THE EFFECT OF ENVIRONMENTAL CONDITIONS ON *SALMONELLA* FIMBRIAE AND BIOFILM FORMATION

by

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ABSTRACT

Salmonella typhimurium is a Gram-negative enteric pathogen that may cause salmonellosis in individuals that consume contaminated food. Triclosan, a broad-spectrum bisphenol, is an antimicrobial agent commonly added to a broad range of products including hand soaps, cleaners and surfaces. Overuse of such products can result in the development of resistant strains able to withstand higher concentrations of the agent than the original parental (Wild Type) strain. We developed two strains of *Salmonella* with reduced susceptibly (SRS) that had triclosan minimum inhibitory concentrations (MIC) of approximately 400 ppm compared to the parental MIC of 1 ppm, indicating increased resistance.

Biofilms produced by bacteria are a public health concern. While in a biofilm, microbes are protected from harmful agents, such as antibiotics and antimicrobials. Abiotic and biotic factors such as temperature and presence of cell structures influence biofilm formation. We used two growth conditions in our study; condition one, 37ºC on Luria Bertani (LB) agar and condition two, 28ºC on LB agar without NaCl. Parental and SRS growth, flagella, motility, fimbriae and biofilm formation were studied under these two conditions.

Cellular structures such as fimbriae and flagella initiate biofilm formation. *S. typhimurium* produces a variety of fimbriae. The type we examined in our study was curli fimbriae. Using transmission electron microscopy and growth on Congo Red agar we observed the lack of curli fimbriae under both growth conditions. Quantitative real time PCR analysis of *fimA* (type 1 fimbriae) and *csgG* (curli fimbriae) genes

indicated that growth conditions used did not have an effect on the presence or absence of fimbriae. All strains possessed flagella. Unlike the parental and SRS strains tested, one SRS strain was more motile at the lower temperature. Motility of all strains was affected by temperature.

A crystal violet assay was used to quantify the amount of biofilm produced as a function of time. Fluorescence microscopy of acridine orange stained biofilms was also carried out. Both studies were done under the two growth conditions. Initially, biofilm formation for all strains was greater in condition one. By hour six, there were no differences between any of the strains under either condition. Seven-hour biofilms of all strains were challenged with 200 ppm triclosan, the concentration of residual triclosan commonly found on surfaces and 2000 ppm triclosan, the concentration found in antimicrobial products. After one hour of exposure, the biofilms were stained with the Live Dead Stain®. All cells in the parental biofilms exposed to both triclosan concentrations were red, indicating cell death. Approximately half of the cells in both SRS biofilms were green, indicating viability at 200 ppm, but all were red at the higher concentration. This indicates that biofilms of SRS strains were resistant to residual levels of triclosan that are found on surfaces. Higher concentrations of triclosan in commercial products are effective in killing microorganisms. However, residual levels left on surfaces may foster the growth of SRS strains. These resistant organisms may develop cross-resistance to other antimicrobials

Chapter 1

INTRODUCTION

1.1 *Salmonella*

The organism used in my studies was *Salmonella enterica* subsp. *enterica* serovar Typhimurium (American Type Culture Collection 14028). *Salmonella* is a non-spore forming Gram-negative rod that possesses both flagella and fimbriae [1]. It causes the diarrheal illness salmonellosis, which is a major public health concern [2-4]. Every year, over 40,000 cases of salmonellosis are reported in the United States and millions of cases are reported worldwide [5, 6]. The organism is found in the intestinal tract of birds and other animals. Salmonellosis is associated with the consumption of contaminated poultry products, which have been undercooked [1, 7]. Contaminated beef products, or even water may also serve as another source for the organism [8]. After ingestion, the incubation time is only eight to 48 hours. The bacteria invade the intestinal mucosa producing an enterotoxin and a cytotoxin that destroy the epithelial cells [9]. During the acute phase of the disease, as many as one billion *Salmonella* cells can be found per gram of feces [10].

1.2 Pathogenicity

In addition to the toxins that are produced, *Salmonella* have a variety of virulence associated surface structures: lipopolysaccharides, capsules, flagella, and fimbriae [11, 12]. Adhesion to the intestinal epithelium is considered to be the first step in pathogenesis preceding invasion. Fimbriae are known to mediate this process.

Salmonella can produce nine different fimbrial types and can often express more than one type of fimbrium at any given time [3, 13, 14]. The characterization of fimbrial structures, their genes, and their distribution among the salmonellae are of importance in understanding their pathogenesis [15]. Enteric bacteria express different morphotypes, which correspond to differences seen in the extracellular matrix. *S. typhimurium* produces an extracellular matrix that features curli fimbriae as the major proteinaceous component. Cellulose is a second component of this matrix. Curli fimbriae with cellulose form a honeycomb like structure that results in the formation of biofilms due to bacteria-bacteria interactions. This then allows for adhesion to surfaces and protection against the environment which is a virulence attribute [16]. Since *Salmonella* is capable of colonizing different hosts and occupying different niches within a host, the capacity to produce several environmentally regulated yet distinct fimbriae may contribute to *Salmonella's* success as a pathogen.

1.3 Flagella and Fimbriae

Salmonella flagella and fimbriae are two important structures involved in biofilm formation and attachment [17, 18]. Flagella are thin, rigid surface appendages that are about 20 nm across and 15 to 20 μm long. They are responsible for motility and chemotaxis [19]. The arrangement of flagella around a bacterial cell is used in identification. *Salmonella* possess peritrichous flagellation [10]. Motile cells are able to carry out chemotaxis. Adsorbed organics on surfaces can serve as chemoattractants for flagellated cells, resulting in initial contact between the bacterium and the surface [20].

Fimbriae are fine, hair like protein appendages Gram-negative bacteria; they play a role in biofilm formation, colonization and invasion of cells [19, 21, 22]. These

structures are approximately three to ten nanometers in diameter and up to several micrometers long. There may be as many as 1,000 fimbriae per cell. They are composed of helically arranged protein subunits. They foster the attachment of cells to solid surfaces in such diverse environments as rocks in streams, host tissues and in dwelling devices such as shunts [10, 23]. Fimbriae are under complex regulatory controls that involve physiological and environmental input [24].

Type 1 and curli are two types of fimbriae that are associated with *Salmonella* [25]. Type 1 fimbriae are approximately seven nanometers in diameter and 0.5 to 2.0 μm in length [26]. They have a channeled appearance due to the arrangement of subunits around a hollow core [11]. These subunits are composed of 17,000 Dalton protein subunits, called pilin [26]. Type 1 fimbriae play a role in attachment of *Salmonella* to epithelial cells and aid in biofilm formation on abiotic surfaces [11, 24, 27, 28]. Type 1 fimbriae require an operon that has eight structural genes, *fimAICDFGH* [29-33]. Fimbriae expression is activated at body temperature and is repressed by high osmolarity, low pH and low temperatures [24].

Curli fibers are coiled surface structures that are composed of a single subunit, curlin, and are involved in adhesion to surfaces, cell aggregation, and biofilm formation [19, 34]. Curli fimbriae are considered to be a part of the extracellular matrix [35] that helps promote host colonization. They mediate host cell adhesion and invasion, and are inducers of the host inflammatory response [36, 37]. *Salmonella* produce curli fimbriae under nutrient limiting conditions such as low osmolarity, growth temperatures below 30°C, low nitrogen, phosphate, and iron [4, 24, 38]. Curli fimbriae expression occurs maximally in media without salt [36]. The genes encoding for the biosynthesis of curli fimbriae are arranged in two transcribed operons,

csgDEFG and *csgBAC [39]*. Operon expression can vary considerably and is usually expressed at temperatures below 30°C [40, 41]. The gene products of *csgEFG* and putative *csgC* have an accessory function in the formation of curli fimbriae [34, 42-44]. Scientists have noted the structural and biochemical similarity between prokaryotic curli fimbriae and eukaryotic amyloid fibers [45-49]. Amyloid formation is responsible for several human diseases including Alzheimer's, Huntington's, and prion diseases [36, 50-52].

1.4 Antimicrobial Agents

Microorganisms form biofilms on a variety of surfaces [53]. There is interest in biofilms because they can serve as a reservoir for infection. It is important that suitable biocides and infection control procedures are used to limit this risk of infection [54, 55]. The introduction of products containing antibacterial agents into healthy households has escalated from a few dozen products in the mid 1990s to more than 700 today. Antibacterial products were developed to prevent transmission of disease causing microorganisms, particularly in hospital settings. These products are being used in homes where there is no added health benefit. There is a concern that antibacterial agents will select for bacteria resistant to the agent [54, 56]. Overuse in the home can be expected to result in the development of resistant microbial variants [57]. The mechanism of resistance can result in the development of cross-resistance to antibiotics. Bacterial antibiotic resistance has become a global health crisis [58].

Antimicrobial agents are agents that kill microorganisms or inhibit their growth. There are factors that influence how effective an antimicrobial agent will be. Population size is a crucial factor; it takes more time to kill a larger population of organisms than a smaller one. Population composition is important because the

effectiveness of an agent varies depending on the species of organisms that are present. Different organisms will differ in their susceptibility to a specific agent. Bacterial endospores are more resistant to antimicrobial agents than are vegetative forms and organisms in lag or log phase are more readily destroyed than organisms in stationary phase [59]. Usually, the more concentrated the agent, the more rapidly microorganisms are destroyed. However, this is not always true. Sometimes an agent is more effective at lower concentrations (example: ethanol at 70% versus at 95%). An increase in temperature at which a chemical acts often enhances its activity. The environment that an organism is in may aid in its destruction or offer protection. An example of this is that heat kills more readily at an acidic pH; acidic foods and beverages are easier to pasteurize than foods with higher pHs. An example of protection is organic matter, which can protect microorganisms against heating and chemical disinfectants. A biofilm is also an example of this. It has been documented that bacteria in biofilms are altered physiologically and this makes them less susceptible to antimicrobial agents. In addition, the extracellular organic material found in biofilms may serve as a barrier to antimicrobial agents [10].

Antimicrobial agents fall into one of two classes: antibiotics and biocides. Antibiotics are microbial products that kill or inhibit the growth of other organisms. Antibiotics have a single specific target that affects a specific cellular function such as transcription, translation, enzymatic activity or cell permeability. The term biocide refers to a chemical agent, usually broad spectrum in nature that inhibits or kills microorganisms [57, 60-62]. Biocides have a broader effect on the cell such as coagulation of the cytosol, membrane damage, and leakage of cellular constituents.

Bacteria use similar mechanisms, such as cell wall impermeability and efflux systems to resist the harmful effects of both biocides and antibiotics [63-66].

The biocide employed in medical and home settings that was used in the studies performed was triclosan.

1.5 Triclosan

Triclosan has been widely used since its introduction about 45 years ago. It has been used as a preservative, antiseptic, and disinfectant [67]. Triclosan is a trichloro derivative of two hydroxydiphenyl ether (Figure 1) [68, 69]. It has broad-spectrum activity against many microorganisms, including *Salmonella enterica* serovar Typhimurium [70]. Due to this broad spectrum of activity, triclosan can be found in various products such as soaps, detergents, dishwashing liquids, lotions, deodorants, mouth rinses, toothpastes, toothbrush handles, slippers, towels, pillows, mattresses, carpets, fabrics, baby toys, plastics, chopping boards, chop sticks, pizza cutters, food storage containers, garbage bags, kitty litter and even paints (Table 1) [71, 72]. These different products have varying concentrations of triclosan in them [73]. At low concentrations (<1000mg/L), triclosan inhibits the growth of many bacteria and is bacteriostatic, and at higher concentrations such as those found in soaps and hand washes it is bactericidal [69, 74, 75].

In triclosan susceptible organisms the mode of action is that growth inhibitory activities of the phenylether result from blocking lipid synthesis by specifically inhibiting a NADH-dependent enoyl-acyl carrier protein reductase or FabI [72, 76]. Previous work has suggested that substitutions within FabI are the primary mechanism mediating triclosan resistance [6, 7]. This process will then affect other processes and cell structures that depend on lipid synthesis. Therefore, triclosan's effect may impact

the cell membrane structure and function, aerobic respiration and membrane protein function [70]. This process occurs at lower concentrations of triclosan. At higher concentrations of triclosan, there is damage to the bacterial cell membrane and disruption of protein and lipid synthesis [77].

1.6 Development of Resistance to Antimicrobials

The major driving force for development of resistance in bacteria to antimicrobial agents is overuse of the agent; this results in the selection of resistant organisms [71, 78, 79]. There is an ongoing problem with using triclosan in soap products. It is difficult to find a soap product without this agent even though studies have shown that there is no additional health benefit of using hand washes containing triclosan over just using soap and water [7]. Data has indicated that high levels of triclosan are present in groundwater in the United States of America and in Europe. A Swedish study has demonstrated significant accumulation of triclosan in the bloodstream and breast milk. This was found in people who did not routinely use products that contained triclosan [7]. This constant exposure to triclosan has raised a concern about triclosan resistant bacteria.

A major mechanism associated with bacterial resistance is the use of efflux pumps, which eject substances from the cell through a series of proteins in the cytoplasmic membrane [6, 60, 68]. Efflux pumps were not thought to have evolved as a response to antimicrobial agents, however they have been shown to be up regulated in triclosan resistance. *Salmonella* strains resistant to triclosan can be obtained by repeated passage in gradually increasing concentrations of triclosan. The development of resistance is associated with the overexpression of the AcrAB efflux pump [64, 68, 70].

1.7 Consequences of Reduced Susceptibility

Cross-resistance occurs when resistance to one antimicrobial agent also results in resistance or decrease in susceptibility to another when both agents attack the same target, initiate a common pathway to cell death or share a common route of access to the target [75]. For example, organisms resistant to triclosan exhibit cross-resistance to tetracycline [68, 80]. This is because the efflux pump that removes triclosan from the cell also removes tetracycline. This is how the use of household products results in the selection of bacteria resistant to triclosan as well as resistance to other antimicrobials [36, 60, 71, 81, 82].

The typical in-use concentration of triclosan is substantially higher than the concentration required for inhibiting most bacteria. Therefore, development of triclosan resistance has been regarded unlikely under conditions of normal use of cosmetics or health care formulations. However, triclosan containing consumer products leave residues on kitchen and bathroom surfaces and in the environment, where they will be diluted to sub lethal concentrations [6, 76]. Prolonged exposure could lead to the development of resistance [71]. Studies have shown that bacteria with increased resistance to triclosan can also have cross resistance to antibiotics [58].

1.8 Biofilm Formation

In a variety of environments, microorganisms can attach to solid surfaces conditioned with nutrients and form communities called biofilms [83-86]. These are characterized by the extracellular matrices present as three-dimensional structures that help promote the survival of the organisms during environmental stresses [36, 87]. *Salmonella* has been observed to attach to various surfaces where it can form these structures [5].

Biofilm formation is a multistep process involving initial surface attachment, monolayer formation, migration to form multilayered microcolonies, production of extracellular matrix and biofilm maturation with a three dimensional architecture [24, 88, 89]. A small number of bacterial cells must adhere to a surface. This process is facilitated by bacterial motility. The cells that attach irreversibly to the surface will begin to divide, forming microcolonies. Once in a microcolony, extracellular polymers are produced. These extracellular polymeric substances (EPS) are primarily polysaccharides [90]. The EPS helps anchor the cells to the surface and to stabilize the colonies. As the biofilm ages, attached bacteria must be able to detach and disperse from the biofilm in order to survive and colonize new niches [84].

Understanding the formation of biofilms is important for their control [91]. Biofilms are between 50 and 100 times less susceptible to antimicrobials than are planktonic cells [57, 85, 92]. Bacteria within a biofilm are more resistant because of limited availability of key nutrients [93-95]. The extracellular matrix also contributes to the increased resistance of biofilm-associated cells [5]. This resistance is attributed to the conditions associated with biofilms including; reduced diffusion, physiological changes due to reduced growth rates and production of enzymes which degrade antimicrobial substances [96]. In the food industry, attachment of bacteria to the food or on the surfaces of equipment can lead to illness, outbreaks and economic losses [84, 97, 98]. Medical concerns occur because bacterial biofilms form on the surfaces of medical devices and on tissue surfaces within compromised organs [99]. Biofilms grow similarly in the environment and in industrial systems [100]. Biofilm associated organisms can easily withstand the attack of commonly used natural and pharmacological agents [100-102].

1.9 Hypotheses

- *Salmonella typhimurium* parental strains can be repeatedly passaged to generate strains with reduced susceptibility (SRS) to triclosan.
- The growth of parental and SRS strains will be similarly affected by environmental conditions.
    - When cells are grown on Luria Bertani (LB) agar at 37ºC, curli fimbriae will not be produced. When cells are grown on LB without NaCl agar at 28ºC, curli fimbriae will be produced.
    - Biofilm formation will be greater when curli fimbriae are present as it fosters attachment to surfaces.
    - Motility of parental and SRS strains will be greater when the cells are grown at 37ºC.
- Biofilms formed by resistant strains should be more resistant to treatments with triclosan than biofilms formed by parental strains.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial Strains

Salmonella typhimurium ATCC® number 14028™ was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This strain was used as the parental strain to generate *Salmonella* with Reduced Susceptibility (SRS) strains B and D. All strains were passaged each week and stored at room temperature. All strains were frozen in glycerol at -80°C in aliquots for storage longer than two months. Purity was checked weekly by plating on Xylose Lysine Desoxycholate Agar (XLD) agar (Becton, Dickinson, and Company, Sparks, MD) as well as stabbing and streaking a Triple Sugar Iron Agar (TSIA) slant (Becton, Dickinson, and Company Sparks, MD). Bacterial cells were grown in two different environmental conditions. In condition one, cells were grown in Luria Bertani (LB) broth at 37°C. In condition two, cells were grown in LB broth without NaCl at 28°C. These conditions will be referenced as condition one and condition two throughout this thesis.

2.2 Development of *Salmonella* strains with Reduced Susceptibility to Triclosan

The *Salmonella* parental strain was grown in condition one without shaking overnight in LB broth. An aliquot was removed from the tube with a sterile cotton swab and used to prepare a bacterial lawn on a LB plate. After 10 minutes of drying at room temperature, Whatman Paper Disks containing varying concentrations of triclosan (Whatman International Ltd., Maidstone, England) were placed onto the LB

agar surface. These disks were prepared using a standard one hole punch (5 mm) on Whatman Filter Paper. After sterilization, five microliters of increasing stock solutions of triclosan were pipetted onto the disks. To control for the possible antimicrobial effects of ethanol, control disks with 5 µl of ethanol were tested during each passage. Triclosan stock solutions were prepared by dissolving the chemical in ethanol. After incubation for 24 hours in condition one, a zone of inhibition was observed. Colonies from the zone or around the edges were inoculated into 5 ml of LB and incubated overnight at 37°C. These cultures were used to prepare bacterial lawns and the above procedure was repeated until there was no zone of inhibition around the disk. The final concentrations of triclosan on the Whatman disks were, 0 ppm, 3000 ppm, 6000 ppm and 9000 ppm.

2.3 Culture Conditions and Media

2.3.1 Parental Strain

The parental *Salmonella* strain was grown at 37°C without shaking in LB Broth (condition one). When needed, the parental strain was grown at 28°C without shaking in LB broth without NaCl (condition two).

2.3.2 SRS Strains

Strains with Reduced Susceptibility (SRS) Strains were grown in condition one without shaking in LB broth. When needed, SRS strains were grown in condition two without shaking.

2.4 Quantifying Resistance

2.4.1 Triclosan Agar Plates

Triclosan infused agar plates were used to quantify triclosan resistance. These plates were prepared by mixing cooled LB agar with triclosan to obtain a variety of concentrations. To control for the ethanol used to dissolve triclosan, plates were also prepared from agar to which only ethanol was added. Once the agar had solidified, the parental and SRS strains were quadrant streaked onto the plates. Growth on the plates indicated resistance to that concentration of triclosan.

2.4.2 Microtiter Plates

Twenty four well flat bottom tissue culture treated polystyrene microtiter plates (Corning Inc., Corning, NY) were used to determine the triclosan resistance of the parental and SRS strains. Parental strains were inoculated into wells containing 0-50 ppm triclosan. SRS strains were inoculated into wells containing 0- 400 ppm. The inoculated microtiter plate was incubated at 37°C for 24 hours. Each well was then examined for turbidity indicating the culture was able to grow in the corresponding concentration of triclosan.

2.5 Growth Curves of Parent and SRS Strains

Cultures were grown overnight in condition one and two in a six well non tissue culture treated microtiter plate (Becton, Dickinson, and Company, Franklin Lakes, NJ). Two hundred microliters aliquots were removed every 30 minutes and transferred to a 96 well flat bottom polystyrene non tissue culture treated microtiter plate. Optical density (O.D.) was recorded at 595 nm immediately after aliquoting using an MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA). Results

were compared using the GraphPad Software unpaired student t test with a 95% confidence interval (http://graphpad.com/quickcalcs/ttest1.cfm).

2.6 Biofilm Formation

2.6.1 Crystal Violet Assay

A crystal violet binding assay was used to determine the time course of *Salmonella* parental and SRS strain attachment to an abiotic surface [69]. Conditions one and two were used for all strains. This experiment was repeated in triplicate and each sample was setup in quadruple wells. An overnight culture of each strain was prepared and diluted 1:100 in the desired medium. One hundred microliters of each diluted culture was pipetted into four wells of a 96 well non tissue culture treated polyvinyl chloride (PVC) microtiter plate (Becton, Dickinson, and Company, Franklin Lakes, NJ). The microtiter plate was covered and incubated at the appropriate temperature. This assay was done hourly over an eight-hour period. After each hour, the microtiter plate was removed from the incubator and planktonic cells were removed from each well by shaking the plate over an established waste tray. The wells were then washed with ddH_2O and 125 µl of 0.1% (w/v) crystal violet solution in water was added to each well for ten minutes. The crystal violet was then removed and the plate was washed twice. It was then inverted on a paper towel to remove any excess liquid. The crystal violet was solubilized by the addition of 200 µl of 95% ethanol to each well for 15 minutes at room temperature. The crystal violet/ethanol solution in each well was mixed by pipetting and then 125 µl was transferred to an optically clear flat bottom non tissue culture treated polystyrene 96 well microtiter plate (Becton, Dickinson, and Company, Franklin Lakes, NJ). The $O.D._{595nm}$ of the

samples was measured using a MRX Microplate Reader (Dynex Technologies, Inc., Chantilly, VA). Results were compared using GraphPad Software unpaired student t test with a 95% confidence interval (http://graphpad.com/quickcalcs/ttest1.cfm).

2.6.2 Microscopic Observation of Biofilms Over Time

An overnight culture of each *Salmonella* strain was prepared in a two chambered Lab-Tek® permanox slide (Nunc, Rochester, NY). Both conditions one and two were studied. Slides were removed from the incubator every two hours for eight hours. Planktonic cells were removed and the chambers were rinsed twice with ddH_2O. Acridine orange (AO) was added to cover the bottom of the chamber slide and was incubated for five minutes at room temperature. After removal of the AO, chambers were rinsed with ddH_2O until there was no longer any orange color. The chamber was removed and the slides were observed at 1000x on a fluorescent Zeiss Axioscope (Carl Zeiss, Inc., Thornwood, NY).

2.7 Motility

2.7.1 Motility Determination on Semi-Solid Agar

Modified Rappaport Vassiliadis Medium (MSRV) was used to compare the motility of the parental and SRS strains in both conditions. MSRV was supplemented with novobiocin per protocol to prevent any possible contamination from outside sources of *Escherichia coli.* Six serial dilutions of overnight cultures of the parental and SRS strains were prepared in EPA dilution water in a 24 well microtiter plate. One microliter of each dilution was plated onto the center of a MSRV plate. Plates were incubated in each strain's given condition for 48 hours. After incubation, the diameter of the zone of growth, which resulted from the bacterial migration from the

original point of inoculation, was measured. Colony forming units for each dilution was also determined by plating 10 µl of each of the six ten-fold dilutions onto LB and LB without NaCl agar plates. The number of cells in 1ul of the inoculums was plotted against the zones of growth. A line of best fit was drawn through the data points. The size of the zone of growth using $1x10^5$, $1x10^6$, $1x10^7$, and $1x10^8$ cells as inoculums was determined. Each experiment was repeated three separate times. Results were compared using GraphPad Software unpaired student with a 95% confidence interval (http://graphpad.com/quickcalcs/ttest1.cfm).

2.7.2 Microscopic Observation of Flagella

An overnight culture of each strain was prepared in conditions one and two. One microliter of each culture was pipetted onto the center of a 0.25% agar plate. Plates were incubated at their appropriate temperatures for 12 hours. A loop full of agar was taken from the edge of the zone of growth and placed in a 2 mL Eppendorf tube with 300 µl of EPA dilution water and was held at room temperature for five minutes. Ten microliters were removed from each Eppendorf tube and placed onto a glass slide and covered with a 22x30 mm cover slip. After 15 minutes, five microliters of RYU flagella stain (Remel) was placed at the edge of the coverslip and allowed to wick under the edge of the coverslip. The slides were then observed at 1000x on a Zeiss Axioscope (Carl Zeiss, Inc., Thornwood, NY).

2.8 Detection of Fimbriae

2.8.1 Congo Red Binding Assay

Curliated bacteria stain red when grown on plates supplemented with diazo dye Congo Red because curli are able to bind to the dye [36, 103]. LB and LB without

NaCl was prepared and autoclaved. After autoclaving, the agar was supplemented with Congo Red (40ug/ml) [104-106]. The agar plates were quadrant streaked with each strain and left in the incubator at 37°C and 28°C for 48 hours, and the morphology of the colonies was observed. *Salmonella* that express curli fimbriae and cellulose display a red, dry and rough colony morphology (RDAR) on Congo Red indicator plates [107, 108]. If there is no curli fimbriae or cellulose present, a colony will appear smooth and white (SAW). If there is no cellulose then the colony will appear brown, dry and rough (BDAR). If there is cellulose but no curli fimbriae, then the colony will appear pink, dry and rough (PDAR) [36, 104, 105]. Carolina *Salmonella typhimurium* was used as a control as the RDAR morphotype was present (Carolina, Burlington, NC

2.8.2 Transmission Electron Microscopy (TEM)

Three overnight cultures were prepared for each parental and SRS strain. The cultures were grown in their respective conditions. They were then quadrant streaked onto the two agars and incubated at the two temperatures indicated. A single colony from each plate was transferred to each medium and grown overnight. These overnight cultures were then plated onto XLD agar to ensure purity. Cultures in which all colonies were black, indicating hydrogen sulfide production were transferred one more time and used for further imaging. Three hundred mesh copper grids with formvar carbon support films (Electron Microscopy Sciences Hatfield, PA) were treated with poly-L-lysine by floating the grids on 19 µl of 0.01% poly-L-lysine solution (Sigma Aldrich St. Louis, MO) for 30 minutes. After air-drying for half an hour, they were floated on 19 µl of overnight culture for one minute. They were then washed by floating on filtered ddH_2O for 20 seconds and stained by floating on two

separate 19 µl drops of five percent filtered ammonium molybdate for ten seconds. All staining steps were followed by dabbing with filter paper to remove excess liquid. The grids were air dried for one hour before microscopic observation. Samples were observed using the LIBRA 120 transmission electron microscope (TEM) with Gatan Ultrascan 1000 2kx2k CCD camera (Carl Zeiss, Inc., Thornwood, NY) with Gatan Digital Micrograph imaging software. At least ten images of each strain were taken for each overnight culture. The bacteria were divided into two groups based on the presence or absence of fimbriae.

2.9 Regulation of Fimbriae Genes

2.9.1 Primer Design

Primers were designed using the SciTools application on the Integrated DNA Technology website (http://www.idtdna.com/scitools/scitools.aspx). The parameters used for the primer design were as follows: 1) Primer dimers and hairpin structures had a ΔG value more positive than negative nine kcal/mole. 2) The T_m for all primers was between 59°C and 61°C. 3) No primer had a sequence similarity greater than 90% to non target DNA regions as reported by Basic Local Alignment Search Tool (BLAST). The genotype of *Salmonella enterica* subsp. enterica serovar *Typhimuruim str.* 14028, complete genome was used in the BLAST (http://blast.ncbi.nlm.nih.gov/) search. Primers were stored as 100uM stock solutions. All primers and cDNA were stored at -20°C.

2.9.2 Primer Sequences

The primer sequences for *gyrB*, *fimA*, and *csgG* are listed in Table 2.

2.9.3 Primer Testing

DNA was prepared using the BioRad Instagene™ Matrix and quantified using a NanoDropR ND-1000 Spectrophotometer and NanoDrop 3.1.2 Software (Thermo Fisher Scientific Inc. (BioRad Hercules, CA). HotStarTaq® DNA Polymerase was used in PCR. Primers were tested by performing reverse transcription PCR on a PTC-100™ Programmable Thermal Controller (MJ Research, Inc. Waltham, MA). PCR products were run on a 2% ethidium bromide agarose gel. The band size of each amplicon was determined using a 100bp DNA ladder (Promega Madison, WI). The expected product lengths for each primer set were 195 base pairs (bp) for *gyrB*, 190 bp for *csgG*, and 103 bp for *fimA*.

2.9.4 RNA Extraction

The parental and SRS strains were grown in the two conditions until mid log phase. RNA was extracted using RNAprotect™ Bacteria Reagent and the RNeasy Mini Kit (Qiagen Valencia, CA).

2.9.5 RNA Quantifications

RNA concentrations were measured in triplicate with a NanoDropR ND-1000 Spectrophotometer and NanoDropR 3.1.2 Software (Thermo Fisher Scientific Inc. Wilmington, DE). The average of the triplicate concentration readings was used for reverse transcription.

2.9.6 Reverse Transcription

Reverse transcription was performed with the Omniscript RT Kit (Qiagen, Valencia, CA) on a PTC-100™ Programmable Thermal Controller (MJ Research,

Inc., Waltham, MA). A random nonomer primer was used. A 1:100 dilution of the reverse transcription reaction was used in the real time PCR assay.

2.9.7 Quantitative Real Time PCR

Four replicates of each sample were ran using the QuantiTect SYBR Green PCR Kit (Qiagen Valencia, CA). The cycling temperatures were 95°C for 15 minutes, then 40 rounds of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. A disassociation curve was performed at the end of each run. SYBR® Green (Qiagen, Valencia, CA) was used to detect the levels of DNA. The ABI Prism 7000 Sequence Detection System was used to perform quantitative real time PCR. The ABI Prism 7000 SDS Software was used to analyze the results (Applied Biosystems, Carlsbad, CA). In each well, five ul of cDNA (diluted 1:100 from the RT reaction) was added and the final concentration of 0.5 ng/ul for each primer was used. The gene *gyrB* was used as an internal control.

2.9.8 Efficiency Values

QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) was used with cDNA concentrations of 1, 5, 25, 60 and 250 ng. These concentrations were obtained by diluting the cDNA concentration from the parental strain determined by using a NanoDropR ND-1000 Spectrophotometer and NanoDropR 3.1.2 Software (Thermo Fisher Scientific Inc. Wilmington, DE). The cDNA was obtained from the parental strain grown in LB at 37°C. Standard curves for each of the primers was used to determine the efficiency of each gene being tested; *csgG, fimA,* and *gyrB*. Ct values were plotted against cDNA concentrations. The trend line was added and the slope of

this line was used in the equation $E = 10^{\left(\frac{-1}{-slope}\right)}$ to obtain the efficiency value of each gene [109].

2.10 Triclosan Treatment on Preformed Biofilms

Biofilms were grown in triplicate in Lab Tek® two chambered permanox chambered slides (Nunc, Rochester, NY) for each strain in its appropriate condition. After eight hours of incubation, biofilms were treated at concentrations of 200 ppm and 2000 ppm of triclosan. Slides were washed three times with 2 ml of sterile ddH_2O to remove any planktonic bacterial cells. The slides were stained with Molecular Probes Live Dead Stain® Baclight™ Bacterial Viability Kit (Invitrogen Eugene, OR**).** One and a half microliters of SYTO 9 (3.34mM) and 1.5 µl of Propidium Iodide (20mM) were added to 997 µl of sterile ddH_2O. After the addition of 50 microliters of the solution, a cover slip was added and incubated in the dark for 15 minutes at room temperature. Slides were observed on a fluorescent microscope at 1000x magnification on a Zeiss Axioscope (Carl Zeiss, Inc., Thornwood, NY).

Chapter 3

RESULTS

3.1 Development of SRS Strains with Reduced Susceptibility to Triclosan

Two strains of *Salmonella enterica* serovar Typhimurium with reduced susceptibility (SRS) to triclosan were developed from the original parental strain (Figure 2). The minimum inhibitory concentration (MIC) of triclosan for this strain was 1 ppm. With each passage in the serial passage process, the zone of inhibition around the triclosan-containing disk continued to decrease. Using this procedure, SRS B and D strains were trained up to a MIC of 400 ppm Triclosan (Figure 3 and 4). There was no zone of inhibition around the control disks, which contained only ethanol. This indicates that is possible to create strains with an increased resistance to triclosan.

3.2 Stability of Parental and SRS Strains to Triclosan

Before each experiment, parental and SRS strains were tested for their MIC and purity. A lawn of each strain was prepared on LB agar plates. Whatman paper disks containing 0 ppm, 3000 ppm, 6000 ppm and 9000 ppm triclosan were placed on the lawn. The parental strain remained sensitive to triclosan and the SRS strains continued to grow to the edge of the 9000 ppm disk indicating resistance.

3.3 Growth Curves of Parental and SRS Strains

Growth curves of all strains were performed by growing the organisms in conditions one and two (Figure 5). The O.D.s of all strains was determined over an eight hour time period. The average O.D. for all strains at time zero was 0.070, with a range of 0.065 to 0.078. At the end of the eight hours, the average O.D. at the end of eight hours was 0.467, with a range from 0.386 to 0.522. The log phase began at approximately 60 minutes for all strains in both conditions. The stationary phase began at approximately 240 minutes for all strains in both conditions.

At each hour for eight hours, the following were compared for both growth conditions: 1) the parent and the SRS strains 2) the parent in each of the conditions and 3) the two SRS strains in each of the conditions.

3.3.1 Growth Curve: Hour Zero

During the initial reading under condition one, there was no significant difference in growth between the parental and either SRS B strain ($p= 0.096$) or SRS D ($p=0.085$). During the initial reading under condition two, there was no significant difference between the parental strain and either SRS B ($p=0.176$) or SRS D ($p=0.246$).

During the initial reading, the parental strain grown in the two conditions were not significantly different ($p=0.598$). There was no significant difference between the SRS B strain between conditions ($p=0.505$). SRS D growth was not significantly different between conditions ($p=0.2150$).

3.3.2 Growth Curve: Hour One

At one hour under condition one, there was no significant difference in growth by the parental and SRS B strain ($p= 0.273$). There was a significant difference

between the parental and SRS D strain (*p=0.041*). At one hour under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.054*) or SRS D (*p=0.181*).

At one hour, the parental strain grown in the two conditions were significantly different (*p=0.009*). There was no significant difference between the SRS B strain between conditions (*p=0.285*). SRS D growth was significantly different between conditions (*p=0.236*).

3.3.3 Growth Curve: Hour Two

During the second hour under condition one, there was no significant difference in growth by the parental and either SRS B strain (*p= 0.112*) or SRS D (*p=0.714*). During the second hour under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.100*) or SRS D (*p=0.471*).

During the second hour, the parental strain grown in the two conditions were significantly different (*p=0.0001*). There was a significant difference between the SRS B strain between conditions (*p=0.004*). SRS D growth was significantly different between conditions (*p=0.019*).

3.3.4 Growth Curve: Hour Three

During the third hour under condition one, there was no significant difference in growth by the parental and either SRS B strain (*p= 0.119*) or SRS D (*p=0.452*). During the third hour under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.686*) or SRS D (*p=0.952*).

During the third hour, the parental strain grown in the two conditions were significantly different (*p=0.022*). There was no significant difference between the SRS B strain between conditions (*p=0.166*). SRS D growth was significantly different between conditions (*p=0.019*).

3.3.5 Growth Curve: Hour Four

During the fourth hour under condition one, there was a significant difference in growth by the parental and either SRS B strain (*p= 0.006*) or SRS D (*p=0.039*). During the fourth hour under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.241*) or SRS D (*p=0.591*).

During the fourth hour, the parental strain grown in the two conditions were significantly different (*p=0.0001*). There was a significant difference between the SRS B strain between conditions (*p=0.001*). SRS D growth was significantly different between conditions (*p=0.007*).

3.3.6 Growth Curve: Hour Five

During the fifth hour under condition one, there was a significant difference in growth by the parental and SRS B strain (*p= 0.038*). There was no significant difference between the parental and SRS D strain (*p=0.848*). During the fifth hour under condition two, there was a significant difference between the parental strain and SRS B (*p=0.004*). There was no significant difference between the parental and SRS D (*p=0.054*).

During the fifth hour, the parental strain grown in the two conditions were significantly different (*p=0.0002*). There was a significant difference between the

SRS B strain between conditions (*p=0.005*). SRS D growth was significantly different between conditions (*p=0.048*).

3.3.7 Growth Curve: Hour Six

During the sixth hour under condition one, there was no significant difference in growth by the parental and either SRS B strain (*p= 0.442*) or SRS D strain (*p=0.259*). During the sixth hour under condition two, there was a significant difference between the parental strain and either SRS B (*p=0.0002*) or SRS D (*p=0.0005*).

During the sixth hour, the parental strain grown in the two conditions were significantly different (*p=0.0006*). There was no significant difference between the SRS B strain between conditions (*p=0.455*). SRS D growth was significantly different between conditions (*p=0.102*).

3.3.8 Growth Curve: Hour Seven

During the seventh hour under condition one, there was no significant difference in growth by the parental and SRS B strain (*p= 0.315*). There was a significant difference between the parental and SRS D strain (*p=0.025*). During the seventh hour under condition two, there was a significant difference between the parental strain and either SRS B (*p=0.0039*) or SRS D (*p=0.0046*).

During the seventh hour, the parental strain grown in the two conditions were significantly different (*p=0.0021*). There was no significant difference between the SRS B strain between conditions (*p=0.356*). SRS D growth was significantly different between conditions (*p=0.180*).

3.3.9 Growth Curve: Hour Eight

During the eighth hour under condition one, there was a significant difference in growth by the parental and either SRS B strain ($p= 0.035$) or SRS D strain ($p=0.010$). During the eighth hour under condition two, there was a significant difference between the parental strain and either SRS B ($p=0.004$) or SRS D ($p=0.0019$).

During the eighth hour, the parental strain grown in the two conditions were significantly different ($p=0.0001$). There was no significant difference between the SRS B strain between conditions ($p=0.108$). SRS D growth was significantly different between conditions ($p=0.034$).

Based off of the growth curve, we begin to see significant differences after four hours. This may be attributed to the different environmental conditions. However, the differences seen in the stationary and death phases, did not affect the growth rate. As each strain had a generation time of approximately one hour.

3.4 Biofilm Crystal Violet Assay

The crystal violet assay was used to assess biofilm formation as a function of time and growth condition (Figure 6). At each hour for eight hours, the following were compared for both growth conditions: 1) the parent and the SRS strains 2) the parent in each of the conditions and 3) the two SRS strains in each of the conditions (Figure 7). All of the optical densities are show in Table 3. During the first hour of biofilm formation, there were significant differences seen. A few comparisons that were made up to hour five were significantly different. However, after hour five there were no more significant differences seen and biofilm formation was similar between all strains and all conditions.

3.4.1 Biofilm Formation: Hour One

At one hour under condition one, there was no significant difference in biofilm formation by the parental and SRS B strains ($p= 0.077$). There was a significant difference between the parental and SRS D strains ($p=0.011$). The parental formed biofilm similarly to one SRS strain and not the other. At one hour under condition two, there was a significant difference between the parental strain and SRS B ($p=0.033$). There was no significant difference between the parental strain and SRS D ($p=0.152$). The parental formed biofilm similarly to one SRS strain and not the other.

At one hour, the parental strain grown in the two conditions were significantly different ($p=0.010$). There was no significant difference between the SRS B strain between conditions ($p=0.434$). SRS D biofilm formation was significantly different between conditions ($p=0.006$). Both the parental and SRS D strain produced significantly different amounts of biofilm in each condition and there were no differences for SRS B.

3.4.2 Biofilm Formation: Hour Two

During the second hour under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain ($p=0.289$) or SRS D strain ($p=0.954$). At hour two under condition two, there was no significant difference between the parental strain and either SRS B ($p=0.086$) or SRS D in LB ($p=0.113$). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the second hour, the parental strains in condition one and condition two were not significantly different ($p=0.074$). There was no significant difference between the SRS B strain between conditions ($p=0.917$). SRS D was also not

significantly different between conditions (*p=0.140*). There were no significant differences seen in biofilm formation when each strain was compared in both conditions.

3.4.3 Biofilm Formation: Hour Three

During hour three under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain (*p=0.343*) or SRS D strain (*p=0.618*). At hour three under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.522*) or SRS D in LB (p=0.783). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the third hour of biofilm formation, the parental strain in condition one and condition two were not significantly different (*p=0.369*). There was no significant difference between the SRS B strain between conditions (*p=0.527*). SRS D was also not significantly different between conditions (*p=0.270*). There were no significant differences seen in biofilm formation when each strain was compared in both conditions.

3.4.4 Biofilm Formation: Hour Four

During hour four under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain (*p=0.274*) or SRS D strain (*p=0.900*). At hour four under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.070*) or SRS D in LB (*p=0.070*). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the fourth hour of biofilm formation, the parental strains in condition one and condition two were not significantly different (*p=0.170*). There was no significant difference between the SRS B strain between conditions (*p=0.571*). SRS D was also not significantly different between conditions (*p=0.150*). There were no significant differences seen in biofilm formation when each strain was compared in both conditions.

3.4.5 Biofilm Formation: Hour Five

During hour five under condition one, there was no significant difference between biofilm formation between the parental and SRS B strain (*p=0.309*). There was a significant difference between the parental and SRS D strain (*p=0.002*). At hour five under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.188*) or SRS D (*p=0.111*). In condition one, the parental strain produced a different amount of biofilm than SRS D, but not SRS B. In condition two, the parental strain produced the same amount of biofilm as both SRS B and D.

During the fifth hour of biofilm formation, the parental strain in condition one and condition two were not significantly different (*p=0.071*). There was no significant difference between the SRS B strain between conditions (*p=0.241*). SRS D was significantly different between conditions (*p=0.005*). There were no significant differences seen in biofilm formation when each the parental and SRS B strain were compared in both conditions. However, SRS D produced different amounts of biofilm between conditions.

3.4.6 Biofilm Formation: Hour Six

During hour six under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain (*p=0.389*) or SRS D strain (*p=0.875*). At hour six under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.345*) or SRS D (*p=0.483*). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the sixth hour of biofilm formation, the parental strains in condition one and condition two were not significantly different (*p=0.560*). There was no significant difference between the SRS B strain between conditions (*p=0.199*). SRS D was not significantly different between conditions (*p=0.828*). There were no significant differences seen in biofilm formation when each strain was compared in both conditions.

3.4.7 Biofilm Formation: Hour Seven

During hour seven under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain (*p=0.786*) or SRS D strain (*p=0.614*). At hour seven under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.459*) or SRS D (*p=0.265*). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the seventh hour of biofilm formation, the parental strains in condition one and condition two were not significantly different (*p=0.824*). There was no significant difference between the SRS B strain between conditions (*p=0.715*). SRS D was not significantly different between conditions (*p=0.324*). There were no

significant differences seen in biofilm formation when each strain was compared in both conditions

3.4.8 Biofilm Formation: Hour Eight

During hour eight under condition one, there was no significant difference between biofilm formation between the parental and either SRS B strain (*p=0.962*) or SRS D strain (*p=0.676*). At hour eight under condition two, there was no significant difference between the parental strain and either SRS B (*p=0.198*) or SRS D (*p=0.172*). In either condition, there were no differences in biofilm formation between the parental strain and SRS B and D.

During the eighth hour of biofilm formation, the parental strains in condition one and condition two were not significantly different (*p=0.943*). There was no significant difference between the SRS B strain between conditions (*p=0.381*). SRS D was not significantly different between conditions (*p=0.110*). There were no significant differences seen in biofilm formation when each strain was compared in both conditions

3.5 Microscopic Observation of Biofilms

The results of microscopic observation of biofilms were similar to those obtained by the crystal violet assay. Initially condition one appeared to foster more biofilm formation than condition two for all strains. A higher O.D. reading in the crystal violet assay and the clustering of *Salmonella* cells under the microscope in the AO study indicated that more biofilm was produced. However, as time progressed, biofilm formation under the two conditions was similar. It appears that there is no difference between the parental and SRS strain biofilm formation (Figure 8).

3.6 Motility

3.6.1 Motility Determination on Semi-Solid Agar

The comparison of growth of the parental and SRS strains under the two conditions was done as described for the crystal violet assay of biofilm formation (Figure 9). Optical densities are shown in Table 4. The average zone size is shown for inoculums of $1x10^2$, $1x10^3$, $1x10^4$ and $1x10^5$ cells (Figure 10).

In condition one, the parental strain was significantly different than either SRS B (*p=0.0001*) or SRS D (*p=0.0001*). The parental strain showed more motility than either SRS B or SRS D. In condition two, there was no significant difference between the parental strain and SRS B (*p=0.1603*). The parental strain was as motile as SRS B. There was a significant difference between the parental strain and SRS D (*p=0.0392*). The parental strain was less motile than SRS D in condition two.

In condition one, the parental strain was significantly different than the parental strain in condition two (*p=0.0001*). The parental strain showed more motility in condition one than condition two. The SRS B strain in condition one was significantly different than SRS B in condition two (*p=0.002*). SRS B was more motile in condition one than in condition two. The SRS D strain in condition one was significantly different than SRS D strain in condition two (*p=0.0068*). SRS D was more motile in condition two than in condition one. Temperature affected motility for all strains; the parental and SRS B were more motile in condition one and SRS D was more motile in condition two.

3.6.2 Microscopic Observation of Flagella

Flagella stains of the parental, SRS B and SRS D strains grown in both conditions are shown in Figure 11. All strains possessed flagella.

3.7 Fimbriae

3.7.1 Congo Red Binding Assay

All of the strains grown in each condition on Congo Red indicator plates expressed the SAW (smooth and white) morphology suggesting that there were no curli fimbriae present (Figure 12).

3.7.2 Transmission Electron Microscopy

TEM was used to determine the presence of fimbriae on parental and SRS strains grown under both conditions (Figure 13). There were numerous cells that lacked fimbriae. Curli fimbriae were not present in any of the strains under either condition. Condition two was not able to foster curli fimbriae production. These results confirm that the SAW morphology seen on the Congo Red indicator plates represents a lack of curli fimbriae present on the cells for all strains.

3.8 Quantitative Real Time PCR

The comparison of growth of the parental and SRS strains under the two conditions was done as described for the crystal violet assay of biofilm formation. The level of *fimA* messenger RNA (mRNA) is similar between comparisons of all strains in both conditions. There was very little up or down regulation and all fold differences were close to one. There was also very little increase or decrease in gene expression of *csgG* and all fold differences were close to one. There were no differences between *fimA* and *csgG* mRNA levels normalized to *gyrB* mRNA levels (Figure 14). Each strain in condition one was compared to that strain in condition two. The parental strain fold difference for *csgG* was 0.5185 and 0.0100 for *fimA*. SRS B's fold difference for *csgG* was 0.1240 and 0.0821 for *fimA*. SRS D's fold

difference for *csgG* was 0.3163 and 0.8129 for *fimA*. The lack of differences concludes that there are no differences in the presence of fimbriae between the strains. All strains possessed similar fimbriae, independent of which condition the strain was grown in. PCR products of each of the genes were ran on a 2% gel are shown in Figure 15.

3.9 Triclosan Treatment on Preformed Biofilms

Using the Live Dead Stain®, cells that are alive stained green and those that are dead stained red. All parental biofilm cells grown under both conditions were red after the addition of 200 or 2000 ppm triclosan. Exposing SRS B and D to 200 ppm in each condition resulted in approximately half the cells staining red and half of the cells staining green. Exposing SRS B and D to 2000 ppm in each condition resulted in all of the cells staining red. As a control, no triclosan was added. All cells in the control remained green (Table 5). When a biofilm is treated with 2000 ppm, an amount that is commonly found in household products, cells in that biofilm will not survive. However, when a biofilm is treated with a concentration that is similar to a residual that may be on a surface or in a sink, resistant cells will still thrive. Resistant organisms that can live in these residuals may have cross-resistance to antibiotics, which lead to difficulties in finding treatments.

Chapter 4

DISCUSSION

4.1 Strains with Reduced Susceptibility to Triclosan

SRS strains B and D were developed by sequentially culturing *Salmonella* in increasingly higher concentrations of triclosan. The SRS strains maintained higher MICs than the parental strain. Both SRS strains had similar MICs that were >400 ppm triclosan. The parental strain's MIC was <1 ppm. The development of SRS strains demonstrates the ability of *Salmonella* to develop resistance to antimicrobials, like triclosan. When bacteria are exposed to triclosan, cross-resistance may occur to biocides such as antibiotics [110, 111]. It is important to study these resistant organisms that are generated as a result of exposure to products that are used to clean our house, hands, and used in hospital or industrial settings, as they are a potential public health concern.

4.2 Growth Curves

The parental strain grew similarly to each SRS strain in all conditions tested. Even though initially in condition two, which had a lower temperature, the organisms would typically grow more slowly, it was found that the log and stationary phases began at approximately similar times. The two environmental conditions resemble that of the human body and room temperature. Even though condition one would be more suitable for bacterial growth, at lower temperatures the microbes were still able to thrive. This was found to be true for biofilm production. Initially, in all three

scenarios, organisms in condition two started off growing more slowly. However, has time passed, growth under both conditions was similar.

4.3 Motility Evaluation

4.3.1 Motility on Semi-Solid Agar

When *Salmonella* was grown on MSRV, the zone of growth increased with increasing numbers of cells in the initial inoculum. The zone of growth on semi-solid media is an indication of motility. This was the general trend for the parental and SRS strains in both conditions. This was also observed with *Salmonella enterica* parental and quaternary ammonium SRS strains [112].

In condition one, the parental strain was significantly different than either SRS B ($p=0.0001$) or SRS D ($p=0.0001$). The parental strain based on zone of growth was more motile. In condition two, there was no significant difference between the parental strain and SRS B ($p=0.1603$) and motility was similar between the two strains. There was a significant difference between the parental strain and SRS D ($p=0.0392$) because SRS D was much more motile. Even though the SRS strains shared a commonality of resistance, there may be other differences between the strains that were not examined in this project. There may be an increase in gene expression of a flagella gene at a lower temperature.

In condition one, the parental strain was significantly different than the parental strain in condition two ($p=0.0001$). The parental strain in condition one was more motile. The SRS B strain in condition one was significantly different than SRS B in condition two ($p=0.002$). SRS B was more motile in condition one. The SRS D

strain in condition one was significantly different than SRS D strain in condition two (*p=0.0068*), as SRS D was more motile in condition two.

SRS D in condition one had a zone of growth that was very small and did not extend outward from the initial point of inoculation. In condition two, SRS D had a larger zone of growth, which was greater than either the parent or SRS B. The parental and SRS B strain were more motile in condition one. QAC SRS strains have also been observed to be different in motility when compared to each other [112]. With the exception of SRS D, condition one fostered the better environment for motility.

Flagella mediated motility plays an important role in biofilm formation. The parental and SRS strains showed differences in motility when plated on semi-solid media. These differences were not observed when biofilm formation was assayed using crystal violet and microscopic observation. This may be because many factors play a role in biofilm formation. Non-motile species and motile species are both capable of forming biofilm. In non-motile organisms, cells increase the expression of adhesins on their outer surface. This increases their ability to promote cell to cell and cell to surface adherence. In motile species, when conditions favor biofilm formation, individual bacteria are able to localize to a surface and produce an extracellular matrix that holds the cells together [24].

4.3.2 Flagella Stain

The production of flagella by *Salmonella* was not dependent upon the environmental condition in which the cells were grown. Using the flagella stain, all strains were observed to possess these structures under both conditions. In addition, all strains were able to grow on MSRV. The lack of movement of SRS D on MSRV

cannot be attributed to a lack of flagella. As typical with *Salmonella,* all strains' showed peritrichous flagellation.

4.4 Biofilms

4.4.1 Crystal Violet Assay

The crystal violet assay produced similar results as the bacterial growth curve. Within the first hour of biofilm or bacterial growth there was a significant difference between strains in both conditions. However, as time passed, there was no significant difference indicating there was a similar amount of biofilm that was produced. At hour one, almost every comparison that was made showed a significant difference. The lower temperature in condition two may be a contributor to these differences as cells grow more slowly at this temperature. By hour two this difference was no longer observed. From hour one until hour five in the biofilm forming study, there were a few significant differences observed. However, after five hours there were no significant differences in any of the comparisons and the biofilms seem similar to one another. These results were supported by microscopic observation of biofilm over time.

Based on the lack of curli fimbriae that is supported by the results of both the Congo Red indicator plates and TEM analysis, the lack of significant differences seen in biofilm formation may be attributed to the lack of differences in fimbriae types. Curli fimbriae have been shown to foster better attachment and none of these cells possessed curli fimbriae [111]. In a study by Bokranz et al., it was found that cells expressing curli fimbriae (RDAR morphotype) produced more biofilm than cells lacking curli fimbriae (SAW morphotype) [104]. Fimbriae are under regulatory

controls that involve physiological and environmental inputs [24]. In our study, the environments used did not result in the production of curli fimbriae.

4.5 Detection of Fimbriae

4.5.1 Congo Red Indicator Plates

The Congo Red indicator plates are used to detect curli fimbriae on cells. Curli are able to bind to the Congo Red forming the wrinkled aggregated colonies. When curli is present, colony morphology is red, dry and rough (RDAR). However, when curli is not present, colonies will look smooth and white (SAW) [111]. The SAW colonies present on our Congo Red indicator plates demonstrated that the strains did not express curli or cellulose. Therefore, the strains did not possess the ability to form patterned, aggregative colonies [104].

4.5.2 TEM

Microscopic observation indicated that curli fimbriae were not present on any of the strains in either condition. This confirmed the SAW morphologies seen on the Congo Red indicator plates. Condition two has been reported to foster production of curli fimbriae; however all strains did not produce curli fimbriae [113]. This may be attributed to strain variation.

4.6 Quantitative Real Time PCR

Quantitative Real Time PCR confirmed the lack of effect of the two different conditions on fimbriae formation. It may be that for the strains we used, other environmental conditions would have been more effective in fostering the production of the two fimbrial types. For non-curli fimbriae, these conditions include, low pH

and temperature. For curli fimbriae these conditions include, low oxygen levels, nitrogen, phosphate and iron [24, 81, 114]. There were no differences in mRNA levels of *fimA* and *csgG* between strains under either condition. This was supported by the microscopic examination of the cells and the SAW morphology observed on Congo Red agar plates. Therefore, there are no differences in the expression of the genes for two different fimbriae. Similar biofilm formation as determined by the crystal violet assay would be expected because all cells did not express curli fimbriae.

4.7 Triclosan Treatment on Preformed Biofilms

Although attachment has been reported to enhance resistance to antimicrobials, the parental strain biofilms cells were red after the addition of 200 ppm triclosan. The SRS strains however, independent of environmental condition, were approximately half red and half green. At 2000 ppm triclosan, all strains were red and therefore, dead. The higher concentration is typically found in household products. Therefore, a typical household product would be able to destroy all organisms including the SRS strains. However, this quick fix of using a highly concentrated product will not solve the problems and concerns associated with resistant organisms; it is actually the residue that is left behind after a product is wiped onto a surface or released into the sink that needs to be addressed. This low level residue may play a role in the development of resistant strains. Even more importantly, there may be cross-resistance to antibiotics with these resistant strains that will make them difficult to treat.

4.8 Conclusions

- It is possible to repeatedly passage *Salmonella* in the presence of triclosan to survive in the presence of increasing concentrations.
- Flagella production was not influenced by the environmental conditions used in the current study.
- Motility of all strains was affected by temperature. The parental and SRS B were more motile in condition one. SRS D was more motile in condition two. This indicates that SRS strains may differ in characteristics unrelated to resistance.
- The most significant difference in growth between strains grown under both conditions occurred in the first hour of biofilm formation. By six hours, there were no differences between strains under either condition.
- Curli fimbriae were not produced by any of the strains under condition two.
- SRS strains growing as biofilms would be killed by levels of triclosan found in household products. They would survive in the levels of triclosan found in residuals on counters and sinks.

Table 1: Triclosan Containing Products. Common antibacterial household products that use triclosan as their active ingredient.

Product	Amount of Triclosan
Bath and Body Works ® Hand Soap	0.30%
Bacdown® Hand Soap	0.50%
Equate® Hand Soap	0.12%
Dawn®Dishwashing Soap	0.10%

Table 2: Crystal Violet Assay Optical Densities. Biofilms were formed as a function of time. After eight hours, planktonic cells were removed; each well was stained with crystal violet, dried and resolubilized with ethanol. An aliquot was taken from each strain and Optical Density was read at 595 nm.

		Optical Density (595nm)	
Time	Strain	Condition 1	Condition 2
One Hour	Parental	0.326	0.142
	SRS B	0.266	0.152
	SRS D	0.382	0.089
Two Hours	Parental	0.483	0.272
	SRS B	0.355	0.364
	SRS D	0.489	0.339
Three Hours	Parental	0.666	0.49
	SRS B	0.508	0.625
	SRS D	0.735	0.541
Four Hours	Parental	0.859	0.541
	SRS B	0.608	0.663
	SRS D	0.833	0.583
Five Hours	Parental	0.72	0.608
	SRS B	0.584	0.829
	SRS D	0.824	0.677
Six Hours	Parental	0.825	0.72
	SRS B	0.627	0.813
	SRS D	0.865	0.818
Seven Hours	Parental	0.738	0.681
	SRS B	0.797	0.868
	SRS D	0.831	0.975
Eight Hours	Parental	0.741	0.753
	SRS B	0.755	1.04
	SRS D	0.781	1.13

Table 3: Motility on Semi-Solid Agar. Cells were standardized to four given inoculum sizes. The zone of growth of these inoculums is given as millimeters. The zone of growth represents the distance that an organism moves from original point of inoculation.

		Cells in Inoculum			
Condition	Zone of Growth (mm)	1+E02	1+E03	1+E04	1+E05
One	Parental	48.6	48.7	49.3	55.6
	SRS B	14.9	15	15.9	24.9
	SRS D	0.59	0.6	0.69	1.59
Two	Parental	5.52	5.58	6.12	11.5
	SRS B	2.21	2.26	2.71	7.21
	SRS D	14.9	15.1	16.9	34.9

Table 4: Primer Sequences. The forward and reverse primer sequences for *gyrB* (control), *fimA* (type 1 fimbriae), and *csgG* (curli fimbriae).

Gene	Primer Sequence
gyrB forward	5'- AAT GAC AGT TCA CGC AGG CGT TTC-3'
gyrB reverse	5'- ACT GGT TAT CCA GCG AGA TGG CAA-3'
fimA forward	5'- TCC ATC GTC CTG AAT GAC TGC GAT-3'
fimA reverse	5'-AGG AGA CAG CCA GCA AAT TAG GGT-3'
csgG forward	5'-ACT GGT CAC CGA GGA AAG GAT-3'
csgG reverse	5'-GCT GAC GGC AAA TAT TAT GGT-3'

Table 5: Triclosan Treatment of Biofilms. Eight hour biofilms were treated with 0 ppm, 200 ppm, and 2000 ppm triclosan. The Live Dead Stain® was used to evaluate the viability of the cells. Cells that were green were alive and cells that were dead were red.

	Live/Dead Stain Reaction		
	0 PPM (control)	200 PPM	2000 PPM
Organism-Condition One			
Parent	Green	Red	Red
SRS B	Green	Mostly Green, few cells Red	Red
SRS D	Green	Mostly Green, few cells Red	Red
Condition Two			
Parent	Green	Red	Red
SRS B	Green	Mostly Green, few cells Red	Red
SRS D	Green	Mostly Green, few cells Red	Red

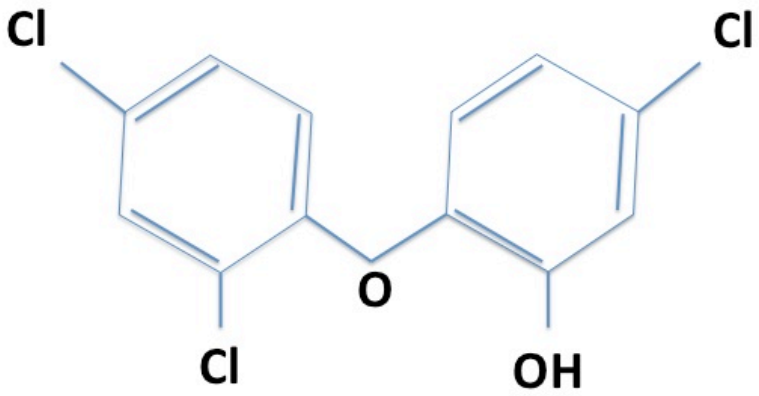
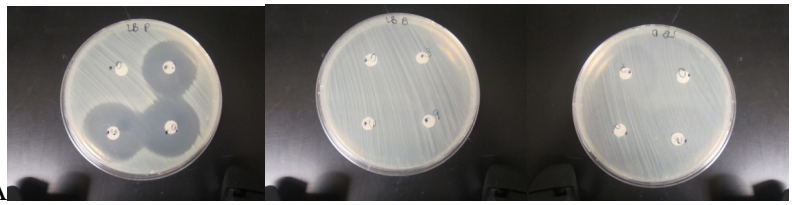
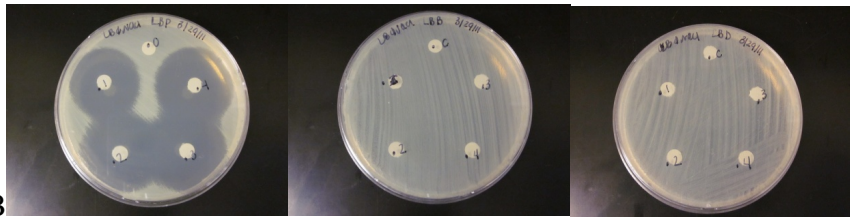


Figure 1: Triclosan Structure. The molecular structure of triclosan, a chlorinated bisphenol used in many household cleaning products, plastics and surfaces as the active antimicrobial ingredient.

A 

B 

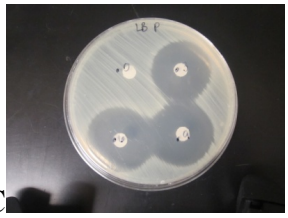
C 

Figure 2: Development of *Salmonella* strains with Reduced Susceptibility to Triclosan. A bacterial lawn of the parental strain was exposed to increasing concentrations of triclosan disks. The zone of inhibition was scraped and subcultured until there was no longer a zone of inhibition. (A) The parental strain and two SRS strains grown in condition one. (B) The parental strain and two SRS strains grown in condition two. (C) The ethanol control. The parental strains remained sensitive to the disks, whereas, the SRS strains remained resistant.

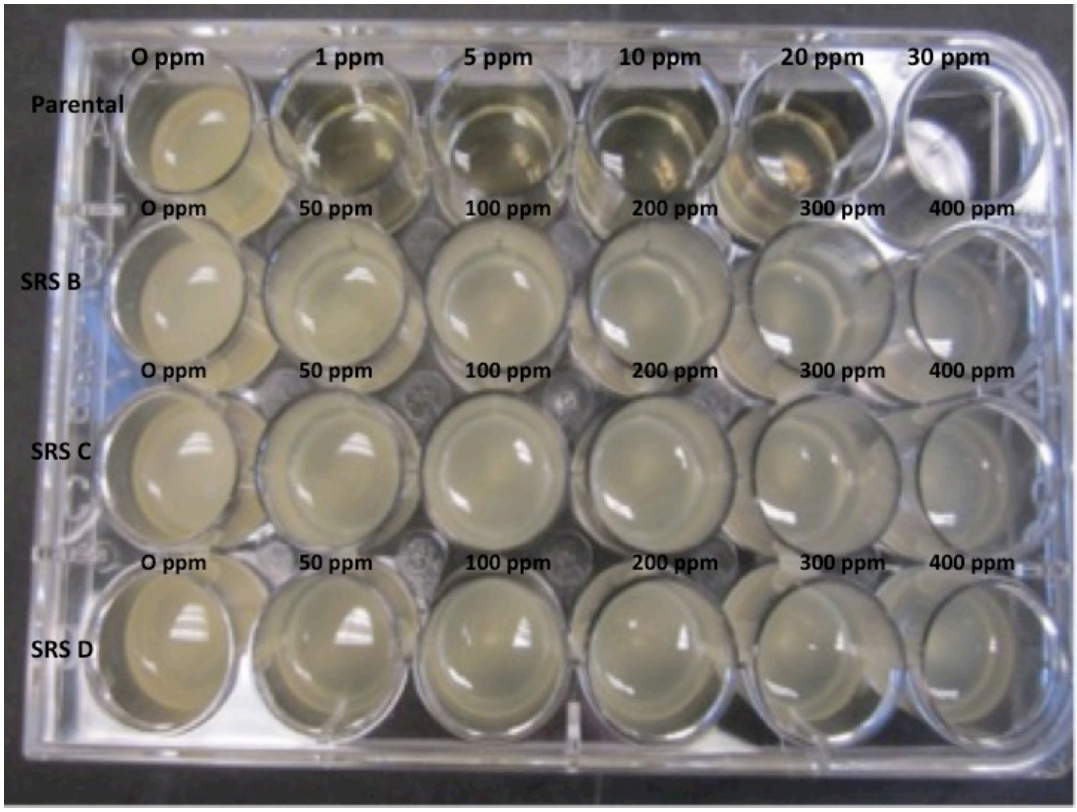


Figure 3: Minimum Inhibitory Concentration of Triclosan for Parental and SRS Strains. Each strain was grown in increasing concentrations of triclosan until there was no longer any growth, the minimum inhibitory concentration (MIC). Turbidity in the well indicated that growth had occurred. The parental strain's MIC was 1 ppm and the SRS strains were 400 ppm triclosan. SRS C was not used in this study.

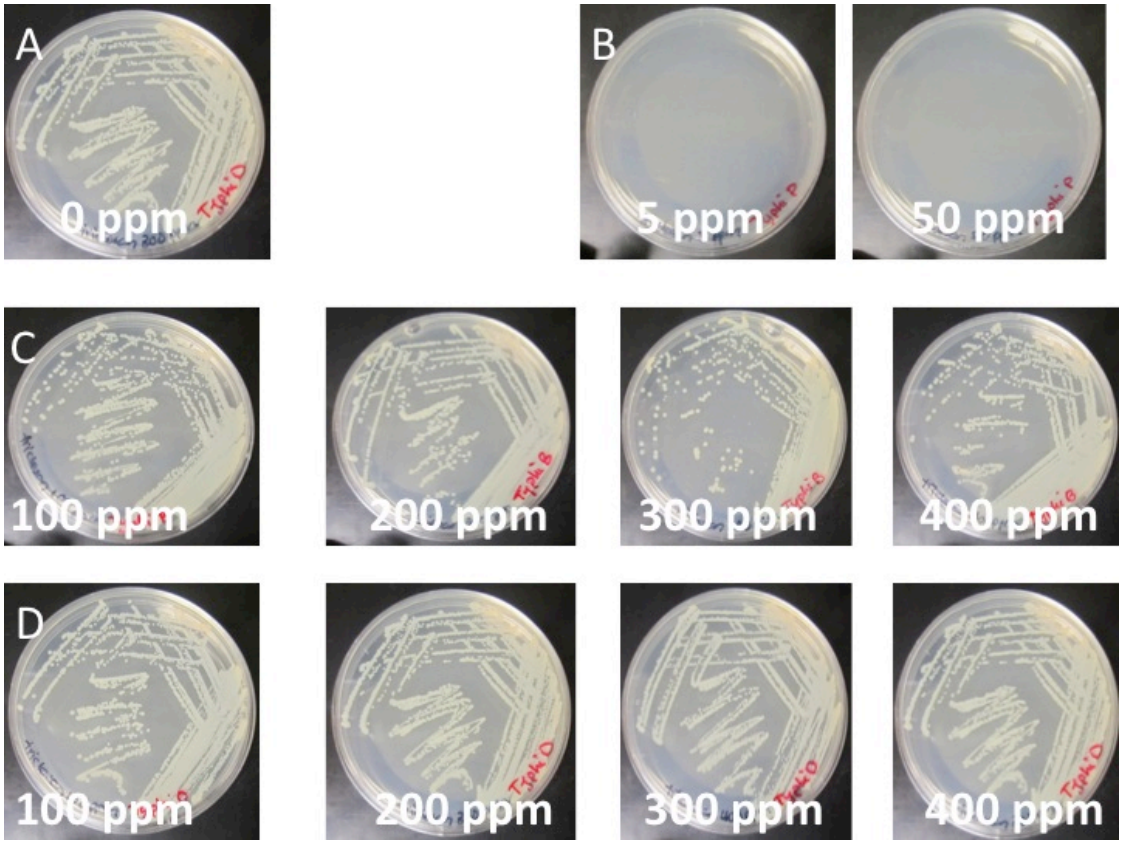
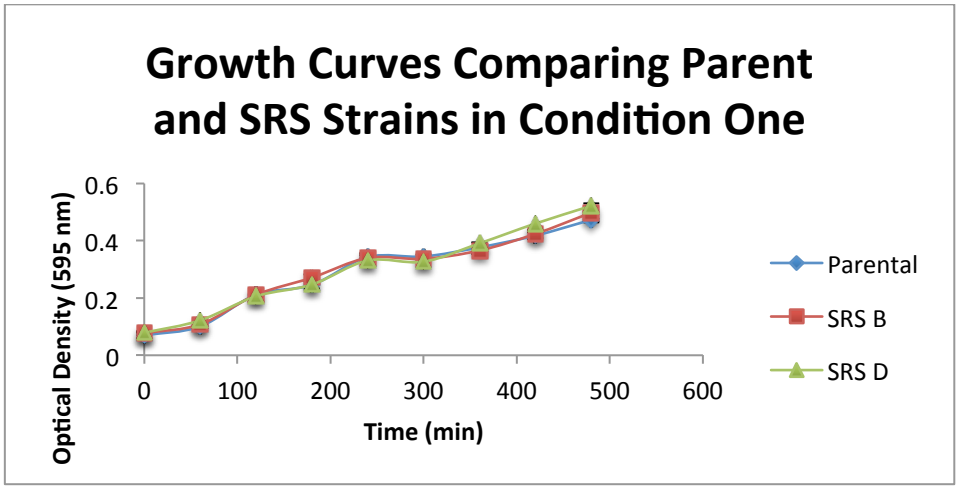


Figure 4: Parental and SRS Strains Grown on Triclosan LB Agar Plates. LB agar with the addition of triclosan was used to quadrant streak each strain to establish a minimum inhibitory concentration (MIC). (A) All strains grew on the control, 0 ppm. (B) The parental strain was not able to grow on 5-50 ppm triclosan. (B) SRS B was able to grow on 100 to 400 ppm triclosan. (C) SRS D was able to grow on 100 to 400 ppm triclosan.

A.



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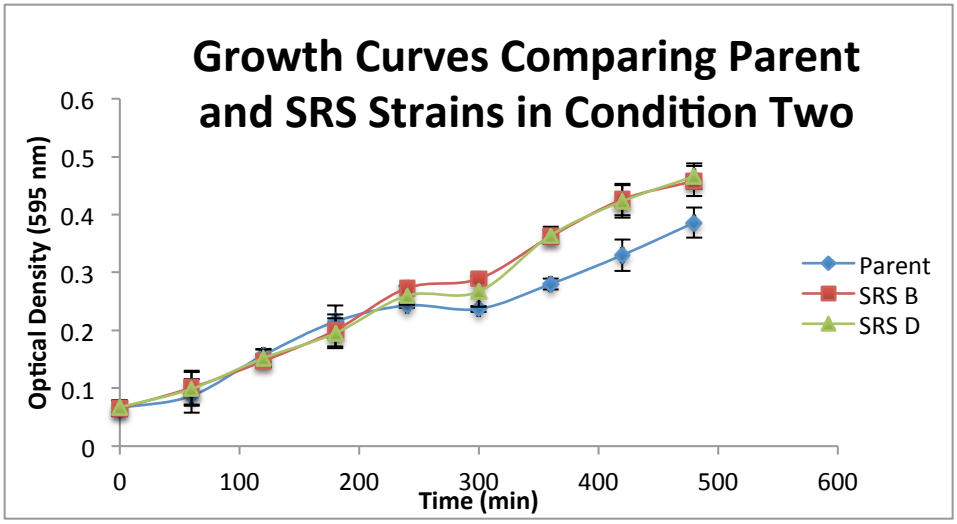


Figure 5: Growth Curves of Parental and SRS Strains in Conditions One and Two. The parental and SRS strains were grown in two environmental conditions. Condition one was Luria Bertani (LB) broth at 37°C and condition two was LB without NaCl at 28°C. Optical Density (O.D.) readings were taken every thirty minutes. (A) Growth curves of all strains in condition one. (B) Growth curves of all strains in condition two. Each experiment was setup in triplicate and repeated three times. Error bars represent standard deviation.

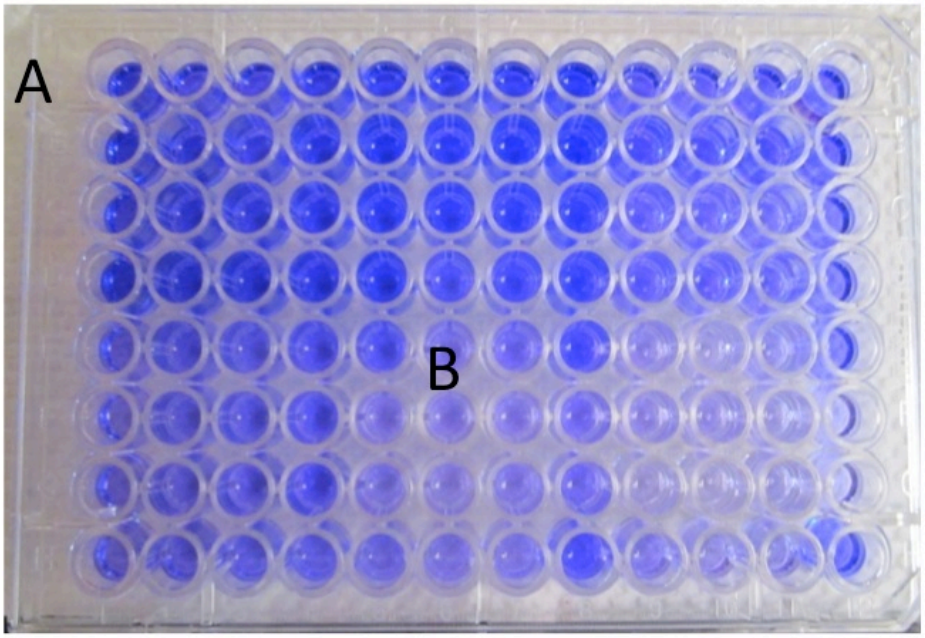


Figure 6: Crystal Violet Binding Assay. After eight hours incubation, planktonic cells were removed, crystal violet was added, and resolubilized with ethanol. Aliquots of the crystal violet and ethanol were read in a microtiter plate reader. (A) A darker crystal violet color indicates that more biofilm was formed occurred. (B) A lighter color indicates that less biofilm attachment occurred.

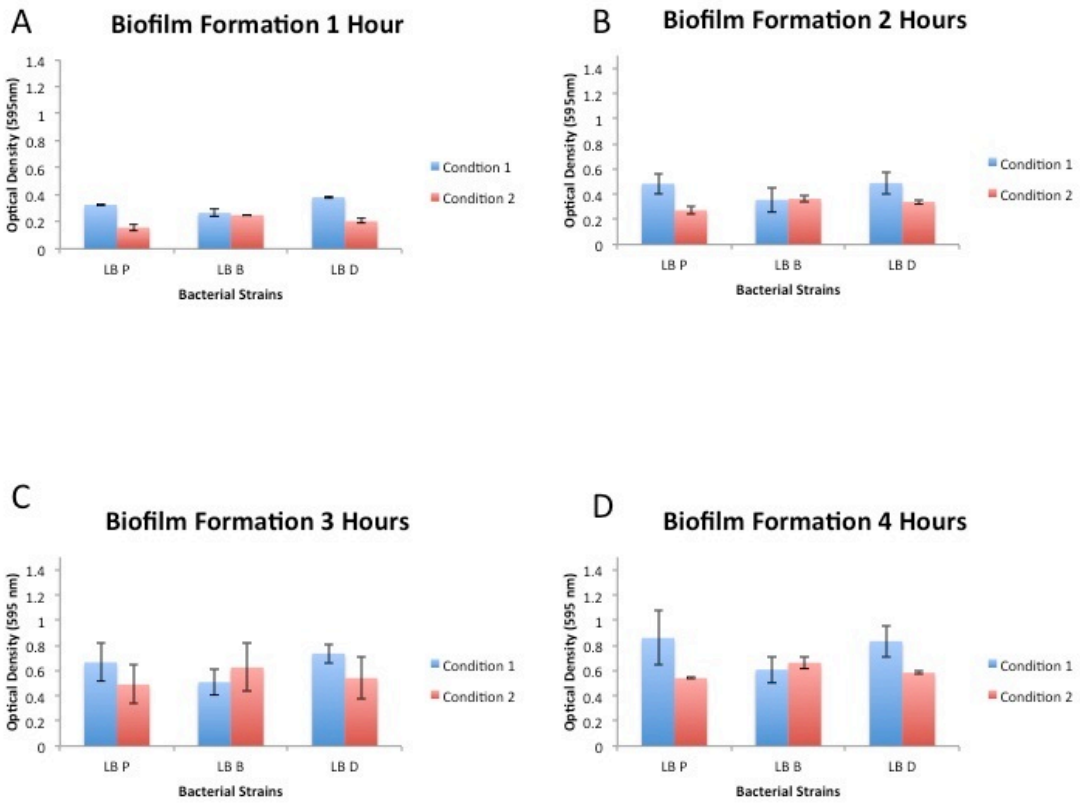
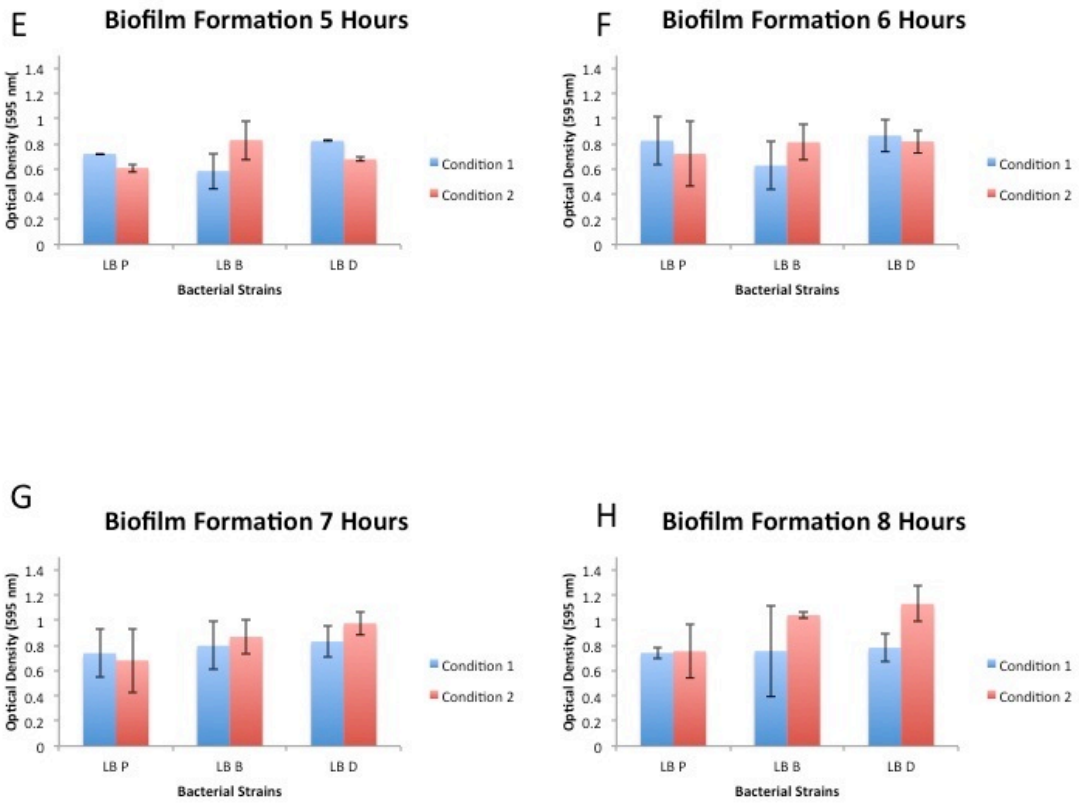


Figure 7: Biofilm Formation as a Function of Time. Each strain was grown in the two environmental conditions over an eight hour time period and Optical Densities were measured at 595 nm. (A) Biofilm formation after one hour. (B) Biofilm formation after two hours. (C) Biofilm formation after three hours. (D) Biofilm formation after four hours. (E) Biofilm formation after five hours. (F) Biofilm formation after six hours. (G) Biofilm formation after seven hours. (H) Biofilm formation after eight hours. There were no significant differences seen after five hours. Each experiment was setup in triplicate and repeated three times. Error bars represent standard deviation.



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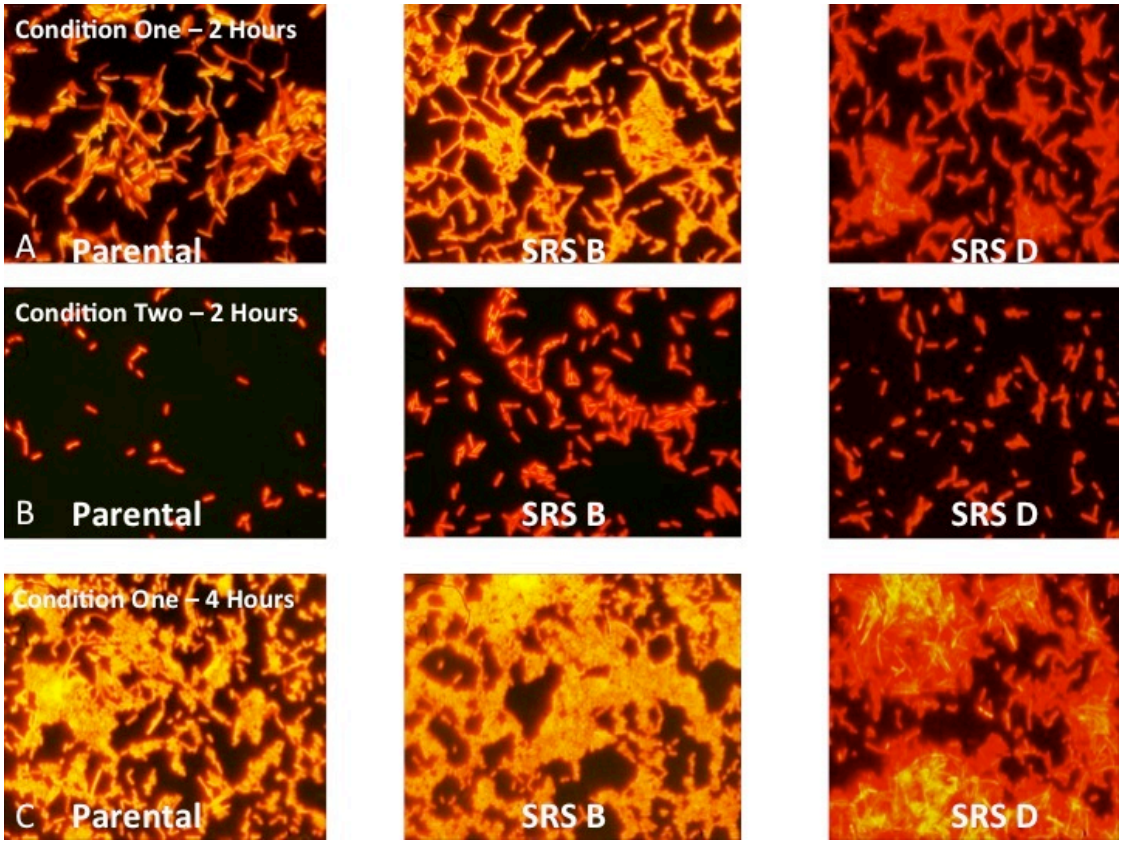
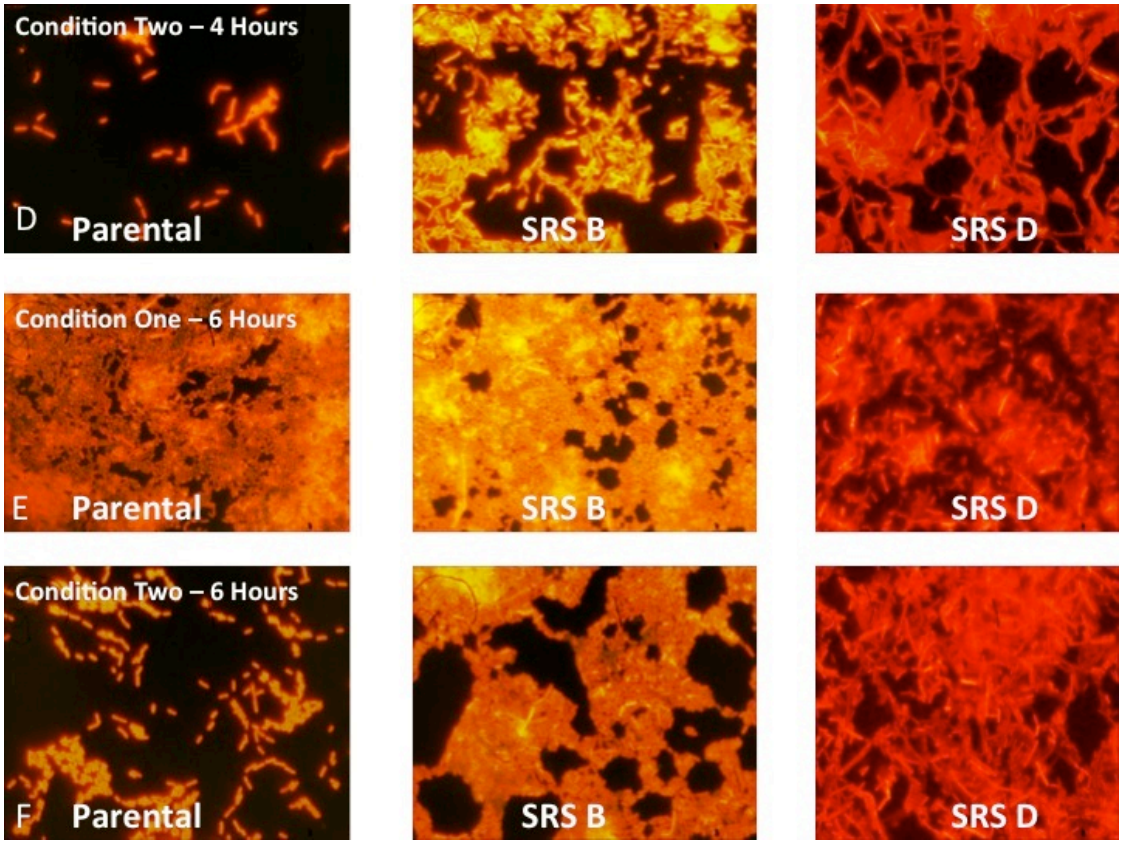


Figure 8: Microscopic Observation of Biofilms. Biofilms were grown for eight hours on a two-chambered permanox slide. Every two hours, planktonic cells were removed and the biofilm was stained with Acridine Orange. Biofilms were then observed under 1000x using a fluorescent microscope. (A) Parental and SRS strains in condition one at two hours. (B) Parental and SRS in condition two at two hours. (C) Parental and SRS strains in condition one at four hours. (D) Parental and SRS strains in condition two at four hours. (E) Parental and SRS strains in condition one at six hours. (F) Parental and SRS strains in condition two at six hours. (G) Parental and SRS strains in condition one at eight hours. (H) Parental and SRS strains in condition two at eight hours. It was observed that overtime biofilms became more similar. Each experiment was setup in duplicate and repeated two times.



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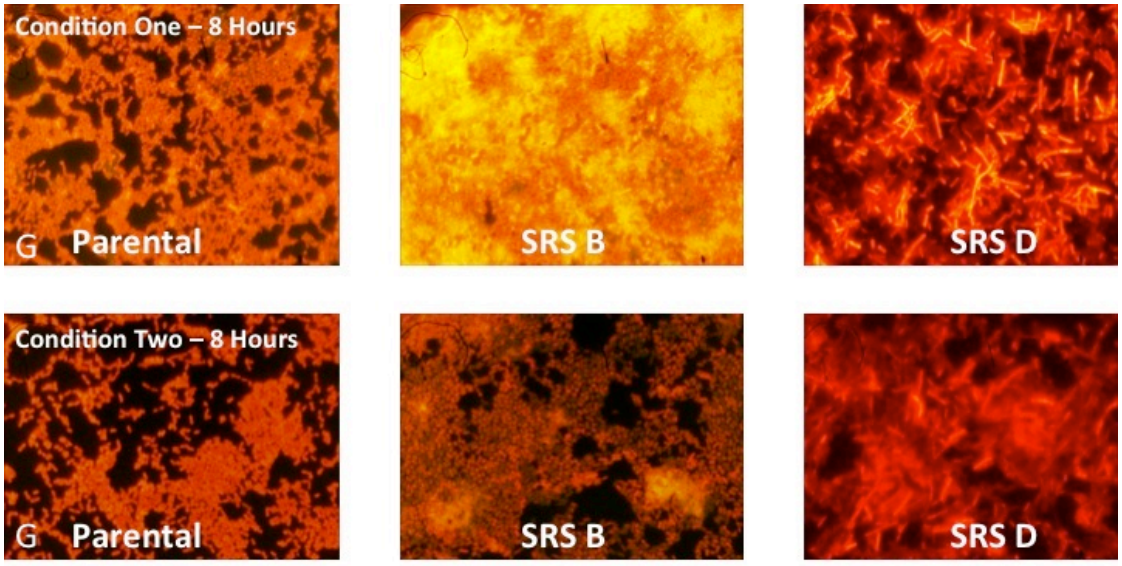

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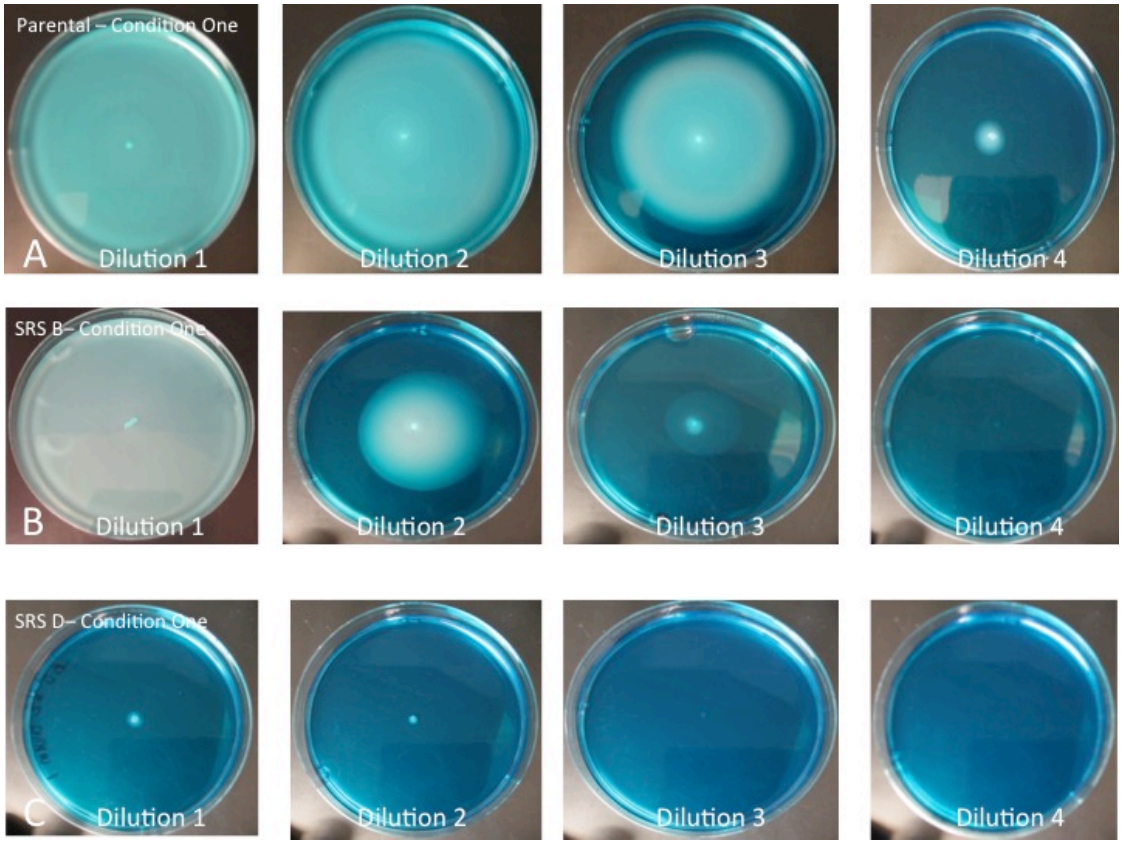
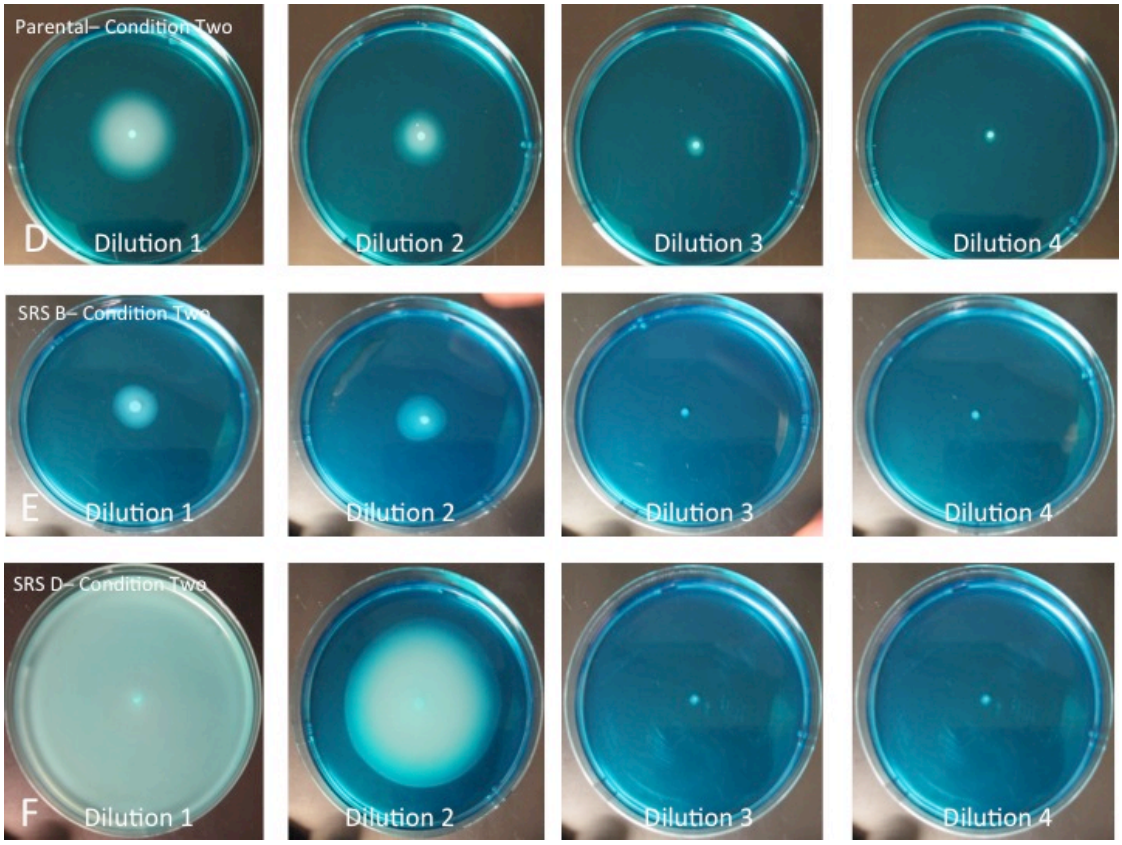


Figure 9: Growth of Parental and SRS Strains on Motility Agar. All strains were diluted and grown on MSRV to observe motility. (A) Parental strain in condition one. (B) SRS B in condition one. (C) SRS D in condition one. (D) Parental strain in condition two. (E) SRS B in condition two. (F) SRS D in condition two. The parental and SRS B were more motile in condition one. SRS D was more motile in condition two. Each experiment was setup in triplicate and repeated three times.



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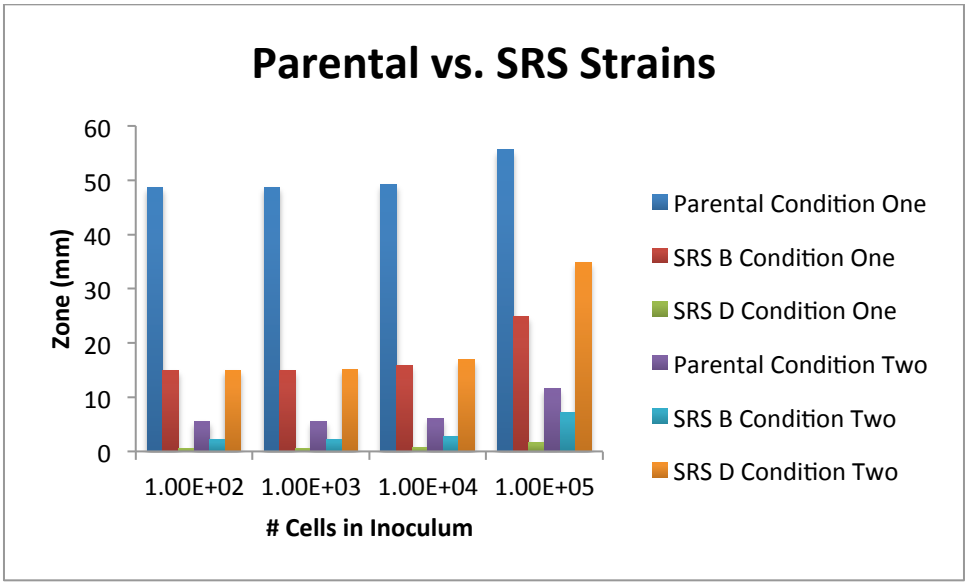


Figure 10: Motility of Parental and SRS Strains on Semi-Solid Agar. Zone of growth on motility agar was determined for a given cell inoculum for each strain. The parental strain in condition one was significantly different than SRS B and D. The parental strain in condition two was not significantly different than SRS B, but was significantly different than SRS D. There were significant differences between each strain in condition one compared to the same strain in condition two.

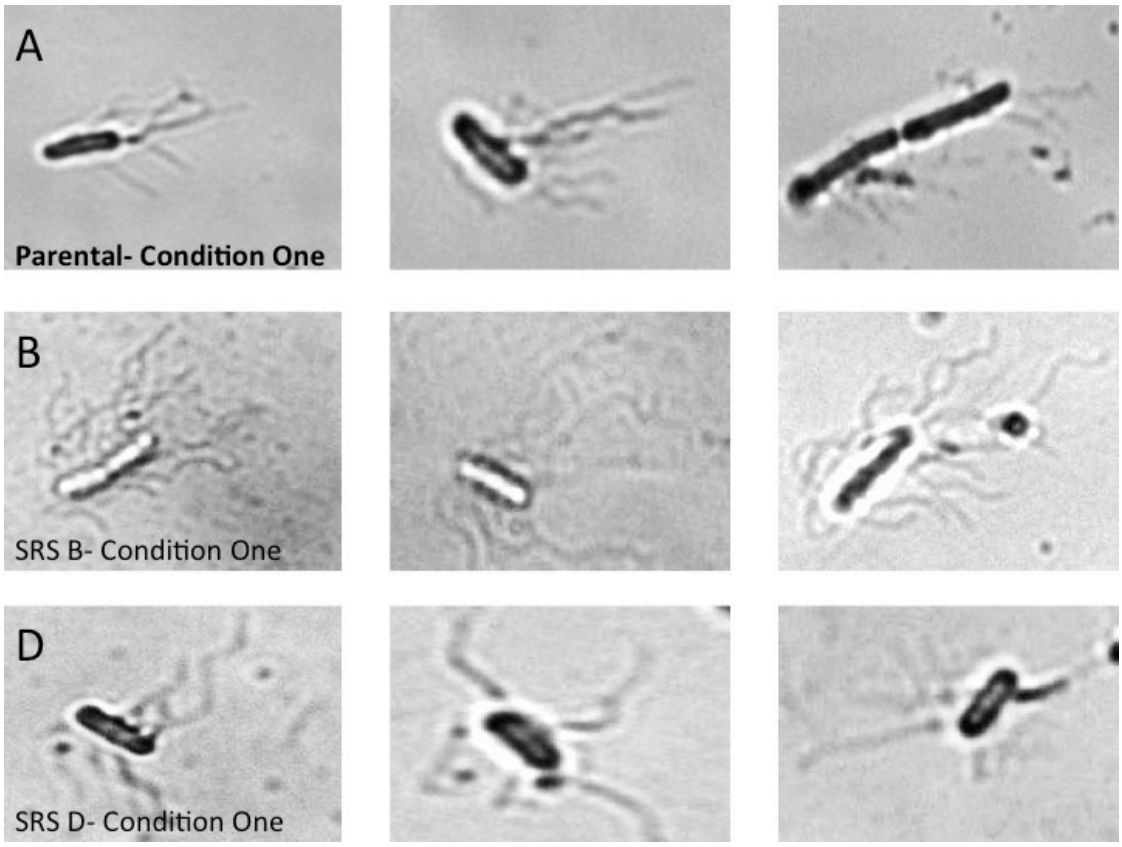
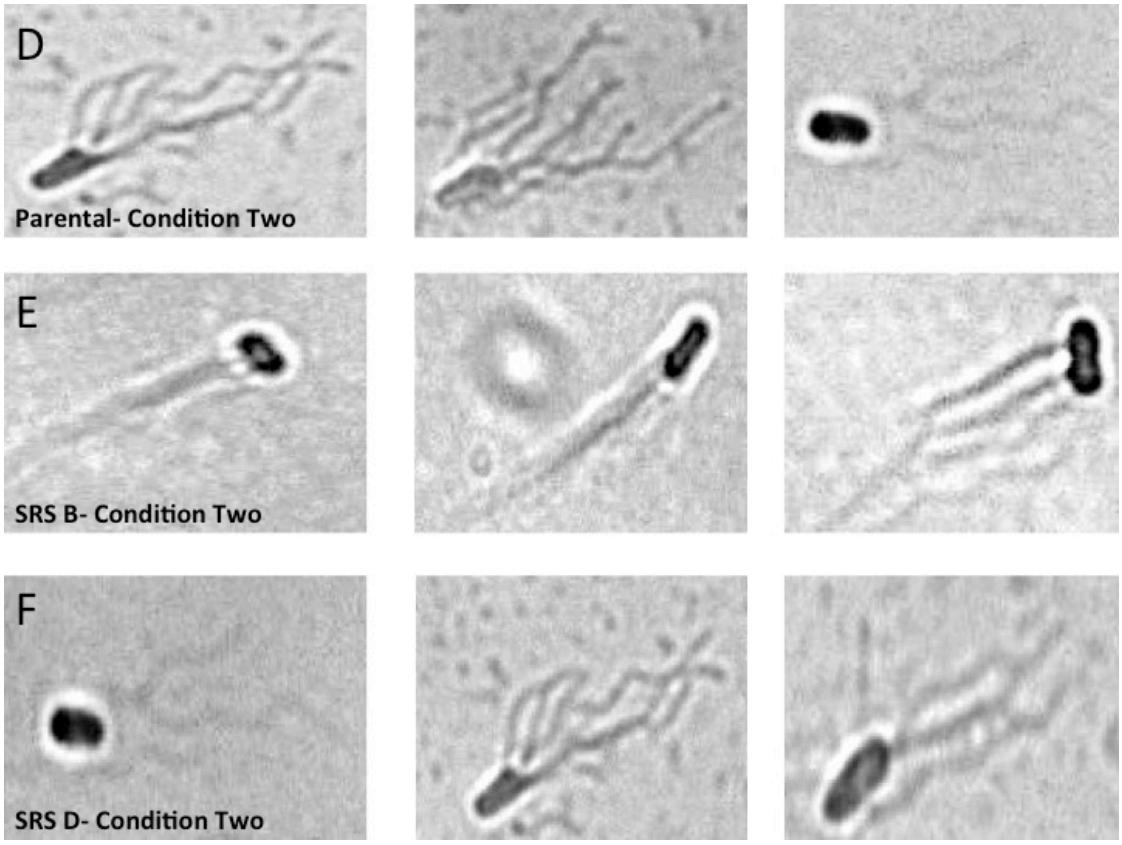


Figure 11: Flagella Stain of Parental and SRS Strains. The RYU flagella stain was used to stain the flagella of the parental and SRS strains. Stained cells were examined at 1000x. (A) The parental strain's flagella in condition one. (B) SRS B flagella in condition one. (C) SRS D flagella in condition two. (D) Parental strain flagella in condition two. (E) SRS B flagella in condition two. (F) SRS D flagella in condition two. All strains produced flagella independent of environment. Each experiment was setup in triplicate and repeated three times.



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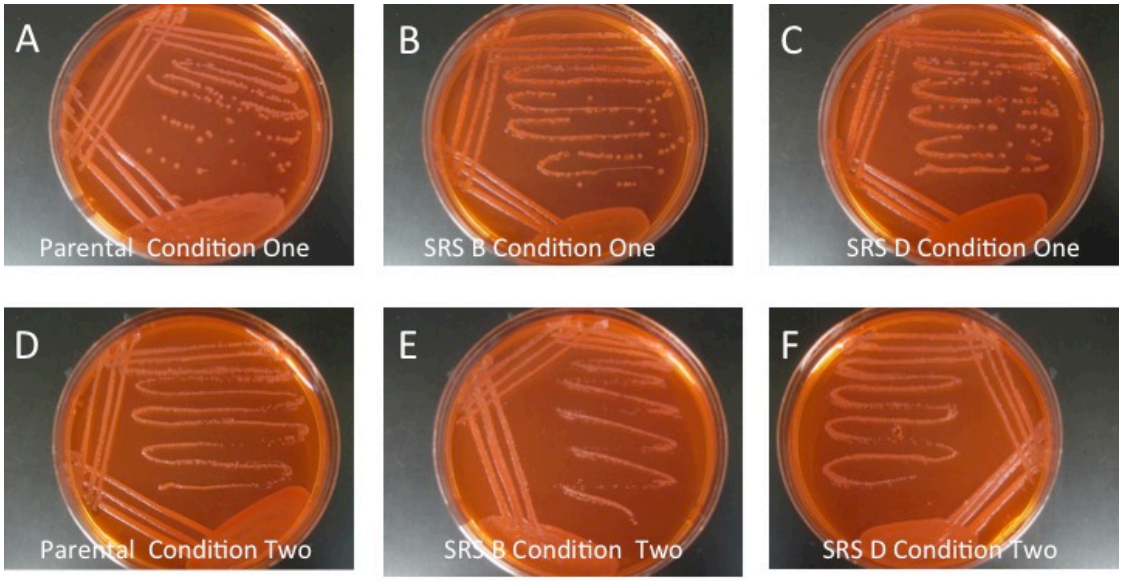


Figure 12: Parental and SRS Strains on Congo Red Agar. Strains were quadrant streaked onto LB without NaCl supplemented with Congo Red. (A) Parental strain in condition one. (B) SRS B in condition one. (C) SRS in condition one. (D) Parental strain in condition two. (E) SRS B in condition two. (F) SRS D in condition two. All strains expressed the saw (smooth and white) morphology. This morphology indicates that there is a lack of curli fimbriae and cellulose. Each experiment was setup in triplicated and repeated three times.

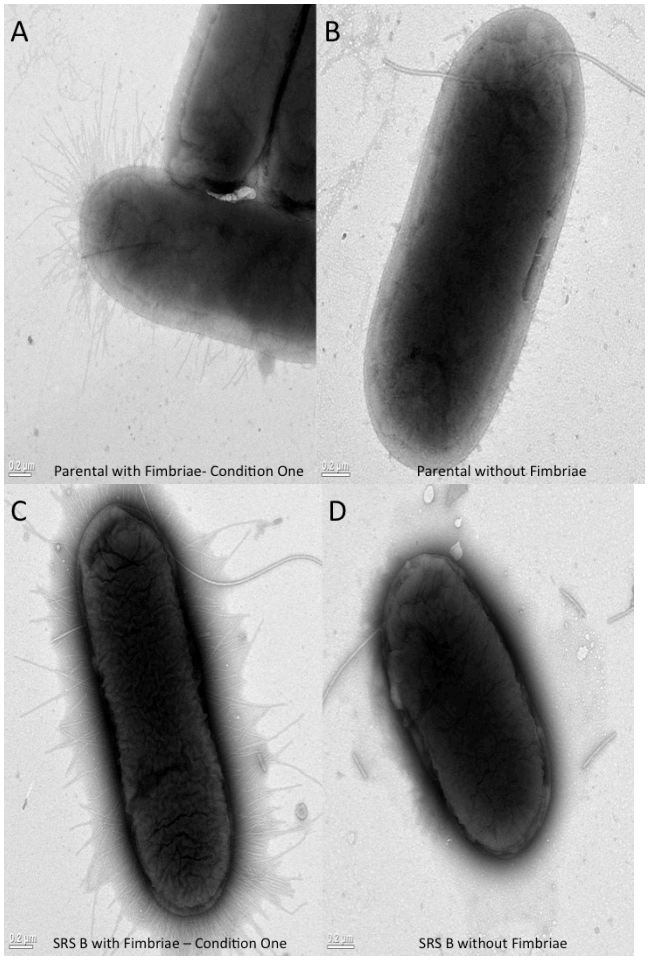
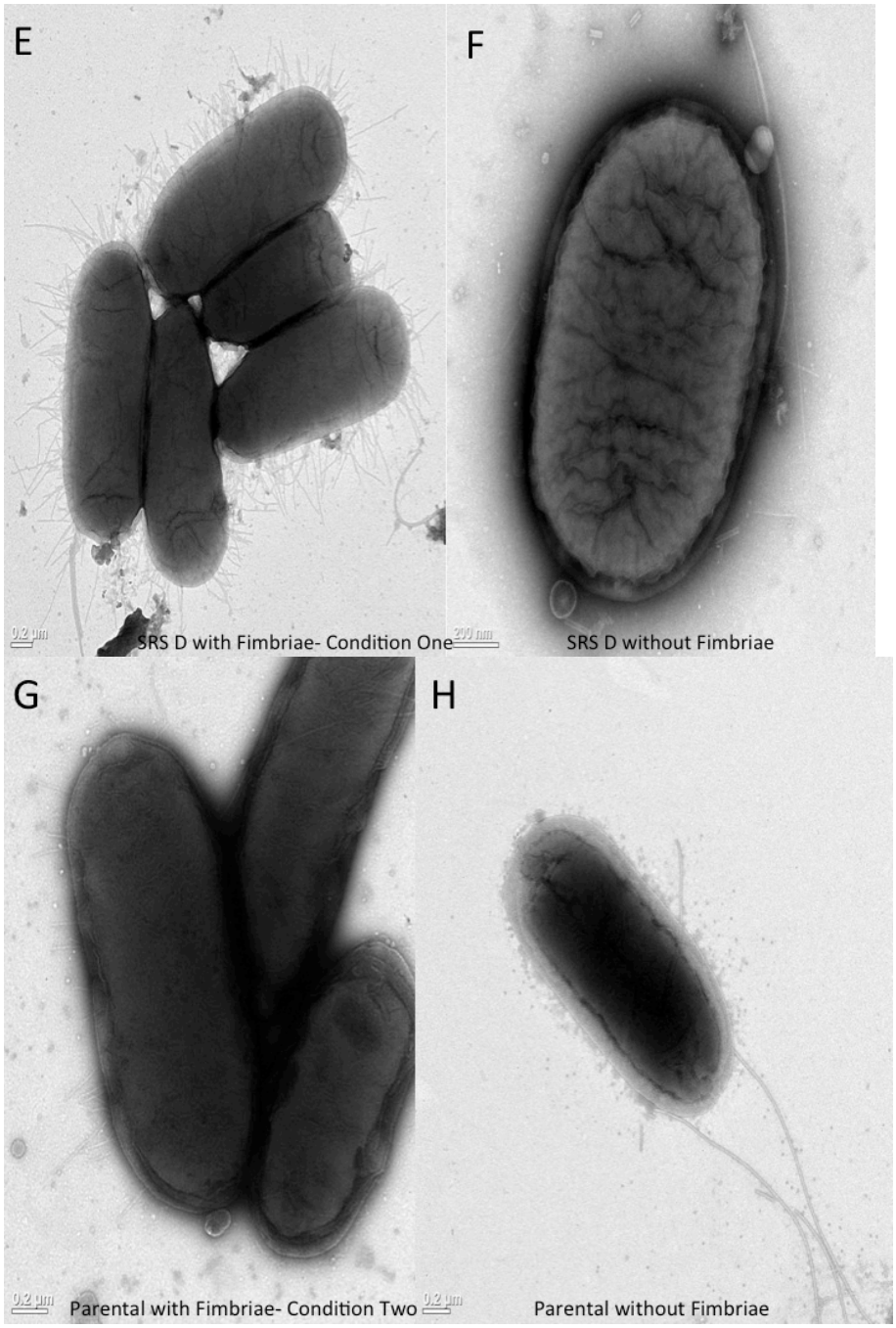


Figure 13: Transmission Electron Microscopy of Parental and SRS Strains. Strains were grown under the two conditions used in this study. Fimbriae were microscopically observed using TEM. (A-F were strains in condition one, G-L were in condition two) (A) Parental strain with fimbriae. (B) Parental strain without fimbriae. (C) SRS B with fimbriae. (D) SRS B without fimbriae. (E) SRS D with fimbriae. (F) SRS D without fimbriae. (G) Parental strain with fimbriae. (H) Parental strain without fimbriae. (I) SRS B with fimbriae. (J) SRS B without fimbriae. (K) SRS D with fimbriae. (L) SRS D without fimbriae. (M) Magnified image of non-curli fimbriae on SRS D. All fimbriae observed were not curli fimbriae.



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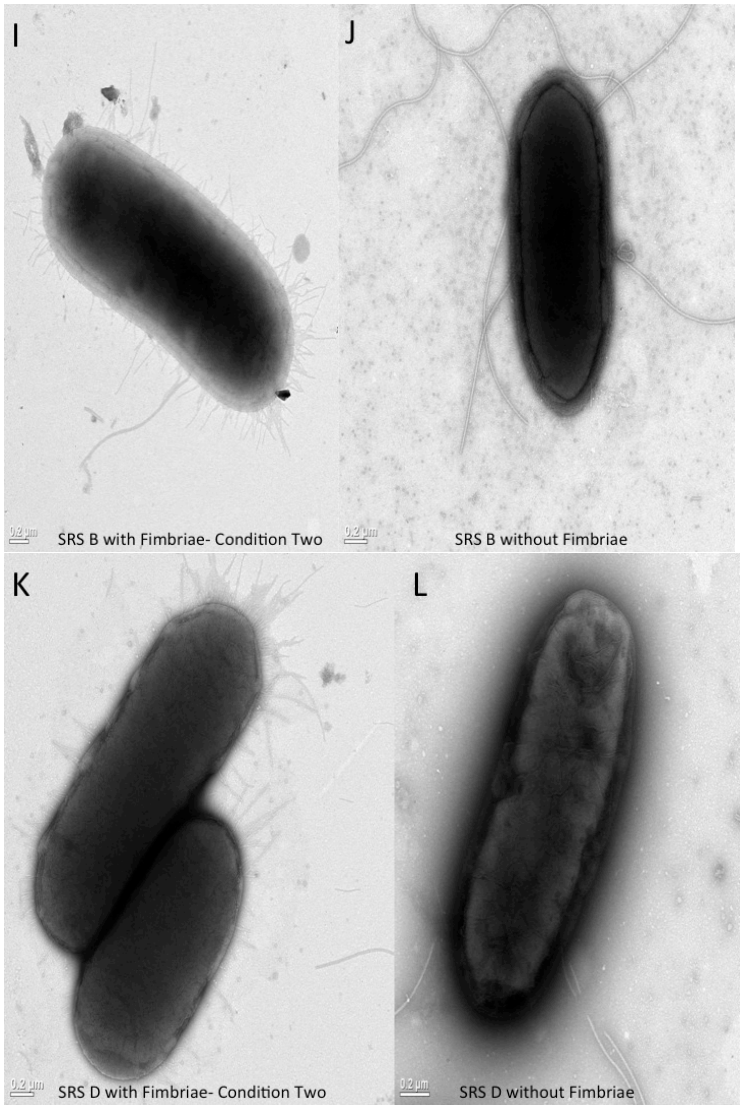

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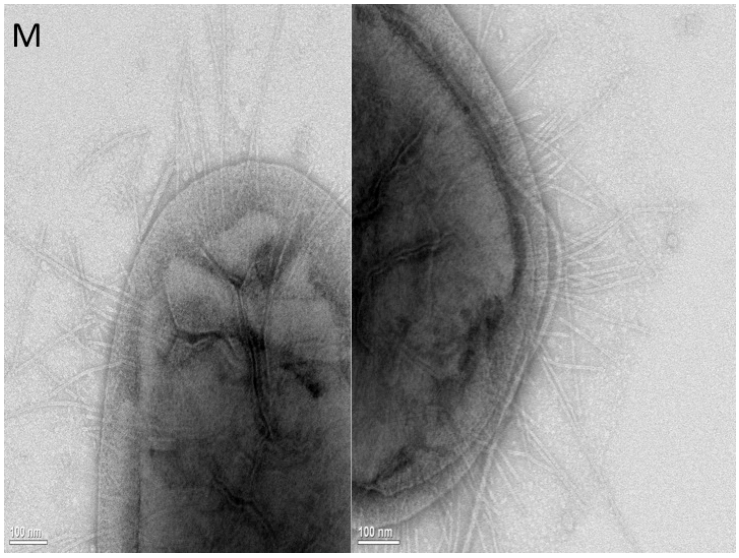

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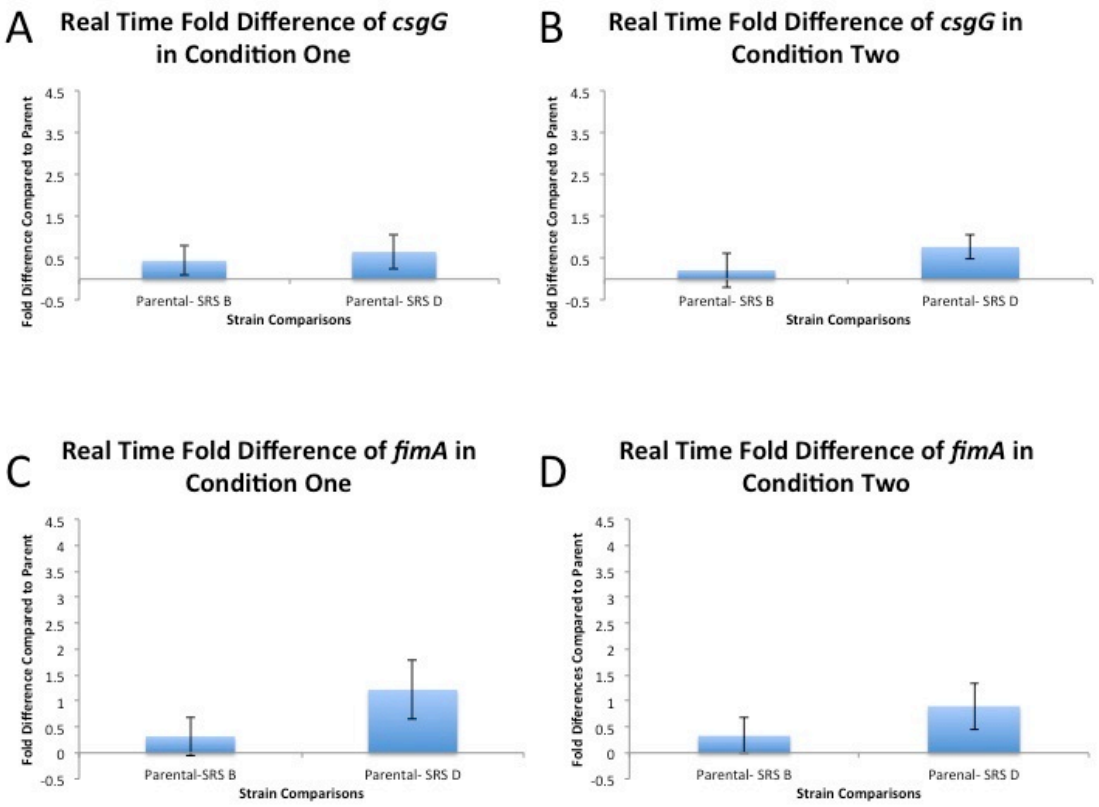

Figure 14: Fold Differences of Fimbriae Genes. Quantitative Real Time PCR was performed to determine the levels of mRNA for two fimbriae genes; *fimA* (type 1 fimbriae) and *csgG* (curli fimbriae). mRNA levels of each gene were the same for all strains. Each experiment was setup in quadruple and repeated three times. Error bars represent standard deviation.

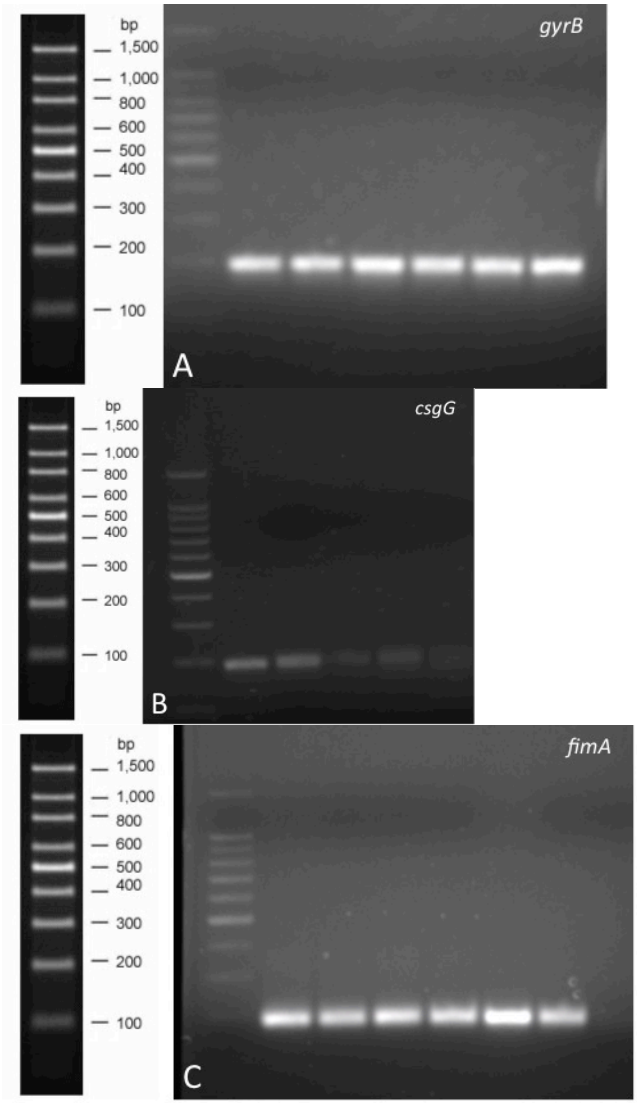

Figure 15: PCR Products. PCR products were ran on a 2% agarose gel with ethidium bromide. (A) PCR products of the housekeeping control gene *gyrB* with base pair length of 195. (B) PCR products of curli fimbriae gene *csgG* with base pair length of 190. (C) PCR products of type 1 fimbriae gene *fimA* with base pair length of 103.

REFERENCES

1. Dibb-Fuller, M.P., et al., *Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by Salmonella enteritidis.* Microbiology-Uk, 1999. **145**: p. 1023-1031.
2. Aiello, A.E., et al., *Relationship between triclosan and susceptibilities of bacteria isolated from hands in the community.* Antimicrobial Agents and Chemotherapy, 2004. **48**(8): p. 2973-2979.
3. Humphries, A., S. DeRidder, and A.J. Baumler, *Salmonella enterica serotype typhimurium fimbrial proteins serve as antigens during infection of mice.* Infection and Immunity, 2005. **73**(9): p. 5329-5338.
4. White, A.P., et al., *Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella.* Journal of Bacteriology, 2006. **188**(9): p. 3219-3227.
5. Castelijn, G.A.A., et al., *Diversity in biofilm formation and production of curli fimbriae and cellulose of Salmonella Typhimurium strains of different origin in high and low nutrient medium.* Biofouling, 2012. **28**(1): p. 51-63.
6. Levy, S.B., *Antibacterial household products: Cause for concern.* Emerging Infectious Diseases, 2001. **7**(3): p. 512-515.
7. Webber, M.A., et al., *Triclosan resistance in Salmonella enterica serovar Typhimurium.* Journal of Antimicrobial Chemotherapy, 2008. **62**(1): p. 83-91.
8. Kuijpers, A.F.A. and K.A. Mooijman, *Detection of Salmonella in food, feed and veterinary samples by EU laboratories.* Food Research International, 2012. **45**(2): p. 885-890.
9. Baumler, A.J., R.M. Tsolis, and F. Heffron, *Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by Salmonella typhimurium.* Infection and Immunity, 1996. **64**(5): p. 1862-1865.
10. Muller, K.H., et al., *TYPE-1 FIMBRIAE OF SALMONELLA-ENTERITIDIS.* Journal of Bacteriology, 1991. **173**(15): p. 4765-4772.
11. Chu, D. and D.J. Barnes, *Modeling fimbriae mediated parasite-host interactions.* Journal of Theoretical Biology, 2010. **264**(4): p. 1169-1176.
12. Althouse, C., et al., *Type 1 fimbriae of Salmonella enterica serovar typhimurium bind to enterocytes and contribute to colonization of swine in vivo.* Infection and Immunity, 2003. **71**(11): p. 6446-6452.
13. Clayton, D.J., et al., *Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by Salmonella enterica serovar Enteritidis reveals a role for a novel locus.* Bmc Microbiology, 2008. **8**.
14. van der Velden, A.W.M., et al., *Multiple fimbrial adhesins are required for full virulence of Salmonella typhimurium in mice.* Infection and Immunity, 1998. **66**(6): p. 2803-2808.

15. Saldana, Z., et al., *Synergistic role of curli and cellulose in cell adherence and biofilm formation of attaching and effacing Escherichia coli and identification of Fis as a negative regulator of curli.* Environmental Microbiology, 2009. **11**(4): p. 992-1006.
16. Lane, M.C., A.N. Simms, and H.L.T. Mobley, *Complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic Escherichia coli.* Journal of Bacteriology, 2007. **189**(15): p. 5523-5533.
17. Romling, U. and M. Rohde, *Flagella modulate the multicellular behavior of Salmonella typhimurium on the community level.* Fems Microbiology Letters, 1999. **180**(1): p. 91-102.
18. Olsen, A., A. Jonsson, and S. Normark, *Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli.* Nature, 1989. **338**(6217): p. 652-655.
19. Allen-Vercoe, E. and M.J. Woodward, *The role of flagella, but not fimbriae, in the adherence of Salmonella enterica serotype Enteritidis to chick gut explant.* Journal of Medical Microbiology, 1999. **48**(8): p. 771-780.
20. Gibson, D.L., et al., *AgfC and AgfE facilitate extracellular thin aggregative fimbriae synthesis in Salmonella Enteritidis.* Microbiology-Sgm, 2007. **153**: p. 1131-1140.
21. Ugorski, M., D. Kisiela, and A. Wieliczko, *Fimbriae of Salmonella enterica serovar Enteritidis.* Medycyna Weterynaryjna, 2001. **57**(10): p. 714-718.
22. Wagner, C. and M. Hensel, *Adhesive Mechanisms of Salmonella enterica*, in *Bacterial Adhesion: Chemistry, Biology and Physics*, D.G.A. Linke, Editor 2011. p. 17-34.
23. Goller, C.C. and T. Romeo, *Environmental influences on biofilm development.* Bacterial Biofilms, 2008. **322**: p. 37-66.
24. Thanassi, D.G. and S.J. Hultgren, *Assembly of complex organelles: Pilus biogenesis in Gram-negative bacteria as a model system.* Methods-a Companion to Methods in Enzymology, 2000. **20**(1): p. 111-126.
25. Buchanan, K., et al., *Frequenc among Enterobacteriaceae of the DNA sequences- sequences encoding type 1 pili* .Journal of Bacteriology, 1985. **162**(2): p. 799-803.
26. Korhonen, T.K., et al., *Characterization of type 1 pili of Salmonella – typhimurium LT2.* Journal of Bacteriology, 1980. **144**(2): p. 800-805.
27. Ugorski, M., M. Kuzminska-Bajor, and D. Kisiela, *The role of type 1 fimbriae in Salmonella infections.* Postepy Mikrobiologii, 2011. **50**(1): p. 59-68.
28. Saini, S., J.A. Pearl, and C.V. Rao, *Role of FimW, FimY, and FimZ in Regulating the Expression of Type I Fimbriae in Salmonella enterica Serovar Typhimurium.* Journal of Bacteriology, 2009. **191**(9): p. 3003-3010.
29. Chuang, Y.-C., et al., *Identification of the genetic determinants of Salmonella enterica serotype Typhimurium that may regulate the expression of the type 1 fimbriae in response to solid agar and static broth culture conditions.* Bmc Microbiology, 2008. **8**.

30. Doran, J.L., et al., *FimA and TCTC based DNA diagnostics for Salmonella.* Molecular and Cellular Probes, 1994. **8**(4): p. 291-310.
31. Dwyer, B.E., et al., *Single nucleotide polypmorphisms of fimH associated with adherence and biofilm formation by serovars of Salmonella enterica.* Microbiology-Sgm, 2011. **157**: p. 3162-3171.
32. Hakalehto, E., et al., *Aerobic and anaerobic growth modes and expression of type 1 fimbriae in Salmonella.* Pathophysiology : the official journal of the International Society for Pathophysiology / ISP, 2007. **14**(1): p. 61-9.
33. Gerstel, U. and U. Romling, *The csgD promoter, a control unit for biofilm formation in Salmonella typhimurium.* Research in Microbiology, 2003. **154**(10): p. 659-667.
34. Romling, U., et al., *Multicellular and aggregative behaviour of Salmonella typhimurium strains is controlled by mutations in the agfD promoter.* Molecular Microbiology, 1998. **28**(2): p. 249-264.
35. Barnhart, M.M. and M.R. Chapman, *Curli biogenesis and function*, in *Annual Review of Microbiology*2006. p. 131-147.
36. Collinson, S.K., et al., *Purification and characterization of thin, aggregative fimbriae from Salmonella enteritidis.* Journal of Bacteriology, 1991. **173**(15): p. 4773-4781.
37. Goulter, R.M., I.R. Gentle, and G.A. Dykes, *Characterisation of Curli Production, Cell Surface Hydrophobicity, Autoaggregation and Attachment Behaviour of Escherichia coli O157.* Current Microbiology, 2010. **61**(3): p. 157-162.
38. Taylor, J.D., et al., *Atomic Resolution Insights into Curli Fiber Biogenesis.* Structure, 2011. **19**(9): p. 1307-1316.
39. Dueholm, M.S., et al., *Fibrillation of the Major Curli Subunit CsgA under a Wide Range of Conditions Implies a Robust Design of Aggregation.* Biochemistry, 2011. **50**(39): p. 8281-8290.
40. Maurer, J.J., et al., *The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin Tsh among avian Escherichia coli.* Avian Diseases, 1998. **42**(1): p. 106-118.
41. Bougdour, A., C. Lelong, and J. Geiselmann, *Crl, a low temperature-induced protein in Escherichia coli that binds directly to the stationary phase sigma subunit of RNA polymerase.* Journal of Biological Chemistry, 2004. **279**(19): p. 19540-19550.
42. Gerstel, U., C. Park, and U. Romling, *Complex regulation of csgD promoter activity by global regulatory proteins.* Molecular Microbiology, 2003. **49**(3): p. 639-654.
43. Robinson, L.S., et al., *Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein.* Molecular Microbiology, 2006. **59**(3): p. 870-881.
44. Wang, X. and M.R. Chapman, *Curli provide the template for understanding controlled amyloid propagation.* Prion, 2008. **2**(2): p. 57-60.

45. Blanco, L.P., et al., *Diversity, biogenesis and function of microbial amyloids.* Trends in Microbiology, 2012. **20**(2): p. 66-73.
46. Epstein, E.A. and M.R. Chapman, *Polymerizing the fibre between bacteria and host cells: the biogenesis of functional amyloid fibres.* Cellular Microbiology, 2008. **10**(7): p. 1413-1420.
47. Gebbink, M., et al., *Amyloids - A functional coat for microorganisms.* Nature Reviews Microbiology, 2005. **3**(4): p. 333-341.
48. Lim, J.Y., J.M. May, and L. Cegelski, *Dimethyl Sulfoxide and Ethanol Elicit Increased Amyloid Biogenesis and Amyloid-Integrated Biofilm Formation in Escherichia coli.* Applied and environmental microbiology, 2012. **78**(9): p. 3369-78.
49. Romling, U., et al., *Occurrence and regulation of the multicellular morphotype in Salmonella serovars important in human disease.* International Journal of Medical Microbiology, 2003. **293**(4): p. 273-285.
50. Bieler, S., et al., *Amyloid formation modulates the biological activity of a bacterial protein.* Journal of Biological Chemistry, 2005. **280**(29): p. 26880-26885.
51. Larsen, P., et al., *Amyloid adhesins are abundant in natural biofilms.* Environmental Microbiology, 2007. **9**(12): p. 3077-3090.
52. Castonguay, M.H., et al., *Biofilm formation by Escherichia coli is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria.* Research in Microbiology, 2006. **157**(5): p. 471-478.
53. Smith, K. and I.S. Hunter, *Efficacy of common hospital blocides with biofilms of multi-drug resistant clinical isolates.* Journal of Medical Microbiology, 2008. **57**(8): p. 966-973.
54. Legendre, G., et al., *Evaluation of Antibacterial Activity against Salmonella Enteritidis.* Journal of Microbiology, 2011. **49**(3): p. 349-354.
55. Angel Fernandez-Fuentes, M., et al., *Isolation and identification of bacteria from organic foods: Sensitivity to biocides and antibiotics.* Food Control, 2012. **26**(1): p. 73-78.
56. White, D.G. and P.F. McDermott, *Biocides, drug resistance and microbial evolution.* Current Opinion in Microbiology, 2001. **4**(3): p. 313-317.
57. Aiello, A.E. and E. Larson, *Antibacterial cleaning and hygiene products as an emerging risk factor for antibiotic resistance in the community.* Lancet Infectious Diseases, 2003. **3**(8): p. 501-506.
58. Russell, A.D., *Mechanisms of bacterial resistance to antibiotics and biocides.* Progress in medicinal chemistry, 1998. **35**: p. 133-97.
59. Braoudaki, M. and A.C. Hilton, *Adaptive resistance to biocides in Salmonella enterica and Escherichia coli O157 and cross-resistance to antimicrobial agents.* Journal of Clinical Microbiology, 2004. **42**(1): p. 73-78.
60. Karatzas, K.A.G., et al., *Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic*

resistance, increased efflux and reduced invasiveness. Journal of Antimicrobial Chemotherapy, 2007. **60**(5): p. 947-955.
61. Escalada, M.G., et al., *Triclosan-bacteria interactions: single or multiple target sites?* Letters in Applied Microbiology, 2005. **41**(6): p. 476-481.
62. Levy, S.B., *Active efflux, a common mechanism for biocide and antibiotic resistance.* Journal of Applied Microbiology, 2002. **92**: p. 65S-71S.
63. Thorrold, C.A., et al., *Efflux pump activity in fluoroquinolone and tetracycline resistant Salmonella and E. coli implicated in reduced susceptibility to household antimicrobial cleaning agents.* International Journal of Food Microbiology, 2007. **113**(3): p. 315-320.
64. Braoudaki, M. and A.C. Hilton, *Mechanisms of resistance in Salmonella enterica adapted to erythromycin, benzalkonium chloride and triclosan.* International Journal of Antimicrobial Agents, 2005. **25**(1): p. 31-37.
65. Martins, M., et al., *Mechanisms of Antibiotic Resistance in Salmonella: Efflux Pumps, Genetics, Quorum Sensing and Biofilm Formation.* Letters in Drug Design & Discovery, 2011. **8**(2): p. 114-123.
66. Moretro, T., et al., *Control of Salmonella in food related environments by chemical disinfection.* Food Research International, 2012. **45**(2): p. 532-544.
67. Birosova, L. and M. Mikulasova, *Development of triclosan and antibiotic resistance in Salmonella enterica serovar Typhimurium.* Journal of Medical Microbiology, 2009. **58**(4): p. 436-441.
68. Bailey, A.M., et al., *Exposure of Escherichia coli and Salmonella enterica serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification.* Journal of Antimicrobial Chemotherapy, 2009. **64**(5): p. 973-985.
69. Saleh, S., et al., *Triclosan - an update.* Letters in Applied Microbiology, 2011. **52**(2): p. 87-95.
70. Yazdankhah, S.P., et al., *Triclosan and antimicrobial resistance in bacteria: An overview.* Microbial Drug Resistance-Mechanisms Epidemiology and Disease, 2006. **12**(2): p. 83-90.
71. Hay, A.G., P.M. Dees, and G.S. Sayler, *Growth of a bacterial consortium on triclosan.* Fems Microbiology Ecology, 2001. **36**(2-3): p. 105-112.
72. Moretro, T., et al., *Assessment of the antibacterial activity of a triclosan-containing cutting board.* International Journal of Food Microbiology, 2011. **146**(2): p. 157-162.
73. Russell, A.D., *Whither triclosan?* Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 693-695.
74. Chapman, J.S., *Disinfectant resistance mechanisms, cross-resistance, and co-resistance.* International Biodeterioration & Biodegradation, 2003. **51**(4): p. 271-276.
75. McBain, A.J., et al., *Selection for high-level resistance by chronic triclosan exposure is not universal.* Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 772-777.

76. Cottell, A., et al., *Triclosan-tolerant bacteria: changes in susceptibility to antibiotics.* Journal of Hospital Infection, 2009. **72**(1): p. 71-76.
77. Meyer, B. and B. Cookson, *Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control?* Journal of Hospital Infection, 2010. **76**(3): p. 200-205.
78. Russell, A.D., *Mechanisms of bacterial resistance to biocides.* International Biodeterioration & Biodegradation, 1995. **36**(3-4): p. 247-265.
79. D'Arezzo, S., et al., *High-level tolerance to triclosan may play a role in Pseudomonas aeruginosa antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation.* BMC research notes, 2012. **5**: p. 43.
80. Gilbert, P. and A.J. McBain, *Biocide usage in the domestic setting and concern about antibacterial and antibiotic resistance.* Journal of Infection, 2001. **43**(1): p. 85-91.
81. Kumar, C.G. and S.K. Anand, *Significance of microbial biofilms in food industry: a review.* International Journal of Food Microbiology, 1998. **42**(1-2): p. 9-27.
82. Mah, T.F.C. and G.A. O'Toole, *Mechanisms of biofilm resistance to antimicrobial agents.* Trends in Microbiology, 2001. **9**(1): p. 34-39.
83. O'Toole, G., H.B. Kaplan, and R. Kolter, *Biofilm formation as microbial development.* Annual Review of Microbiology, 2000. **54**: p. 49-79.
84. Crull, K., et al., *Biofilm formation by Salmonella enterica serovar Typhimurium colonizing solid tumours.* Cellular Microbiology, 2011. **13**(8): p. 1223-1233.
85. Merritt, J.H., D.E. Kadouri, and G.A. O'Toole, *Growing and analyzing static biofilms.* Current protocols in microbiology, 2005. **Chapter 1**.
86. Smirnova, T.A., et al., *Study of the Structures of Biofilms Formed by Salmonella typhimurium Bacteria on Abiotic Surfaces by the Methods of Light and Transmission Electron Microscopy.* Applied Biochemistry and Microbiology, 2010. **46**(7): p. 706-711.
87. Donlan, R.M. and J.W. Costerton, *Biofilms: Survival mechanisms of clinically relevant microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-+.
88. Steenackers, H., et al., *Salmonella biofilms: An overview on occurrence, structure, regulation and eradication.* Food Research International, 2012. **45**(2): p. 502-531.
89. Gilbert, P. and A.J. McBain, *Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance.* Clinical Microbiology Reviews, 2003. **16**(2): p. 189-+.
90. Xu, H., et al., *Effect of NaCl on the Biofilm Formation by Foodborne Pathogens.* Journal of Food Science, 2010. **75**(9): p. M580-M585.
91. Speranza, B., M.R. Corbo, and M. Sinigaglia, *Effects of Nutritional and Environmental Conditions on Salmonella sp Biofilm Formation.* Journal of Food Science, 2011. **76**(1): p. M12-M16.

92. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: A common cause of persistent infections.* Science, 1999. **284**(5418): p. 1318-1322.
93. Van Houdt, R. and C.W. Michiels, *Biofilm formation and the food industry, a focus on the bacterial outer surface.* Journal of Applied Microbiology, 2010. **109**(4): p. 1117-1131.
94. Condell, O., et al., *Efficacy of Biocides Used in the Modern Food Industry To Control Salmonella enterica, and Links between Biocide Tolerance and Resistance to Clinically Relevant Antimicrobial Compounds.* Applied and environmental microbiology, 2012. **78**(9): p. 3087-97.
95. Costerton, J.W., *Introduction to biofilm.* International Journal of Antimicrobial Agents, 1999. **11**(3-4): p. 217-221.
96. Kaplan, J.B., *Methods for the treatment and opinion prevention of bacterial biofilms.* Expert Opinion on Therapeutic Patents, 2005. **15**(8): p. 955-965.
97. Russell, A.D., *Bacterial resistance to disinfectants: present knowledge and future problems.* Journal of Hospital Infection, 1999. **43**: p. S57-S68.
98. Aiello, A.E., E.L. Larson, and S.B. Levy, *Consumer antibacterial soaps: Effective or just risky?* Clinical Infectious Diseases, 2007. **45**: p. S137-S147.
99. Wong, H.S., et al., *Comparative susceptibility of Salmonella Typhimurium biofilms of different ages to disinfectants.* Biofouling, 2010. **26**(7): p. 859-64.
100. Rodrigues, D., et al., *Salmonella enterica Enteritidis Biofilm Formation and Viability on Regular and Triclosan-Impregnated Bench Cover Materials.* Journal of Food Protection, 2011. **74**(1): p. 32-37.
101. Tabak, M., et al., *Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms.* Fems Microbiology Letters, 2007. **267**(2): p. 200-206.
102. Romling, U., et al., *Curli fibers are highly conserved between Salmonella typhimurium and Escherichia coli with respect to operon structure and regulation.* Journal of Bacteriology, 1998. **180**(3): p. 722-731.
103. Bokranz, W., et al., *Expression of cellulose and curli fimbriae by Escherichia coli isolated from the gastrointestinal tract.* Journal of Medical Microbiology, 2005. **54**(12): p. 1171-1182.
104. Jonas, K., et al., *Roles of curli, cellulose and BapA in Salmonella biofilm morphology studied by atomic force microscopy.* Bmc Microbiology, 2007. **7**.
105. Hammar, M., et al., *Expression of two csg operons is required for production of fibronectin- and Congo red-binding curli polymers in Escherichia coli K-12.* Molecular Microbiology, 1995. **18**(4): p. 661-670.
106. Zogaj, X., et al., *Production of cellulose and curli fimbriae by members of the family Enterobacteriaceae isolated from the human gastrointestinal tract.* Infection and Immunity, 2003. **71**(7): p. 4151-4158.
107. Romling, U., *Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae.* Cellular and Molecular Life Sciences, 2005. **62**(11): p. 1234-1246.

108. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Research, 2001. **29**(9).
109. Russell, A.D. and M.J. Day, *Antibiotic and biocide resistance in bacteria.* Microbios, 1996. **85**(342): p. 45-65.
110. Russell, A.D., *Do biocides select for antibiotic resistance?* Journal of Pharmacy and Pharmacology, 2000. **52**(2): p. 227-233.
111. Gerstel, U. and U. Romling, *Oxygen tension and nutrient starvation are major signals that regulate agfD promoter activity and expression of the multicellular morphotype in Salmonella typhimurium.* Environmental Microbiology, 2001. **3**(10): p. 638-648.
112. Olsen, A., et al., *Environmental regulation of curli production in Escherichia coli.* Infectious Agents and Disease-Reviews Issues and Commentary, 1993. **2**(4): p. 272-274.
113. Kolter, R., D.A. Siegele, and A. Tormo, *The stationary phase of the bacterial life cycle.* Annual Review of Microbiology, 1993. **47**: p. 855-874.
114. White, A.P., et al., *Structure and characterization of AgfB from Salmonella enteritidis thin aggregative fimbriae.* Journal of Molecular Biology, 2001. **311**(4): p. 735-749.

1. Dibb-Fuller, M.P., et al., *Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by Salmonella enteritidis.* Microbiology-Uk, 1999. **145**: p. 1023-1031.
2. Aiello, A.E., et al., *Relationship between triclosan and susceptibilities of bacteria isolated from hands in the community.* Antimicrobial Agents and Chemotherapy, 2004. **48**(8): p. 2973-2979.
3. Humphries, A., S. DeRidder, and A.J. Baumler, *Salmonella enterica serotype typhimurium fimbrial proteins serve as antigens during infection of mice.* Infection and Immunity, 2005. **73**(9): p. 5329-5338.
4. White, A.P., et al., *Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella.* Journal of Bacteriology, 2006. **188**(9): p. 3219-3227.
5. Castelijn, G.A.A., et al., *Diversity in biofilm formation and production of curli fimbriae and cellulose of Salmonella Typhimurium strains of different origin in high and low nutrient medium.* Biofouling, 2012. **28**(1): p. 51-63.
6. Levy, S.B., *Antibacterial household products: Cause for concern.* Emerging Infectious Diseases, 2001. **7**(3): p. 512-515.
7. Webber, M.A., et al., *Triclosan resistance in Salmonella enterica serovar Typhimurium.* Journal of Antimicrobial Chemotherapy, 2008. **62**(1): p. 83-91.
8. Kuijpers, A.F.A. and K.A. Mooijman, *Detection of Salmonella in food, feed and veterinary samples by EU laboratories.* Food Research International, 2012. **45**(2): p. 885-890.

9. Baumler, A.J., R.M. Tsolis, and F. Heffron, *Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by Salmonella typhimurium.* Infection and Immunity, 1996. **64**(5): p. 1862-1865.
10. Willey, J.M., Sherwood, L.M. and Woolverton, C.J., *Microbiology.* 2008.
11. Muller, K.H., et al., *TYPE-1 FIMBRIAE OF SALMONELLA-ENTERITIDIS.* Journal of Bacteriology, 1991. **173**(15): p. 4765-4772.
12. Chu, D. and D.J. Barnes, *Modeling fimbriae mediated parasite-host interactions.* Journal of Theoretical Biology, 2010. **264**(4): p. 1169-1176.
13. Althouse, C., et al., *Type 1 fimbriae of Salmonella enterica serovar typhimurium bind to enterocytes and contribute to colonization of swine in vivo.* Infection and Immunity, 2003. **71**(11): p. 6446-6452.
14. Clayton, D.J., et al., *Analysis of the role of 13 major fimbrial subunits in colonisation of the chicken intestines by Salmonella enterica serovar Enteritidis reveals a role for a novel locus.* Bmc Microbiology, 2008. **8**.
15. van der Velden, A.W.M., et al., *Multiple fimbrial adhesins are required for full virulence of Salmonella typhimurium in mice.* Infection and Immunity, 1998. **66**(6): p. 2803-2808.
16. Saldana, Z., et al., *Synergistic role of curli and cellulose in cell adherence and biofilm formation of attaching and effacing Escherichia coli and identification of Fis as a negative regulator of curli.* Environmental Microbiology, 2009. **11**(4): p. 992-1006.
17. Lane, M.C., A.N. Simms, and H.L.T. Mobley, *Complex interplay between type 1 fimbrial expression and flagellum-mediated motility of uropathogenic Escherichia coli.* Journal of Bacteriology, 2007. **189**(15): p. 5523-5533.
18. Romling, U. and M. Rohde, *Flagella modulate the multicellular behavior of Salmonella typhimurium on the community level.* Fems Microbiology Letters, 1999. **180**(1): p. 91-102.
19. Olsen, A., A. Jonsson, and S. Normark, *FIBRONECTIN BINDING MEDIATED BY A NOVEL CLASS OF SURFACE ORGANELLES ON ESCHERICHIA-COLI.* Nature, 1989. **338**(6217): p. 652-655.
20. Allen-Vercoe, E. and M.J. Woodward, *The role of flagella, but not fimbriae, in the adherence of Salmonella enterica serotype Enteritidis to chick gut explant.* Journal of Medical Microbiology, 1999. **48**(8): p. 771-780.
21. Gibson, D.L., et al., *AgfC and AgfE facilitate extracellular thin aggregative fimbriae synthesis in Salmonella Enteritidis.* Microbiology-Sgm, 2007. **153**: p. 1131-1140.
22. Ugorski, M., D. Kisiela, and A. Wieliczko, *Fimbriae of Salmonella enterica serovar Enteritidis.* Medycyna Weterynaryjna, 2001. **57**(10): p. 714-718.
23. Wagner, C. and M. Hensel, *Adhesive Mechanisms of Salmonella enterica*, in *Bacterial Adhesion: Chemistry, Biology and Physics*, D.G.A. Linke, Editor 2011. p. 17-34.
24. Goller, C.C. and T. Romeo, *Environmental influences on biofilm development.* Bacterial Biofilms, 2008. **322**: p. 37-66.

25. Thanassi, D.G. and S.J. Hultgren, *Assembly of complex organelles: Pilus biogenesis in Gram-negative bacteria as a model system.* Methods-a Companion to Methods in Enzymology, 2000. **20**(1): p. 111-126.
26. Buchanan, K., et al., *FREQUENCY AMONG ENTEROBACTERIACEAE OF THE DNA-SEQUENCES ENCODING TYPE-1 PILI.* Journal of Bacteriology, 1985. **162**(2): p. 799-803.
27. Korhonen, T.K., et al., *CHARACTERIZATION OF TYPE-1 PILI OF SALMONELLA-TYPHIMURIUM LT2.* Journal of Bacteriology, 1980. **144**(2): p. 800-805.
28. Ugorski, M., M. Kuzminska-Bajor, and D. Kisiela, *The role of type 1 fimbriae in Salmonella infections.* Postepy Mikrobiologii, 2011. **50**(1): p. 59-68.
29. Saini, S., J.A. Pearl, and C.V. Rao, *Role of FimW, FimY, and FimZ in Regulating the Expression of Type I Fimbriae in Salmonella enterica Serovar Typhimurium.* Journal of Bacteriology, 2009. **191**(9): p. 3003-3010.
30. Chuang, Y.-C., et al., *Identification of the genetic determinants of Salmonella enterica serotype Typhimurium that may regulate the expression of the type 1 fimbriae in response to solid agar and static broth culture conditions.* Bmc Microbiology, 2008. **8**.
31. Doran, J.L., et al., *FIMA AND TCTC BASED DNA DIAGNOSTICS FOR SALMONELLA.* Molecular and Cellular Probes, 1994. **8**(4): p. 291-310.
32. Dwyer, B.E., et al., *Single nucleotide polypmorphisms of fimH associated with adherence and biofilm formation by serovars of Salmonella enterica.* Microbiology-Sgm, 2011. **157**: p. 3162-3171.
33. Hakalehto, E., et al., *Aerobic and anaerobic growth modes and expression of type 1 fimbriae in Salmonella.* Pathophysiology : the official journal of the International Society for Pathophysiology / ISP, 2007. **14**(1): p. 61-9.
34. Gerstel, U. and U. Romling, *The csgD promoter, a control unit for biofilm formation in Salmonella typhimurium.* Research in Microbiology, 2003. **154**(10): p. 659-667.
35. Romling, U., et al., *Multicellular and aggregative behaviour of Salmonella typhimurium strains is controlled by mutations in the agfD promoter.* Molecular Microbiology, 1998. **28**(2): p. 249-264.
36. Barnhart, M.M. and M.R. Chapman, *Curli biogenesis and function*, in *Annual Review of Microbiology*2006. p. 131-147.
37. Collinson, S.K., et al., *PURIFICATION AND CHARACTERIZATION OF THIN, AGGREGATIVE FIMBRIAE FROM SALMONELLA-ENTERITIDIS.* Journal of Bacteriology, 1991. **173**(15): p. 4773-4781.
38. Goulter, R.M., I.R. Gentle, and G.A. Dykes, *Characterisation of Curli Production, Cell Surface Hydrophobicity, Autoaggregation and Attachment Behaviour of Escherichia coli O157.* Current Microbiology, 2010. **61**(3): p. 157-162.
39. Taylor, J.D., et al., *Atomic Resolution Insights into Curli Fiber Biogenesis.* Structure, 2011. **19**(9): p. 1307-1316.

40. Dueholm, M.S., et al., *Fibrillation of the Major Curli Subunit CsgA under a Wide Range of Conditions Implies a Robust Design of Aggregation.* Biochemistry, 2011. **50**(39): p. 8281-8290.
41. Maurer, J.J., et al., *The occurrence of ambient temperature-regulated adhesins, curli, and the temperature-sensitive hemagglutinin Tsh among avian Escherichia coli.* Avian Diseases, 1998. **42**(1): p. 106-118.
42. Bougdour, A., C. Lelong, and J. Geiselmann, *Crl, a low temperature-induced protein in Escherichia coli that binds directly to the stationary phase sigma subunit of RNA polymerase.* Journal of Biological Chemistry, 2004. **279**(19): p. 19540-19550.
43. Gerstel, U., C. Park, and U. Romling, *Complex regulation of csgD promoter activity by global regulatory proteins.* Molecular Microbiology, 2003. **49**(3): p. 639-654.
44. Robinson, L.S., et al., *Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein.* Molecular Microbiology, 2006. **59**(3): p. 870-881.
45. Wang, X. and M.R. Chapman, *Curli provide the template for understanding controlled amyloid propagation.* Prion, 2008. **2**(2): p. 57-60.
46. Blanco, L.P., et al., *Diversity, biogenesis and function of microbial amyloids.* Trends in Microbiology, 2012. **20**(2): p. 66-73.
47. Epstein, E.A. and M.R. Chapman, *Polymerizing the fibre between bacteria and host cells: the biogenesis of functional amyloid fibres.* Cellular Microbiology, 2008. **10**(7): p. 1413-1420.
48. Gebbink, M., et al., *Amyloids - A functional coat for microorganisms.* Nature Reviews Microbiology, 2005. **3**(4): p. 333-341.
49. Lim, J.Y., J.M. May, and L. Cegelski, *Dimethyl Sulfoxide and Ethanol Elicit Increased Amyloid Biogenesis and Amyloid-Integrated Biofilm Formation in Escherichia coli.* Applied and environmental microbiology, 2012. **78**(9): p. 3369-78.
50. Bieler, S., et al., *Amyloid formation modulates the biological activity of a bacterial protein.* Journal of Biological Chemistry, 2005. **280**(29): p. 26880-26885.
51. Larsen, P., et al., *Amyloid adhesins are abundant in natural biofilms.* Environmental Microbiology, 2007. **9**(12): p. 3077-3090.
52. Romling, U., et al., *Occurrence and regulation of the multicellular morphotype in Salmonella serovars important in human disease.* International Journal of Medical Microbiology, 2003. **293**(4): p. 273-285.
53. Castonguay, M.H., et al., *Biofilm formation by Escherichia coli is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria.* Research in Microbiology, 2006. **157**(5): p. 471-478.
54. Smith, K. and I.S. Hunter, *Efficacy of common hospital blocides with biofilms of multi-drug resistant clinical isolates.* Journal of Medical Microbiology, 2008. **57**(8): p. 966-973.

55. Legendre, G., et al., *Evaluation of Antibacterial Activity against Salmonella Enteritidis.* Journal of Microbiology, 2011. **49**(3): p. 349-354.
56. Angel Fernandez-Fuentes, M., et al., *Isolation and identification of bacteria from organic foods: Sensitivity to biocides and antibiotics.* Food Control, 2012. **26**(1): p. 73-78.
57. White, D.G. and P.F. McDermott, *Biocides, drug resistance and microbial evolution.* Current Opinion in Microbiology, 2001. **4**(3): p. 313-317.
58. Aiello, A.E. and E. Larson, *Antibacterial cleaning and hygiene products as an emerging risk factor for antibiotic resistance in the community.* Lancet Infectious Diseases, 2003. **3**(8): p. 501-506.
59. Russell, A.D., *Mechanisms of bacterial resistance to antibiotics and biocides.* Progress in medicinal chemistry, 1998. **35**: p. 133-97.
60. Braoudaki, M. and A.C. Hilton, *Adaptive resistance to biocides in Salmonella enterica and Escherichia coli O157 and cross-resistance to antimicrobial agents.* Journal of Clinical Microbiology, 2004. **42**(1): p. 73-78.
61. Karatzas, K.A.G., et al., *Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness.* Journal of Antimicrobial Chemotherapy, 2007. **60**(5): p. 947-955.
62. Escalada, M.G., et al., *Triclosan-bacteria interactions: single or multiple target sites?* Letters in Applied Microbiology, 2005. **41**(6): p. 476-481.
63. Levy, S.B., *Active efflux, a common mechanism for biocide and antibiotic resistance.* Journal of Applied Microbiology, 2002. **92**: p. 65S-71S.
64. Thorrold, C.A., et al., *Efflux pump activity in fluoroquinolone and tetracycline resistant Salmonella and E. coli implicated in reduced susceptibility to household antimicrobial cleaning agents.* International Journal of Food Microbiology, 2007. **113**(3): p. 315-320.
65. Braoudaki, M. and A.C. Hilton, *Mechanisms of resistance in Salmonella enterica adapted to erythromycin, benzalkonium chloride and triclosan.* International Journal of Antimicrobial Agents, 2005. **25**(1): p. 31-37.
66. Martins, M., et al., *Mechanisms of Antibiotic Resistance in Salmonella: Efflux Pumps, Genetics, Quorum Sensing and Biofilm Formation.* Letters in Drug Design & Discovery, 2011. **8**(2): p. 114-123.
67. Moretro, T., et al., *Control of Salmonella in food related environments by chemical disinfection.* Food Research International, 2012. **45**(2): p. 532-544.
68. Birosova, L. and M. Mikulasova, *Development of triclosan and antibiotic resistance in Salmonella enterica serovar Typhimurium.* Journal of Medical Microbiology, 2009. **58**(4): p. 436-441.
69. Bailey, A.M., et al., *Exposure of Escherichia coli and Salmonella enterica serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification.* Journal of Antimicrobial Chemotherapy, 2009. **64**(5): p. 973-985.

70. Saleh, S., et al., *Triclosan - an update.* Letters in Applied Microbiology, 2011. **52**(2): p. 87-95.
71. Yazdankhah, S.P., et al., *Triclosan and antimicrobial resistance in bacteria: An overview.* Microbial Drug Resistance-Mechanisms Epidemiology and Disease, 2006. **12**(2): p. 83-90.
72. Hay, A.G., P.M. Dees, and G.S. Sayler, *Growth of a bacterial consortium on triclosan.* Fems Microbiology Ecology, 2001. **36**(2-3): p. 105-112.
73. Moretro, T., et al., *Assessment of the antibacterial activity of a triclosan-containing cutting board.* International Journal of Food Microbiology, 2011. **146**(2): p. 157-162.
74. Russell, A.D., *Whither triclosan?* Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 693-695.
75. Chapman, J.S., *Disinfectant resistance mechanisms, cross-resistance, and co-resistance.* International Biodeterioration & Biodegradation, 2003. **51**(4): p. 271-276.
76. McBain, A.J., et al., *Selection for high-level resistance by chronic triclosan exposure is not universal.* Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 772-777.
77. Cottell, A., et al., *Triclosan-tolerant bacteria: changes in susceptibility to antibiotics.* Journal of Hospital Infection, 2009. **72**(1): p. 71-76.
78. Meyer, B. and B. Cookson, *Does microbial resistance or adaptation to biocides create a hazard in infection prevention and control?* Journal of Hospital Infection, 2010. **76**(3): p. 200-205.
79. Russell, A.D., *Mechanisms of bacterial resistance to biocides.* International Biodeterioration & Biodegradation, 1995. **36**(3-4): p. 247-265.
80. D'Arezzo, S., et al., *High-level tolerance to triclosan may play a role in Pseudomonas aeruginosa antibiotic resistance in immunocompromised hosts: evidence from outbreak investigation.* BMC research notes, 2012. **5**: p. 43.
81. White, A.P., et al., *Structure and characterization of AgfB from Salmonella enteritidis thin aggregative fimbriae.* Journal of Molecular Biology, 2001. **311**(4): p. 735-749.
82. Aiello, A.E., E.L. Larson, and S.B. Levy, *Consumer antibacterial soaps: Effective or just risky?* Clinical Infectious Diseases, 2007. **45**: p. S137-S147.
83. Gilbert, P. and A.J. McBain, *Biocide usage in the domestic setting and concern about antibacterial and antibiotic resistance.* Journal of Infection, 2001. **43**(1): p. 85-91.
84. Kumar, C.G. and S.K. Anand, *Significance of microbial biofilms in food industry: a review.* International Journal of Food Microbiology, 1998. **42**(1-2): p. 9-27.
85. Mah, T.F.C. and G.A. O'Toole, *Mechanisms of biofilm resistance to antimicrobial agents.* Trends in Microbiology, 2001. **9**(1): p. 34-39.
86. O'Toole, G., H.B. Kaplan, and R. Kolter, *Biofilm formation as microbial development.* Annual Review of Microbiology, 2000. **54**: p. 49-79.

87. Crull, K., et al., *Biofilm formation by Salmonella enterica serovar Typhimurium colonizing solid tumours.* Cellular Microbiology, 2011. **13**(8): p. 1223-1233.
88. Merritt, J.H., D.E. Kadouri, and G.A. O'Toole, *Growing and analyzing static biofilms.* Current protocols in microbiology, 2005. **Chapter 1**.
89. Smirnova, T.A., et al., *Study of the Structures of Biofilms Formed by Salmonella typhimurium Bacteria on Abiotic Surfaces by the Methods of Light and Transmission Electron Microscopy.* Applied Biochemistry and Microbiology, 2010. **46**(7): p. 706-711.
90. Donlan, R.M. and J.W. Costerton, *Biofilms: Survival mechanisms of clinically relevant microorganisms.* Clinical Microbiology Reviews, 2002. **15**(2): p. 167-+.
91. Steenackers, H., et al., *Salmonella biofilms: An overview on occurrence, structure, regulation and eradication.* Food Research International, 2012. **45**(2): p. 502-531.
92. Gilbert, P. and A.J. McBain, *Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance.* Clinical Microbiology Reviews, 2003. **16**(2): p. 189-+.
93. Xu, H., et al., *Effect of NaCl on the Biofilm Formation by Foodborne Pathogens.* Journal of Food Science, 2010. **75**(9): p. M580-M585.
94. Wong, H.S., et al., *Comparative susceptibility of Salmonella Typhimurium biofilms of different ages to disinfectants.* Biofouling, 2010. **26**(7): p. 859-64.
95. Rodrigues, D., et al., *Salmonella enterica Enteritidis Biofilm Formation and Viability on Regular and Triclosan-Impregnated Bench Cover Materials.* Journal of Food Protection, 2011. **74**(1): p. 32-37.
96. Speranza, B., M.R. Corbo, and M. Sinigaglia, *Effects of Nutritional and Environmental Conditions on Salmonella sp Biofilm Formation.* Journal of Food Science, 2011. **76**(1): p. M12-M16.
97. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: A common cause of persistent infections.* Science, 1999. **284**(5418): p. 1318-1322.
98. Van Houdt, R. and C.W. Michiels, *Biofilm formation and the food industry, a focus on the bacterial outer surface.* Journal of Applied Microbiology, 2010. **109**(4): p. 1117-1131.
99. Condell, O., et al., *Efficacy of Biocides Used in the Modern Food Industry To Control Salmonella enterica, and Links between Biocide Tolerance and Resistance to Clinically Relevant Antimicrobial Compounds.* Applied and environmental microbiology, 2012. **78**(9): p. 3087-97.
100. Costerton, J.W., *Introduction to biofilm.* International Journal of Antimicrobial Agents, 1999. **11**(3-4): p. 217-221.
101. Kaplan, J.B., *Methods for the treatment and opinion prevention of bacterial biofilms.* Expert Opinion on Therapeutic Patents, 2005. **15**(8): p. 955-965.

102. Tabak, M., et al., *Effect of triclosan on Salmonella typhimurium at different growth stages and in biofilms.* Fems Microbiology Letters, 2007. **267**(2): p. 200-206.
103. Romling, U., et al., *Curli fibers are highly conserved between Salmonella typhimurium and Escherichia coli with respect to operon structure and regulation.* Journal of Bacteriology, 1998. **180**(3): p. 722-731.
104. Bokranz, W., et al., *Expression of cellulose and curli fimbriae by Escherichia coli isolated from the gastrointestinal tract.* Journal of Medical Microbiology, 2005. **54**(12): p. 1171-1182.
105. Jonas, K., et al., *Roles of curli, cellulose and BapA in Salmonella biofilm morphology studied by atomic force microscopy.* Bmc Microbiology, 2007. **7**.
106. Hammar, M., et al., *Expression of two csg operons is required for production of fibronectin- and Congo red-binding curli polymers in Escherichia coli K-12.* Molecular Microbiology, 1995. **18**(4): p. 661-670.
107. Zogaj, X., et al., *Production of cellulose and curli fimbriae by members of the family Enterobacteriaceae isolated from the human gastrointestinal tract.* Infection and Immunity, 2003. **71**(7): p. 4151-4158.
108. Romling, U., *Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae.* Cellular and Molecular Life Sciences, 2005. **62**(11): p. 1234-1246.
109. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Research, 2001. **29**(9).
110. Russell, A.D., *Do biocides select for antibiotic resistance?* Journal of Pharmacy and Pharmacology, 2000. **52**(2): p. 227-233.
111. Gerstel, U. and U. Romling, *Oxygen tension and nutrient starvation are major signals that regulate agfD promoter activity and expression of the multicellular morphotype in Salmonella typhimurium.* Environmental Microbiology, 2001. **3**(10): p. 638-648.
112. Weaver-Stevenson, N., *Reduced susceptibility of Salmonella enterica to biocides*, in *Biological Sciences*2008, Univeristy of Delaware.
113. Olsen, A., et al., *ENVIRONMENTAL-REGULATION OF CURLI PRODUCTION IN ESCHERICHIA-COLI.* Infectious Agents and Disease-Reviews Issues and Commentary, 1993. **2**(4): p. 272-274.
114. Kolter, R., D.A. Siegele, and A. Tormo, *THE STATIONARY-PHASE OF THE BACTERIAL LIFE-CYCLE.* Annual Review of Microbiology, 1993. **47**: p. 855-874.