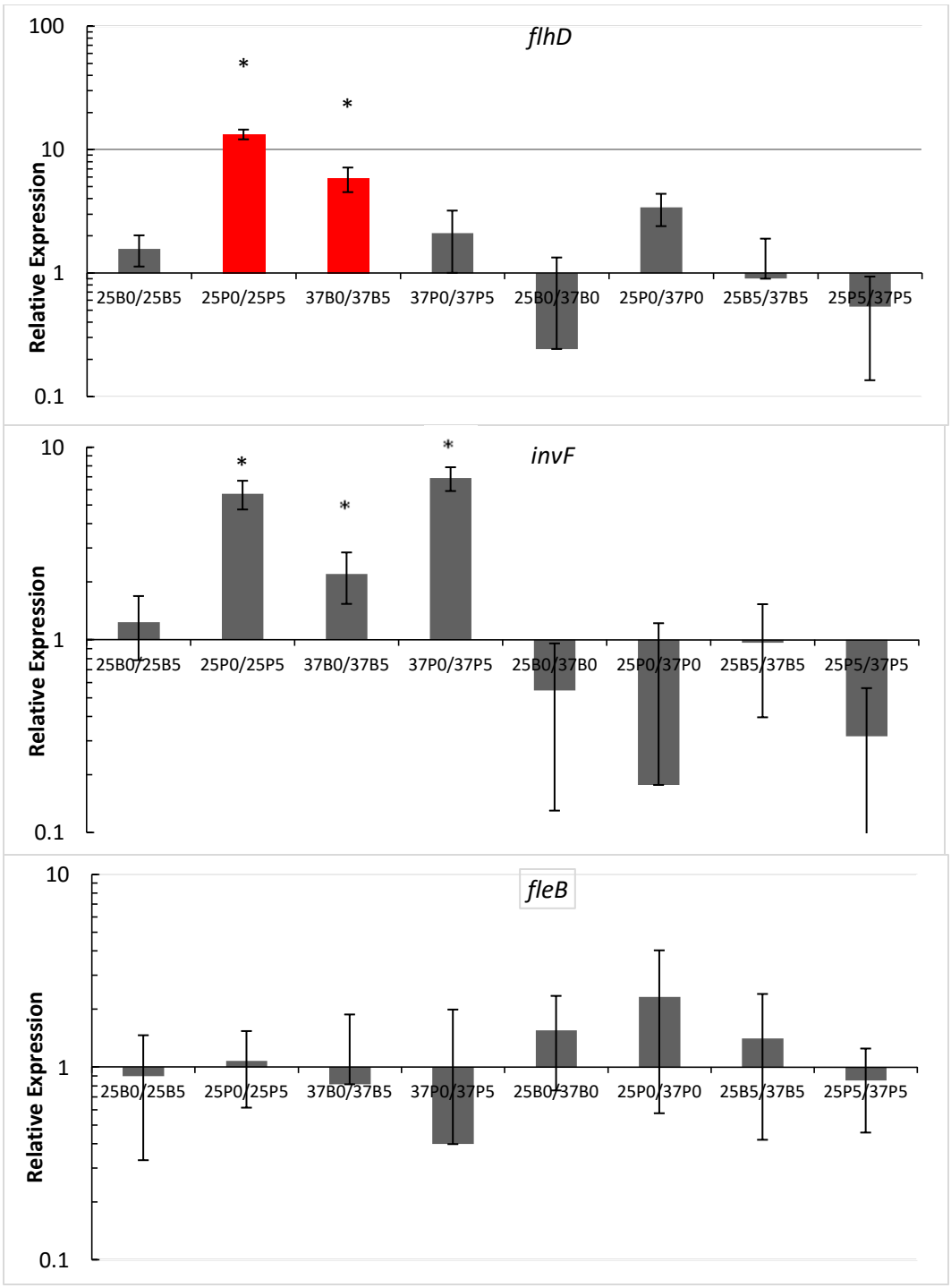

Figure 6: Relative transcription of early virulence genes. In this figure, the X axis indicates the sample conditions being compared, and the Y axis represents the relative gene transcription calculated using the $2^{-\Delta Ct}$ method plotted on a logarithmic scale. *Astericks signify a *p*-value <0.05. Data from the two red bars were later investigated and confirmed phenotypically.

At 25°C, *flhD* showed approximately a 10-fold reduction in transcription in planktonic bacterial cells when AAA was added, while the biofilm bacterial cells, at 37°C, showed a 5-fold reduction in *flhD* transcription when exposed to AAA. The biofilm bacterial cells at 25°C and the planktonic cells at 37°C, could not be said to show significant changes in their *flhD* transcription levels. The transcription levels of *invF* showed a reduction when cultures were supplemented with AAA. There is approximately a 7-fold reduction in transcription by AAA in the planktonic bacteria at both 25 and 37°C. There was also another decrease in transcription by AA seen in the biofilm cells growth at 37°C. The *fleB* gene transcription seems to not be effected by the additon of AAA as shown by the lack of significant differences in transcription.

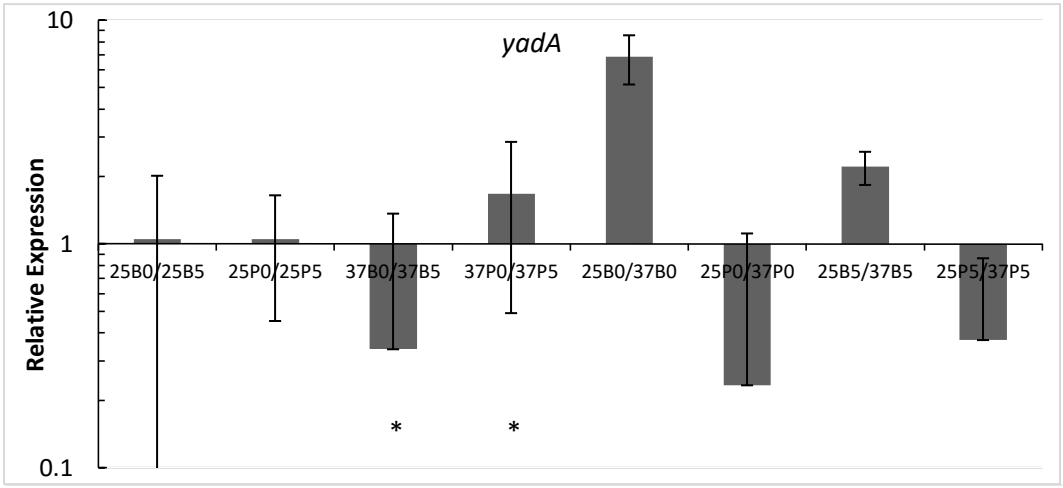

Figure 7: Relative transcription of late virulence genes. In this figure, the X axis indicates the sample conditions being compared, and the Y axis represents the relative gene transcription calculated using the $2^{-\Delta Ct}$ method plotted on a logarithmic scale. *Astericks signify a *p*-value <0.05.

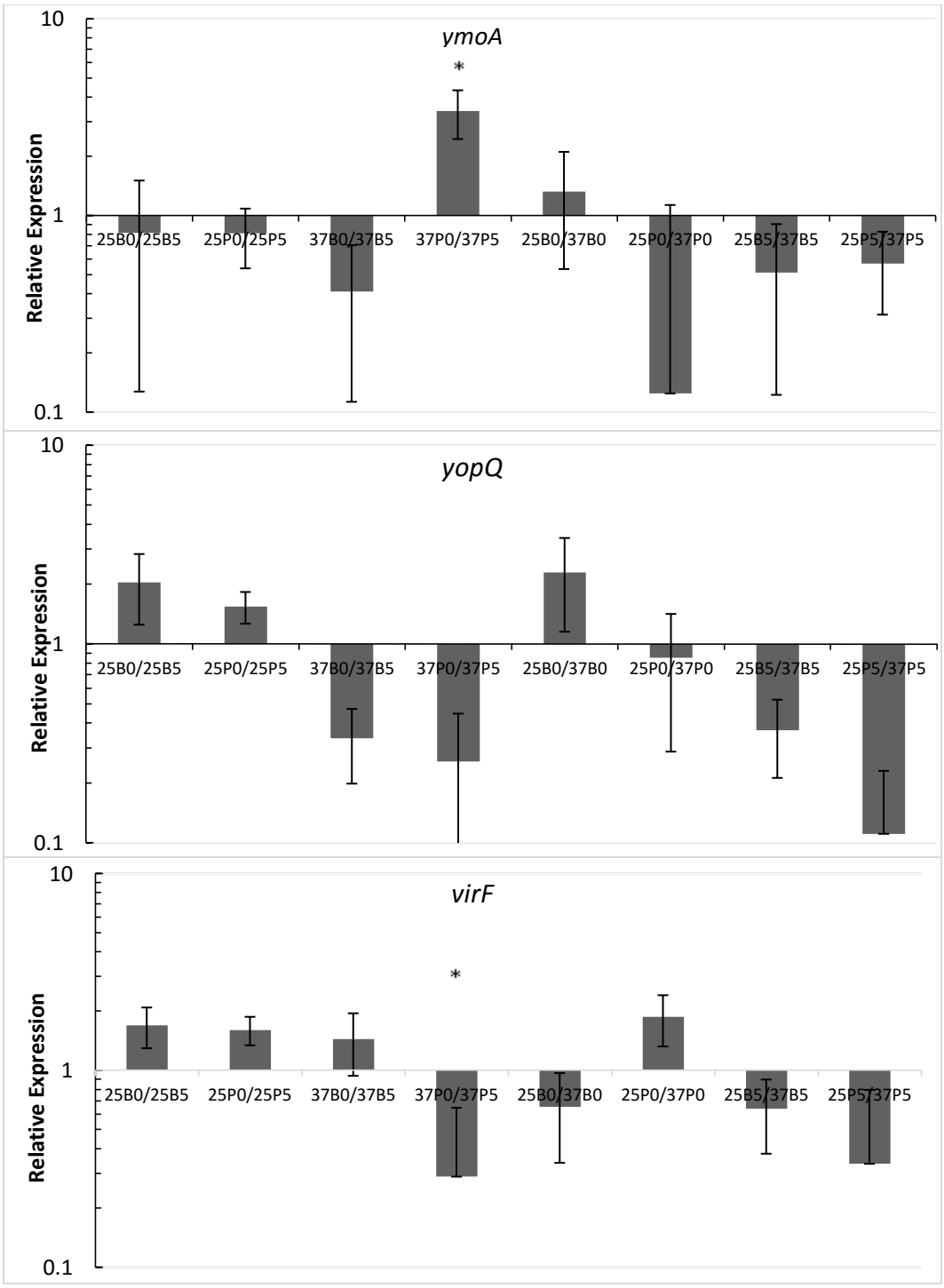

Figure 7: Relative transcription of late virulence genes (continued).

The evaluation of the relative transcription of the *yadA* gene yielded no significant changes with the addition of AAA at either temperature, or in biofilm/planktonic bacterial cells.

The relative transcription of *ymoA* did show a significant decrease in the planktonic cells at 37°C. The addition of AAA seemed to have little effect on the relative transcription levels of *yopQ* when the cells were grown at 25°C, but at 37°C both cells types, biofilm and planktonic, showed a significant increase in the relative transcription. The *virF* gene also shows a significant increase in relative transcription, but only in the planktonic cells at 37°C. There was no difference noted in the 25°C cells, or in the 37°C biofilm bacterial cells.

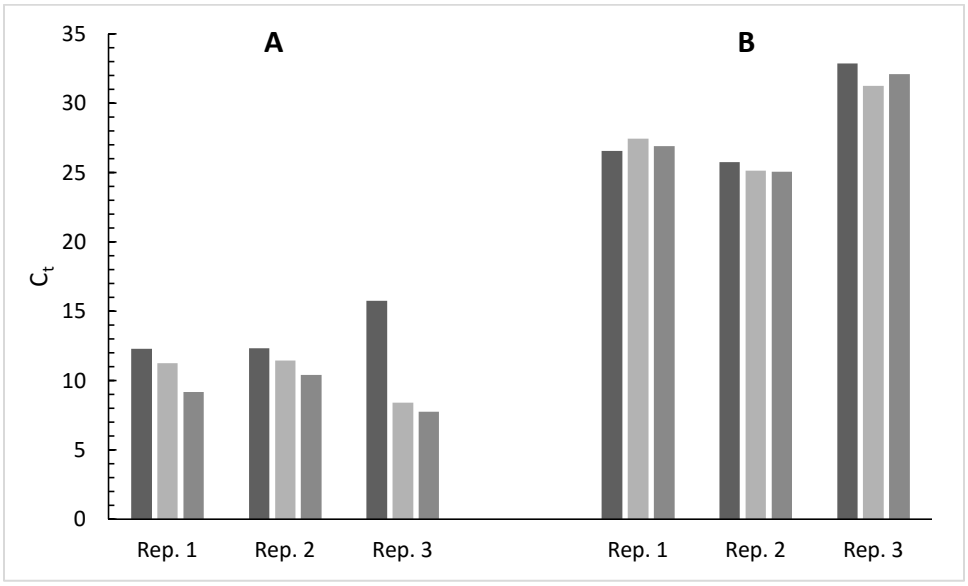

Figure 8: Cycle thresholds for biological and technical replicates of *fleB* (A) and *flhD* (B).

The first qPCR experiment that evaluated the effect of AAA on the transcription of the *fleB* gene in *Y. enterocolitica* 8081v shows a large amount of variation in the technical replicates. Improved pipetting skills led to a decrease in the variation seen in the technical replicates but could not reduce the variation in the biological replicates, as seen in the *flhD* data that were produced last.

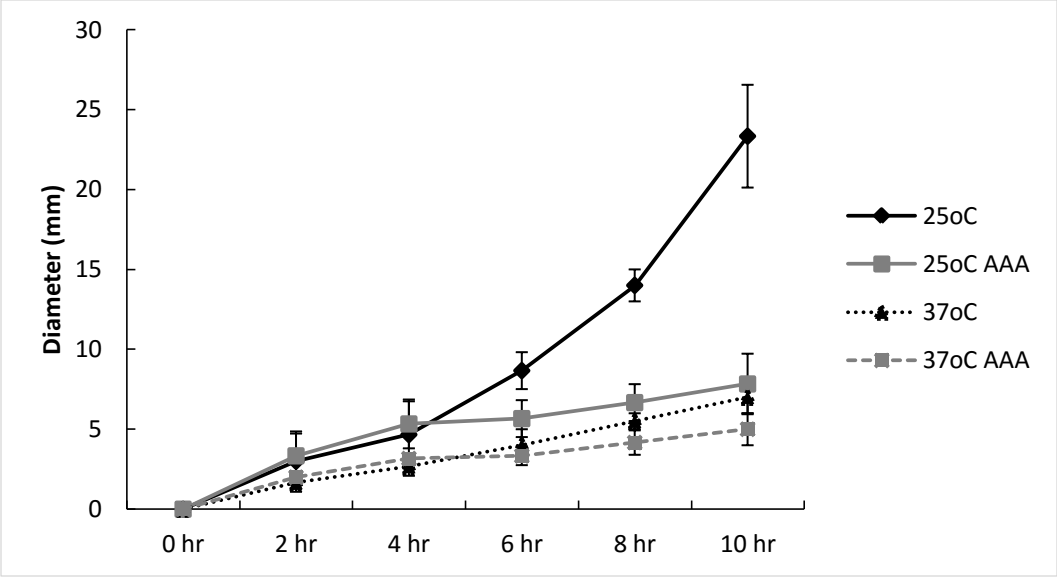

Figure 9: Effect of 5 mg/ml AAA on *Y. enterocolitica* 8081v motility at 25 and 37°C. The diameter of the swim ring, in mm, was recorded. The solid black line represents swimming at 25°C with no AAA, the solid grey line 25°C with AAA. The dotted black line represents swimming at 37°C with no AAA, and the dotted grey line 37°C with AAA.

The motility of the bacterium was measured in mm every 2 hrs for 10 hrs. For the first 4 hrs, there was little difference in the size of either of the rings because bacteria have to grow first. After these first 4 hrs, motility of the bacterial isolate was greatest at 25°C without AAA. At this temperature, motility was greatly reduced by the addition of AAA. At 37°C, little motility was seen on the plate without AAA, but there was still a possible slight reduction with AAA.

DISCUSSION

The first objective of this study was to investigate the feasibility of using AAA to reduce growth in planktonic bacteria and reduce biofilm amounts. The data show that AAA was able to reduce both planktonic bacterial growth and biofilm amounts in all isolates tested for this study, with the most striking reduction occurred in *Y. enterocolitica* 8081v at 37^{o}C. In addition to this objective we also wanted to understand how AAA was effecting a selection of early and late expressed virulence genes. The qPCR experiments showed that the transcription of some of these genes was being changed by the addition of AAA. The effect AAA had on the gene transcription varied between biofilm and planktonic cells, and also showed a difference in regulation at the two temperatures.

Bacterial growth and biofilm amounts

AAA has shown to be effective in inhibiting growth and biofilm amounts and therefore is a plausible choice for prevention of bacterial biofilms. In the growth patterns across the concentration range of AAA, *C. sakazakii*, *S. marcescens*, and *Y. enterocolitica* isolates all show a slight increase in growth at the lowest concentrations, before decreasing as the concentration was increased. It could be possible that the bacterium are able to utilize the molecule in low concentrations as a nutrient source. A similar trend was also seen in the biofilm amounts of all the isolates, there seems to be a modest increase in the amounts before decreasing. Interestingly, *S. marcescens*, *Y. enterocolitica* 8081c at both 37^{o}C and 25^{o}C, and *Y. enterocolitica* 8081v at 25^{o}C show an increase in growth at around 25-27.5 mg/ml, 231-255 mM, of AAA, with *S. marcescens* showing only a small bump in growth in comparison to the more pronounced peak seen in the *Y. enterocolitica* isolates. The specific reason for this is unknown, but it is

possible that at these cells are using a different nutrient and therefore showing an increase in growth. Another possibility is that once a certain concentration is reached a stress response is triggered which could lead to a change in phenotype [76]. There are many known environmental stressors that may cause a change in phenotype, such as starvation or oxidative stress, but the exact mechanism of this response, if that is what is taking place, is currently unknown. This increase in growth was a confounding factor when calculating the IC_{50}'s for bacterial growth for *Y. enterocolitica* 8081v at 25^{o}C. The increase in growth was large enough to make any such calculation inaccurate.

Relative gene transcription in the presence of AAA

In our investigation into the changes in virulence gene transcription AAA was shown to induce changes in both early and late virulence genes. In the early virulence gene subset, there was a significant reduction in *flhD* transcription in the planktonic bacteria grown at 25^{o}C, and in the biofilm cells grown at 37^{o}C. This master regulator plays a major role in *Y. enterocolitica's* motility and its ability to colonize surfaces[77]. Motility has been shown to be necessary for host cell invasion, so a decrease in transcription due to AAA could prove advantageous [78]. Previous studies also concluded that the loss of *flhD* results in a decrease in virulence in a chicken embryo model [79]. Although this qPCR experiment can only investigate gene transcription, it gives reassurance that the AAA is not increasing *flhD* mediated virulence. There was also a significant reduction in *invF* in the 25^{o}C planktonic cells, and in both planktonic and biofilm cells at 37^{o}C. Conversely, there was no regulation seen in the *fleB* gene transcription. This is unusual as the flagellin genes are known to be regulated in part by *flhDC* [54]. It would be expected that if the *flhD* shows a change in gene transcription, that there may be a resultant

change in *fleB* transcription, but this was not the case. A possible reason for a lack of significant regulation seen in *fleB* is human error while performing the qPCR. The *fleB* gene was the first gene tested for and the qPCR pipetting technique was not mastered at this time. This allowed for errors in the data that made conclusions on the relative gene transcription difficult to make. Subsequent qPCR experiments showed an improvement in the consistency in the technical replicates (see Fig. 8).

In the late virulence gene category, regulation by AAA was seen in *ymoA*, *yopQ*, and *virF*, but not in *yadA*. Gene transcription of *ymoA* was significantly reduced in the planktonic cells grown at 37^{o}C. Transcription of *yopQ* was increased in both planktonic and biofilm cells at 37^{o}C, while *virF* transcription was increased only in the planktonic cells grown at 37^{o}C. A previous study was able to show that when the *flhDC* master regulator is knocked out there is a resultant increase in both the *yop* regulon and *virF* transcription [60]. This is in line with our gene transcription data where there was a decrease in *flhD* transcription with a resultant increase in *yopQ* and *virF* transcription, although the whether this is direct regulation by *flhD* cannot be determined with the current experiment.

The qPCR experiments done in this study were able to investigate the gene transcription levels when in the presence of AAA, but could not inform us about the biological significance of the results seen. To explore the correlation between the relative gene transcription seen in the qPCR and the biological relevance of the data, we performed a phenotypic assay on swim plates with and without 5 mg/ml of AAA, at 25 and 37^{o}C. The results of this assay confirmed what was seen in gene transcription experiment. At 37^{o}C, very small rings were seen, which is what was expected as *Y. enterocolitica* are not motile at this temperature, and was no change with

the addition of AAA at this temperature. A major difference was seen at 25^{o}C between the plates with AAA and without AAA. At this temperature the bacterium are motile and large swim rings were seen in all three plates without AAA. With the addition of AAA the rings are appreciably smaller, and are approximately the same size as was seen at 37^{o}C.. This phenotypic assay strongly supports what we saw in the gene transcription experiment.

When performing studies like qPCR, where the researcher is looking at relative transcription levels for samples taken on different days, it is necessary to normalize this data somehow to account for the differences that may be seen due to the growth of the samples or the RNA preparation. To normalize the data there is a couple of main techniques favored across the research community. The first technique uses a standard curve to quantify the amount of template in the sample [80]. In this study, a standard curve was done with the pPM61 plasmid with a known copy number to aid in normalizing. Unfortunately, the standard curve was not linear across the whole range used and therefore could not be used to quantify the amount of template in the samples. A second technique is the use of housekeeping genes. The goal is to find genes that are constantly transcribed by the cells regardless of the conditions being used in the study. For the selection of these housekeeping genes, central metabolism or genes needed for cell maintenance are usually considered possible candidates [81]. If these genes are not being regulated by the test conditions, the levels present in the biological replicates can then be used to normalize the data. It can be difficult to find genes that are consistently being transcribed and a shortfall of this study is that appropriate housekeeping genes could not be found that were not regulated by the conditions being investigated. We investigated *dnaE*, *polA*, and *gapA* possible housekeeping genes as they are not regulated by

temperature. Unfortunately, they did show regulation by AAA and therefore could not be used to normalize the qPCR data.

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