PREVALENCE AND MICROBIAL ECOLOGY OF ENTEROBACTERIACEAE ON TEXAS PRODUCE AND THE SURVIVAL OF *SALMONELLA* ON PARSLEY AS AFFECTED BY PROCESSING AND STORAGE

A Dissertation

by

ELIZABETH ANNE DUFFY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Food Science and Technology

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ABSTRACT

Prevalence and Microbial Ecology of Enterobacteriaceae on

Texas Produce and the Survival of *Salmonella* on Parsley as Affected by

Processing and Storage. (August 2004)

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To assess the risk factors involved in the contamination of fresh produce with human pathogenic organisms, a total of 1,257 samples were collected from cantaloupe, oranges, and parsley in the field and after processing, and the environment. Samples were collected twice in a season from two farms with operating packing sheds per commodity and analyzed for the presence of *Salmonella*. Sixteen, 6, and 3 isolates were obtained from irrigation water, packing shed equipment, and washed cantaloupe, respectively. *Salmonella* was not detected on oranges or parsley. Serotyping, pulsed-field gel electrophoresis, and repetitive sequence-based polymerase chain reaction assays were applied to *Salmonella* isolates to evaluate their genetic diversity and to determine if there are relationships between sources of contamination. Using PFGE, all *Salmonella* isolates obtained from irrigation water and equipment were determined to be different from cantaloupe isolates. Only one equipment isolate was related to isolates from

irrigation water. Rep-PCR demonstrated some similarity between equipment and cantaloupe isolates, but this technique is less discriminatory. DNA fingerprinting did not conclusively determine relationships between sources of contamination. Isolates were also subjected to antimicrobial susceptibility testing using the disk diffusion method. Five out of 25 of the isolates demonstrated intermediate sensitivity to streptomycin and one isolate was resistant to streptomycin.

Green fluorescent protein was an effective marker system when monitoring the survival of *Salmonella* on parsley as affected by processing. Dip temperature had little effect on the attachment and survival of *Salmonella* on parsley. Regardless of the temperature or duration of dip, *Salmonella* were internalized. Immersion for longer times resulted in higher numbers of attached and internalized cells. Microscopic observations agreed with these results and showed *Salmonella* near the stomata and within cuticle cracks. *Salmonella* increased over 7 storage days at 25°C and decreased at 4°C. After 7 days at 4°C, no internalized *Salmonella* were detected.

Examination of the native microflora of parsley showed that bacterial populations were similar for parsley collected in the field and packing shed. Higher bacterial populations and fungi were observed at retail with *Pseudomonas* the predominant organism. Parsley supports the growth of a diverse group of microorganisms.

DEDICATION

This study is dedicated to my fiancé, John Wayne Ellebracht, in appreciation of his unending encouragement, love, support, and patience.

ACKNOWLEDGEMENTS

The author wishes to express her utmost appreciation to her advisor, Dr. Gary R. Acuff, for his guidance and patience throughout her research. She would also like to express gratitude to Dr. Alejandro Castillo, Dr. Luis Cisneros-Zevallos, and Dr. Leon H. Russell, Jr. for serving as members of her committee and providing assistance in completing this research.

An additional thanks needs to be extended to Lisa Lucia for her instruction and advice on many subjects and to the graduate students including, but not limited to: Jennifer Beach, Elisa Cabrera, Heather Martin, Mangesh Palekar, Alex Puerta, Paphapit Ungkuraphinunt, Kyle Wright, and Maria Belem Magaña Yépez for their assistance in the field and in the laboratory. Gratitude is also expressed to Dr. Suresh Pillai, Dr. Steven Ricke, and Cassie Woodward for use of equipment and additional technical assistance.

TABLE OF CONTENTS

Page

LIST OF TABLES

TABLE Page

TABLE Page

LIST OF FIGURES

FIGURE Page

INTRODUCTION

Raw fruits and vegetables may potentially harbor a variety of foodborne pathogens. Consequently, fresh produce has been repeatedly implicated as a vehicle in the transmission of foodborne gastroenteritis. Salmonellosis outbreaks have been epidemiologically associated with the consumption of cantaloupes, celery, watermelon, tomatoes, lettuce, alfalfa sprouts, parsley, and other raw salad vegetables. Tracing the source of the *Salmonella* or any other pathogen implicated in an outbreak can be difficult. Outbreaks associated with produce have been linked to fecal contamination during postharvest handling, shipping, or processing under circumstances permitting bacterial contamination. Produce contamination has also been linked to contaminated dump tanks used by packers, carriage of the contaminant from the surface into the interior tissues, contaminated seeds, and exposure to warm storage temperatures.

Several explanations exist for the increased number of foodborne illness outbreaks linked to the consumption of fresh produce. The increase may be due to the globalization of the food supply. Virtually any commodity is available for purchase in the United States, regardless of the season, due to the ease with which products can be imported from around the world. This is desirable, but also a concern because of some countries adhering to lower level sanitation standards. Reports emphasizing the health benefits of consuming raw fruits and vegetables may be affecting the consumption habits of the American population. There may also be an increase in the number of produce-

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This dissertation follows the style and format of the *Journal of Food Protection*.

associated illnesses, due to increased microbial detection methods and knowledge that fruits and vegetables can support the growth of these pathogens.

In order to assess the risk factors involved in the contamination of fresh produce with human pathogenic organisms, it is important to understand the microbial ecology of the various products as well as possible vectors during harvesting and processing that may be contributing to the product contamination. Every fruit and vegetable has a unique microenvironment including varying pH levels, different surface structures, and the presence of antimicrobial compounds. Each of these fruits and vegetables are also harvested, processed, and marketed using unique methods. To understand the attachment and survival of pathogens each of these unique scenarios needs to be considered.

The goals of the present investigation were: (a) to determine the prevalence of *Salmonella* and *Escherichia coli* on cantaloupes, oranges, and parsley produced in Texas, before and after processing; (b) to determine the prevalence of *Salmonella* and *E. coli* on environmental samples in order to determine where pathogens are being introduced; (c) to use phenotypic and genotypic methods to examine the genetic diversity of *Salmonella* isolates obtained in the growing and processing environment; (d) to transform *Salmonella* strains to express enhanced green fluorescent protein and examine the stability and growth of these organisms; (e) to study the effect of processing and storage parameters on the survival of *Salmonella* on parsley; and (f) to study the microbial ecology of parsley before and after processing and in the retail environment.

REVIEW OF LITERATURE

Microbial ecology of fresh produce. Fresh fruits and vegetables, an essential component of the human diet, also serve as an ecosystem for the growth and survival of numerous types of living organisms. The plant ecosystem, as described by Lund *(120)*, consists of the microorganisms present, the structure of the plant, the pH of the plant tissue, presence of organic acids and antimicrobial compounds, the state of the plant tissue at harvest, and the extrinsic factors such as temperature, water activity, and gaseous environment. The diverse microflora present on fresh produce, as influenced by the plant ecosystem, has a direct effect on issues of postharvest quality and safety.

The microbial population is not static, but constantly changing due to environmental factors and handling. The presence and numbers of organisms on fresh produce differ depending on the type of produce, agricultural practices, geographical area, and weather conditions before harvest *(28, 120, 143)*. Rain and insects are suspected to be involved in the transfer of bacteria on and among plants in the field *(110)*. Fruits and vegetables are frequently in contact with insects, soil, and animals, both wild and domestic, during growing and harvesting in the field *(80, 151)*. Janisiewicz et al. *(98)* showed that fruit flies contaminated with a fluorescent-tagged nonpathogenic strain of *Escherichia coli* O157:H7 served as a vector in the transmission of this microorganism in wounded apples. Insects, particularly beetles and grasshoppers, may contaminate leafy vegetables, but fecal coliforms are not part of the permanent flora of insects. These bacteria are transferred transiently by way of hairy exoskeletons during contact with feces or are ingested by the insect *(80)*.

Raw fruits and vegetables may potentially harbor many foodborne pathogens *(52, 120)*. Pathogens that have been isolated from minimally-processed fresh vegetables include *Listeria monocytogenes*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Staphylococcus aureus*, *E. coli*, and *Salmonella*. However, the incidence of human pathogens is lower on vegetables than on meat, seafoods, or dairy products. Hudson and De Lacy *(93)* reported that only 1% of vegetable products were contaminated with *Aeromonas* spp., whereas 20% of meat products and 66% of shellfish products were contaminated. Farber et al. *(61)* isolated *L. monocytogenes* from meat and poultry, but not vegetables; however, other researchers have isolated this microorganism from fresh produce *(20, 100, 181)*. This is not surprising because *L. monocytogenes* is one of the most prevalent human pathogens in soil *(208)*. Many of the human pathogens do not grow on produce unless the plant tissue is damaged. *E. coli* O157:H7 does not grow on freshly picked, intact apples; however, this organism grew well in bruised apple tissue independent of the date of harvest *(51)*. Wells and Butterfield *(207)* found that the incidence of *Salmonella* on produce affected by bacterial soft rot was twice that of produce not affected by soft rot.

Human pathogenic bacteria on fruits and vegetables can occur from the application of feces or untreated sewage as well as from contact with contaminated irrigation and surface run-off waters. Irrigation water containing untreated sewage may contain pathogenic bacteria, viruses, and parasites *(19)*. Interestingly, detection of pathogens on farm produce growing in contact with contaminated water or soils treated with manure is infrequent unless the plant is grossly contaminated *(80)*. Transmission of

pathogens by irrigation and organic fertilizers has been reported *(2, 164, 176)*. Solomon et al. *(176)* demonstrated the transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants using laser scanning confocal microscopy, epifluorescence microscopy, and recovery of viable cells from the inner tissues of plants. However, Dunlop and Wang *(56)* attempted to contaminate crops by irrigating with water inoculated with *Salmonella* and failed.

Cultivation, harvesting, and retail preparation of fruits and vegetables allow for numerous human and equipment contact opportunities *(19, 80)*. Typical hand labor operations including picking, sorting, trimming, tying, bunching, and pre-cleaning of soil particles in a rinse water which subject the product to extensive handling and exposure to a variety of microorganisms. Furthermore, rinse water can be of questionable bacteriological quality *(80)*. Reina et al. *(161)* determined that recycled water reached Enterobacteriaceae populations of 10^6 CFU/g during a typical day's operation. Worker handling and improperly sanitized equipment further increase risk for contaminated products *(19)*. King et al. *(104)* reported that the end product was frequently less contaminated than the raw vegetable after sampling different stages of processing. Pao and Brown *(151)* reported average APCs and yeast and mold counts on fruit surfaces were reduced from 4.0 log CFU/cm^2 to 2.1 log CFU/cm^2 and 3.3 log CFU/cm^2 and 1.3 log CFU/cm^2, respectively, by processing in the packinghouse. Processing does not appear to change the mesophilic microflora as it remains similar in end products and in unprocessed vegetables, but processing and handling could increase the possibility of contamination by human pathogens *(122)*. Garg et al. *(76)* found that shredders and

slicers were more significant sources of contamination than conveyor belts and washing, and chlorinated dips only partially removed organisms.

The exact microbial composition of fruits and vegetables cannot be described because it is possible for almost any organism to be present at some time; however a characteristic flora can be described for fresh produce *(29)*. Surface flora on fresh produce is generally reflective of the environmental flora where it is grown and processed. Mundt et al. *(134)* states that because a field of plants does not represent a fluid mixture, the frequency of a given microorganism and its population on the surfaces of individual plants is dictated by its frequency in the environment and its ability to reproduce on that surface. Splittstoesser *(179)* found that the kinds of microorganisms on raw and blanched vegetables were similar and recontamination following blanching resulted in a higher proportion of catalase-negative cocci. Blanching destroys the plants natural barrier protecting against microbial invasion and also breaks down complex carbohydrates into forms more readily usable by the organisms *(121)*. Microorganisms on a packaged, fresh-cut product not exposed to a blanching step may reflect the microflora on the vegetables growing in the field as well as any contamination during processing *(76, 95)*. Consequently, potentially human pathogenic bacteria associated with the environment are occasionally found on produce *(100, 120)*. There is a potential for fruits and vegetables to become contaminated with human pathogens because they are exposed to a variety of conditions during growth, harvest, processing, and distribution *(121)*.

The type of produce will also have an effect on what groups of microorganisms will be present. For example, yeasts, molds and lactic acid bacteria will have the competitive advantage in fruits due to a lower pH. Vegetables have a pH closer to neutral, resulting in a flora dominated by bacteria. The acidic pH of most fruits prevents the development of most human pathogens and these pathogens rarely grow well on vegetables because the normal spoilage flora will predominate and have the competitive advantage *(29, 36, 90, 178)*. Leafy vegetables, which have the greatest surface area, are often the most heavily contaminated. Group-D *Streptococcus* contamination in commercially available parsley was substantial *(102)*. Spinach received from the grower had relatively high counts, generally over 10^6/g *(76)*. Contamination of plant tissue is largely associated with the surfaces of the produce and the inner cores are often considered sterile *(136)*. However, Lund *(120)* and Robbs et al. *(163)* reported low levels of bacteria in the internal tissues of intact vegetables. Garg et al. *(76)* reported that field contamination was reduced by the removal of outer leaves from lettuce, cabbage, and cauliflower and the peeling step of onions and carrots. The level of organisms on peas protected by a husk or pod were reported to be as high as those potatoes, carrots, and other vegetables that were grown in close contact with the soil *(179)*. Furthermore, peas, which had been essentially sterile up until their removal from the pod, yielded counts much higher than green beans which had been exposed to contamination from the soil and air throughout their growth history *(179)*.

The flora of fresh produce may also be affected by the location where they are growing (i.e., vegetables growing close to the soil versus fruit growing from a tree), as

well as how they are stored *(29)*. As would be expected, storage temperature selects for certain microorganisms. Growth of mesophilic microflora on shredded lettuce was significantly reduced as storage temperature decreased (between 10°C and 2°C) *(22, 26)*. Manvell and Ackland *(126)* reported that in packaged mixed vegetables, lactic acid bacteria multiplied very slowly at 7°C, but dominated after storage at 30°C. Populations of aerobic mesophiles, psychrotrophs, and Enterobacteriaceae increased, regardless of washing treatment, as storage time at 5°C and 15°C increased *(112)*. Low humidity discourages growth of microorganisms on the surface of produce, but also leads to the product shrinking from dehydration. Dehydration may create a situation that allows bacteria to infect the product more easily *(196)*. High humidity allows moisture to condense on the produce surface creating accumulated water on the product which helps microbial growth and aids in the spreading of bacteria *(81)*. Cut products will be more perishable due to the release of tissue fluids with nutrients which aid bacterial growth *(29)*.

Microorganisms present on fruits and vegetables can change their environment. The growth of some microorganisms can make conditions better or worse for the growth of other organisms *(29)*. Mixed populations on minimally processed lettuce diminished *L. innocua* growth, but varying the size of the indigenous populations had no effect on its survival or growth *(70)*. *Enterobacter cloacae* and lactic acid bacteria were effective in reducing *L. innocua* growth on minimally processed lettuce *(70)*. Liao and Fett *(113)* found that some strains of *Bacillus* spp. and *Pseudomonas fluorescens* inhibited the

growth of *L. monocytogenes*, *E. coli*, and *Erwinia carotovora* subsp. *carotovora* on some types of fresh produce.

Some of the organisms found in the typical microflora of fresh fruits and vegetables include *Pseudomonas* spp., *E. herbicola*, *Flavobacterium*, *Xanthomonas*, and *E. agglomerans* as well as various yeasts and molds. Additionally, lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus* spp. are also common *(113, 215)*. Green plants have a resident microflora which survives on the small traces of carbohydrates, protein, and inorganic salts which dissolve in the water that exudes from the epidermis or condenses on the plant *(121)*. *P. fluorescens*, *E. herbicola*, and *E. agglomerans* are major components of the epiphytic microflora of most vegetables *(79, 171, 172)*. Coryneforms and spores of *Clostridium* species and *Bacillus* are commonly found in soil, so their presence on fruits and vegetables should be expected *(19, 121)*. Vegetables acquire most of their flora from the soil and processing area. The main groups on freshly harvested vegetables are the gram-positive coryneforms, lactic acid bacteria, *Bacillus* and *Micrococcus*, gram-negative *Pseudomonas* and Enterobacteriaceae, and yeasts and molds *(90)*. Liao and Fett *(113)* found that fluorescent pseudomonads accounted for 23-73% of aerobic plate counts (APCs) recovered from carrots, pepper, and lettuce. Fluorescent pseudomonads actively participate in the breakdown of plant tissues releasing potential nutrients for pathogenic microorganisms. The presence of pseudomonads on vegetables probably does not depend on external contamination because they are probably endemic on the product *(143)*. Pectinolytic *P. fluorescens*, pectinolytic *Xanthomonas* spp., *Cytophaga* spp., and

Flavobacterium spp. have been isolated from various raw unprocessed vegetables at the retail outlet *(114, 115, 116)*. Coliforms are also commonly isolated from plants *(121)*.

Microorganisms on minimally processed fresh vegetables are similar to what is found in the field or after harvest and are probably from the epiphytic microflora of the raw materials. Splittstoesser and Gadjo *(180)* found similar distributions of microorganisms on vegetables processed at different facilities, as well as on vegetables in different stages of processing. Thunberg et al. *(189)* evaluated ready-to-use vegetables at retail and reported the APCs for sprouts, lettuce, celery, cauliflower, and broccoli to be 8.7, 8.6, 7.5, 7.4, and 6.3 log CFU/g, respectively. Pseudomonads are major components of the indigenous microflora of ready-to-use vegetables *(143)*. *P. fluorescens*, *P. putida*, *P. aeroginosa*, *E. cloacae*, *E. agglomerans*, *E. sakazakii*, *L. citreum* and *L. brevis* were isolated from lettuce *(70)*. Babic et al. *(8)* examined and identified microbial populations on fresh-cut spinach leaves that were stored in gas permeable bags at 10°C for 12 days. Populations of mesophiles, psychrotrophs, Pseudomonadaceae, and Enterobacteriaceae increased sharply during the storage period. Initial populations were 10^7, 10^6, 10^6, and 10^4 CFU/g, respectively. Populations reached 10^{10} for mesophiles, psychrotrophs and Pseudomonadaceae and 10^7 CFU/g for Enterobacteriaceae after 12 days of storage. Micrococcaceae, lactic acid bacteria and yeasts remained constant (10^3-10^4 CFU/g) *(8)*.

Salmonella. Nontyphoidal salmonellae are important foodborne pathogens that cause gastroenteritis, bacteremia, and subsequent focal infection *(91)*. Focal infections

can include meningitis, septic arthritis, osteomyelitis, cholangitis, and pneumonia *(91, 216)*. More than 95% of cases of *Salmonella* infections are foodborne, and salmonellosis accounts for approximately 30% of deaths resulting from foodborne illnesses in the United States *(130)*. Symptoms, however, are frequently mild and mortality is low among healthy individuals *(159)*. Symptoms of salmonellosis include diarrhea, abdominal pains, chills, fever, vomiting, dehydration, and headache. The incubation period for the illness is between 5-72 h and lasts approximately 12-36 h *(53)*. *Salmonella* should be completely destroyed by proper cooking and so the risk lies in foods not subjected to a heat treatment before consumption as well as cross contamination *(159)*. Bayre et al. *(13)* reported that five minutes at 60°C destroyed 10^8 cells of *S.* Typhi in chicken meat, while 10-15 min at 65°C was required for *S.* Typhimurium.

As of the year 2000, there are 2,463 serotypes of *Salmonella* as determined by serologic identification *(31, 156)*. The nomenclature system used for *Salmonella* has changed many times over the years and there is not a lot of consistency among the nomenclature used worldwide in various reported research. Originally, the naming system was based on one serotype with one species on the basis of the serologic identification of the somatic (O) and flagellar (H) antigens as developed by Kauffman *(31, 103)*. The nomenclature system defined by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*, as utilized by the Centers for Disease Control and Prevention, places all salmonellae in two species (*S. enterica* and *S. bongori*). *S. enterica* is divided into six subspecies, which are referred to

by a Roman numeral and a name (I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *indica*) *(31)*.

Description of organism. Salmonellae are mesophilic, gram-negative, non-sporeforming, straight rods (0.7-1.5 x 2-5 *u*m) that appear very similar to *E. coli* when viewing microscopically or on any nonselective growth media *(99)*. They are facultatively anaerobic and usually motile by peritrichous flagella. Biochemically, they are characterized as follows: oxidase negative, catalase positive, indole and Voges-Proskauer negative, and methyl red and Simmons citrate positive, lysine and ornithine decarboxylase positive; there is a variable arginine dehydrolase reaction. Hydrogen sulfide (H_2S) is produced and urea is not hydrolyzed *(92)*.

There has a lot of attention given to the worldwide spread of *S.* Typhimurium definitive type 104 (DT104), which carries chromosomally based resistance to ampicillin, chloramphenicol, TMP-SMZ, streptomycin, and tetracycline. Researchers have presented evidence that connect the antimicrobial resistance of human *Salmonella* isolates with agriculture use of antibiotics *(6)*. More recently, there have been reports of an emergence of *Salmonella* species that are resistant to extended-spectrum cephalosporins *(64, 91)*.

Outbreaks. The most commonly implicated sources in salmonellosis outbreaks are poultry and other meat products, eggs, and dairy products; however, several large

outbreaks have been associated with fresh fruits and vegetables *(21)*. Egg-associated illness caused by *S.* Enteritidis accounts for 25% of all human salmonellosis reported in the United States *(4)*. Anderson et al. *(3)* described an outbreak of *S.* Typhimurium where veal was the food vehicle of transmission. The largest outbreak of salmonellosis occurred in 1994 from the consumption of ice cream made from milk that had been transported in tanker trucks that had previously hauled liquid eggs *(99)*. Salmonellosis is a challenge to the public health due to its ability to cause enormous, widespread outbreaks, which is likely attributed to the trend towards centralized, large-scale food processing with wide distribution *(187).*

Outbreaks associated with fresh produce. Fresh produce increasingly gains attention as an important cause in foodborne illness outbreaks. Salmonellosis outbreaks have been epidemiologically associated with the consumption of cantaloupes, watermelon, tomatoes, lettuce, celery, alfalfa sprouts, parsley, unpasteurized orange juice, and other raw salad vegetables *(39, 40, 42, 75, 77, 96, 123, 152)*. Outbreaks associated with fresh produce have been linked to fecal contamination during postharvest handling, shipping, or processing under circumstances permitting bacterial multiplication *(123).*

Several outbreaks of salmonellosis in the United States have been connected to product at salad bars or product subjected to cutting. *Salmonella* can survive and grow on cut fruit *(59)*. In Massachussets in 1955, 17 people suffered from an outbreak of gastroenteritis caused by the consumption of sliced watermelon containing *S.* Miami

(77). An outbreak of salmonellosis associated with diced tomatoes occurred in the United States in 1999 *(206)*. In 1991, more than 400 cases of *S.* Poona infections were linked to pre-sliced cantaloupe *(42)*. Tracing the source of the *Salmonella* implicated in the outbreak is difficult. Hypotheses for causes of outbreaks have included contaminated water-baths used by packers, carriage of the contaminant from the rind or surface to the interior meat of the product, contaminated seeds, and exposure to warm storage temperatures *(136)*.

Reservoirs. The natural reservoirs of *Salmonella* are the intestinal tracts of animals and birds, and sometimes insects *(21, 99)*. Fowls are the greatest reservoir of salmonellae both in the United Kingdom and in the United States *(89)*. Lofton et al. *(119)* reported a 6.3% incidence of *Salmonella* in the intestinal flora of 127 wild birds captured near farms in Colorado and Wobeser and Finlayson *(212)* isolated *S.* Typhimurium strains from moribund house sparrows. *Salmonella* was isolated from 7.5% of 253 wild mammals located on seven Illinois farms *(170)*. From the vertebrate reservoirs, salmonellae are spread to the environment (air, food, feed, soil, water, etc.) where they can be transmittted to other hosts *(159)*. *Salmonella* has the ability to survive for months in sewage sludge applied to agricultural soils *(202)*. Microorganisms transported via floodwaters can survive for months or years on croplands following the flood *(19)*. Human pathogenic bacteria can become established in a packing plant and serve as a potential source of cross-contamination *(28)*. Additionally, people recovering

from salmonellosis may continue to shed the organism in their feces and remain long-term carriers for weeks to months *(53)*.

Presence in foods. *Salmonella* has been isolated from a variety of food products. Lammerding et al. *(106)* sampled meat and poultry products in Canada and detected salmonellae on pork, turkey, chicken, and beef samples (17.5%, 69.1%, 60.9%, and 2.6%; respectively). Duffy et al. *(54)* detected salmonellae on 9.6% of samples obtained from various ready-to-cook pork retail products. *Salmonella* is also present on chilled lamb and beef carcasses produced in the United States *(55, 175)*. A variable proportion of imported and home-produced frozen and dried egg products and meats, desiccated coconut, and other foods have been shown to contain small to large numbers of salmonellae *(90)*. Additionally, undercooked eggs have been linked to salmonellosis and it has been shown that *Salmonella* can be transferred transovarially from chickens to egg *(91)*.

Typing of microorganisms. Typing systems are based on the principle that related isolates share certain phenotypic and/or genotypic characteristics by which they can be differentiated from unrelated isolates *(7)*. Typing can be used to link bacterial strains in foodborne illness outbreak investigations as well as to track sources of contamination in food processing. Typing of microbial strains can be completed using a variety of techniques. The efficiency of each of these methods can be compared based on factors such as typeability, reproducibility**,** and discrimination *(94)*. Typeability is

the ability to obtain a clear, positive result for each isolate analyzed; nontypeable isolates are those that give an uninterpretable result *(7)*. Reproducibility refers to the ability of a technique to yield the same result when the same strain is tested repeatedly *(7)*. The discriminatory power of a typing method refers to its ability to distinguish between unrelated strains as determined by the number of types defined by the test method and the relative frequencies of these types *(94)*. Typing systems are often classified as a phenotypic or genotypic. Phenotypic techniques (i.e., serotyping and antimicrobial susceptibility testing) are those that detect characteristics expressed by the microorganism, whereas the genotypic techniques (i.e., rep-PCR and PFGE) involve direct DNA-based analyses of chromosomal or extrachromosomal genetic elements *(7)*.

Antimicrobial susceptibility testing. In addition to selecting appropriate antimicrobial agents to control infections, antimicrobial susceptibility testing is also commonly used to identify new or unusual patterns of antibiotic resistance among cultures *(7)*. Many *S.* Typhimurium DT104 isolates possess a multiple antibiotic resistance phenotype to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT); however, not all DT104 isolates express this penta-resistant phenotype *(209)*. Spika et al. *(177)* reported an investigation of an outbreak of Salmonellosis where approximately 1,000 people were infected with *S.* Newport exhibiting chloramphenicol resistance. Chloramphenicol-resistant *S.* Newport was traced from infected people through processing of contaminated ground beef to a dairy farm area *(177)*. Antibiotic susceptibility testing is often not very useful for detailed

epidemiological studies due to the multiple genetic mechanisms by which a given strain can become abruptly resistant to a particular antibiotic *(7)*. Resistance to antimicrobial agents is considered to be relatively unstable because plasmids carrying resistance factors are often transferable between strains *(147)*. Olsen et al. *(147)* reported that reproducibility of antibiotic typing had low reproducibility if anything other than complete resistance to an antibiotic was considered for typing. Although antibiotic susceptibility testing is not a very effective typing method, it is necessary for the control of infection as well as monitoring unusual resistance patterns.

Serotyping. Serotyping refers to the serological procedures that are used to differentiate strains of a species based on differences in the antigenic composition of their cell surfaces. *Salmonella* can have three kinds of antigens including H or flagellar antigens; O or somatic antigens which are the lipopolysaccharide component of the cell wall; and K or the capsular antigen. Most *Salmonella* do not possess a capsule and identification is determined based on the identification of O and H antigens following the Kauffmann-White scheme *(27, 157)*.

Serotyping normally forms a background for other more advanced typing methods. Serotyping can be inadequate as a single typing method when isolates to be compared are of the same serotype. However, the appearance of isolates with an unusual serotype can be indicative of an epidemiological relationship between isolates *(147)*. The majority of outbreaks of salmonellosis in livestock are caused by only a few serotypes *(147)*. In contrast, surveys of fresh produce have reported the presence of

several different *Salmonella* serotypes capable of causing human infection (*21*). Due to the relatively poor discriminatory power of serotyping and the high frequency of nontypeable isolates, DNA-based typing techniques are becoming the more popular for typing of isolates *(7)*.

Repetitive sequence-based polymerase chain reaction (Rep-PCR). Rep-PCR is a typing method that examines strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes *(146, 197)*. This method uses oligonucleotide primers matching the interspersed repetitive DNA sequences to yield DNA fingerprints of individual bacterial isolates after separating the amplified products using electrophoresis *(197)*. Repetitive extragenic palindromic sequences have been identified for many members of the Enterobacteriaceae family as well as for some gram positive bacteria *(7)*. Rep-PCR can be completed with DNA extracted from bacterial colonies or a modified method using whole cells *(146, 213)*. Rep-PCR correlates well with pulsed-field gel electrophoresis (PFGE) results, but with slightly less discriminatory power; however, rep-PCR is much less labor intensive *(118)*.

Pulsed-field gel electrophoresis (PFGE). Pulsed-field gel electrophoresis (PFGE) is considered to be the "gold standard" of molecular typing methods due to its excellent ease of interpretation, discriminatory power and reproducibility *(7, 146)*. Lee et al. *(111)* applied PFGE to confirm the chain of transmission of *S*. Javiana from restaurant food handlers or food to consumers. Refsum et al. *(160)* investigated the

molecular epidemiology of *S.* Typhimurium isolates from wild birds, domestic animals and the environment by using PFGE.

For this technique, bacterial isolates are combined with molten agarose and poured into small plug molds. The agarose plugs embedded with bacteria are then subjected to detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. Plugs are then inserted into an agarose gel and subjected to electrophoresis *(146)*. The large DNA fragments are separated using a variation of agarose gel electrophoresis where the orientation of the electric field across the gel is changed periodically ("pulsed") rather than being kept constant *(7)*. Chu et al. *(44)* found that the migration pattern and resolution of DNA molecules could be greatly improved by applying a contour-clamped homogenous electric field (CHEF) and recirculate the buffer to dissipated uneven heating across the gel.

Microbial attachment on produce. The colonization of bacteria on fresh produce has a profound effect on the safety and quality of the product. Numerous types of fresh fruits and vegetables are available in the retail market, each possessing unique surface characteristics affecting the attachment and/or infiltration abilities of microorganisms. Fruits and vegetables exist in various forms including stems, roots, florets, and leaves. All of these forms offer different ecological niches for microorganisms. Microbial attachment and colonization on fresh produce have significant public health implications as these processes may be related to the inability of sanitizer and washing treatments to remove or inactivate human pathogens *(17, 71)*.

A microorganism's ability to attach to different surfaces will affect its survival, especially in low-nutrient environments *(86)*. Bacteria use numerous mechanisms to bind to plant surfaces. Attachment is affected by cell surface charge, hydrophobic effects, bacterial structures, excreted extracellular polysaccharides, and the surrounding environment *(50, 71, 129, 142, 165, 195)*. Detachment is also important to the survival of microorganisms as they may need to detach should an environment become unfavorable *(71, 194)*. Sometimes detachment is not possible due to physical entrapment of cells by the capillary action of the food tissue or when the cell has produced large amounts of extracellular binding polymers referred to as glycocalyx *(71)*.

Bacterial cells exhibit a net negative charge on the cell wall as do most plant surfaces. In order for the bacteria to attach to a plant surface, it must overcome the electrostatic forces through bridging by divalent cations, ionic bridging between local positively charged groups on one surface, hydrogen bonding, and using van der Waals forces *(15, 165)*. It has been reported that the adhesion of *P. fluorescens* increases as the ionic strength of the solution increases regardless of the cations present *(154)*. Ions in solution reduce the thickness of the electrical diffuse double layer on each surface which allows negatively charges cells to move closer to a negatively charged surface so that van der Waals forces or bacterial surface appendages can overcome the electrostatic repulsion forces *(71)*. Dickson and Koohmaraie *(50)* established that the magnitude of the bacterial cell surface charge is an important factor in attachment to meat surfaces. It has also been reported that motile *L. monocytogenes*, which has a greater surface charge than nonmotile cells, attaches more readily to glass *(49)*. Ukuku and Fett *(191)* reported

that both negative and positive charges are correlated with the strength of attachment of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* to cantaloupe rind. Van der Waals attraction forces and electrostatic forces are part of the initial weak interactions between the bacterial cells and plant surface that are referred to as reversible adhesion. When bacteria are reversibly attached they can be removed very easily by fluid shear forces such as rinsing *(128).*

In addition to cell charge, hydrophobicity also plays a role in the attachment and colonization of bacteria on a plant surface. A plant surface consists of an epidermis, followed by a pectin layer, and finally a multilayered hydrophobic cuticle than is 1 to 15 *u*m thick *(144, 166)*. Hydrophobic plant surfaces and hydrophobic regions of bacterial surface proteins pull cells closer together to facilitate further interactions *(15)*. By measuring the contact angle of water on a bacterial layer, Van Loosdrecht et al. *(195)* showed that hydrophobic cells adhered to a greater extent to negatively charged sulfated polystyrene than the hydrophilic cells. Ukuku and Fett *(191)* showed that *Salmonella*, which had the most hydrophobic surface of the three genera they tested, bound the strongest to the hydrophobic, intact surface of a cantaloupe. Lindow *(117)* speculates that differences in hydrophobicity on leaves, due to varying patterns of wax structures and composition, could affect the locations that bacteria may select for attachment. This may result in the preferential colonization of the most hydrophobic sites by bacteria possessing the most hydrophobic cell surface *(117)*. However, Fett *(63)* found there was no clear correlation between hydrophobicity and adherence potential even though he found differences in hydrophobicity among bacterial species which occur on leaf

surfaces. Although cell surface charge and hydrophobicity are important in attachment, it can be difficult to predict the surface properties of human pathogenic bacteria because environmental conditions can alter the surface properties of these bacteria when they are first exposed to a plant surface *(32, 48)*.

Many plant-associated (epiphytic) bacteria are surrounded by layers of extracellular polysaccharide (EPS) when observed on leaf surfaces *(117)*. EPS helps anchor the bacterial cells to the leaf surface and may also alter the physical and chemical environment around the cell to improve growth and survival, as well as prevent cells from dessication *(14, 109, 117)*. The EPS fibers are involved in the initial attachment and become thicker with time resulting in a biofilm matrix *(65, 74)*. Glycocalyx, slime, capsule, and sheath are other terms that have been used to describe EPS *(43, 78)*. Within this group are S-layers (subunit surface layers), capsules, and slime layers *(27)*. Capsule polymers usually contain acidic residues which contribute to the negative charge to the cell surface *(47)*.

Some bacteria have proteinaceous, threadlike, nonflagellar attachment structures such as fimbriae or pili *(66, 149)*. These structures range in length from 0.2 to 20 *u*m and in width from 3 to 14 nm *(149)*. Common pili are adhesins that are often the first step in establishing infection when attaching to an animal host *(68)*. Pili also cause certain bacteria to aggregate and form pellicles on a surface of a liquid medium assisting with oxygen delivery to the cell. They are more commonly found on gram-negative bacteria, especially members of the Enterobacteriaceae family; however, similar structures have been observed on gram-positive bacteria such as *Corynebacteria* and

Streptococcus (27). Pili contribute to the hydrophobicity of the cell as the amino acids on some of the proteins contain numerous nonpolar side chains *(45)*.

Bacteria do not colonize plant surfaces uniformly. On leaf surfaces, bacteria usually are seen in depressions of the cuticular layer of the epidermis and at the bases of trichomes *(110, 117, 127)*. Trichomes are protuberances from the surface of the cuticle and when many are present they provide a hairy surface that acts as an anti-wetting agent. Gas exchange occurs through the pores in the epidermis called stomata which are surrounded by guard cells that open in response to environmental stimuli *(71)*. Mansvelt and Hattingh *(127)*, using scanning electron microscopy, demonstrated that *P. syringae* pv. *syringae* colonizes and multiplies on the surface of pear leaves, particularly on trichomes and in cuticular depressions and were able to enter the leaf tissue through the open base of trichomes. Bacteria are also found at stomata and at epidermal cell wall junctions, particularly in the grooves along the veins and on cut surfaces, and broken trichomes *(127, 173)*. Babic et al. *(8)* found that during storage, spinach leaves were colonized predominately in areas where the waxy cuticle was broken. Adams et al. *(1)* reported that microorganisms were protected in the hydrophobic pockets or folds in the leaf's surface and not exposed to disinfectants in wash solutions. Microorganisms have also been found on areas of leaves where the cuticle is broken and, with the use of low temperature scanning electron microscopy (LTSEM), microorganisms could be seen infecting the internal palisade parenchyma *(8)*. Population density appears to affect the attachment and survival of bacteria. Wilson and Lindow *(211)* reported that cells in inocula with high cell concentrations exhibited up to 100-fold better survival on leaves

than cells in inocula of low cell concentration suggesting that leaves colonized by bacteria provide a habitat that is different from uncolonized leaves.

Several studies using confocal scanning laser microscopy (CSLM) have demonstrated that human bacterial pathogens have the ability to become internalized within lettuce tissue *(173, 176, 183, 185, 201)*. Menely and Stanghellini *(131)* detected enteric bacteria within the internal tissue of healthy cucumbers. There have been several reports that bacteria can be become internalized in fruit through immersion when there is a negative temperature differential *(34, 37, 217)*. Zhuang et al. *(217)* demonstrated the internalization of *Salmonella* in tomatoes.

Methods to determine microbial attachment. In order to effectively develop and evaluate decontamination methods for fresh produce it is important to understand the mechanisms of attachment as well as be able to qualitatively and quantitatively evaluate the attachment and or internalization of the microorganisms on the product. There are a variety of methods available including both plating and microscopic methods; however, there are inherent advantages and disadvantages of the various methods.

Cell detachment. Cell detachment is a conventional method that relies on the detachment of the microorganisms using a swab or a sponge, stomaching the sponge in a peptone solution, and then plating serial dilutions of the homogenate to produce countable colonies. The enumerated count represents the cells that were attached to the

swabbed surface *(87)*. Another cell detachment method involves submerging the sample in inoculum, followed by a rinse with sterile water to remove loose cells, and then stomaching to release attached cells *(50, 185, 191)*. Using both plate counts as well as confocal scanning laser microscopy, Takeuchi et al. *(185)* determined that *E. coli* O157:H7 and *L. monocytogenes* attached preferentially to cut edges of lettuce leaves, while *P. fluorescens* attached preferentially to intact surfaces of lettuce.

In order to measure the relative strength of attachment to different surfaces using a plating procedure, Firstenberg-Eden et al. *(65)* determined an *S* value that has been used to determine strength of attachment to lean and fat tissues as well as to cantaloupe *(50, 62, 65, 191)*. As described by Dickson and Koohmaraie *(50)*, the *S* value measures the difference between bacteria which are physically attached to a surface and those which are loosely associated with a surface, [$S = \log_{10}$ (physically attached bacteria) - $\log_{10}$ (loosely attached bacteria)]. Therefore, an increase in the *S* value indicates an increase in the numbers of bacteria which are physically attached to the surface under defined conditions. Using the *S*-value, Dickson and Koohmaraie *(50)* also calculated a S_R value which represents the percentage of the total population of bacteria associated with the tissue surface which is physically attached to the surface [S_R = (physically attached bacteria)/(physically attached + loosely associated bacteria)]. Ukuku and Fett *(191)* used S_R values to describe populations of bacteria remaining on melon surfaces after washing treatments.

Plating procedures can also be used to determine if bacteria have become trapped or internalized within the inner tissues of fruits and vegetables. It is important to

consider that some of the surface bacteria are bound or entrapped in the surface of the fruit or vegetable and are not actually attached *(71)*. Surface sterilization techniques are crucial when trying to examine internal populations *(210)*. Menely and Stanghellini *(131)* detected enteric bacteria within healthy cucumbers by sanitizing the outside surface of cucumbers using a hot detergent solution and soaking in 10% chlorox for 0.5 h before taking a sample from the inside after aseptically snapping the cucumber in half. Solomon et al. *(176)* recovered viable *E. coli* O157:H7 cells from the inner tissue of lettuce after surface disinfecting by dipping the lettuce in 80% ethanol for 5 s followed by immersion in 0.1% (wt/vol) $HgCl_2$ and washing twice in sterile water. Zhuang et al. *(217)* determined the uptake of *S*. Montevideo by core tissue of tomatoes by submerging tomatoes in varying temperature dip suspensions, storing them, and then removing the core and the external stem scar area using a sterile scalpel, and then excision sampling the underlying conical area of tissue. The challenge in enumerating the internal bacteria is to avoid the addition of surface bacteria which, of course, will inflate the counts.

Agar overlay and imprinting. Direct agar overlay and agar imprinting techniques are simple procedures, but can be ineffective if the bacteria form spreading colonies or there are high numbers of microorganisms present. Low numbers of viable attached microflora can be detected by pouring nutrient agar media directly on the test surface and incubating *(5)*. This technique has also been used to determine residual attached microflora on different surfaces to test the efficacy of various sanitizing treatments *(23, 72)*. Solidified nutrient agar can also be used to detect attached bacteria by pressing the

test surface onto the agar and then incubating. The plant print method has been used to study the location and movement of bacterial populations on plants *(107, 108)*. Solomon et al. *(176)* detected internalized *E. coli* O157:H7 expressing green fluorescent protein by sanitizing lettuce seedlings, slicing them longitudinally to the base of the cotyledons and pressing the inner surfaces onto tryptic soy agar surfaces to detect any internalized bacteria. A variation of the leaf print technique is the "balloon print" method developed by Rusch and Leben *(167)*. This method uses a large toy rubber balloon to transfer microorganisms from the leaf to the printing medium. The balloon is sterilized and the plant is pressed gently onto the balloon, and then the balloon is inflated and pressed onto the surface of the agar printing medium. This technique is useful if there are both fast and slow growing residents on the plant surface and also separates the colonies to reduce the effects of any antagonistic effects between the colonies *(167)*.

Direct microscopic examination using confocal scanning laser microscopy.

Direct microscopic examination is probably the most effective method to discriminate between attached, entrapped, and/or internalized cells as well as to understand exactly how bacteria are actually colonizing the plant surface. Morris et al. *(133)* used epifluorescence microscopy, scanning electron microscopy, and confocal laser scanning microscopy to observe microbial biofilms directly on leaf surfaces. Confocal Scanning Laser Microscopy (CSLM) is preferred to conventional light microscopy and electron microscopy due to the ability of being able to observe the three-dimensional structure in a fully hydrated state with little or no sample preparation as well as being able complete

optical sectioning *(25, 84)*. CSLM can actually be considered a form of light microscopy where a narrow range of wavelengths of laser light excites a specific fluoresence material. With the confocal system, a point in the object is optimally illuminated and imaged in a detector pinhole, which leads to an increased resolution and a reduced depth of field because off-focus levels in the specimen will not contribute to the image *(84)*. Specimens can be viewed without any prior dehydration steps and sections as thin as 2-3 um may be used which would be advantageous when studying leafy vegetables (e.g., parsley) *(199)*.

When using CSLM, the bacteria need to be stained or transformed in a manner that will allow the organism to be easily viewed. Commonly used tools include fluorescent molecular probes or fluorescent dyes *(71)*. Dual staining with fluorescein isothiocyanate-labeled antibody and propidium iodide can be used to visualize cells and also determine the viability of the cells *(173)*. Bacterial strains can also be transformed to produce green fluorescent protein so that organisms can be viewed and monitored noninvasively *(192)*.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been used as a fluorescent tag for pathogens in various fruit and vegetable internalization and attachment studies *(37, 150, 201)*. Colonies of bacterial cells expressing GFP can be easily viewed and counted by illumination with ultraviolet light (365 nm) *(148)*. Seo and Frank *(173)* observed *E. coli* O157:H7 expressing green fluorescent protein, attached to the surface, stomata, and cut edges of lettuce leaves, as well as entrapped 20 to 100 *u*m below the surface. The fluorescence expressed by GFP is stable, species-

independent and can be monitored noninvasively using confocal scanning laser microscopy and epifluorescence microscopy *(192, 214)*. However, Burnett and Beuchat *(35)* found that it may not be ideal when it is necessary to microscopically differentiate viable and dead cells on produce following sanitizing treatments. Several researchers have reported that GFP expression will not affect the biochemical, morphological, or growth and survival characteristics of the microorganism *(24, 73, 158, 190)*. In contrast, Oscar *(148)* found that GFP expression altered lag time and specific growth rate on sterile chicken burgers and that it altered the maximum population density on sterile chicken burgers and in broth starter cultures.

Introducing plasmid DNA into microbial cells. Plasmids are extrachromosomal, double-stranded, circular molecules of DNA, 1 kb to more than 200 kb in size, found in bacteria and yeast cells *(168, 200)*. Both gram-positive and gram-negative bacteria can take up and establish exogenous plasmid DNA; however, bacteria need to be in a state of competence for transformation to occur *(83)*. Artificial transformation can be used to incorporate plasmid DNA into microbial cells. Either chemical or physical methods are necessary to enable the DNA to cross cell membranes to reach the intracellular region where they can be expressed and replicated. Other methods that bacteria can use to exchange genetic material besides transformation include transduction which involves the transfer of genes from one cell to another by bacterial viruses and conjugation which is the direct transfer of bacterial genes from cell to another via cell-to-cell contact *(97)*.

E. coli cells can be artificially induced to enter a state of competency using simple salt solutions containing metal cations such as calcium or magnesium *(168)*. Mandel and Higa *(124)* demonstrated that when bacterial cells and DNA were mixed together in ice-cold calcium chloride solutions and then exposed to a brief heat pulse at 37°C to 42°C, they could be transfected with bacteriophage DNA. Oscar *(148)* introduced a plasmid encoding green fluorescent protein and ampicillin resistance into *S.* Typhimurium, *S.* Dublin, and *S.* Enteritidis by calcium chloride precipitation. Artificial transformation is generally inefficient meaning that plasmids only become stably established in a small minority of the bacterial population and so plasmid vectors often contain a genetic marker such as resistance to a particular antibiotic (e.g., ampicillin) to give growth advantages to the plasmid-bearing bacteria *(168)*.

Electroporation is a physical method that can be used to incorporate nucleic acids into the bacterial cell. Transformation is much more efficient using electroporation with 10^{10} cells transformed per each microgram of superhelical plasmid DNA versus 10^{6} to 10^{7} transformed cells per microgram of superhelical plasmid DNA using chemical methods *(168)*. Electroporation involves exposing the bacterial cells to an electrical charge with destabilizes the cell membranes and induces the formation of transient membrane pores through which the plasmid DNA can pass *(204)*.

MATERIALS AND METHODS

Produce and environmental sample collection. Production farms with operating packing sheds in Texas were selected according to their production of cantaloupes, oranges, and parsley. For each type of product sampled, a minimum of two farms were selected, with 25 samples collected in the field and 25 samples collected after processing. Samples were collected twice per season, resulting in a total of 100 samples per product tested. Global positioning system (GPS) coordinates were recorded for all field sampling locations using a hand-held Magellan® 315 GPS receiver (Thales Navigation, Santa Clara, Calif., Table 1). Parsley samples were collected in bunches using a gloved hand, slicing the stems with a disposable knife and each bunch (approximately 50 g) was transferred to a sterile (17.8 x 27.9-cm) Whirl-Pak® bag (International BioProducts, Redmond, Wash.). Cantaloupe samples were collected using a BioPro Sampling System which consisted of a sponge sampling kit with supplied sterile gloves (International BioProducts). Sponges were rehydrated with 25 ml of sterile buffered peptone water (Mega Reg 25™, International BioProducts) immediately before sampling. Using both sides of the sponge, the entire cantaloupe surface was swabbed and the sponge returned to the sample bag and sealed for transport to the laboratory. For orange samples, an orange was selected with a gloved hand and placed in a sterile (17.8 x 27.9-cm) Whirl-Pak® bag. Following the addition of a 25-ml aliquot of sterile buffered peptone water, the bag was shaken vigorously 10 times, rubbed forcibly by hand for 1 min, and shaken an additional 15 times. The orange was then discarded and the sample bag sealed for transport to the laboratory.

TABLE 1. *Global Positioning System (GPS) coordinates for field sampling locations*[a].

Field Description	Latitude	Longitude	Elevation (m)
Orange grove (1)[b]	26°22.107 N	98°16.200 W	54
Orange grove (1)	26°21.124 N	98°16.426 W	56
Orange grove (2)	26°25.744 N	98°23.506 W	95
Orange grove (2)	26°26.378 N	98°23.407 W	92
Parsley field (3)	26°07.934 N	98°16.948 W	41
Parsley field (3)	26°08.351 N	98°16.881 W	34
Parsley field (4)	26°09.180 N	98°18.016 W	37
Parsley field (4)	26°10.086 N	98°18.629 W	32
Cantaloupe field (5)	29°08.242 N	99°44.496 W	261
Cantaloupe field (5)	29°07.183 N	99°44.951 W	264
Cantaloupe field (3)	26°05.822 N	98°10.048 W	20
Cantaloupe field (3)	26°19.539 N	98°14.356 W	19

[a]Produce and soil samples were collected from these designated fields and irrigation water samples were collected from the nearest irrigation source supplying the designated field.
[b]Within field description, fields with the same number in parentheses are managed by the same packing shed.

Environmental samples, including water, soil, and surface samples, were also collected during each sampling trip. Water samples were obtained from irrigation sources and wash water sources, as available, for a total of 10 samples per source. Samples were collected using a 100-ml disposable, long-handled "Dippa" sampler (International BioProducts) and transferred into a sterile, 150-ml Oxford® sterile polypropylene specimen container (International BioProducts) and sealed for transport to the laboratory. Irrigation water samples were collected from the Rio Grande, as well as from dirt canals, cement canals, water reservoirs, and furrows. However, the majority of irrigation water supplying all sources originates from the Rio Grande. Soil samples consisted of a 100-g composite sample per each sampling trip for a total of 12 composite soil samples. Samples were collected using a 150-ml Oxford® sterile polypropylene specimen container. Seven scoops of soil from random surface locations (< 5 cm deep) from each field at each farm were placed in a Ziploc® bag. The bag was mixed by rotating the bag 25 times and then transferring enough of the mixed soil to fill one 150-ml Oxford® container before sealing for transport to the laboratory. Surface samples were collected using a BioPro Sponge Sampling System. Sponges were rehydrated with 25 ml of sterile buffered peptone water immediately before sampling. A 400-cm^2 area was selected from surfaces that come in contact with the product, both in the field and the packing sheds (i.e., trailers used for transport, harvesting bins, washing tanks, conveyor belts, etc.). Each selected surface was vigorously sponged with 10 passes of the sponge in 3 directions, using both sides. The sponge was then returned to the bag and sealed for transport to the laboratory.

Samples were placed loosely in a Freezsafe® insulated container (Polyfoam Packers Corp., Wheeling, Ill.) to allow for proper airflow between samples. Frozen Polyfoam Utek® refrigerant packs (VWR Scientific, Suwanee, Ga.) were placed in the container with a piece of cardboard placed in between to prevent the direct contact of samples with the ice-packs. Samples were shipped to the laboratory by overnight courier and analyzed within 24 h of sample collection.

Detection of *Salmonella* and *E. coli*. After receipt at the laboratory, all samples were processed and then examined for the presence of *Salmonella* and *E. coli*. For parsley samples, 50 ml of sterile buffered peptone water were added to 12.5 g of parsley and pummeled for 1 min using a Tekmar® Stomacher Lab-Blender 400 (Tekmar Co., Cincinnati, Ohio) before pipetting the homogenate for analysis. Each sponge sample was squeezed and released 10 times within the bag and each water sample was inverted 10 times before analysis. For soil samples, 20 g of the composite soil sample was added to 100 ml of 0.1% sterile peptone diluent (Bacto™ Peptone, Difco), shaken well and allowed to settle for 1 min before pipetting for analysis.

E. coli were enumerated using Petrifilm™ *E. coli*/Coliform Count Plates (3M™ Microbiology Products, St. Paul, Minn.). One-ml volumes of appropriate decimal dilutions of the samples were plated. Following incubation for 48 h at 35°C, *E. coli* colonies (blue colonies associated with a gas bubble) were counted. Presumptive positive *E. coli* colonies from 10% of presumptive positive samples were picked from the Petrifilm™ and were streaked onto Tryptic Soy Agar (TSA, Difco Laboratories,

Detroit, Mich.) plates and incubated overnight at 35°C for biochemical confirmation. One presumptive *E. coli* colony was selected from each TSA plate and streaked onto a plate containing TSA (Difco) plus 5% sheep's blood (Cleveland Scientific, Bath, Ohio) and a TSA slant and incubated at 35°C for 24 ± 2 h. Growth on the TSA slant was tested for the production of catalase, an enzyme that breaks down hydrogen peroxide (H_2O_2) into water and oxygen gas (O_2) and the production of oxidase, an enzyme involved in electron transport. Production of the catalase enzyme was detected by using a loop to transfer a small amount of culture onto a sterile petri dish and then introducing a drop of hydrogen peroxide (3% solution). Immediate production of oxygen bubbles was designated as a catalase positive result. The lack of catalase was evident by a lack of or weak bubble production. Oxidase production was detected by using a platinum loop to transfer a small amount of growth onto a ready-made reagent-impregnated strip (Pathotec Cytochrome Oxidase Strips, General Diagnostics, Morris Plains, N.J.). The inoculated area was observed for a color change to a deep blue within 20 s which indicated an oxidase positive result. Oxidase negative and catalase positive cultures were also gram stained. Gram stains were examined with a compound microscope (Leica Microsystems, Buffalo, N.Y.).

Catalase positive and oxidase negative gram-negative rods were biochemically confirmed to be *E. coli* using an Industrial Vitek® (BioMérieux, Hazelwood, Mo.), a fully automated *in vitro* testing system for biochemical confirmation. From the blood agar plates containing the presumptive *E. coli* culture (less than 24 h of growth), 2 to 3 colonies were selected from each plate with a sterile cotton tipped applicator (Harwood

Products Co., Guilford, Maine) and suspended in 2 ml of 0.45% sodium chloride solution (Allegiance Healthcare Corp., McGaw Park, Ill.) in a 12 x 75-mm sterile, disposable clear polystyrene culture tube (VWR Scientific). Using a Vitek® colorimeter (Hach Co., Loveland, Colo.), the turbidity of each suspension was adjusted to between a 67 to 76% transmittance (visually corresponded to a 1.0 McFarland). Each individual suspension was inoculated into a GNI+ card (BioMérieux) using the Industrial Vitek® vacuum chamber and the card was read and results interpreted by the Industrial Vitek® automated *in vitro* testing system to confirm the *E. coli* isolates.

Salmonellae were detected qualitatively by using an enrichment procedure consisting of a pre-enrichment, a selective enrichment, and a post-enrichment. Ten milliliters from each sample were transferred into 90 ml of Universal Pre-enrichment Broth (Difco) and incubated at 37°C for 18 to 22 h. Following incubation, for each sample, 1 ml of pre-enrichment was transferred to 9 ml of Tetrathionate Broth (Difco) and an additional 0.1 ml of pre-enrichment was transferred into 9.9 ml of Rappaport-Vassiliadis Broth (Difco). Both broths were incubated in a MagniWhirl® constant temperature bath (Blue M, Blue Island, Ill.) at 41°C to 43°C for 16 to 20 h. Following the selective enrichment, each sample was post-enriched by transferring 1 ml of each of the Tetrathionate and Rappaport-Vassiliadis Broth enrichments into separate 10-ml aliquots of Bacto™ M Broth (Difco) and incubated at 37°C for 6 to 8 h. Following post-enrichment, a *Salmonella* Tecra® (Tecra®, International BioProducts) visual immunoassay was used to screen the post-enrichment samples for *Salmonella*. Samples testing positive by the Tecra® visual immunoassay were streaked for isolation on Xylose

Lysine Desoxycholate Agar (XLD, Difco), Hektoen Enteric Agar (HE, Difco), and Bismuth Sulfite Agar (BS, Difco) from the M Broth post-enrichment. Plates were incubated at 37°C for 24 ± 2 h and examined for the presence of typical colonies. Typical *Salmonella* colonies on XLD appeared pink with or without black centers, but most cultures of *Salmonella* did produce colonies with large, glossy black centers. On HE, typical colonies appeared blue-green to blue with or without black centers. Typical *Salmonella* colonies on BS were brown, gray, or black colonies and commonly had a metallic sheen. When *Salmonella* is growing on BS, the surrounding medium is usually brown initially, but later turns black with continued incubation producing a halo effect. For each sample, one typical colony was selected from each of XLD, HE, and BS agars and used to inoculate both a Triple Sugar Iron Agar (TSIA, Difco) slant and a Lysine Iron Agar (LIA, Difco) slant. TSIA slants were inoculated by touching the center of the typical colony with a sterile loop and streaking the slant and then stabbing the butt. Immediately after inoculating the TSIA slant, without flaming, the LIA slant was inoculated by stabbing the butt twice and then streaking the slant. TSIA and LIA slants were incubated at 37°C for 24 ± 2 h and then examined for reactions typical for *Salmonella*. For TSIA slants, *Salmonella* typically produced an alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar). For LIA, *Salmonella* typically produced an alkaline (purple) reaction in the butt usually with H_2S production. For isolates exhibiting typical *Salmonella* reactions on LIA, regardless of TSIA reactions, a loopful of growth from the LIA slant was transferred onto a TSA slant as well as a Urea Agar slant (Difco) and incubated at 37°C for 24± 2 h. Most

salmonellae are urease-negative. TSA slants from urease-negative cultures were retained and used to test for catalase and oxidase. Salmonellae produce catalase, but do not produce cytochrome oxidase. Procedures for testing for catalase and oxidase enzymes used were the same as described previously. Only those isolates exhibiting catalase positive and oxidase negative results were retained. These isolates were transferred to fresh TSA slants and incubated overnight at 37°C and then gram stained. Only cultures appearing as gram-negative rods were retained for final biochemical confirmation.

Retained cultures were subjected to biochemical confirmation using the Vitek®, as described previously. Eighteen- to 24-h cultures of the confirmed pure *Salmonella* isolates were inoculated onto Protect™ Bacterial Preservers (Key Scientific Products, Round Rock, Tex.) and stored at -80°C. Protect™ is a sterile vial containing chemically treated porous beads stored in a cryopreservative fluid of tryptic soy broth and glycerol with a hypertonic additive. Each bead served as a carrier for the *Salmonella* culture during storage.

Serotyping of *Salmonella* isolates. Biochemically confirmed *Salmonella* isolates were transferred onto TSA slants, grown overnight at 37°C and shipped by overnight courier to the Diagnostic Bacteriology Laboratory of the National Veterinary Services Laboratory (NVSL) in Ames, Iowa for serotyping. Upon receipt at the laboratory, each isolate was logged in and transferred according to the NVSL Standard Operating Procedure, *Salmonella* serotyping – Logging in and transferring *(141)*. As

outlined in this procedure, each *Salmonella* isolate was transferred to Trypticase Soy Tryptose Broth (TST, NVSL media number 10120), a Nutrient Agar slant (NVSL media number 10220), and a blood agar base slant (BAB, NVSL media number 10008) and incubated at 37°C overnight. To the TST broth culture, saline containing 0.6% formaldehyde was added to adjust the cell density to match a MacFarland standard between 1 and 2. Using a pasteur pipette, approximately 1 ml of 0.85% sterile saline was used to wash the cells from the bottom 1/3 of the BAB slant culture into a 13 x 100-mm tube.

The determination of the O antigen was completed following the NVSL Standard Operating Procedure, *Salmonella* serotyping – Determination of O antigen *(140)*. Following this procedure, *Salmonella* O antisera (rabbit origin) was diluted to a working dilution with saline containing 0.5% phenol (NVSL medium number 30092). A drop of Group 18, B, C1, C2, D, and E antisera was placed on a glass slide. Approximately the same size drop of antigen (saline suspension of *Salmonella* cells from BAB slants) was also placed on the glass slide and mixed with the antisera using a sterile inoculating loop. The slide was rocked for 1 to 2 min and observed for agglutination, evident by the visible clumping of the cells. Positive (agglutination) and negative (no agglutination) reactions were recorded. If group B was positive, the single factor 5 was tested; if group C1 was positive, factors 7 and 14 were tested; if group C2 was positive, factors 6, 8, and 20 were tested; if group D was positive, factors 9 and 46 were tested; and if group E was positive, factors 10, 15, 19, and 34 were tested. If the antigen was negative in all 6

antisera (18, B, C1, C2, D, and E) the procedure described above was repeated using pools with higher O antisera.

The components of the positive pool were tested with the antigen, as described previously, to determine the O group. If the positive component of the pool was a complex antigen, such as 13, 23 or 6, 14, 24, the single factor antisera was tested to determine the O group. If the antigen was negative in all of the pools and also negative for the first 6 antisera tested, the antigen was tested for agglutination using a 1:500 solution of acriflavin (NVSL medium number 30002). If an antigen was agglutinated using acriflavin, the O antigen was reported as "rough". Smooth to rough variation occurs from a gradual, nonreversible process which alters the cell wall and causes the antigen to react in either many antisera or none.

An alcohol-killed antigen was prepared from antigens negative in all of the pools and the first 6 antisera and was tested in the grouping antisera. The alcohol-killed antigen was prepared by suspending the remaining *Salmonella* cells on the BAB slant in a 13 x 100-mm tube containing 1 ml of 95% ethyl alcohol. This suspension was heated in a 60°C water bath for 30 min and then centrifuged to help settle the antigen to the bottom of the tube. The alcohol was decanted and the antigen resuspended in 1 ml of saline with 0.5% phenol and filtered though cotton to repeat testing with O antisera. If the alcohol-killed antigen was again negative in all antisera, the isolate was streaked onto a Nutrient Agar plate and incubated overnight at 37°C. Two colonies were picked and transferred to BAB slants and TST broth and incubated overnight at 37°C and tested as done before. If there was still no O reaction and acriflavin was positive, then the

isolate was determined to be untypable, but, in this case, H (flagellar) reactions were still reported.

Following the identification of the O antigen, the H antigen was determined following the NVSL Standard Operating Procedure, *Salmonella* serotyping – Determination of H antigen *(139)*. Single H factors were selected from a list suggested by NVSL based on O antigen group reactions *(139).* After selecting the appropriate H antigens to test for, 0.02 ml of a 1:20 dilution of the antisera with saline containing 0.5% phenol was added to a 13 x 100-mm tube containing 1 ml of antigen. The tubes were incubated in a rack in a 50°C water bath for 0.5 to 1 h. After incubation, the tubes were gently shaken and observed for flocculent agglutination. Single H factors were selected from a list suggested by the NVSL *(139)* and tested using the procedures described previously. If there was no agglutination after 1 h, additional H antisera were tested as suggested by the NVSL *(139),* repeating the same procedure. If there was no agglutination after 1 h from any of these antisera, H pools or polyvalent antiserum were used to test the antigen. If the polyvalent antiserum was positive, then the antigen was tested in H pools. If one of the pools was positive, then all of the components of that pool were tested to determine the H antigen. If the antigen was negative in all of the H pools or polyvalent antisera after 1 h, then one of three procedures was followed: 1) specific biochemical tests were completed to definitively identify the *Salmonella* serotype as Pullorum or Gallinarum, both nonmotile serotypes; 2) complete a full set of biochemical tests to confirm if the isolate is *Salmonella*; or 3) perform a motility test to determine if the isolate is nonmotile or poorly motile, and if this was true, then the O

antigen was reported and “nonmotile” or “poorly motile” was reported. If the colonies were negative in both O and H antisera and biochemical tests indicated that isolate was *Salmonella*, then motility was improved by inoculating a tube of motility test medium and the H antigens were tested again.

DNA-based typing of *Salmonella* isolates. *Salmonella* isolates obtained from samples collected from various produce and environmental sources were subjected to DNA characterization using repetitive sequence-based polymerase chain reaction (Rep-PCR) and pulsed-field gel electrophoresis (PFGE) to evaluate the genetic diversity of the isolates and to determine if there were relationships between sources of contamination.

Repetitive sequenced-based polymerase chain reaction (Rep-PCR). Twenty-five *Salmonella* isolates were shipped to Bacterial BarCodes, Inc. (Houston, Tex.), a commercial laboratory specializing in using molecular technology, specifically Rep-PCR, for microbial typing and identification. *Salmonella* isolates were subjected, in duplicate, to the Rep-PCR technique using a Bacterial Barcodes repPRO™ DNA fingerprinting kit (Bacterial BarCodes). Samples were prepared and analyzed following protocols described in the repPRO™ DNA fingerprinting kit package insert and technical bulletins provided by Bacterial BarCodes, Inc.

DNA was isolated from *Salmonella* isolates using an Ultraclean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, Calif.). Extracted DNA was diluted in TE buffer (10 mM Tris · Cl, 1 mM EDTA; pH 8.0) and its concentration was determined using the following formula.

[DNA concentration (ng/µl) = A_{260} reading x conversion factor x dilution factor]

DNA purity was determined by measuring the absorbance of diluted DNA with a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, Calif.) using the ratio of A_{260}/A_{280} = 1.7 to 1.9. Ratios of less than 1.8 are indicative of protein contamination or residual solvents from the extraction process. Ratios of greater than 2.0 are indicative of RNA contamination in the sample. All samples were tested in duplicate using two primers, Uprime-B1 and Uprime-E. Master mix and primers were prepared according to the repPRO™ DNA fingerprinting kit package insert. A fresh PCR tube was set up for each duplicate *Salmonella* sample as well as for positive and negative controls. Master mix was mixed and centrifuged at 8,000 rpm for 15 s using a Quick-Spin Minifuge (ISC Bioexpress, Kaysville, Utah). Twenty-four microliters of master mix and 1 µl of genomic DNA (100 ng/µl) were added to each corresponding sample PCR tube and centrifuged at 2,000 rpm for 15 s using a Marathon 3000 centrifuge (Fisher Scientific, Pittsburgh, Pa.). After centrifuging, PCR tubes were inserted into an aluminum 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, Calif.) for amplifications using a specific program for each primer. Amplification products were separated using a Bio-Rad Electrophoresis Sub-Cell Model 192 with a 25 x 25-cm gel tray (Bio-Rad). Electrophoresis of gels was completed using Bio-Rad Power Pac 300 electrophoresis power supply (Bio-Rad) set at 120 V for 6 h. After the run was complete, the gel was exposed to ultraviolet (UV) light using a TFM-30 UV Transilluminator (ISC Bioexpress) and gel images were digitally captured using an AlphaImager™ Model 2200 (Alpha Innotech Corp., San Leandro, Calif.). Images

were analyzed using Bionumerics software program version 2.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) which compared the positions of bands between samples. A high number of matching bands, or bands with the same molecular weight, indicated that two samples were highly related or had a high degree of similarity. Parsimonious solutions were found using the Pearson correlation coefficient to express the degree of similarity between the fingerprints. The fingerprints generated for each of the 25 *Salmonella* isolates were grouped into clusters using unweighted pairwise grouping with mathematical averaging (UPGMA). Data generated from the gel images of both primers was combined to construct a composite dendrogram.

Pulsed-field gel electrophoresis (PFGE). Twenty-five *Salmonella* isolates were also analyzed by PFGE. PFGE procedures used were similar to those used by the Centers for Disease Control and Prevention as described in the *Compendium of Methods for the Microbiological Examination of Foods (60)*. For each *Salmonella* isolate, an isolated colony was streaked onto TSA plates for confluent growth. Using the same loop, a TSA slant was also inoculated to ensure that the same colony could be retested, if necessary. Cultures were incubated at 37°C for 14 to 18 h.

To prepare DNA plugs, a sterile, cotton tipped applicator (Harwood Products Co.) was used to transfer some growth from the inoculated TSA plate into a 12 x 75-mm polysterene tube (VWR Scientific) containing 2 ml of cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0; Sigma-Aldrich Corp. St. Louis, Mo.). The concentrations of the cell suspensions were adjusted to a 20% transmittance with a Vitek® colorimeter

(Hach Co., Loveland, CO). Two-hundred microliters of the adjusted cell suspensions were transferred to labeled 1.5-ml microcentrifuge tubes (VWR Scientific). Ten microliters (20 mg/ml) of GeneMate® Proteinase K (ISC Bioexpress) were added to each tube and mixed gently with the pipet tip. Two-hundred µl of melted plug agarose (1% Seakem® Gold agarose; Cambrex Bioscience, Rockland, Maine: 1% GeneMate® Sodium Dodecyl Sulfate; ISC Bioexpress), were cooled to 55 to 60°C and added to the 200-µl cell suspension. The mixture was mixed gently by pipetting the mixture up and down a few times and then was quickly dispensed into the appropriate, pre-labeled well of a CHEF Disposable Plug Mold (Bio-Rad). Plugs were allowed to solidify at room temperature for 10 to 15 min. At least 3 plugs were made using the amounts of cell suspension and agarose described previously.

Up to three plugs of the same *Salmonella* strain were lysed in the same 50-ml Falcon® polypropylene screw cap tube (Becton Dickinson, Sparks, Md.) containing a lysis buffer/enzyme solution. The lysis buffer/enzyme solution consisted of 5 ml of cell lysis buffer (50 mM Tris: 50 mM EDTA, Sigma Aldrich Corp.; pH 8.0 plus 1% Sarcosyl, Fisher Scientific) and 25 µl of Proteinase K solution (20 mg/ml). After ensuring that all the plugs were under the lysis buffer/enzyme solution, the tubes were placed in a 54°C Yamato Shaking Water Bath YB-521 (Fisher Scientific) for 2 h with constant, vigorous agitation (175 to 200 rpm).

After at least 2 h of cell lysis, the temperature of the shaker water bath was lowered to 50°C. The tubes were removed and the lysis buffer was poured out using a screened plug cap (Bio-Rad). Fifteen ml of sterile deionized water (pre-heated to 50°C)

were added to each tube and vigorously shaken in the shaker water bath for 10 to 15 min. Using the screened plug cap, water was poured from the plugs and the wash step repeated following the protocol described previously. After 2 water washes, the plugs were washed 4 additional times (15 min of vigorous shaking per wash) using 15 ml of sterile TE buffer (ISC Bioexpress) pre-heated to 50°C. After pouring off the last wash, 5 ml of sterile TE buffer were added to each tube and the plugs were stored at 4°C until proceeding with the restriction digestion.

For restriction digestion, a sterile spatula was used to carefully remove a plug from the TE buffer and place it on a sterile disposable Petri dish (VWR Scientific). Using a sterile scalpel, a 2.5- to 3.0-mm wide slice of the plug was transferred to a 1.5-ml microcentrifuge tube (VWR Scientific) with 200 µl of 1X H buffer diluted from 10X H buffer (Roche Molecular Biochemicals, Indianapolis, Ind.) using nuclease-free water (Ambion, Austin, Tex.). Samples were incubated in a MagniWhirl® constant temperature bath at 37°C for 5 to 10 min. Following this pre-restriction incubation, a pipet was used to remove the buffer. A restriction enzyme mixture was prepared by combining 175 µl of nuclease free water/plug slice, 20 µl of 10X H buffer/plug slice, and 5 µl of X*ba*I restriction endonuclease/plug slice (10 U/µl; Roche Molecular Biochemicals). Two-hundred µl of the restriction enzyme mixture were added to each tube containing a plug slice. Each tube was gently finger-vortexed and incubated in a MagniWhirl® constant temperature bath at 37°C for 4 h to overnight.

Approximately 1 to 2 h before the restriction digest reaction was finished, the electrophoresis gel was cast. The electrophoresis gel was prepared from 1% Seakem®

Gold agarose (SKG) in 0.5X Tris-Borate EDTA (TBE) buffer, prepared from 10X TBE buffer (Sigma-Aldrich). For the 14-cm-wide-gel form (15 wells), 1.0 g SKG was mixed with 100 ml 0.5X TBE and heated in the microwave for 60 s, mixed gently and repeated for 15-s intervals until the agarose was completely melted. The agarose was cooled to 50 to 55°C before casting into the leveled casting stand provided with the CHEF Mapper® XA Pulsed-Field Electrophoresis System (Bio-Rad). The gel was allowed to harden for 30 to 45 min before carefully removing the comb. Restricted plugs were removed from the water bath and the enzyme/buffer mixture removed by pipet and replaced with 200 µl of 0.5X TBE and incubated at room temperature for 15 min. A 2.5- to 3.0-cm wide slice of a Lambda DNA molecular size standard (Bio-Rad) was loaded into lanes 1, 8, and 15 of the gel and the remaining lanes were loaded with the samples. After loading, the wells of the gel were filled with melted 1% SKG.

The gel was carefully placed inside the electrophoresis chamber containing 2.2 L of freshly prepared 0.5X TBE buffer maintained at 14°C by the cooling module and circulating at a flow rate of approximately 1 L/min (a setting of approximately 70 on the pump regulator). Restriction fragments of DNA were separated using the CHEF Mapper® XA system programmed for an 18-h run time (initial switch time, 2.20 s; final switch time, 63.80 s) at 6 V/cm. After the run was completed, the gel was stained for 30 min in 500 ml of a 0.5µl/ml solution of ethidium bromide (Sigma-Aldrich) and destained in 500 ml of deionized water for 60 to 90 min; changing the water every 20 min. Gels were exposed to UV light using an Ultra Lum™ Electronic UV Transilluminator (Ultra-Lum Inc., Carson, Calif.) and gel images were digitally captured using a Kodak® DC

120 camera (Eastman Kodak Co., Rochester, N.Y.). Gel images were analyzed using Bionumerics software program version 2.0 (Applied Maths). Similarity between isolate fingerprints was determined on the basis of the Dice correlation coefficient. A band position tolerance of 1% was used for the analysis of PFGE patterns. Dendrograms were generated by the unweighted pairwise grouping with mathematical averaging (UPGMA). Capital letters (A to L) were used to designate main cluster lineages of the *Salmonella* isolates in the dendrogram, while subclusters were given numerical suffixes.

Antibiotic resistance profiling of *Salmonella* isolates. The susceptibility of *Salmonella* isolates to various antibiotics was tested using the disk diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS). Antibiotics tested were selected based on the recommendations by the NCCLS and a review of the current literature regarding the antibiotic resistance of *Salmonella (6, 64, 137, 162)*. The antibiotics (BBL® Sensi-Disc®, Becton Dickinson) tested and their corresponding doses tested were as follows: amikacin 30 µg, amoxicillin/clavulanic acid 20/10 µg, ampicillin 10 µg, cefazolin 30 µg, cefoxitin 30 µg, ceftriaxone 30 µg, cephalothin 30µg, chloramphenicol 30 µg, ciprofloxacin, 5 µg, gentamicin 10 µg, kanamycin 30 µg, nalidixic acid 30 µg, streptomycin 10 µg, tetracycline 30µg, and trimethoprim/sulfamethoxazole 1.25/23.75 µg. Ampicillin, chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole, and tetracycline were tested as part of the R-type ACSSuT resistance pattern commonly associated with multidrug-resistant *S.* Typhimurium definitive type 104 (DT104) *(162)*. Fluoroquinolones (e.g., ciprofloxacin)

and broad and expanded-spectrum cephalosporins (e.g., cephalothin and ceftriaxone, respectively) were tested as they are commonly used for antibiotic treatment for invasive *Salmonella* infections in humans *(6, 64)*. All antibiotics tested are part of the list of antimicrobials monitored for resistance to *Salmonella* by the National Antimicrobial Resistance Monitoring System - Enteric Bacteria (NARMS) of the Antimicrobial Resistance Research Unit *(67)*.

Each antimicrobial was tested in duplicate following the procedures described in the NCCLS Performance standards for antimicrobial disk susceptibility tests; approved standard – 7th edition *(138)*. Each *Salmonella* isolate tested was streaked to a TSA plate and incubated overnight at 37°C. From each plate, 3 to 5 well-isolated colonies were transferred into a tube containing 5 ml of tryptic soy broth (TSB, Difco). The broth culture was then incubated at 37°C until the culture achieved or exceeded the turbitity of the 0.5 McFarland standard. A sterile cotton tipped applicator (Harwood Products Co.) was dipped into the broth inoculum and rotated several times and pressed firmly on the inside wall of the tube to remove excess inoculum from the swab. The swab was then used to inoculate the dried surface of a 150-mm Mueller-Hinton Agar (Difco) plate by streaking the swab over the entire agar surface. This streaking procedure was repeated by streaking the plate 2 more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. After streaking the entire plate, the rim of the agar was swabbed and the plate set aside for 3 to 5 min to allow for any excess surface moisture to be absorbed before applying the drug-impregnated disks. A second cotton tipped applicator was used to inoculate a 100-mm Mueller-Hinton Agar plate using the

procedure described previously. Reference strains *E. coli* American Type Culture Collection® (ATCC®) 35128 and *E. coli* ATCC® 25922 were used as quality control strains and were tested using the same procedures as the *Salmonella* isolates. NCCLS recommended *E. coli* ATCC® 35218 as a control organism for the β-lactam and β-lactamase inhibitor combinations, such as those containing clavulanic acid and *E. coli* ATCC® 25922 to monitor the remaining antibiotic-impregnated disks that did not contain these inhibitors.

The antimicrobial disks were dispensed onto the inoculated agar plates using BBL® Sensi-Disc 12-place and 6-place Self-Tamping Dispensers (Becton Dickinson) making sure each disk was pressed down to ensure complete contact with the agar surface. The plates were inverted and incubated at 37°C within 15 min after applying the disks. After 16 to 18 h of incubation, each plate was examined. The diameters of the zones of complete inhibition, including the diameter of the disk, were judged visually and measured using a Scienceware® Digi-max, caliper, slide with LCD readout (Bel-Arts Products, Pequannock, N.J.). The zone margin of inhibition was defined as the area showing no obvious, visible growth that could be detected with the unaided eye.

The sizes of the zones of inhibition were interpreted by referring to Table 2A (Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae) of the NCCLS Performance standards for antimicrobial susceptibility testing; twelfth informational supplement *(137)*. *Salmonella* isolates were reported as susceptible, intermediate, or resistant to the antibiotic tested.

Transformation of *Salmonella* to express enhanced green fluorescent protein (EGFP). Three *Salmonella* strains were transformed to express EGFP for use in attachment and survival studies on fresh parsley. Transformed strains included *S.* Javiana isolated from the surface of a cantaloupe, *S.* Rubislaw isolated from irrigation water, and *S.* Anatum isolated from irrigation water. The plasmid vector EGFP (pEGFP; 3.4 kb; Clontech Laboratories, Inc., Palo Alto, Calif.) which carries a red-shifted variant of wild-type GFP was inserted into the *Salmonella* strains using either a calcium chloride method or electroporation. The plasmid vector pEGFP encodes a variant of the *A. victoria* GFP that has been optimized for brighter fluorescence and higher expression. The EGFP gene was inserted in the frame with the *lacZ* initiation codon from pUC19, an *E. coli* cloning vector, so that the protein is expressed from the *lac* promoter. The donor (pUC) backbone also contains an ampicillin resistance gene for propagation and selection.

S. Javiana was transformed using a variation of the calcium chloride method as described by Sambrook and Russell *(168)*. *S.* Javiana was streaked onto a Luria-Bertani (LB, Fisher Scientific) plate and incubated for 16 to 20 h at 37°C. A single colony (2 to 3 mm in diameter) was picked from this plate and transferred into 100 ml of LB broth in a 1-L flask. This culture was incubated for approximately 3 h at 37°C in a Classic C76 Water Bath Shaker (New Brunswick Scientific Co., Edison, N.J.) shaking at 250 rpm. The density of the culture was monitored using a Bausch and Lomb Spectronic 20 Colorimeter-Spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.) and cells were harvested when the optical density (OD_{600}) of the culture reached 0.35 which was

equivalent to approximately 10^8 CFU/ml. The culture was transferred to ice-cold, sterile, disposable Corning® 50-ml polypropylene centrifuge tubes (Corning, Inc., Corning, N.Y.). Cultures were cooled by storing the centrifuge tubes on ice for 10 min. Cells were then recovered by centrifugation at 2,700 x *g* for 10 min at 4°C using an Eppendorf® Refrigerated Multipurpose Centrifuge Model 5804R (Brinkmann Instruments, Inc., Westbury, N.Y.). The supernatant was decanted from the cell pellet and the pellet was gently resuspended by swirling in 20 ml of ice-cold $MgCl_2$ solution (80 mM $MgCl_2$, Sigma Aldrich Corp.; 20 mM $CaCl_2$, Fisher Scientific). The supernatant was decanted from the pellet again and the pellet was gently resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. Competent cells were dispensed immediately in 200-µl aliquots in pre-chilled 1.5-ml microcentrifuge tubes using chilled micropipette tips and stored immediately at -80°C.

$CaCl_2$-treated (competent) *S.* Javiana cells and the plasmid vector (pEGFP) were thawed on ice for 10 to 15 min. Two-hundred µl of competent cells were transferred to sterile, chilled 17 x 100-mm polypropylene tubes (VWR) using a chilled micropipette tip. Ten µl (50 ng) of pEGFP vector were added to each tube and mixed by gently swirling. Tubes were stored on ice for 30 min. Tubes containing cells/DNA mixture were placed in a rack in a pre-heated 42°C MagniWhirl® constant temperature bath for exactly 90 s to heat shock the cells. Tubes were then immediately transferred to an ice bath and allowed to chill for 1 min. One ml of SOC medium (2% tryptone peptone, Difco; 0.5% yeast extract, Difco; 10 mM NaCl, EMD™ Chemicals, Inc., Gibbstown, N.J.; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$, Sigma-Aldrich; and 20 mM glucose,

Difco) was transferred into each tube. Cultures were incubated in a Classic C76 Water Bath Shaker at 37°C at 100 rpm for 45 min to allow the bacteria to recover and express the ampicillin resistance marker. One hundred µl and 10 µl were surface plated onto LB agar supplemented with ampicillin (100 µg/ml, Sigma-Aldrich) (LBA-amp) and 200 µg/ml Novagen® isopropyl thiogalactoside (IPTG; Novagen®, Madison, Wis.). Plates were incubated for 12 to 16 h at 37°C and examined for fluorescing colonies using UV light (365 nm; Model UVGL-58, UVP Inc., San Gabriel, Calif.). Transformed colonies were picked and transferred into LB broth containing ampicillin (100 µg/ml) (LBB-amp) and incubated for 12 to 16 h and then streaked onto LBA-amp and incubated again for 12 to 16 h at 37°C. Transformed colonies were inoculated onto Protect™ Bacterial Preservers.

Due to the inability to transform *S.* Rubislaw and *S.* Anatum using the calcium chloride method, these isolates were transformed to express EGFP using electroporation. Electroporation was completed using a MicroPulser™ electroporator (Bio-Rad). Electrocompetent *Salmonella* cells were prepared following procedures in The MicroPulser™ Electroporation Apparatus Operating Instructions and Applications Guide (Bio-Rad) and methods described by O'Callaghan and Charbit *(145)*. For each *Salmonella* isolate, 100 ml of LB broth was inoculated with 1/100 volume of 12 to 16 h culture. The culture was grown at 37°C at 250 rpm in a Classic C76 Water Bath Shaker to an OD_{600} of approximately 0.6 to 0.7. The culture was transferred into two ice-cold, sterile, disposable Corning® 50-ml polypropylene centrifuge tubes (VWR Scientific) and allowed to chill in ice for approximately 20 min before centrifuging. Cultures were

centrifuged at 4,000 x *g* for 15 min at 4°C using an Eppendorf® Refrigerated Multipurpose Centrifuge Model 5804R (Brinkmann Instruments, Inc., Westbury, N.Y.). Following careful decanting of the supernatant, the pellet was gently resuspended by swirling in 25 ml of sterile, ice-cold 10% glycerol (Sigma-Aldrich). Cells were centrifuged again at 4,000 x *g* for 15 min at 4°C. Cells were centrifuged and resuspended in 25 ml of ice-cold 10% glycerol in the same manner three additional times. Following the last centrifuging, the supernatant was decanted and the cells were very gently resuspended in 1 ml of ice-cold 10% glycerol. Electrocompetent cells were dispensed immediately in 100-µl aliquots in pre-chilled 1.5-ml microcentrifuge tubes using chilled micropipette tips and stored immediately at -80°C.

Electrocompetent cells and pEGFP plasmid vector were thawed on ice. For each sample that was electroporated, a 1.5-ml microcentrifuge tube and a 0.1 cm electroporation MicroPulser™ 0.1 cm gap cuvette (Bio-Rad) were placed on ice. In an ice-cold microcentrifuge tube, 40 µl of the cell suspension were mixed with 2 µl of DNA. The cell/DNA suspension was gently mixed by pipetting up and down and allowed to chill on ice for approximately 1 min. The cell/DNA suspension was then transferred into a cold electroporation cuvette (0.1 cm gap cuvette) without creating bubbles and tapped gently to move the suspension to the bottom of the cuvette. The cuvette was then placed into the chamber slide and pulsed once with 1.8 kV. The cuvette was quickly removed from the chamber and 1 ml of pre-warmed SOC medium was immediately added to the cuvette. Cells were very gently resuspended using a pasteur pipette and transferred to a 17 x 100-mm polypropylene tube and incubated at

37°C for 1 h at 200 rpm in a Classic C76 Water Bath Shaker. After incubation, cell suspensions were surface plated onto LB agar. One hundred µl and 10 µl were surface plated onto LBA-amp supplemented with 200 µg/ml IPTG. Plates were incubated for 12 to 16 h at 37°C and examined for fluorescing colonies using UV light (365 nm). Transformed colonies were picked and transferred into LBB-amp and incubated for 12 to 16 h and then streaked onto LBA-amp and incubated again for 12 to 16 h at 37°C. Transformed colonies were inoculated onto Protect™ Bacterial Preservers and stored at -80°C.

Determination of stability of *Salmonella*/pEGFP strains. Transformed *Salmonella* strains maintained at -80°C were transferred to 10 ml LBB-amp containing and incubated in a Classic C76 Water Bath Shaker at 37°C for 12 to 16 h with shaking (200 rpm). Using a sterile, cotton-tipped applicator a portion of each liquid culture was applied onto LBA-amp plates. The culture was then streaked for isolation using a sterile loop. Inoculated LBA-amp plates were incubated at 37°C and examined for fluorescing colonies using UV light (365 nm). A fluorescent colony was picked from each plate and used to inoculate a fresh LBA-amp plate. This was repeated for 10 days to observe whether the *Salmonella* colonies maintained and/or continued to express the pEGFP plasmid.

Additionally, LBA-amp plates inoculated with each of the transformed *Salmonella* strains were incubated at 37°C for 12 to 16 h and, following that incubation period, the plates were transferred to 4°C storage and observed periodically over a 2-

month period to monitor whether the recombinant *Salmonella* strains continued to express the green fluorescence.

Growth comparison of transformed *Salmonella* versus parent strains. The growth kinetics of the parent and recombinant strains of *Salmonella* were studied by constructing growth curves in order to ensure that the GFP expression did not affect the growth of the transformed *Salmonella* cultures. Growth was measured by enumerating viable counts of cells using plate counts of the liquid culture after 0, 1, 2, 3, 4, 6, 8, 12, and 24 h of incubation at 37°C.

For each of the three parent strains and their respective transformed strains, 10-ml aliquots of LB broth and LBB-amp were inoculated with 1% (vol/vol) of an overnight culture (18 to 24 h at 37°C) and allowed to incubate for 18 to 24 h at 37°C. This culture was then diluted with sterile 0.1% sterile peptone water to an approximate final concentration of 10^6 CFU/ml. One-tenth ml of the adjusted culture was dispensed into 100-ml aliquots of LB broth in 500-ml Erlenmeyer flasks when testing the parent strains or LBB-amp when testing the transformed strains. The flasks were incubated at 37°C in a Classic C76 Water Bath Shaker (200 rpm) and at each time increment (0, 1, 2, 4, 6, 8, 12, and 24 h), 5 ml were removed from each flask and plated onto LB agar for parent *Salmonella* strains and LBA-amp for transformed *Salmonella* strains. Following incubation at 37°C for 24 h, plates were counted.

Attachment of *Salmonella*/pEGFP to parsley. The attachment, survival, and growth of *Salmonella*/pEGFP on parsley as affected by processing and storage were examined. Following harvesting, parsley, along with other leafy greens, are commonly submerged in a wash tank to remove both field heat and visible soil contamination. Following this step, there may or may not be a spray wash. Should a human pathogen be introduced into the wash tank, there may be ample opportunity for product to be inoculated through commingling. Parsley bunches were dipped in inoculum to simulate a contaminated wash tank to examine the contamination, attachment, growth, and survival of *Salmonella* on parsley.

Preparation of parsley and inoculum. Three cases of Italian or flat leaf parsley (*Petroselinum crispum* var. *neapolitanum*) were obtained from a packing shed. Parsley had been harvested within 24 h and had been subjected to a wash consisting of a dip in water chlorinated at a level of 5 ppm (city water supply). Upon arrival at the laboratory, bunches were placed onto aluminum foil, and retied into 10-g bunches. Five cm of the stem end were removed using scissors sterilized by flaming with 95% alcohol. Parsley bunches were stored at 4°C in sterile plastic tubs prior to treatments.

A 3-strain inoculum was prepared from cultures of *S.* Anatum (isolated from irrigation water), *S.* Rubislaw (isolated from irrigation water), and *S.* Javiana (isolated from cantaloupe) that were previously transformed to express enhanced green fluorescent protein (EGFP). When preparing the inoculum, each strain was grown individually in 10 ml of LBB-amp and incubated overnight with shaking (200 rpm) at

37°C in a water bath shaker. Ten ml of each *Salmonella* culture were transferred to a 15-ml centrifuge tube and centrifuged 3 times (2,000 x *g*; 10 min; 21°C) with sterile 0.1% peptone water. Following the final wash, the cells were resuspended in 10 ml of sterile 0.1% peptone water to yield a final concentration of 10^9 CFU/ml with and OD_{600} approximately equal to 0.7. Equal volumes of each of the 3 *Salmonella*-pEGFP strains were combined. Eight ml of the 3-strain suspension were transferred to 8 L of sterile 0.1% peptone water pre-equilibrated to 5°C, 25°C, or 35°C in a 54 x 43.5 x 13-cm Nalgene® polypropylene sterilizing pan (VWR) to create an 8-L dip suspension (inoculum) with a cell density of approximately 10^5 CFU/ml. The cell density of the dip suspension was confirmed by surface plating appropriate decimal dilutions onto LBA-amp plates.

Processing and storage. Twelve 10-g parsley bunches (pre-equilibrated to 25°C) were placed in a sterile basket made from 19 gauge galvanized wire and then submerged in the 8-L 3-strain dip suspension pre-equilibrated to 5°C, 25°C or 35°C for 3 or 15 min and rotated approximately every 1.5 min using sterile forceps. After dipping, the parsley was shaken gently to remove excess inoculum and allowed to stand for 1 h to allow the excess inoculum to drain off, as well as allow the leaves to dry, before sampling or storing at 4°C or 25°C. Parsley samples were stored in oxygen permeable Sysco® clear plastic, 21.1-cm x 21.1-cm x 7.3-cm, 1 compartment, hinged containers (Sysco, Bryan, Tex.). Because parsley has such a high respiration rate (22 to 38 mg CO_2 kg^{-1} h^{-1} at 5°C; 176 to 221 CO_2 kg^{-1} h^{-1} at 20°C), 6 additional holes were created in the containers to

allow ample oxygen transmission. Samples were analyzed on the day of inoculation (day 0) and after 1, 7, 14, and 30 days of storage at 4°C and 25°C. For 4°C storage, containers with parsley were placed in a low temperature incubator (Fisher Scientific) in a manner allowing uniform air flow between containers. For 25°C storage, containers of parsley were placed in a closed, metal storage cabinet. Ten-g parsley bunches not exposed to inoculum were sampled on day 0, and after 1, 7, 14, and 30 days of storage at 4°C and 25°C to serve as the controls.

Microbiological analyses. Loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella* were enumerated using the following procedures. To enumerate loosely attached and/or associated *Salmonella*, a 10-g bunch of parsley was added to 90 ml sterile 0.1% peptone water in a sterile (17.8 x 27.9-cm) Whirl-Pak® bag and inverted 25 times. Bacteria in the peptone rinse were enumerated by surface plating appropriate decimal dilutions onto LBA-amp plates. To enumerate the strongly attached and/or associated bacteria, the peptone rinse was decanted and an additional 90 ml sterile 0.1% peptone water was added to the 10 g of parsley and pummeled in a Tekmar® Stomacher Lab-Blender 400 for 2 min. The resulting peptone rinse was surface plated onto LBA-amp plates as described above. LBA-amp plates were incubated at 37°C for 16 to 24 h and following incubation, plates were counted by examining under UV light (365 nm).

A procedure employed by Buchanan et al. *(34)* to study the infiltration of *E. coli* O157:H7 into apples was used in this study to enumerate the internalized and/or

entrapped *Salmonella* cells. A 10-g bunch of parsley was surface sanitized by immersion in 2.0 L of a 2,000-mg/L sodium hypochlorite solution (22°C) for 3 min followed by draining and immersion in sterile distilled water for 2 successive 1-min rinses. The 2,000-mg/L sodium hypochlorite solution was prepared by adding 66.7 ml of commercial unscented bleach (6.00% sodium hypochlorite; Clorox® Co., Oakland, Calif.) to 1933.3 ml of sterile distilled water. The pH of the chlorine solution was adjusted to 6.9 ± 0.1 pH unit using a pH meter (model 612; Markson Science, Inc., Henderson, N.C.). The effectiveness of the chlorine decreases with increasing pH and at a pH close to neutral (pH 7.0) approximately 75-80% of chlorine is in the form of hypochlorous acid which is the form with the strongest antibacterial activity *(155)*. The 10-g parsley bunch was then placed in a stomacher bag with 90 ml sterile 0.1% peptone water and pummeled for 2 min. Appropriate decimal dilutions of the homogenate were then surface plated onto LBA-amp plates and incubated at 37°C for 16 to 24 h. Following incubation, fluorescent colonies were identified and counted by examining the plates under UV light (365 nm).

To evaluate the background microflora, aerobic plate counts as well as yeast and mold counts were determined for the control samples. Ten g of parsley were placed in a stomacher bag with 90 ml of sterile 0.1% peptone water and pummeled for 1 min. The decimal dilutions of the homogenate were plated onto Petrifilm™ Aerobic Count Plates (3M™ Microbiology Products) and Petrifilm™ Yeast and Mold Count Plates (3M™ Microbiology Products). Petrifilm™ Aerobic Count Plates were incubated at room temperature (25°C) for 48 h. Following incubation, all colonies were counted.

Petrifilm™ Yeast and Mold Count Plates were incubated at 22°C for 72 ± 3 h, yeasts were identified as small colonies with defined edges, pink-tan to blue-green in color, usually a raised appearance, and usually without a focus (dark center in the middle of the colony). Molds appeared as large, flat colonies with diffuse edges, having variable color and, usually with a focus in the center of a colony. In addition, samples were surface plated onto LBA-amp plates to determine if any of the background flora had the ability to grow and fluoresce on the LBA-amp media thus damaging the validity of the *Salmonella*-pEGFP counts.

Visualization of Salmonella*/pEGFP on parsley.* Four leaf sections (0.5-cm x 0.5-cm) obtained from samples from Day 0, 1, and 7 treatment groups were placed on a Rite-On® microslide (GoldSeal® Products, Portsmouth, N.H.) slide and a cover slip (VWR Scientific) was gently placed over the leaf sections. Each coverslip was secured by applying clear nail polish to the edge of the cover slip. Slides were transported to the Image Analysis Laboratory at the College of Veterinary Medicine at Texas A&M University, College Station, Tex., where they were observed using a Bio-Rad Radiance 2000 Scanning Laser Confocal Microscope (Bio-Rad) equipped with an Krypton/Argon ion lasers interfaced with a fully equipped Nikon T300 inverted microscope. EFGP-expressing *Salmonella* cells were detected using an excitation wavelength of 488 nm and observed using the 60x oil immersion objective. Images were obtained and recorded of fields containing cells in which the leaf structure was also apparent. Images were captured using the Lasersharp Capture Software (Bio-Rad).

Isolation and identification of parsley microflora. Enumeration of lactic acid bacteria, yeasts and molds, mesophilic and psychrotrophic bactera, as well as the development of a microbial type distribution, were completed on Italian (plain leaf) parsley collected from 3 stages of production (field, packing shed, and retail). Three bunches (approximately 50 g per bunch) were collected from 3 locations, including the field, after processing in the packing shed, and the retail environment. Parsley samples were collected in bunches using a gloved hand, slicing the stems with a disposable plastic knife. Each bunch was transferred to a sterile (17.8 x 27.9-cm) Whirl-Pak® bag. After receipt at the laboratory, using a sterile scalpel and gloved hands, leaves were separated by slicing through the petiole ≤ 1 cm below the base of the leaf. Three 20-g samples of leaves from each of the 3 sampling locations were placed in sterile (17.8 x 27.9-cm) Whirl-Pak® bags. Two-hundred ml of sterile 0.1% peptone water were added to each 20-g sample and then pummeled for 1 min in a Tekmar® Stomacher Lab-Blender 400 before pipetting analysis.

To enumerate lactic acid bacteria, decimal dilutions of the homogenate were surface plated onto acidified MRS agar (de Man Rogosa Sharpe) prepared from Lactobacillus MRS broth (55 g/L; Difco) and Bacto™ agar (15 g/L; Difco). MRS agar was acidified to pH 5.5 ± 0.1 with sterile glacial acetic acid (Birko Corp., Henderson, Colo.) *(135)*. To the MRS agar, 0.01% 2,3,5-triphenyltetrazolium HCl (TTC; United States Biochemical Corp., Cleveland, Ohio) was also added to favor the enumeration of lactic acid bacteria commonly associated with plants *(82)*. Plates were incubated anaerobically at 35°C for 72 ± 3 h using a BBL™ GasPak™ jar (Becton Dickinson) with

a palladium catalyst (Becton Dickinson) and a GasPak™ anaerobic indicator (Becton Dickinson).

Yeasts and molds were enumerated by plating appropriate decimal dilutions of the homogenate onto Petrifilm™ Yeast and Mold Count plates. Following incubation at 22°C for 72 ± 3 h, yeast and mold colonies were counted. On Petrifilm™ Yeast and Mold Count plates, yeasts were identified as small colonies with defined edges, pink-tan to blue-green in color, usually a raised appearance, and usually without a focus (dark center in the middle of the colony). Molds appeared as large, flat colonies with diffuse edges, having variable color and, usually with a focus in the center of a colony.

Homogenate was also surface plated in duplicate onto TSA agar to enumerate mesophiles, psychrotrophs, as well as to complete a type distribution for both of these microbial populations on parsley collected from different points of production. One set of TSA plates was incubated at 35°C for 48 h to examine the mesophilic population, while the second set of plates was incubated at 7°C for 10 days to study the psychrotrophic population. From both sets of plates (35°C and 7°C), for each sample, each different colony type on the countable plate (25 to 250 colonies) was described in detail (considering pigment, size, shape, texture, etc.) and enumerated. One colony of each described colony type was carefully picked using a sterile inoculating loop, transferred to a TSA slant and incubated at 35°C for mesophilic bacteria or at room temperature (22°C) for psychrotrophic bacteria for 24 h (up to 48 h for slow-growing organisms). Each isolate was gram stained and tested for the production of catalase and oxidase using the materials and procedures for these tests as described previously. All

isolates that were gram-positive rods were examined for spores by staining. A small amount of the culture was mixed with a drop of deionized water on a microscope slide and allowed to air dry. After drying completely, the slide was flooded with a 5.0% (w/v) aqueous solution of malachite green (Allied Chemical Corp., New York, N.Y.) and stained for 10 min. Following a thorough rinse with deionized water, the slide was counterstained with a 0.5% aqueous solution of safranin (Biochemical Sciences, Inc., Swedesboro, N.J.) for 15 s. The slide was rinsed thoroughly with deionized water and blotted dry with bibulous paper (Scientific Products, Evanston, Ill.). Cells were observed at 100x using a compound microscope. Using this procedure, bacterial cells stained red and spores stained green *(169).*

After completion of these initial tests, isolates were categorized as gram-negative, gram-positive, presumptive *Bacillus* spp., and yeasts. Further biochemical identification was completed using the Industrial Vitek® automated *in vitro* testing system. Isolates were streaked onto plates containing TSA plus 5% sheep's blood and incubated at 35 to 37°C for 24 ± 2 h. Two to 3 colonies were selected from each plate with a sterile cotton tipped applicator and suspended in 2 ml of 0.45 % sodium chloride solution in a 12 x 75-mm sterile, disposable clear polystyrene culture tube. Using a Vitek® colorimeter, the turbidity of each suspension was adjusted to transmittance appropriate for the Vitek® Test Card for the isolate. Vitek® Test Cards used in this study and their respective required cell suspension transmittance levels (or McFarland standards) were as follows: GNI+ Cards (Gram-Negative Plus), 67 to 77% (1.0 McFarland); GPI Cards (Gram-Positive Identification), 80 to 88% (0.5 McFarland);

BAC Cards (Bacillus Identification), 80 to 88% (0.5 McFarland); YBC Cards (Yeasts), 46-56% (2.0 McFarland). Each individual cell suspension was inoculated onto the appropriate test card using the Industrial Vitek® vacuum chamber and the cards were read and results interpreted by the Industrial Vitek® automated *in vitro* testing system. Verification of questionable isolates and determination of isolates unable to be confirmed using the Vitek® system was performed using standard biochemical tests following the identification schemes described by Vanderzant and Nickelson *(193)* and additional texts *(68, 92, 169)*. Percentage distribution of microbial types and the frequency of occurrence of each type within the total number of isolates were calculated.

Statistical analyses. Microbiological plate count data were converted to $\log_{10}$ CFU/ml or cm^2 or gram before analysis. Data were analyzed using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, N.C.). The Bonferroni least significant difference method was used to determine whether there were significant differences ($P < 0.05$) between means.

RESULTS AND DISCUSSION

Prevalence of *Salmonella* on produce and in the growing and processing environment. Prevalence of *Salmonella* on samples collected from cantaloupe, oranges, and parsley, as well as from the environment are presented in Table 2. A total of 25 *Salmonella* isolates were collected from 1,257 samples. Sixteen, 6, and 3 isolates were obtained from irrigation water, packing shed equipment, and washed cantaloupe, respectively. Of all the types of samples collected, *Salmonella* was most commonly isolated from irrigation water. Within the types of irrigation water sources sampled (canal, furrow, reservoir, river, and well), *Salmonella* was detected more frequently in samples obtained from reservoirs followed by dirt canals, furrows, and cement canals. *Salmonella* was not detected in any of the samples collected from the river (Rio Grande); however, the majority of irrigation water supplying all sources originates from the Rio Grande. Irrigation water samples were collected from the edge of the source at the water-soil or water-cement interface. Geldreich and Bordner *(80)* reported that the water-soil interface of an irrigation ditch can be a reservoir for fecal pollution.

Six of the total 25 or 24% of the *Salmonella* isolates were obtained from swab samples of equipment in the packing shed, as described in Table 2. Five of these isolates were obtained from equipment surfaces that were exposed to washed product not intended for any further processing before boxing. Parish and Higgins *(153)* reported that total microbial counts increased on commercially prepared grapefruit sections during processing, indicating a buildup of contamination on the processing equipment. Detection of *Salmonella* in the processing environment indicates a need for improved

TABLE 2. *Prevalence of* E. coli *and* Salmonella *on cantaloupe, oranges, and parsley, as well as in the growing and processing environment.*

Source	*E. coli*[a]		*Salmonella*[a]	
Commodity (Field)				
Cantaloupe	13/100	(13.0)	0/100	(0.0)
Oranges	0/100	(0.0)	0/100	(0.0)
Parsley	1/100	(1.0)	0/100	(0.0)
Commodity (Shed)				
Cantaloupe	21/100	(21.0)	3/100	(3.0)
Oranges	6/100	(6.0)	0/100	(0.0)
Parsley	3/100	(3.0)	0/100	(0.0)
Environment				
Irrigation water	67/170	(39.4)	16/170	(9.4)
canals (cement)	3/50	(6.0)	2/50	(4.0)
canals (dirt)	15/30	(50.0)	4/30	(13.3)
furrow	15/20	(75.0)	2/20	(10.0)
reservoir	15/30	(50.0)	8/30	(26.7)
river	9/30	(30.0)	0/30	(0.0)
well	10/10	(100.0)	0/10	(0.0)
Wash water	2/165	(1.2)	0/165	(0.0)
Packing ice	0/30	(0.0)	0/30	(0.0)
Equipment	26/280	(9.3)	6/280	(2.1)
Boxing ramp	1/30	(4.0)	2/30	(6.7)
Conveyors				
cups	0/5	(0.0)	0/5	(0.0)
belts	5/70	(7.1)	1/70	(1.4)
rollers	2/20	(10.0)	2/20	(10.0)
Dump tank auger	0/5	(0.0)	0/5	(0.0)
Hands	0/10	(0.0)	0/10	(0.0)
Harvest bag/plastic	2/5	(40.0)	0/5	(0.0)
Harvest baskets/plastic	0/5	(0.0)	0/5	(0.0)
Harvest bins/plastic	1/40	(2.5)	0/40	(0.0)
Harvest bins/wood	0/5	(0.0)	0/5	(0.0)
Ice chutes	0/20	(0.0)	0/20	(0.0)
Plastic packing crates	0/5	(0.0)	0/5	(0.0)
Railguard (boxing)	0/5	(0.0)	0/5	(0.0)
Receiving hopper	2/10	(20.0)	0/10	(0.0)
Trailer	7/20	(35.0)	0/20	(0.0)
Unloading ramp	6/15	(40.0)	1/15	(6.7)
Wall	0/10	(0.0)	0/10	(0.0)
Soil	1/12	(8.3)	0/12	(0.0)
Overall	140/1257	(11.1)	25/1257	(2.0)

[a] Number of positive samples, percent samples with detectable numbers is in parentheses.

cleaning and sanitation protocols, as this human pathogen could be transferred to the final product. Birds and rodents are known to harbor *Salmonella*, which may be transmitted to equipment through their feces *(212)*. A large population of birds was observed in the vicinity of one of the packing sheds where *Salmonella* was isolated from equipment. The construction of the shed allowed for bird infestation and which, although not verified, may have been the source of the *Salmonella* on the equipment. In an attempt to inhibit bird infestation, the packing shed employed the use of visual bird scaring devices suspended from the ceilings at packing shed entrances. These devices were brightly colored inflatable balls with images of large glaring eyes on the surface. This type of bird deterrent is commonly used in agricultural production.

The remaining 3 isolates were obtained from the surfaces of washed cantaloupe. There have been several outbreaks of salmonellosis epidemiologically linked to cantaloupe. Surveys of cantaloupe following these outbreaks revealed the presence of additional *Salmonella* serotypes on the surfaces of cantaloupe not associated with the serotype attributed to the outbreaks *(21)*. Higher prevalence of *Salmonella* on cantaloupe versus the other commodities sampled may be due to surface characteristics. The raised net surface has a roughness which favors microbial attachment *(191)*. In addition, cantaloupe grows in direct contact with soil which may be contaminated with pathogens originating from a number of possible sources such as wildlife or contaminated irrigation water. *Salmonella* was not isolated in any of the composite soil samples; however, there were only 12 total soil samples collected. Several studies have reported the survival of *Salmonella* in soil for time periods ranging from 46 days to more

than 200 days *(16, 33, 203)*. It is important to note that the salmonellae were only detected on washed cantaloupe sampled in the packing shed, suggesting that the fruit was contaminated during processing. An alternate explanation for the presence of *Salmonella* on washed cantaloupe could be that the washing process exposed the bacteria already entrapped in the net surface, allowing the organisms to be detected by swabbing.

No salmonellae were isolated from the samples collected from oranges and parsley. Some hypotheses as to why *Salmonella* was not present may be attributed to the structures of these commodities as well as how these products are irrigated and harvested. Oranges are rarely in direct contact with the soil or irrigation water. Orange groves were irrigated via drip and furrow irrigation systems. Irrigation methods that subject the plant to direct contact with contaminated water increase the risk of contamination *(136)*. Parsley, similar to cantaloupe, grows in direct contact with the soil; however, this product is harvested by slicing through the stems approximately 5 cm above the soil. Parsley crops were typically irrigated using a furrow system. Although, *Salmonella* was isolated in the furrow irrigation water, it was not found on the parsley. Plant surfaces are covered with a waxy cuticle that does not allow bacteria to attach as easily and uniformly as surfaces such as animal tissue *(71)*. Due to differences in surface morphology, it may not be as easy for *Salmonella*, if present in the environment, to attach to parsley and oranges as it is for this organism to attach or become entrapped within the cantaloupe surface.

Prevalence and counts of *E. coli* on produce and in the growing and processing environment. The presence and concentration of *E. coli* was determined for all produce and environmental samples. *E. coli* has been shown to be an effective indicator of fecal contamination on fresh produce *(80)*. *E. coli* was detected on all types of commodities (cantaloupe, oranges, and parsley) as well as in irrigation water samples and on equipment surface swabs (Table 2). Table 3 shows the prevalence and means for *E. coli* counts detected on samples of commodities in the field and in the shed after all processing. For samples collected from product in the field, *E. coli* was detected on 13.0% of cantaloupe samples versus 0.0% and 1.0% for oranges and parsley, respectively. For all three types of commodities sampled, *E. coli* was detected more frequently on product sampled in the packing shed following sorting and washing. This agrees with Shapiro and Holden *(174)* who found that microbial populations on vegetable products increased during processing. Prevalence of *E. coli* was highest on cantaloupe samples for both sampling locations (field and in the shed). Mean *E. coli* counts on samples with counts above or equal to the minimum detection level, collected from the field and the packing shed, were the same (2.1 $\log_{10}$ CFU/fruit) for cantaloupe and oranges. Parsley samples collected from the field (above or equal to the minimum detection level) had higher *E. coli* counts than cantaloupe and oranges; however, *E. coli* was only detected on only 1% of these samples. In another study, *E. coli* was generally detected in parsley with the roots intact *(102)*. In the same study, *E. coli* was detected in 74% of commercially available parsley samples, but only in 5% of aseptically harvested samples, indicating that the contamination may be of human origin *(102)*. Parsley

TABLE 3. *Prevalence and mean* E. coli *counts for commodity surface samples with counts above or equal to the minimum detection level*[a] *collected from commodities in the field and in the packing shed following all processing.*

		Prevalence[b]		Log_{10} CFU/commodity[c]		
Commodity	Location			Mean[d]	Min.	Max.
Cantaloupe[e]	Field	13/100	(13.0)	2.2 ± 0.8	1.4	3.5
	Shed	21/100	(21.0)	2.1 ± 0.7	1.4	3.3
	Field + Shed	34/200	(17.0)	2.1 ± 0.7	1.4	3.5
Oranges[f]	Field	0/100	(0.0)	-	-	-
	Shed	6/100	(6.0)	2.1 ± 0.4	1.7	2.8
	Field + Shed	6/200	(3.0)	2.1 ± 0.4	1.7	2.8
Parsley[g]	Field	1/100	(1.0)	2.4 ± 0.0	2.4	2.4
	Shed	3/100	(3.0)	0.9 ± 0.6	0.6	1.6
	Field + Shed	4/200	(2.0)	1.3 ± 0.9	0.6	2.4

[a] Minimum detection levels for cantaloupe, oranges, and parsley were 1.4 log_{10} CFU/cantaloupe, 1.4 log_{10} CFU/orange, and 0.6 log_{10} CFU/g of parsley, respectively.
[b] Percent samples with *E. coli* counts above the minimum detection level.
[c] Means reported log_{10} CFU/cantaloupe, log_{10} CFU/orange, and log_{10} CFU/g of parsley.
[d] Mean ± standard deviation.
[e] Cantaloupe were sampled by swabbing the entire surface.
[f] Oranges were sampled by rinsing with 25-ml buffered peptone water.
[g] Parsley samples consisted of a 12.5-g sample taken from a 50-g bunch collected from each source.

collected in the present study was harvested without the roots, which may be one explanation for the low prevalence of *E. coli*. In another study involving 5 types of produce, *E. coli* was detected on only 1 out of 50 samples obtained at the retail level *(189)*. Fowler and Foster *(69)* reported *E. coli* counts on fresh green salad to be low. In contrast, a survey of vegetables in the Netherlands revealed greater than 100 *E. coli*/g on 11% of samples *(186)*.

E. coli was detected in 39.4% of irrigation water samples, including all sources of irrigation water (Table 4). The mean concentration of *E. coli* in water samples with counts above or equal to the minimum detection level was 0.4 $\log_{10}$ CFU/ml (Table 4). Geldreich and Bordner *(80)* found that when the fecal coliform density per 100 ml was above 1,000 organisms in various stream waters, *Salmonella* occurrence reached almost 100% frequency. One-hundred percent samples of irrigation water obtained from wells were contaminated with *E. coli* at a mean concentration of 0.7 $\log_{10}$ CFU/ml. The concentration of *E. coli* was significantly higher for water sampled from wells and reservoirs ($P < 0.05$) which both had the same concentration of *E. coli* ($P > 0.05$). *E. coli* was detected in only 3 out of 50 samples or 6.0% of irrigation water collected from cement irrigation canals whereas *E. coli* was detected in 15 out of 30 samples or 50.0% of irrigation water samples collected from dirt irrigation canals. The water-soil interface of the irrigation canal can be a reservoir for fecal pollution transported in the channel *(80)*.

E. coli was detected on 26 out of 280 or 9.3% of swabs of equipment surfaces sampled in the field and in the packing shed (Table 5). The overall mean count of

TABLE 4. *Prevalence and mean* E. coli *counts for water samples with counts above or equal to the minimum detection level (1 CFU/ml) collected in the field and in the packing shed following all processing.*

	Prevalence[a]		Log_{10} CFU/ml		
Source			Mean[bc]	Min.	Max.
Canals (cement)	3/50	(6.0)	0.1 ± 0.2B	0.0	0.3
Canals (dirt)	15/30	(50.0)	0.3 ± 0.4B	0.0	1.3
Furrow	15/20	(75.0)	0.2 ± 0.3B	0.0	1.1
Reservoir	15/30	(50.0)	1.0 ± 0.7A	0.0	1.7
River	9/30	(30.0)	0.1 ± 0.2B	0.0	0.5
Well	10/10	(100.0)	0.7 ± 0.3A	0.3	1.1
Overall	67/179	(39.4)	0.4 ± 0.5	0.0	1.7

[a] Number of positive/number of samples, percent samples with detectable numbers is in parenthesis.

[b] Mean ± standard deviation.

[c] Means with different letters in the same column (excluding row containing 'overall') are significantly different ($P < 0.05$).

TABLE 5. *Prevalence and mean* E. coli *counts for equipment surface swab samples with counts above or equal to the minimum detection level (1.4 log_{10} CFU/400 cm^2) collected in the field and in the packing shed.*

	Prevalence[a]		Log_{10} CFU/400 cm^2		
Source			Mean[bc]	Min.	Max.
Boxing ramp	1/30	(4.0)	1.7 ± 0.0A	1.7	1.7
Conveyors					
cups	0/5	(0.0)	-	-	-
belts	5/70	(7.1)	1.8 ± 0.4A	1.4	2.2
rollers	2/20	(10.0)	1.4 ± 0.0A	1.4	1.4
Dump tank auger	0/5	(0.0)	-	-	-
Hands[d]	0/10	(0.0)	-	-	-
Harvest bag/plastic	2/5	(40.0)	2.1 ± 0.1A	2.1	2.2
Harvest baskets/plastic	0/5	(0.0)	-	-	-
Harvest bins/plastic	1/40	(2.5)	1.4 ± 0.0A	1.4	1.4
Harvest bins/wood	0/5	(0.0)	-	-	-
Ice chutes	0/20	(0.0)	-	-	-
Plastic packing crates	0/5	(0.0)	-	-	-
Railguard (boxing)	0/5	(0.0)	-	-	-
Receiving hopper	2/10	(20.0)	2.7 ± 0.4A	1.4	2.9
Trailer[e]	7/20	(35.0)	1.8 ± 0.6A	1.4	2.9
Unloading ramp	6/15	(40.0)	2.8 ± 1.1A	1.4	3.8
Wall[f]	0/10	(0.0)	-	-	-
Overall	26/280	(9.3)	2.1 ± 0.8	1.4	3.8

[a] Number of positive/number of samples, percent samples with detectable numbers is in parenthesis.

[b] Mean ± standard deviation.

[c] Means with different letters in the same column (excluding row containing 'overall') are significantly different ($P < 0.05$).

[d] Bare hands or gloves of workers in the packing shed boxing the previously washed commodities.

[e] Trailer used to transport harvested product to the packing shed.

[f] Surface of a citrus de-greening room wall.

E. coli on samples with counts above or equal to the minimum detection level (1.4 $\log_{10}$ CFU/400 cm^2) was 2.1 $\log_{10}$ CFU/400 cm^2. Surfaces where *E. coli* was detected included boxing ramps, conveyor belts, plastic bags and bins used for harvesting, a receiving hopper, trailers used for transport, and an unloading ramp used to move harvested product onto the processing line. Similarly, Parish *(152)* detected *E. coli* on samples of conveyor belts in an orange juice processing facility. Many of the sources of *E. coli* contamination found in this study, such as transport containers and vehicles, sorting and packing equipment, have been traditionally recognized as postharvest control points for access of pathogens to whole or cut produce *(19*). Mean *E. coli* counts for samples with counts above or equal to the detection level were the same for each source sampled ($P > 0.05$). The counts ranged from 1.4 $\log_{10}$ CFU/400 cm^2 to 3.8 $\log_{10}$ CFU/400 cm^2. *E. coli* was detected most frequently on samples obtained from the surfaces of trailers (7/20 or 35.0%), unloading ramps (6/15 or 40.0%) and plastic harvest bags (2/5 or 40.0%). Trailers and trucks used for transport were open, used repeatedly between washing, and exposed to dust, road dirt, insects, and rodents *(80)*. Harvesting bags are more difficult to clean and sanitize. When equipment is not effectively cleaned and sanitized, biofilms may develop on this harvesting equipment *(17, 23, 46)*. It is interesting to note that in this study, *E. coli* was not detected on the surfaces of employee gloves or hands, which is similar to the results reported by Parish *(152*). However, hands of workers obviously should not be overlooked as a vehicle for the transmission of pathogens to the product, as fresh produce harvesting and processing involves an extensive amount of hand labor.

Serotyping of *Salmonella* isolates. Twenty-five *Salmonella* isolates were obtained from samples collected from fresh produce, irrigation water, and surface swabs of equipment in the growing and processing environment (Table 6). Seven different serotypes were found with two of the isolates remaining untypeable. Serotypes detected were *S.* Anatum, *S.* Arizona, *S.* Javiana, *S.* Muenchen, *S.* Newport, *S.* Rubislaw, and *S.* Texas. *S.* Anatum, *S.* Javiana, *S.* Muenchen, *S.* Newport, and *S.* Rubislaw are all serotypes that have been linked to foodborne illness outbreaks associated with fresh produce *(41*, *122)*. The Centers for Disease Control and Prevention *(38)* reported that infections caused by *S.* Javiana and *S.* Newport, 2 of the 5 most common infection-causing serotypes, have increased significantly. The 2 most commonly isolated serotypes were *S.* Javiana (9 out of 25; 36.0%) and *S.* Arizona (7 out of 25 isolates; 28.0%) which were both found on cantaloupe, in irrigation water, and on equipment surfaces (Table 5). *S.* Texas, isolated from cantaloupe, was not found on any other type of samples. Seven out of 16 or 43.8% of the serotypes found in irrigation water were serotype Javiana with the remaining isolates being relatively diverse. *S.* Arizona was the serotype most commonly isolated from equipment surfaces (5 out of 6; 83.3%). Due to the poor discriminatory power of serotyping and high frequency of untypeable isolates, all 25 *Salmonella* isolates were also compared using DNA typing methods.

Antibiotic resistance profiles of *Salmonella* isolates. Nineteen out of the 25 *Salmonella* isolates or 76% were susceptible to all antimicrobials tested (Table 7). Twenty percent (5 out of 25) of the isolates demonstrated intermediate sensitivity to

TABLE 6. Salmonella *serotypes detected on cantaloupe surfaces, irrigation water, and on equipment in the growing and processing environment*

	No. (%) of isolates of serotype from:			
Salmonella Serotype	Cantaloupe (n = 3)	Irrigation water (n = 16)	Surfaces (n = 6)	Total (n = 25)
Anatum	0 (0.0)	1 (6.3)	0 (0.0)	1 (4.0)
Arizona	1 (33.3)	1 (6.3)	5 (83.3)	7 (28.0)
Javiana	1 (33.3)	7 (43.8)	1 (16.7)	9 (36.0)
Muenchen	0 (0.0)	1 (6.3)	0 (0.0)	1 (4.0)
Newport	0 (0.0)	3 (18.8)	0 (0.0)	3 (12.0)
Rubislaw	0 (0.0)	1 (6.3)	0 (0.0)	1 (4.0)
Texas	1 (33.3)	0 (0.0)	0 (0.0)	1 (4.0)
Untypeable	0 (0.0)	2 (12.5)	0 (0.0)	2 (8.0)

TABLE 7. *Results of antibiotic susceptibility testing of* Salmonella *isolates obtained from cantaloupe surfaces, irrigation water, and swabs of equipment surfaces.*

Antimicrobial agent	No. (%) of resistant isolates from:							
	Cantaloupe (n = 3)		Irrig. water (n = 16)		Surfaces (n = 6)		Total (n = 25)	
Amikacin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Amoxicillin/clavulanic acid	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Ampicillin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Cefazolin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Cefoxitin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Ceftriaxone	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Cephalothin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Chloramphenicol	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Ciprofloxacin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Gentamicin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Kanamycin	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Nalidixic acid	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Streptomycin	1	(33.3)	4[a]	(25.0)	1[a]	(16.0)	6[b]	(24.0)
Tetracycline	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)
Trimethoprim/sulfamethoxazole	0	(0.0)	0	(0.0)	0	(0.0)	0	(0.0)

[a]These isolates demonstrated intermediate sensitivity to the antimicrobial agent
[b]This number includes isolates demonstrating resistance and intermediate sensitivity

streptomycin. It is not uncommon for many organisms to be resistant to streptomycin *(9)*. One *Salmonella* isolate from cantaloupe demonstrated resistance to streptomycin. The resistant isolate was determined to be serotype Texas and was untypeable by PFGE and not genetically similar to any of the other isolates by Rep-PCR. Four of the 5 isolates demonstrating intermediate sensitivity to streptomycin were isolated from irrigation water with the remaining fifth isolate originating from an unloading ramp in the packing shed. Three of these 5 strains were determined to be *S.* Javiana and were also genetically indistinguishable by PFGE and Rep-PCR.

DNA-based typing of *Salmonella* isolates. In addition to the phenotypic analyses, including serotyping and antimicrobial susceptibility, the molecular epidemiology of the 25 *Salmonella* isolates obtained from irrigation water, equipment surfaces, and cantaloupe surfaces was also investigated using pulsed-field gel electrophoresis (PFGE) and repetitive sequence-based polymerase chain reaction (rep-PCR). Genotypic methods have much higher discrimination indices than the traditional phenotypic methods *(94)*.

The fingerprints generated by PFGE using the restriction enzyme X*ba*I are shown in the gel image in Figure 1 and the resulting dendrogram generated using unweighted pairwise grouping with mathematical averaging (UPGMA) is displayed in Figure 2. As depicted in Figure 2, there were 12 main clusters, designated A to L, discernible at the 90% similarity level. Isolates are considered to be closely related if their PFGE patterns differ by only 2 or 3 fragment differences *(188)*. These differences

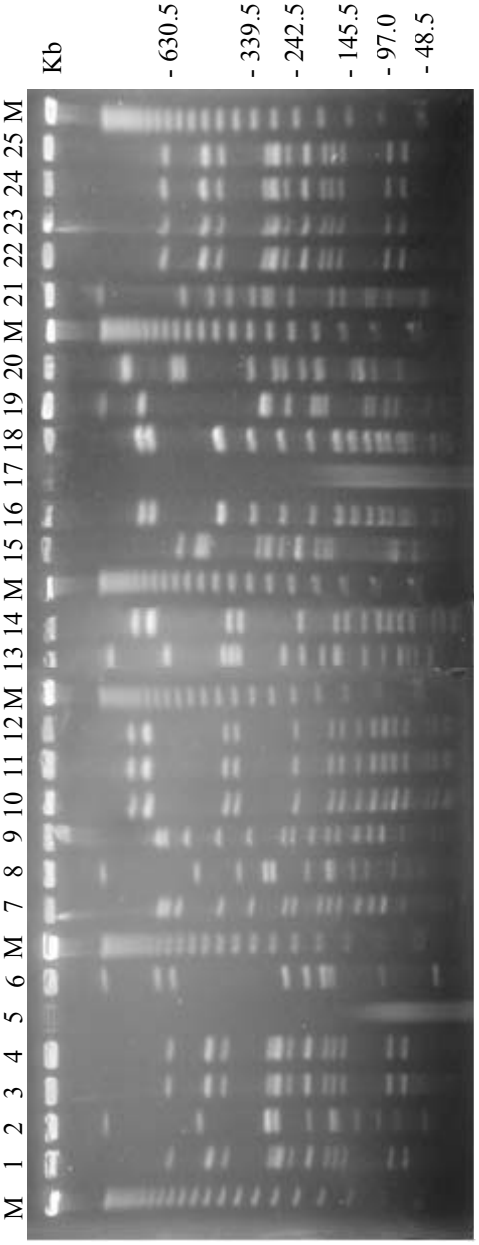

FIGURE 1. *PFGE of* XbaI*-digested genomic DNA from isolates of* Salmonella *obtained from irrigation water, packing shed equipment, and cantaloupe surfaces. Lanes designated M are molecular weight markers, lambda ladders (Bio-Rad); lanes 1-3,5-8, 13,15, and 19-25 are isolates from irrigation water; lane 4 is an isolate from an unloading ramp; lanes 9 and 10 are isolates from a ramp to boxing; lane 11, 12, and 14 are isolates from conveyors; and lanes 16-18 are isolates from cantaloupe surfaces.*

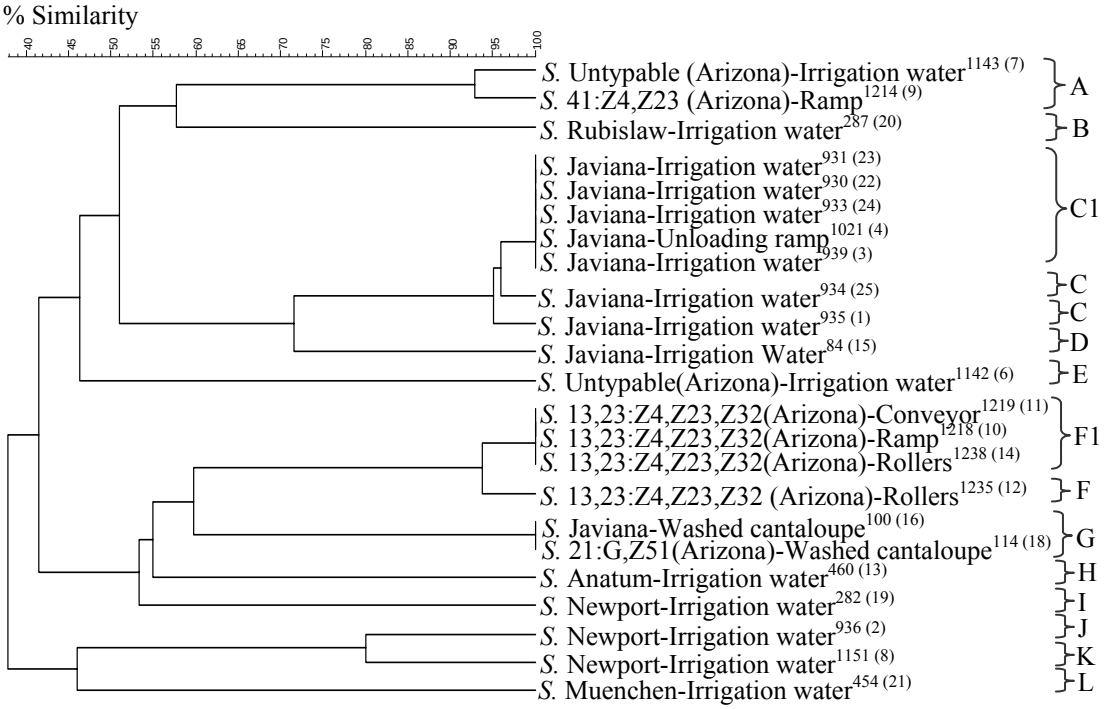

FIGURE 2. *Dendrogram based on PFGE profiles of* Salmonella *isolates. Capital letters (A to L) were used to designate main cluster lineages (greater than 90% similarity level), while subclusters (greater than 99% similarity level) were given numerical suffixes. The superscript number next to the isolate description is the sample identification and the number in parentheses corresponds to the lane on the gel image in FIGURE 1.*

may be present due to a single genetic event such as a point mutation or an insertion or deletion of DNA *(188)*. There were 2 subclusters with greater than 99% similarity which were designated with numerical suffixes. Isolates were designated genetically indistinguishable if their restriction patterns had the same numbers of bands and the corresponding bands were the same size (*188)*. One subcluster included irrigation water samples while the other subcluster included various equipment swabs from the same packing shed. Restriction profiles of *Salmonella* isolates obtained from the surface of cantaloupe were identical to each other (zero fragment differences; Figure 1); however, they were different from isolates obtained from equipment and irrigation water. An isolate obtained from an unloading ramp was genetically similar to isolates obtained from irrigation water. The restriction patterns for the isolates obtained from cantaloupe surfaces had more than 7 fragment differences compared to the isolates obtained from equipment and irrigation water (Figure 1). Tenover et al. *(188)* designated isolates as unrelated when investigating a possible outbreak strain, if its PFGE pattern differed from the outbreak pattern by changes consistent with 3 or more independent genetic events which typically resulted in 7 or more band differences.

Although 16 out of the 25 or 64% of *Salmonella* isolates were obtained from irrigation water, all of these isolates were different, as determined by PFGE, from the *Salmonella* isolates obtained from the surface of the cantaloupe. Furthermore, *Salmonella* isolated from the surfaces of equipment including ramps and conveyors were unrelated to isolates obtained from irrigation water. Although irrigation water is commonly thought to be the source of pathogenic contamination on fresh produce, this

was not supported in the present study. Dunlop *(56)* was also unable to demonstrate transmission of *Salmonella* to crops through contaminated irrigation water. PFGE did not conclusively determine relationships between sources of contamination.

Two of the *Salmonella* strains persistently appeared as smears of degraded DNA in lanes 5 and 17 (Figure 1), regardless of storage time of DNA agarose plugs or length of digestion. The inability to produce discernible bands for those isolates may be due to the presence of endogenous nucleases. Other studies have also reported occurrence of extensive DNA degradation during the PFGE procedure hypothesized to be attributed to endogenous nuclease activity *(85, 101, 105, 132)*. Klaassen et al. *(105)* found that the addition of 100 μM thiourea to the electrophoresis buffer enabled some untypeable strains to yield very faint, but recognizable DNA fingerprinting patterns.

In addition to PFGE, genetic relatedness of *Salmonella* isolates was also examined using Rep-PCR. Results acquired with Rep-PCR were very similar to the PFGE results. Liu et al. *(118)* also found that Rep-PCR results were similar to PFGE, in general, but with slightly less discriminatory power. Fingerprints generated with Rep-PCR using Uprime-B1 and Uprime-E primers are shown in the gel images in Figure 3 and Figure 4, respectively. The corresponding dendrogram in Figure 5 was created using UPGMA of the fingerprints obtained using the Uprime-E primer is presented in Figure 5. The dendrogram resulting from the fingerprints obtained from the Uprime-B1 primer, not pictured, is the same as Figure 5. Using Rep-PCR, ten main clusters (designated A to J; Figure 5) were evident at the 87% similarity level with the majority

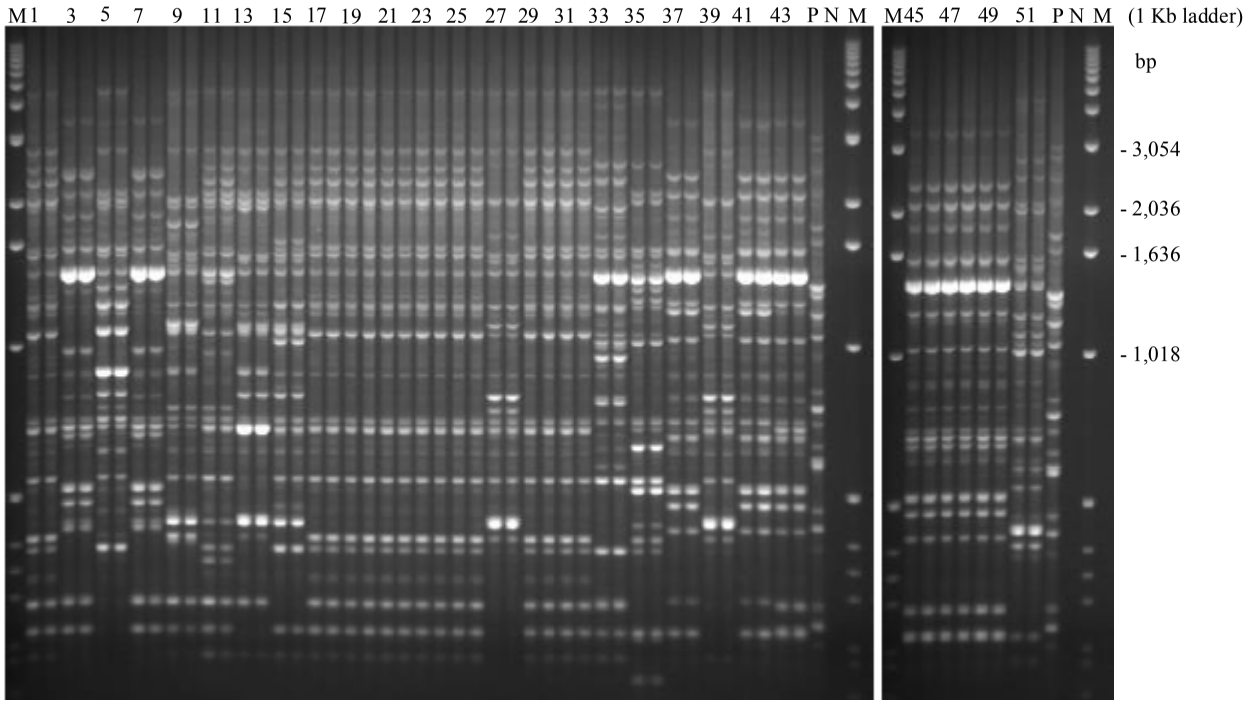

FIGURE 3. *Rep-PCR generated genomic fingerprints (using Uprime-B1 primer) of* Salmonella *strains isolated from irrigation water, packing shed equipment, and cantaloupe surfaces. Samples were run in duplicate. Lanes designated M are molecular weight markers; lanes designated P are positive controls and N are negative controls. Lanes 1-2, 9-30, and 33-40 are isolates from irrigation water; lanes 3-8 are isolates from cantaloupe surfaces; lanes 31-32 is an isolate from an unloading ramp; lanes 41-44 are isolates from a ramp to boxing; lanes 45-50 are isolates from conveyors; and lanes 51-52 is an additional positive control.*

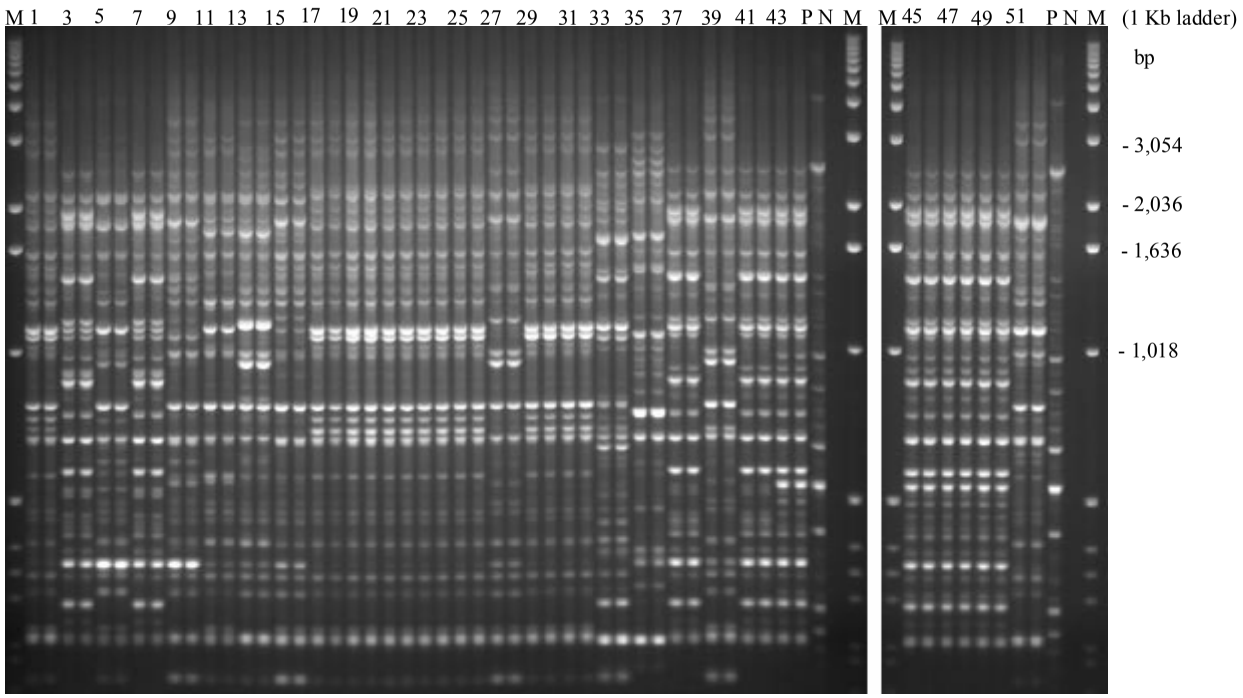

FIGURE 4. *Rep-PCR generated genomic fingerprints (using Uprime-E primer) of* Salmonella *strains isolated from irrigation water, packing shed equipment, and cantaloupe surfaces. Samples were run in duplicate. Lanes designated M are molecular weight markers; lanes designated P are positive controls and N are negative controls. Lanes 1-2, 9-30, and 33-40 are isolates from irrigation water; lanes 3-8 are isolates from cantaloupe surfaces; lanes 31-32 is an isolate from an unloading ramp; lanes 41-44 are isolates from a ramp to boxing; lanes 45-50 are isolates from conveyors; and lanes 51-52 is an additional positive control.*

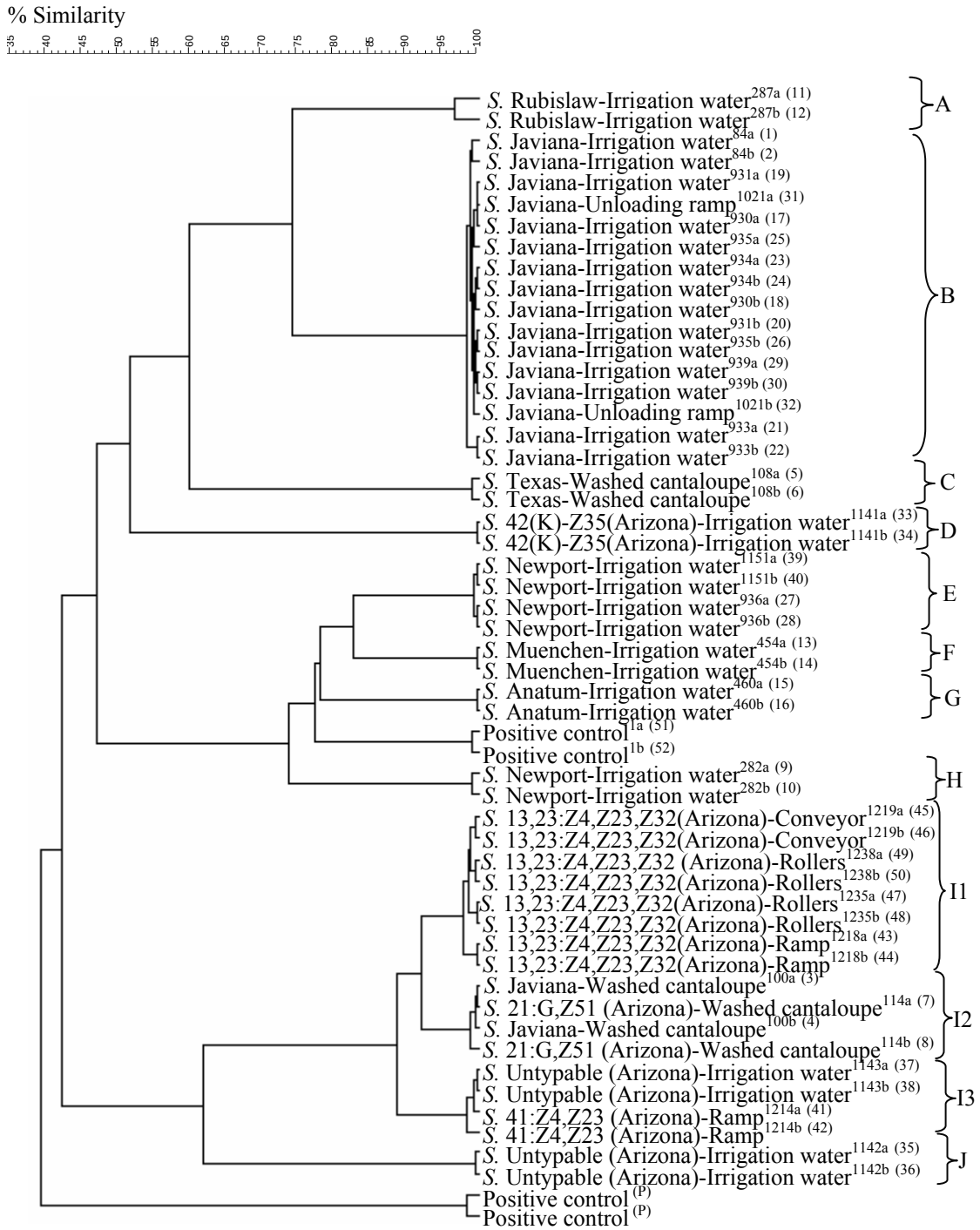

FIGURE 5. *Dendrogram based on the Rep-PCR analysis of* Salmonella *isolates. Capital letters (A to J) were used to designate the main cluster lineages (greater than 87% similarity level), while subclusters (greater than 95% similarity level) were given numerical suffixes. The superscript number next to the isolate description is the sample identification and the number in parentheses corresponds to the lane on the gel images in* FIGURE 3 *and* FIGURE 4.

of the isolates (64%) being grouped into two main clusters B (32%, n = 8) and I (32%; n = 8). Rep-PCR was less discriminatory than the PFGE. Using Rep-PCR, there were some equipment samples showing greater than 95% similarity to an isolate obtained from irrigation water. Using PFGE, the isolates obtained from cantaloupe were shown to be different from isolates collected from other sources; however, when using Rep-PCR fingerprints obtained from cantaloupe isolates were shown to be 87% similar to isolates obtained from the surfaces of ramps and conveyors. It is generally common to find more differences between isolates with PFGE than Rep-PCR; however, Weigel et al. *(205)* identified 14 pairs of *Salmonella* isolates obtained from swine farms as genetically different that PFGE identified as genetically identical. Using the results obtained from PFGE and Rep-PCR, it was not possible to adequately infer epidemiologic relationships between the isolates.

Characteristics of the 25 *Salmonella* isolates and their PFGE and Rep-PCR profiles are presented in Table 8. PFGE had the ability to discriminate between strains that were indistinguishable by Rep-PCR and serotyping. There were instances where strains were designated different serotypes that PFGE and Rep-PCR designated as genetically identical. Serotyping results can be easily affected by environmental selective pressures which can influence the stability of antigenic traits *(57)*. Furthermore, serotyping is based on subjective observations for agglutination, introducing further possibility for human error.

TABLE 8. *Twenty-five* Salmonella *isolates from irrigation water, packing shed equipment, and fresh produce according to origin, serotype, Rep-PCR cluster, and PFGE cluster.*

Origin	Serotype	Rep-PCR cluster	PFGE cluster
Irrigation water (84)	Javiana	B	D
Irrigation water (282)	Newport	H	I
Irrigation water (287)	Rubislaw	A	B
Irrigation water (454)	Muenchen	F	L
Irrigation water (460)	Anatum	G	H
Irrigation water (930)	Javiana	B	C1
Irrigation water (931)	Javiana	B	C1
Irrigation water (933)	Javiana	B	C1
Irrigation water (934)	Javiana	B	C
Irrigation water (935)	Javiana	B	C
Irrigation water (936)	Newport	E	J
Irrigation water (939)	Javiana	B	C1
Irrigation water (1141)	42(K) – Z35 (Arizona)	D	Untypeable
Irrigation water (1142)	Untypeable[a]	J	E
Irrigation water (1143)	Untypeable[a]	I3	A
Irrigation water (1151)	Newport	E	K
Ramp (1214)	41:Z4, Z23 (Arizona)	I3	A
Ramp (1218)	13, 23:Z4, Z23, Z32 (Arizona)	I1	F1
Conveyor (1219)	13, 23:Z4, Z23, Z32 (Arizona)	I1	F1
Rollers (1235)	13, 23:Z4, Z23, Z32 (Arizona)	I1	F
Rollers (1238)	13, 23:Z4, Z23, Z32 (Arizona)	I1	F1
Unloading ramp (1021)	Javiana	B	C1
Cantaloupe (114)	21:G, Z51 (Arizona)	I2	G
Cantaloupe (100)	Javiana	I2	G
Cantaloupe (108)	Texas	C	Untypeable

[a] rough O:Z4, Z32

Transformation of *Salmonella* to express EGFP. EGFP was used as a marker system to monitor the attachment and survival of *Salmonella* on parsley. When marking pathogens to use as predictive models, it is important that the marked strain demonstrate similar growth and survival characteristics as the parent strain from which it originated. Fratamico et al. *(73)* found that *E. coli* strains transformed to express GFP were indistinguishable from their parent strains in biochemical and immunological assays, and also there were no notable differences in their growth rates. Prachaiyo and McLandsborough *(158)* reported that the expression of EGFP did not affect the hydrophobicity or surface charge of the strains. The marker system used in this study was evaluated to ensure that the plasmid could be maintained and also the growth kinetics of both the parent and recombinant strains were compared to confirm that the bacterial transformation to express green fluorescence did not alter the growth kinetics of the *Salmonella*.

Stability of EGFP expression by *Salmonella* strains. Transformed *Salmonella* strains continued to express the pEGFP plasmid through 2 consecutive cycles of subculturing in broth supplemented with antibiotic (ampicillin), followed by 10 cycles of subculturing onto LBA-amp plates. Other studies have also demonstrated that at 37°C, recombinant strains are able to maintain plasmid and express fluorescence when grown with antibiotic selection *(73, 198)*. *Salmonella*/pEGFP strains were only grown on media supplemented with antibiotic. Prachaiyo and McLandsborough *(158)* found that when grown without antibiotic selection, approximately 90% of the cell population lost

the plasmid over the course of an 8-h growth curve *(158)*. Transformed *Salmonella* strains inoculated onto LBA-amp plates, incubated for 12 to 16 h and stored at 4°C, stably maintained the pEGFP plasmid for over 30 days, which conflicts with Takeuchi and Frank *(182)* who found that a percentage of *E. coli* O157:H7 transformed to express GFP, lost or failed to express the pEGFP during refrigeration. Vialette et al. *(198)* reported that only 0 to 10% of *E. coli* O157:H7/pEGFP transformed strains lost the plasmid during storage at 10°C for 5 days.

Growth comparison of transformed *Salmonella* versus parent strains.

Several studies have concluded that the growth and/or survival of transformed strains does not differ from their parent strains *(24, 73, 88, 158, 190, 198)*. However, studies completed by Oscar *(148)* indicated that the growth kinetics (i.e., lag time, specific growth rate, and maximum population density) of the GFP strains tested were different from those of the parent strains.

Growth curves comparing the growth of the parent *Salmonella* strains with their respective *Salmonella*/pEGFP strains are presented in Figures 6, 7, and 8. The doubling time or generation time (g), was determined for each of the recombinant and parent strains, to serve as an index of the growth rate. Doubling times for *S.* Anatum, *S.* Javiana, and *S.* Rubislaw were all 0.3 h. Doubling times for the EGFP-expressing *Salmonella* strains were 0.4 h, 0.4 h, and 0.3 h, respectively. In this study, there were only minimal differences between the growth of 2 of the *Salmonella* strains (Anatum and

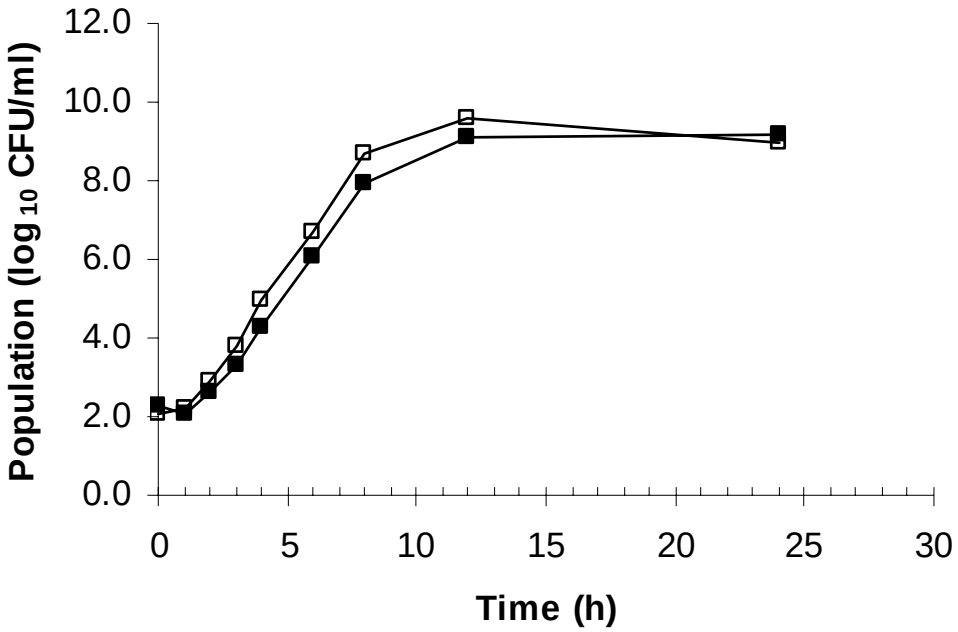

FIGURE 6. *Growth curves for* Salmonella *Anatum transformed to express enhanced green fluorescent protein* (■) *and the parent* S. *Anatum strain* (□).

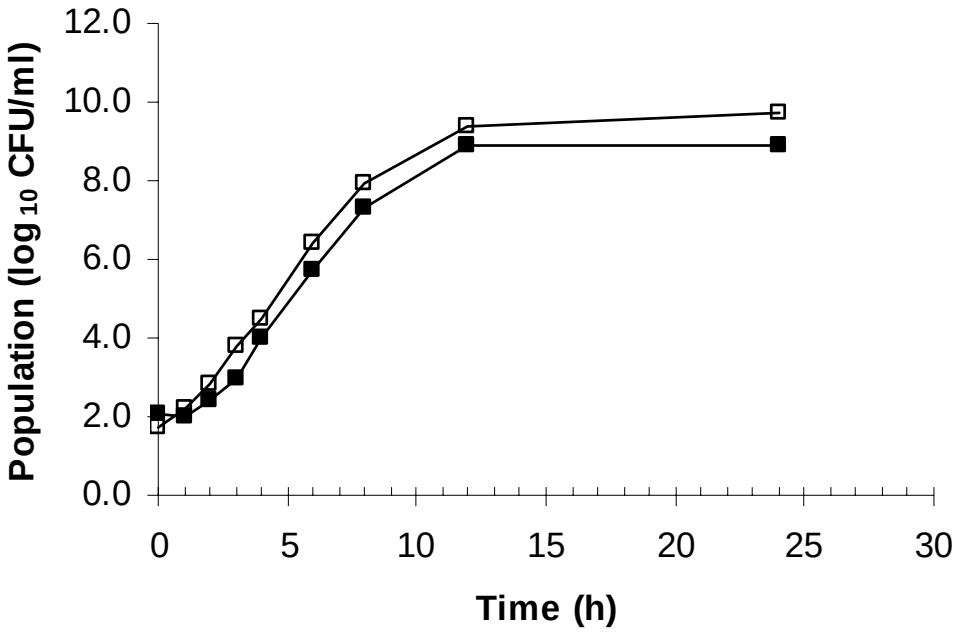

FIGURE 7. *Growth curves for* Salmonella *Javiana transformed to express enhanced green fluorescent protein (■) and the parent* S. *Javiana strain* (□).

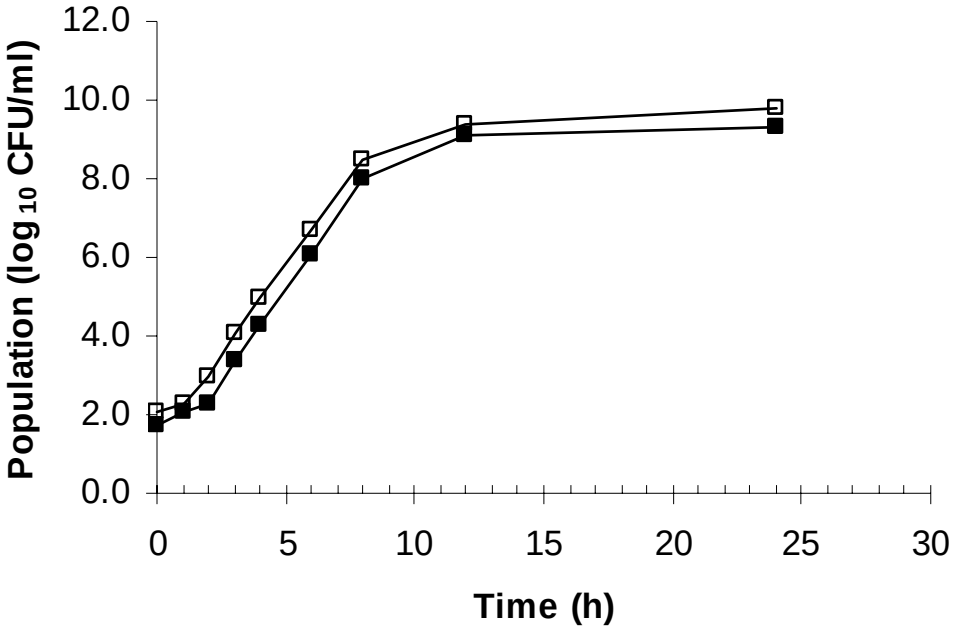

FIGURE 8. *Growth curves for* Salmonella *Rubislaw transformed to express enhanced green fluorescent protein* (■) *and the parent* S. *Rubislaw strain* (□).

Javiana) and their accompanying transformed strains. There was no difference between the growth of *S.* Rubislaw/pEGFP and its parent strain.

Attachment of *Salmonella*/pEGFP to parsley. To simulate the immersion of parsley in a contaminated dump tank, parsley bunches were dipped in 0.1% peptone water inoculated with 5.6 ± 0.5 $\log_{10}$ CFU/ml of a 3-strain suspension prepared from cultures of *S.* Anatum (isolated from irrigation water), *S.* Rubislaw (isolated from irrigation water), and *S.* Javiana (isolated from cantaloupe) that were previously transformed to express enhanced green fluorescent protein (EGFP). The aqueous peptone solution was used as a carrier for the inoculum to imitate practical conditions *(18)*. As product is submerged in the dump tank, soil, leaves, and other organic material are also introduced into the water and it is common for more than one commodity to be processed in the same dump tank on the same day of processing.

Dip suspensions were pre-equilibrated to 5°C, 25°C, or 35°C to examine the effect of temperature on the attachment and infiltration of *Salmonella* in parsley. Studies have demonstrated that a negative temperature differential increases the infiltration of microorganisms into the stem scar of fruits *(12, 217)*. This study examined whether a temperature differential affects the attachment and internalization of pathogens on leafy vegetables with stems, such as parsley. Populations of loosely attached and/or associated, strongly attached and/or associated, and internalized and/or entrapped *Salmonella*/pEGFP cells were enumerated for each treatment. Internalized and/or entrapped cells were enumerated by disinfecting the surface of the parsley with a 2,000

ppm chlorine solution, which has been shown to be an effective surface disinfectant. Seo and Frank *(173)* observed that a 20 ppm chlorine treatment destroyed bacteria on the surface of lettuce, some bacteria inside the stomata, but had little effect on cells that had penetrated the lettuce tissue. Analogous to these results, Zhuang et al. *(217)* reported that chlorine was effective at killing *S.* Montevideo on the surface, but did not significantly ($P < 0.05$) reduce the number of viable cells in the core tissue.

The effects of different processing and storage parameters, including the temperature of the dip suspension, duration of the dip, and days of storage at 4°C or 25°C are presented in Tables 9 and 10, respectively. Mean $\log_{10}$ counts ($\log_{10}$ CFU/g) of loosely attached/associated, strongly attached/associated, and internalized and/or entrapped *Salmonella*/pEGFP are presented, stratified by the individual effects. Each of these individual treatment effects, averaged over each of the other variables, were significant ($P < 0.05$). Although, the 3-way interactions of the main effects were significant ($P < 0.05$), the main effects have been discussed individually because of the inability to discern very many meaningful interpretations from the 3-way interactions. Means, across all effects, for parsley stored at 4°C and 25°C, were significantly different (data not shown). Means for each storage temperature and the 2 and 3-way interactions of the main effects are presented in Tables 11 through 18.

For both storage temperatures, across all effects, differences in *Salmonella*/pEGFP populations, as affected by the dip temperature, were slight. Mean counts for loosely and strongly attached cells on parsley dipped in the 25°C suspensions, were lower than parsley dipped in 5°C and 35°C suspensions, for both storage

TABLE 9. *Mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 4°C stratified by dip temperature, duration of dip, and days of storage.*

Effect	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
Temperature of dip (°C) (n = 60)	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
5	2.1B	1.9B	1.4A
25	1.7C	1.7C	1.2B
35	2.6A	2.4A	1.5A
Duration of dip (min) (n = 270)	$P = 0.0001$	$P = 0.0001$	$P = 0$ .0001
3	1.9B	1.8B	1.2B
15	2.3A	2.1A	1.6A
Storage (days) (n = 36)	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
0	4.5A	4.0A	2.5A
1	3.2B	3.1B	2.2B
7	1.3C	1.2C	< 1.0C
14	< 1.0D	< 1.0D	< 1.0C
30	< 1.0D	< 1.0D	< 1.0C

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Within a column and for each effect, means with different letters are significantly different ($P < 0.05$).

TABLE 10. *Mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 25°C stratified by dip temperature, duration of dip, and days of storage.*

Effect	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
Temperature of dip (°C) (n = 36)	$P = 0.0001$	$P = 0.0001$	$P = 0.1581$
5	5.5A	5.2A	4.6A
25	4.9B	4.9B	4.3A
35	5.6A	5.6A	4.5A
Duration of dip (min) (n = 54)	$P = 0.0001$	$P = 0.0001$	$P = 0$ .0001
3	5.0B	4.9B	4.1B
15	5.6A	5.5A	4.8A
Storage (days) (n = 36)	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
0	4.3C	3.9C	2.7C
1	5.4B	5.4B	4.8B
7	6.2A	6.3A	5.9A

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Within a column and for each effect, means with different letters are significantly different ($P < 0.05$).

TABLE 11. *Two-way interactions of dip temperature (°C) and duration of dip (min) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 4°C.*

Dip temp. x dip time (n = 30)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0233$	$P = 0.0283$	$P = 0.1563$
5°C x 3 min	1.9^{C}	1.7^{DE}	1.2^{C}
5°C x 15 min	2.2^{BC}	2.1^{BC}	1.6^{AB}
25°C x 3 min	1.4^{D}	1.4^{E}	1.1^{D}
25°C x 15 min	2.0^{C}	1.9^{CD}	1.4^{BC}
35°C x 3 min	2.5^{AB}	2.3^{AB}	1.3^{CD}
35°C x 15 min	2.7^{A}	2.4^{A}	1.8^{A}

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 12. *Two-way interactions of dip temperature (°C) and duration of dip (min) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 25°C.*

Dip temp. x dip time (n = 18)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	P = 0.0233	*P* = 0.0264	*P* = 0.0398
5°C x 3 min	5.4^{AB}	5.1^{B}	4.4^{BC}
5°C x 15 min	5.6^{AB}	5.4^{B}	4.8^{AB}
25°C x 3 min	4.4^{C}	4.4^{C}	3.8^{C}
25°C x 15 min	5.4^{AB}	5.3^{B}	4.7^{AB}
35°C x 3 min	5.3^{B}	5.2^{B}	3.9^{C}
35°C x 15 min	6.0^{A}	6.0^{A}	5.1^{A}

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 13. *Two-way interactions of dip temperature (°C) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella/p*EGFP on parsley stored at 4°C.*

Dip temp. x storage (n = 12)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0001$	$P = 0.0001$	$P = 0.0001$
5°C x 0 days	4.5^{AB}	3.8^{B}	2.3^{A}
5°C x 1 day	3.0^{D}	3.0^{C}	2.5^{A}
5°C x 7 days	1.3^{FG}	1.2^{EF}	< 1.0^{C}
5°C x 14 days	< 1.0^{G}	< 1.0^{G}	< 1.0^{C}
5°C x 30 days	< 1.0^{G}	< 1.0^{G}	< 1.0^{C}
25°C x 0 days	3.8^{C}	3.4^{BC}	2.3^{A}
25°C x 1 day	2.4^{DE}	2.6^{D}	1.6^{B}
25°C x 7 days	< 1.0^{G}	< 1.0^{FG}	< 1.0^{C}
25°C x 14 days	< 1.0^{G}	<1.0^{FG}	< 1.0^{C}
25°C x 30 days	< 1.0^{G}	< 1.0^{G}	< 1.0^{C}
35°C x 0 days	5.0^{A}	4.8^{A}	2.8^{A}
35°C x 1 day	4.2^{B}	3.7^{B}	2.6^{A}
35°C x 7 days	1.9^{EF}	1.5^{E}	< 1.0^{C}
35°C x 14 days	1.0^{G}	1.0^{FG}	< 1.0^{C}
35°C x 30 days	< 1.0^{G}	< 1.0^{FG}	< 1.0^{C}

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 14. *Two-way interactions of dip temperature (°C) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 25°C.*

Dip temp. x storage (n = 12)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.2000$	$P = 0.0574$	$P = 0.3104$
5°C x 0 days	4.5^{DE}	3.8^{EF}	2.7^{D}
5°C x 1 day	5.4^{CD}	5.5^{BC}	5.1^{BC}
5°C x 7 days	6.5^{A}	6.4^{A}	5.9^{A}
25°C x 0 days	3.7^{E}	3.4^{F}	2.4^{D}
25°C x 1 day	5.0^{D}	5.1^{CD}	4.6^{C}
25°C x 7 days	6.0^{ABC}	6.1^{AB}	5.8^{AB}
35°C x 0 days	4.8^{D}	4.6^{DE}	3.0^{D}
35°C x 1 day	5.8^{BC}	5.8^{AB}	4.7^{BC}
35°C x 7 days	6.3^{AB}	6.3^{A}	5.8^{AB}

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 15. *Two-way interactions of duration of dip (min) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 4°C.*

Dip time x storage (n = 18)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0002$	$P = 0.0001$	$P = 0.0001$
3 min x 0 days	4.0$_{B}$	3.6$_{B}$	1.9$_{C}$
3 min x 1 day	2.8$_{C}$	2.8$_{C}$	1.9$_{C}$
3 min x 7 days	1.2$_{DE}$	1.2$_{DE}$	< 1.0$_{D}$
3 min x 14 days	< 1.0$_{E}$	< 1.0$_{EF}$	< 1.0$_{D}$
3 min x 30 days	< 1.0$_{E}$	< 1.0$_{F}$	< 1.0$_{D}$
15 min x 0 days	4.9$_{A}$	4.4$_{A}$	3.0$_{A}$
15 min x 1 day	3.5$_{B}$	3.4$_{B}$	2.6$_{B}$
15 min x 7 days	1.5$_{D}$	1.3$_{D}$	< 1.0$_{D}$
15 min x 14 days	< 1.0$_{E}$	< 1.0$_{F}$	< 1.0$_{D}$
15 min x 30 days	< 1.0$_{E}$	< 1.0$_{F}$	< 1.0$_{D}$

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 16. *Two-way interactions of duration of dip (min) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 25°C.*

Dip time x storage (n = 18)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0169$	$P = 0.1410$	$P = 0.0130$
3 min x 0 days	4.1D	3.6D	2.4D
3 min x 1 day	4.9C	5.0C	4.2C
3 min x 7 days	6.1AB	6.1AB	5.6AB
15 min x 0 days	4.6CD	4.2D	3.0D
15 min x 1 day	5.9B	5.9B	5.4B
15 min x 7 days	6.4A	6.5A	6.1A

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 17. *Three-way interactions of dip temperature (°C), duration of dip (min) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 4°C.*

Dip temp. x dip time x storage (n = 6)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0228$	$P = 0.0015$	$P = 0.0001$
5°C x 3 min x 0 days	4.4^{AB}	3.6^{BCD}	1.9^{EF}
5°C x 3 min x 1 day	2.7^{DEF}	2.7^{DE}	2.3^{DE}
5°C x 3 min x 7 days	< 1.0^{HIJ}	< 1.0^{IJ}	< 1.0^{G}
5°C x 3 min x 14 days	< 1.0^{J}	< 1.0^{J}	< 1.0^{G}
5°C x 3 min x 30 days	< 1.0^{IJ}	< 1.0^{J}	< 1.0^{G}
5°C x 15 min x 0 days	4.7^{AB}	4.1^{ABC}	2.8^{ABCD}
5°C x 15 min x 1 day	3.3^{CD}	3.2^{CD}	2.7^{BCD}
5°C x 15 min x 7 days	1.7^{FGHI}	1.6^{GHI}	< 1.0^{G}
5°C x 15 min x 14 days	< 1.0^{HIJ}	< 1.0^{J}	< 1.0^{G}
5°C x 15 min x 30 days	< 1.0^{J}	< 1.0^{J}	< 1.0^{G}
25°C x 3 min x 0 days	2.9^{CDE}	2.7^{DEF}	1.6^{F}
25°C x 3 min x 1 day	1.8^{FGHI}	2.1^{EFG}	1.7^{F}
25°C x 3 min x 7 days	< 1.0^{HIJ}	< 1.0^{IJ}	< 1.0^{G}
25°C x 3 min x 14 days	< 1.0^{J}	< 1.0^{IJ}	< 1.0^{G}
25°C x 3 min x 30 days	< 1.0^{J}	< 1.0^{J}	< 1.0^{G}
25°C x 15 min x 0 days	4.7^{AB}	4.1^{ABC}	3.0^{ABC}
25°C x 15 min x 1 day	3.0^{CDE}	3.2^{CD}	1.6^{F}
25°C x 15 min x 7 days	< 1.0^{HIJ}	1.0^{IJ}	< 1.0^{G}
25°C x 15 min x 14 days	< 1.0^{J}	< 1.0^{J}	< 1.0^{G}
25°C x 15 min x 30 days	< 1.0^{J}	< 1.0^{J}	< 1.0^{G}
35°C x 3 min x 0 days	4.6^{AB}	4.5^{AB}	2.3^{CDE}
35°C x 3 min x 1 day	4.1^{BC}	3.6^{BC}	1.8^{EF}
35°C x 3 min x 7 days	2.0^{EFG}	1.8^{FGH}	< 1.0^{G}
35°C x 3 min x 14 days	1.1^{GHIJ}	1.0^{IJ}	< 1.0^{G}
35°C x 3 min x 30 days	< 1.0^{HIJ}	< 1.0^{J}	< 1.0^{G}
35°C x 15 min x 0 days	5.4^{A}	5.1^{A}	3.2^{AB}
35°C x 15 min x 1 day	4.4^{AB}	3.9^{BC}	3.5^{A}
35°C x 15 min x 7 days	1.8^{FGH}	1.3^{GHIJ}	< 1.0^{G}
35°C x 15 min x 14 days	1.0^{GHIJ}	1.0^{HIJ}	< 1.0^{G}
35°C x 15 min x 30 days	< 1.0^{HIJ}	1.0^{IJ}	< 1.0^{G}

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

TABLE 18. *Three-way interactions of dip temperature (°C), duration of dip (min) and length of storage (days) for mean log_{10} counts (log_{10} CFU/g) of loosely attached, strongly attached, and internalized and/or entrapped* Salmonella*/pEGFP on parsley stored at 25°C.*

Dip temp. x dip time x storage (n = 6)	Loosely attached[ad]	Strongly attached[bd]	Internalized/ entrapped[cd]
	$P = 0.0071$	$P = 0.0034$	$P = 0.0414$
5°C x 3 min x 0 days	4.5FGHI	3.7IJ	2.5DE
5°C x 3 min x 1 day	5.1DEFGH	5.2CDEFGH	4.9BC
5°C x 3 min x 7 days	6.6AB	6.4AB	5.7AB
5°C x 15 min x 0 days	4.6EFGHI	3.9HIJ	2.8DE
5°C x 15 min x 1 day	5.7BCDEFG	5.7BCDE	5.3AB
5°C x 15 min x 7 days	6.4ABC	6.5AB	6.1A
25°C x 3 min x 0 days	3.0I	2.7J	1.9E
25°C x 3 min x 1 day	4.1HI	4.3FGHI	3.8CD
25°C x 3 min x 7 days	6.0ABCDE	6.1ABC	5.9AB
25°C x 15 min x 0 days	4.4GHI	4.0GHIJ	3.0DE
25°C x 15 min x 1 day	5.8ABCDEF	5.8BCD	5.4AB
25°C x 15 min x 7 days	5.9ABCDE	6.1ABC	5.8AB
35°C x 3 min x 0 days	4.7EFGH	4.4EFGHI	2.7DE
35°C x 3 min x 1 day	5.3CDEFG	5.4BCDEF	3.9CD
35°C x 3 min x 7 days	5.7ABCDEFG	5.7BCDE	5.2AB
35°C x 15 min x 0 days	4.8DEFGH	4.7DEFGHI	3.3DE
35°C x 15 min x 1 day	6.2ABCD	6.2ABC	5.6AB
35°C x 15 min x 7 days	6.9A	7.0A	6.3A

[a]Loosely attached *Salmonella* were enumerated by rinsing 10 g of parsley with 90 ml 0.1% peptone water.
[b]Strongly attached *Salmonella* were enumerated by pummeling 10 g of parsley (pre-rinsed with 90 ml 0.1% peptone water) with 90 ml 0.1% peptone water.
[c]Internalized/entrapped *Salmonella* were enumerated on parsley that was surface-disinfected with a 2,000 mg/L sodium hypochlorite solution.
[d] Means within a column with different letters are significantly different ($P < 0.05$).

temperatures ($P < 0.05$). Differences in the means ranged from 0.2 to 0.9 $\log_{10}$ CFU/g. The number of internalized/entrapped cells on parsley stored at 4°C was significantly lower for parsley dipped at 25°C ($P < 0.05$); however, for parsley stored at 25°C, there were no differences between the means for internalized/entrapped cells on parsley. Regardless of the temperature differential, *Salmonella*/pEGFP cells were entrapped or internalized in the parsley structure. Similar to the results found in this study, Burnett and Beuchat *(35)* reported the infiltration of *E. coli* O157:H7 through the apple floral tube and its attachment to seeds, pericarp, and internal trichomes, regardless of the temperature differential of the inoculum.

In packinghouses, parsley is likely to be immersed in a dump tank for less than 3 min, but there are situations that may cause the product to be exposed to longer immersion times (i.e., employee breaks, equipment malfunctions slowing down the line, or random chance). Parsley is not necessarily removed from the tank in a "first in, first out" basis. Bartz *(11)* reported that when keeping dipping times to less than 2 min and immersion depths to less than 17 cm, infiltration of *Erwinia carotovora* subsp. *carotovora* into tomatoes could be prevented. As shown in Table 9 and 10, parsley submerged for 15 min had higher populations of loosely attached, strongly attached, and internalized *Salmonella*/pEGFP cells, which agrees with Bartz (*11*) findings. However, when considering the 3-way interactions of dip temperature, dip time, and days of storage, there were less differences seen between the 3 and 15 min dip (Tables 17 and 18). As shown in Table 17, for parsley stored at 4°C, numbers of strongly attached *Salmonella* cells were significantly higher for parsley dipped for 15 min after 0 and 1

day of storage. After 1 day of storage at 4°C or 25°C there were no differences between immersion time, regardless of the temperature of the dip suspension (Tables 17 and 18).

Growth and survival of *Salmonella*/pEGFP on parsley as affected by immersion time in the dip suspension, as well as the changes in the population over time at 4°C or 25°C storage, are illustrated in Figures 9, 10, and 11 for loosely attached, strongly attached, and internalized cells, respectively. Populations of loosely attached, strongly attached, and internalized/entrapped cells significantly increased ($P < 0.05$) during storage at 25°C over days 0, 1, and 7, regardless of the duration of immersion (Figure 9, 10, and 11). In contrast, during storage at 4°C, all populations significantly decreased over days 0, 1, and 7, regardless of immersion time ($P < 0.05$). For parsley stored at 4°C, numbers of *Salmonella* were significantly higher for parsley dipped for 15 min ($P < 0.05$); however, after day 7 there were no differences between immersion times ($P > 0.05$). For parsley stored at 25°C and sampled after day 0 and 1, populations of loosely attached cells were higher on parsley dipped for 15 min but, as seen on parsley stored at 4°C, there was no difference on and following day 7. After 7 days of storage, no internalized cells were detected on parsley stored at 4°C; whereas, viable loosely and strongly attached *Salmonella* cells were detected after 30 days of storage at 4°C. *Salmonella* cells rapidly died on parsley stored at 4°C.

As shown in Table 16, for parsley dipped for 15 min, after 1 day of storage at 25°C the mean counts for loosely attached, strongly attached, and internalized/entrapped *Salmonella* cells were 5.9, 5.9, and 5.4 $\log_{10}$ CFU/g, respectively. However, after only 1 day of storage at 4°C, the mean counts for loosely attached, strongly attached, and

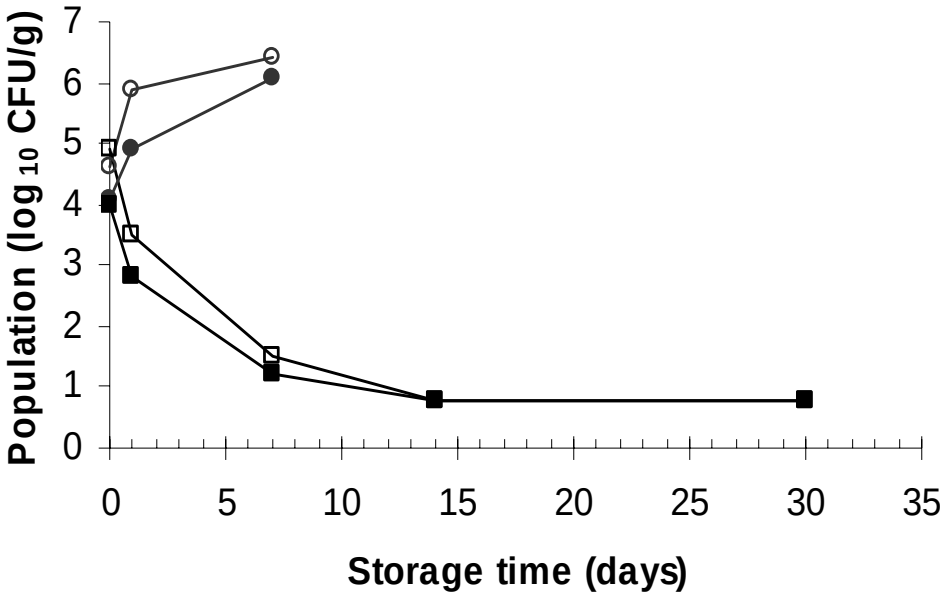

FIGURE 9. *Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of* Salmonella/*pEGFP (loosely attached) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log10 CFU/g.*

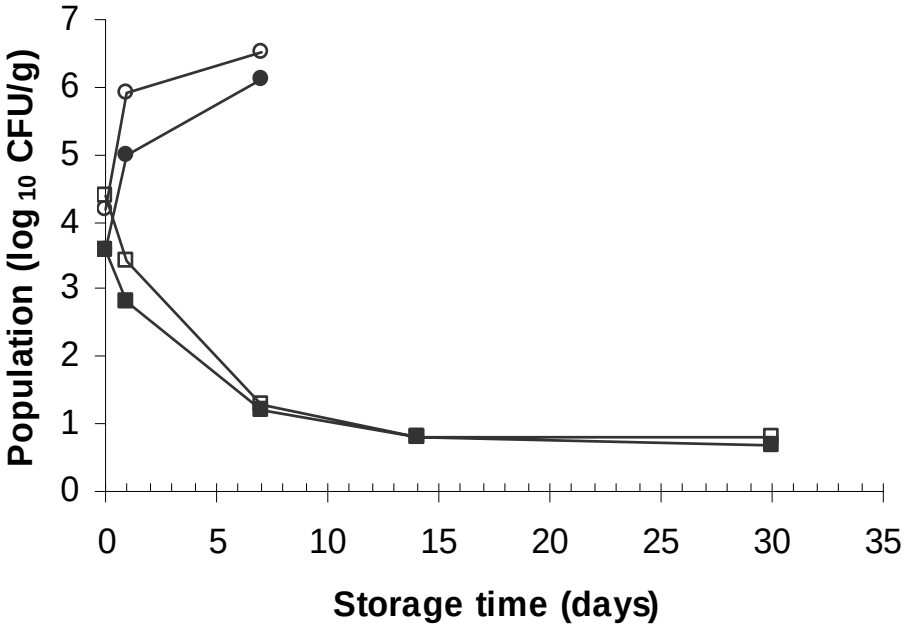

FIGURE 10. *Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of* Salmonella/*pEGFP (strongly attached) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log_{10} CFU/g.*

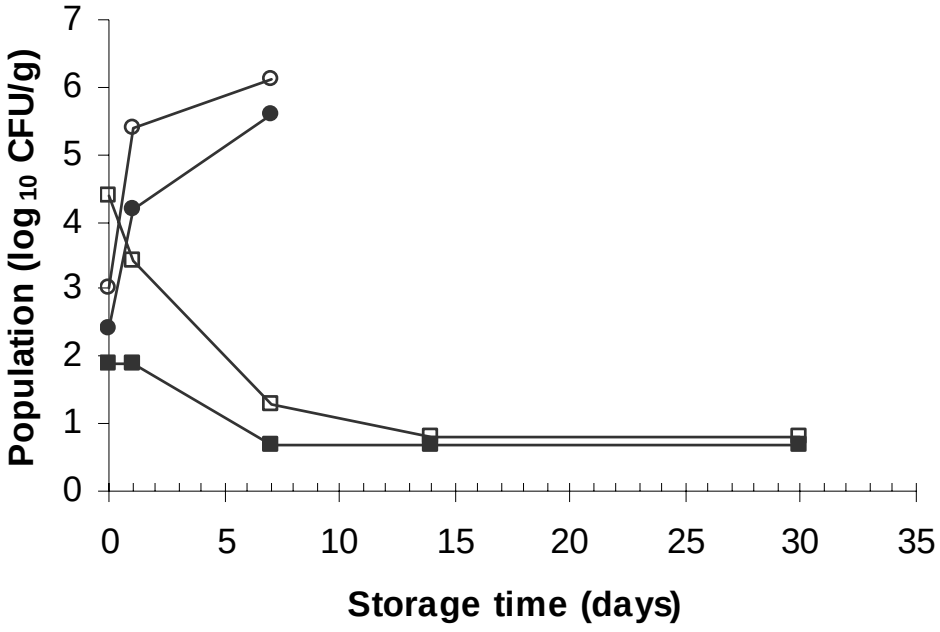

FIGURE 11. *Effect of immersion time in dip suspension (3 or 15 min) and storage temperature (4 or 25°C) on the growth and survival of Salmonella/pEGFP (internalized) cells on parsley. Parsley was dipped for 15 min with storage at 25°C (○), 15 min with storage at 4°C (□), 3 min with storage at 25°C (●), and 3 min with storage at 4°C (■). The minimum detectable limit was 0.7 log_{10} CFU/g.*

internalized cells were reduced to 3.5, 3.4, and 2.6 $\log_{10}$ CFU/g, respectively (Table 15). Storage temperature is an important control measure for maintaining the safety of fresh parsley. Additionally, as the parsley ages and begins to decay, *Salmonella* cells may be exposed to more of the phenolic acids which are known to have antimicrobial effects. Phenolic compounds, such as photoreactive furocoumarins, extracted from parsley have been shown to inhibit human pathogens, such as *E. coli* O157:H7, *L. monocytogenes*, and various spoilage organisms, such as *E. carotovora* and *L. innocua (125)*. Elgayyar et al. *(58)* reported that oil extracted from parsley was strongly inhibitory against *S. aureus*, *Y. enterocolitica*, and fungi and moderately inhibitory against *S.* Typhimurium.

Background or natural microflora on non-inoculated parsley was monitored over 0, 1, and 7 days of storage at 25°C and over 0, 1, 7, 14, and 30 days of storage at 4°C. Aerobic plate counts (APCs) and yeasts and molds were determined and are displayed in Table 19. APCs on parsley stored at 4°C and 25°C were not significantly different ($P >$ 0.05) and remained constant over the storage period. During the course of the storage period, parsley dehydrated considerably, possibly inhibiting the growth of the normal flora. The average APC for parsley, including both storage temperatures and across all days of storage was 6.4 $\log_{10}$ CFU/g. Garg et al. *(76)* reported similar counts for spinach received from the grower. Average populations of yeasts and molds, as well as APCs, remained constant over the storage period. Only one significant difference was observed on day 7. On day 7, numbers of molds on parsley stored at 4°C were significantly lower than parsley stored at 25°C; however, this may be due to sample variability. Babic et al. *(8)* also reported relatively constant yeast populations over storage for spinach leaves,

TABLE 19. *Aerobic plate counts (APCs), yeasts, and mold counts (log_{10} CFU/g) on non-inoculated parsley samples (n =3) over 0, 1, 7, 14, and 30 days of storage at 4° or 25°C.*

Organism	Storage temp (°C)	Storage time (days)				
		0[ab]	1	7	14	30
APC	4	6.4AY	6.0AY	6.4AY	6.2A	6.4A
	25	7.0AY	6.1AY	6.6AY	-	-
Yeasts	4	5.2AY	5.4AY	5.3AY	5.2A	5.6A
	25	5.5AY	5.5AY	5.5AY	-	-
Molds	4	4.5AY	4.4AY	3.9AY	4.3A	4.2A
	25	4.1AY	4.1AY	4.8AZ	-	-

[a] Means within rows, storage temperature, and organism with different letters (A,B) are significantly different ($P < 0.05$).
[b] Means within columns, storage time and organism with different letters (Y,Z) are significantly different ($P < 0.05$).

another leafy green vegetable.

Visualization of *Salmonella*/pEGFP on parsley. *Salmonella*/pEGFP cells were observed on leaf sections collected from samples of parsley after 0, 1, and 7 days of storage from each treatment. Observations on leaves submerged at different temperatures did not reveal any obvious difference in cell populations, although populations were not enumerated. Higher populations of *Salmonella* were observed on leaves that had been submerged in the dip suspension for 15 min compared to 3 min. Cells were observed near the stomata of the leaf and in fissures on the leaf surface, as shown in CSLM images in Figure 12. Cells were only observed near stomata and within cracks of the waxy cuticle. No cells were observed near the leaf edges or on intact leaf surfaces. These observations agree with the observations of *E. coli* O157:H7 on lettuce surfaces *(173, 183, 184)*. For leaf surfaces that had been surface disinfected with a 2000 mg/L sodium hypochlorite solution, no cells were observed, regardless of immersion time. Seo and Frank *(173)* reported that a 20 mg/L chlorine treatment easily killed bacteria on the surface of lettuce leaves. After 7 days of storage at room temperature it was difficult to observe cells due to a high level of plant autofluorescence which may be attributed to the decay of the plant structure and increased exposure of phenolic acids.

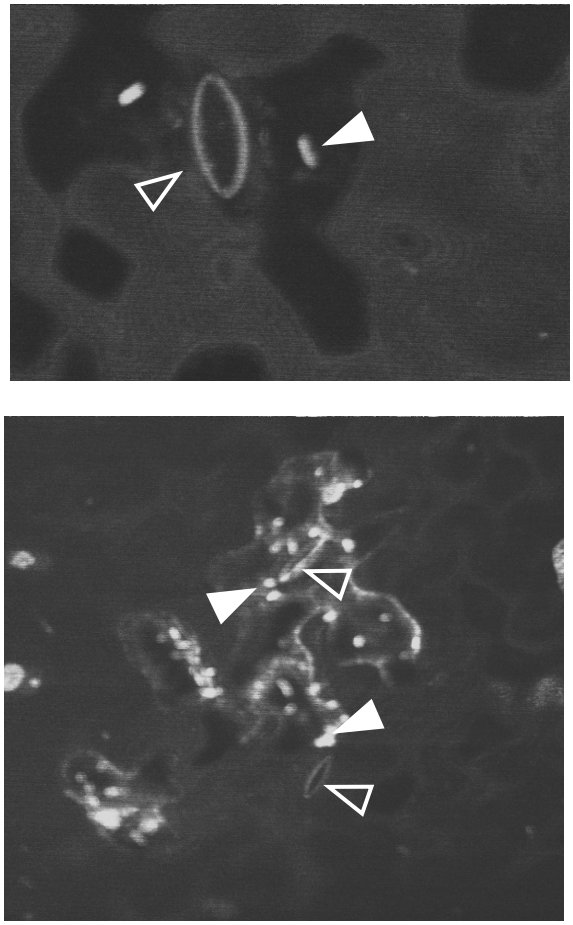

FIGURE 12. *CSLM images (*X*600) illustrating the attachment of* Salmonella*/pEGFP on parsley leaves associated with the parsley leaf stomata. Open arrows show the stomata and closed arrows indicate* Salmonella *cells.*

Changes in the microflora of parsley. The native microflora of parsley was determined on parsley samples collected from 3 different points of production including: 1) growing in the field before harvesting; 2) in the packing shed after all sorting, washing and handling; and 3) from the retail display case. Mean log counts (log_{10} CFU/g) of different bacterial groups (mesophiles, psychrotrophs, lactic acid bacteria, yeasts, and molds) on parsley are presented in Table 20. Populations of each of the bacterial groups on parsley collected in the field and shed were similar, varying by less than 0.5-log cycle. It has been reported that the bacteria normally found on fresh-cut products are the same as those normally found on produce in the field *(215)*. Higher numbers of the mesophilic and psychrotrophic bacterial populations were observed on the retail samples. As shown in Table 20, mean counts for mesophiles and psychrotrophs at retail were 7.5 log_{10} CFU/g and 8.7 log_{10} CFU/g, respectively. Babic et al. *(8)* found that initial populations of mesophilic and psychrotrophic aerobic microflora on fresh-cut spinach leaves were 10^6 to 10^7 CFU/g. Mesophilic bacteria counts on minimally processed fruits and vegetables sampled at retail have been found to be highly variable, ranging from 10^3 to 10^9 CFU/g *(143, 181)*. Although, the counts on the parsley at retail were high, the parsley exhibited good visual quality with only some small regions of minor discoloration. Barriga et al. *(10)* reported high visual quality ratings associated with psychrotrophic populations of 10^6 to 10^7. Very commonly, total microbial numbers have little or no relationship to the product quality or shelf-life of fresh produce *(215)*. Mean populations for lactic acid bacteria were 1 to 2 log_{10} CFU/g at each sampling location, which agrees with the 1 to 100 CFU/g range Li et al. *(112)*

TABLE 20. *Mean log counts (log_{10} CFU/g) of bacterial groups on parsley collected from 3 points of production.*

Group	Mean log_{10} CFU/g ± SD		
	Field[a]	Shed	Retail
Mesophiles	4.0 ± 0.7	4.8 ± 0.2	7.5 ± 0.1
Psychrotrophs	4.8 ± 0.2	5.2 ± 0.4	8.7 ± 0.2
Lactic acid bacteria	1.5 ± 0.7	1.5 ± 0.8	2.2 ± 0.2
Yeast	5.0 ± 0.2	4.7 ± 0.5	5.8 ± 0.2
Mold	4.7 ± 0.1	4.3 ± 0.4	3.3 ± 0.6

[a] Figures are averages of 3 parsley samples from each location (field, shed, and retail).

detected on iceberg lettuce obtained from a retail store. Yeast mean counts were determined to be 5.0, 4.7, and 5.8 $\log_{10}$ CFU/g for parsley collected in the field, in the shed, and at retail, respectively. Mold mean counts on parsley were similar in the field and in the packing shed (4.7 and 4.3 $\log_{10}$ CFU/g, respectively), but were lower at retail (3.3 $\log_{10}$ CFU/g). Most fresh vegetable commodities generally have fungal populations around 10^3 CFU/g with numbers usually increasing as a product moves through distribution *(30)*. In the present study, mold counts decreased while yeast counts increased (Table 20).

Percent distribution of the mesophilic microflora on parsley analyzed by sample and the average percentage distribution of organisms across the 3 samples are presented in Tables 21 and 22, correspondingly. Mesophilic microflora were determined by describing the population on TSA plates incubated for 48 h at 37°C. Populations were only described on the countable plate or plated containing between 25 and 250 colonies. For parsley collected in the field, gram-positive bacteria were predominant (53.1%; Table 22), while in the shed, gram-negative bacteria were predominant (50.5%; Table 22). When examining the percent distribution of mesophilic microflora by individual parsley samples (Table 21), it appears that there were no apparent patterns to the occurrence of any one particular organism on parsley samples collected in the field compared to the packing shed. In contrast, *Pseudomonas* was clearly predominant at retail with distributions of 90.3%, 67.6%, and 49.4% for parsley samples 1, 2, and 3 (Table 21). In Table 22, it appears that as the parsley moves through different stages of

TABLE 21. *Percent distribution of mesophilic[a] microflora on parsley samples (1, 2, and 3) collected from different stages of production.*

Microbial types	% Distribution[b]								
	Field			Shed			Retail		
	1	2	3	1	2	3	1	2	3
Gram-negative									
Achromobacter	-	-	-	19.3	-	-	-	-	-
Acinetobacter	3.2	-	6.8	3.9	-	-	-	-	-
Delftia	-	-	-	-	-	-	-	2.9	-
Enterobacter	-	-	-	-	-	-	3.2	-	-
Flavimonas	-	62.3	-	11.6	-	8.3	-	-	-
Flavobacterium	-	-	-	-	-	8.3	-	-	-
Klebsiella	-	-	-	21.2	-	-	-	5.9	-
Pantoea	-	-	-	2.0	-	-	-	-	6.2
Pasteurella	-	12.5	-	-	-	1.4	-	-	-
Pseudomonas									
aeruginosa	-	-	-	-	-	-	48.4	-	-
fluorescens	-	-	-	-	-	-	41.9	23.5	49.4
pseudoalcaligenes	-	-	-	3.9	-	-	-	-	-
putida	-	-	-	-	-	26.4	-	20.6	-
saccharophila	-	-	-	-	-	-	-	23.5	-
stutzeri	12.9	-	-	-	-	-	-	-	-
(All *Pseudomonas)*	12.9	-	-	3.9	0.0	26.4	90.3	67.6	49.4
Sphingomonas	-	16.0	-	7.7	-	37.4	-	-	-
Stenotrophomonas	-	-	-	-	-	-	-	14.7	-
Vibrio	-	-	-	-	-	-	-	-	12.3
Xanthomonas	-	-	-	-	-	2.8	-	-	-
All Gram-negative	16.1	90.8	6.8	69.6	0.0	84.6	93.5	91.1	67.9
Gram-positive									
Bacillus	5.8	2.5	58.1	5.2	13.6	4.2	-	-	0.6
Corynebacterium	29.0	3.2	-	-	66.7	1.4	-	-	-
Listeria	-	-	-	-	-	2.8	-	-	-
Micrococcus	35.5	-	8.5	2.0	5.3	-	-	-	-
Staphylococcus	-	-	10.3	5.8	-	4.2	-	-	-
Streptococcus	6.5	-	-	-	-	-	-	-	-
All Gram-positive	76.8	5.7	76.9	13.0	85.6	12.6	0.0	0.0	0.6
Other									
Yeasts, mold	-	-	0.2	0.2	-	0.1	-	-	18.5
Unidentified	7.1	3.6	16.1	17.4	14.4	2.8	6.5	8.8	13.0

[a] Organisms selected from TSA plates incubated at 37°C for 48 h.

[b] Percentages of the total number of colonies on the countable plate for each of three parsley samples (1, 2, 3) collected from each of three locations (field, shed, retail).

TABLE 22. *Average percent distribution of mesophilic*[a] *microflora on parsley (n = 3) collected from different stages of production.*

Organism	% Distribution[b]		
	Field	Shed	Retail
Gram-negative			
Achromobacter	-	6.4	-
Acinetobacter	3.3	1.3	-
Delftia	-	-	1.0
Enterobacter	-	-	1.1
Flavimonas	20.8	6.6	-
Flavobacterium	-	2.8	-
Klebsiella	-	7.1	2.0
Pantoea	-	0.7	2.1
Pasteurella	4.2	0.5	-
Pseudomonas			
aeruginosa	-	-	16.1
fluorescens	-	-	38.3
pseudoalcaligenes	-	1.1	-
putida	-	8.8	6.9
saccharophila	-	-	7.8
stutzeri	4.3	-	-
All *Pseudomonas*	4.3	10.1	69.1
Sphingomonas	5.3	15.0	-
Stenotrophomonas	-	-	4.9
Vibrio	-	-	4.1
Xanthomonas	-	0.9	-
All Gram-negative	37.9	51.2	84.3
Gram-positive			
Bacillus	22.1	7.7	0.2
Corynebacterium	10.7	22.7	-
Listeria	-	0.9	-
Micrococcus	14.7	2.4	-
Staphylococcus	3.4	3.3	-
Streptococcus	2.2	-	-
All Gram-positive	53.1	37.0	0.2
Other			
Yeasts, mold	0.1	0.1	6.2
Unidentified	8.9	11.5	9.4

[a] Organisms selected from TSA plates incubated at 37°C for 48 h.
[b] Figures are averages of three parsley samples for each sampling location.

production, the microflora becomes predominantly gram-negative, largely *Pseudomonas*. Numerous studies on various types of fresh produce report *Pseudomonas* to be the predominant microorganism of the flora *(8, 10, 79, 109, 113, 215)*. In this study, *Pseudomonas* was detected on parsley from all 3 sampling locations. Along with *Pseudomonas*, the fungi population also increased at retail from less than 1% to more than 6%. The most common organisms detected on parsley sampled from the field were *Bacillus*, *Flavimonas*, and *Micrococcus*. The most common organisms detected on parsley sampled in the packing shed were *Corynebacterium*, *Sphingomonas*, and *P. putida*. At retail, the most common organisms detected were *P. fluorescens*, *P. aeruginosa*, and *P. saccharophila*. Other organisms isolated from mesophilic plates included but were not limited to *Achromobacter*, *Flavobacterium*, *Klebsiella*, *Stenotrophomonas*, *Staphylococcus*, and *Streptococcus. Vibrio* was isolated from one retail sample. Although not expected to be present on fresh parsley, *Vibrio* has been associated with cabbage that had been irrigated with polluted water *(143)*.

Percent distribution of the psychrotrophic microflora on parsley by sample and the average percentage distribution for the three samples are presented in Tables 23 and 24, respectively. Psychrotrophic microflora was determined by describing the population isolated on TSA plates incubated for 10 days at 7°C. Populations were only described on the countable plate or plated containing between 25 and 250 colonies. For field samples, yeast and molds comprised 82.3% of the psychrotrophic microflora. Similar to what was found when TSA plates were incubated at 37°C, *Pseudomonas* were the predominant organism on retail samples plated onto TSA and incubated at 7°C for 10

TABLE 23. *Percent distribution of psychrotrophic[a] microflora on parsley samples (1, 2, and 3) collected from different stages of production.*

Microbial types	% Distribution[b]								
	Field			Shed			Retail		
	1	2	3	1	2	3	1	2	3
Gram-negative									
Actinobacillus	-	-	-	5.9	-	-	-	-	-
Cedecea davisae	-	-	-	-	1.8	-	-	-	-
Chromobacterium	-	-	-	-	-	-	-	1.7	11.4
Enterobacter	-	19.7	-	-	-	-	-	-	-
Flavimonas	2.8	-	-	-	1.8	9.1	-	-	-
Flavobacterium	-	-	-	-	-	-	14.5	20.0	-
Pantoea	-	-	-	-	3.5	-	-	10.0	-
Pasteurella	-	-	-	-	-	-	-	-	-
Pseudomonas									
aeruginosa	-	-	-	-	-	4.5	-	-	-
fluorescens	-	-	-	11.8	-	-	58.1	13.3	37.1
Putida	-	-	-	-	-	-	-	1.7	11.4
Stutzeri	-	-	-	-	-	-	-	-	5.7
Syringae	-	-	-	-	-	-	-	35.0	-
(All *Pseudomonas)*	-	-	-	11.8	-	4.5	58.1	50.0	54.2
Sphingomonas	-	1.6	2.5	-	-	-	-	-	-
Xanthomonas	-	3.2	-	-	-	4.5	-	5.0	17.1
All Gram-negative	2.8	24.5	2.5	17.7	7.1	13.6	72.6	86.7	82.7
Gram-positive									
Bacillus	-	-	-	-	7.0	4.6	3.2	-	-
Clavibacter	-	1.6	-	2.9	50.9	4.5	-	-	-
Corynebacterium	-	-	-	5.9	-	18.2	1.6	-	-
Gemella	-	-	1.3	-	-	-	-	-	-
Micrococcus	-	1.6	0.1	-	-	-	-	-	-
Staphylococcus	-	3.2	-	-	-	-	-	-	-
Streptococcus	-	-	-	-	-	-	-	-	-
All Gram-positive	-	4.8	1.4	8.8	57.9	27.3	4.8	-	-
Other									
Yeasts, mold	88.9	65.6	92.4	50.0	22.8	40.9	-	-	-
Unidentified	8.3	4.9	3.8	23.5	12.3	18.2	22.6	13.3	17.1

[a] Organisms selected from TSA plates incubated at 7°C for 10 days.

[b] Percentages of the total number of colonies on the countable plate for each of three parsley samples (1, 2, 3) collected from each of three locations (field, shed, retail).

TABLE 24. *Average percent distribution of psychrotrophic[a] microflora on parsley (n = 3) collected from different stages of production.*

Organism	% Distribution[b]		
	Field	Shed	Retail
Gram-negative			
Actinobacillus	-	2.0	-
Cedecea	-	0.6	-
Chromobacterium	-	-	4.4
Enterobacter	6.6	-	-
Flavimonas	0.9	3.6	-
Flavobacterium	-	-	11.5
Pantoea	-	1.2	3.3
Pseudomonas			
aeruginosa	-	1.5	-
fluorescens	-	3.9	36.2
putida	-	-	4.4
stutzeri	-	-	1.9
syringae	-	-	11.7
All *Pseudomonas*	-	5.4	54.2
Sphingomonas	1.4	-	-
Xanthomonas	1.1	1.5	7.4
All Gram-negative	10.0	14.3	80.8
Gram-positive			
Bacillus	-	3.9	1.1
Clavibacter	0.5	19.4	
Corynebacterium	-	8.0	0.5
Gemella	0.4	-	-
Micrococcus	0.6	-	-
Staphylococcus	1.1	-	-
All Gram-positive	2.6	31.3	1.6
Other			
Yeasts, mold	82.3	36.7	-
Unidentified	5.7	18.0	17.7

[a] Organisms selected from TSA plates incubated at 7°C for 10 days.
[b] Figures are averages of three parsley samples for each sampling location.

days. The 3 most common species of *Pseudomonas* found on retail samples were *P. fluorescens* (36.2%), *P. syringae* (11.7%), and *P. putida* (4.4%), (Table 24). For samples collected in the shed, the most commonly isolated organisms were gram-positive and fungi. Other organisms isolated from psychrotrophic plates included *Chromobacterium*, *Clavibacter*, *Flavimonas*, *Flavobacterium*, *Corynebacterium*, *Pantoea agglomerans,* and *Xanthomonas*.

Clearly, parsley has the ability to support the growth of a diverse population of microorganisms. This product is exposed to a variety of conditions and handling as it moves from the field, through processing and packing in the shed, to the retail display. Each of these stages of processing affects the natural microflora of parsley and there exists a potential for parsley to become contaminated with human pathogens.

CONCLUSIONS

A total of 25 *Salmonella* isolates were obtained from 1,257 samples. Sixteen, 6, and 3 isolates were obtained from irrigation water, packing shed equipment, and washed cantaloupe, respectively. *Salmonella* was not detected on oranges and parsley. *Salmonella* was most commonly isolated from irrigation water and, more specifically, from water reservoirs. Isolates obtained from packing shed equipment were most frequently detected on surfaces exposed to washed product, not intended for any further processing before boxing. *Salmonella* was detected on washed cantaloupe, but not detected on the surfaces of oranges or parsley likely attributed to the rough surface characteristics of the cantaloupe and its close proximity to the soil and irrigation water.

Serotyping and DNA-based typing showed the 25 *Salmonella* isolates to be genetically diverse. Serotypes included *S*. Anatum, *S*. Arizona, *S*. Javiana, *S*. Muenchen, *S*. Newport, *S*. Rubislaw, and *S*. Texas. The two most commonly isolated serotypes were *S*. Javiana and *S*. Arizona. The majority of these isolates were susceptible to all antibiotics tested. Five of the isolates demonstrated intermediate sensitivity to streptomycin and one isolate was resistant to streptomycin. Using PFGE, all of the *Salmonella* isolates obtained from irrigation water and equipment surfaces were different from the *Salmonella* isolates obtained from the surface of the cantaloupe. The majority of isolates from equipment surfaces were unrelated to isolates obtained from irrigation water. Only one isolate obtained from an unloading ramp was found to be genetically similar to *Salmonella* isolates obtained from irrigation water.

Results obtained using Rep-PCR were less discriminatory than results obtained using PFGE. PFGE was able to discriminate between strains that by Rep-PCR and serotyping were indistinguishable. Rep-PCR showed more similarity between isolates from irrigation water with isolates obtained from equipment. Isolates obtained from cantaloupe were 87% similar to isolates obtained from equipment surfaces. This study did not demonstrate a connection between irrigation water and pathogenic contamination on fresh produce.

E. coli, which has been shown to be an effective indicator of fecal contamination on fresh produce (*82*), was detected on all type of commodities (cantaloupes, oranges, and parsley) as well as in irrigation water samples and on equipment surface swabs. *E. coli* was detected most frequently on cantaloupe, followed by parsley and oranges. As demonstrated with *Salmonella* prevalence, cantaloupe tended to be a more contaminated product. For all 3 types of commodities sampled, *E*. coli was detected more often on product sampled in the packing shed, following all sorting and washing, indicating that the packing shed is an important source of bacterial contamination on produce. *E. coli* was detected in all sources of irrigation water, but more likely to be detected in irrigation water collected from dirt canals than cement irrigation canals. *E. coli* may be concentrating in the water-soil interface.

Green fluorescent protein served as an effective marking system to monitor the attachment and survival of *Salmonella* on parsley. Transformed *Salmonella* strains continued to express the pEGFP plasmid through consecutive subculturing and storage at 4°C. Numerous studies have concluded that organisms transformed to express green

fluorescent protein have the same growth kinetics as their parent strains. This study demonstrated the same results and revealed only minimal differences between the growth of the parent and recombinant strains.

After arriving at the packing shed, it is common for harvested fruit and vegetables to be submerged in a dump tank to remove field heat and visible soil contamination. As more than one commodity may be run through the tank during one processing day, there are opportunities for commingling of product and, consequently, spreading of pathogens. In this study, it was found that the temperature of a simulated dump tank or dip suspension had very little effect on the attachment, internalization, and survival of *Salmonella* on fresh Italian parsley. Regardless of the temperature of the dip suspension, *Salmonella* cells were internalized in the structure of the parsley. It was found that immersion for longer times in the dip suspension resulted in higher numbers of loosely attached, strongly attached, and internalized *Salmonella*. Observations of leaf sections using confocal scanning laser microscopy confirmed these findings. *Salmonella* cells were observed near stomata and within cracks of the waxy cuticle.

Storage temperature had the greatest impact on the survival of *Salmonella* on parsley. When stored at 25°C, parsley had a shelf-life of 7 days with the population of *Salmonella* significantly increasing over the 7 days of storage. For parsley stored at 4°C, numbers of *Salmonella* decreased over days 0, 1, and 7. After 7 days of storage, there were no viable, internalized *Salmonella* detected. Storage temperature represents an important control point for the safety of fresh parsley.

Examination of the native microflora of parsley revealed that the populations of mesophilic and psychrotrophic bacteria, lactic acid bacteria, yeasts, and molds on product collected in the field and the packing shed were similar. However, higher numbers of mesophilic and psychrotrophic bacteria and fungi were observed on retail samples. There were no apparent patterns to the occurrence of any one particular organism on parsley collected in the field compared to the packing shed. In contrast, *Pseudomonas* was the predominant organism on parsley at retail. As parsley moved through different stages of production, the microflora became predominantly gram-negative, largely *Pseudomonas*. It was shown that parsley has the ability to support the growth of a diverse population of microorganisms.

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