

**A Transcriptomic Analysis of *Salmonella enterica* Newport *In Planta* and after Postharvest
Sanitization**

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Degree

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Dedication

To my best friend

John Quillen

and my parents

Lewis & Jean Dunn

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Abstract

The consumption of fresh produce is increasingly linked to incidence of foodborne illness. Pathogens including *Salmonella enterica*, Shiga toxigenic *Escherichia coli*, and *Listeria monocytogenes* are exposed to produce crops through direct human or animal contact, contaminated agricultural water, bioaerosols, run-off, and improperly treated compost. *S. enterica* has demonstrated exceptional tolerance to the stresses encountered in the environment, on plant tissue, and from postharvest antimicrobial mitigation strategies. Understanding the transcriptomic mechanisms *S. enterica* employs to survive these hazards is integral for the development of more effective preventive controls and post-harvest steps to prevent the pathogen from infecting consumers.

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Introduction

Preventive measures to mitigate food safety risks associated with commercially processed products were addressed by the passing of the Food Safety Modernization Act, which was signed into law in 2011. The new legislation was geared to change the way the industry addresses safety risks, by stressing prevention instead of a purely retroactive post-outbreak response (159). Produce growers have perhaps one of the greatest challenges when it comes to ensuring a safe product. Their product is typically grown outdoors, exposed to the environment and animals that readily spread contamination. Furthermore, their products frequently do not undergo further processing that may include a kill step. In this case, preventing, or in reality, limiting initial contamination is one of the only ways to ensure the microbial safety of their fresh produce.

Novel techniques are being explored to address pathogen contamination of fresh produce. Most commonly, sanitizers are incorporated into washing systems to limit cross-contamination. Chlorine is most frequently used due to its low cost, but concentrations approved for postharvest washing achieve limited bacterial inactivation and, in many instances, do not surpass removal seen with water alone (164). Ozone, essential oils, and aqueous and gaseous chlorine dioxide have been examined on several commodities, including cilantro, lettuce, and carrots (100, 139, 164). However, organoleptic properties are of concern with implementation of these compounds (164). Irradiation has also been extensively explored (51, 110, 112), but consumer perceptions surrounding the safety of irradiation make it unlikely to be accepted by the public. More novel methods examined include the use of ultrasound, the chlorine stabilizer T128, or vapor from antimicrobials such as acetic acid and hydrogen peroxide

(106, 131, 173). However, as of yet, no clear solution has been identified to effectively remove enteric pathogens from produce without either compromising quality, being cost-prohibitive, requiring extensive set-up and monitoring, posing a hazard to workers, facing resistance from consumers, or any combination of these hurdles.

In the absence of a solution, attention is turning to the examination of the pathogens themselves to determine the intrinsic tools they employ to make them so resistant to environmental stresses. The gene expression of enteric pathogens under stressed conditions has been thoroughly evaluated (87, 89, 165, 166). However, studies evaluating their transcriptomic response on living plants is limited (161), and even more so when post-harvest sanitization occurs. To address this knowledge gap, *Salmonella enterica* serovar Newport, a pathogen previously associated with outbreaks on tomatoes, was inoculated onto the fruit of live tomato plants. These inoculated plants were exposed to field humidity and temperature conditions in an environmental chamber, harvested, and sanitized with water, chlorine, or peracetic acid. The subsequent transcriptomic examination identified the differential regulation of individual and clusters of genes involved in the *S. Newport* stress response.

Chapter 1: Review of Literature

Food Safety: A Growing Concern

The increase in epidemiological methodologies and a multitude of large scale outbreaks recently in the United States has led to the perception that incidence of foodborne illness is on the rise (114). While the number of food related cases has remained relatively stable, an estimated 48 million cases with roughly 3,000 deaths occur per annum in the United States (132, 133). The economic burden per case has been estimated between \$1,068 and \$1,626, resulting in a gross loss ranging from \$51 to \$77.7 billion annually, depending on the analytical model applied (134). Consumers of lower socio-economic status and minority groups are more prone to foodborne illness due to food specific hazards as well as decreased knowledge of food safety (121). Food trends towards increased food preparation outside of the home and the growing demand and availability of fresh-cut produce have put more pressure on food processors and growers to ensure microbiologically safe products. For example, the typical American grocery store stocked roughly 300 food items in the 1950's. By the 1990's, the food stock of grocery stores in the United States ballooned to anywhere from 25,000 to 50,000 items (45).

Bacterial Contamination of Fresh Produce- Microorganisms of Concern

Escherichia coli O157:H7 and *Salmonella enterica* are both members of the family *Enterobacteriaceae*, they are commonly referred to as enteric pathogens as they typically inhabit and infect the intestinal tracts of humans and animals. Upon elimination from an infected host, these pathogens can persist and, in favorable conditions, replicate in the environment (128). Roughly 8% of beef and dairy cattle may carry *E. coli* O157:H7, which is then disseminated asymptotically through the bovines feces (141) into the soil or water.

Poultry are considered a predominant reservoir of *Salmonella* (21), but in reality, virtually all species can and do carry the pathogen, which generally demonstrates little host specificity (47, 54, 151). Both *E. coli* and *Salmonella* are readily spread to subsequent hosts through water, soil, vegetative matter, or feed that becomes contaminated by pathogen containing fecal matter (47, 55).

Plants are not particularly ideal hosts for *Salmonella* and *E. coli*, but when they encounter the plant environment, their tenacity allows them to persist and in some instances, colonize plant tissue. *Salmonella* in compost amended soil used for growing parsley and lettuce persisted for 231 days in the soil and on the produce itself (81). Another study analyzing *Salmonella* and *E. coli* survival in sandy loam and loamy sand after fertilization with liquid hog manure. *Salmonella* was detected in the soil 54 days after fertilization, while *E. coli* was recovered up to 77 days post-fertilization (34). Additionally, the proximity of cattle, swine or poultry on mixed crop-animal farms to fields used for fruit and vegetable production can increase the likelihood of enteric pathogens contacting crops (43, 125, 126).

Pathogenic *E. coli* has only been recognized as a produce safety concern in recent history. Largely considered a commensalist by microbiologists and food scientists throughout much of the 20th century, realization of the destructive tendencies some strains of *E. coli* came about in a 1982 ground beef outbreak from a fast food chain on the West Coast. While the strain known as O157:H7 had been previously recognized, it had not until this outbreak been identified as pathogenic (6). The strain was once more thrust into public knowledge in 1993; once again, ground beef was the culprit. The fast food chain Jack-in-the-Box was incriminated this time in an outbreak that resulted in over six hundred reported cases and the deaths of four

children who succumbed to hemolytic uremic syndrome. The pathogen surfaced yet again in the consumer conscience in 1996 when a “new” commodity was implicated in *E. coli* O157:H7 transmission. Unpasteurized apple juice produced by the California company Odwalla, sickened at least 65 people, more than 12 of those developed hemolytic uremic syndrome and one of who died. Odwalla was fined \$1.5 million and in response to the outbreak began pasteurizing their juice products (58). This outbreak led to the realization that pathogenic *E. coli* was not a concern isolated to producers who use ingredients of animal origin in their products.

The prevalence of Shiga-toxin producing *E. coli* outbreaks has become such a concern that in September of 2011, the United States Department of Agriculture added six more non-O157:H7 Shiga-toxin producing *E. coli* (STEC) species to its “Zero Tolerance Policy” in ground beef: these include *E. coli* O103, O111, O145, O26, O45 and O121. This means that beef for commercial use may not enter the food supply if any of these pathogens are found as adulterants in the product (155).

However, since 1996, Shiga toxigenic *E. coli* contamination has occurred increasingly in fresh produce, resulting in a rash of outbreaks. Pathogenic strains of *E. coli* have a particular affinity for leafy greens, as evidence by a bagged spinach outbreak in 2006 (28) and a mesclun lettuce outbreak in 1996 (141). The success of *E. coli* on leafy greens is likely due to the variable surface area and structure of the leaves and the fact that soil frequently encounters the low growing leaves. These produce outbreaks appear to occur primarily when fecal material from cattle, wildlife, or human sources comes in contact with production fields, via water, soil or direct contact.

It has been demonstrated that lettuce is able to internalize *E. coli* from contaminated soil or water, but only when present at levels higher than would typically be present in most farm scenarios (141). Much of the threat *E. coli* poses comes from its direct contact with consumable portions of fruits and vegetables where it can penetrate into micro-niches depending on the plants' topography (163). For example, the rough, netted surface of cantaloupe melons readily allows attachment by *Salmonella*, *E. coli*, and *Listeria*, making these melons more susceptible to contamination than smooth-skinned melons (154). Damaged plant tissue is also more susceptible to colonization, as *E. coli* (and other pathogens) preferentially colonize cut surfaces that leach higher levels of nutrients (19, 137, 150, 171). This is concerning particularly for products that do not require unblemished fruit, such as juices and ciders, since damaged regions of a piece of produce are more likely to harbor pathogenic bacteria.

Salmonella enterica

Soil and water are the primary sources of produce contamination pre-harvest. *Salmonella* serovars are reported to survive for long periods of time in both manure amended soil [up to 332 days (172)] and in water (82). Several factors influence the ability of *Salmonella* to attach to a produce surface, including the *Salmonella* serovar and the plant species, genotype or age (82, 118). It has been shown that *Salmonella* serovars capable of forming biofilms have greater ability to attach to surfaces of lettuce and cabbage (118). When exposed to produce, *S. enterica* can attach to the plant surface within minutes (104).

While gene expression varies greatly depending on serovar and plant interactions, there is no consensus on whether genes utilized during colonization of warm-blooded hosts are employed during colonization of plants. Jacobsen and Bech published that there was no

evidence these genes were employed (82), while Barak and others (2009) have stated that *S. enterica* virulence genes are employed during plant colonization. It has been shown that *Salmonella* uses similar proteins for communication and colonization of both mammalian and plant host species (135). Colonization of plant tissue involves a collection of genes and mechanisms, including swarming genes (11), O-antigen, fimbriae, and virulence genes. Whether plant tissue serves as a true host or merely a vector to perpetuate the spread of foodborne pathogens is still up for debate.

Like *E. coli* O157:H7, *S. enterica* was initially associated with foodborne illness related to consumption of animal products. The largest salmonellosis outbreak in the US was the result of contaminated milk from Hillfarm Dairy in Illinois in 1985. Estimates for illnesses due to consumption of the contaminated milk approach 200,000 individual cases (162). Despite the scale of the 1985 outbreak, the organism still tends to be associated with the consumption of poultry and eggs (2, 16). However, fresh produce is increasingly implicated as a vehicle for *Salmonella* transmission. In 2006, an *S. Typhimurium* outbreak on restaurant-served tomatoes sickened 183 people in 21 states (27). From 2010-2011, three *Salmonella* outbreaks from alfalfa sprouts occurred in the US, sickening a total of 209 people (31). *Salmonella* Newport sickened 275 people exposed to the pathogen on cucumbers (5). Cucumbers were again the source of illness for 907 cases that resulted in 204 hospitalizations and 6 deaths in a 2015 *Salmonella* Poona outbreak (30).

Contamination by Irrigation Water

Fresh produce is increasingly implicated as the vehicle for transmission of enteric disease, information that is due in large part to increased epidemiologic methods and genomic

mapping/cataloguing. The presence of enteric pathogens often indicates fecal contamination, including contamination from humans and domesticated and wild animals. Irrigation water is a predominant source of contamination of produce and plant sourced foods as it readily serves as a vehicle for pathogen transmission. Agricultural run-off, wildlife and birds, and improperly treated human waste all can contribute enteric species, and when contamination is introduced into water sourced for irrigation, pathogens are readily inoculated onto irrigated plants.

Guidelines suggested for surface water quality are based on indicator organisms, not the pathogens of interest themselves (63). According to the Produce Safety Rule within the Food Safety Modernization Act, water that contacts the edible portion of a crop must have a rolling geometric mean (GM) of 126 or fewer CFU generic *E. coli* and a statistical threshold value (STV) of less than 410 CFU of generic *E. coli* for every 100 mL of water. The GM value serves as a baseline to monitor the average quantity of generic *E. coli* within a water source at any given time, while the STV indicates the amount of variability within the supply. For surface water sources, the initial GM and STV values are calculated using at least 20 samples collected over the course of two to four years as close to harvest as possible. After these initial values are calculated, a minimum of five new samples must be tested yearly and compiled with the fifteen previous most recent samples to create a continuously adjusting data set. Furthermore, water that may come in direct contact with produce during or after harvest (postharvest water) is required to be free of generic *E. coli* (158). While these indicators are species typically present in fecal material, as are pathogens, opinions on the effectiveness of using indicators to predict pathogenic hazards are mixed (3, 7, 49, 73, 99).

Salmonella and *E. coli* rapidly attach to plant surfaces when exposed, and anywhere from 30-80% of the plant microbiome exists in biofilms, making the removal of all pathogenic species virtually impossible using current practices (105). Enterics, as with many bacteria, are tenacious and highly adaptable, able to alter their gene expression within seconds when presented with a new stressing agent such as a chemical sanitizer or antibiotic.

The best course of action to ensure public safety while implementing a financially viable solution to produce growers is a multi-faceted approach. Irrigation water must be of good quality prior to application to food crops. Municipal or ground water sources are generally considered the safest sources of water, but cost and accessibility are limiting factors. Water of questionable quality may be improved through the application of sanitizers such as chlorine, peracetic acid, or chlorine dioxide, or through the use of ultraviolet radiation treatment of water. Uncontaminated irrigation water decreases the likelihood of pathogens contacting produce and making it to the consumer. If an appropriate antimicrobial agent is applied to wash water, and if fewer contaminated produce pieces due to cleaner irrigation water would enter the wash system, lower populations of pathogenic bacteria would survive to reach the consumer. A hurdle type system such as this could greatly reduce the occurrence of produce associated foodborne outbreaks and increase consumer confidence in the safety of the products they feed their families.

More than 90 people were infected during the summer of 1995 by *Escherichia coli* 0157:H7 in a Montana lettuce outbreak (1, 143). In 2004, an outbreak in Norway, Sweden, and the United Kingdom involving several *Salmonella* serovars and *Campylobacter* was traced back to lettuce sourced from Italy (64, 115). A *Salmonella* Saintpaul outbreak four years later

sickened 1,442 people in the United States and Canada. Tomatoes and peppers were identified as the primary vectors (29, 64). A 2005 *Salmonella* Newport outbreak in the US involving tomatoes sickened over 70 people in 16 states (68, 82). In all instances, the contamination was determined to come from irrigation water.

In the United States, approximately 39% of fresh water is used for crop irrigation (160). The method by which water is applied to crops is a great risk factor in produce contamination. Drip and furrow methods result in little to no water contact on aerial crops, and are thus much less likely to result in product contamination. Drip systems also result in higher yield and more efficient water utilization (127); this is largely due to evaporative water loss due to pooling on foliage and soil, as well as dispersion of air-borne water droplets beyond the application zone when water is sprayed on a crop (152). Sprinkler irrigation is still largely used, and accounted for approximately 44% of irrigation systems in the US in 1994, compared to just over 4% that were drip or sub irrigation (77). This disparity is due in large part to large capital investment required for a drip system versus a sprinklers system. Surface irrigation is the most widely implemented system worldwide (77); a surface system employs gravity to flood a plot or field (160), and is used for crops such as rice or sugar cane.

Delaying harvest for a period of time after the last irrigation event is an important step in reducing the likelihood of pathogenic bacteria reaching the consumer. As environmental stressors are known to reduce the viability of human enteric pathogens on plant surfaces, a period of six days has been suggested after the last irrigation event to allow sufficient time for natural control of contamination (170). Unfortunately, growers in dry regions with scarce rainfall may not be able to abide by this guideline and still have a marketable crop.

Contamination by Domesticated Animals

As *Escherichia coli*, including Shiga toxigenic strains, comprise a portion of the normal microflora of the bovine intestinal tract, cattle are a primary reservoir for strains pathogenic to humans (14). A survey of *Campylobacter jejuni* prevalence in domesticated animals found that 95% of swine, 19% of cattle, 36% of chickens, and 5% of pet dogs and cats were positive for the pathogen (149). *Salmonella* is thought to be spread to domesticated animals from wild birds (76), and can readily be spread to uninfected animals through water, contaminated grass or feed (75). *Salmonella* infection of pet dogs, as with *C. jejuni*, is likely between 1-5%; however, 40-60% of stray and performance dogs have been shown to shed the pathogen (26, 75, 148).

The California Leafy Green Products Handler Marketing Agreement (LGMA) audit checklist asks if a distance of 120 m (400 ft) exists between cattle operations and produce crops (25). However, Berry and others (2014) examined spinach plots that were 60, 120 and 180 m away from feedlot pens and found statistically similar contamination of *E. coli* O157:H7 in all three plot distances, suggesting that the 120-meter suggestion by LGMA is insufficient.

Contamination from Bioaerosols

As mentioned previously, proximity to land housing cattle and other livestock contributes risk by enteric pathogens. Pathogenic bacteria from animal or human waste are effectively transported via bioaerosolization (48, 91); a qPCR study indicated aerosolized *Salmonella* levels ranging from 2.2×10^1 to 3×10^6 per m^{-3} in poultry house air (50). Ko et al. documented higher levels of fecal indicator bacteria downwind of swine operations than upwind, indicting the farms as the likely cause (91). Bioaerolization may make crop fields located near animal production facilities more susceptible to microbial contamination.

One study evaluated the dust borne transfer and survival of *S. Newport* onto tomato blossoms. One week after exposure to airborne *S. Newport*, 29% of blossoms were contaminated with the pathogen. They took the study further to examine *S. Newport* survival after airborne inoculation on blossoms through fruiting. Tomatoes were harvested 3 weeks later, at which time 66.6% of airborne contaminated blossoms resulted in tomatoes that were *S. Newport* positive, indicating that the pathogen can survive from the time it contacts the blossom until fruiting (95).

Contamination by Wildlife

Direct wildlife exposure to produce fields has been repeatedly identified as a source of contamination in produce. Three deaths out of 205 illnesses were attributed to the introduction of *E. coli* O157:H7 to baby spinach fields in 2006 (83). Feral swine and cattle feces in close proximity to the fields contained the same strain of *E. coli* as that found in the stool samples of case subjects (147). In 2011, fifteen cases of illness caused by *E. coli* O157:H7 in Oregon were attributed to deer feces in a strawberry field. Of these 15 individuals, four developed hemolytic uremic syndrome (HUS), two of whom died (97). Outbreaks have been traced back to and attributed to wildlife in several foodborne outbreaks, including a Finnish carrot outbreak in 2004 attributed to shrews, an Alaskan pea outbreak in 2008 associated with sandhill cranes and a 1995 Florida orange juice outbreak possibly caused by the indigenous amphibian population (98).

The wildlife identified by auditors and inspectors as the largest threat to produce safety included feral swine, deer, birds, rodents, reptiles and amphibians. Deer and feral swine are

considered the highest risk wildlife types to spread pathogenic *E. coli* by the LGMA, a risk that is compounded when these animals have access to areas occupied by cattle (98).

Total exclusion of wildlife from produce fields is impossible. Fences are the most effective means, but are expensive to put up and maintain and do not eliminate all species (e.g. birds, rodents). Habitat destruction has been utilized to minimize crop exposure to feral swine and deer, but is again costly and not very effective. Legitimate concerns have been raised over the efficacy of this methodology and the unanticipated effects caused by the destruction of wetlands and forests adjoining farms (98). Poisoned bait is being utilized with growing frequency, but its use is discouraged by conservationists who fear poison may move through the food chain and affect endangered predators and birds of prey (147).

Another proposed wildlife control method is the use of bare-ground exclusion grounds around crop fields. As these zones are free of vegetation, they can be monitored for wildlife tracks and stool to evaluate risk and monitor potential contamination sources in a field. However, vegetation naturally removes contaminants in water, including pathogenic bacteria. These exclusions zones, by limiting wildlife imposed risks, may in fact be increasing risks associated with reduced water quality (147). Produce farmers have raised concerns about some of these wildlife exclusion methods. They believe the policies intended to reduce the risk of wildlife introduced contamination are not only at odds with wildlife protection laws, but that guidelines by these companies are unattainable and not supported by scientific data (98, 120).

Challenges on Plant Tissue

Once a pathogen, or any microorganism, contacts plant tissue several factors contribute to preventing survivability. A key defense is the plant's immune system. Plants rely on

antimicrobials such as essential oils, which are thought to disrupt the bacterial cell membrane and are continuously present in plant tissue. Other compounds such as salicylic acid are released when bacterial invasion occurs, causing localized necrosis of plant tissue and killing bacteria through the subsequent dehydration occurring on the necrotic region. The plant immune system relies on the recognition of pathogen associated molecular patterns, which include proteins or components of flagellin, elongation factors, secretion systems, lipopolysaccharides, and many others (171). *S. Typhimurium* has been shown to initiate salicylic acid production, which is mediated in part by the signal molecule ethylene (80, 135). Together, ethylene and salicylic acid have been shown to interfere with alfalfa colonization by *Salmonella* (80). When the *Arabidopsis* plant detects the initial 18 amino acid sequence of elongation factor TU, which is the most abundant bacterial protein, ethylene production and an oxidative burst occur (96). Other plant protective mechanisms upon the recognition of pathogenic invasion may include the expression of nitric oxide, reactive oxygen species (153), hydrolytic enzymes (109), reinforcement of plant cell walls through glycoprotein cross-linking (153), and ionic fluxes (171). Self-necrosis induced by expression of many of these compounds, such as reactive oxygen species and salicylic acid, localize infection using “scorched earth” practices. Dehydrated and nutrient starved regions limit a pathogen’s ability to mobilize to other plant tissues (40, 107).

Pathogens, including plant and likely enteric pathogens, employ several mechanisms to avoid triggering these plant defenses. Iniguez et al. (80) found that *S. Typhimurium* mutants devoid of the Type III Secretion System located on Pathogenicity Island I failed to initiate salicylic acid dependent defenses in *Medicago truncatula*; however, the plant promoter

mediating the response was activated by the wild type *S. Typhimurium* strain. However, it is still unclear if enteric pathogens require their type III secretion systems to colonize and invade plant tissue. Many of the other components required for colonization of tissues, including fimbriae, O-antigen, and flagella are responsible for activating the plant immune response (13, 17). As with the secretion systems, it is unclear if it is beneficial for bacteria to express these characteristics when invading host tissue, or if downregulating genes involved in the production of these pathogen associated molecular patterns is more helpful to avoid the host immune response.

The sigma factor responsible for regulating stress response, *rpoS*, is critical for initial attachment. *RpoS*, which is required for virulence, regulates *agfD*, which in turn regulates curli and cellulose expression. Wild-type *S. enterica* had 1 log increased attachment over the *rpoS* mutant after 24 h; within 48 h, however, the mutant caught up to wild-type attachment. Restoring the *rpoS* gene resulted in similar attachment to the wild-type (12). Similarly, the plant pathogen *Erwinia carotovora* requires *rpoS* to survive the stresses involved in plant tissue colonization (4, 12).

rpoS is also crucial for biofilm formation (12). In a microtiter plate assay, *S. Tennessee* and *S. Thompson* were determined to be stronger biofilm producers than *S. Newport*, *S. Braenderup*, and *S. Nege* (118). The formation of thin aggregative fimbriae was required for cell aggregation of *S. Enteritidis*, while fimbriae deficient mutants only formed sparse biofilms on Teflon and stainless steel surfaces. While single cells could attach, the mutant *S. Enteritidis* did not aggregate together (8).

Normal plant microflora also compete with enteric pathogens that find themselves on the plant surface. Plant growth-promoting rhizobacteria antagonize pathogens through the production of antibiotics and similar compounds that prevent colonization (167). Lactic acid bacteria produce bacteriocins and hydrogen peroxide, antimicrobials utilized to control competing species (37, 38). Nutritional and moisture competition is prevalent, with some bacterial species utilizing components such as siderophores to sequester minerals, limiting availability of basic compounds required for survival. For example, *Pseudomonas* secretes a hydroxamate-type siderophore that depletes the immediate area of Fe^{3+} , which *Salmonella* requires for growth (39, 113).

Plant and foliage age plays a role in the attachment of bacterial species to tissues, although results differ among studies. One study found that older lettuce leaves had greater attachment of *Salmonella enterica* Seftenberg than younger leaves (78). However, Brandl and Amundson (2008) found that due to greater quantities of nutrient rich exudates on young leaves, they harbored greater quantities of *Salmonella* and *E. coli* O157:H7 than middle aged or older leaves on romaine lettuce (20). *Salmonella* also attached more readily to lettuce than cabbage. After 5-minute exposure, 3.19 CFU/cm² attached to Romaine and 3.36 CFU/cm² to Iceberg. In contrast, 1.83 CFU/cm² attached to the intact cabbage surface. *Salmonella* attachment strength was greater on both lettuce types than on cabbage, and preferential attachment to damaged product occurred (118).

Attachment to plant tissue involves several structures working in concert. Curli fibers and cellulose production are regulated by AgfD. The significance of the genes encoded on AgfD was evident by the fact that *Salmonella* attached with similar efficiency to alfalfa sprouts as did

plant bacteria, but enterohemorrhagic *E. coli* lacking the *agfD* homologue (*csgD*) were readily washed off. When a nonpathogenic *E. coli* was found to attach as readily as the *Salmonella* strains, it was discovered that the *E. coli* was positive for curli production (12). AgfD also regulates O-antigen capsule production, which is important not only for desiccation stress (65) but for plant colonization as well (13). On alfalfa sprouts, *Salmonella* deficient in *yihO*, the gene encoding capsule and cellulose, were less able to colonize after 24 and 48 h than the wild-type (13). Chemotactic function and flagella are also important for plant colonization. *Salmonella cheY* (chemotaxis protein) and *fliGHI* (flagella associated protein) mutants were less able to internalize through the stomata of lettuce leaves than the wild-type strains. While the *cheY* mutants were unhindered in their ability to attach externally to the lettuce leaves, attachment by *fliGHI* was significantly reduced (by approximately 50%), indicating that flagella are integral to *Salmonella*'s ability to attach to the produce surface (94).

Wash Water

Despite the implementation of mitigation strategies to circumvent pathogen associated risks preharvest, contamination of produce does occur. Most produce is grown in the open and exposed to risks ranging from water, soil, wind, animals, insects, and humans. Proper post-harvest handling intervention strategies are important in the event a crop is unknowingly contaminated with a foodborne pathogen. If a pathogen colonizes a piece of produce in the field and can survive until harvest, in many cases (depending on the commodity and farm practices) it must next survive a cleaning and sanitizing wash step. This wash is applied to many commodities to remove field dirt; however, if not done properly, this practice can result in

localized contamination spreading to the remainder of a crop via pathogen-contaminated water (23, 66, 116).

This route for the spread of pathogens necessitates the use of produce wash systems that utilize sanitizing agents to prevent cross-contamination in the water. The sanitizers most commonly utilized in these wash systems is chlorine and peroxyacetic acid. However, efficacy of these sanitizers is contingent on constant monitoring, as fluctuations in concentration as well as parameters such as pH, temperature, sunlight exposure and organic matter can all interfere with antimicrobial activity. In addition, sanitizers currently do little to inactivate attached pathogenic organisms on the produce itself. While sanitizing agents in wash water do little to remove attached bacteria to produce, they do reduce the likelihood of cross-contamination to clean product if pathogens are introduced into the wash system (108). Chlorine, for example, typically achieves only a 1-2 log reduction on contaminated surfaces; However, it effectively kills planktonic pathogens in wash water (41, 88). A sanitizer aids in keeping contamination limited to one or only a few pieces of produce, instead of an entire lot that shared wash water with the contaminated fruit.

Chlorine

Chlorine is currently the most commonly used sanitizing agent used in wash water due to its low cost and relatively easy application (117). The FDA recommends using 50-200 ppm total chlorine for 1-2 minutes at pH 6-7.5 for the removal of microorganisms in wash water (41, 156). The term 'free chlorine' is the amount of hypochlorous acid (HOCl) present in the solution, the compound with the greatest antimicrobial activity.

HOCl reacts readily with organic matter to form bound chlorine particles with very limited antimicrobial activity. The amount of HOCl that reacts with the organic matter in a solution is called the chlorine demand, and any leftover HOCl is considered free residual chlorine (41, 72). Chlorine levels require constant monitoring to ensure that levels of HOCl remain around 5-7 ppm free residual chlorine; pH monitoring to ensure pH is neutral is essential, as HOCl dissociates to form hypochlorite in basic pH (41, 59).

Concerns regarding the safety and efficacy of chlorine are driving the members of the produce industry to explore alternative sanitizers. A dump tank system containing 200 ppm free chlorine will inactivate most free-floating pathogens and prevent their attachment to clean produce. However, this same solution will only achieve a 1-2 log reduction in microorganisms attached to the surface of already contaminated produce and many times a similar reduction is observed with water alone (108, 169). On apples, a 2,000 ppm solution of sodium hypochlorite only achieved 1-3 log reduction of *E. coli* O157:H7 after 1 min rinse (22).

Chlorine is known to produce harmful by-products, such as trihalomethanes, haloacetic acids, and chloroform (117, 138). However, production of these by-products generally requires greater concentrations and higher temperatures than are used in produce wash systems. Chlorinated systems also produce large quantities of waste water with high biological oxygen demands, making dispersion into waterways environmentally dangerous. These reasons, in conjunction with the need for constant monitoring, are driving the decreased use and even bans of chlorinated water in wash systems in parts of Europe for conventional produce and the entire continent for organic produce (117).

Peracetic Acid

Peracetic (peroxyacetic) acid (PAA) is one of the most promising sanitizers proposed to replace chlorine. PAA is the equilibrium mixture of acetic acid and hydrogen peroxide, and degrades to form water, oxygen and acetic acid without the dangerous disinfection by-products that are produced by chlorine (117). Also unlike chlorine, PAA is not affected by organic matter, and, therefore, does not require the continuous monitoring required for chlorinated systems (10). A concentration of 60 ppm PAA is generally recommended for submersion wash systems (70), but must not exceed 80 ppm (157). PAA has a greater oxidizing potential than both chlorine and chlorine dioxide (90).

An early evaluation of the antimicrobial efficacy of PAA found that 1.3 mmol/l (99 ppm) achieved 6 log reductions of vegetative *Pseudomonas*, *Klebsiella*, *Streptococcus*, and *Staphylococcus* after exposure for 1 min, while also significantly reducing spore and yeast levels (9). In a produce wash system, PAA behaves similarly to chlorine in terms of bacterial reductions once already attached to produce. When *E. coli* O157:H7 had 24 h contact time on the apple surface prior to washing with 80 ppm peracetic acid, less than a 1 log reduction occurred. Concentrations in excess of 2.1 (15 min contact time) to 14 (5 min contact time) times the recommended dose were necessary to achieve a 5-log reduction (169). Their results are consistent with other studies examining PAA efficacy on pathogens attached to produce (15, 129, 130).

Salmonella Response to Oxidative Stress

Salmonella encounters oxidative stress both during production, if an oxidizing agent is applied to wash water, and upon consumption by a mammalian host. *Salmonella* responds to

oxidative stress via several mechanisms, including DNA repair, production of enzymes with antioxidant capacity, and by competing with phagocytes for oxygen (74). The release of strong oxidizers, often described as an “oxidative burst,” is a key component of defense by phagocytic immune cells. For example, a significant increase in the production of reactive oxygen species was observed in a murine macrophage culture within hours of exposure to *Salmonella enterica* serovars Typhimurium and Infantis. It is hypothesized that this early response is crucial in limiting the dissemination of pathogenic bacteria to other tissues (61). When the concentration of radical oxygen species exceeds the capabilities of a bacterial cell’s defenses, damage to physiological and genetic components results in cell death (53, 145).

Gene Regulation of Oxidative Stress Mechanisms

Two regulons control the majority of genes responsible for the mitigation of oxidative stress in *Salmonella* spp. Genes on the OxyR regulon are expressed when a cell is exposed to hydrogen peroxide (H_2O_2) in excess (53, 145). Catalase (*katG*), glutathione reductase (*gorA*), and alkyl hydroperoxide reductase (*ahpFC*) are all under the control of OxyR (60, 111, 146). Deletions of OxyR in *Salmonella* Typhimurium resulted in spontaneous mutagenesis rates 10 to 55 times greater than wild-type *S. Typhimurium* (144). Pretreatment with H_2O_2 has been shown to increase *Salmonella* resistance to oxidation (168), and the expression of about 30 genes appear to be responsible for this resistance (33). *Salmonella* exposed to the oxidative activity of adrenaline, a hormone responsible in part for mediating gut physiology in a normal GI tract, up-regulated OxyR and manganese superoxide dismutase (85). Also, regulated in part by OxyR, is the DNA binding protein in stationary phase (Dps). Upon entering the stationary phase, *E. coli* and *Salmonella* produce copious amounts of Dps making it the most abundant molecule in the

cell. Despite the name, whether or not it is bound to DNA, Dps has been shown to increase *Salmonella* resistance to oxidative stress *in vivo* (71).

The SoxRS operon regulates the stress response when exposed to superoxide (O_2^-) (52, 145), as well as to dissolved nitric oxide, hypochlorous acid, copper sulfate, and others (52), and is activated by *soxS* (46). Though the genes encoded on SoxRS respond to similar forms of stress as OxyR, expression of the genes on one regulon confer no cross-protection if the cell encounters an oxygen species the protection for which is encoded on the other regulon's genes. In *S. Typhimurium*, *soxS* gene is required for the production of Mn-superoxide dismutase. *S. Typhimurium soxS* mutants were hypersensitive when compared to the wild-type to paraquat, but not to 3% H_2O_2 . Additionally, *soxS* mutants were no more susceptible to killing by murine macrophages than wild-type *S. Typhimurium* (52).

The sigma factor RpoS is another regulator identified in *Salmonella* that is activated upon oxidative and other stresses. It shares regulation of *katG* with OxyR and also regulates *katF*, or catalase hydroperoxidase II (60, 93). RpoS plays a greater role in acid induced stress response than oxidative; however, this acid response cross-protects against H_2O_2 and osmotic pressure (142).

Genes and Proteins Involved in Oxidative Stress Response

Sequestration of nutrients and essential elements is a proactive defense against pathogenic invasion for plants and animals. *Salmonella* utilizes Fe^{2+} and Mn^{2+} for the production of protective enzymes including catalase and superoxide dismutase. Increased proton exposure increases *mntH* (H^+ -coupled manganese transport) mediated uptake of Mn^{2+} as a defense against reactive oxygen species. Mn^{2+} is required for production of Mn^{2+} superoxide dismutase.

Two enteric *mntH* mutants, a catalase positive *E. coli* and a catalase negative *S. Typhimurium*, were both sensitive to the oxidative activity of peroxide, indicating that Mn^{2+} exerts catalase independent oxidative protection (86).

Catalase is responsible for breaking down H_2O_2 to water and oxygen and is especially beneficial to the invasion and survival of enteric pathogens (53). Survival of *Salmonella* in a H_2O_2 containing medium was directly proportional to the amount of catalase produced by the bacteria (168). A manganese-containing catalase was identified in *S. enterica* Typhimurium encoded on *katN*; very few of these have been identified (most catalases depend on a heme group at the active site) and no homologue appears to exist in *E. coli* (124).

The protein products of the *Salmonella htrA* gene and its *E. coli* counterpart were found to be nearly 90% homologous. As it was well established in *E. coli* that *htrA* codes for a heat shock protein, Johnson and others (1991) wanted to establish the role that *htrA* plays in the *Salmonella* stress response. They found that mutants were not more susceptible to temperature stress, but that *Salmonella htrA* mutants could not withstand oxidative stress from H_2O_2 as well as the wild-types receiving similar exposure (84).

Genetic response to osmotic stress

A study by Finn et al. found that deletion of any of three *S. Typhimurium* proline transport systems (*proP*, *proU* and *osmU*) involved in osmotic stress response resulted in reduced long-term survival. Proline is thought to slow water loss from cells (44, 57), and supplementation of the amino acid to the media stimulated growth in NaCl containing media (35). Of the 20 common amino acids examined, only proline stimulated growth, while L-cysteine was completely inhibitory (35). All three proline transport systems were upregulated

during desiccation on a stainless steel surface (56). *Salmonella* and *E. coli* also sequester glutamate (18), potassium and betaine (24) osmoprotectants; the transport of which are under control of *proU*. Intracellular concentrations of potassium ions and glutamate were observed to increase linearly as the osmolality of *E. coli*'s environment increased (123), suggesting the cell adjusts its internal ionic gradient to exceed the environmental concentrations, maintaining turgor pressure within the cell (36).

Chen et al. determined that *fabA* is upregulated in *S. enterica* when osmotically stressed in sugar; *fabA* governs unsaturated fatty acid biosynthesis, the upregulation of which might increase membrane fluidity (32, 79). In another study, *S. Tennessee* similarly upregulated *fabA* on paper disks after desiccation (56, 102).

Genes involved in cellular repair

Upon oxidative damage, *S. enterica* employs a DNA repair technique called Base Excision Repair (BER) (119). Most organisms have some homologue of an enzyme called DNA glycosylase, which excises damaged regions into individual bases within the DNA molecule (119, 122). Endo or exonucleases mediate the repair of the damaged portion, which if left unrepaired results can result in splits in the DNA strand, resulting in cell death (136, 140). This DNA repair maintains genomic integrity. In mammalian cells, this process occurs in both the nuclei and mitochondria and protects against cancers and aging (119). Bile salt exposure up-regulates virulence genes in *S. enterica* as their presence indicates to the cell that it has entered the gastrointestinal tract. Wild-type *S. enterica* normally up-regulate genes required for survival of oxidative stress; however, *S. enterica* mutants in exonuclease III (*xthA*) and endoculease IV (*nfo*)

were unable to recover to bile salt induced oxidation, indicating that BER is necessary for survival in the gut (119).

S. enterica also uses homologous recombination to repair oxidative damage to genetic material (62, 119). Damaged DNA strands are repaired through the exchange of nucleotide sequences, fixing double-strand breaks, interstrand crosslinks, or gaps (103). Homologous recombination is encoded on multiple *rec* genes, including *recBCD* and *recF* (62, 92).

Survival of *Salmonella* and enteric bacteria in general depends on its ability to adapt to constantly changing environments. Their intrinsic success as pathogens relies on the genes that are at their disposal and the speed with which they are able to adjust their gene expression.

Cross-protection from multiple stressors

Multiple studies have indicated that some stress responses engaged by bacteria cross-protect against other stress agents. Desiccated *S. enterica* spp. were less susceptible to ethanol, hydrogen peroxide, bile salt, and UV exposure than non-desiccated cells. However, the same study demonstrated that increased tolerance is not a hard and fast rule, as desiccated cells were more susceptible to acetic and citric acid exposure (69). *S. Enteritidis* inoculated into peanut oil transcribed <5% of its genome; active genes included those for heat and cold shock responses and DNA protection. The authors hypothesized that these transcripts offered cross-protection for the starved and desiccated *Salmonella* (42). Leyer and Johnson demonstrated that adaptation to acid appears to make *S. Typhimurium* more resistant to heat, salt, crystal violet and polymyxin B (101). Similarly, another study showed that acetic acid tolerant *S. Typhimurium* is more resistant to NaCl and KCl induced osmotic stress; however, lactic acid

adapted *Salmonella* did not share this increased resistance and were even more sensitive to salt exposure (67).

Conclusions

The transcriptomic mechanisms by which *Salmonella* and similar enteric pathogens survive environmental stresses are still being explored. Subsequent examinations at the genetic level are crucial for our understanding of how these organisms persist in the environment and on food despite mitigation strategies. The risk of enteric disease can be greatly reduced through effective intervention strategies, but elimination of risk requires greater knowledge of the many intricate, intertwined factors that enable bacterial access to consumers, including: pathogen and plant associations, gene responses to stress or predation, means by which pathogens gain access to crops, and factors that as of yet have not been identified. Research in these areas will lead to novel techniques and systems suitable for on-farm implementation, which is the first line of defense to limit food crop exposure to microbial hazards.

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Chapter 2: Transcriptomic Response of *Salmonella enterica* Newport on Tomatoes *In Planta* after Exposure to Postharvest Sanitizers

Abstract

The success of *Salmonella enterica* as a produce pathogen is due in large part to its ability to survive environmental stresses prior to contacting a human host. *Salmonella* Newport was inoculated onto the fruit of live tomato plants, allowed to dry, harvested, and immediately exposed to a 1 min postharvest rinse containing water, peroxyacetic acid (40 ppm; PAA), or chlorine (50 ppm). RNA sequencing determined the genetic response of *S. Newport* to these compounded stresses. Catalase production was upregulated 1.3-fold for water and chlorine treatments, but no significant change was detected from the PAA treatment ($p < 0.05$). Hydroxylamine reductase, an enzyme involved in ammonia oxidation, was upregulated 20.9-fold by PAA and 4.7-fold by water; no differential regulation occurred due to chlorine exposure. Upregulation of both homo- and heterolactic fermentation occurred for chlorine and water exposed *Salmonella*, whereas homolactic fermentation was downregulated by PAA ($p < 0.05$) and no differential expression of heterolactic fermentation occurred. Genes involved in osmotic stress response experience mixed differential regulation. Elongation Factors Tu and G (EL-Tu; EL-G), thought to be involved in response to stress, were upregulated by chlorine and water, but not PAA; however, PAA upregulated stringent starvation protein A in response to acid stress. Downregulation of the virulence transcriptional regulator *hilD* occurred in all treatments and of *invF* in water. Further understanding of the ability of *Salmonella* to survive multiple stressors may facilitate the development of techniques to control *Salmonella* contamination of produce and prevent subsequent outbreaks.

Introduction

Produce related outbreaks of salmonellosis over the past decade in the United States have been well-documented (8-13, 23). In 2011, salmonellosis resulted in an estimated 1.2 million cases of illness and 450 deaths in the United States (55). Serovars implicated include Newport, Typhimurium, Saintpaul, Reading, and Abony on commodities including alfalfa sprouts, cucumbers, cantaloupes, and tomatoes.

Contamination of produce with *Salmonella* can be due to exposure to contaminated irrigation water, run-off, soil, wildlife and domestic animal access or proximity (57), harvesters, packing equipment, and poorly monitored intervention strategies. *Salmonella* is inherently stress tolerant, both within (20) and outside of the host, contributing to its success as a pathogen. *Salmonella* appears to be most successful when exposed to damaged plant tissue, including lesions due to insect damage (57) or microbial induced decay (25), where nutrients are in abundance. Stomata on lettuce leaves have also been documented as an invasion location for *S. Typhimurium* (37). Genetic mechanisms employed by *Salmonella* to survive on plant tissue, however, are poorly understood.

Transcriptomic analysis of pathogens allows scientists to determine not only how they survive under specific conditions of duress, but gives insight into viable mitigation strategies prior to host access. Evaluations of specific mechanisms, including biofilm formation and virulence genes, have been extensively published (20, 29, 33, 38, 43, 53), but generally when encountering only one stressing condition. The current study aimed to determine not only the genetic mechanisms employed upon exposure to oxidative stress from postharvest wash solutions, but also this stress in concert with desiccative and plant immune defense-related

pressure such as *Salmonella* would encounter upon coming in contact with an unharvested tomato in the field.

Materials and Methods

Culture Preparation

Salmonella enterica serovar Newport 0418 (human outbreak strain) was obtained from Dr. Laura Strawn at Virginia Tech University. Culture was revived in tryptic soy broth and incubated at 37 °C for 24 h. Culture was transferred at least 3 times prior to use. Sixteen to eighteen hours prior to use, 300 µL of stationary phase culture was spread evenly onto tryptic soy agar (TSA; BD, Sparks, MD) plates and incubated at 37 °C for 24 h.

Plates were flooded with 3 mL phosphate buffered saline (PBS; Fisher Scientific, Hampton, NH) and scraped to remove bacterial lawn. Culture was collected poured into a 50-mL centrifuge tube, the plates were rinsed with 2 additional mL PBS. *Salmonella* was centrifuged at 8000 x *g* at 4 °C for 5 min and the pellet resuspended in 250 µL PBS per TSA plate scraped.

Inoculation

Dwarf tomato plants (gibberellic acid mutant) were utilized for ease of manipulation and obtained from Dr. Dean Kopsell, Department of Plant Sciences, University of Tennessee. Mature tomatoes were inoculated while still attached to the plant with 15 (+/- 2) 5 µL drops of culture and allowed to dry within a biosafety cabinet for 1 h. Plants were then transferred to an environmental chamber where they remained for 2 h and exposed to a controlled temperature

of 29.4 °C and relative humidity (RH) of 90% (to simulate daytime conditions in East Tennessee in July).

Sanitizer Treatment

A 50-ppm free chlorine solution was made using sodium hypochlorite (Fisher Scientific, Hampton, NH) and verified using a chlorine meter (Hannah Instruments, Woonsocket, RI). PAA (Sanidate 5.0, Biosafe Systems, East Hartford, CT) was combined with sterile DI water to a concentration of 40 ppm per the manufacturer's label. Twenty-five mL portions of chlorine, PAA, or sterile deionized water were aliquoted into individual 50 mL RNase/DNase free centrifuge tubes (Corning, New York, NY). Upon removal from the humidity chamber, individual inoculated tomatoes were snipped off the plants directly into one of the 3 treatments for 1 min. Liquid was decanted from the sample after exposure and each tomato was placed in 1.5 mL RNAprotect Stabilizing Reagent (Qiagen, Germantown, MD). Cells were removed from the fruit surface using vortex bursts for 20 seconds before the RNAprotect and culture were pipetted into 1.5 mL RNase/DNase free microcentrifuge tubes. Tubes were centrifuged for 10 min (8600 x g) and the supernatant was decanted. RNA extraction using the remaining pellet either occurred immediately or within 3 days of collection (stored at 20 °C).

RNA Extraction

RNA extraction utilized the RNeasy Mini Kit (Qiagen, Germantown, MD). One hundred mL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing lysozyme was added to microcentrifuge tube containing cell pellet and vortexed at room temperature for 10 min. RLT Buffer with β -mercaptoethanol was added to the tube (350 μ l) and vortexed vigorously for 15

min. Two hundred and fifty μ l of 200 proof ethanol was added and mixed by pipetting. The entirety of the sample was then placed on RNeasy mini column seated within a 2 mL collection tube. The column was centrifuged for 15 sec at 8,000 x *g* and flow-through was immediately discarded. Buffer RW1 (700 μ l) was added to column, then centrifuged again for 15 sec at 8,000 x *g*. Flow-through was again discarded. Buffer RPE (500 μ l) was added to the column, then incubated at room temperature for 10 min prior to 15 sec centrifugation. RPE addition, incubation, and centrifugation steps were repeated one additional time. The column was finally placed in a clean collection tube and centrifuged at max speed for 15 sec to prevent any ethanol carryover from the RPE buffer. RNA was eluted by pipetting 25 μ l RNase free water directly onto the membrane and centrifuging for 1 min at 8,000 x *g*.

RNA quantity was determined with a Nanodrop (Thermo Scientific, Waltham, MA) to confirm concentration >80 ng/ μ l with a 260/280 ratio ranging from 1.8-2.2. Quality of RNA extract was determined with the Agilent Bioanalyzer (Palo Alto, CA).

The following protocol was supplied by Brian Sereni, GENEWIZ, LLC. (South Plainsfield, NJ). RNA was shipped to GENEWIZ, LLC. for library preparation, sequencing, bioinformatic analysis; protocol of their procedure was provided upon request. Quantification and RNA integrities were checked after shipment using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The Ribozero rRNA Removal Kit (Bacteria Probe) (Illumina, San Diego, CA) was used to enrich the whole RNA transcriptome. The NEBNext Ultra RNA Library Prep Kit for Illumina was used per the manufacturer's recommendations (NEB, Ipswich, MA) to prepare the RNA sequencing library. RNAs were fragmented at 94 °C for 15 min and two strands of cDNA were subsequently

synthesized. cDNA fragments were end repaired and adenylated at 3' ends. Universal adapter was ligated to the fragments. This was followed by index addition and library enrichment using PCR. A DNA Chip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) validated the sequencing libraries; PCR (Applied Biosystems, Carlsbad, CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) quantified libraries.

Sequencing libraries were multiplexed and clustered on one flowcell and one lane. Flowcell and lane were loaded after clustering onto the Illumina HiSeq 2500 instrument per manufacturer's instructions. Sample sequencing used a 1x50 Single Read Rapid Run Configuration. Image analysis and base calling were conducted by the HiSeq Control Software on the HiSeq 2500. This generated raw sequence data as .bcl files, which were converted into fastq files and de-multiplexed using the Illumina bcl2fastq v1.8.4 program. One mis-match was allowed for index sequence identification.

Fastq files were imported into CLC Genomic Workbench 8.0.3. Low quality bases were trimmed off the ends of sequence reads, which were then mapped to the reference genome. Sequence hit account and RPKM values were calculated for genes, and the total gene read count data was normalized using Baggerley's test. Statistical significance was determined based on defined groups of samples. The control was a 2 h sample that received a no rinse treatment. A box plot was generated for each comparison using the original and normalized expression values. To examine sample separation between groups for each comparison, Principal Component Analysis and Hierarchical clustering analysis were also performed.

MetaCyc (MetaCyc.org) and BioCyc (BioCyc.org) Database Collections were used to identify individual gene functions and to cluster multiple genes that were involved in metabolic pathways (7). Venn diagrams were made using Venny 2.1.0 (48).

Results and Discussion

The three wash treatments resulted in the upregulation of 136 genes by *S. Newport* (Figure 1). Water and chlorine shared the similarities as 61 genes were upregulated by both treatments. PAA, on the other hand, only upregulated a total of 23 genes. Two hundred twenty-nine genes were downregulated by all three wash treatments (Figure 2). Ninety-three of them were shared by all treatments. The top ten differentially expressed genes by each treatment are shown in Tables 1-3.

Protective Mechanisms

While catalase production was upregulated similarly (1.3-fold) in *S. Newport* exposed to water and chlorine, the enzyme was not upregulated when exposed to PAA. This is unexpected as catalase breaks down reactive oxygen species, including hydrogen peroxide (H_2O_2), to limit oxidative damage to the cell. As PAA is an equilibrium mixture of acetic acid and H_2O_2 , one would expect to see an increase in catalase production, especially when upregulation occurred for the water treatment. The *S. Newport* in chlorine response to upregulate catalase was expected, as chlorine's mode of action is oxidative damage. Chlorine exposed *S. Newport* also upregulated the production of glutathione peroxidase 1.3-fold, the only treatment to do so. The water treatment resulted in a 1.7-fold upregulation of Glutaredoxin-like protein *nrdH*; however, all treatments downregulated glutaredoxin -1.4 or -1.5-fold (Table 4).

The most catastrophic target of oxidative damage is to an aerobic organism's nucleic acids. Strand breaks in the DNA or RNA backbones and indiscriminate cross-linking to other molecules prevents replication and regulation of cellular processes (35). Mutagenesis and death can occur with 10 min exposure to millimolar quantities of H_2O_2 (31). Lipids and proteins are also targeted. Lipid peroxidation within the membrane causes a loss in membrane fluidity, interfering with molecular transport in and out of the cell. Oxidative stress is not limited to extrinsic factors; radical oxygen species are continuously generated as a by-product of aerobic metabolism (35). Healthy bacteria maintain a balance between radical species and antioxidant compounds because under ambient conditions as it is more biologically efficient to repair damage to biomolecules than it is to scavenge every oxygen radical in the immediate environment or within the cell (26, 50). Catalase and superoxide dismutases also serve as tools in bacterial defense against reactive oxygen species produced by the plant immune system (14). As the PAA treatment did not elicit the production of catalase, it would appear in this study that catalase production was stimulated by the presence of water and chlorine, and not because of the tomato plant's immune system.

The pathways in which catalase were upregulated include ethanol degradation IV and superoxide radical degradation. As for the superoxide degradation, catalase is involved after superoxide dismutases break down reactive oxygen species into hydrogen peroxide and water; the remaining H_2O_2 undergoes catalase mediated disproportionation into water and oxygen. During ethanol degradation, catalase is involved in the conversion of ethanol, to acetaldehyde, which is eventually degraded to acetyl-CoA.

Catalase production assists survival of the phagocytic oxidative burst produced by NADPH oxidase (29) upon host invasion; some research, however, suggests that DNA repair mechanisms appear to play a more significant role in oxidative stress during invasion (5, 18). In contrast, Winquist et al. (63) found that in *S. Typhimurium*, when exposed to subsequent increasing quantities of H₂O₂, resistance was directly proportionate to the quantities of catalase produced. In addition to macrophage induced oxidative stress post-*Salmonellae* consumption, pro-biotics in the human gut utilize H₂O₂ against pathogenic bacteria. Products including lactic acid and bacteriocins secreted by *Lactobacillus johnsonii* NCC 533 were known antimicrobial agents, but Pridmore et al. (51) determined that the lactic acid bacteria also produces H₂O₂. Within the gut, *Salmonella*, therefore, not only encounters oxidative stress upon invasion of epithelial tissue, but also prior to contact with gut tissue as human commensal bacteria are secreting antimicrobial compounds.

Stress Response Proteins

PAA treated *Salmonella* upregulated stringent starvation protein A (SspA) 1.5-fold. No other treatment induced differential regulation of this protein. *sspA* is a transcriptional activator that is inducible upon acid exposure and glucose, nitrogen, phosphate or amino acid deprivation (27, 62). As chlorine and water treatments did not induce *sspA* expression, increased expression due to PAA is likely due to the acidity of the treatment. Increased heat and oxidative stress resistance has been demonstrated in starved *E. coli* cultures when compared to growing cultures that had been previously conditioned against heat or oxidizing agents (32). *sspA* is an inhibitor of H-NS, a repressor for a host of genes that include those involved in osmotic stress, oxidative stress, and acid tolerance in *E. coli* (27, 28). Accordingly,

expression of the DNA binding protein H-NS was also downregulated in PAA treated *Salmonella* -1.4-fold. It has been suggested that increased *sspA* increases a cell's ability to respond to acid-induced stress (28).

While some upregulation of stress response genes occurred in *S. Newport*, many genes involved in the stress response were downregulated. The greatest disparity occurred with the osmotically induced stress response. The osmoprotectant uptake system *yehW* was downregulated -1.4 to -1.5-fold for all three treatments, and PAA downregulated osmotically-inducible protein Y -1.3-fold. This most likely is due to the fact that the cells were rapidly responding to water exposure as compared to the control cells which did not receive this treatment, demonstrating how rapidly cells can adapt to new stimuli. However, water and chlorine upregulated osmoprotectant uptake system substrate-binding protein *osmF* 1.4-fold.

Other stress responses that underwent downregulation included heat shock proteins. These proteins serve as chaperones that bind to non-native sites on damaged proteins during heat stress. This ability prevents irreversible protein aggregation from occurring, which otherwise results in cellular death. Once the heat stress is removed, heat shock proteins then work with other chaperones to refold the damaged proteins back to their former structure (44, 46). Small heat shock protein *ibpB* was downregulated ranging from -1.8 to -2.2-fold for all three treatments, while only PAA downregulated protein *Hsp20* -1.6-fold. While no differential regulation occurred for PAA, water and chlorine resulted in downregulations of zinc resistance and arsenic resistance proteins ranging from -1.6 to -1.9-fold.

Universal stress protein G was downregulated -1.3-fold by PAA and water, but not by chlorine. This stress protein is hypothesized to play a role in iron homeostasis within the cell, which is intimately linked to the cell's ability to avoid to mitigate oxidative stress (60). Multiple stress resistance protein *bhsA* was downregulated -1.8-fold by PAA, -2.0-fold by chlorine, and -2.4-fold by water treated *Salmonella*. In *E. coli*, *bhsA* mutants were more susceptible to acid, heat, and oxidative stress. The protein also appears to regulate biofilm formation by decreasing aggregation and adhesion (54, 67). As with osmotic stress, it is likely that the treatments were not of sufficient concentrations to elicit stress responses by *S. Newport*. It is well documented that chlorine's efficacy on attached pathogens is limited to about a 2-log reduction under ideal wash conditions (35, 41, 45), and most of these studies evaluate using concentrations of 100-200 ppm, much higher than used in the current study. While the FDA recommends using 50-200 ppm, a concentration of 50 ppm is quickly depleted upon addition of organic material (16, 22, 41).

Structural Processes

Glutamate racemase was upregulated 1.7-fold in PAA exposed *Salmonella*. Chlorine and water treated *Salmonella* were devoid of this transcriptomic alteration, which is involved in peptidoglycan biosynthetic pathways. Glutamate racemase synthesizes D-glutamate from L-glutamate. D-glutamate and UDP-N-acetylmuramoyl-L-alanine combine (with the help of ATP) to form UDP-N-acetylmuramoyl-L-alanyl--D-glutamate. This process continues with the synthesis of peptidoglycan as the UDP-N-acetylmuramoyl-pentapeptide is transported and attached to the exterior of the membrane, where glycosyltransferases and transpeptidases polymerize the monomers by forming glycan chains (glycosyltransferases) and start the

crosslinking of peptides. Structurally, peptidoglycan serves as a sort of sieve that limits the flow of compound towards the interior of the cell. While structural variability is great in Gram-positive bacteria, peptidoglycan is compositionally similar in most Gram-negative organisms (35).

Conversely, lipoprotein was downregulated -1.3-fold by PAA and chlorine treated *Salmonella*. Water and chlorine both downregulated lipoprotein *yifL* and lipoprotein *ygdR* flags ($p < 0.05$), while PAA expressed no differential regulation of either. Water and chlorine downregulated *yifL* -1.6 and -1.5-fold, respectively, and *ygdR* -2.7 and -3.5-fold, respectively. Major outer membrane lipoprotein was downregulated in PAA -1.4-fold and -1.5-fold in water treated *S. Newport*.

Lipoprotein is a key component of the outer membrane of *Enterobacteriaceae* and along with lipopolysaccharide is responsible in large part to septic shock, an inflammatory cytokine response induced by the host immune system. Toll-like receptors 2 and 4 are the primary host signaling transducers that detect lipoprotein and activate macrophages and the immune response (39). As a cellular component, lipoprotein is critical in moving undesirable compounds out of the cell via drug efflux pumps, transporting substrates across the membrane, and in overall cellular surface structure (47). Some pathogens alternate lipoprotein expression in order to avoid recognition by the host immune response (52, 64). In *S. Typhimurium*, knocking out certain lipoprotein genes resulted in a reduction of interleukin-8 and proinflammatory cytokine tumor necrosis factor alpha. However, these same mutants were a virulent in intestinal epithelial cells, were less motile, and were no better suited to survive the macrophage consumption than the wild-type (56). One would assume that by simply downregulating

lipoprotein a pathogen could avoid an immune response; however, as lipoprotein is intrinsically linked to virulence, its presence appears to be crucial for invasion of host tissues (4, 21, 42).

Nucleic acid structure

Downregulation of the enzyme carbamoyl-phosphate synthase small chain (*carA*) occurred 1.5-fold for PAA and 1.7-fold for water treated *Salmonella*. This enzyme is involved in the biosynthesis of pyrimidine deoxyribonucleotides and ribonucleotides, precursors to DNA and RNA, respectively. Specifically, the enzyme, alone with its long chain variant, uses ATP to convert hydrogen carbonate (bicarbonate) and L-glutamine (used as a nitrogen source) to carbomyl-phosphate (30). The pathway continues as carbamoyl-phosphate and L-aspartate (derived from the TCA cycle formation of oxaloacetate) together form N-carbamoyl aspartate. Further along the pathway, UMP and finally, CTP and dTTP are synthesized.

Another pathway competes for the *carA* synthesized carbamoyl-phosphate. The arginine biosynthesis IV (archaebacteria) pathway uses carbamoyl phosphate and L-ornithine to make arginine. Due in part to this competition, regulators such as ornithine (an activator) and UMP (an inhibitor) are critical in regulating the use carbamoyl-phosphate; however, if both compounds are bound to the carbamoyl-phosphate, ornithine overrides the repressing activity of UMP (1, 30, 35, 59). An enzyme involved in L-ornithine conversion from L-glutamate, acetylornithine aminotransferase, was upregulated 1.4-fold by PAA. While other enzymes were involved in several arginine biosynthetic pathways, acetylornithine aminotransferase and *carA* were not upregulated from water or chlorine exposure. After CP and L-ornithine join, the enzyme argininosuccinate lyase transforms L-argino-succinate to L-arginine. Expression of the

RNA controlling this enzyme was upregulated 1.4-fold from PAA treatment. Again, no significant change in expression was observed for water or chlorine treatments ($p < 0.05$).

An enzyme involved in the arginine biosynthesis I (via ornithine) and II (acetyl cycle) pathways, amino-acid acetyltransferase, was upregulated 1.4-fold in chlorine and PAA treated *Salmonella*. This enzyme is involved in the first step of ornithine biosynthesis, converting glutamate to N-acetyl-L-glutamate. Ornithine's largest contribution as an amino acid is as an intermediary along the arginine biosynthetic pathway (35). No further proteins were differentially expressed for water or chlorine for either arginine pathway; however, in conjunction with arginosuccinate lyase (1.4-fold upregulation) and *carA* (1.5-fold downregulation), acetylornithine aminotransferase was upregulated 1.4-fold in PAA treated *Salmonella*.

Ammonia Oxidation and Amino Acid Synthesis

Both water and PAA treatments resulted in an upregulation of the hydroxylamine reductase (*hcp*), involved primarily in the ammonia oxidation IV (autotrophic ammonia oxidizers), ammonia oxidation I (aerobic), and nitrifier denitrification pathways. PAA resulted in a 20.9-fold upregulation and water a 4.7-fold upregulation. No significant regulatory change was detected in the chlorine treatment.

All three pathways (as the names imply), are involved at the very least in the conversion of ammonia to nitrite (ammonia oxidation I aerobic), while nitrifier denitrification terminates with the production of nitrogen gas. Hydroxylamine (NH_2OH) is an intermediary compound during nitrification. Hydroxylamine reductase cleaves the hydroxyl group to form nitrite (NO_2).

The amino acid glutamine serves as a storage compound for ammonia (35) and has been suggested to regulate the utilization of other amino acids (15). Dai et al. found that increased glutamine degradation to glutamate was directly related to increased glutamine utilization. In intestinal *E. coli*, increased glutamine resulted in increased ornithine production (15). Results from the current study indicated an increase in ornithine biosynthetic enzymes when degradation of glutamine decreased in PAA treated *Salmonella*. Ornithine biosynthesis was also upregulated in chlorine treated *S. Newport*.

Energy Generation

While water exposed *Salmonella* did not see large fold-changes in regulation of anaerobic respiration, five genes involved in the process were upregulated in six locations. Enolase, responsible for converting 2-phospho-D-glycerate to phosphoenolpyruvate, the first step of pathway, was upregulated 1.4-fold. The pathway then splits, with one route synthesizing succinate from phosphoenolpyruvate. The other route utilizes pyruvate kinase and pyruvate kinase 1 to create pyruvate. Production of the latter enzyme was upregulated 1.3-fold. From here, pyruvate can be converted to formate with the addition of coenzyme A and the assistance of several formate acetyltransferases, eventually terminating with the creation of CO₂ and H₂. Acetyltransferase was upregulated 1.5-fold. Alternatively, pyruvate conversion to acetyl-CoA occurs with the help of pyruvate dehydrogenase E1 component, upregulated 1.4-fold in water treated *Salmonella*. Acetyl-CoA transforms into citrate upon action by citrate synthase 1; citrate is then converted to cis-aconitate and then D-threo-citrate due to the activity of aconitate hydratase 1 (upregulated 1.3-fold) 2. D-threo-citrate, with the addition of

NADP⁺ and the presence of isocitrate dehydrogenase produces 2-oxoglutarate with ancillary CO₂ and NADPH.

No significant upregulation of the respiratory (anaerobic) pathway occurred for PAA treated *Salmonella*, but three enzymes out of the five upregulated by water exposure were also upregulated from chlorine exposure: enolase (1.3-fold), pyruvate kinase 1 (1.3-fold), and formate acetyltransferase (1.5-fold). No differential regulation occurred for pyruvate dehydrogenase E1 component or aconitate hydratase 1.

Microorganisms utilize fermentation for energy generation under anaerobic conditions by exploiting an organic compound as an electron donor (sugars) and using an organic compound of lower energy as an electron acceptor. This anaerobic method to derive energy is relatively inefficient, as very little of the energy produced is involved in the creation of ATP (3). For example, homolactic fermentation transforms glucose into ATP with lactate (lactic acid) produced at the pathway terminus (35). Heterolactic fermentation results in the creation of ATP along with lactic acid, CO₂, and ethanol (17); both forms are hallmark characteristics of lactic acid bacteria, but are readily utilized by other bacteria (3, 24, 34). Products of mixed acid fermentation often include formate or CO₂, lactate, succinate, acetate and ethanol (24, 66). A study involving *Lactococcus lactis* subsp. *lactis*, considered the model homolactic organism, found that the rate of sugar consumption, often mediated by the carbohydrate form or prevalence, caused the bacteria to shift towards either homolactic or mixed-acid fermentation. Glucose induced a higher glycolytic flux, limiting pyruvate formate lyase activity while increasing glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. However, because lactose and galactose supported slower growth and, therefore, a diminished glycolytic flux and

lower NADH/NAD⁺ ratios, mixed-acid fermentation was the predominant pathway. As the authors simply stated, substrates encouraging rapid metabolism encouraged homolactic fermentation, while substrates supporting slower metabolism resulted in bacteria utilizing mixed acid fermentation (24). The tendency towards mixed acid fermentation has also been noted under conditions of high-glucose concentrations in *E. coli* (66).

Differential regulation of genes involved in homolactic fermentation occurred. Production of the enzyme 6-phosphofructokinase was downregulated 1.4-fold on PAA treated tomatoes. However, upregulation of multiple genes involved in homolactic fermentation occurred for both chlorine and water treatments. Chlorine saw 1.3-fold upregulation of glyceraldehyde-3-phosphate dehydrogenase, as well as the enzymes enolase and pyruvate kinase 1, which were previously mentioned as being involved in anaerobic respiration. Like chlorine, water treated *Salmonella* upregulated enolase production (1.4-fold) and pyruvate kinase 1 (1.3-fold), but rather than altering glyceraldehyde-3-phosphate dehydrogenase expression similarly to water treated bacteria, a 1.4-fold downregulation of 6-phosphofructokinase occurred with Cl treatment, behaving similarly to those exposed to the PAA treatment.

Many of the same enzymes overlap into mixed acid fermentation, a pathway upregulated from water and chlorine treatments but not differentially expressed in PAA treated *Salmonella*. The enzymes pyruvate kinase (1.3-fold), formate acetyltransferase (1.5-fold), acetate kinase (1.4-fold), and aldehyde dehydrogenase (1.3-fold) were all similarly upregulated due to water and chlorine treatments. Water treated *Salmonella* further upregulated

phosphate acetyltransferase (1.4-fold) and aconitate hydratase 1 (1.3), which is involved in 2 separate locations in the mixed acid fermentation pathway.

Protein Translation

Both genes regulating EL-Tu were upregulated in chlorine (1.3-fold) and in water (1.3-fold and 1.4-fold) treated *Salmonella*. EL-G was also upregulated for both treatments; 1.3-fold for chlorine and 1.4-fold for the water treated *Salmonella*. No significant differential regulation occurred for PAA treated *S. Newport*. EL-Tu is crucial in translational elongation as it delivers charged tRNA to the ribosome (58). It has also been suggested that EL-Tu in *E. coli* protects proteins from stress, including heat stress, by refolding denatured and damaged proteins (6). EL-G binds to the ribosome to aid in translocation, where the mRNA moves forward one codon as the amino acid advances from the A to P position (2).

Virulence

All three treatments downregulated expression of the virulence transcriptional regulator, *hilD*. PAA treated *Salmonella* downregulated -1.8-fold, chlorine -2.1-fold, and water resulted in a -2.2-fold downregulation. *hilD* works in concert with other regulators, namely *hilC* and *rtsA*, to activate *hilA* that in turn activates the type III secretion system (T3SS) (40). The invasion protein, *invF*, was downregulated -2.0 in water only. The secretion system apparatus protein *ssaP* was downregulated -2.2-fold by chlorine.

Activation of the T3SS is a joint effort predominantly between *hilA* and *invF*, both of which are encoded on the *Salmonella* pathogenicity island 1. Knocking out *invF*, which regulates the externally encoded (from the pathogenicity island) expression of T3SS secreted proteins,

results in significantly impaired host cellular invasion. *Salmonella* devoid of *hilA*, which regulates the mechanics of the T3SS, were still able to effectively invade host tissue (19).

Overall response to water, chlorine and peroxyacetic acid exposure

Three hundred and sixty-five genes were found to be differentially regulated amongst all treatments. Overall, the transcriptomic response of *Salmonella* Newport in this study is greatly diminished compared to another study examining the stress response of planktonic *Salmonella* spp. when exposed to an oxidating sanitizer. Wang et al (2010) found more than 2,000 genes up-regulated and a similar number of genes down-regulated after exposure of *S. Typhimurium* LT2 and *S. Enteritidis* PT4 to a 130 ppm, 30 min chlorine treatment in microbiological media (61).

A general lack of transcriptomic response in this experiment implies one of two scenarios: 1) that treatments were not sufficient from that experienced on the surface of the tomato to elicit a transcriptomic response or 2) cells were in a dormant state commonly observed as a survival mechanism in foodborne pathogens. First studied in response to antibiotic resistance, persister cells have been increasingly shown to be an advantageous mechanism for bacteria in which the cell enters a dormant state, as triggered by production of a toxin that inhibits cellular activity while not causing cell death when exposed to adverse conditions. These toxins can be inactivated by binding of antitoxins in the form of complimentary RNA or proteins depending upon the toxin-antitoxin type (65).

RelE is the toxin component in the RelB/E Type II toxin-antitoxin module. RelE inhibits translation by cleaving mRNAs in the ribosomal A site (49). In this study, *relE* was down-

regulated in all treatments -1.65 to 1.46 as compared to the no rinse controls. At the same time, the antitoxin component, *relB*, was not differentially regulated. This indicates that cells on the tomato surface had an increased level of RelE, potentially resulting in a dormant population that once exposed to a washing system was beginning to downregulate this genetic component to regain metabolic activity.

Dormancy on the surface of produce would help with the explanation of why sanitizing wash treatments show relatively little activity and are many times only as effective as water when inactivating organisms attached to produce, resulting in a 90-99% inactivation (16, 36). Further investigation with better annotated *Salmonella* spp. is needed to elucidate the extent toxin-antitoxin systems may be playing a role in *Salmonella* survival on produce surfaces and when exposed to subsequent inactivation steps, such as postharvest washing.

Conclusions

The ability of *Salmonella* to readily adapt to stress, persist in soil and water, and firmly adhere to surfaces, especially fresh produce, aid its success as a foodborne pathogen. A very limited response of *Salmonella* Newport to the water, chlorine and PAA is a departure from what has been observed with planktonic cells exposed to oxidizing sanitizers in microbiological media. Additionally, the vast majority of transcriptional responses were shared with water, indicating they were not specific to the oxidizing sanitizers. Desiccation on the tomato may have resulted in a dormant state for many *S. Newport* cells. Further exposure to washing treatments was shown to downregulate *relE*, the toxin-component of the one of the primary toxin-antitoxin systems. Further investigation is warranted to determine how desiccation on the

tomato surface may drive cells into a dormant state, subsequently allowing them to survive adverse conditions.

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Appendix

Table 1. Top ten up- and downregulated *Salmonella* Newport genes of known products post-water exposure ($p < 0.05$; hypothetical proteins excluded).

Regulation	Location	Name	Product/Function	Fold Change	Length (bp)
Up	SN31241_t170		tRNA-Lysine	34.1	76
	SN31241_19550		Hydroxylamine reductase	4.7	1644
	SN31241_39290		Reductase	4.0	1440
	SN31241_34490		Phosphotransferase	2.1	273
	SN31241_60		ATP synthase subunit c	1.7	240
	SN31241_38980	<i>nrdH</i>	Glutaredoxin-like protein	1.7	186
	SN31241_38010		Large repetitive protein	1.6	110367
			Peptide transport system ATP-binding protein		
	SN31241_27770	<i>sapD</i>		1.6	993
	SN31241_26330	<i>treY</i>	TreY	1.6	2529
Down	SN31241_7760		30S ribosomal protein S6	1.6	396
	SN31241_21220		bacteriophage protein	-37.6	138
	SN31241_t60		tRNA-Leucine	-30.7	85
	SN31241_t410		tRNA-Tryptophan	-6.4	76
	SN31241_t110		tRNA-Threonine	-6.3	76
	SN31241_32970	<i>yeiS</i>	Protein yeiS	-5.1	246
	SN31241_t540		tRNA-Glutamate	-4.3	73
	SN31241_t310		tRNA-Valine	-4.1	76
			Surface presentation of		
	SN31241_39810	<i>spaQ</i>	antigens protein	-4.0	261
	SN31241_t270		tRNA-Asparagine	-3.7	76
	SN31241_t450		tRNA-Proline	-3.5	77

Table 2. Top ten up- and downregulated *Salmonella* Newport genes of known products post-chlorine exposure (p < 0.05; hypothetical proteins excluded).

Regulation	Location	Name	Product/Function	Fold Change	Length (bp)
Up	SN31241_t180		tRNA-Lysine	31.4	76
			Ethanolamine ammonia-lyase		
	SN31241_35630		heavy chain	1.8	1362
	SN31241_34250		Reductase	1.7	543
	SN31241_4530		30S ribosomal protein S19	1.7	150
			Peptide transport system ATP-binding protein		
	SN31241_27770	<i>sapD</i>	30S ribosomal protein S16	1.6	993
	SN31241_37880		Lysine-sensitive aspartokinase 3	1.6	249
	SN31241_5370		Peptide transport system permease protein	1.6	1350
	SN31241_27760	<i>sapC</i>	50S ribosomal protein L34	1.6	891
	SN31241_340		Glycogen synthase	1.6	141
	SN31241_3580			1.5	1434
Down	SN31241_t740		tRNA-Valine	-65.9	77
	SN31241_38550		bacteriophage protein	-33.2	180
	SN31241_t20		tRNA-Glutamate	-8.3	76
	SN31241_t260		tRNA-Asparagine	-6.0	76
	SN31241_21220		bacteriophage protein	-5.9	138
	SN31241_t760		tRNA-Serine	-5.1	88
	SN31241_t640		tRNA-Glutamate	-5.0	76
	SN31241_t590		tRNA-Serine	-4.6	93
	SN31241_t660		tRNA-Alanine	-4.5	76
	SN31241_t540		tRNA-Glutamate	-4.4	73

Table 3. Top ten up- and downregulated *Salmonella* Newport genes of known products post-PAA exposure ($p < 0.05$; hypothetical proteins excluded).

Regulation	Location	Name	Product/Function	Fold Change	Length (bp)
Up	SN31241_t10		tRNA-Proline	35.0	77
	SN31241_19550		hydroxylamine reductase	20.9	1644
	SN31241_39290		Reductase	16.4	1440
	SN31241_39300		Reductase	6.0	1134
	SN31241_28930	<i>tehB</i>	Tellurite resistance protein	2.8	342
	SN31241_28450		Reductase	2.7	3744
			Potassium-transporting ATPase		
	SN31241_17070		A chain	2.1	1680
	SN31241_19540		Reductase	2.0	972
			HTH-type transcriptional		
Down	SN31241_21880	<i>rutR</i>	regulator	1.7	726
	SN31241_45560		Glutamate racemase	1.7	783
	SN31241_t640		tRNA-Glutamate	-8.0	76
	SN31241_t300		tRNA-Arginine	-7.9	75
	SN31241_t750		tRNA-Serine	-7.0	88
	SN31241_t760		tRNA-Serine	-6.3	88
	SN31241_t320		tRNA-Valine	-5.7	76
	SN31241_t390		tRNA-Glutamate	-5.5	76
	SN31241_t740		tRNA-Valine	-4.6	77
	SN31241_t70		tRNA-Isoleucine	-4.2	77
	SN31241_t590		tRNA-Serine	-4.1	93
	SN31241_t500		tRNA-Threonine	-4.0	76

Table 4. Differential regulation of genes involved in oxidative response by *S. Newport* after 2 h tomato exposure and 1 min sanitizing wash ($p < 0.05$).

Gene	Function	Water	Fold Change	
			40 ppm PAA	50 ppm Cl
SN31241_28140	Catalase	1.3	.	1.3
SN31241_23930	Catalase HPII	1.3	.	.
SN31241_25000	Glutaredoxin	-1.5	-1.4	-1.4
<i>nrdH</i>	Glutaredoxin-like protein	1.7	.	.
SN31241_24130	Glutathione peroxidase	.	.	1.3

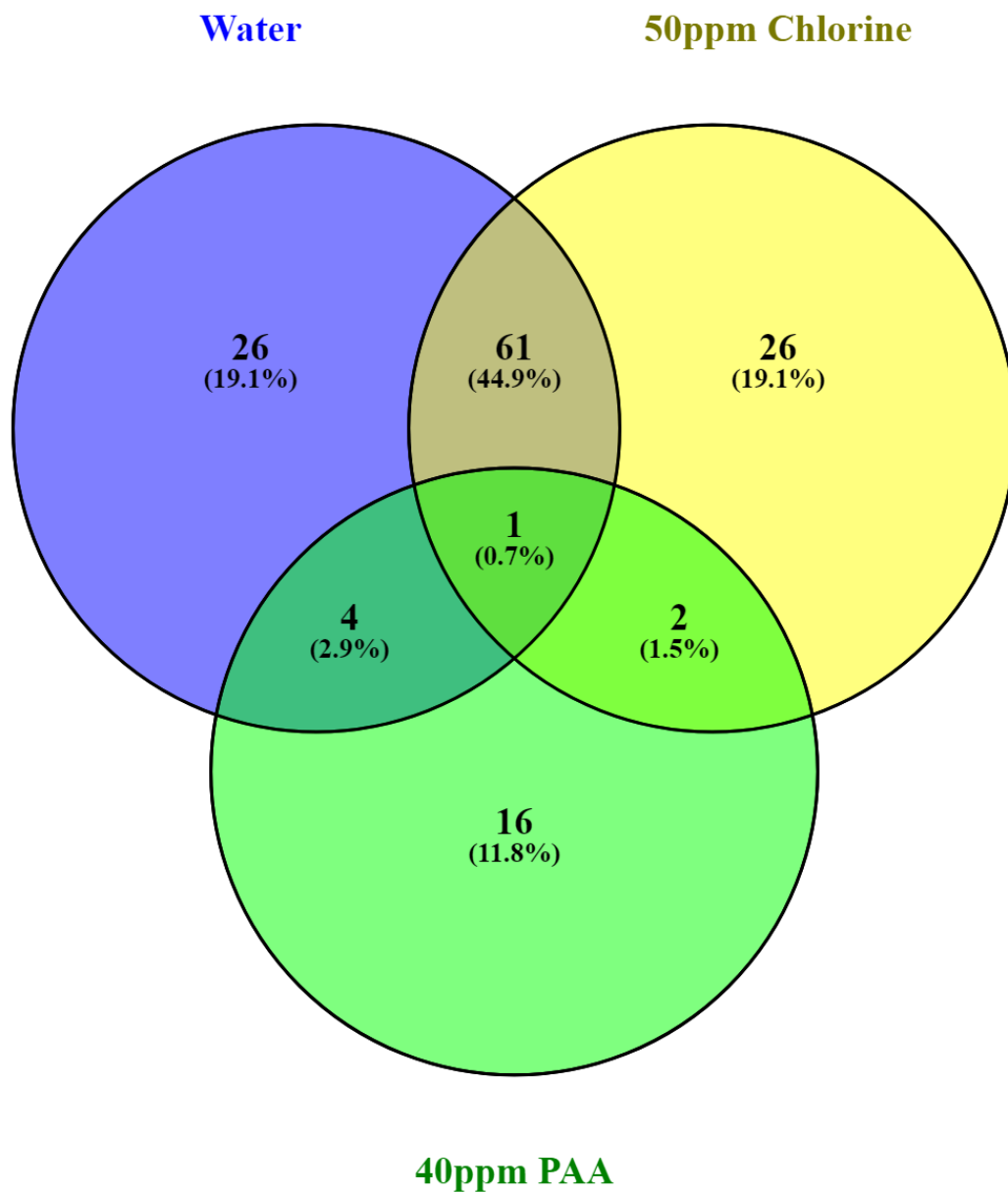


Figure 1. Distribution of upregulated genes amongst all washing treatments.

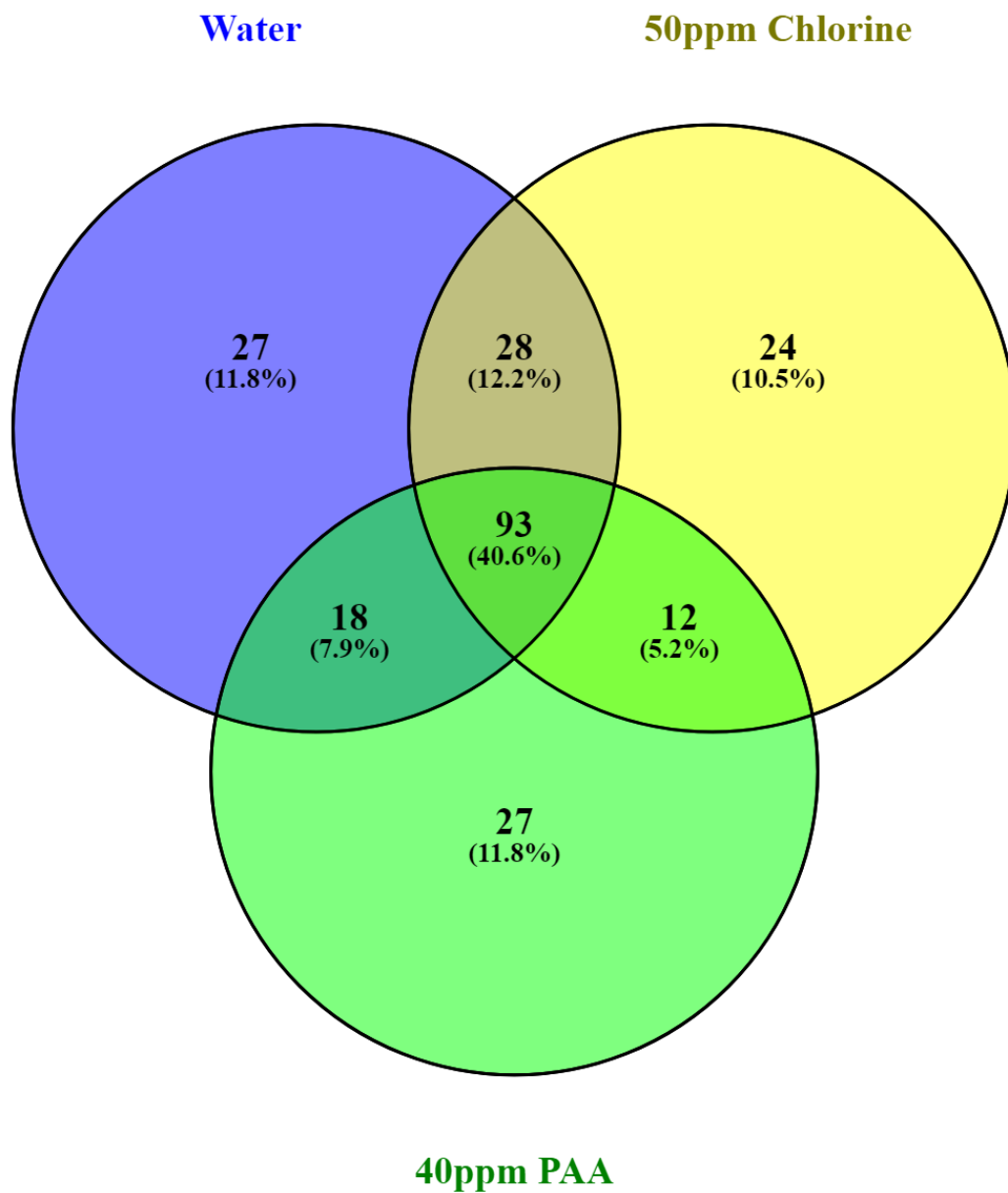


Figure 2. Distribution of downregulated genes amongst all washing treatments.

Chapter 3: Transcriptomic Analysis of *Salmonella enterica* Serovar Newport after 24-hour Environmental Exposure *In Planta* and Postharvest Sanitizing

Abstract

Salmonella enterica has a multitude of survival mechanisms to employ when exposed to environmental stress. The transcriptomic response of *S. Newport* was evaluated after 24 h exposure on a tomato plant within an environmental chamber, followed by subsequent sanitizer treatment. Genes involved in motility, especially involving flagellar function and chemotaxis were significantly downregulated for all treatments, which included a no rinse (NR) treatment, water, 40 ppm peroxyacetic acid (PAA), 85 ppm PAA, and 50 ppm chlorine (Cl). The TCA cycle was upregulated to varying degrees by all treatments, ranging from 11 enzymes upregulated by 85 ppm PAA to only 4 genes upregulated by 40 ppm PAA and chlorine. The six enzymes involved in arginine catabolism via the arginine succinyltransferase system was also upregulated across all five treatments. Oxidative stress response overall was upregulated, including catalase. Superoxide dismutase was upregulated except by water treated *S. Newport*, which experienced no differential regulation. Other stress response proteins, including heat shock, cold shock, phage shock, and osmoprotective enzymes experienced mixed regulation across treatments.

Introduction

Salmonella enterica spp. *enterica*, with its more than 1,500 serovars (6), is one of the leading causative agents of foodborne illness worldwide, with an estimated 1.2 million cases per year in the United States alone (70). Produce contamination from animal reservoirs is increasingly the cause of widespread outbreaks for several reasons, including the increased consumption of fresh, non-processed fruits and vegetables, large-scale production practices, and increased food distribution distances (6, 67, 68). While production and consumption

practices have been changing, surveillance for human illness caused by foodborne pathogens has also been improving, driving a perceived increase in cases and outbreaks (31).

Of the 4,937 genes contained within the *Salmonella enterica* serovar Newport genome, 4,828 code for proteins and 109 are RNA genes. *Salmonella* spp. are tolerant of low water environments and are persistent on a multitude of water scarce surfaces (24); low water activity foods responsible for outbreaks include peanut butter containing *S. Tennessee* (11) and *S. Typhimurium* (12), chocolate contaminated with *S. Eastbourne* (15) and *S. Napoli* (23), cereal with *S. Agona* (10), and infant formula containing *S. Seftenberg* (69). Exposure to one stress and subsequent response may provide cross protection for *Salmonella* to survive pressure from another condition; for example, studies have shown that low water activity creates a more robust survival response to subsequent heat treatment in some, but not all, serovars (37, 46-48).

Stress induced behaviors are regulated by the *rpoS* gene, and *Salmonella* deficient in *rpoS* are less stress tolerant, including that related to low water activity (46). Little is known about how *Salmonella* responds to subsequent stresses, such as chemical sanitation, after exposure to dry or low water activity surfaces. *Salmonella* undergoes morphological changes when exposed to reduced water activity. Filamentation of *Salmonella* cells occurred after exposure to a water scarce surface for a 21-day period in which cells were still able to maintain membrane integrity. It was also observed that these cells were more resistant to sanitation. *S. Enteritidis* cells also elongated to greater than 6 μm upon exposure to 0.94 to 0.95 a_w (35). Moreover, one study by Kieboom et al. (35) did find that *S. Enteritidis* was more tolerant of sodium hypochlorite treatment when pre-exposed to NaCl vs a tryptic soy agar control.

Salmonella is indiscriminate in its host preference, and therefore colonizes a wide array of macroorganisms. It has been suggested that *Salmonella* has greater environmental persistence than *E. coli*, as it appears to be more adept at evading predation, more tolerant to fluctuations in pH, temperature, and relative humidity, and more readily adaptable to starvation stress than *E. coli* (5, 42, 49, 81).

Inoculum preparation

Upon attachment to the tomato plant, especially the tomato fruit itself, *Salmonella* finds itself exposed to a multitude of stressors; these include low water activity, UV radiation, competition from existing microflora, and the plant immune system. If the cells survive the harsh conditions on the plant, they must then survive postharvest sanitation practices that generally involve a CI or PAA rinse. The present study evaluated *Salmonella* Newport response to desiccation and subsequent sanitizer treatment. A transcriptomic analysis evaluated gene expression to make comparisons across sanitizer treatments.

Materials and Methods

A human outbreak strain of *Salmonella enterica* serovar Newport (0418) was obtained from Dr. Laura Strawn at Virginia Tech University. Tryptic soy broth was used to revive culture with three consecutive transfers after incubation at 37 °C for 24 h. Prior to experimental use, 300 µL of 10⁸ CFU/mL culture was spread across tryptic soy agar (TSA; BD, Sparks, MD) plates and incubated for 16-18 h. After incubation, plates were flooded with 3 mL phosphate buffer solution (PBS; Fisher Scientific, Hampton, NH). Sterile L-shaped spreaders scraped bacterial culture from plate surface and culture was aseptically transferred into a 50-mL centrifuge tube. An additional 2 mL of PBS was introduced to the plate to remove residual culture from the agar

surface, which was then added to the initial culture. Collected culture was centrifuged for 5 min at 8000 g; cell pellet was re-suspended in 250 μ L PBS.

Inoculation

Gibberallic acid deficient dwarf tomato plants obtained from Dr. Dean Kopsell, University of Tennessee Department of Plant Sciences, were utilized for the study for ease of manipulation. *Salmonella* culture was applied to mature tomatoes still attached to the plant; 16 (+/- 2) 5 μ L drops of culture were applied to the cheek of the fruit surface, midway between the stem and blossom scar regions. Plants remained in the biosafety cabinet where inoculation occurred for 1 h to allow culture drops to dry, where after they were moved to an environmental chamber for 24 h.

The conditions within the chamber were set to mimic approximate field conditions in the eastern region of Tennessee. The initial time interval assumed contamination occurred mid-morning, and was therefore set for 8 h at 29.4 °C at 60% relative humidity (RH). The chamber then cycled through a 2 h interval with temps dipping to 23.9 °C and RH increasing to 75% to approximate evening cooling. A 10 h nighttime interval maintained the temperature at 20 °C and 89% RH, before warming up with a simulated morning temperature 23.9 °C and 75% RH. The cycle ended with 2 h of daytime temperature at 29.4 °C and 60% RH, as in most operations a tomato harvest would likely occur around mid-morning to mid-day.

Sanitizer Treatment

Sodium hypochlorite (Fisher Scientific, Hampton, NH) was added to sterile deionized water (DI) to a final concentration of 50 ppm and verified using a chlorine meter (Hannah Instruments, Woonsocket, RI). PAA (Sanidate 5.0; Biosafe Systems; East Hartford, CT) and DI

were mixed to final concentrations of 40 and 85 ppm. Chlorine, PAA (both concentrations), and DI were aliquoted into 25 mL volumes in individual 50 mL RNase/DNase free centrifuge tubes (Corning, Corning, NY).

Upon tomato plant removal from the environmental chamber, individual tomatoes were harvested using sanitized scissors and placed directly into one of the four treatments. After 1 min exposure, the treatment was decanted. The tomato was aseptically transferred to a new tube containing 1.5 mL RNAlater (Qiagen; Hilden, Germany). *Salmonella* was removed using vortex bursts for 20 sec, upon which time the culture containing RNAlater was pipetted out of the tube into sterile 1.5 mL RNase/DNase free microcentrifuge tubes. Cells were incubated at room temperature for 10 min. Ten min centrifugation at 8600 x *g* separated the cells from supernatant, which was removed from the tube.

RNA Extraction

RNA extraction occurred either immediately or within 3 days of collection (stored at -20 °C). TE buffer containing lysozyme (6 mg/mL) was added (100 µL) to the pellet and vortexed continuously for 10 min. Three hundred and fifty µL buffer RLT was added and vortexed. Ethanol (96-100%; 250 µL) was added and mixed by pipetting. Each sample was placed in an RNeasy mini column, which was placed in a 2-mL collection tube. The tube was centrifuged for 15 sec at 8,000 x *g*; the collection tube was discarded, and column placed in new tube. Buffer RW1 (700 µL) was applied to the column and centrifuged for 15 secs at 8,000 x *g*. The collection tube was again replaced, and 500 µL Buffer RPE was added to the column, where it remained for 5 min prior to centrifugation (15 secs; 8,000 x *g*). The RPE step was repeated two additional times. The column was placed into a new collection tube and centrifuged one final time to

remove any remaining ethanol. The column was then placed into a 1.5 mL collection tube, and 25 μ L RNase-free water was applied directly to the RNeasy membrane. RNA was eluted via centrifugation for 1 min at 8,000 $\times g$.

RNA was analyzed with Nano drop (Thermo Scientific, Waltham, MA) to ensure the concentration was > 80 ng/ μ L and that the 260/280 ratio was between 1.8 and 2.2. A final volume containing at least 5,000 ng was collected per sample.

RNA-Sequencing

The following protocol was supplied by Brian Sereni, GENEWIZ, LLC. (South Plainsfield, NJ). Library preparation, sequencing, and bioinformatics analysis were performed by GENEWIZ, LLC. (South Plainfield, NJ). Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) quantified RNA and RNA integrity was checked with 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Ribozero rRNA Removal Kit (Illumina, San Diego, CA) was used to perform whole transcriptome RNA enrichment. Manufacturer's recommendations were followed for RNA sequencing library preparation using the NEBNext Ultra RNA Library Prep Kit (NEB, Ipswich, MA) for Illumina. RNAs were fragmented at 94 °C for 15 min. First and second strand cDNA was synthesized subsequently. cDNA fragments ends were repaired and 3' ends adenylated; universal adapter was ligated to cDNA fragments, followed by index addition and library enrichment with limited cycle PCR. A DNA chip on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) validated sequencing libraries, which were then quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and quantitative PCR (Applied Biosystems, Carlsbad, CA).

Sequencing libraries were multiplexed and clustered on one flowcell and one lane. After clustering, the flowcell and lane were loaded on the Illumina HiSeq 2500 per manufacturer's instructions. Samples were sequenced using 1x50 Single Read Rapid Run configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HSC) on the HiSeq 2500 instrument. Raw sequence data (.bcl files) generated from Illumina HiSeq 2500 was converted into fastq files and de-multiplexed using Illumina bcl2fastq v1.8.4 program. One mismatch was allowed for index sequence identification.

Differential Transcriptome Analysis

Fastq files from each sample were imported into CLC Genomics Workbench 8.0.3. Sequence reads were trimmed to remove bases with low quality at ends. Sequence reads were then mapped to the reference genome. The total gene read count data was normalized using Baggerley's test and the statistical significance was calculated on the defined groups of samples. A box plot was generated for each comparison using the original and normalized expression values. A Principal Component Analysis (PCA) and Hierarchical clustering analysis were also performed to examine sample separation between the groups for each comparison. S. Newport exposed for 2 h (29.4 °C, 60% RH) in the environmental chamber and receiving a no-rinse (NR) treatment postharvest served as the control for the study.

The MetaCyc (MetaCyc.org) and BioCyc (BioCyc.org) databases were used to annotate individual genes and to categorize genes and gene clusters within their associated metabolic pathways (9). Venn diagrams generated by Venny 2.1 and InteractiVenn were also utilized to visualize distribution of up- and downregulated genes amongst washing treatments (27, 59).

Results and Discussion

Six hundred eighty-nine genes were upregulated amongst all five treatments (Figure 1). Amongst these, 660 genes were upregulated in response to one of the four washing treatments (Figure 2). Four hundred and five genes were downregulated in response to one or more of the five treatments (Figure 3). Three hundred ninety-one genes were downregulated in one of four washing treatments (Figure 4). Specific themes were found amongst genes involved in stress response, especially with oxidative stress response, starvation, metabolism, outer membrane components, motility and biofilm formation.

Regulation of stress response genes was mixed across treatments

While some upregulation of stress response genes occurred in *S. Newport*, many genes involved in the stress response were downregulated. The greatest disparity occurred with the osmotically induced stress response. The osmoprotectant uptake system *yehW* was downregulated -1.3 to -1.6-fold for all five treatments, while the osmoprotectant uptake system *yehY* was upregulated 1.4 to 1.7-fold by all treatments except NR. Osmoprotectant uptake system ATP-binding protein *YehX* was upregulated 1.6 to 1.7-fold across all treatments except water. All treatments upregulated osmoprotectant uptake system substrate-binding protein *osmF* ranging from 1.3 to 1.7-fold. All treatments also upregulated osmotically-inducible protein Y ranging from 1.5 to 1.8-fold (Table 1).

Other stress responses that underwent downregulation included heat shock proteins. These molecule chaperones bind to non-native sites on heat-damaged proteins to prevent aggregation within the cell, which is irreversible (45). When the heat is removed from the cell, small heat shock proteins, with the help of other chaperones, return the protein to its native

state through ATP dependent hydrolysis. Heat shock proteins are also intimately linked to the maintenance of the cellular membrane, which is sensitive to temperature variation towards either extreme. Therefore, it has been suggested that under heat stress, shock proteins are divided between protecting proteins in the cytoplasm from aggregation and ensuring membrane integrity is maintained (55). Small heat shock protein *ibpA* was upregulated 2.3-fold for NR and 2.1-fold for 85 ppm PAA treated *S. Newport*; NR *S. Newport* also upregulated small heat shock protein *ibpB*. Heat shock protein 20 was upregulated 1.9-fold by both NR and 85 ppm PAA, while *hspQ* was downregulated -1.3-fold by water and -1.4-fold by 85 ppm PAA. The regulation of heat shock proteins, along with other stationary phase stress response proteins, is encoded on the sigma factor encoded on *rpoS*. *rpoS* was upregulated 1.3 and 1.4-fold by 40 ppm PAA and chlorine, respectively.

Universal stress proteins were downregulated for water and both PAA treatments, upregulated by the NR treatment (stress protein F), and underwent no differential regulation by chlorine treated *Salmonella*. The proteins are involved in stress response from several agents, including acid, oxidative, and temperature (18), and are prevalent in all three domains of life. However, their exact physiological role is unclear. It has been hypothesized that universal stress proteins E and C are necessary for motility, as *E. coli* knock-outs were devoid of flagella; proteins F and G appear to be important for iron homeostasis within the cell, a state that is linked to a cell's ability to survive oxidative stress (76).

Phage shock proteins underwent significant upregulation for NR, W, and 85 ppm PAA treatments. It hypothesized that these proteins are expressed during phage infection or

environmental stress in order to stabilize the cytoplasmic membrane (16). In the current study, positive regulation of these proteins ranged from 2.5 to 5.8-fold (Table 1).

S. Newport upregulated catalase in response to oxidative stress

NR, 40 ppm PAA, and chlorine treated *S. Newport* upregulated catalase hydroperoxidase I activity 1.5, 1.4, and 1.5-fold, respectively. Surprisingly, no differential regulation occurred for 85 ppm PAA, but the lack of differential regulation due to water was not surprising as there should have been little presence of radical oxygen species. Two other genes for a manganese containing catalase and catalase hydroperoxidase II (HPH) were upregulated by 40 ppm PAA, water, and chlorine. Catalase HPH was upregulated by 85 ppm PAA, but the Mn-containing catalase was not (Table 2). All treatments except water upregulated at least one of three superoxide dismutases. Superoxide dismutase reduces superoxide to the less reactive H_2O_2 , particularly within the periplasm and inner membrane regions (17). The fact that water did not upregulate one was not surprising, although the 2.5-fold upregulation by the NR treatment was. Superoxide dismutase expression is regulated by *soxR*; however, only chlorine upregulated the transcriptional activator.

All biological macromolecules are susceptible to oxidative stress, including nucleic acids, lipids, and proteins (21). Peroxidation of lipids in the membrane results in diminished membrane fluidity. Strand breaks in the DNA backbone and cross-linking to other molecules interferes with a cell's ability to replicate (7). While H_2O_2 is generally thought to be a significant source of oxidative stress, it is a nonradical and relatively inefficient oxidizer of bacterial components (19), and is even produced in small quantities as a result of aerobic respiration. It is when an excess of iron is available within the cell or the environment that H_2O_2 mediated

oxidative damage can get out of control. H_2O_2 reacts with ferrous iron (Fe^{2+}) via the Fenton Reaction to generate hydroxide radicals, which have much greater oxidative potential and indiscriminately damage bacterial macromolecules (7, 77). It is hypothesized that the generation of these radicals via the Fenton Reaction is why iron is so tightly regulated by bacterial cells (77). Two overlapping systems in *E. coli* and *Salmonella* maintain a reduced cytosolic environment in response to environmental stress. The glutathione and thioredoxin systems are generally not required for growth under non-stressed conditions, but are induced upon oxidative stress (8). Glutathione peroxidase was upregulated 1.3-fold by 40 ppm and 1.5-fold by chlorine treated *S. Newport*. Thioredoxin-2 was downregulated by 85 ppm PAA -1.4-fold. All five treatments upregulated at least one of four glutaredoxins; however, all treatments except NR also downregulated one glutaredoxin. Glutaredoxins were discovered when *E. coli* lacking thioredoxin still maintained a viable hydrogen transport system to ribonucleotide reductases (8, 29). Two alkyl hydroperoxide reductases were upregulated by several of the treatments. These reductases rely on NADH or NADPH as reducing agents to convert lipid or alkyl hydroperoxides to alcohols (8, 32). The peroxiredoxin *osmC* was upregulated 1.4 and 1.5-fold by 40 ppm PAA and chlorine, respectively. *osmC* reduces organic hydroperoxides and to a much lesser extent, H_2O_2 (40).

S. Newport upregulates arginine catabolism via the arginine succinyltransferase system after 24 h tomato surface exposure

The presence of amino acids is critical to the survival of acid stressed bacteria (34, 71). While *Salmonella* has repeatedly been demonstrated to be less acid resistant than *E. coli* under identical conditions (34), arginine supplementation enabled survival of *S. Typhimurium* at pH

2.5. Previously, *S. Typhimurium* had been unable to survive at that pH indicating the presence and imperative nature of an arginine-dependent acid resistance system. A similar study found enhanced survival of previously acid adapted *S. Typhimurium* when growth medium was supplemented with either arginine or lysine, though the same effect was not observed in non-acid adapted cells or when glutamic acid supplementation occurred. Expression of decarboxylases for both arginine and lysine was upregulated, indicating that these systems are important for acid stress response (2). The arginine decarboxylase creates agmatine by adding a cytoplasmic proton to arginine in the location that formerly contained an α -carboxyl group. This removal of protons from the cytoplasm helps the cell maintain a stable pH under acidic conditions (66). In this study, *Salmonella* desiccated for 24 h upregulated arginine decarboxylase 1.4-fold for NR and 85 ppm PAA, 1.5-fold for water, 1.6-fold for 40 ppm PAA, and 1.7-fold in chlorine.

The necessity of arginine in the avian lumen by *Salmonella* was evident in a poultry study that found upregulation of *metE*, *metF*, and *metR*, *adiA*, *speA*, *argA*, and *argS*; meanwhile in the gut mucosa, only 1 amino acid transport gene, *yhiP*, was upregulated, although genes encoding *carB* and 2 ABC transporter proteins were downregulated (26). Utilization of arginine as a nitrogen, carbon, and energy source by *Pseudomonas* (25, 80) and *Klebsiella aerogenes* (71) and as a sole nitrogen source for *E. coli* (but not carbon source; (71)) via the arginine succinyltransferase system (*ast* operon) has been well documented (41). Prior to the discovery of the AST pathway it was unknown how ammonia, which was required for glutamine biosynthesis was generated from amino acid degradation. The previously identified catabolic pathway for arginine, arginine decarboxylation, was determined to only utilize 3% of the

broken-down arginine (71). Schneider et al. (71) identified the *astCADBE* operon in *E. coli*, which encodes five enzymes involved in the catabolism of arginine to succinate and glutamate with ancillary ammonia production. They also determined that AST enzymes were upregulated due to nitrogen deprivation, while Lu et al. (41) found carbon deprivation stimulated the AST system; the operon under both conditions is regulated by the *argR* operon.

S. Newport in the present study encountered nitrogen and carbon starvation as well as acid stress when treated with PAA. *Salmonella* also has six enzymes involved in the AST pathway (Figure 5) to *E. coli*'s five, all of which were significantly upregulated when exposed to 40 (range 1.5 to 2.5-fold) and 85 (range 1.5 to 1.9-fold) ppm PAA treatments (Table 3). NR, chlorine, and water treatments experienced similar upregulation. *Salmonella* that remained on the tomato surface for only 2 h prior to 40 ppm PAA treatment did not differentially express any enzymes in this pathway. Apparently, prolonged desiccation on the tomato plays a significant role in the upregulation of the pathway.

Juxtaposed to degradation pathways, arginine biosynthetic pathways were significantly downregulated for 24 h treatments ($p < 0.05$). Of the 11 enzymes involved in arginine biosynthetic pathways I (via L-ornithine) and ten involved in pathway II (acetyl cycle), eight were downregulated from the water treatment (range -1.5 to -2.2) and 85 ppm PAA (range -1.7 to -2), seven were downregulated for the NR (range -1.5 to -1.8) and 40 ppm PAA (range -1.7 to -2.7), and six were downregulated for chlorine (range -1.6 to -2.4). A similar trend was observed for the arginine biosynthetic pathway IV (archaeobacteria) as most enzymes are shared among the pathways.

The TCA cycle and its precursors were upregulated by all treatments

Energy generation via the TCA cycle occurs in heterotrophic cells through an intricate pathway of enzymes and proteins (Figure 6) (22). One iteration of the cycle generates one molecule of ATP, three molecules of NADH, and one molecule of FADH²⁺. NADH and FADH²⁺ serve as electron carriers to aid in the creation of more ATP through oxidative phosphorylation. The cycle's other predominant function in bacteria is the provision of precursors that are necessary for the synthesis of other cellular structures (4, 75).

The TCA cycle I (prokaryotic), was significantly upregulated when 24 h S. Newport encountered 85 ppm PAA treatment (Table 4; $p < 0.05$). Of the 13 genes involved in the TCA cycle, 11 were upregulated ranging from 1.4-fold (aconitate hydratases 1 and 2) to 3.1-fold (Succinyl-CoA ligase [ADP-forming] subunit beta); fumarate hydratase and fumarate hydratase II underwent no significantly different changes in expression ($p < 0.05$). While fewer genes were upregulated in the NR and water treatments, nine and seven, respectively, ranging from 1.5 to 2.2-fold. Chlorine and the 40 ppm PAA treatments only upregulated four genes; curiously, chlorine and 40 ppm PAA upregulated expression of fumarate hydratase class II, the expression of which all other treatments failed to induce.

Prior to induction of TCA, several pyruvate synthesizing pathways were upregulated. The D-fructonate catabolic pathway involves the dehydrogenation (D-mannonate oxidoreductase), dehydration (mannonate dehydratase), and phosphorylation (2-dehydro-3-deoxygluconokinase) of D-fructonate. KHG/KDPG aldolase acts upon the resultant molecule to create pyruvate, which is shunted to the pyruvate decarboxylation pathway which creates acetyl-CoA prior to TCA, and D-glyceraldehyde 3-phosphate, an intermediate in glycolysis.

Twenty-four h *Salmonella* exposed to 85 ppm PAA, water, and NR upregulated the initial two enzymes, D-mannonate oxidoreductase (5.1, 3.4, and 4.7-fold, respectively) and mannonate dehydratase (5.0, 3.0, and 4.3-fold, respectively). No part of the pathway was upregulated in chlorine or 40 ppm PAA treated *Salmonella*. Pyruvate enters an upregulated decarboxylation pathway, which was upregulated for all chemical treatments. Upregulation for all three enzymes, pyruvate dehydrogenase, dihydrolipoamide acetyltransferase, dihydrolipoyl dehydrogenase, ranged from 1.4 to 2.4-fold (NR had no differential regulation of dihydrolipoamide acetyltransferase).

Upregulation of the TCA cycle serves several purposes in a stressed cell. Excess cytoplasmic ATP can be applied to power efflux pumps, which remove unwanted or destructive ions or molecules from the cell (79), including antimicrobial compounds, free fatty acids, and detergents (43, 56). In the case of acid stress, active transport can remove excess H^+ to maintain cytoplasmic homeostasis. The TCA cycle also contributes to precursors necessary for biosynthesis of lipids and amino acids (75). A cell undergoing damage to nucleic acids, proteins, and structural components benefits from an influx of these components for rapid self-repair.

Outer Membrane Components

The outer membrane of a Gram-negative cell requires highly selective permeability in order to limit permeation by hydrophilic compounds that would otherwise readily access and potentially damage the cell (73, 79). Outer membrane protein C (*ompC*) was upregulated 1.8 to 2.2-fold across all treatments (Table 5). The positive transcription regulator of *ompC* and outer membrane protein F (*ompF*), OmpR, was upregulated 1.4-fold by water and 1.6-fold by chlorine treated *S. Newport*. Both *ompC* and *ompF* passively diffuse small hydrophilic molecules through

the cell membrane, and expression is determined by osmolality. These proteins are important in antibiotic and other chemical resistance as they form pores by which these compounds are removed from the cell. *ompC* is also highly antigenic (64). Increased osmolality favors *ompC*, while decreased osmolality favors *ompF*. OmpR is modulated by the environmental sensor EnvZ; both work in tandem in response to osmotic conditions. Signaling between the two requires ATP-sourced phosphorylation onto EnvZ, at which point the phosphoryl group is passed on to OmpR (1).

Outer membrane protein A (*ompA*) and DNA protection during starvation protein (*Dps*) were also upregulated ranging from 1.7 to 2.3-fold. Conversely, outer membrane protein W (*ompW*) was down regulated -1.5-fold by water, -1.9-fold by 40 ppm PAA, and -2.2-fold by chlorine treated *S. Newport*. All three outer membrane proteins are considered antigenic and capable of inducing an immune response. Neutrophil elastase-mediated attack on *E. coli* acts by degrading OmpA, which results in misshaped cells (3, 83). As such, they are considered potential targets for vaccine development (13).

Several lipoproteins, also frequently antigenic, were differentially down and upregulated across all treatments. In Gram-negative bacteria, lipoproteins are imbedded in either the cytoplasmic membrane or on either side of the outer membrane (38). Lipoproteins are involved in a variety of cellular functions. *nlpE*, an outer membrane protein, is involved in adhesion (62). *nlpE* was downregulated -1.4-fold by water and 85 ppm PAA treatment. Overexpression of the lipoprotein Nlpl resulted in the loss of rod shape in *E. coli* K12, suggesting it plays a role in cell morphology (57). Both 40 and 85 ppm PAA resulted in -1.4-fold

downregulation of *nlp*. Other lipoproteins are involved in the attachment of peptidoglycan to the outer membrane (14).

In 40 ppm PAA and chlorine treated *S. Newport*, the phospholipid-binding protein *mlaC* was upregulated 1.3 and 1.4-fold, respectively. This protein functions by relocating misplaced phospholipids from the outer membrane (where the primary lipid should be in the form of lipopolysaccharide) to the inner membrane (where the primary lipid is phospholipid). This process ensures stability and maintains integrity of the cellular membrane; disrupting *mlaC* results in non-exclusive permeability and increases bacterial susceptibility to extraneous compounds (30).

Treatment with both 40 and 85 ppm PAA resulted in a 1.7-fold upregulation of Glucose-1-phosphate thymidyltransferase, a key enzyme in O-antigen biosynthesis; specifically, the synthesis of rhamnose. Interestingly, NR treated *Salmonella* with 24 h environmental chamber exposure, saw no significant changes in expression of this enzyme ($p < 0.05$).

O antigenic variations within lipopolysaccharide (LPS) exhibit great variability among serovars while the core features of the remainder of the outer membrane remain relatively homogenous (6). The presence of O-antigen and the specific variations within elicit a host immune response upon infection. Complement resistance, for instance, is affected by the length of the O-antigenic LPS side chains in *Salmonella*. Immediately upon detecting the LPS structure, complement resistance becomes activated and initiates the membrane attack complex (28). Comprised of five complement proteins, the membrane attack complex forms a pore in the bacterial cell by inserting itself into the outer lipid membrane (53). This pore formation frequently results in cell lysis; however, if lysis does not occur, the MAC created pore

also interferes with ion gradients and signaling pathways within the bacterial cell (52). The bacterial cell's efforts to stabilize itself, combined with the ion flux, result in the initiation of a host-mediated pro-inflammatory response (52, 78). Activation of complement (and subsequently, MAC) is one of the first lines of defense once a pathogen enters the host, but overexpression can result in host tissue damage as complement binding is unspecific (72).

Motility

Genes involved in chemotaxis and flagellar function were, for the most part, downregulated. The flagella are comprised of three major regions; the basal body, the hook, and the filament. The basal body contains the motor machinery, the direction of which is reversible. The hook attaches the filament, which serves as external propeller.

The basal body is embedded within cell itself, and subsequently has a series of rings that bind it to the different layers of cellular membranes. The cytoplasmic (C) ring is located in the cytoplasm, the membrane/supramembrane (MS) ring is bound to the cytoplasmic membrane, the peptidoglycan ring is bound to the peptidoglycan layers, and the lipopolysaccharide ring is attached to the outer membrane (51). The interface between the MS and C rings is a motor. Also along this interface are several non-rotating membrane embedded regions called the stators, within which motility proteins A and B are contained. Their primary function is the movement of ions across the membrane, resulting in proton motive propulsion (54, 63) of up to ~300 Hz (51). Motility protein A was downregulated in all 24 h treatments. NR treatment resulted in a 2.2-fold downregulation of motility protein A, while all other 24 h treatments ranged from 3.5-5.3- fold downregulations. Water, 40 ppm PAA, and 50 ppm Cl resulted in -2.2, -3.3, and -3.5-fold downregulations of motility protein B, while NR and 85 ppm PAA resulted in

no differential regulation ($p < 0.05$). The chemotaxis signaling protein *cheY* works with other proteins to allow the flagellar motor to spin clockwise. *cheY* and several other chemotaxis proteins including *cheA*, *cheR*, *cheW*, and *cheZ* were downregulated ranging from -1.8 to -4.1-fold for all treatments (Table 6). Two methyl-accepting chemotaxis proteins and a chemotaxis regulator also experienced significant downregulations. The gene encoding an alternative RNA polymerase sigma factor for flagellar operon (*fliA*) was downregulated for three of the 24 h treatments; water (3.6-fold), 40 ppm PAA (4.6-fold), and chlorine (5.6-fold). *fliA* serves as a positive regulator for *mot* and *che* genes (58).

The flagellar hook-associated proteins one and three were downregulated 2.5-5.0-fold, while for 2 h treatments no significant differential regulation of the gene occurred ($p < 0.05$). No differential regulation of flagellar hook-associated protein two was observed for 2 or 24 h treatments. No significant differential regulation of this gene occurred for NR or 85 ppm PAA treatments ($p < 0.05$). Chlorine and 40 ppm PAA treated *S. Newport* downregulated phase II flagellin -1.8 and -1.9-fold, respectively.

The flagellar brake protein, *ycgR*, was downregulated by all treatments ranging from -2.2 to -2.8-fold. Conversely, cyclic di-GMP-binding protein (bis-(3'-5')-cyclic dimeric guanosine monophosphate) was upregulated 1.8-fold by NR and water and 2.0-fold by 85 ppm PAA. Both proteins work to move *Salmonella* from a planktonic lifestyle to a biofilm, or vice versa. Cyclic di-GMP, a secondary messenger, binds to the PilZ region on the gene product of *ycgR*; this action interferes with flagellar rotor proteins *fliM* and *fliG* (84), behavior that has been seen in both *Salmonella* and *Escherichia coli* (63). Cyclic di-GMP also promotes the synthesis of capsule and fimbriae, compounds required for the sedentary lifestyle within a biofilm (82).

Flagella are pathogen associated molecular patterns (PAMPs), which readily stimulate the host innate and adaptive immune systems. Toll-like receptor-5 binds flagellar material, inciting pro-inflammatory cytokine production, including the cytokine interleukin-6 (IL-6). Downregulation of flagellar machinery and other PAMPs aids in avoidance of the immune system, but can also be a hindrance in the early stage of infection as flagella are important for virulence (61). *S. Typhimurium* containing flagellar deficiencies resulted in a significantly diminished IL-6 induction; however, the same mutation in *S. Dublin* had a similar IL-6 response as did a wild-type strain (60).

Biofilm Formation

Pathogenic *Salmonella* contain several toxin-antitoxin (TA) systems which appear to serve several different functions. As non-pathogenic strains have fewer TA systems, they are thought to play a role in pathogenicity, and they have been demonstrated to be integral in biofilm formation and response to environmental stress (50). The systems encode 2 proteins; one is a toxin that disrupts cellular function and results in cell dormancy in a certain percentage of the population, while the other serves as an antitoxin that binds the toxin and prevents it from acting on the cell (44). For example, in the RelE/RelB system, the RelE toxin cleaves the stop codons from mRNAs at the ribosome, resulting in incomplete mRNAs, arresting transcription. The RelB antitoxin prevents this action (74). Deletion of five TA systems in *E. coli* resulted in decreased initial biofilm formation (after 8 h), followed by increased biofilm formation after 24 h. However, deleting individual toxins resulted in increased biofilm formation after 8 h. Additionally, overexpression of the antitoxin *yefM* also resulted in significantly greater biofilm formation (36). In the current study, *S. Newport* downregulated the

toxin *RelE* in the *RelE/RelB* system, and the antitoxin *YefM* in the *YoeB/YefM* system across all treatments. The downregulation of *RelE* might suggest an increase in biofilm formation; this is corroborated in NR, W, and 85 ppm PAA treatments that also significantly upregulate the production of curlin, a thin aggregative fimbriae required for adherence to plant and mammalian cells (39) and integral in the formation of biofilms (20). Bacteria also use curli to adhere to surrounding bacterial cells, forming stable bundles that can form into biofilms (65). Upregulation by NR, W, and 85 ppm PAA ranged from 6.5 to 71.1-fold for the minor and major curlin subunit (Table 7). Biofilm modulated protein *bdm* was upregulated 2.3 to 2.9-fold for all treatments except NR. Chlorine treated *S. Newport* also positively upregulated *entR*, a transcriptional regulator of colonic acid synthesis. Colanic acid is not required for initial biofilm adhesion but rather is involved in the structural integrity of the biofilm and contributes to the formation of large biofilms (65). However, the positive regulator fimbriae Y protein (FimY) was downregulated -1.7 to -2.6-fold for all treatments except NR.

Conclusion

Salmonella's vast genetic arsenal was evident in the organism's transcriptomic response during tomato colonization and sanitization treatment as over 1,000 genes were differentially expressed. *Salmonella's* ability to survive outside of its traditional hosts, persist throughout the stresses encountered on plant tissue, and avoid inactivation during sanitation continue to make it a public health hazard. Understanding the tools employed by *Salmonella* that enable this tenacity is the first step towards developing efficient methods for its limitation as a food contaminant. While many differentially expressed genes were shared across treatments, 41% of upregulated and 34% of downregulated genes were as a result of one of the three oxidizer-

containing washes. The organism's stress response systems apparently offered some cross-protection, as proteins generally associated with temperature stress or phage infection were significantly upregulated. Further examination regarding the multi-faceted stress response is critical in understanding the many survival mechanisms available to *S. Newport*.

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Appendix

Table 5. Differential regulation of genes involved in heat shock, osmotic stress, and environmental pressure for 24 h *S. Newport* exposed to NR, Water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Change		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
<i>ibpA</i>	small heat shock protein	2.3	.	.	2.1	.
<i>ibpB</i>	small heat shock protein	1.6	.	.	.	.
<i>hsp20</i>	heat shock protein	1.9	.	.	1.9	.
<i>hspQ</i>	heat shock protein	.	-1.3	.	-1.4	.
<i>pspA</i>	phage shock protein	2.5	.	.	3.3	.
<i>pspB</i>	phage shock protein	.	.	.	3	.
<i>pspC</i>	phage shock protein	3.8	3.5	.	5.8	.
<i>pspD</i>	phage shock protein	3.5	2.6	.	4.2	.
<i>cspD</i>	cold shock protein	.	.	1.5	.	1.7
<i>cspE</i>	cold shock protein	.	-1.5	.	-1.4	.
<i>yecG</i>	universal stress protein C	.	-1.6	-1.5	.	.
<i>ydaA</i>	universal stress protein E	.	-1.3	.	-1.4	.
<i>ynaF</i>	universal stress protein F	1.6	.	.	.	.
<i>ybdQ</i>	universal stress protein G	.	.	.	-1.3	.
<i>osmF</i>	osmoprotectant uptake system substrate-binding protein	1.3	1.4	1.6	1.4	1.7
SN31241_9360	osmotically-inducible protein Y	1.8	1.6	1.5	1.7	1.5
<i>yehW</i>	osmoprotectant uptake system permease protein	-1.3	-1.5	-1.4	-1.6	-1.5
<i>yehX</i>	osmoprotectant uptake system ATP-binding protein	1.7	.	1.7	1.6	1.7
<i>yehY</i>	osmoprotectant uptake system permease protein	.	1.5	1.6	1.4	1.7

Table 6. Differential regulation of genes involved in oxidative stress for 24 h *S. Newport* exposed to NR, Water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Change		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
SN31241_28140	catalase	.	1.4	1.7	.	1.8
SN31241_45790	catalase-peroxidase	1.5	.	1.4	.	1.5
SN31241_23930	catalase hydroperoxidase II	1.8	1.9	2.0	1.9	2.1
<i>soxR</i>	redox-sensitive transcriptional activator	.	.	.	.	1.7
SN31241_24980	superoxide dismutase	2.5	.	.	2.0	.
SN31241_25070	superoxide dismutase	.	.	1.5	.	1.6
SN31241_46750	superoxide dismutase	.	.	1.5	.	1.6
SN31241_1780	glutaredoxin	1.5	1.9	2.0	1.6	1.9
SN31241_19270	glutaredoxin	1.5	.	.	.	1.5
SN31241_25000	glutaredoxin	.	-1.6	-1.6	-1.9	-1.5
SN31241_22330	glutaredoxin-2	1.2	.	.	1.6	.
SN31241_37650	thioredoxin-2	.	.	.	-1.4	.
<i>osmC</i>	peroxiredoxin	.	.	1.4	.	1.5
SN31241_24130	glutathione peroxidase	.	.	1.3	.	1.5
SN31241_14020	alkyl hydroperoxide reductase/ thiol specific antioxidant/ Mal allergen	.	1.4	1.6	.	1.8
SN31241_16120	alkyl hydroperoxide reductase/ thiol specific antioxidant/ Mal allergen	1.5	1.5	1.9	.	1.8

Table 7. Regulation of enzymes involved in expression of the arginine succinyltransferase system for 24 h exposed *S. Newport* after exposure to NR, Water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Genes	Enzymes	Fold Change				
		NR	Water	40 ppm PAA	85 ppm PAA	50 ppm Cl
SN31241_23770	Arginine N-succinyltransferase	2.1	2.1	2.5	1.9	2.5
SN31241_23790	N-succinylarginine dihydrolase	1.7	1.9	2.3	1.6	2.5
SN31241_23760	Succinylornithine transaminase	2.1	2.1	2.3	1.9	2.4
SN31241_23780	N-succinylglutamate 5-semialdehyde dehydrogenase	1.8	1.9	2.3	1.6	2.5
SN31241_2030	Aldehyde dehydrogenase	1.3	1.6	1.7	1.5	1.8
SN31241_23800	Succinylglutamate desuccinylase	1.7	2	2.2	1.9	2.3

Table 8. Regulation of enzymes involved in the TCA cycle for 24 h exposed *S. Newport* after exposure to NR, Water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Changes		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
SN31241_25370	Fumarate hydratase class II	.	.	1.4	.	1.5
SN31241_17610	Fumarate hydratase	.	.	.	.	.
SN31241_44830	Malate dehydrogenase	1.9	1.6	1.5	1.8	1.5
SN31241_17300	Citrate synthase I	1.9	.	.	1.7	.
SN31241_27940	Aconitate hydratase 1	1.5	1.5	1.6	1.4	1.6
SN31241_11450	Aconitate hydratase 2	.	.	.	1.4	.
SN31241_23060	Isocitrate dehydrogenase 2-oxoglutarate	.	.	.	1.6	.
SN31241_17350	dehydrogenase E1 component	2.5	2.2	1.7	2.8	1.7
SN31241_17380	Succinyl-CoA ligase [ADP-forming] subunit alpha	2.0	1.6	.	1.9	.
SN31241_17370	Succinyl-CoA ligase [ADP-forming] subunit beta	2.8	2.2	.	3.1	.
SN31241_17310	Succinate dehydrogenase cytochrome b556 subunit	1.8	.	.	1.7	.
SN31241_17330	Succinate dehydrogenase flavoprotein subunit	2.6	2.1	.	2.7	.
SN31241_17340	Succinate dehydrogenase, iron-sulfur	2.5	1.8	.	2.4	.

Table 9. Differential regulation of genes involved in outer membrane function for 24 h *S. Newport* exposed to NR, water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Changes		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
<i>ompA</i>	outer membrane protein A	2.0	2.2	2.1	2.3	2.2
<i>ompC</i>	outer membrane protein C	2.1	2.0	1.8	2.2	1.9
<i>ompD</i>	outer membrane porin protein D	-1.6	-2.3	-2.3	-2.6	-2.3
<i>ompR</i>	transcriptional regulation protein	.	1.4	.	.	1.6
<i>ompW</i>	outer membrane protein W	.	-1.5	-1.9	.	-2.2
<i>ompX</i>	outer membrane protein X	.	.	1.3	.	1.4
<i>ompD</i>	outer membrane porin protein	-1.6	-2.3	-2.3	-2.6	-2.3
<i>Dps</i>	DNA protection during starvation protein	1.9	1.9	1.7	2.1	1.8
<i>yaeT</i>	outer membrane protein assembly factor	.	1.6	.	1.8	.
<i>Slp</i>	outer membrane protein	.	.	.	-1.5	.
<i>yhjG</i>	outer membrane biogenesis	.	.	.	.	1.3
<i>tolC</i>	type I secretion outer membrane protein	.	.	1.7	1.4	1.8
<i>slyB</i>	outer membrane lipoprotein	-1.4	-1.6	-1.4	-1.8	-1.3
SN31241_650	conserved outer membrane protein	.	.	.	-1.5	.
SN31241_23860	osmotically-inducible lipoprotein E	.	.	1.3	.	1.4
SN31241_24490	major outer membrane lipoprotein	.	.	.	-1.5	.
SN31241_17520	peptidoglycan-associated lipoprotein	.	.	1.3	.	1.4
SN31241_2360	lipoprotein	.	.	.	-1.4	1.4
SN31241_7200	lipoprotein	.	.	.	-1.3	.
SN31241_12400	lipoprotein 28	.	1.4	.	.	1.5
SN31241_22190	lipoprotein	1.3	.	.	.	.
<i>nlpE</i>	outer membrane lipoprotein	.	-1.4	.	-1.4	.
<i>nlpI</i>	outer membrane lipoprotein	.	.	-1.4	-1.4	.
<i>yceB</i>	lipoprotein	.	-1.4	-1.4	-1.4	-1.3
<i>ydjL</i>	lipoprotein	2.2	2.1	1.6	2.6	1.7
<i>yfgL</i>	lipoprotein	.	.	.	.	1.5

Table 9. Continued.

Gene	Function	NR	Water	Fold Changes		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
<i>ygdI</i>	lipoprotein	1.6	1.4	1.4	1.4	1.5
<i>yifL</i>	lipoprotein	.	-1.5	-1.4	-1.5	-1.5
<i>ygdR</i>	lipoprotein flags	.	.	.	-2.8	-3.1
<i>mlaC</i>	phospholipid-binding protein	.	.	1.3	.	1.4

Table 10. Differential regulation of genes involved in chemotaxis, flagella, and motility for 24 h S. Newport exposed to NR, water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Changes		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
<i>cheA</i>	chemotaxis protein <i>cheA</i>	-1.8	-2.4	-2.5	-1.9	-2.6
<i>cheR</i>	chemotaxis protein <i>cheR</i>	-1.8	-2.9	-4.0	-2.5	-3.2
<i>cheW</i>	chemotaxis protein <i>cheW</i>	-2.0	-2.9	-3.1	-2.7	-2.7
SN31241_34190	<i>cheW</i> chemotaxis signal transduction protein	-2.1	-2.9	-3.7	-2.5	-4.0
<i>cheY</i>	chemotaxis protein <i>cheY</i>	-2.1	-2.5	-3.4	-2.4	-3.4
<i>cheZ</i>	chemotaxis protein <i>cheZ</i>	-1.8	-2.0	-2.2	-2.1	-2.8
<i>Tcp</i>	methyl-accepting chemotaxis protein	-2.0	-2.8	-3.6	-2.2	-4.1
<i>Tar</i>	methyl-accepting chemotaxis protein II	-3.2	-5.5	-6.4	-4.1	-5.9
SN31241_30170	chemotaxis response regulator protein-glutamate methylesterase DNA-binding	-2.2	-3.0	-3.7	-2.4	-6.0
<i>IrhA</i>	transcriptional repressor of flagellar, motility and chemotaxis genes	.	-1.7	-1.8	-2.0	-1.9
<i>motA</i>	motility protein A	-2.2	-3.6	-5.3	-3.5	-4.8
<i>motB</i>	motility protein B	.	-2.2	-3.3	.	-3.5
<i>flgK</i>	flagellar hook-associated protein I	-2.7	-3.9	-5.0	-2.9	-4.5
<i>flgL</i>	flagellar hook-associated protein III	-2.5	-3.5	-4.1	-3.4	-3.5
<i>flhD</i>	flagellar transcriptional regulator	.	.	-1.6	.	.
SN31241_30560	phase II flagellin	.	.	-1.9	.	-1.8
<i>fliA</i>	RNA polymerase sigma factor for flagellar operon	.	-1.7	-4.6	.	-5.6
<i>ycgR</i>	flagellar brake protein	-2.7	-2.8	-2.2	-2.7	-2.2
SN31241_2700	cyclic di-GMP binding protein	1.8	1.8	.	2.0	.

Table 11. Differential regulation of toxin-antitoxin system related genes and genes involved in biofilm formation for 24 h *S. Newport* exposed to NR, water, 40 ppm PAA, 85 ppm PAA, and 50 ppm Cl.

Gene	Function	NR	Water	Fold Change		
				40 ppm PAA	85 ppm PAA	50 ppm Cl
<i>relE</i>	toxin (<i>relE/relB</i> system)	-1.3	-1.7	-1.5	-2	-1.5
<i>yefM</i>	antitoxin (<i>yoeB/yefM</i> system)	-2	-2.3	-2.4	-3.5	-2.7
SN31241_22110	major curlin subunit	6.5	9.1	.	19.7	.
SN31241_22100	minor curlin subunit	20.3	36.9	.	71.1	.
<i>fimY</i>	fimbriae Y protein	.	-1.7	-1.7	-2.6	-1.9
<i>Bdm</i>	biofilm modulated protein	.	2.7	2.9	2.3	2.7
<i>entR</i>	transcriptional regulation of colanic acid capsular biosynthesis activation protein A	.	.	.	.	1.5

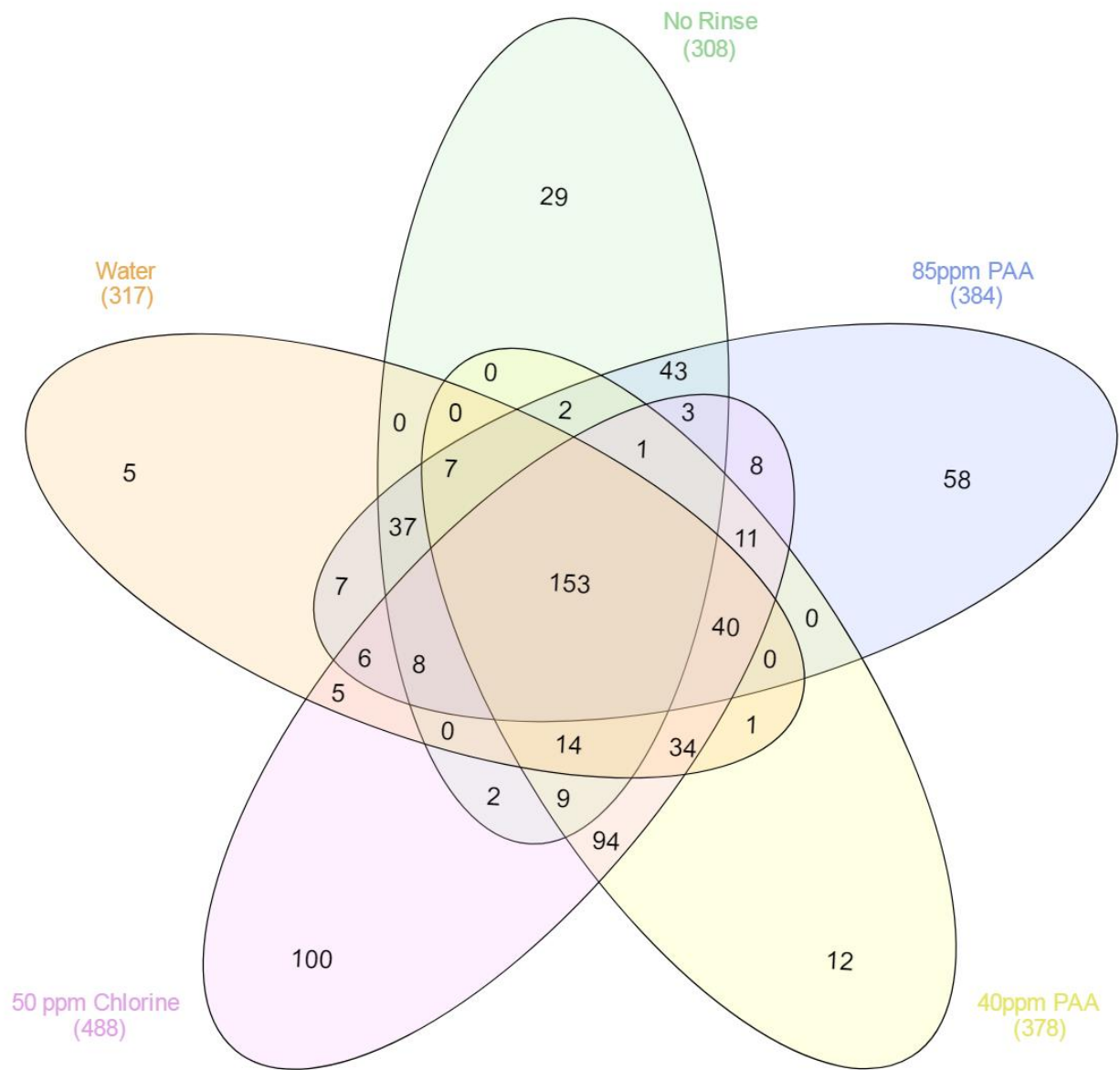


Figure 3. Distribution of upregulated genes amongst all five treatments.

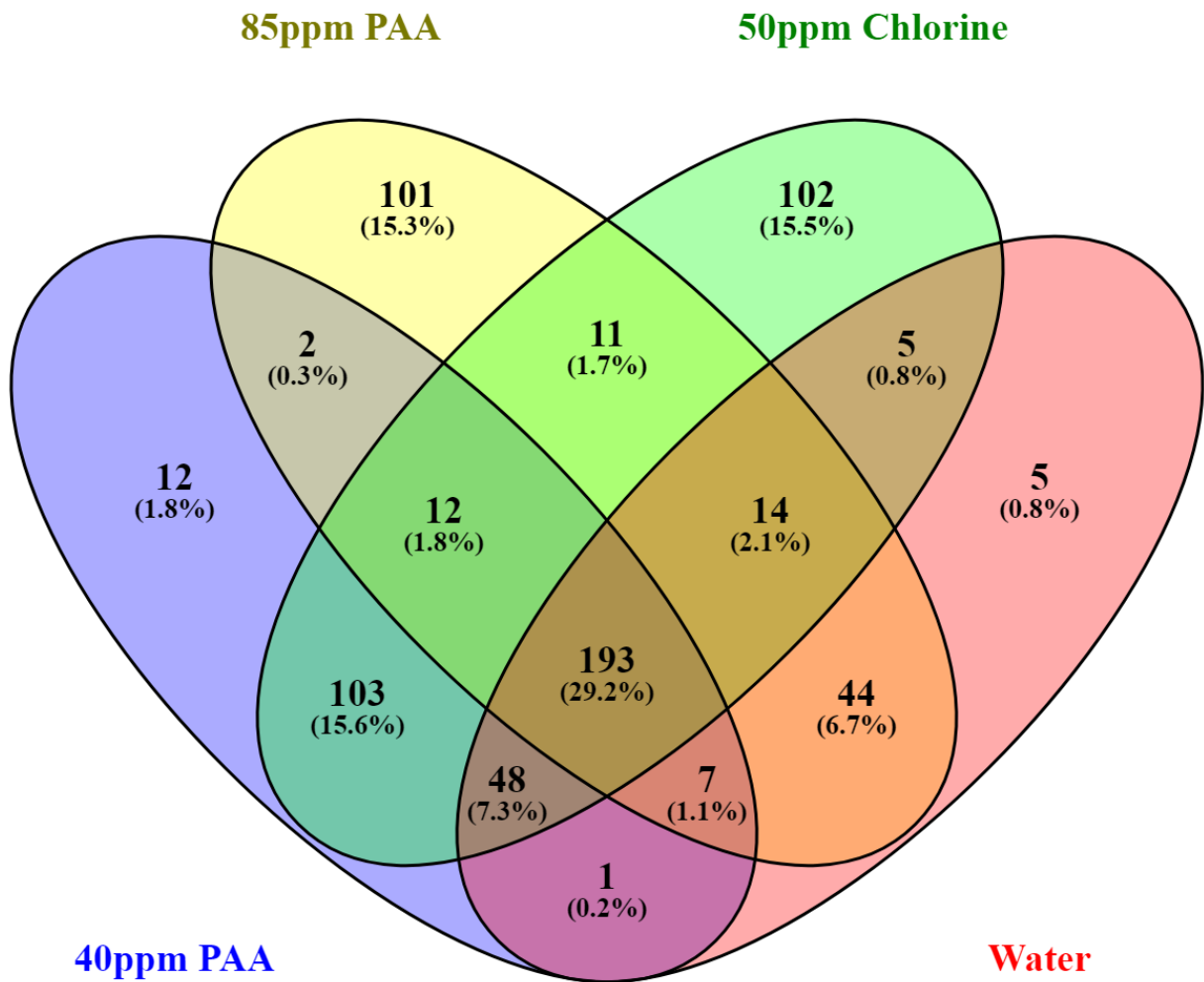


Figure 4. Distribution of upregulated genes amongst all washing treatments.

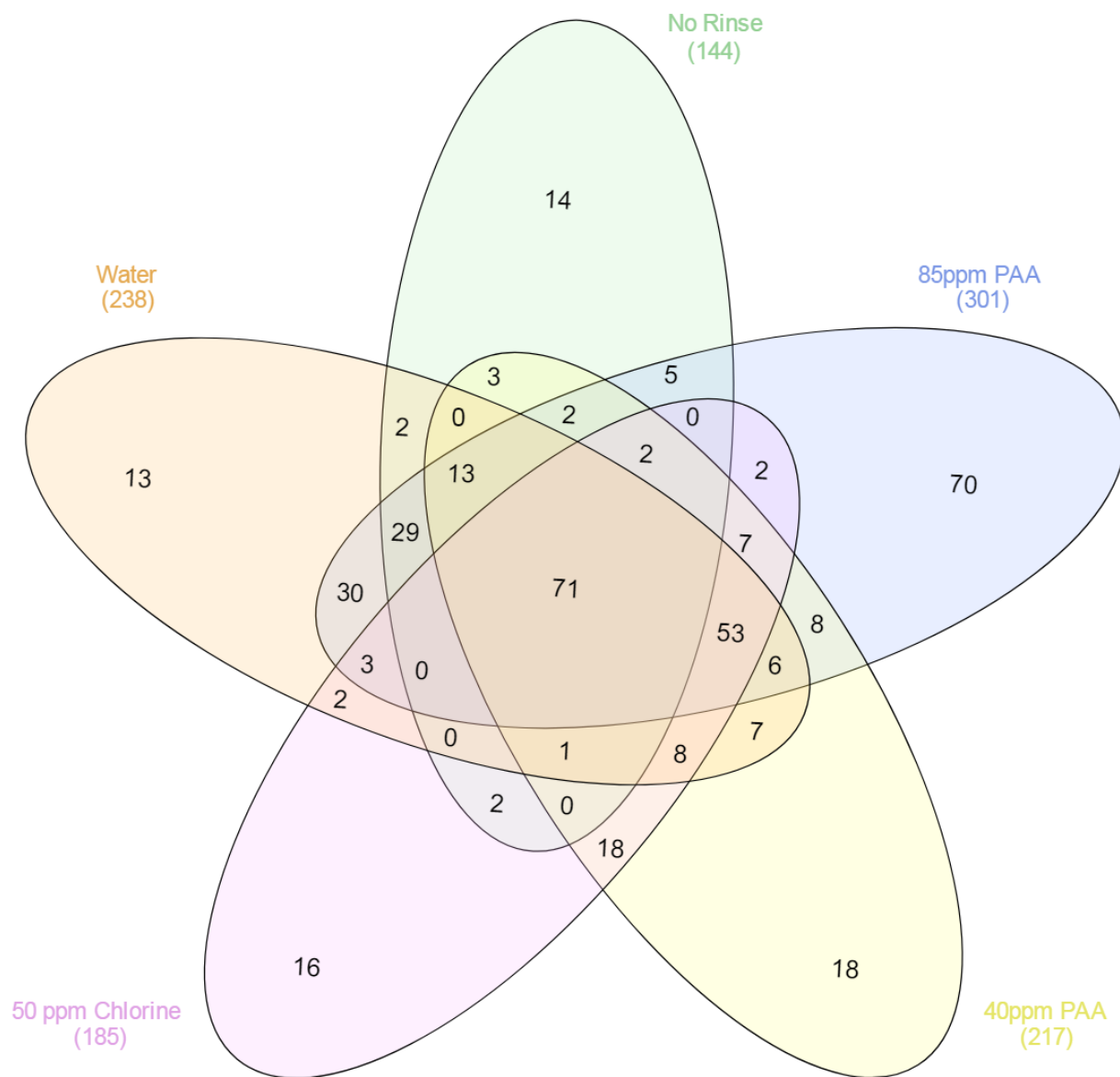


Figure 5. Distribution of downregulated genes amongst all five treatments.

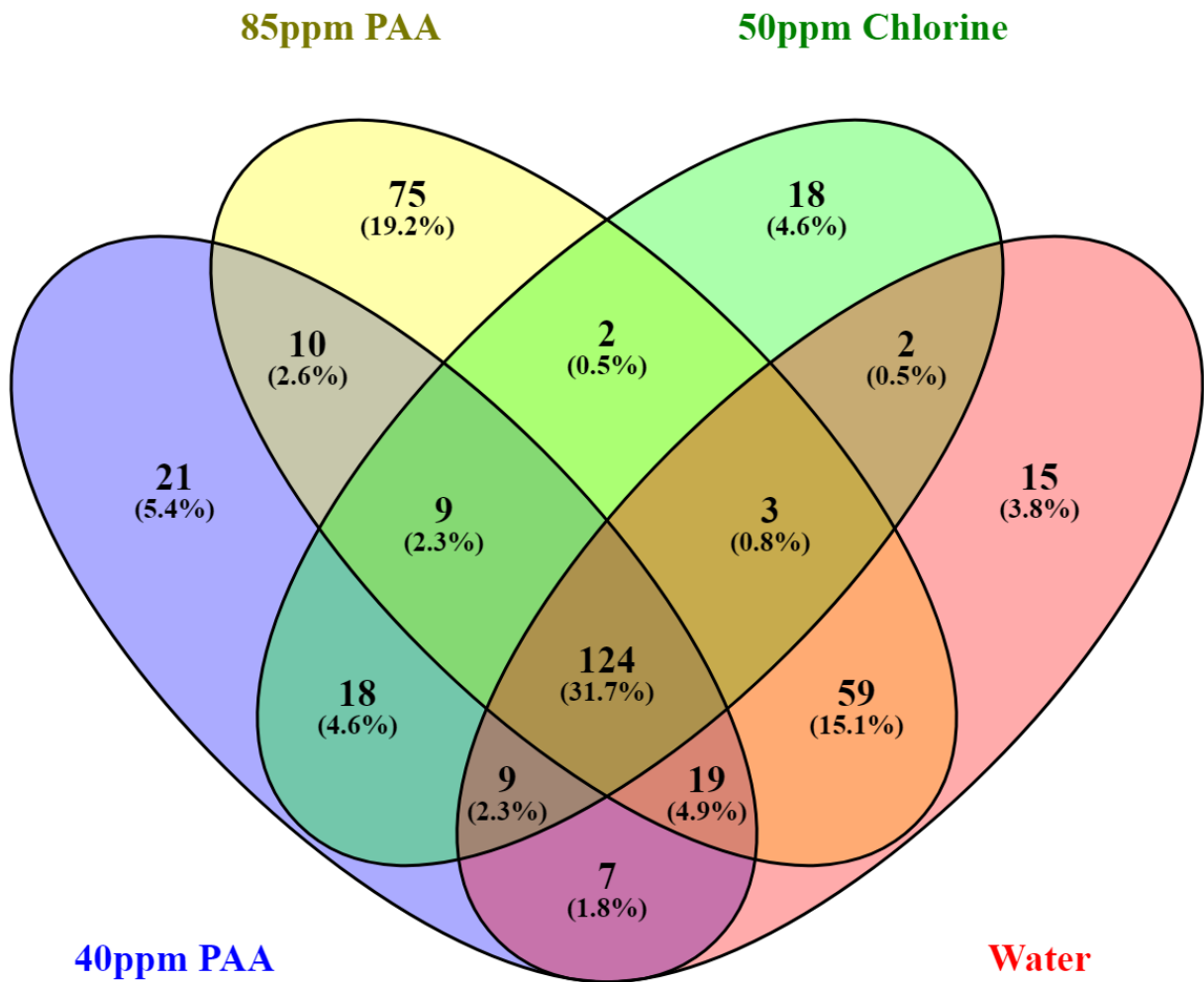


Figure 6. Distribution of downregulated genes amongst all washing treatments.

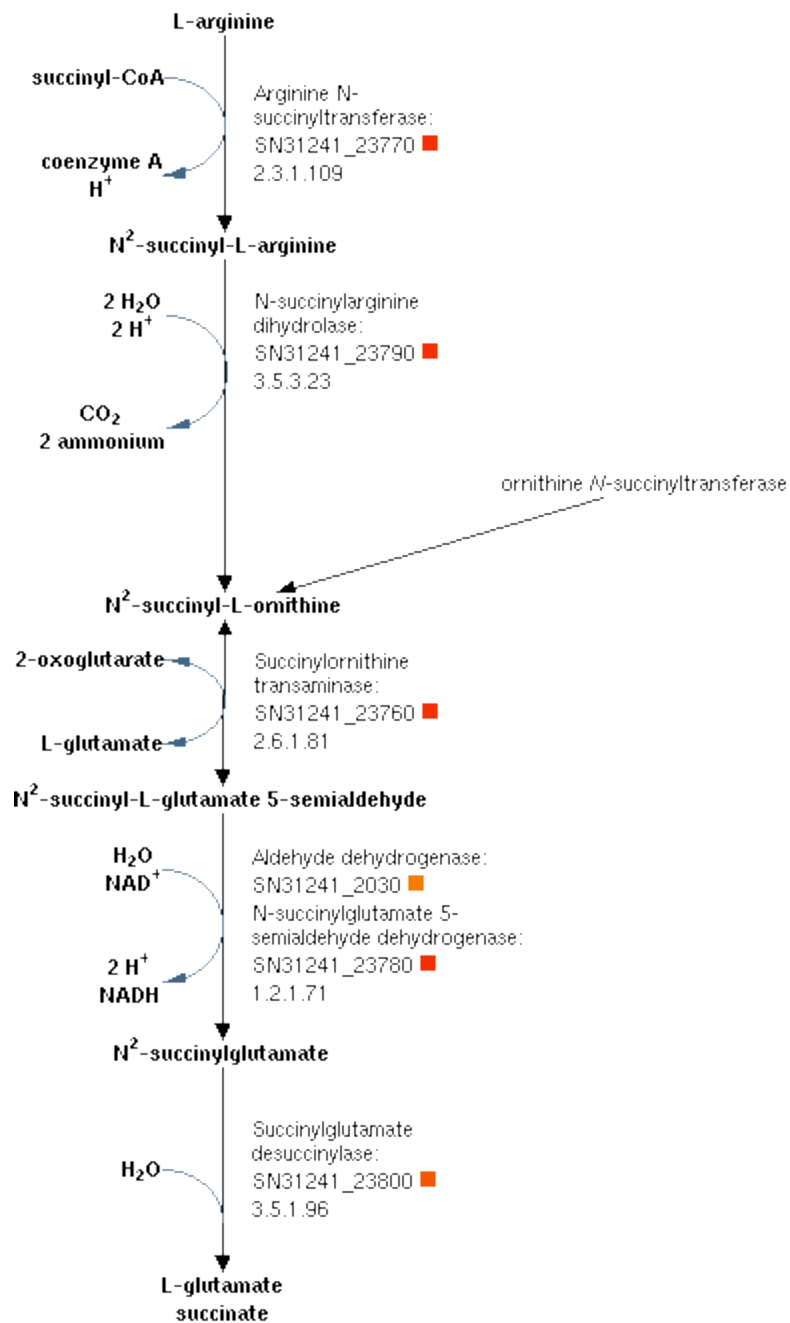


Figure 7. Arginine succinyltransferase system in *S. Newport* (33).

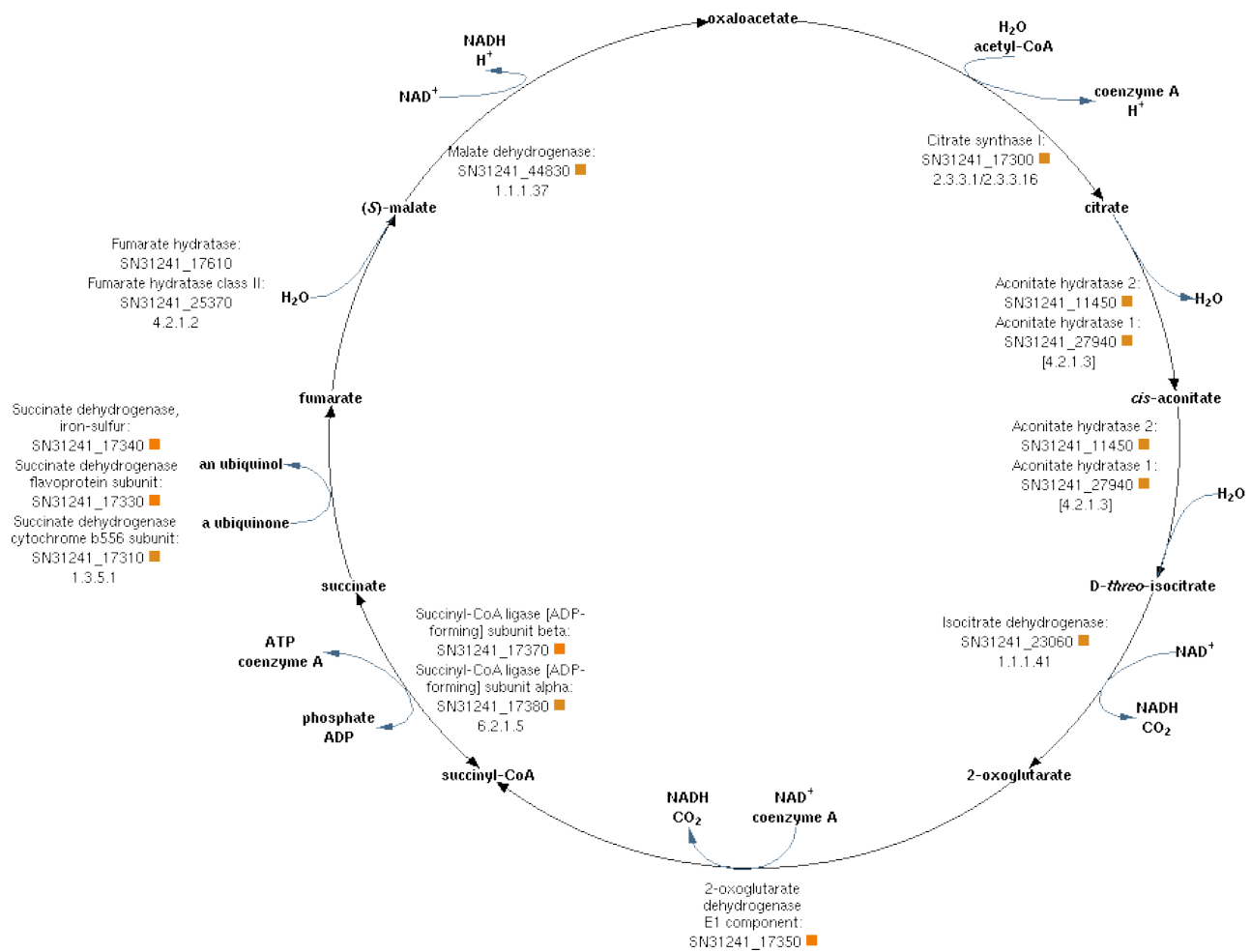


Figure 8. Tricarboxylic acid cycle I (prokaryotic) (33).

Conclusion

Understanding the genetic mechanisms that *Salmonella* spp. employ to survive environmental and postharvest stresses is critical to ensure public health by reducing the prevalence of foodborne outbreaks. Building on the knowledge gained from this and similar studies will aid in the development of better methods to limit the pathogen's access to food crops, as well as the creation or discovery of more effective sanitization procedures against *Salmonella* while still maintaining produce quality.

Vita

Laurel Dunn was born in 1987 in Gretna, Louisiana to Lewis and Jean Dunn. The family eventually moved to Cleveland, Tennessee, where Laurel spent her elementary through high school years. After graduating from Cleveland High School in 2006, Laurel attended the University of Tennessee where she completed her Bachelor's degree in food science and Technology in 2009. Laurel returned to the University of Tennessee to complete her Master's degree in food science in 2013 and her Ph.D. in food microbiology in 2017. She plans to remain in academia in research, teaching, and/or extension.