ATTACHMENT AND BIOFILM FORMATION OF FOODBORNE PATHOGENS

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ABSTRACT

Outbreaks of *Listeria monocytogenes, Salmonella,* and *Escherichia coli* are increasingly attributed to fresh produce. Current control measures have been assessed for decades, with no alternatives adopted. Sources were identified, reducing *flhD* transcription and biofilm amounts nearly 2-fold. β-phenylethylamine (PEA), reduced growth and biofilm 96% and 70%, respectively. Curli production was assessed and found to be microorganism-, strain-, and/or serotype-dependent.

Reporter fusions were constructed, evaluating expression of *Listeria* cellulose protein (Lcp). P_{lcp} was not impacted by conditions used. Conditions were then used in attachment of *L. monocytogenes* to stainless steel. Attachment was significantly reduced by 5 ppm chlorine and 2% lysate.

Small molecules could be alternatives to current control measures. More research is needed on what induces curli production. Controls confirm that reporter fusions are an effective way to discover signals impacting gene expression. Attachment/expression assays, indicate that something other than Lcp are responsible for changes in attachment to stainless steel.

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DEDICATION

I dedicate this work to my loving family and friends, especially my husband, Ronald Smith, and my two children, Soraea and Cassius, for supporting, inspiring, and encouraging me all along the way, but most of all for believing in me and the value of a higher education.

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INTRODUCTION

In today's health conscious world, ready-to-eat fresh produce has been in high demand year-round. With this high demand, there has also been a significant increase in foodborne illness and outbreaks associated with fresh produce that has been seen since the 1970's (1-3). According to the Center for Science in the Public Interest (CSPI), as a single commodity, fresh produce, had the greatest number of outbreaks in the U.S. between 2002 and 2011, but the CSPI recommends eating "more, not less" fresh produce to maintain one's health (4). One contribution to this increase is thought to be the trend in consuming produce raw or without additional processing (5-9). The Centers for Disease Control and Prevention (CDC) has estimated that 48 million Americans become ill, 128,000 people are hospitalized, and 3,000 deaths result from foodborne illness each year (10, 11). Between 1998 and 2008, 46% of foodborne illnesses and 23% of foodborne related deaths were attributed to produce contaminated with foodborne pathogens (12).

Salmonella and enterohemorrhagic *Escherichia coli* (EHEC) are among the top five foodborne pathogens that result in hospitalizations and *Salmonella* and *Listeria monocytogenes* are among the top five foodborne pathogens that result in death (10). It is estimated that each case of foodborne illness costs $1,626, with a total healthcare and economic cost of approximately $78 billion annually (13).

According to the CDC, *Salmonella,* EHEC, and *L. monocytogenes* have been responsible for 36 multi-state outbreaks between 2014 and 2017, and 14 of these involved fresh fruits and vegetables. Outbreaks were linked to papaya, cucumbers, bean sprouts, and alfalfa sprouts contaminated with *Salmonella* (14). Outbreaks were also associated with apples, soy sprouts, bean sprouts, frozen vegetables, celery, cantaloupe, and packaged salads contaminated by *L.*

monocytogenes (15-18). In addition, outbreaks of EHEC were linked to raw clover and alfalfa sprouts (19). The most severe outbreak was in 2015, a salmonellosis outbreak involving cucumbers in 40 states causing 907 illnesses, 204 hospitalizations, and 6 deaths (20).

As enteric pathogens, *Salmonella* and EHEC thrive and are virulent in the gastrointestinal tract of their selected host and *L. monocytogenes,* a saprophyte, is prevalent in soil. However, as shown previously, *Salmonella*, EHEC, and *L. monocytogenes* can be associated with fresh produce and it has been shown that all three microorganisms, once deposited on produce, can persist (survive long-term) in less than ideal and even hostile non-host environments (21-30). For example, Erickson and colleagues detected *E. coli* O157:H7 on the surface of field-grown lettuce leaves 27 days after being sprayed with contaminated irrigation water (21). Islam and colleagues found that *Salmonella* persisted for 161 and up to 231 days in soils amended with contaminated composts on which lettuce and parsley, respectively, were grown (23). *Salmonella* was also detected for up to 63 days and 231 days on the lettuce and parsley, respectively (23). Milillo and colleagues was not only able to show survival of *L. monocytogenes* after ten days on contaminated *Arabidopsis thaliana*, but growth of the pathogen (30).

Bacteria are known to respond to stresses in their environment. Stress can be defined as any departure from optimal conditions, with the potential to decrease or inhibit bacterial growth (31). Stress situations can induce or repress the expression of genes that respond to specific environmental cues. Along the farm-to-fork continuum there are generally three categories of stress – physical, chemical, or nutritional, that can all lead to different types of bacterial cell damage (32). Stresses include osmotic stress, oxidizers, changes in pH, temperature changes, humidity changes, antibiotic exposure, antiseptic exposure, and starvation. With time, tolerance and eventually resistance can develop if exposure is at a sub-lethal level, allowing the pathogens

to survive and possibly grow slowly under these extreme conditions until a more ideal environment comes along in which they can thrive and/or become virulent, *i.e.* in the human gastrointestinal tract (32).

One mechanism that bacteria can use to survive stressful conditions is by formation of biofilms. Biofilms can be defined as complex aggregations of single or multi-species bacterial communities that can form on a solid substrate, at a liquid-air interface or intracellularly (33). Once a biofilm forms, eradicating the bacteria is nearly impossible (2). Biofilms provide increased tolerance and often resistance, 100 to 1000 times greater, against the various stresses encountered in the pre- and postharvest environments compared to their planktonic counterparts (34).

Biofilm formation requires bacteria to irreversibly attach to a conditioned surface. Curli, flagella, and pili overcome repulsive forces and these appendages allow attachment to the conditioning layer matrix (35). It is known that flagella are required for *L. monocytogenes* to form biofilms (36). Curli was shown to significantly enhance the attachment of *E. coli* O157:H7 to spinach leaves and stainless steel surfaces by 5-fold. Curli was also required for *E. coli* O157:H7 biofilm formation on stainless steel and enhanced biofilm production on glass by 19-27 fold (37). The conditioning matrix or substrate characteristics have also been shown to have a large effect on attachment and subsequent biofilm formation. *Salmonella* was shown to preferentially attach to Romaine lettuce over cabbage and to cut surfaces over intact surfaces (38). It is the differences in the bulk (planktonic) culture and the conditioning layer that causes stress that I am interested in and the effect that these stresses have on biofilm production, growth, gene expression, and attachment of common foodborne pathogens.

LITERATURE REVIEW

Contamination of the pre- and postharvest environment

Contamination of fresh produce by *Salmonella,* EHEC, and *L. monocytogenes* can happen in either the pre-harvest or postharvest environment. Two infamous examples of pre-harvest and postharvest handling resulting in contamination of fresh produce are the 2006 *E. coli* O157:H7 outbreak involving spinach and the 2011 *L. monocytogenes* outbreak attributed to cantaloupe (39-41). The pre-harvest environment has many variables that contribute to produce contamination, such as fecal deposition from intruding domesticated and wild animals, surface and ground waters, soil, plant matter, manure, and equipment (5, 12, 41-44). Metagenomics and Geospatial Information Systems (GIS) have been used to predict the prevalence of pathogens in the pre-harvest environment (45, 46). However, these environmental factors are continuously fluctuating and less is understood about the implications for pre-harvest contamination than postharvest contamination (16), other than that fresh produce can act as vectors for contamination in the processing environment (6).

Contamination in the processing or postharvest environment is many times due to the contamination of equipment and/or food product via pre-harvest contaminated produce into wash water or onto processing equipment (7, 47, 48). Contamination can also occur between raw and processed storage, within wash systems, and on processing equipment surfaces (5, 26, 49, 50). This contamination can ultimately lead to foodborne illness. Once contamination occurs, eradication can be very difficult despite intensive equipment decontamination efforts using best generally accepted cleaning and prevention practices.

Biofilm formation and attachment

Bacterial attachment and biofilm formation has been a significant problem for many industries including the food industry and is a considerable topic of scientific research (2, 36, 51). This is because food surfaces and the processing environments that contain pathogens can cross-contaminate other parts of the food processing chain causing postprocessing contamination and potential foodborne illness (7, 52-55). Biofilms are defined as cells, single species or multi-species, immobilized at a surface and embedded in an organic polymer matrix of microbial origin. They are a biologically active matrix of cells and extracellular substances, or EPS, in association with this surface, having an altered phenotype compared to their planktonic counterparts and lack Brownian motion. Composition usually entails 10 to 25% cells and 75 to 90% EPS matrix with a mushroom-like shape (35, 56). Biofilm formation offers many advantages including protection from antibiotics, disinfectants, and dynamic environments. This is because of rapid up-and-down regulation of gene expression enabling temporal adaptation through intracellular communications (35).

There are many genes (greater than 250) involved in attachment and biofilm formation many of them making up two-component signaling systems that produce specialized mechanisms that bacteria use to respond to changes in their environment (57). For *E. coli,* there are four important two-component signal transduction systems that regulate motility and biofilm development. EnvZ/OmpR, involved in osmolarity, is also important for the transition from reversible to irreversible attachment, inhibiting *flhDC*, and increasing synthesis of type I fimbriae and curli. RcsCDB activates colanic acid (biofilm matrix) synthesis and down-regulates *csgD* and *flhD* in both EHEC and *S. enterica* (58). CheA/CheY/CheB controls the direction of the flagellar motor rotation, and QseC/QseB connects quorum sensing with motility, biofilm

development, and virulence (57). PhoPR, a phosphate-sensing two-component system, has also been shown to regulate biofilm formation in *L. monocytogenes* (36).

BapL, in *L. monocytogenes* and *S. enterica*, and BapA, in EHEC, are biofilm-associated proteins that have been well classified (58-60). Other proteins important for biofilm formation in *L. monocytogenes* are InlA, PlcA (phospholipase), FlaA, PBP (putative penicillin-binding protein), ActA, Lmo2504 (putative cell wall binding protein), PrfA and Lmo0753 (a novel Crp/Fnr family transcription factor) (60, 61).

Characklis and Marshal described biofilm formation as eight steps (62). The first step involves conditioning of the surface on which the biofilm is to grow. This can include both organic or inorganic matter, and anything that settles onto the surface can become part of the conditioning layer. This conditioning layer provides anchorage and nutrients for the bacterial community. The second step is reversible attachment in which cells are transported from bulk liquid to the conditioned surface by physical forces or by appendages such as such flagella. At this point, there are weak physical forces such as van der Waals forces, and if repulsive forces are greater than the attractive forces the bacteria will detach from the surface. If this is not the case, the third step in biofilm formation is irreversible attachment. It is thought that at this stage bacterial physical appendages such as flagella, fimbriae, and pili overcome the repulsive forces. The fourth step is exponential population growth with the excretion of EPS forming stronger bonds between cells. The fifth and six stages are stationary phase growth and quorum sensing, respectively. The seventh and eighth steps are the death phase and active release of surface bacteria for colonization of fresh substrates, respectively (35, 56).

Of great interest is the environmental conditions, physical, chemical, and biological, that influence the transition between reversible attachment and irreversible attachment. Again,

reversible attachment, with the aid of motility elements like flagella, mimics van der Waal interactions or hydrogen bonding, the two surfaces having a weak electrostatic attraction that can be easily broken (63, 64). Irreversible attachment involves the formation of cellulose fibrils which aid in a stronger adhesion that can only be broken by shear force (64, 65).

There are many signals that cause bacteria to lock onto a surface and begin the production of EPS, and/or specific ligands, such as pili of fimbriae that then require stronger physical and chemical forces to remove the bacteria from the surface, such as scraping, scrubbing, or chemical cleaners (56). For example, something as simple as temperature can signal this switch between reversible and irreversible attachment (66). The attachment-upon-starvation response, observed in many bacteria including pathogenic bacteria, results in more biofilm biomass than nutrient rich environments (67). Carbon sources that result in acetate metabolism also produced more biomass, this was supported by a reduced biomass phenotype in strains in which genes associated with acetate metabolism were inactivated. Suggesting that acetate metabolism may act as a metabolic sensor, communicating changes in environmental cues to mechanisms that regulate biofilm biomass and structure (68). Extracellular DNA (eDNA) was important for irreversible attachment and immature biofilm formation in several bacteria species (60, 67, 69). It is still unclear how other environmental signals found in the processing environment, such as lettuce lysate, impact the switch between reversible and irreversible attachment.

The process of switching from reversible to irreversible attachment has been reported to be completed in as little as 5 min. for *L. monocytogenes* (70). There are many theories emphasizing the importance of changes in surface charges and pH, allowing two negatively charged surfaces to lock together. That, however, treats the whole cell as a particle, rather than

the dynamic, complicated structure that it is. Jones and colleagues were able to show that *S. epidermidis* revealed marked interaction with a cation-exchange resin, a negatively charged resin, even though the overall cell charge was negative, suggesting that different regions on the surface can present different surface charges (71).

Salmonella, EHEC, and *L. monocytogenes* all have surface components such as pili, curli, and fimbriae that aid in attachment and flagella that aid in motility (46, 72, 73). The cell walls of *Salmonella* and EHEC (gram negative) are different from *L. monocytogenes* (gram positive). Gram-negative bacteria have a lipid bilayer (lipopolysaccharide; LPS) outer membrane, a thin peptidoglycan layer, and an inner membrane phospholipid bilayer. Gram-positive bacteria lack an outer membrane, but have a thicker peptidoglycan layer that contains lipoteichoic acids, and an inner cell membrane. There is some question as to whether these differences impact attachment as they do with virulence.

Attachment and survival on produce surfaces

While the mechanisms of *Salmonella*, EHEC, and *L. monocytogenes* are known in relation to virulence, the plant-specific interactions that allow attachment and biofilm production are less understood and have largely been the subject of recent research (74-76). For example, *flaA* and *motAB* are genes that encode flagellin and part of the flagellar motor respectively, in *L. monocytogenes.* Deletion mutants were constructed for both genes, and the Δ*flaA* mutant did not produce flagella whereas the Δ*motAB* mutant had nonfunctional flagella. A reduced fitness phenotype for colonization of alfalfa, radish and broccoli sprouts was seen for the Δ*flaA* mutant only, showing that the presence of flagella is important for the colonization of some produce but not motility itself (75). Lcp, a cellulose binding protein in *L. monocytogenes*, was found to play an important role in the attachment to produce (1). When evaluating the role of Lcp in

attachment to lettuce leaves, the percent adherence by the Δ*lcp* mutant was significantly reduced compared to the wild-type (WT) strain, 0.3% ± 0.05% vs. 2.97% ± 0.37% ($P < 0.001$), respectively. The role of Lcp was also evaluated in the attachment to baby spinach and cantaloupe, where the Δ*lcp* mutant showed a similar phenotype to lettuce leaves. For baby spinach, there was an approximately 6% overall difference between the Δ*lcp* mutant and the WT and for cantaloupe a nearly 16% difference. To determine the mechanism between Lcp and cellulose, Bae and colleagues performed a cellulose binding assay in which they found that the WT optical density was significantly higher than the Δ*lcp* mutant (1). Lmo0753, as mentioned as previously being important for biofilm production, was also found to be significant in attachment to lettuce leaves and cantaloupe rinds (61).

Impaired colonization and diminished attachment ability by 1 logCFU/cm^2 to lettuce leaves was seen for EHEC using a *csgA* mutant strain compared to the WT (77). A *csgA* mutant along with a *fliN* (flagellar synthesis) mutant had a reduced capacity to attach to lettuce roots (78). A similar case was made for *Salmonella* and *agfB*, a gene that encodes a surface-exposed aggregative fimbria (curli) nucleator. The *agfB* deletion mutant showed a reduced-attachment phenotype to alfalfa sprouts (79). In another study, *agfBA* and *bcsA* genes for curli and cellulose respectively, were deleted. The double mutant had a 1 log reduction of cell counts within parsley leaves compared to the WT strain. The *agfBA* gene for the mutant curli phenotype showed a higher reduction than the *bcsA* gene for the mutant cellulose phenotype, but both showed reduced cell counts compared to the WT, showing their importance for survival and transfer of the pathogen from soil to the produce surface (63).

Many of the genes that are important for attachment on produce surfaces are known virulence genes, such as *misL*, *sirA*, and *yigG* in *Salmonella*, *espA* and *yadK* in EHEC, and *lapB*

in *L. monocytogenes* (1, 61, 79-85). In EHEC, *yadK* encodes a putative fimbrial adhesion protein, shown to be turned on by acid stress (81). Another study looking at genes *bcsA,* along with *yidR,* and *misL,* which encode a putative ATP/GTP-binding protein and an adhesin of the autotransporter family expressed from the *Salmonella* pathogenicity island-3, respectively. All three genes were shown to be induced by cold stress and knock-out mutants had impaired phenotypes in both attachment and biofilm formation (84). In yet another study, a putative stress protein highly conserved in both *Salmonella* and EHEC and encoded by *ycfR,* when mutated via in-frame deletion, had a significant reduction in attachment to plant surfaces and exhibited reduced chlorine resistance (61).

Several of the studies that focus on attachment to produce surfaces focus on the genes that encode fimbriae, flagella, and cellulose, or their common regulator, CsgD (75-77). As mentioned previously, these factors are known to be involved in attachment, but as deletion mutants show, attachment is not completely eliminated. Attachment is a complex process that involves many mechanisms and factors. Survival on a produce surface is difficult and dependent on, as with any surface, available nutrients, microbial competition, and environmental conditions. For produce other factors come into play such as cultivar, whether produce is injured/cut or intact, and even ripeness of produce (86-89). The bacteria, once irreversibly attached will produce an exopolysaccharide matrix that aids in their protection from any unfavorable conditions or stresses, such as humidity, pH, temperature, and UV radiation (2). It has also been suggested that these environmental stresses may in fact expedite attachment and/or biofilm formation, especially those that inhibit motility, and contribute to overall persistence of the pathogen (38).

Attachment and survival on abiotic surfaces

It has been proposed that, with some overlap, attachment to different surfaces each require a specific set of genes and bacteria are likely to rely on more than one mechanism for attachment to different surfaces, allowing them to switch mechanisms in response to environmental changes (64). For example, genes *ycfR*, *sirA*, and *yigG*, when deleted in *Salmonella*, the mutant strains not only showed reduced attachment to produce surfaces, but showed reduced chlorine resistance and attachment to glass and polystyrene (90). In EHEC, *csgA* was found to be just as important for attaching to abiotic surfaces like glass, Teflon, and stainless steel as it was for attachment to lettuce leaves (91). As with attachment to produce, it is known that virulence factors contribute to attachment to abiotic surfaces. For *L. monocytogenes*, the over-expression of internalins improves attachment to abiotic surfaces especially at elevated incubation temperatures (59). Two genes, *inlA* and *inlB*, that encode internalins that promote adhesion to and invasion into host epithelial and liver cells, respectively, have also been shown to promote attachment to glass surfaces (59). Along with six virulence and virulence-like genes, five other adhesion-like genes with unknown protein function were found that were important for attachment to abiotic surfaces (lmo0723, lmo0585, lmo0587, lmo1068, and lmo2656) (59).

Abiotic surfaces do not provide a source of nutrients to sustain bacteria, as plants surfaces can, yet *Salmonella*, EHEC, and *L. monocytogenes* are known to persist in processing plants for years (26). *Salmonella* persists in a food processing environment for 10 years despite intensive decontaminating efforts (49). Nutrients may be accessible in other ways though, such as plant lysate material on the blades that cut fresh produce, much like what has been seen in meat processing plants (92). A study was conducted in which one inoculated onion was sliced using a mechanical slicer followed by 20 uninoculated onions. Scollon and colleagues found that the last

onion still had up to 2.7 logCFU/onion with an initial inoculation level of 8.6 logCFU/onion. Their lowest inoculation level of 5.9 logCFU/onion still yielded nearly 1 logCFU/onion on the twentieth onion (54). Direct attachment of *Salmonella* to salad leaves has been shown to increase > 350% when only 2% salad leaf juice was used as an available nutrient (93).

Control measures for biofilms/attachment

The most commonly used sanitizer in the fresh produce industry is chlorine, in the form of sodium hypochlorite (94, 95). It is recognized that chlorine and similar washes are minimally effective for product decontamination, but when levels are maintained correctly during washing the chlorine is effective at preventing cross-contamination (96). The problem with chlorine is that it loses its effectiveness when organic matter is present. This is a problem because the product itself, the adhering soil, and the microbes all constitute a source of organic matter. This is especially the case with fresh-cut produce that leaches organic matter into the wash water. The processing water is recycled for reuse and as organic matter accumulates the chlorine can reach sublethal levels (96, 97). If levels decrease to this point, pathogens will no longer be inactivated. This could then lead to the spread of not only contamination, but pathogens that are primed to acquire resistance to chlorine and other environmental stresses. Studies have looked at the transfer of EHEC from inoculated leaf pieces to the wash water and then to uninoculated leaf pieces during washing with chlorine. Using a 10% organic load and 30 ppm free chlorine produced the same effects as washing the lettuce with water alone (98). This is equivalent to transfer of 3 logCFU/mL to the wash water and 2 logCFU/mL of pathogen from inoculated leaf to uninoculated leaves (96). Another study evaluated whether whole leaf washing would improve chlorine efficacy over post cut leaf washing, and while it did by 3.3%, cross-contamination did still occur (97).

Again, subminimal inhibitory levels of sanitizer can lead to resistance strains and increased biofilm production (99). Benzalkonium chloride (BAC) is another commonly used sanitizer used in processing. A BAC-resistant strain was characterized in *L. monocytogenes*, and produced significantly more biofilm at a subminimal inhibitory concentration of 5 mg/L than its BAC-sensitive parent strain (99). Biofilm formation is not only most likely what enables persistence in the processing environment, but also acts as a reservoir for reoccurring contamination and potential foodborne illness (49). Current relied-upon methods, such as chlorine washes, have shown to be effective to some degree in preventing biofilm formation on food processing surfaces. But once a mature biofilm is able to form, resistance can develop quickly leading to persistence of pathogens. It was shown that chlorine concentrations of up to 200 µg/mL (200 ppm) will not eliminate EHEC in biofilms on stainless steel (100).

Reversible attachment precedes irreversible attachment and biofilm formation. At this point bacteria are at lower numbers than biofilms and attached by weak electrostatic forces, like Van der Waals forces that are able to be removed by other simple forces, such as rinsing (56). It is this reversible attachment stage that we believe is important for targeted control methods of foodborne pathogens. It is therefore important to understand the mechanisms and the gene expression/regulation that causes the pathogen to initially attach so that biofilms, cross-contamination, and foodborne illness can be prevented.

SCREENING OF SMALL MOLECULES AND CURLI PRODUCTION

Abstract

Outbreaks due to *L. monocytogenes, Salmonella*, and EHEC are an increasing problem in the fresh produce industry. Current control measures have failed to completely eradicate these harmful pathogens from contamination of the food processing environment and product. Attachment and biofilm formation lead to this contamination and to foodborne illness. Targeted control measures are needed that prevent attachment, proliferation, and biofilm formation. Flagella, encoded by *flhD,* and curli, encoded by *csgA,* are important for attachment and biofilm, respectively, and are promising targets in prevention techniques. Small molecules, a promising area of research, can be a nutrient at certain levels but toxic at other levels. They may also be less likely to cause adaptations for resistance. This study screened several small molecules for their effect on growth rate, *flhD* transcription, and biofilm formation of *E. coli*. Five carbon sources and two nitrogen sources are promising with a nearly 2-fold reduction in *flhD* transcription and biofilm amounts. PEA is another small molecule that has shown to be effective at reducing cell counts on beef pieces. When exposing *L. monocytogenes*, *Salmonella*, and EHEC to various concentrations of PEA, reductions in growth and biofilm by as much as 96% and 70%, respectively, were seen. Impact of temperature on curli production of *Salmonella* and EHEC was also screened using a Congo red assay. Findings were strain dependent, with some strains primarily producing curli at lower temperatures of 10°C and 25°C, and other strains only produced curli at 37°C.

Introduction

Salmonella and EHEC are among the top five foodborne pathogens that result in hospitalizations and *Salmonella* and *L. monocytogenes* are among the top five foodborne

pathogens that result in death (10). According to the CDC, *L. monocytogenes, Salmonella,* and EHEC have been responsible for several outbreaks and are a concern for many industries, including the produce industry. *Salmonella,* EHEC, and *L. monocytogenes* are associated with fresh produce which acts as a vehicle of contamination into the food processing environment. Once these pathogens enter the processing environment they become very difficult to erradicate because they attach and form biofilms. Biofilms allow pathogens to survive long-term in less than ideal and even hostile environments until more ideal conditions come along such as the human gastrointestinal tract (21, 23, 26).

Curli, encoded by *csgA*, has been studied for decades and it is known that curli production is necessary for biofilm formation and maturation in both EHEC and *Salmonella*. Curli synthesis is required for both primary adhesion to inert surfaces and development of multilayered cell clusters. Curli also provide direct interactions with the substratum and form interbacterial bundles, allowing a cohesive and stable association of cells (101). Curli are coiled filamentous surface structures, which are assembled by an extracellular nucleation/precipitation pathway and heir production is strain dependent (102). Curli production and the resulting biofilm production aid in the resistance to antimicrobials, such as chlorine on stainless steel coupons (100).

Current methods used to control biofilm formation include mechanical and manual cleaning like pressure spraying, scrubbing and scraping, chemical cleaning and sanitation like chlorine and other detergents or acids, and application of hot water (103). However, with these current methods, if biofilm is not completely erradicated, resistant microrganisms can be spread by the shear forces of the cleaning action. Also, if biofilm is not completely removed from the

contaminated surface, left over EPS, curli, and eDNA can act as signals for new microrganisms to attach (103).

It is unclear whether there are other alternatives to the current use of chlorine that can target well-known attachment factors like *flhD* or biofilm production factors such as *csgA* that are effective without the risk of resistance development or spread of damaged but active cells. The present study will look at the effect of small molecules on growth, *flhD* transcription, and biofilm formation along with the impact of temperature on curli production.

Materials and Methods

Bacterial strains and growth conditions

Escherichia coli AJW678 (104) transformed with pPS71(33), a derivative of pUA66 with a *flhD::gfp* fusion inferring kanamycin resistance, was used in Phenotype MicroArray^{TM} (PM) technology (Biolog, Hayward, CA) studies. The strain was stored in lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl (Difco^{TM}, Sparks, MD)) with dimethyl sulfoxide (DMSO) at -80°C. Before use, the strain was plated onto LB agar plates incubated overnight at 37°C.

Two strains of *E. coli* O157:H7 (105, 106), *E. coli* O26:H11 (107), *Salmonella enterica* Typhimurium, and *Salmonella enterica* Newport and 4 strains of *Listeria monocytogenes* were used to study the effects of PEA at 37°C. Two different *E. coli* O157:H7 (108) were chosen for the 10°C study because they were curli-positive at 10°C, along with the two *E. coli* O26:H11 and four strains of *L. monocytogenes* used in the 37°C study. Seven additional *E. coli* O157:H7, and 70 *S. enterica* (Table 1) were screened for curli production. The strains were stored in LB or Brain Heart Infusion broth (BHI; 14.5 g/L casein peptone, 10 g/L brain heart infusion from

Table 1. List of strains and their sources

Strains for the PEA experiment		Source	EPI/OUT
TB40	*Salmonella* Typhimurium FSL P3-1552	soil	
TB42	*Salmonella* Typhimurium FSL R6-0207		
TB49	*Salmonella* Newport FSL R8-2543	human feces	human sporadic
TB51	*Salmonella* Newport FSL R8-4110	bovine feces	
TB60	*E. coli* O157:H7 Sakai TWO8264	human	outbreak
TB62	*E. coli* O26:H11 TWO9184	human	
TB63	*E. coli* O157:H7 TW10045	human	outbreak
TB65	*E. coli* O157:H7 Spinach TW14359	human	outbreak
TB66	*E. coli* O157:H7 TW14584	human (Caucasia)	outbreak
TB70	*E. coli* O26:H11 TW16501	human	outbreak
TB1	*L. monocytogenes* 10403S	skin lesion	
TB5	*L. monocytogenes* FSL J1-194	human, CSF	
TB7	*L. monocytogenes* H7858	hot dog	outbreak
TB8	*L. monocytogenes* EGDe	human	

Strains for the Congo red experiment		Source	EPI/OUT
TB40	*Salmonella* Typhimurium FSL P3-1552	soil	
TB42	*Salmonella* Typhimurium FSL R6-0207		
TB49	*Salmonella* Newport FSL R8-2543	human feces	human sporadic
TB51	*Salmonella* Newport FSL R8-4110	bovine feces	
TB60	*E. coli* O157:H7 Sakai TWO8264	human	outbreak
TB62	*E. coli* O26:H11 TWO9184	human	
TB65	*E. coli* O157:H7 Spinach TW14359	human	outbreak
TB70	*E. coli* O26:H11 TW16501	human	outbreak
TB54	*E. coli* O157:H7 TWO2302	hamburger	outbreak
TB55	*E. coli* O157:H7 TWO4863	human, diarrhea	outbreak
TB59	*E. coli* O157:H7 TWO8263	human	outbreak
TB63	*E. coli* O157:H7 TW10045	human	outbreak
TB64	*E. coli* O157:H7 TW14313	human	
TB66	*E. coli* O157:H7 TW14584	human (Caucasia)	outbreak
TB67	*E. coli* O157:H7 TW14585	human	outbreak
TB68	*E. coli* O157:H7 TW14588	human	outbreak
TB69	*E. coli* O157:H7 TW16133	human	outbreak
TB313	*Salmonella* Agona FSL R8-8615	environmental	
	Salmonella Agona FSL R8-8619	environmental	
TB339	*Salmonella* Agona FSL S10-1750	environmental, produce preharvest soil	
TB342	*Salmonella* Agona FSL S10-1759	environmental, produce preharvest swab	
TB343	*Salmonella* Agona FSL S10-1760		
TB344	*Salmonella* Agona FSL S10-1761	environmental, produce preharvest water	

Table 1. List of strains and their sources (continued)

Strains for the Congo red experiment		Source	EPI/OUT
TB276	*Salmonella* Enteriditis FSL F6-963		
TB284	*Salmonella* Enteritidis FSL R8-288	human, clinical	
TB323	*Salmonella* Enteritidis FSL R9-566	environmental, food	
TB334	*Salmonella* Enteritidis FSL S10-1621	environmental, produce preharvest soil	
TB335	*Salmonella* Enteritidis FSL S10-1623	environmental, produce preharvest soil	
TB336	*Salmonella* Enteritidis FSL S10-1644	environmental, produce preharvest swab	
TB337	*Salmonella* Enteritidis FSL S10-1646	environmental, produce preharvest swab	
TB293	*Salmonella* Montevideo FSL R8-2812	environmental, farm	
TB297	*Salmonella* Montevideo FSL R8-3417	environmental, farm	
TB300	*Salmonella* Montevideo FSL R8-3658	environmental, farm	
TB301	*Salmonella* Montevideo FSL R8-3659	environmental, farm	
TB305	*Salmonella* Montevideo FSL R8-4923	human, clinical	
TB324	*Salmonella* Montevideo FSL R9-1588	environmental, farm swab	
TB277	*Salmonella* Newport FSL R6-186	human	
TB279	*Salmonella* Newport FSL R6-204		
TB280	*Salmonella* Newport FSL R6-241		
TB281	*Salmonella* Newport FSL R6-388		
TB282	*Salmonella* Newport FSL R6-776	environmental, farm	
TB283	*Salmonella* Newport FSL R6-777	environmental, farm	
TB289	*Salmonella* Newport FSL R8-1598	human, clinical stool	
TB290	*Salmonella* Newport FSL R8-1637	environmental, farm	
TB292	*Salmonella* Newport FSL R8-2690	bovine fecal	
TB302	*Salmonella* Newport FSL R8-3994	equine fecal	
TB306	*Salmonella* Newport FSL R8-5020	human, clinical	
TB286	*Salmonella* Newport FSL R8-802	human, clinical stool	
TB287	*Salmonella* Newport FSL R8-830	bovine fecal	
TB315	*Salmonella* Newport FSL R8-9630	bovine fecal	
TB338	*Salmonella* Newport FSL S10-1743	environmental, produce preharvest soil	
TB340	*Salmonella* Newport FSL S10-1755	environmental, produce preharvest swab	
TB331	*Salmonella* Newport FSL S10-985	environmental, produce field soil	
TB299	*Salmonella* Saintpaul FSL R8-3582	human, clinical stool	
TB303	*Salmonella* Saintpaul FSL R8-4484	human, clinical stool	
TB307	*Salmonella* Saintpaul FSL R8-5023	human, clinical urine	
TB308	*Salmonella* Saintpaul FSL R8-5029	human, clinical stool	
TB309	*Salmonella* Saintpaul FSL R8-5077	human, clinical stool	
TB325	*Salmonella* Saintpaul FSL R9-1724	human, clinical	

Table 1. List of strains and their sources (continued)

Strains for the Congo red experiment		Source	EPI/OUT
TB330	*Salmonella* Saintpaul FSL S5-649		
TB294	*Salmonella* Stanley FSL R8-2954	human, clinical stool	
TB295	*Salmonella* Stanley FSL R8-2955	human, clinical stool	
TB296	*Salmonella* Stanley FSL R8-2966	human, clinical stool	
TB298	*Salmonella* Stanley FSL R8-3511	human, clinical	
TB304	*Salmonella* Stanley FSL R8-4894	human, clinical stool	
TB317	*Salmonella* Stanleyville FSL R9-145	human, clinical	
TB278	*Salmonella* Tennessee FSL R6-198		
TB291	*Salmonella* Tennessee FSL R8-2240	environmental, farm	
TB326	*Salmonella* Tennessee FSL R9-2434	unspecified	
TB327	*Salmonella* Tennessee FSL R9-2435	unspecified	
TB328	*Salmonella* Tennessee FSL R9-2436	unspecified	
TB341	*Salmonella* Tennessee FSL S10-1757	environmental, produce preharvest swab	
TB310	*Salmonella* Typhimurium FSL R8-5469	equine feces	
TB285	*Salmonella* Typhimurium FSL R8-784	human, clinical stool	
TB288	*Salmonella* Typhimurium FSL R8-865	bovine fecal	
TB316	*Salmonella* Typhimurium FSL R9-0042	human, clinical	
TB318	*Salmonella* Typhimurium FSL R9-148	human, clinical	
TB319	*Salmonella* Typhimurium FSL R9-436	human, clinical	
TB320	*Salmonella* Typhimurium FSL R9-441	human, clinical	
TB322	*Salmonella* Typhimurium FSL R9-532	equine fecal	
TB332	*Salmonella* Typhimurium FSL S10-1134	environmental, farm water	
TB333	*Salmonella* Typhimurium FSL S10-1269	environmental, farm standing water	
TB345	*Salmonella* Typhimurium FSL S10-1766	environmental, produce preharvest water	
TB329	*Salmonella* Typhimurium FSL S5-384	bovine fecal	
TB321	*Salmonella* Typhimurium var.O:5- FSL R9-460	human, clinical	
TB311	*Salmonella* Typhimurium var.O:5- FSL R8-7281	bovine feces	
TB312	*Salmonella* Typhimurium var.O:5- FSL R8-7950	human clinical	

FSL numbers provided by the Food Safety Lab at Cornell University, foodmicrobetracker.com
TW numbers provided by the STEC Center at Michigan State University
EPI/OUT indicates whether the strain was part of an epidemic or outbreak, respectively

solids, 5 g/L animal tissue peptone, 5 g/L NaCl, 2.5 g/L Na_2PO_4, and 2 g/L dextrose (Criterion™, Santa Maria, CA)) with 20% glycerol at -80°C. The strains were plated onto LB or BHI agar for EHEC/*S. enterica* or *L. monocytogenes*, respectively, and incubated overnight at 37°C before use.

Screening of 95 carbon, 95 nitrogen, and 95 phosphorus/sulfur sources for their effect on *E. coli* growth, biofilm amounts, and *flhD* transcription

PM technology was developed for the determination of bacterial growth phenotypes (109). Using a 96-well format, where individual nutrients or chemicals are dried in the base of each well, this PM technology permits the testing of more than 2000 bacterial phenotypes by way of respiration. As in Lynnes et al., this PM technology can also be used to determine biofilm amounts (110).

E. coli AJW678 was removed from the LB plates with a nylon flocks swab and suspended in sterile tryptone broth (TB; pancreatic digest of casein (Bacto™, Sparks, MD)) to an OD_{600} of 0.1. A 100 µL aliquot of this solution was used to inoculate each well of PM 1 (carbon sources), PM 3B (carbon and nitrogen sources), and PM 4A (phosphorus/sulfur sources) plates. All plates were then incubated at 37°C for 16 hours. Each experiment was done in six replicates. For the determination of growth, the OD_{600} was determined by taking readings every two hours using a Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT). For the determination of biofilm amounts, the TB was removed from the wells. Biofilms were gently rinsed with 100 µL PBS and resuspended in 100 µL of TB. The bacteria were homogenized by carefully pipetting the media up and down. Homogenized biofilms were then transferred to 96-well black plates (Greiner bio-one, Monroe, NC) in which both an OD_{600} reading for biofilm and

a fluorescence reading (emission wavelength 528, excitation wavelength 485) representing transcription from *flhD*::*gfp* was taken.

Data were analyzed as follows: reductions of OD_{600} and fluorescence by each respective nutrient were determined as ratios, dividing the value for the respective carbon, nitrogen, or phosphorus/sulfur source by that of the negative control that contained an unsupplemented TB. Ratios below one were indicators of reductions (of growth, biofilm amounts, and transcription of *flhD*) and inhibitory effectiveness of the respective nutrient. Nutrients were arranged by increasing ratios or decreasing effectiveness and the nutrients that yielded the largest reduction in growth, biofilm amounts, or *flhD* transcription were presented and discussed. Carbon, nitrogen, and phosphorus/sulfur sources were analyzed separately as well as growth, biofilm amounts and *flhD* transcription.

Effect of β-phenylethylamine (PEA) on *E. coli* O157:H7, *E. coli* O26:H11, *S. enterica* Typhimurium, *S. enterica* Newport, and *L. monocytogenes*

Cultures were prepared by inoculated in tryptic soy broth (TSB; 17 g pancreatic digest of casein, 3 g papaic digest of soybean, 2.5 g dextrose, 5 g NaCl, 2.5 g K_2PO_4 (Bacto™)) with the respective bacterial strain and incubated at 37°C for 16 hours with shaking at 225 rpm. Overnight cultures were centrifuged at 4500 rpms for 10 minutes. Supernatants were discarded, and cell pellets were resuspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer (Amresco, Solon, OH)). Cultures were normalized to an OD_{600} reading of 1.0. One hundred microliters of normalized culture was then transferred into 10 mL of 2X TSB. Dilutions of PEA were prepared with initial concentrations of: 40, 20, 17.5, 15, 13.5, 10, 8, 6, 4, 2, and 0.5 mg/mL in PBS. To respective wells of a 24-well plate, 500 µL of culture and 500 µL of PEA was added. Plates were then incubated statically at 37°C and OD_{600}

readings were taken every two hours for 16 hours to measure growth. After 16 hours, liquid culture was removed, and each biofilm was gently rinsed with PBS three times. Biofilms were allowed to dry and then stained with 1% crystal violet for 15 min. Crystal violet was removed, and biofilms were gently rinsed with PBS three times. Biofilms were again allowed to dry, and crystal violet was extracted from the biofilms by adding 1 mL of 80% ethyl alcohol/20% acetone. After a 5 min incubation, an aliquot of 150 µL of extracted crystal violet was transferred to a 96-well plate and an OD_{600} reading was taken to determine biofilm amounts. The process was repeated at 10°C and 10 days for *E. coli* and 10°C and 4 days for *L. monocytogenes*, with the exception that Bactiter Glo™ (Promega, Madison, WI) was used to measure ATP activity in biofilm instead of crystal violet for biofilm amounts for *L. monocytogenes* only. Bactiter Glo™ was used instead of crystal violet due to inconsistencies with crystal violet measurements for *L. monocytogenes*, so measurement of ATP activity was thought to be a more accurate measurement of biofilm. This was accomplished by replacing crystal violet with 100 µL of Bactiter Glo™ to each well of a 96-well white plate with 100 µL of biofilm suspension and incubating for 10 min at room temperature. Luminescence was then read using the Synergy H1 Hybrid Reader.

Three replicates of each strain were performed. Growth and biofilm amounts obtained from each dilution were compared to those of the untreated but inoculated positive control for each of the three replicate experiments. Percent reduction was calculated ((0 mg/mL PEA – 10 mg/mL PEA/ 0 mg/mL PEA)* 100), and a Student's T-test was performed using Excel to determine significance of the difference between 0 mg/mL and 10 mg/mL concentrations. P values of <0.05 were considered statistically significant.

Congo red screening for curli production

Congo red indicator agar (YESCA agar; 10 g/L Casamino acids (Difco), 1 g/L yeast extract (Difco), and 20 g/L Bacto agar (Difco), 20 mg/L Congo red (Sigma) and 10 mg/L Coomassie brilliant blue G (Sigma)) (111), was used to screen 74 strains of *S. enterica* and 13 strains of *E. coli* serotypes O157:H7 and O26:H11 (Table 1) for their expression of curli in cells grown as colonies. Strains were incubated on the agar plates at 25ºC for 48 h and 10ºC for 10 days. A subset of strains used in the PEA assay were also evaluated at 37°C for 24 h. Strains were classified as positive (++), slightly positive (+), or negative (-) based on colony color of red, pink, or white, respectively.

Results

Effect of 95 carbon, 95 nitrogen, and 95 phosphorus/sulfur sources on growth, biofilm formation, and *flhD* transcription of *E. coli*

I found five carbon sources, acetoacetic acid (AAA), α-ketobutyric acid, D-xylose, glucuronamide, and L-proline (Table 2) with reductions in both *flhD* transcription and biofilm amounts. AAA was able to reduce both *flhD* transcription and biofilm amounts by 1.7 and 1.4 fold, respectively. α-ketobutyric acid was slightly better than AAA and was the best overall at reducing *flhD* transcription and growth by 1.8 and 1.9 fold, respectively. D-xylose had the greatest effect on reducing biofilm by 1.7 fold. I also found two nitrogen sources of interest. These were D,L-α-amino-caprylic acid and hydroxylamine (Table 2). Both were similar in their reductions of *flhD* transcription and biofilm to the five carbon sources, but they both had an higher, 3.7 and 4.5 fold, reduction in growth of *E. coli*, respectively. There were no phosphous/sulfur sources that caused a reduction of *flhD* transcription and biofilm amounts.

Table 2. Reductions in *flhD* transcription, growth, and biofilm of *E. coli* AJW678.

Carbon sources	*flhD*::*gfp*	Growth	Biofilm
Acetoacetic acid	0.59 ± 0.28 (1.7)	0.82 ± 0.05 (1.2)	0.69 ± 0.29 (1.4)
α-ketobutyric acid	0.57 ± 0.12 (1.8)	0.54 ± 0.09 (1.9)	0.62 ± 0.19 (1.6)
D-xylose	0.56 ± 0.25 (1.8)	0.91 ± 0.04 (1.1)	0.60 ± 0.22 (1.7)
Glucuronamide	0.59 ± 0.23 (1.7)	0.98 ± 0.05 (1.0)	0.62 ± 0.20 (1.6)
L-proline	0.59 ± 0.31 (1.7)	0.98 ± 0.06 (1.0)	0.64 ± 0.28 (1.6)
Nitrogen sources			
D, L-α-Amino-Caprylic Acid	0.56 ± 0.07 (1.8)	0.27 ± 0.07 (3.7)	0.85 ± 0.09 (1.2)
Hydroxylamine	0.59 ± 0.13 (1.7)	0.22 ± 0.02 (4.5)	0.76 ± 0.10 (1.3)

Data obtained from *flhD* transcription, growth, and biofilm was divided by the data obtained from the untreated TB control. Averages and standard deviations are reported, with the fold change compared to the control in parentheses.

Effect of PEA on *E. coli* O157:H7, *E. coli* O26:H11, *S. enterica* Typhimurium, *S. enterica* Newport, and *L. monocytogenes*

Among *S. enterica* Typhimurium and *S. enterica* Newport (Fig. 1, a-d), there was a significant reduction of 22.7% (P = 0.0033) and 22.1% (P = 0.0286) of growth rate and biofilm at 37 ℃, respectively, at 10 mg/mL PEA compared to the untreated control (0 mg/mL PEA) for TB40 (Fig. 1a), and a significant reduction of 70.2% (P = 0.0326) for biofilm of TB42 (Fig. 1b). No significant change in growth rate was seen for TB42.

Effect on growth rate of *L. monocytogenes* at 37°C varied from a reduction of 48.6% for strain 10403S (Fig. 2a) to an increase of 41.9% for strain H7858 (Fig. 2c). Biofilm also showed an increase of 40.9% for strain EGDe (Fig. 2d). Biofilm reduction varied across strains from 22.2% to 56.6%. The only significant reductions were found to be reductions in biofilm of 56.6% (P = 0.0309) for strain 10403S (Fig. 2a) and 48.3% (P = 0.0242) for strain H7858 (Fig. 2c).

At 10°C, growth rate of *L. monocytogenes* increased at 10 mg/mL PEA, compared to the control, with the exception of 10403S. For FSL J1-194 and EGDe, growth rate increased significantly by 111% (P = 0.0129) (Fig. 4b) and 166% (P = 0.0033) (Fig. 4d), respectively.

There were no significant reductions in biofilm although they ranged from a reduction of 68.5% for H7858 (Fig. 4c) to 4.6% for EDGe (Fig. 4d).

EHEC had the most significant reductions in growth rate at both 37°C and 10°C. Sakai had a 76.7% reduction (37°C; P = 0.0009) (Fig. 3a), while TB65 had the highest significant reduction in growth rate overall of 95.4% (37°C; P = 0.00006) (Fig. 3c). TB62 had a significant reduction of 80.3% (P = 0.0017) (Fig. 5a) at 10°C but an insignificant reduction of only 4% at 37°C. TB66 had an increase in both growth rate and biofilm at 10°C, but only growth rate was significant at 96.2% (P = 0.0043), this may be due to the fact that it is positive for curli production at 10°C. Of interest, TB70, had significant reductions in both growth and biofilm at both 37°C and 10°C of 69.7% (P = 0.0298), 54.6% (P = 0.0073) (Fig. 3d), 80.1% (P = 0.0010), and 39.7% (P = 0.0346) (Fig. 5d), respectively. This may be due to the fact that this strain is negative for curli production at 10°C and only slightly positive at 37°C (see Congo red results).

Impact of temperature on curli production

I screened 13 EHEC (Table 3) and 74 *S. enterica* (Table 3 and Appendix A) of various serotypes for their ability to produce curli at temperatures of 10°C or 25°C. A subset of strains used in the PEA assay were also evaluated at 37°C. For three of the EHEC strains, temperature had no effect; TB54, TB55, and TB68 were slightly positive for curli production for both temperatures tested. Five of the EHEC strains, TB59, TB63, TB64, TB66, and TB67, were slightly positive for curli production at 10°C, but negative at 25°C. TB69 was strongly positive at 25°C and slightly positive at 10°C. Of interest, the infamous Spinach and Sakai strains were negative for curli production at 10° and 25°C, but slightly positive at 37°C. Also of interest, EHEC O26:H11 serotypes were both negative at 10°C, strongly positive at 25°C, and then

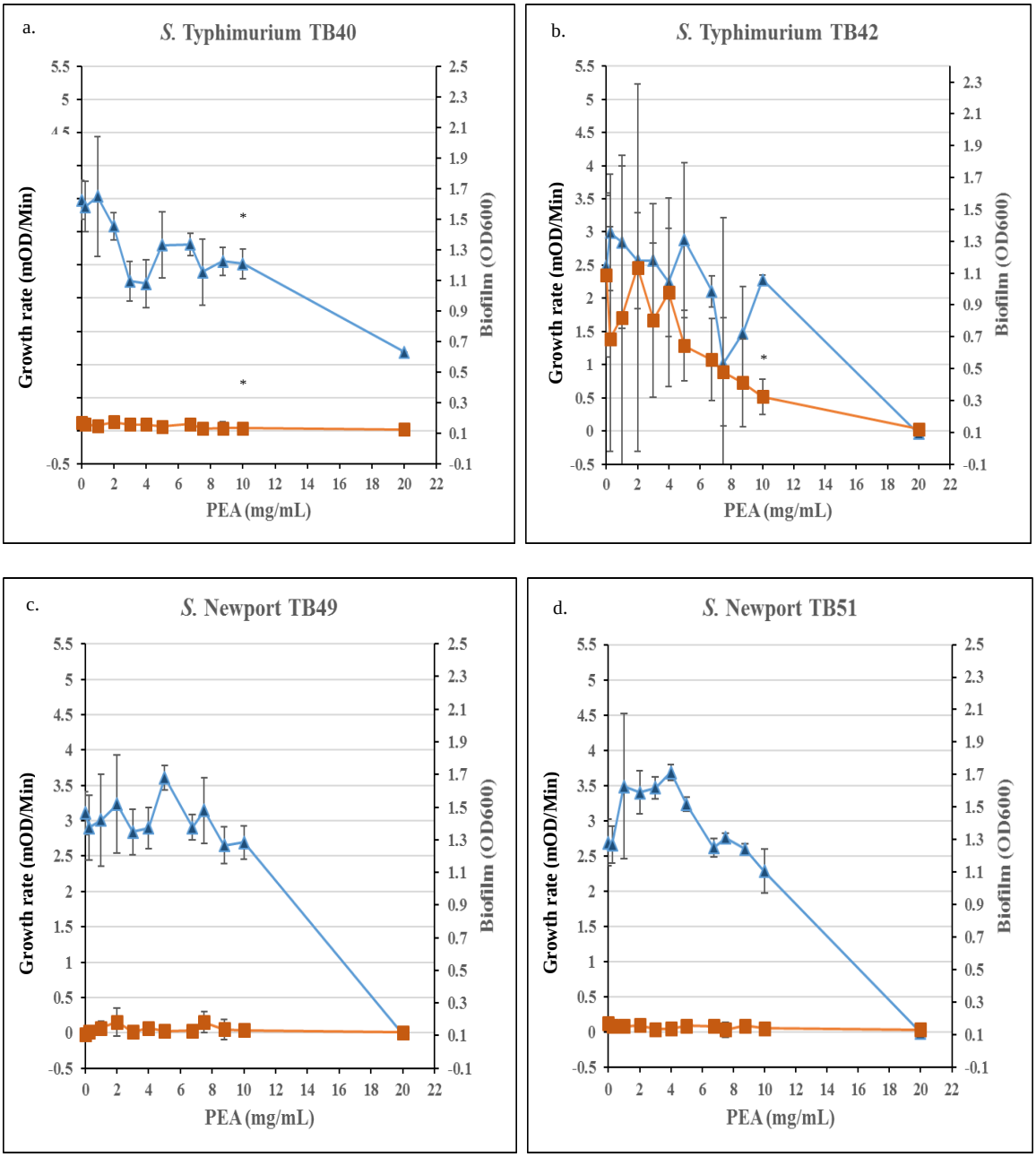

Fig. 1. Effect of PEA on growth rate and biofilm amounts of *S. enterica* Typhimurium (a and b) and *S. enterica* Newport (c and d) at 37°C across increasing concentrations. Average and standard deviation were calculated across the three replicate experiments. Growth is represented by the blue triangles and biofilm by the orange squares. * indicates that value is significantly different then 0 mg/mL.

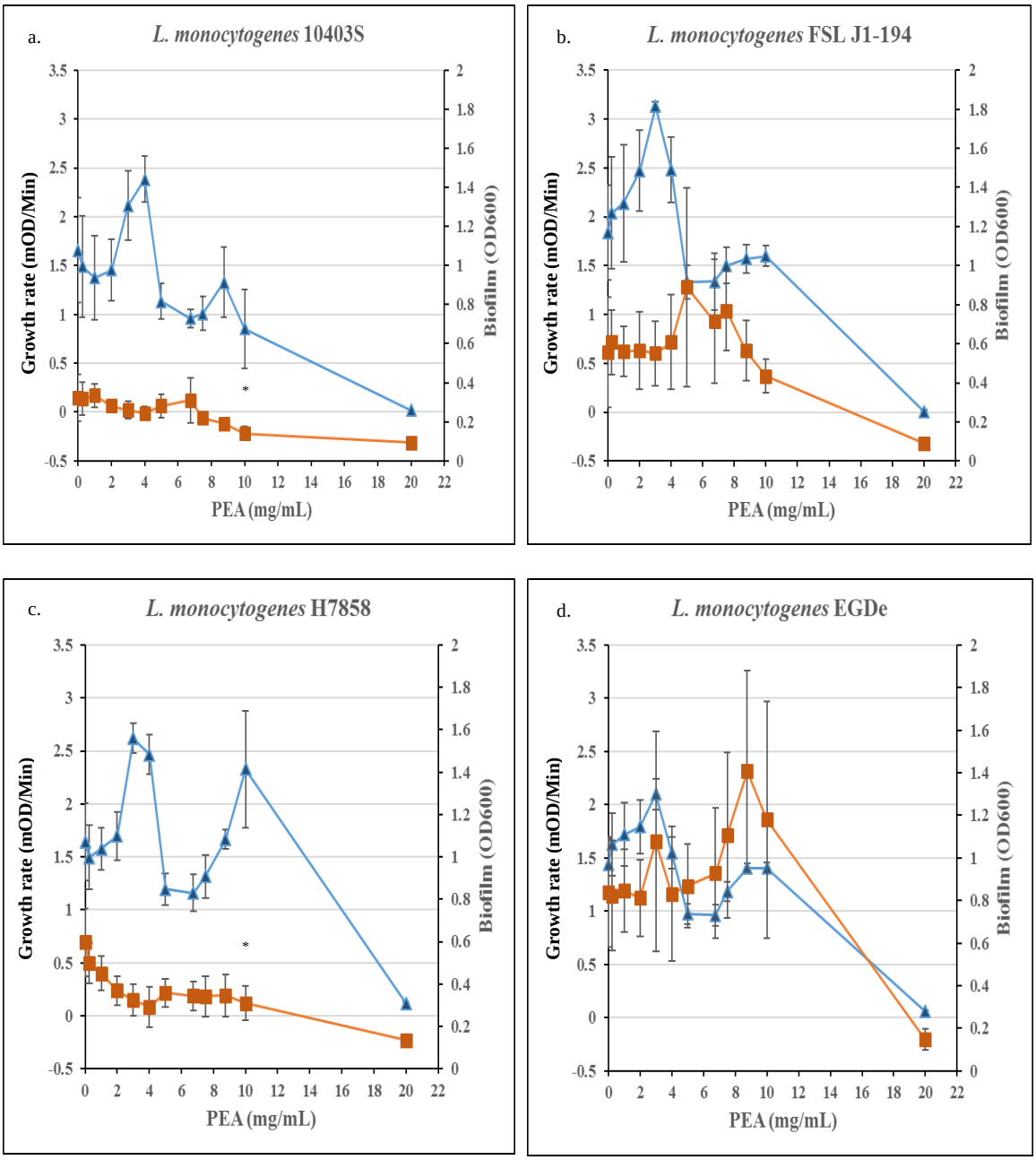

Fig. 2. Effect of PEA on growth rate and biofilm amounts of *L. monocytogenes* at 37°C across increasing concentrations. Average and standard deviation were calculated across the three replicate experiments. Growth rate is represented by the blue triangles and biofilm by the orange squares. * indicates that value is significantly different then 0 mg/mL.

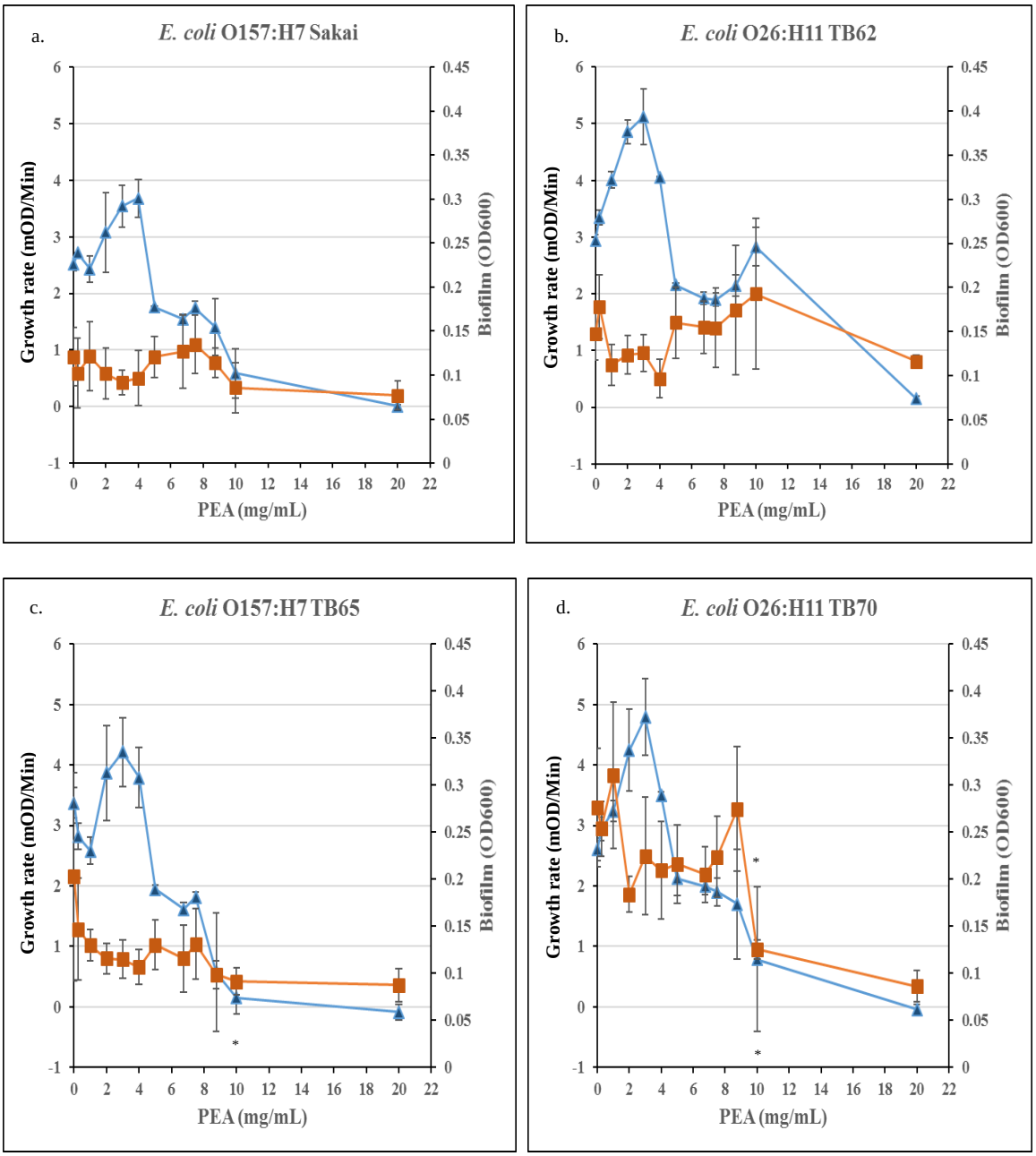

Fig. 3. Effect of PEA on growth rate and biofilm amounts of *E. coli* O157:H7 (a and c) and *E. coli* O26:H11 (b and d) at 37°C across increasing concentrations. Average and standard deviation were calculated across the three replicate experiments. Growth rate is represented by the blue triangles and biofilm by the orange squares. * indicates that value is significantly different then 0 mg/mL.

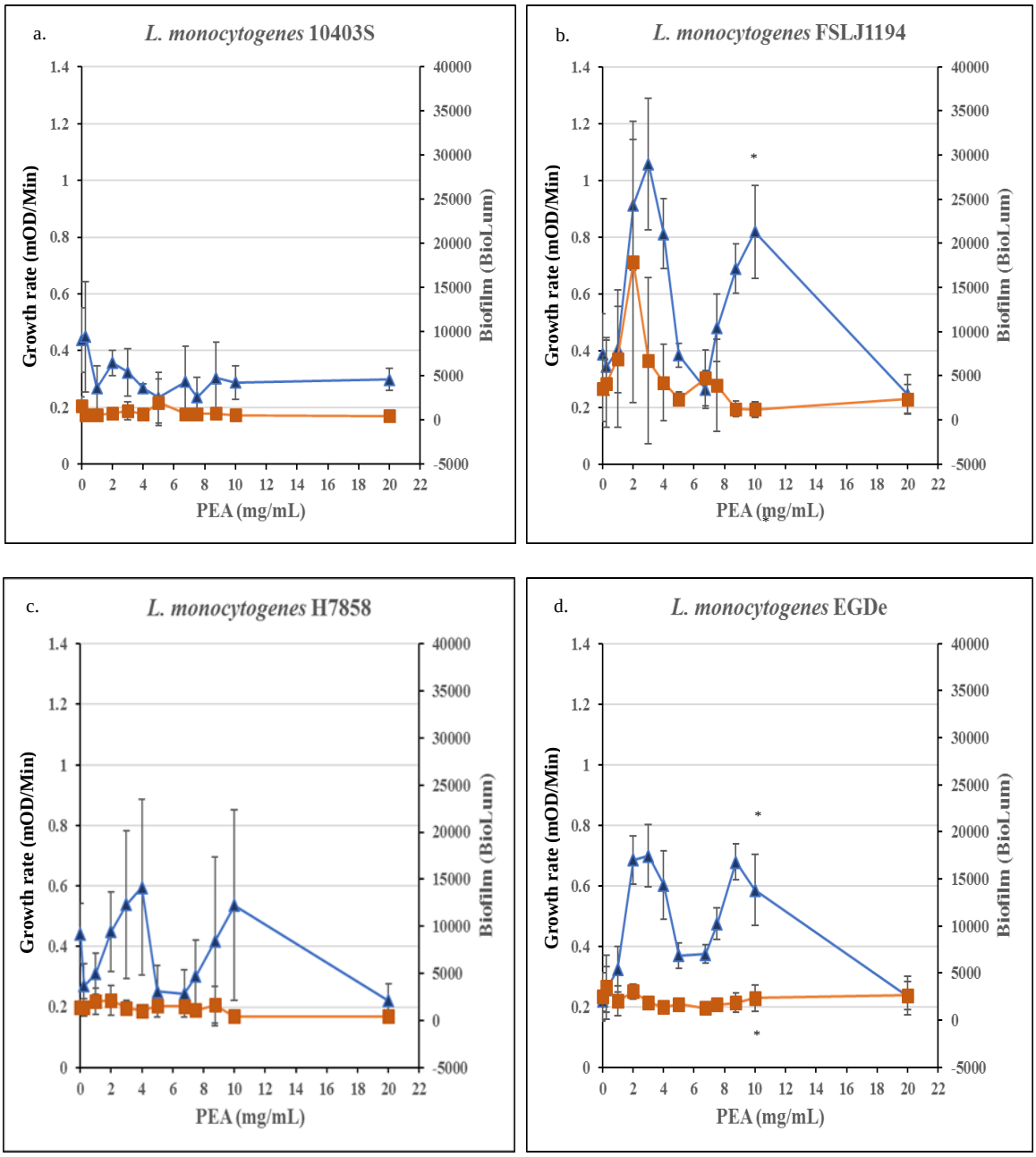

Fig. 4. Effect of PEA on growth rate and biofilm amounts of *L. monocytogenes* at 10°C across increasing concentrations. Average and standard deviation were calculated across the three replicate experiments. Growth rate is represented by the blue triangles and biofilm by the orange squares. * indicates that value is significantly different then 0 mg/mL.

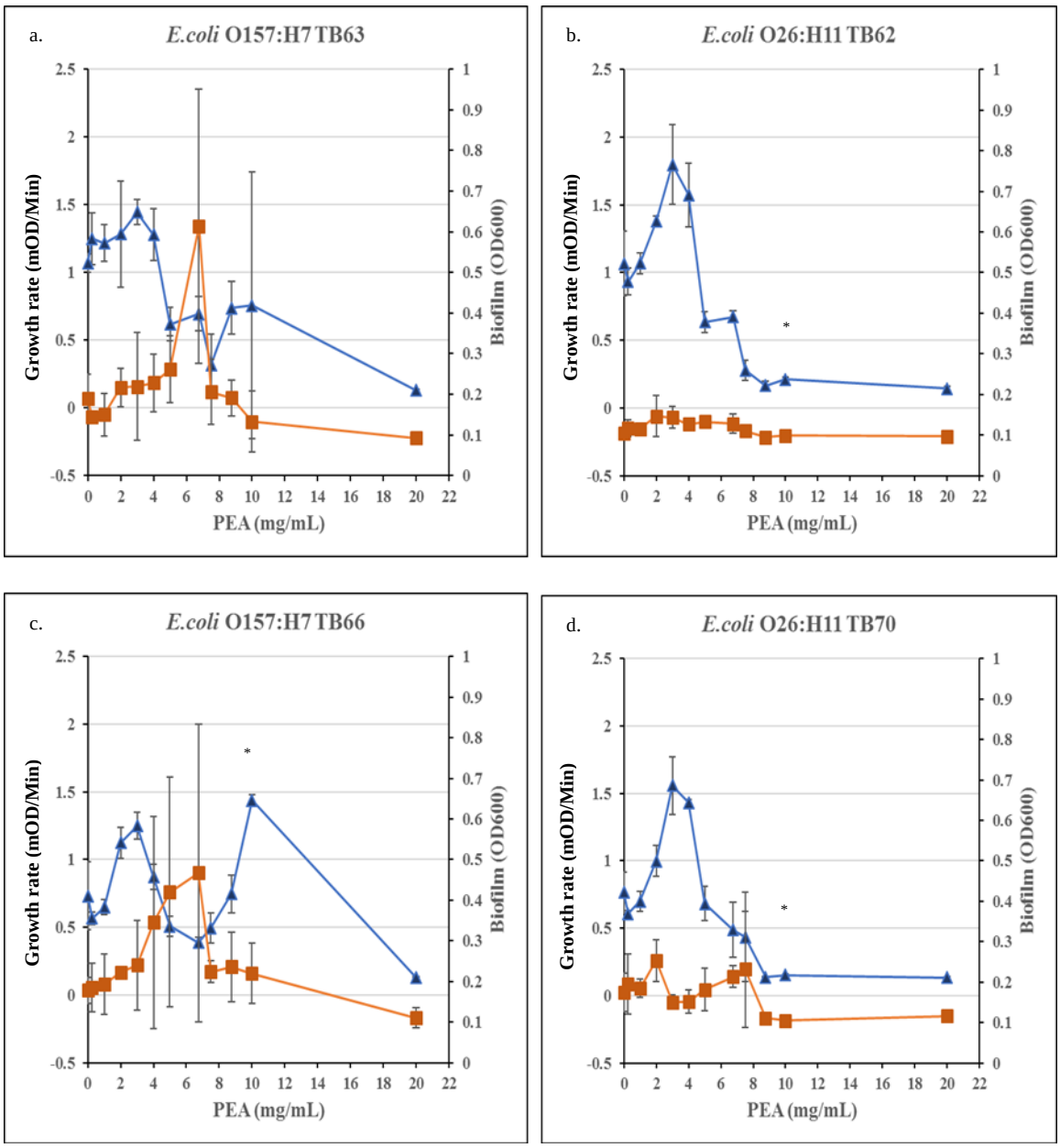

Fig. 5. Effect of PEA on growth rate and biofilm amounts of *E. coli* O157:H7 (a and c) and *E. coli* O26:H11 (b and d) at 10°C across increasing concentrations. Average and standard deviation were calculated across the three replicate experiments. Growth rate is represented by the blue triangles and biofilm by the orange squares. * indicates that value is significantly different then 0 mg/mL.

Table 3. Strain, temperature (°C), and result of Congo red screen for curli production. Strains were classified as strongly positive (++), slightly positive (+), or negative (-) for curli production.

Strain		Temp.	Result (++, +, OR -)
TB40	*Salmonella* Typhimurium FSL P3-1552	10	(+)
		25	(-)
		37	(+)
TB42	*Salmonella* Typhimurium FSL R6-0207	10	(++)
		25	(+)
		37	(+)
TB49	*Salmonella* Newport FSL R8-2543	10	(++)
		25	(++)
		37	(+)
TB51	*Salmonella* Newport FSL R8-4110	10	(+)
		25	(+)
		37	(+)
TB54	*E. coli* O157:H7 TWO2302	10	(+)
		25	(+)
TB55	*E. coli* O157:H7 TWO4863	10	(+)
		25	(+)
TB59	*E. coli* O157:H7 TWO8263	10	(+)
		25	(-)
TB60	*E. coli* O157:H7 Sakai TWO8264	10	(-)
		25	(-)
		37	(+)
TB62	*E. coli* O26:H11 TWO9184	10	(-)
		25	(++)
		37	(+)
TB63	*E. coli* O157:H7 TW10045	10	(+)
		25	(-)
TB64	*E. coli* O157:H7 TW14313	10	(+)
		25	(-)
TB65	*E. coli* O157:H7 Spinach TW14359	10	(-)
		25	(-)
		37	(+)
TB66	*E. coli* O157:H7 TW14584	10	(+)
		25	(-)
TB67	*E. coli* O157:H7 TW14585	10	(+)
		25	(-)
TB68	*E. coli* O157:H7 TW14588	10	(+)
		25	(+)
TB69	*E. coli* O157:H7 TW16133	10	(+)

Table 3. Strain, temperature (°C), and result of Congo red screen for curli production (continued). Strains were classified as strongly positive (++), slightly positive (+), or negative (-) for curli production.

Strain		Temp.	Result (++, +, OR -)
		25	(++)
TB70	*E. coli* O26:H11 TW16501	10	(-)
		25	(++)
		37	(+)

slightly positive at 37°C. For the 74 *S. enterica* strains all were slightly or strongly positive for curli production at all temperatures tested except for two strains that were negative at 25°C alone. These two strains, TB40 and TB312, are from soil and human sources, respectively.

Discussion

Effect of 95 carbon, 95 nitrogen, and 95 phosphorus/sulfur sources on the growth, biofilm formation, and *flhD* transcription of *E. coli*

In the process of screening the effects of 95 carbon, 95 nitrogen, and 95 phosphorus/sulfur-sources using PM technology for their effects on growth, biofilm formation, and *flhD* transcription of *E. coli*, I found five carbon and two nitrogen sources (Table 2) with promising reductions in both *flhD* transctription and biofilm amounts. AAA (PubChem CID 96) is the simplest beta-keto acid and has been used as an antibacterial and antifungal for ear infections. It is important for lipid production especially in newborns, but can be a toxic by-product of the liver in diabetics. AAA is expensive, averaging $165/g. Regardless, AAA, is continuing to be studied in our lab as a biofilm inhibitor. α-ketobutyric acid (PubChem CID 58) is involved in the metabolism of several amino acids and plays a part in the citric acid cycle. It is primarily used as a flavoring agent in the food industry and is comparatively inexpensive averaging $15/g. D-xylose, glucuronamide, and L-proline were all very similar to both AAA and

α-ketobutyric acid for reductions in *flhD* transcription and biofilm, except that D-xylose is the least expensive at $0.10/g. Although slightly less than 2 fold in reductions of *flhD* transcription, growth, and biofilm formation, any one of these carbon sources has the potential to be used as an inhibitor of biofilm in the food industry. D,L-α-amino-caprylic acid could be useful but no further studies have been done on its toxicity. As for hydroxylamine, it is very toxic to humans with a oral lethal dose of 50 - 500 mg/kg. To date there has been no other studies that have looked at these nitrogen or carbon sources for inhibition of biofilm formation.

Medium-chain fatty acids (MCFAs) and organic acids (OAs) are classes of compounds that have also been largely researched for their effectiveness in bactericidal effects especially in a synergistic matter (112). There is a possibility that the carbon sources discovered in this study can also have an increased effectiveness when combined with another compound or small molecule that shows inhitory affects. The mechanism proposed is that one antimicrobial disrupts the cell membrane allowing entry of the other antimicrobial into the cell (112). Small molecules have been used to block virulence mechanisms (113). Inhbitory mechanisms of the small molecules include targeting toxins, inhibiting adhesions, inhibiting specialized bacterial secretory systems, inhibiting organism-specific virulence gene expression, inhibiting cell-to-cell signalling, and broad-spectrum inhibition of virulence and signalling. For example, broad-spectrum inhibition of virulence and signalling involves using small molecules to target a common signalling pathway that starts with the membrane-bound QseC histidine sensor kinase. Common to at least 25 human and plant pathogens, QseC, involves a complex phosphorylation cascade in the bacterial cell that regulates the expression of virulence genes. By targeting these bacterial-specific mechanisms, therapies avoid severe side effects because the targets are nonexistant in the host cell. Also, many of these mechanisms do not impact growth, so inhibition

of these pathways may not exert strong selective pressure towards development of antimicrobial resistance (113). Use of small molecules could therefore be a promising mechanism for targeted control measures in the food industry.

Effect of PEA on *E. coli* O157:H7, *E. coli* O26:H11, *S. enterica* Typhimurium, *S. enterica* Newport, and *L. monocytogenes*

PEA is a trace amine that acts as a neurotransmitter and as a result of microbial metabolism it can be found in fermented food and meat. PEA is also found in chocolate as the result of thermal processing (114). PEA was identified previously to be the most successful at reducing cell counts of *E. coli* O157:H7 on beef, leading to a 90% reduction in cell counts at 10°C (110). PEA has not been evaluated against any other pathogens besides *E. coli*. For this reason, I further evaluated the effects of PEA on growth and biofilm formation across a range of foodborne pathogens.

The effect of PEA on growth and biofilm varied across strain, microorganism, and concentration of PEA. For the majority of strains tested, at 20 mg/mL bacteria did not grow and biofilm formation was very low to nonexistent. For this reason, I evaluated the percent reduction between 0 – 10 mg/mL PEA for each strain of each organism and performed a Student's T-test on each. A peak in growth and/or biofilm at low concentrations (0.25-5 mg/mL) of PEA occurred with the majority of strains. This could be explained in that PEA is a secondary metabolite and may be used as a nutrient source at these concentrations. For this reason, at concentrations of 10 mg/mL or higher, PEA may act as an inhibitor of biofilm for *Salmonella* and EHEC. *L. monocytogenes* does not have the same global regulator complex as *Salmonella* and EHEC, and no one has examined in detail with respect to flagellar genes *motAB* and *flaA* if the same response can be made for *L. monocytogenes.* Of interest, at 10°C there is a second peak

in which 10 mg/mL PEA seems to act as a nutrient for *L. monocytogenes* FSL J1-194 and EDGe and EHEC TB66 by increasing growth significantly.

Impact of temperature on curli production

Curli production, along with EPS and cellulose, have been shown to be important for attachment and biofilm formation for both EHEC and *Salmonella* on biotic and abiotic surfaces (37, 76, 77, 115-118). Curli are adhesive fimbriae that are encoded by *csgA*. Affinity for Congo red dye is a common way to measure curli production, cellulose, and colanic acid (119). There are contradicting studies on the impact of temperature on curli production. Some suggest that curli production is more abundant at the higher temperatures of 30°C to 37°C vs 25°C, but is strain and serotype dependent (119). Others suggest that curli production is greater at temperatures less than 32°C (111). Many *E. coli* O157:H7 strains have disrupted regulators for curli, specifically MlrA, a transcription regulator, which binds the promoter of *csgD* to enhance the RpoS-dependent transcription of *csgD*. The stx_1 gene carrying bacteriophage, utilize an insertion site in the proximal *mlrA* coding region of *E. coli* O157:H7 strains. The major factor contributing to poor curli and biofilm expression would be expected to be the loss of *mlrA* function in *E. coli* O157:H7 strains (120). Therefore, positive results at 10°C are most likely not due to curli production.

There have not been any studies using Congo red to assess curli production at temperatures lower than 25°C. My results for EHEC support that curli production is strain and serotype dependent. There are several (5 of 13) strains that were negative at 25°C but positive at 10°C. One strain was more positive at 25°C vs. 10°C. Two strains were negative at 10°C and 25°C but positive at 37°C. Three strains were the same at 25°C and 10°C. The last two strains were negative at 10°C, and were positive at both 25°C and 37°C, but had greater curli production

at 25°C vs 37°C. For *Salmonella*, there were two strains negative for curli production at 25°C, but positive at 10°C and 37°C. Two other strains had greater curli production at temperatures lower than 37°C. The rest of the *Salmonella* strains were positive for curli production at 25°C. This suggests that the impact of temperature on curli production is microorganism, strain, and possibly serotype dependent. The slightly positive (+) results could be negative for curli but positive for either cellulose or colonic acid instead.

Conclusions

Five carbon sources and two nitrogen sources showed effective reductions of nearly two logs of biofilm and *flhD* transcription. This indicates that small molecules can be effective at reducing biofilm amounts suggesting less risk of adaptations that lead to resistance because of its nutrient properties than other antimicrobials. PEA, another small molecule, was able to reduce growth at 10 mg/mL for most strains and significantly by as much as 95.38% for EHEC TB65. PEA also had the ablity to reduce biofilm formation significantly by as much as 70.23% for *S. enterica* Typhimurium TB42. Of interest, significant increases of growth were also seen, mainly for *L. monocytogenes* at 10°C, by 110.99% (FSL J1-194) and 165.91% (EGDe), but still had nonsignificant reductions in biofilm formation. Curli production, known to be improtant for biofilm formation, showed no evidence of temperature dependence, but rather, dependence on the microorganism, strain, and/or serotype was indicated.

Acknowledgements

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EFFECT OF ENVIRONMENTAL TEST CONDITIONS ON EXPRESSION OF AN ATTACHMENT FACTOR, LCP, AND ATTACHMENT TO STAINLESS STEEL

Abstract

Rich in iron and many other vitamins and minerals, fresh produce such as leafy greens are a healthy part of our diet. Outbreaks of *L. monocytogenes* are also being increasingly attributed to contamination of leafy greens. Attachment is the first step in biofilm production, and once a mature biofilm is established they are nearly impossible to erradicate. For this reason it is important to understand the mechanisms of attachment and find targets that could be used in prevention measures. *Lcp* encodes a protein that contains a cellulose binding domain, and has been shown to be an important factor for attachment of *L. monocytogenes* to fresh produce and cantaloupe. Using a single-copy chromosomal reporter fusion, the change in GUS expression driven by P_{lcp} when exposed to test conditions found in the processing environment was observed. P_{lcp} was not significantly up or down-regulated by any of the conditions tested. Attachment assays were then conducted on stainless steel using the same test conditions with the addition of 10 mg/mL PEA. Chlorine at 5 ppm and 2% lettuce lysate significantly reduced loosely and strongly attached cells compared to the control by 0.7, 0.5, 1, and 0.5 logCFU/cm^2, respectively. This suggests together suggests that not Lcp, but other protiens are responsible for attachment under the selected test conditions.

Intrduction

Unlike EHEC and *Salmonella*, flagella are necessary for *L. monocytogenes* biofilm formation (121). It has been suggested that *L. monocytogenes* forms biofilms more effectively in coculture by utilizing a primary colonizing microrganism and taking advantage of their EPS production in flowing systems such as a lettuce washing facility (122). Some even question

whether *L. monocytogenes* forms a true biofilm or simply adheres, however, one of the main components of biofilm is the production of EPS and *L. monocytogenes* is capable of producing EPS (123). Similar to EHEC and *Salmonella* curli production, *L. monocytogenes* biofilm formation is strain dependent and persistent strains seem to form biofilms more efficiently than non-persistent strains (123).

L. monocytogenes can grow at 4°C, pH levels as low as 4.5, under salt stress, and in high humidity conditions. It can also survive frozen and low water activity conditions. Biofilms formed by *L. monocytogenes* also allow for increased resistence to chlorine (124) and other antimicrobials such as individual organic acids, nisin (125), Benzalkonium chlorine (99).

Unknown are the signals that influence irreversible attachment. It is also unknown how these signals affect the regulation of known attachment factors such as *lcp*. In this study single-copy chromosomal reporter fusions will be used to determine the effect of select signals on the expression of *lcp*. These expression patterns can then be correlated to physical attachment by the conduction of attachment assays under the same select signals.

Materials and Methods

Bacterial strains and GUS reporter fusion construction

Listeria monocytogenes strain H7858 was used as a background strain for all GUS reporter fusions, which were maintained in brain heart infusion (BHI) broth at -80°C with 20% glycerol. Single-copy chromosomal reporter fusions in H7858 were constructed using pMJG2, a derivative of pPL2 (126) with β-glucuronidase (GUS) as the reporter protein and chloramphenicol (Chl) selection. This vector integrates into the $tRNA^{Arg}$ – *attBB'* site on the chromosome. Reporter fusions were constructed for two different promoter regions: that of *lcp* encoding a cellulose binding protein (1), and of *uspA* encoding a SigB-regulated general stress

response protein (127). Promoters were amplified from H7858 DNA with the primers listed in Table 1, using AccuStart Taq DNA polymerase HiFi (Quanta BioSciences, inc., Gaithersburg, MD) master mix with concentrations of 10 mM dNTPs, 2 mM $MgSO_4$, and 10 µM forward and reverse primers. Touchdown PCR was used to amplify the promoter regions, and thermocycling conditions were as follows: 95°C for 5 min, followed by 20 cycles of 95°C for 45 s, annealing temperatures (Table 1) for 45 s, and 72°C for 45 s. This was followed by an additional 20 cycles of 95°C for 45 s, a final annealing temp (Table 4) for 45 s, then 72°C for 45 s, and finally 72°C for 5 min. Amplified promoters (inserts) and pMJG2 were then digested using the restriction enzymes *Kpn*I and *Sal*I (New England BioLabs, Inc., Ipswich, MA), cut ends of pMJG2 were dephosphorylated using rSAP (New England BioLabs, Inc.), and the insert and cut pMJG2 were ligated with T4 DNA Ligase (New England Biolabs, Inc.). The ligated vector was then transformed, using heat shock, into *E. coli* chemically competent cells. Plasmids were extracted using E.Z.N.A plasmid DNA Mini kit I (Omega bio-tek, Norcross, GA). Inserts were confirmed by sequencing with primers TB138 and TB139 (Table 4), and aligned using Geneious R6.1.8 software (Biomatters Inc., Newark, NJ), and then electroporated into respective background *L. monocytogenes* strains. GUS reporter fusions were stored in BHI broth + 10 µg/mL chloramphenicol at -80°C with 20% glycerol.

Preparation of reporter fusion cultures

Prior to each experiment, reporter fusion strains were streaked onto BHI + 10 µg/mL chl agar and incubated at 37°C for 24 h. A single colony was used to inoculate 5 mL BHI + 10 µg/mL chl, followed by incubation at 37°C for 24 h with shaking (230 rpm). A 1 mL aliquot of the overnight culture was then used to inoculate a second 50 mL overnight culture of BHI broth followed by incubation at 37°C for 16 h with shaking (230 rpm). A 10 mL aliquot of the second

overnight culture was then used to inoculate 190 mL of BHI broth without chl followed by incubation at 37°C for 4 h with shaking (230 rpm). Cells were pelleted by centrifugation at 13,000 x g for 5 minutes at room temperature. Pellets were washed with 20 mL of 1.5% glucose-defined minimal media (GDMM), modified from Schneebeli et al., 2013 (128), pelleted again, and suspended in 200 mL GDMM.

Preparation of test conditions

Sodium chloride (NaCl), 9% was prepared in GDMM and then filter-sterilized using a 0.22 µm vacuum filter. Chlorine wash (XY-12 liquid sanitizer, EcoLab, St. Paul, MN; 8.4% sodium hypochlorite), with 2 ppm free chlorine or 10 ppm free chlorine was also prepared in GDMM. Lettuce, for 4% and 40% leaf lysate, was purchased from a local supermarket and used within 24 h. Lettuce was juiced using a standard countertop juicer. Juice extract was then centrifuged at 13,000 x g for 30 min. Supernatant was then filtered through a 0.22 µm vacuum filter for sterilization, aliquoted into 2 mL sterile tubes, and stored at -80°C until needed. Cellulose at 2% wt/vol was added to GDMM (acetylcellulose, Sigma-Aldrich, St. Louis, MO).

Cell harvest and GUS assay

GUS reporter fusions were grown as stated above. A 1 mL aliquot was taken from both the initial 18 h BHI and GDMM culture and centrifuged at 10,000 x g for 3 min. Cells were washed with ABlight (0.06 M K2HPO4 + 0.04 M KH2PO4 + 0.1 M NaCl + 1 L ddH2O, pH 7), pelleted again, then flash-frozen using liquid nitrogen. The remaining GDMM culture was then combined 1:1 with test conditions for a total volume of 40 mL and final concentrations of: 4.5% NaCl, 1 ppm or 5 ppm free Chlorine, 2% or 20% leaf lysate, GDMM, and phosphate buffered saline (PBS). Environmental conditions represented osmotic stress, oxidation, organic matter/nutrient source, control, and nutrient limitation, respectively. Exposure was done at two

different temperatures, 25°C and 4°C, and for 120 minutes (150 minutes for 4°C) for each condition. A 1 mL aliquot was taken at 15, 30, 60, 90, 120, and 150 (4°C) minutes for each condition, centrifuged at 10,000 x g for 3 min, and cells were then washed with ABlight, pelleted again, then flash-frozen with liquid nitrogen. Cells were stored at -80°C until needed. Bacterial numbers were determined by spread-plating sample aliquots, for each condition at each time point, on BHI agar plates. Plates were incubated at 37°C for 24 h and CFU/ml was obtained by counting colonies using Color Qcount (Spiral Biotech, Inc., England, UK). GUS measurements were performed as described by Ollinger, 2009, with few modifications (129). Prior to GUS measurements, cell pellets were thawed and suspended in 1 mL ABlight. Cells were lysed by the addition of 135 µL CellLytic B reagent (Sigma-Aldrich), followed by incubation for 10 min at room temperature. Duplicate samples of bacterial lysates (80 µL) and appropriate dilutions (in ABlight) were pipetted into 96-well flat-bottomed black polystyrene plates (Greiner Bio-One, Monroe, NC). The enzymatic reaction was initiated by addition of 20 µL of 0.4 mg/mL 4-methylumbelliferyl-β-D-glucuronide (MUG: Sigma-Aldrich) in dimethyl sulfoxide. A standard curve corresponding to 3.75, 1.88, 0.94, 0.47, 0.23, 0.12, 0.06, and 0.03 µM 4-methylumbelliferone (MU; Sigma-Aldrich) was included with every plate. The enzymatic reaction was stopped after 30 min by the addition of 50 µL of 1 M Na_2CO_3 stop solution. Immediately after the addition of stop solution, fluorescence was measured at 460 nm (with an excitation wavelength of 365 nm), using a Synergy H1 hybrid reader (BioTek, Winooski, VT). The amount of background fluorescence determined for a given sample was subtracted from the fluorescence measurement in the corresponding experimental well, and the concentration of liberated MU was calculated using the standard curve. The GUS activity for each strain was

Table 4. Primers and annealing temperatures used in this study

Gene	Forward primer‡	Reverse primer‡	Strain/ Purpose	Annealing Temperature	Reference
lcp	ATT ATA TAA ACA CAG AAA TCA CT	TCT AGT TGC TTT AAT CAT AAA	H7858	61-51°C*	This study
uspA	CAT TGC CAC CCT ATT CTC GT	TCT TTG GAT CCA TCA ACT GCT	H7858	60-50°C*	This study
TB138/ 139	AAT TGC CCG GCT TTC TTG TAA C	TGC ATG TGT CAG AGG TTT TCA	check presence/absence of insert in pMJG2	55°C	
TB140/ 141	ACA TAA TCA GTC CAA AGT AGA TGC	GTC AAA ACA TAC GCT CTT ATC	confirm vector integration into chromosome	51°C	
TB140/ 142	ACA TAA TCA GTC CAA AGT AGA TGC	GAA TAA GGG ACA GTG AAG AAG G	determine if vector has integrated multiple times	51°C	

‡ all sequences are shown 5' → 3'

* Touchdown PCR reaction

measured for three independent biological replicates and reported as (μM MU/30 min)/(log CFU/mL).

Attachment assays

Wt H7858 was grown up as before except for the absence of chloramphenicol. Test conditions were also prepared as stated above with the addition of PEA at an initial concentration of 20 mg/mL and a final concentration of 10 mg/mL. All attachment assay test conditions were done at 25°C. Stainless steel coupons were sterilized by placing a 2 cm x 2 cm square coupon in a 50 mL centrifuge tube filled with acetone until the coupon was submerged. Coupons were vortexed for 10 min then rinsed with distilled water. Process was repeated with 2% aqueous solution of commercial detergent. Coupons were rinsed until distilled water ran clear and then rinsed 3 times with RO-water and autoclaved at 15 psi, 121°C, for 20 min.

All attachment assays were done in six-well plates on a belly-dancer set to 6. To each well of a sterile six-cell culture plate the following was added: 0.8 mL bacterial suspension, 3.2 mL of GDMM, and 4 mL of the test-condition-modified GDMM for a 1:1 dilution. Stainless steel coupons were aseptically submerged into the bacterial suspensions that have been modified by the test conditions and incubated at 25°C for 30, 60, 90, and 120 min. As each time point was reached, 100 μL of the test solution was removed and placed into 900 μL PBS, serially diluted to 10^{-6} and plated in duplicate on BHIA to measure unattached bacterial growth. Inoculated stainless steel coupons were removed from suspension and dipped-rinsed for 2-3 s to remove residual cells carried over from the inoculum. Each stainless steel coupon was then transferred into a sterile 50 mL centrifuge tube containing 10 mL sterile PBS with 0.1% Tween 20 and vortexed for 20 s to remove loosely attached cells. The same stainless steel coupon was removed from Tween 20 wash and dip rinsed for 2-3 s to remove residual cells carried over from

inoculum. Again, stainless steel coupon was transferred to a new sterile 50 mL centrifuge tube with 10 mL PBS and 6-10 sterile glass beads. To recover strongly attached bacteria, each tube was vortexed for 120 s. For each solution, Tween 20 and PBS with beads, 100 µL was added to 900 µL PBS and serially diluted to 10^{-4} for loosely attached bacteria and 10^{-1} for strongly attached bacteria, and 0.1 mL was plated in duplicate.

Statistical analysis

To determine the statistical significance of differences in GUS activity a two-way ANOVA was used to determine if the GUS activity was significantly affected by the presence or absence of each condition, using the following model:

GUS activity = Condition + Time + (Condition x Time) + rep (biological replicate).

Tukey's multiple comparison correction was applied to all ANOVA results to determine significant differences among the strains. Adjusted *P* values of < 0.05 were considered statistically significant.

To determine the statistical significance of differences in attachment, cell counts by enumeration of CFU/mL were measured. For the 120 min time point, the test condition CFU/mL measurement was subtracted from the GDMM CFU/mL measurement and a Student's T-test was performed using Excel.

Results

GUS expression assays

GUS expression driven by P_{lcp}, P_{uspA}, and $P_{uspA::\Delta sigB}$ was tested across eight conditions (4.5% NaCl, 1 ppm chlorine, 5 ppm chlorine, 2% lettuce lysate, 20% lettuce lysate, 1% cellulose, PBS, and GDMM). GUS expression was measured as (µM MU/30Min)/(LogCFU/mL) across

time exposed to test conditions. P_{uspA} was used as a control and *uspA* encodes a SigB regulated general stress response protein.

The expression of GUS driven by P_{uspA} upon exposure to 5 ppm chlorine at 4°C over 150 min significantly decreased expression from 0.3384 ± 0.0926 (µM MU/30Min)/(LogCFU/mL) at time zero to 0.2380 ± 0.0486 (µM MU/30Min)/(LogCFU/mL) (P = 0.0045) (fig. 9 a). At 25°C there were several significant changes to GUS expression. For GDMM, expression increased significantly from 0.1685 ± 0.0201 (µM MU/30Min)/(LogCFU/mL) at time zero to 0.2262 ±0.0319 (µM MU/30Min)/(LogCFU/mL) (P = 0.0003) (fig. 8 d) in just 15 min, with an overall significant increase to 0.2834 ± 0.0261 (µM MU/30Min)/(LogCFU/mL) (P = <0.0001) after 120 min. The increase in expression was also significant from time point 0 to time points 30, 60, and 90 min with all P-values <0.0001. Similar significant increases were seen for 2% lettuce lysate, reaching 0.2582 ± 0.0298 (µM MU/30Min)/(LogCFU/mL) (P = 0.0001) after 120 min with an initial increase to 0.2312 ± 0.0346 (µM MU/30Min)/(LogCFU/mL) (P = 0.0083) after 15 min (fig. 8 c). Again, each time point from time 0 were also significant with P-values of 0.0002, 0.0003, and 0.0023 for time points 30, 60, and 90, respectively. The same pattern of significance was seen for 1 ppm chlorine with a final expression level of 0.2155 ± 0.0313 (µM MU/30Min)/(LogCFU/mL) (P = <0.0001) after 120 min from the initial expression of 0.1685 ± 0.0201(µM MU/30Min)/(LogCFU/mL) (fig. 8 b). Finally, for 20% lettuce lysate at 25°C, there was a significant increase at 30 min and again at 120 min. The initial expression being 0.3384 ± 0.0926 (µM MU/30Min)/(LogCFU/mL) increasing to 0.4021 ± 0.0418 (µM MU/30Min)/(LogCFU/mL) (P = 0.0369) and 0.4122 ± 0.0461 (µM MU/30Min)/(LogCFU/mL) (P = 0.0110) for 30 and 120 min, respectively (fig. 9 b).

Although the expression of GUS driven by $P_{uspA::\Delta sigB}$ had significant changes, these changes were much lower compared to GUS levels in the normal *sigB* background. GUS was upregulated significantly at 4°C and 25°C for all of the following test conditions: 1 ppm chlorine, 2% lettuce lysate, GDMM, and PBS. With an initial expression level of 0.0022 ± 0.0002 (µM MU/30Min)/(LogCFU/mL), increases varied only slightly with the largest expression level being for both GDMM 0.0037 ± 0.0002 (µM MU/30Min)/(LogCFU/mL) (P = <0.0001) (fig. 10 d) and 2% lettuce lysate 0.0037 ± 0.0005 (µM MU/30Min)/(LogCFU/mL) (P = 0.0009) (fig. 10 c) at 25°C over 120 min and the smallest expression level being for both GDMM 0.0031 ± 0.0002 (µM MU/30Min)/(LogCFU/mL) (P = <0.0001) (fig. 10 d) and 2% lettuce lysate 0.0031 ± 0.0013 (P = 0.0466) (fig. 10 c) at 4°C over 150 min. For each test condition, all time points were significantly different from time zero. GUS expression driven by $P_{uspA::\Delta sigB}$ was also significant for all time points under 4.5% NaCl and 20% lettuce lysate at 25°C, but not 4°C, with a total increase of 0.0012 (µM MU/30Min)/(LogCFU/mL) (P = <0.0001) (fig. 10 a) and 0.0015 (µM MU/30Min)/(LogCFU/mL) (P = 0.0009) (fig. 11 b), over 120 min respectively. Expression was significant in the prescence of 1% cellulose from time point 0 to 120 min only at 25°C, where expression increased from 0.0081 ± 0.0029 (µM MU/30Min)/(LogCFU/mL) at time zero to 0.0109 ± 0.0038 (µM MU/30Min)/(LogCFU/mL) (P = 0.0056) (fig. 11 c) for the largest overall expression seen in this study. GUS expression was downregulated for one test condition, 5 ppm chlorine at 4°C, expression was again significant for all time points compared to time point 0 which was measured at 0.0081 ± 0.0029 (µM MU/30Min)/(LogCFU/mL). After 150 min expression was 0.0066 ± 0.0027 (µM MU/30Min)/(LogCFU/mL) (P = 0.0002) (fig. 11 a). Taken together these data show that the reporter fusions are effective in measuring regulation differences.

The GUS expression driven by P_{lcp} under 4.5% NaCl stress at 4°C had a significant increase from 0.0042 ± 0.0004 (µM MU/30Min)/(LogCFU/mL) at time zero to 0.0046 ± 0.0004 (µM MU/30Min)/(LogCFU/mL) (P = 0.0444) and 0.0045 ± 0.0005 (µM MU/30Min)/(LogCFU/mL) (P = 0.0299) at 120 min and 150 min, respectively (fig. 6 a). Howerver, this increase is unlikely to be biologically significant. Increases were also seen at 25°C for 2% lettuce lysate, 20% lettuce lysate, and GDMM, but the differences were not significant with P-values of 0.0872, 0.0720, and 0.0817, respectively.

Attachment assays

Attachment assays were conducted on stainless steel, incubated at 25°C for 120 min under the following conditions: GDMM (control), 5 ppm chlorine, PBS, 10 mg/mL PEA, 4.5% NaCl, and 2% lettuce lysate. After 120 min aliquots of loosely attached and strongly attached cells were collected, serially diluted to 10^{-4} and 10^{-1}, respectively, and enumerated by cell counts on BHI. The differences in attachment to stainless steel in GDMM were then compared to the attachment on stainless steel in each test condition and measured in logCFU/cm^2. At 5 ppm a significant reduction in both loosely attached and strongly attached cells to stainless steel compared to the control by 0.7 ± 0.55 logCFU/cm^2 (P = 0.0206) and 0.55 ± 0.44 logCFU/cm^2 (P = 0.0223), respectively. Of interest, 2% lettuce lysate also reduced both loosely and strongly attached cells to stainless steel, compared to the control, by 0.49 ± 0.30 logCFU/cm^2 (P = 0.0241) and 1.02 ± 0.48 logCFU/cm^2 (P = 0.0109).

Discussion

GUS expression assays

GUS reporter fusions have been described as a tool that has been used effectively in *L. monocytogenes* previously. For example, a GUS reporter fusion was utilized to determine

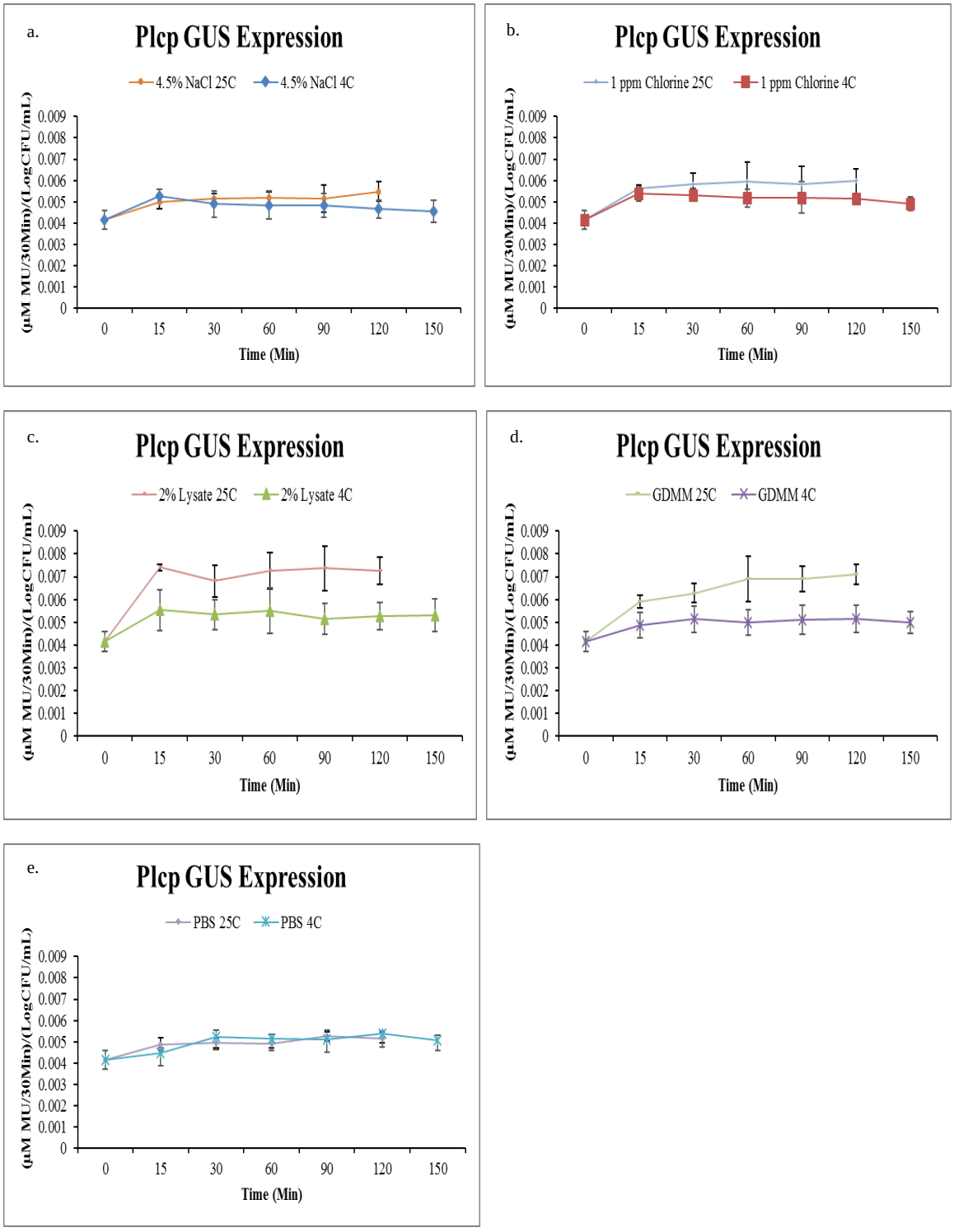

Fig. 6. Effect of test conditions 4.5% NaCl (a), 1 ppm chlorine (b), 2% lysate (c), GDMM (d), and PBS (e); and temperatures 4°C and 25°C on P_{lcp} GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across two replicate experiments.

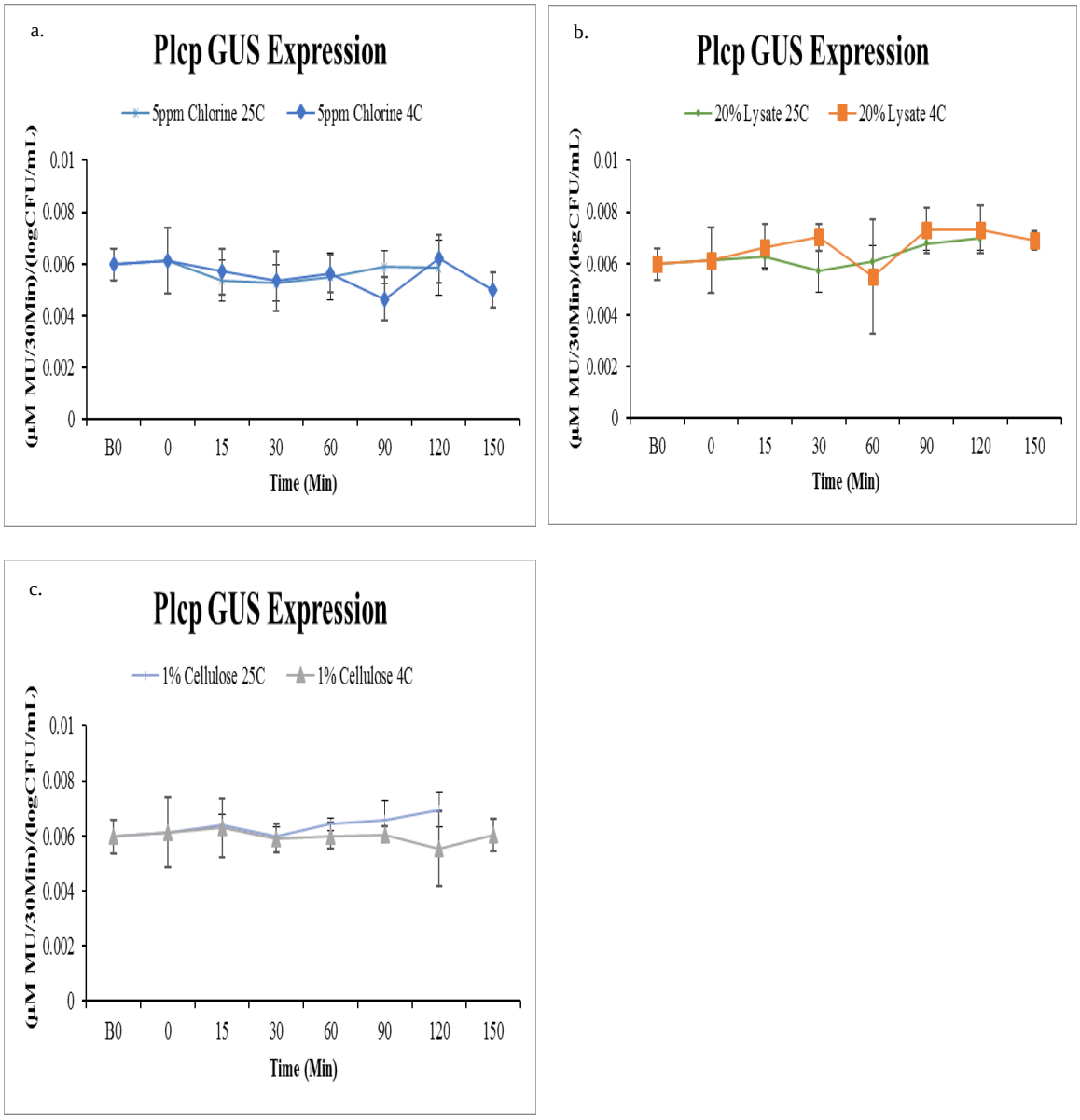

Fig. 7. Effect of test conditions 5 ppm (a), 20% lysate (b), and 1% cellulose (c); and temperatures 4°C and 25°C on P_{lcp} GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across three replicate experiments.

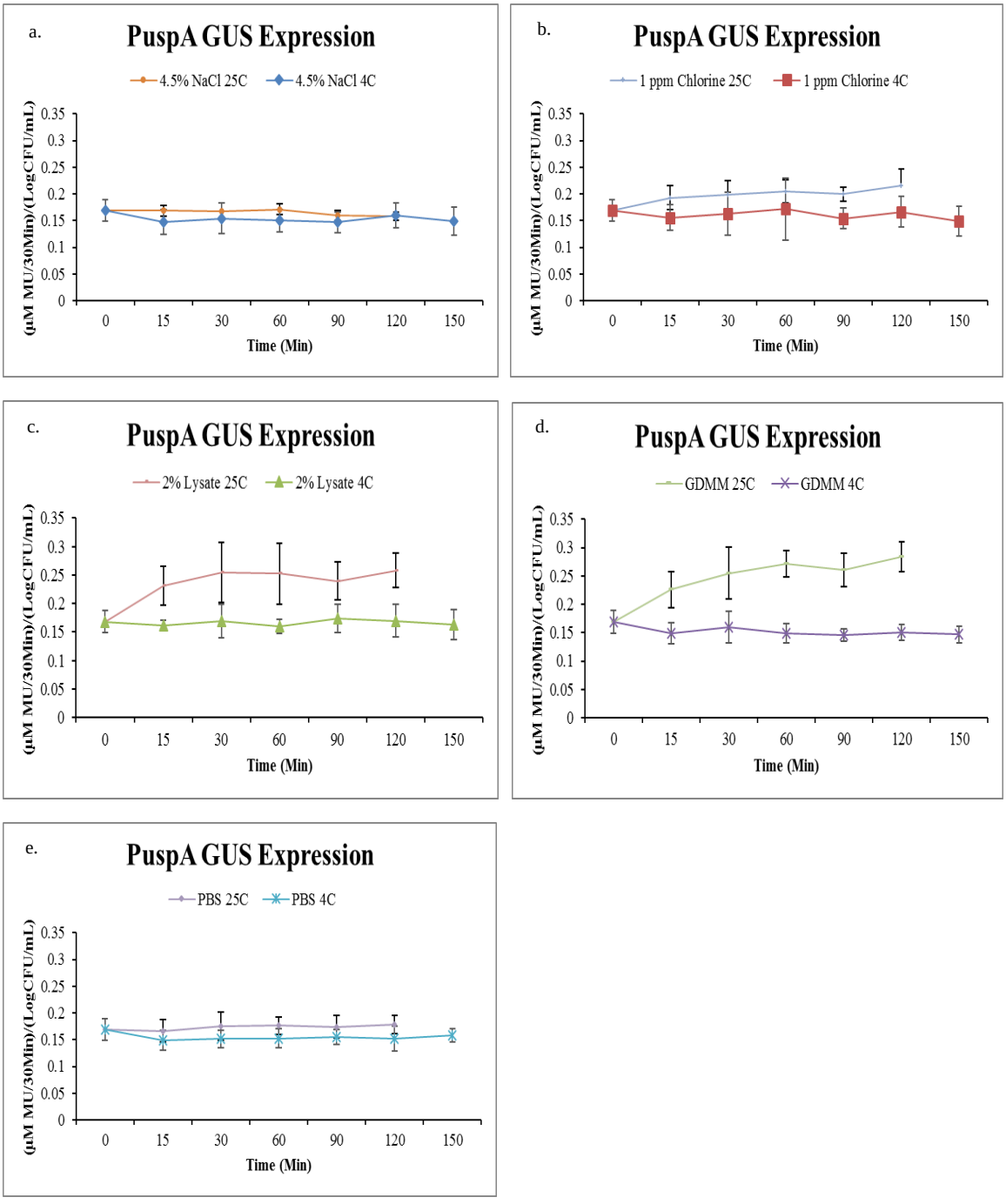

Fig. 8. Effect of test conditions 4.5% NaCl (a), 1 ppm chlorine (b), 2% lysate (c), GDMM (d), and PBS (e); and temperatures 4°C and 25°C on P_{uspA} GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across three replicate experiments.

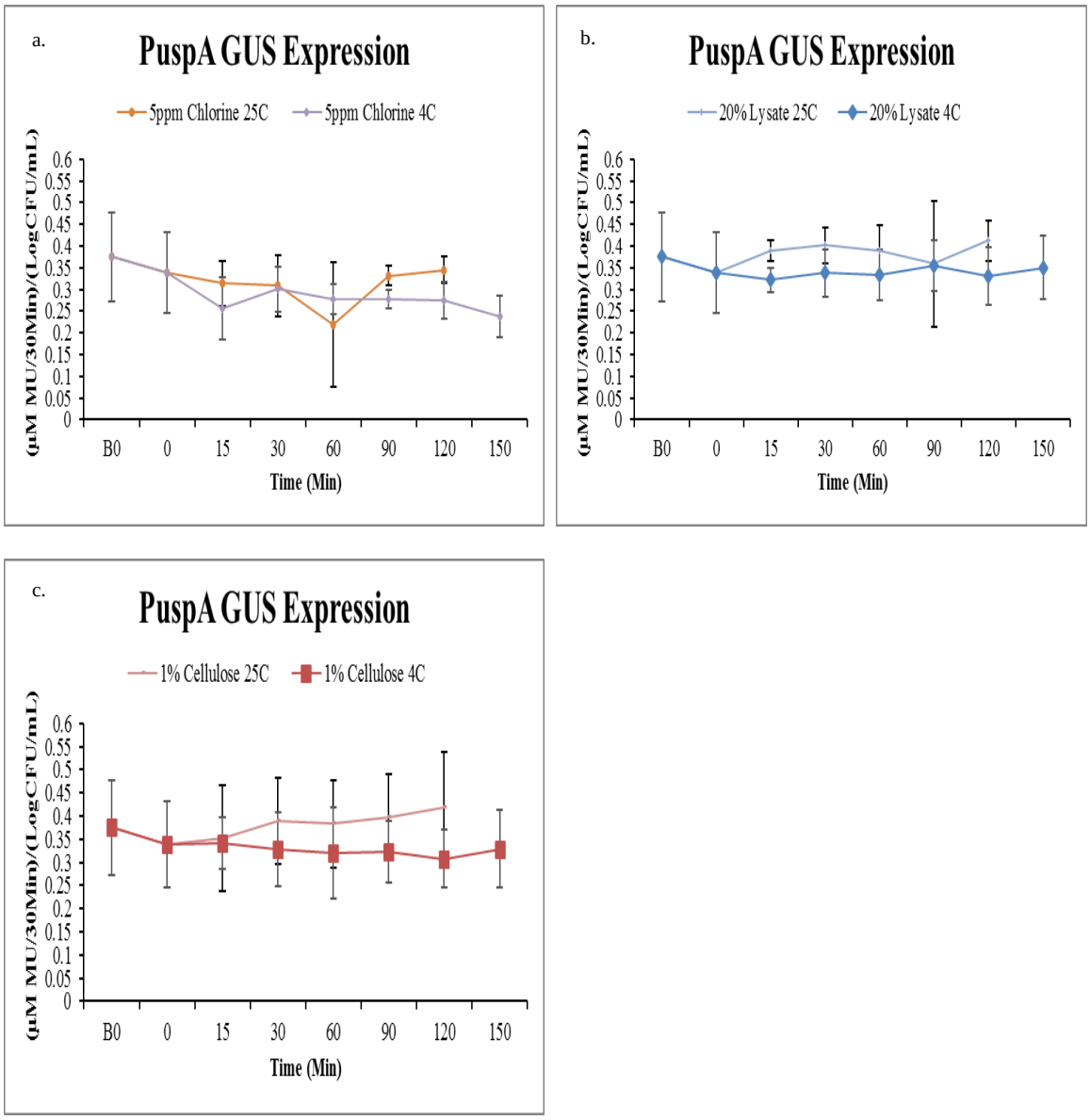

Fig. 9. Effect of test conditions 5 ppm (a), 20% lysate (b), and 1% cellulose (c); and temperatures 4°C and 25°C on P_{uspA} GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across three replicate experiments.

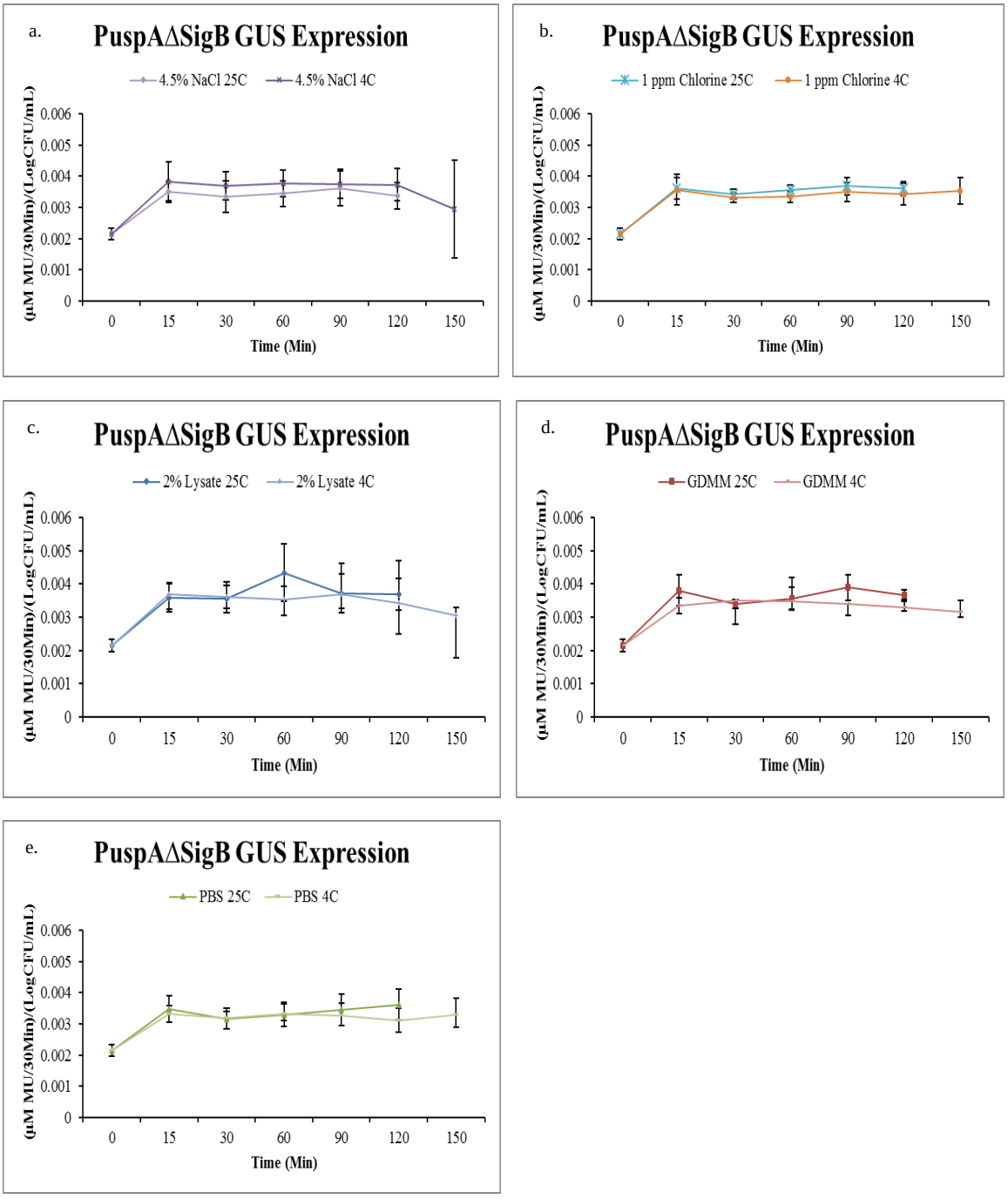

Fig. 10. Effect of test conditions 4.5% NaCl (a), 1 ppm chlorine (b), 2% lysate (c), GDMM (d), and PBS (e); and temperatures 4°C and 25°C on $P_{uspA::\Delta sigB}$ GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across two replicate experiments.

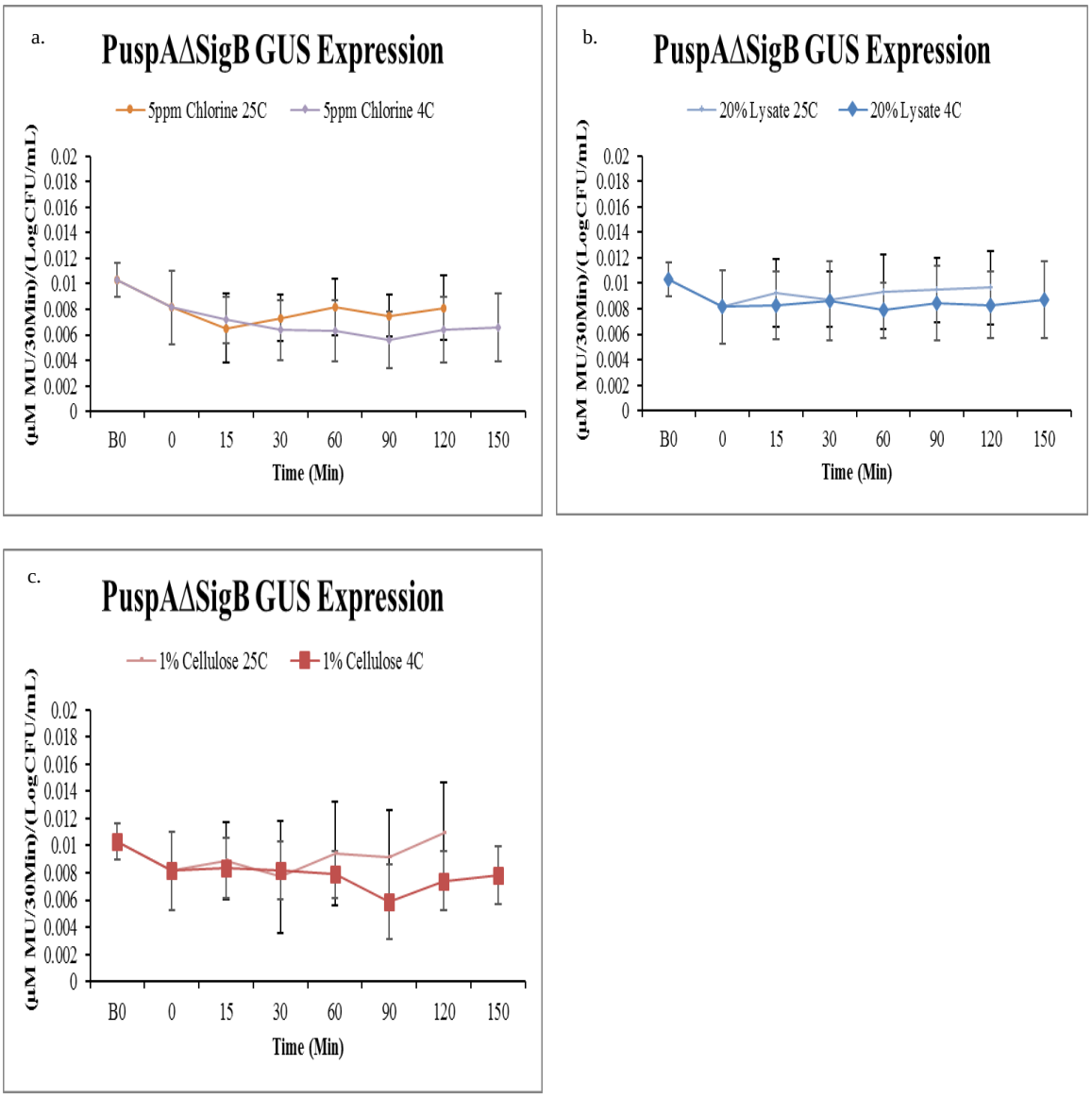

Fig. 11. Effect of test conditions 5 ppm (a), 20% lysate (b), and 1% cellulose (c); and temperatures 4°C and 25°C on $P_{uspA::\Delta sigB}$ GUS expression. Average and standard deviation of (µM MU/30Min)/LogCFU/mL) were calculated across three replicate experiments.

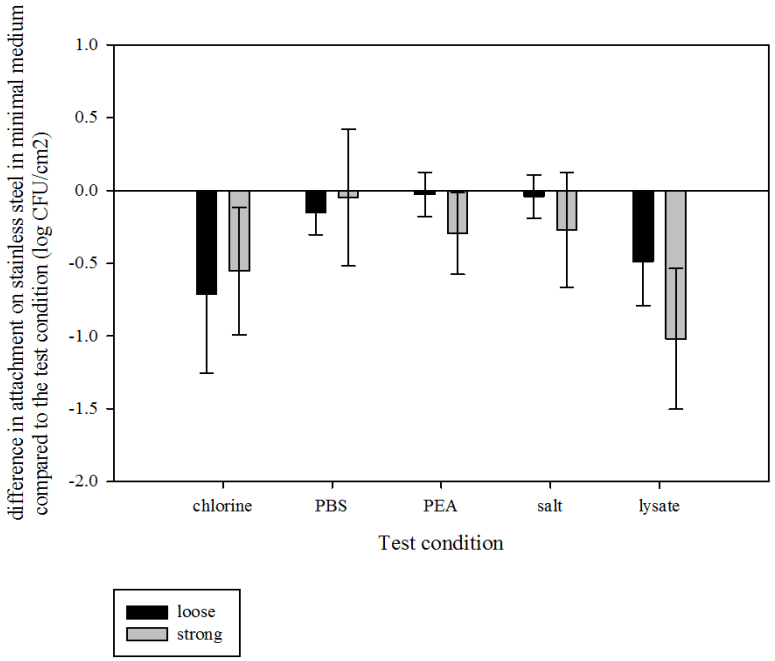

Fig. 12. Attachment of *L. monocytogenes* H7858 to stainless steel coupons. Differences in loosely and strongly attached cells in the presence of test conditions compared to the control. Average of three replicates with error bars representing standard deviation.

whether resistance to Nisin is the consequence of transcriptional regulation of VirR-regulated genes, such as *dltABCD*. This operon has previously been reported to be dependent on VirR. This dependence was confirmed by the lack of GUS expression by the *dltABCD* GUS fusion in the absence of VirR and VirS. It was also reported that Nisin does not specifically induce VirR-mediated upregulation of *dltABCD* (125).

The *lcp* promoter was chosen for the GUS expression assays for two reasons: the cellulose binding domain that has been shown to be important for attachment to fresh produce, and because there has only been one other study looking at Lcp as a protein of interest (1). UspA is a general stress protein that is known to be regulated by SigB. I included the promoter of *uspA* in both the parent strain and in a Δ*sigB* mutant of the same parent strain as controls.

P_{uspA} driven GUS expression was significantly down-regulated in the presence of 5 ppm chlorine at 4°C, but significantly upregulated under several of the test conditions at 25°C. These test conditions included GDMM, 2% lettuce lysate, 20% lettuce lysate, and 1 ppm chlorine. $P_{uspA::\Delta sigB}$ GUS expression was significantly upregulated by nearly all test conditions, but levels of expression were much lower than those of in the normal *sigB* background. There were no significant changes for 4.5% NaCl, 20% lettuce lysate, or 1% cellulose at 4°C. For 5 ppm chlorine there was no significant change at 25°C but, significant downregulation was observed at 4°C. Together these data show the GUS fusions used do in fact work for the purpose of this experiment.

I expected that Plcp, because of the cellulose binding domain, would be upregulated by the two lettuce lysate concentrations and cellulose. Although GUS expression was upregulated by 2% lettuce lysate and 1% cellulose after 120 min at 25°C, along with GDMM, none of the resulting data was significant at the P = 0.05 level.

Attachment assays

Of interest, 2% lettuce lysate significantly reduced attachment to stainless steel compared to the GDMM control, loosely attached by nearly 0.5 logCFU/cm^2 and strongly attached by the greatest reduction observed, nearly 1.1 logCFU/cm^2. I expected that, as a nutrient source, it would have been a signal to attach. One explaination for these unexpected results could be that the bulk liquid contained the majority of the lysate so there is no need to become sessile and attach because they are not stressed. Had we increased the observation time past 120 min and allowed the lettuce lysate to become more of a conditioning layer on the stainless steel we may have seen increased attachment.

The chlorine concentration used (5 ppm) is considered a subminimal inhibitory concentration (124). Significant reduction in attachment to stainless steel compared to the control was observed. Reduction of loosely attached cells reached 0.7 logCFU/cm^2 and strongly attached cells reached 0.55 logCFU/cm^2. This reduction, although significant, shows that a concentration of 5 ppm chlorine is not a high enough concentration to completely eliminate *L. monocytogenes* attachment to stainless steel. Also of importance, the cells were not being killed by 5 ppm chlorine over the 120 min. This is a problem because cells that are not inactivated and allowed to attach could develop resistance to chlorine and potentially form a mature biofilm that would be extremely difficult to erradicate. This can happen because wash water in the processing plant is recycled, and if chlorine levels are not monitored, the prescence of organic matter can reduce the concentration of free chlorine to these subminimal inhibitory concentrations. The resulting contamination could become dispersed throughout the product, leading to potential foodborne illness and outbreaks.

Conclusions

This study showed that the single-copy reporter fusions constructed for observation of *lcp* and *uspA* do work effectively,thus supporting the conclusion that *lcp* expression was not induced under the selected test conditions. Attachment, however, was influenced by both 5 ppm chlorine and 2% lettuce lysate. With the lack of induction of *lcp* expression under the same conditions it is unlikely that observed differences in attachment are due to changes in *lcp* expression. Other attachment factors are most likely to be involved. Further reseach is needed to discover conditions that do induce the expression of the attachment factor *lcp,* and to identify the factors that are involved in attachment of *L. monocytogenes* exposed to chlorine or lettuce lysate

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CONCLUSIONS

Small molecules, such as AAA and PEA, could be promising alternatives to conventional chemical disinfectants with more research. AAA, individually, was able to reduce biofilm amounts and *flhD* transcription significantly by nearly 2 fold. Four other carbon sources had similar effects: α-ketobutyric acid, D-xylose, glucuronamide, and L-proline. PEA, individually, was able to reduce growth by more than 95% and biofilm by more than 70%, although reductions were microorganism, strain, and temperature dependent. One advantage over common disinfectants, such as sodium hypochlorite, that small molecules could offer is a lower chance of developing resistance as they often are nutrients at lower concentrations.

For decades curli production has been known to be important for biofilm formation. *csgA*, which encodes the major subunit of curli, continues to be a target for biofilm inhibition. Congo red agar is a common method to test the presence of curli due to its afinity to Congo red. Upon screening EHEC and *Salmonella* for curli production at temperatures of 10°C, 25°C and 37°C, I found that production of curli is not dependent on temperature itself, but rather microorganism, strain, and/ or serotype.

Single-copy chromosomal reporter fusions are an excellent tool to measure promoter activity. This is supported by the successful construction of three GUS reporter fusions: P_{lcp}, P_{uspA}, and $P_{uspA::\Delta sigB}$. The two controls confirm that they work by the expression of $P_{uspA::\Delta sigB}$ being much lower than P_{uspA}. This was expected as *uspA* is a SigB regulated universal stress response protein. It can also be concluded that *lcp* expression was not induced by any of the selected test conditions. Attachment was influenced by different conditions, but it is unlikely that these differences in attachment are due to changes in *lcp* expression.

FUTURE STUDIES

We found several small molcules that individually reduce biofilm amounts and *flhD* transcription for *E. coli* AJW678. Further research could be done to see if effects are reproducable on other pathogens. Experiments could also be done in order to see if there are any synergistic effects by combining the small molecules, such as AAA and PEA.

There are many more attachment factors for *L. monocytogenes*, such and LapB and BapL, a cell wall protein known for attachment to epithelial cells and a biofilm associated protein, respectively (82, 130), that single-copy chromosomal fusions could be constructed for. In this study we used only one background strain so further construction of these attachment factor reporter fusions could be constructed in outbreak or other strains of interest. Similar constructs could also be created for EHEC and *Salmonella* using *lacZ* as a reporter protein and red recombinase technology.

Attachment assays in this study were conducted on only one abiotic surface, stainless steel. F urther research on other abiotic surfaces such as plastic and rubber would be beneficial as these surfaces are also found in the processing environment. Attachment assays should also be conducted on biotic surfaces such as lettuce or spinach. Overall, these additional studies would further our understanding of the proteins involved in attachment and if select conditions create signals that allow pathogens to switch from reversible attachment to irreversible attachment.

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APPENDIX

Table A1. Strain, temperature, and result of Congo red screen. Strains were classified as strongly positive (++), slightly positive (+), or negative (-).

Strain		Temp.	Result (++, +, -)
TB276	*Salmonella* Enteriditis FSL F6-963	25	(++)
TB277	*Salmonella* Newport FSL R6-186	25	(++)
TB278	*Salmonella* Tennessee FSL R6-198	25	(++)
TB279	*Salmonella* Newport FSL R6-204	25	(++)
TB280	*Salmonella* Newport FSL R6-241	25	(++)
TB281	*Salmonella* Newport FSL R6-388	25	(++)
TB282	*Salmonella* Newport FSL R6-776	25	(++)
TB283	*Salmonella* Newport FSL R6-777	25	(++)
TB284	*Salmonella* Enteritidis FSL R8-288	25	(++)
TB285	*Salmonella* Typhimurium FSL R8-784	25	(++)
TB286	*Salmonella* Newport FSL R8-802	25	(++)
TB287	*Salmonella* Newport FSL R8-830	25	(++)
TB288	*Salmonella* Typhimurium FSL R8-865	25	(++)
TB289	*Salmonella* Newport FSL R8-1598	25	(++)
TB290	*Salmonella* Newport FSL R8-1637	25	(++)
TB291	*Salmonella* Tennessee FSL R8-2240	25	(++)
TB292	*Salmonella* Newport FSL R8-2690	25	(++)
TB293	*Salmonella* Montevideo FSL R8-2812	25	(++)
TB294	*Salmonella* Stanley FSL R8-2954	25	(++)
TB295	*Salmonella* Stanley FSL R8-2955	25	(++)
TB296	*Salmonella* Stanley FSL R8-2966	25	(++)
TB297	*Salmonella* Montevideo FSL R8-3417	25	(++)
TB298	*Salmonella* Stanley FSL R8-3511	25	(++)
TB299	*Salmonella* Saintpaul FSL R8-3582	25	(++)
TB300	*Salmonella* Montevideo FSL R8-3658	25	(++)
TB301	Salmonella Montevideo FSL R8-3659	25	(++)
TB302	*Salmonella* Newport FSL R8-3994	25	(++)
TB303	*Salmonella* Saintpaul FSL R8-4484	25	(++)
TB304	*Salmonella* Stanley FSL R8-4894	25	(+)
TB305	*Salmonella* Montevideo FSL R8-4923	25	(++)
TB306	*Salmonella* Newport FSL R8-5020	25	(++)
TB307	*Salmonella* Saintpaul FSL R8-5023	25	(++)
TB308	*Salmonella* Saintpaul FSL R8-5029	25	(++)
TB309	*Salmonella* Saintpaul FSL R8-5077	25	(++)
TB310	*Salmonella* Typhimurium FSL R8-5469	25	(++)
TB311	*Salmonella* Typhimurium var.O:5- FSL R8-7281	25	(++)
TB312	*Salmonella* Typhimurium var.O:5- FSL R8-7950	25	(-)
TB313	*Salmonella* Agona FSL R8-8615	25	(++)

Table A1. Strain, temperature, and result of Congo red screen (continued). Strains were classified as strongly positive (++), slightly positive (+), or negative (-).

Strain		Temp.	Result (++, +, -)
TB314	*Salmonella* Agona FSL R8-8619	25	(++)
TB315	*Salmonella* Newport FSL R8-9630	25	(++)
TB316	*Salmonella* Typhimurium FSL R9-0042	25	(++)
TB317	*Salmonella* Stanleyville FSL R9-145	25	(++)
TB318	*Salmonella* Typhimurium FSL R9-148	25	(++)
TB319	*Salmonella* Typhimurium FSL R9-436	25	(++)
TB320	*Salmonella* Typhimurium FSL R9-441	25	(++)
TB321	*Salmonella* Typhimurium O:5- FSL R9-460	25	(++)
TB322	*Salmonella* Typhimurium FSL R9-532	25	(++)
TB323	*Salmonella* Enteritidis FSL R9-566	25	(++)
TB324	*Salmonella* Montevideo FSL R9-1588	25	(++)
TB325	*Salmonella* Saintpaul FSL R9-1724	25	(++)
TB326	*Salmonella* Tennessee FSL R9-2434	25	(++)
TB327	*Salmonella* Tennessee FSL R9-2435	25	(++)
TB328	*Salmonella* Tennessee FSL R9-2436	25	(++)
TB329	*Salmonella* Typhimurium FSL S5-384	25	(+)
TB330	*Salmonella* Saintpaul FSL S5-649	25	(++)
TB331	*Salmonella* Newport FSL S10-985	25	(++)
TB332	*Salmonella* Typhimurium FSL S10-1134	25	(++)
TB333	*Salmonella* Typhimurium FSL S10-1269	25	(++)
TB334	*Salmonella* Enteritidis FSL S10-1621	25	(++)
TB335	*Salmonella* Enteritidis FSL S10-1623	25	(++)
TB336	*Salmonella* Enteritidis FSL S10-1644	25	(++)
TB337	*Salmonella* Enteritidis FSL S10-1646	25	(++)
TB338	*Salmonella* Newport FSL S10-1743	25	(+)
TB339	*Salmonella* Agona FSL S10-1750	25	(+)
TB340	*Salmonella* Newport FSL S10-1755	25	(++)
TB341	*Salmonella* Tennessee FSL S10-1757	25	(+)
TB342	*Salmonella* Agona FSL S10-1759	25	(+)
TB343	*Salmonella* Agona FSL S10-1760	25	(+)
TB344	*Salmonella* Agona FSL S10-1761	25	(+)
TB345	*Salmonella* Typhimurium FSL S10-1766	25	(++)