ESCHERICHIA COLI O157:H7 CELL SURFACE MOIETIES TRIGGER PLANT DEFENSE RESPONSE INFLUENCING SURVIVAL OF THAT PATHOGEN ON PLANTS

by

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ABSTRACT OF THE DISSERTATION

Escherichia coli O157:H7 cell surface moieties trigger plant defense response influencing survival of that pathogen on plants

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Previous studies have shown that human pathogens are able to colonize plants epiphytically and endophytically by utilizing similar mechanisms as phytopathogens. In addition, it has been suggested that colonization of plants by human pathogens induces plant defense responses, although some of their pathogenic mechanisms differ from those of phytopathogens. However, it is not well understood what factors are involved in intimate association of foodborne pathogens with plants and the plant response to enteric bacteria.

To address these questions, we investigated whether *Escherichia coli* O157:H7 cell surface structures induce plant host defense responses and subsequently affect colonization or survival of the pathogen on plants. Also, the influence of growth medium

or exposure conditions on bacterial cell surface structures, particularly in exopolysaccharides, was investigated.

Among the pathogen associated molecular patterns (PAMPs), flagella made a substantial impact on survival of *E. coli* O157:H7 on the *Arabidopsis* plant. Curli were recognized by the *Arabidopsis* plant and induced plant defense responses. *E. coli* O157:H7 mutants lacking flagella or curli induced lower PR (pathogenesis-related) genes based on the weak β-glucuronidase (GUS) activity compared to flagella or curli positive *E. coli* O157:H7, resulting in increased survivals of those mutants on the plant.

E. coli O157:H7 grown in Luria-Bertani (LB) broth supplemented with manure extracts showed a significant 58% increase in capsular polysaccharides (CPS) production as compared to cells grown in LB medium alone. Exposure of *E. coli* cells to soil and manure extracts also stimulated the CPS compared to the non-exposed control. Plants inoculated with *E. coli* O157:H7 with a greater amounts of CPS resulted in less inductions of PR genes compared to those with less CPS, contributed to the enhanced survivals on the *Arabidopsis* plant.

Cell surface structures of *E. coli* O157:H7 have a significant impact on the induction of differential plant defense responses, thereby impacting survival of the pathogen on plants. Growth medium or exposure environment conditions showed a great impact on bacterial CPS, which enabled the human pathogen to persist longer on the plant by possibly evading plant defense responses.

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Dedication

This work is dedicated to my parents, my wife Joo-Yeon, and my two lovely sons, Minsung and Minkyu.

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List of Abbreviation

BGL2	β-1,3-glucanase
COR	Coronatine
CPS	Capsular polysaccharides
CRI	Congo red indicator
EPS	Exopolysaccharides
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FLS2	Flagellin-sensitive 2
GUS	β-glucuronidase
HC	Hemorrhagic colitis
HR	Hypersensitive response
HUS	Hemolytic uremic syndrome
ISR	Induced systemic resistance
JA	Jasmonic acid
LB	Luria-Bertani
LPS	Lipopolysaccharides
MAMPs	Microbe-associated molecular patterns
4-MU	4-Methyl umbelliferone
MUG	4-Methylumbelliferyl- β-D-glucuronide
NB	Nutrient broth

NB-LRR	Nucleotide binding- leucine rich repeat
NOS	Nitric oxide synthase
PAMPs	Pathogen associated molecular patterns
PR	Pathogenesis related
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species
RPs	Resistance proteins
SA	Salicylic acid
SAR	Systemic acquired resistance
SPI	*Salmonella* pathogenicity island
Tafi	Thin aggregative fimbriae
TLR2	Toll-like receptor 2
TSA	Trypric soy agar
TSB	Tryptic soy broth
TTSS	Type three secretion system

CHAPTER 1

Introduction

1.1. Background and Significance

During the past decade, microbial safety of fresh produce has become a major concern in the United States. Microbiological contamination of foods seem to be more threatening than chemical hazards with respect to safety of public health since microbial risks are more frequently reported and can generally make a large number of people sick with acute symptoms in a short period of time. Over the past several decades, the number of outbreaks caused by foodborne pathogens associated with consumption of fresh fruits and vegetables has substantially increased in the United States. Between 1973 and 1997, 190 produce-associated outbreaks were reported to the Center for Disease Control and Prevention (CDC), resulting in 16,058 illnesses, 598 hospitalizations, and eight deaths (Sivapalasingam et al., 2004). During 5 subsequent years (1998-2002), 279 produce-associated outbreaks were reported, involved with 10,533 illnesses and seven deaths (CDC, 2006A). In 2011, a large Shiga toxin-producing *Escherichia coli* O104:H4 outbreak in Germany resulting in 852 HUS cases and 32 HUS associated deaths (CDC, 2011). Consumption of sprouts has been implicated as a possible source of the German outbreak.

Consumption of fresh fruits and vegetables in the United States has dramatically increased with the increased awareness of the health benefits of fresh produce. However, many raw fresh produce items, especially leafy greens have been recognized as important carriers of enteric pathogens that were traditionally associated with animal-based foods in

the past. Thus, special attention has been given to the safety of fresh produce with respect to public health. Reasons for increased foodborne outbreaks might be attributed in large part to increased consumption of fresh fruits and vegetables, changes in agronomic practices, post-harvest practices, distribution patterns, as well as better surveillance and detection methods (Beuchat, 2002). The U.S. Food and Drug Administration (FDA) announced a guidance for five commodity groups that are responsible for the massive produce-associated outbreaks: cantaloupes, lettuce and leafy greens, tomatoes, green onions, and herbs (FDA, 2007). Among all produce commodities, leafy greens including lettuce, and tomatoes are top two items, accounting for 47% of all produce associated outbreaks during 1998-2006 (Buchanan, 2006). Various pathogens have been implicated in outbreaks associated with fresh fruits and vegetables, with *Salmonella* and *E. coli* O157:H7 being the most dominant (Sivapalasingam et al., 2004).

Recent studies have shown that human pathogens utilize similar mechanisms as plant pathogens to invade or colonize plants although the mechanism of pathogenicity may differ. Plants are able to recognize enteric pathogens and initiate defense systems to avoid bacterial proliferation on plants. Knowledge about the mechanism by which human pathogens colonize and persist on or in fruits and vegetables is limited. Also it is not clear how bacterial surface structures influence bacterial colonization or persistence on plant surfaces. Moreover, previous studies were mainly focused on genetic analyses rather than practical behaviors of foodborne pathogens in field conditions. Therefore, further research on the Plant-Human pathogen interaction was needed to better understand the behaviors of human pathogens on the plant.

1.2. Objectives

The objectives of this research are to provide a better understanding of the factors involved in intimate association of foodborne pathogens with plants and the plant response to enteric bacteria.

Specific objectives of this research are;

1. To investigate whether *E. coli* O157:H7 cell surface structures influence plant host defense responses and subsequently affect colonization or survival of the pathogen on plants.

2. To investigate whether growth medium or exposure conditions influence the bacterial exopolysaccharides, and whether that precipitates changes in plant defense responses, consequently affecting bacterial survival on plants.

These objectives are presented in Chapters three and four in this dissertation.

CHAPTER 2

Literature Reviews

2.1. *Escherichia coli* O157:H7 and Foodborne Illness

Escherichia coli is a Gram-negative, facultative anaerobic bacterium, and one of the many groups of bacteria that live in the intestines of healthy humans and most warm-blooded animals. Most strains of *E. coli* are considered as harmless; however, a particular strain of *E. coli*, such as *E. coli* O157:H7 can cause severe illness in human. Diarrheagenic *E. coli* are categorized into six classes: enterohemorrhagenic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), enteropathogenic (EPEC), and diffusely adherent (DAEC), based on their virulence, pathogenicity, or clinical symptoms (Buchanan and Doyle, 1997). *E. coli* O157:H7 infection can cause severe bloody diarrhea and abdominal cramps. Hemolytic uremic syndrome (HUS) is a severe complication of *E. coli* O157:H7 infection, characterized by hemolytic anemia, thrombocytopenia, leading to renal failure (Banatvala et al., 2001). *E. coli* O157:H7 was first recognized as a human pathogen in 1982 during investigation of outbreaks of hemorrhagic colitis (HC) from eating improperly cooked hamburger patty that was contaminated with the bacteria (Riley et al., 1983). Produce-associated outbreaks of *E. coli* O157:H7 were first reported in 1991 and is second most common vehicle among foodborne outbreaks followed by outbreaks linked to ground beef (Figure 2-1). *E. coli* O157:H7 outbreak associated with fresh produce accounted for 38 (21%) of 183 foodborne outbreaks and 34% of 5,269 foodborne outbreak-related cases during 1982-2002 (Rangel et al., 2005). One of the deadliest outbreaks in the United States

occurred in 2006; a multistate outbreak associated with consumption of pre-packed spinach resulting in 203 cases, 102 hospitalizations, 31 patients developed HUS and 3 deaths (CDC, 2006B).

2.2. Sources of Contamination

Fruits and vegetables can become contaminated at any point in the production chain; pre-harvesting, harvesting, post-harvesting processing, handling, and distribution. A number of studies have investigated the potential contamination sources of produce at the pre-harvest stage (field). Contaminated irrigation water, inadequately composted animal manures, feces of wild animals, and insects have been considered as potential sources of contamination (Figure 2-2) (Brandl, 2006). Water is most likely to be an important source of contamination in the field via direct deposition of fecal material into agriculture surface water or run-off water from the nearby livestock facilities by flooding or rainfall. *E. coli* O157:H7 was reported to persist in municipal well water for over 40 days (Rice et al., 1992) and in distilled or deionized water over 50 days (Kolling and Matthews, 2001). Animal manure is commonly applied to agricultural fields to fertilize soils. Thus, the use of improperly treated feces from domestic livestock may increase the risk of microbial contamination of crops in the field. *E. coli* O157:H7 can survive more than 5 months in soils amended with contaminated composts or with contaminated irrigation water, regardless of source or crop type (Isalm et al., 2004, Wang et al., 1996). Extended survival of enteric pathogens in an agricultural environment will increase the chance of crop contamination, although the survival of an enteric pathogen outside the host or on crops may be greatly affected by environmental factors such as temperature

fluctuation, nutrient availability, and UV radiation (Brandl, 2006). After harvest, enteric pathogens are able to survive for an extended period of time, depending on the environmental conditions, such as temperature, water availability, available nutrients, and the presence of commensal microflora on the plant (Brandl, 2006; Cooley et al., 2006). In addition, insects have been considered as a possible source of contamination. Talley et al. (2009) showed that files caught in leafy green production fields near rangeland habitats were positive (11 of 18 flies) for *E. coli* O157:H7 and transfer of the pathogen to the leaf surface resulted in contamination of crops.

2.3. Plant-Microbe Interactions

2.3.1. Attachment

Attachment is the first step in plant-microbe interactions and an essential process for subsequent colonization or survival of enteric pathogens within or outside plant tissue. Physicochemical properties of the bacterial surface structures, properties of the attachment surface, and the surrounding medium influence bacterial attachment to the surface (Frank, 2001). Bacterial adherence to plant host tissues is a complicated process and it can be divided into two major stages; primary or docking stage and the secondary or locking phase (Dunne, 2002). Primary stage is reversible and is dictated by physiochemical variables such as electrostatic, van der Waal, and hydrophobic interactions between planktonic microorganisms and surface. Attached bacterial cells in this stage can be easily removed by mild mechanical forces. In the secondary phase, binding is irreversible and molecularly mediated between specific bacterial adhesions and the surface. Bacterial surface structures such as pili, curli fimbriae, and flagella may

involve in secondary step. At the conclusion, adhesion becomes irreversible and the organism is firmly attached to the surface. At this stage, attached cells are very difficult to remove from the surface, and strong shear force or chemical/biological reagents are needed to detach the cells from the surface (Bower et al., 1996).

2.3.2. Roles of Bacterial Cell Surface Structures in Plant-microbe Interactions

Research demonstrates that bacterial surface components, such as curli fimbriae, flagella, capsular polysaccharides (CPS), or exopolysaccharides (EPS) play an important role in interactions between bacteria and abiotic or biological surfaces. These bacterial surface structures may directly or indirectly influence initial adhesion or development of biofilm formation. Effects of bacterial surface structures on adherence to abiotic or biological surfaces have been investigated by several authors.

Curli are a long, thin, coiled extracellular amyloid structure on cell surface of most pathogenic *E. coli* strains (Olsen et al., 1989), and homologous surface proteins are expressed in *Salmonella* spp. known as thin aggregative fimbriae (Tafi). Two curli gene operons (*csg*BA and *csg*DEFG) are involved in curli biogenesis (Figure 2-3) (Barnhart and Chapman, 2006). CsgD is a positive transcriptional regulator of the *csg*BA operon encoding the major structural units of curli. Curli-positve (curli expressor) bacterium typically produces dark red or brownish colonies, while curli-negative bacterium (non-curli expressor) forms white colonies on Congo red indicator agar (Figure 2-4). Curli plays an important role in the adhesion of *E. coli* to its contact hosts (Gophna et al., 2002) and in the development of biofilm on inert surfaces (Vidal et al., 1998). Previous studies showed that curli are typically expressed under stressful conditions, such as low osmotic

pressure, suboptimal growth temperature below 30°C, and nutrient limitations (Olsen et al., 1993). Starvation was shown to induce expression of fimbriae controlled by the sigma factor RpoS, produced during entry into stationary phase (Moreno et al., 2000). Curli fibers are expressed by many pathogenic isolates of *E. coli* and *Salmonella* spp. as a survival mechanism (Boyer et al., 2007). Solomon et al (2005) reported that 100% of *Salmonella* tested clinical isolates and meat-related isolates produced curli, and 80% of produce-related isolates showed curli biosynthesis. White et al (2006) found that fimbriae positive *Salmonella* Typhimurium showed increased survival, up to several months in the absence of nutrients compared to mutants deficient in fimbriae and/or cellulose production. Introduction of the gene encoding for curli production into the non pathogenic *E. coli* K12 strain resulted in significantly higher ability of attachment to plant surfaces while the wild-type *E. coli* K12 failed to adhere (Jeter and Matthysse, 2005). However, mutation of *csgA*, the regulator of curli expression in the pathogenic *E. coli* strain, did not show significant changes in attachment, suggesting that curli are not necessary for attachment of pathogenic strains of *E. coli* indicating more than one mechanism is involved in the binding process. Lapidot and Yaron (2009) reported that extracellular structures, curli and cellulose are important factors in bacterial transfer or survival on parsley plants. Significantly higher numbers of bacteria were found in the edible portion when plants were irrigated with strains that produce cellulose and curli than strains negative for cellulose and curli production. The *agfB* mutant of *Salmonella enterica* Newport, which lacks curli and the surface-bound protein, showed reduced initial attachment and colonization onto alfalfa sprouts compared to the wild type strain (Barak et al., 2005).

Capsular polysaccharides (CPS) are highly hydrated molecules (95% water) and covalently attached to the outer membrane of the cell wall. CPS is produced by the cell in response to environmental conditions and generally regarded as important virulence factors. They are composed of repeating monosaccharide units with glycosidic linkage (Roberts, 1996). Capsules may protect the bacterial surface from desiccation, promote adherence of bacteria to both surfaces and each other, and confer resistance to the host immune system (Jann and Jann, 1990; Roberts, 1996). Hassan and Frank (2004) found that *E. coli* O157:H7 grown in TSB were more encapsulated compared to the same strain grown in nutrient broth. They also reported that the more encapsulated organism showed higher adherence to lettuce and apple surfaces. However, conflicting results were reported indicating that CPS does not enhance or mediate bacterial adhesion but contributes to voluminous three dimensional biofilm structures or maturation of biofilms (Prigent-Combaret et al., 2000; de Rezende et al., 2005). Hanna et al. (2003) reported that capsular polysaccharide colonic acid does not enhance bacterial adhesion but rather inhibits the establishment of specific binding between pathogens and inert substrates. LPS and O-antigen have been associated with cell adherence and colonization. O-antigen deletion mutant of *E. coli* O157:H7 exhibited less persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa (Sheng et al., 2008). Barak et al. (2007) reported that mutations in the O-antigen capsule of *Salmonella enterica* resulted in reduced attachment of that pathogen to alfalfa sprouts.

Flagella are a structural component of bacteria providing the function of bacterial motility. In addition, flagella have been shown to mediate adherence of bacteria to surfaces and to affect biofilm formation on abiotic or biological surfaces. Flagella of

enteropathogenic *E. coli* were shown to mediate adherence to cultured human epithelial cells (Girón, 2002). Expression of flagella protein seems to be an important factor that affects interactions between the cells and contact surfaces. Flagella mediated motility has been known to play an important role in the initial steps of biofilm development in *Pseudomonas* sp. (DeFlaun et al., 1994). Knockout of the flagellin gene of *Salmonella* Typhimurium DT104 resulted in decreased biofilm formation on contact surfaces (Kim and Wei, 2009), and mutation of *fliC* flagellin gene in *E. coli* O157:H7 rendered the organism significantly less adherent to baby spinach and lettuce leaf surfaces (Xicohtencatl-Cortes et al., 2009). Cooley et al. (2003) reported that non-motile *Salmonella* mutant exhibited decreased colonization and limited migration of bacteria from roots to edible portion of the plant compared to the wild-type strain.

2.3.3. Behavior of Enteric Pathogens on the Plant

Enteric pathogens on the leaf surface encounter unfavorable environment conditions, i.e., fluctuations in temperature, large shift in osmotic conditions, lack of nutrients, as well as presence of UV radiation (Brandl, 2006). Human pathogens have not evolved to be as well adapted as plant-colonizing endophytes, and are not considered to be a part of the phyllosphere microbial habitat. However, many studies have shown that enteric pathogens, e.g., *E. coli* O157:H7 and *Salmonella* can persist on the leaf surface for an extended period of time. *E. coli* O157:H7 were able to persist in the lettuce and parsley phyllosphere for 77 and 177 days, respectively, following exposure of plants to contaminated manure or irrigation water (Islam et al., 2004A). Another study showed that *S. enterica* Typhimurium on carrots and radishes survived for 203 and 84 days,

respectively, after seed sown in contaminated soil (Islam et al., 2004B). Persistence or survival of the enteric pathogen on plant surface was influenced by method of inoculation, presence of epiphytes, as well as location at which the pathogen was applied. Lettuce plants inoculated using spraying irrigation resulted in greater chance of transmission of *E. coli* O157:H7 onto plants than those inoculated with surface irrigation (Solomon et al., 2002). Co-inoculation of *S*. Newport and *E. coli* O157:H7 with *Enterobacter asburiae* on lettuce plants resulted in a 10-fold decreased growth of enteric pathogens possibly due to the competition of available carbon and nitrogen nutrients from the plant (Cooley et al., 2006). Ericson et al. (2010) reported that survival of *E. coli* O157:H7 was higher when the pathogen was applied on the leaf abaxial (lower) side than when applied on the adaxial (upper) side. Unlike plant pathogens, enteric pathogens are unable to penetrate or degrade the leaf epidermis; they rely entirely on natural openings, such as stomata and accidental wounds to enter internal tissues. The presence of phytopathogens may enhance growth of enteric pathogens on the plant surface because destruction of the physical outer barrier by phytopathogens allows enteric pathogens to enter internal tissue and gain access to nutrient sources for growth (Wells and Butterfield, 1999).

2.3.4 Endophytic Colonization of Enteric Pathogens

Many studies have investigated the internalization of enteric pathogens in plant tissue, and reported that internalization may occur in a number of types of fruits and vegetables via various mechanisms at different sites. However, how the bacteria gain access to internal tissue and where they are located within plants are not clearly understood. Internalization of human pathogens into produce commodities has been a

particular concern with respect to microbial safety of produce since internalized cells may be protected from sanitizing treatments during post-harvest processing. In addition, internalized cells on growing plants are less likely to experience environmental stresses, thus cells may survive or persist longer on the plant. Disrupted leaf surface and structural components of intact plant tissue, such as stomata, hydathodes, and trichomes are considered as potential sites of bacterial entry. Dense population of *E. coli* O157:H7 were observed in both internal and external lettuce tissue at the site of leaf tip burn lessions (Brandl, 2008). Shoot injury on lettuce plants increased persistence of *E. coli* most likely due to the increased nutrients from damaged tissue (Harapas et al., 2010). Several studies have reported evidence of internalization of lettuce plants by *E. coli* O157:H7. Solomon et al. (2002) found that *E. coli* O157:H7 can enter the lettuce plant through the root system and migrate throughout the edible portion of the plant. Internalized cells were observed in intracellular space at depth of 45 μm. Another study showed that *E. coli* O157:H7 preferentially bind to roots of lettuce from contaminated irrigation water, and cells were observed in the xylem (Wachtel et al., 2002). Methods of irrigation were shown to influence transmission of human pathogens to growing plants in the field. A study has shown that internalization of *E. coli* O157:H7 did not occur in spinach plants when plants were exposed to contaminated soil, while internalization was observed in plants inoculated through the leaf surface drop method (Mitra et al., 2009). *Salmonella* were also shown to endophytically colonize plants. Significant levels of internalized *S.* Typhimurium cells were observed in lettuce seedlings grown in contaminated hydroponic and soil systems (Franz et al., 2007). *S. enterica* Dublin endophytically colonized the lettuce plants grown in contaminated soil (Klerks et al.,

2007). Root inoculation of *Arabidopsis* plants with *E. coli* O157:H7 and *Salmonella* resulted in internalization in the lettuce plant via invasion of the roots at lateral root junctions (Cooley et al., 2003).

2.4. Plant Defense System

2.4.1. Innate Immune System

In nature, plants are continuously threatened by various pathogens, including viruses, bacteria, fungi, oomycetes, and insects. Unlike animals, plants do not have an adaptive immune system, therefore, they solely rely on the innate immunity (Jones and Dangl, 2006). To protect themselves against the wide variety of microbial and fungal pathogens, plants utilize multiple physical barriers and preformed antimicrobial metabolites to attenuate invasion by potential attackers (Pieterse et al., 2009). Unlike fungal pathogens, enteric bacteria are unable to penetrate the physical barrier of the leaf. However, bacteria can enter the plant host through natural openings and accidental wounds. As a first line of defense, plants have evolved elaborate strategies to recognize their attackers and translate this recognition into an effective immune response. Jones and Dangl (2006) proposed four phased 'zigzag' model plant immune system (Figure 2-5). In phase 1, primary innate immunity is achieved through the pattern recognition receptors (PRRs) that recognize conserved microbe-associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs). Upon recognition, plants initiate diverse downstream signaling events eventually resulting in an activation basal resistance, called PAMP-triggered immunity (PTI). PAMPs that trigger innate immune response include flagellin, LPS fraction of Gram-negative bacteria as well as chitins, and glucans

from fungi (Nürnberger et al., 2004). Typical PTI responses include deposition of callose in the cell wall, production of reactive oxygen species, and defense-related proteins, which negatively affect bacterial colonization (Nicaise et al., 2009). In phase 2, pathogens inject multiple effectors into the plant host by using the type three secretion system (TTSS), which suppresses the PTI responses and promotes virulence of the pathogen, resulting in effector-triggered susceptibility (ETS). In phase 3, plants in turn acquired an NB (nucleotide binding)-LRR (leucine rich repeat) R protein that recognizes specific effectors, activating effector-triggered immunity (ETI). ETI is an accelerated PTI response, resulting in enhanced disease resistance. In phase 4, pathogens acquire new effectors through natural selection to avoid ETI, resulting in suppression of ETI. Natural selection results in new plant NB-LRR alleles (new R protein) that can recognize one of the new effectors so that plants gain ETI again. The balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to activate effective defenses determines the fate of pathogens and plants (Pieterse et al., 2009).

2.4.2. Induced Plant Defense Responses

Plants are also protected by induced defense responses, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Figure 2-6). A systemic defense involves triggering defense responses in distal parts of the plant once plant defenses are activated at the site of infection, resulting in protection throughout the plant against subsequent attacks by a broad spectrum of microorganisms (Durant and Dong, 2004). SAR is triggered by PTI- and ETI-mediated pathogen recognition and characterized by increased levels of endogenous salicylic acid (SA) (Mishina and Zeier, 2007). Salicylic

acid (SA) is an essential signal molecule for the onset of SAR and required for the expression of PR (pathogenesis related) genes. SAR is a SA-dependent pathway. *Arabidopsis* mutant plants that are impaired in SA signaling failed to activate SAR and a set of PR genes upon pathogen recognition by plants (Durant and Dong, 2004). ISR is induced by colonization of roots by rhizosphere bacteria in a similar manner as SAR, and is regulated by jasmonic acid (JA) and ethelene (ET) (Pieterse et al., 1998). Recognition of soil-borne microbes by plants results in a mild, but effective activation of the plant immune responses and is associated with priming for enhanced defenses (Van Wees et al., 2008).

2.4.3. Plant Defense Response to Plant and Enteric Pathogens

Induction of the plant defense response is dependent on the nature and pathogenicity of microorganisms. In general, for the successful colonization of pathogens on plants, pathogens should overcome or suppress the host defense mechanism. For instance, the plant pathogen *Pseudomonas* syringae is able to inject multiple effector proteins into the plant host by utilizing TTSS to suppress PAMP-triggered immune responses (Li et al., 2005). Recent studies have shown that not only plant pathogens induce plant defense responses but also human pathogens induce defense responses upon colonization. Klerks et al. (2007) reported that colonization of lettuce plants with *Salmonella enterica* Serovar Dublin induced increased expression pathogenicity-related (PR) genes. Schikora et al. (2008) showed that *Salmonella* can colonize *Arabidopsis* plants through intact shoots or roots and proliferate in intracellular space, resulting in wilting, chlorosis, and even death. *Arabidopsis* plants infected with *Salmonella* induced

the expression of SA- and JA-regulated pathogenesis-related (PR) genes. Recognition of *E. coli* O157:H7 by *Arabidopsis* plant resulted in stomatal closure which is a part of SA-regulated plant innate immune response to restrict bacterial entry (Melotto et al., 2006). TTSS are found in many pathogens, such as *Salmonella*, EHEC, and EPEC, and *Shigella* species. These structures mediate the transfer of protein effectors directly from the bacterial to the host cell cytoplasm. TTSS plays an important role in their enhanced pathogenicity in the host and is conserved across the Gram-negative plant and enteric pathogens although the function of effectors may differ. Transcriptional gene expression analysis revealed that the gene induction of *Arabidopsis* plants inoculated with *E. coli* O157:H7 was strikingly similar to the induction elicited by the *Pst* DC3000 *hrpA* mutant (defective in COR toxin production), implying that the enteric pathogen does not produce or carry COR toxins contributing to the suppression of SA-dependent host defense mechanism (Thilmony et al., 2006). A recent study revealed that tobacco plants inoculated with live *S.* Typhimurium did not induce oxidative burst, while plants treated with killed *S.* Typhimurium and purified LPS induced plant defense responses, suggesting that *S.* Typhimurium can actively suppress the plant response and successfully colonized tobacco plants (Shirron and Yaron, 2011). However, the precise role and the mechanism of effector proteins of enteric pathogens in plant defense system during bacterial infection are not well understood.

Plants recognize PAMPs, such as flagellin, LPS, chitins, and glucans from fungi and these components were considered as potential inducers of plant defense responses (Nürnberger et al., 2004). *Arabidopsis* flagellin receptor, FLS2 (Flagellin-sensitive 2), recognizes a 22 amino acid residue of the most conserved part in the N terminus of

flagella (Felix et al., 1999). *Arabidopsis* plants treated with flg22 (a peptide representing the elicitor-active epitope of flagellin) induced the expression of many defense-related genes and exhibited increased resistance to the plant pathogen, but not in mutant plants lacking flagellin receptor gene *FLS2* (Zipfel et al., 2004). Local infection of *Arabidopsis* leaf with flagellin induced SAR (Mishina and Zeier, 2007). Lipopolysaccharides (LPS) are the cell surface component of Gram-negative bacteria that contribute to the barrier function of the outer membrane and also can interact with host responses. LPS can be recognized by plants to induce some defense-related responses in plants (Dow et al., 2000). *Arabidopsis* plants treated with LPS induced nitric oxide synthase (NOS) and activated an array of defense-related genes (Zeidler et al., 2004). Induction of oxidative burst response with increased H_2O_2 production was observed in tobacco plants treated with LPS (Meyer et al., 2001). Mishina and Zeier (2007) found that local treatment of *Arabidopsis* with LPS induced SA-mediated PR gene expression and SAR.

Many studies utilizing bacterial mutants deficient in one of the PAMPs and mutant plants impaired in defense-related mechanism were conducted to elucidate the role of plant defense response in persistence or colonization by pathogens. *Salmonella* mutant lacking flagellin and TTSS showed significantly higher endophytic colonization on *Arabidopsis* plants compared to the wild-type strain, suggesting that flagella and TTSS are recognized by the plant, and induce plant defense response(s) (Iniguez et al., 2005). Moreover, enhanced colonization of *Salmonella* was observed in both *Arabidopsis* mutants *NahG* (deficient in SA accumulation) and *npr*1-1 (nonexpresser of PR genes) compared with the wild-type plant, implying that both SA-dependent and SA-independent defense pathways were involved in restriction of microbial colonization.

Psudomonas aeruginosa PA14 colonized an *Arabidopsis* mutant impaired in defense system (*npr1*) to a greater extent than wild-type *Arabidopsis* plant (Volko et al., 1998). These studies suggests that the lack of one of the outer structures of a microorganism may contribute to enhanced persistence through escaping detection by plant defenses.

2.4.4. Evasion and Suppression of Host Basal Defenses

Many pathogenic and symbiotic bacteria have developed a wide range of strategies to avoid the plant defense system and to protect themselves. Moletto et al. (2006) showed that the phytotoxin coronatine (COR) secreted from *P*. syringae pv. tomato DC3000 suppresses SA-dependent defenses, resulting in increased virulence to the plant. The phytotoxins COR contribute to virulence and disease symptom development. Structural changes or loss of PAMPs may result in evasion of the immune response to pathogens. Post-translational modification of flagellin allows pathogens to avoid PRR-mediated recognition, resulting in increased susceptibility to pathogens (Taguchi et al., 2003; Felix et al., 1999). Bacteria are able to modify the structure of LPS, including the lipid A component, in response to host microenvironments (Darveau, 1998) and modification to the O-antigen during symbiotic relationship between bacteria and plants has been reported (Lerouge and Vanderleyden, 2002). Variations in O-antigen during the infection enable pathogenic bacteria to escape the plant host immune system. Bacterial polysaccharides, such as exopolysaccharides (EPS), capsular polysaccharides (CPS), and lipopolysaccharides (LPS), play an important role in evasion of the defense responses. Bacterial polysaccharides provide protection against host defenses, such as reactive oxygen species (ROS), antimicrobial peptides and acidification. Biofilm, a

structured community enclosed in self-produced exopolysaccharide matrix that confers resistance against the host's defense response (D'Haeze and Holsters, 2004). Bacterial EPS or CPS may completely mask the underlying antigenic molecules. Expression of EPS for some phytopathogenic bacteria have been shown to be essential for virulence. EPS on *Erwinia amylovora* may act by masking cell surface molecules that might be recognized by plants to elicit a defense response. *E. amylovora* mutant lacking EPS production resulted in pathogen recognition by the plant defense mechanism (Bugert and Geider, 1995). In addition, Aslam et al. (2008) reported that bacterial EPS suppress PAMP-induced signaling in plants through chelating divalent calcium ions, required for defense induction. EPS-deficient *Xanthomonas campestris pv. campestris* (*Xcc*) elicited calcium influx and expressed defense-related genes in *Arabidopsis*, while wild-type strain suppressed induced defense responses without interfering PAMP-receptor bindings.

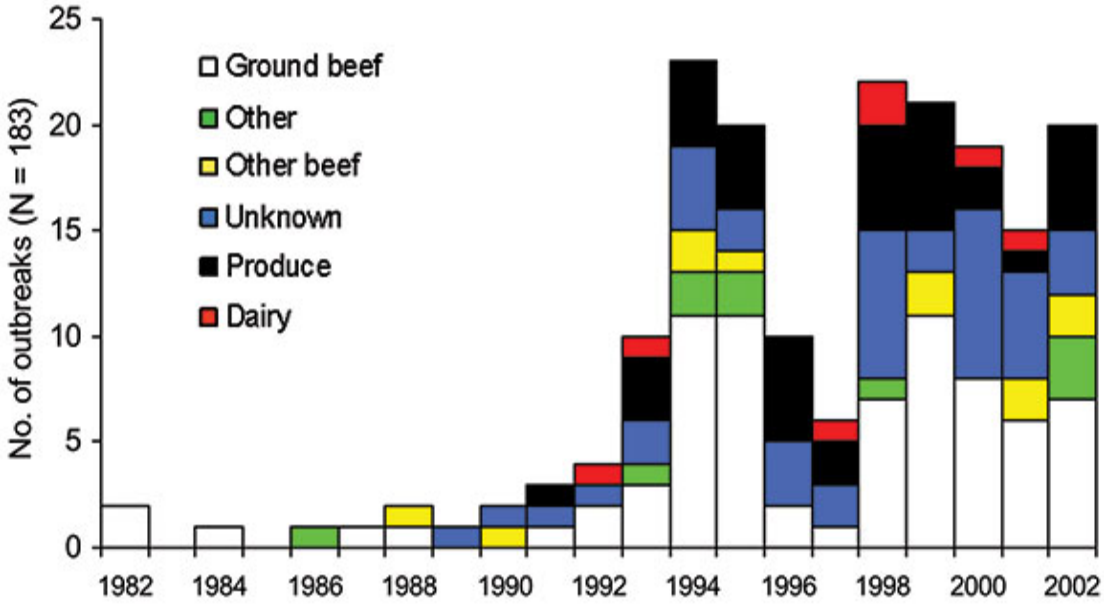

Figure 2-1. Vehicle of foodborne *E. coli* O157:H7 outbreaks during 1982-2002.

Adapted from Rangl et al., 2005.

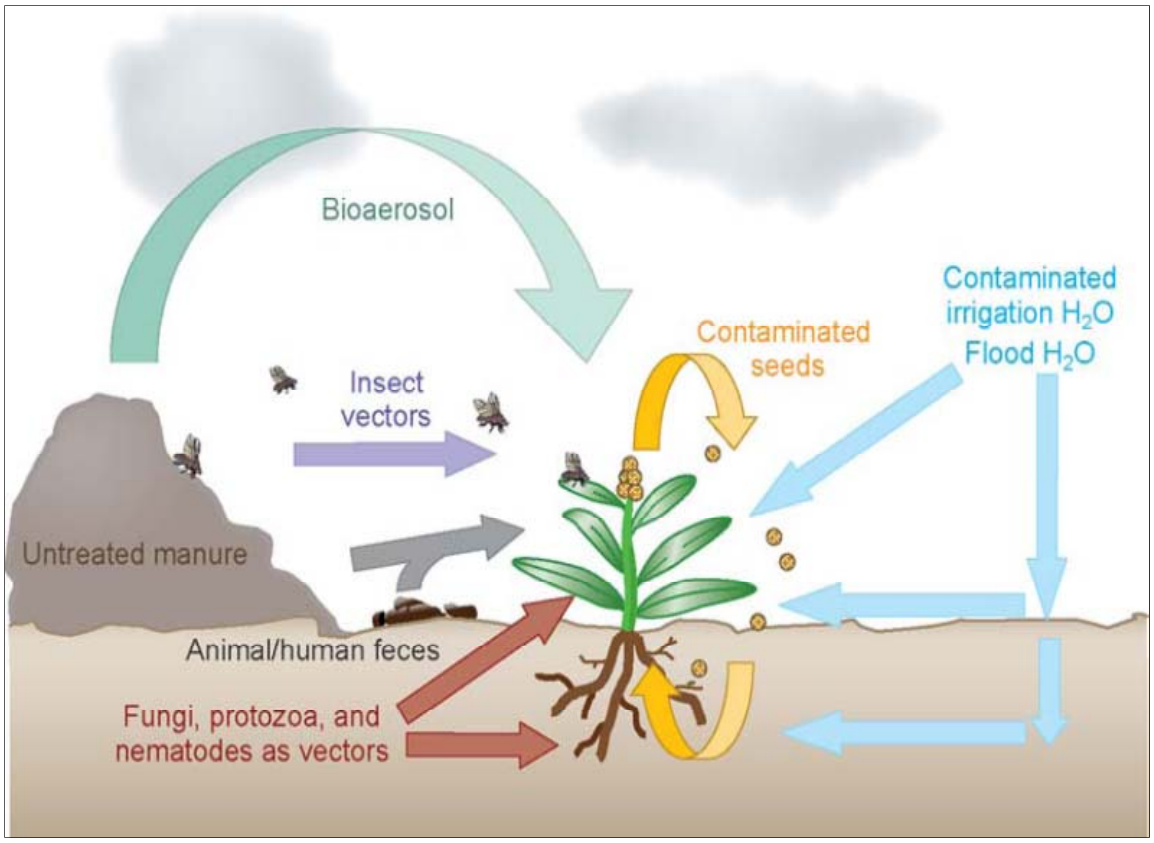

Figure 2-2. Schematic illustration of factors that can contribute to the contamination of fruits and vegetables with enteric pathogens in the field. *Adapted from Brandl, 2006.*

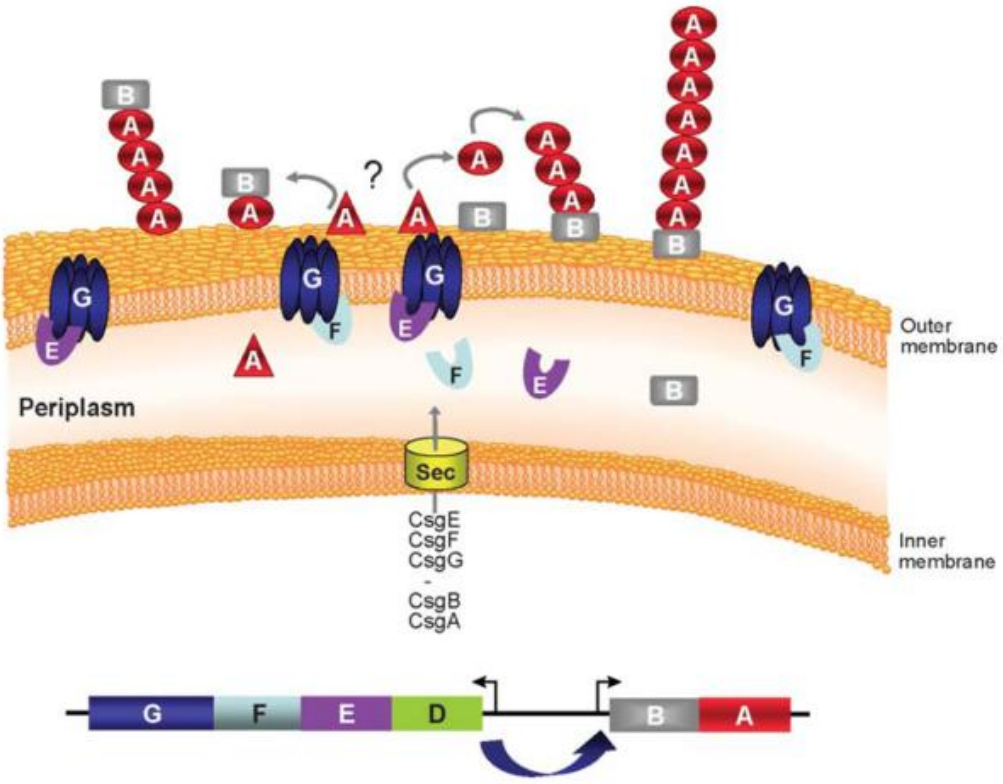

Figure 2-3. Schematic diagram of curli biogenesis. The *csg*BA operon encodes the major structural subunit, CsgA, and the nucleator protein CsgB. The *csg*DEFG encodes four accessory proteins required for curli assembly. CsgD is a positive transcriptional regulator of the *csg*BA operon. *Adapted from Barnhart and Chapman (2006).*

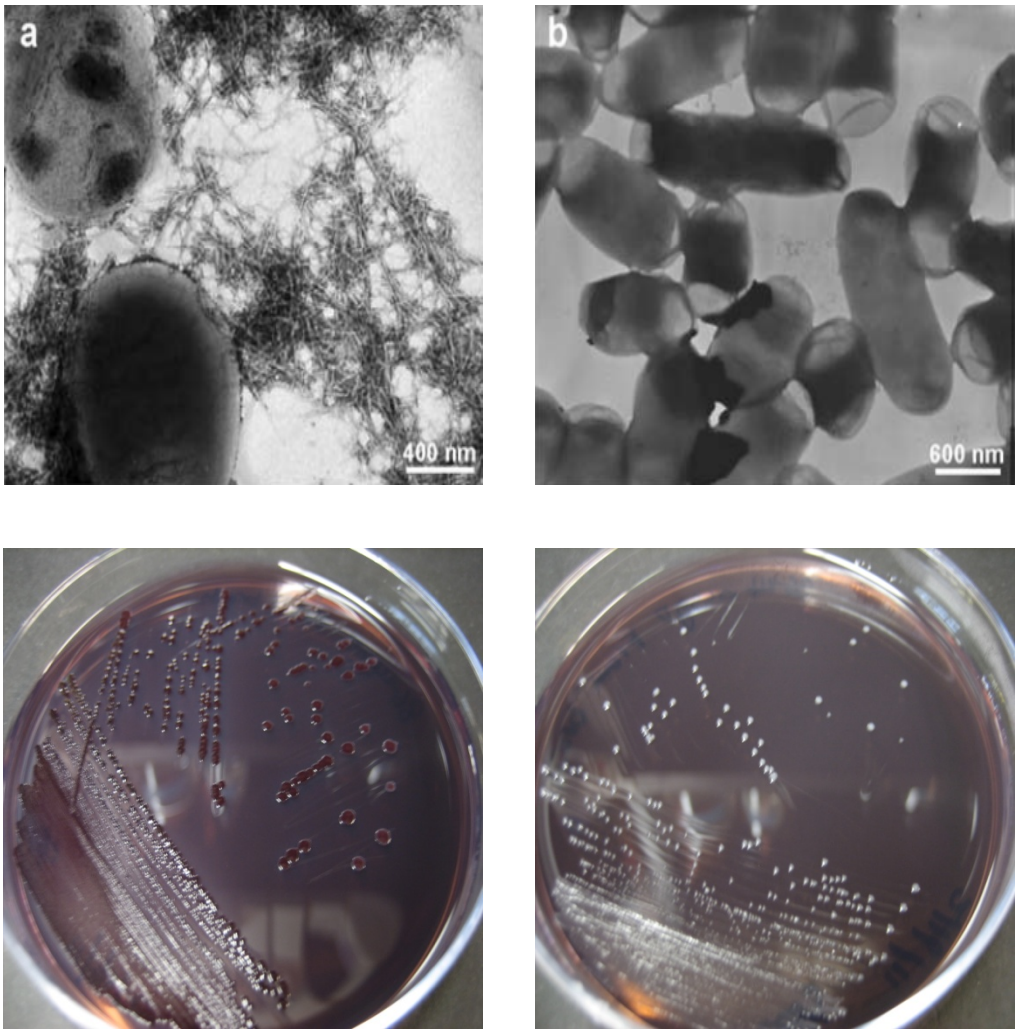

Figure 2-4. Electron micrograph of curli and colony morphology of *E. coli* O157:H7 on Congo red indicator agar. Curli-Positive bacterium (top left) and curli-negative bacterium (top right). *Images adapted from Barnhart and Chapman (2006).* Curli-positive *E. coli* O157:H7 86-24 wild type (bottom left) forms red/dark brown colonies, and curli deficient mutant of *E. coli* O157:H7 86-24 Δ*csgD* forms white colonies (bottom right).

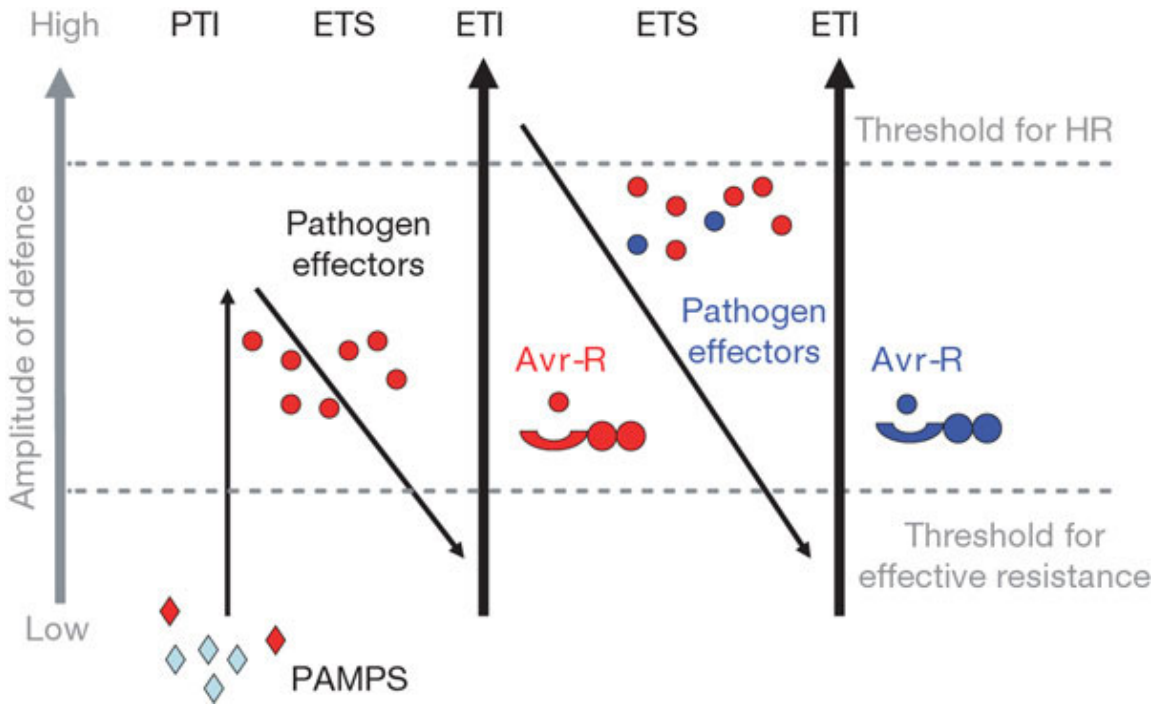

Figure 2-5. A zigzag model illustrates the quantitative output of the plant immune system. The definition of basal defense would be PTI - ETS + ETI. Plant detects PAMPs (red diamond) via PRR to trigger PTI (Phase 1). Pathogens deliver effectors that interfere PTI, resulting in ETS (Phase 2). Recognition of one effector (red) by disease resistance R protein, activating ETI (Phase 3). Pathogenic isolates are selected that have lost red effector, obtained new effectors (blue), help pathogens to suppress ETI (Phase 4). New R protein (new R specificities) resulted from natural selection recognizes one of the newly acquired effector, triggering ETI again. Figure adapted from Jones and Dangl (2006).

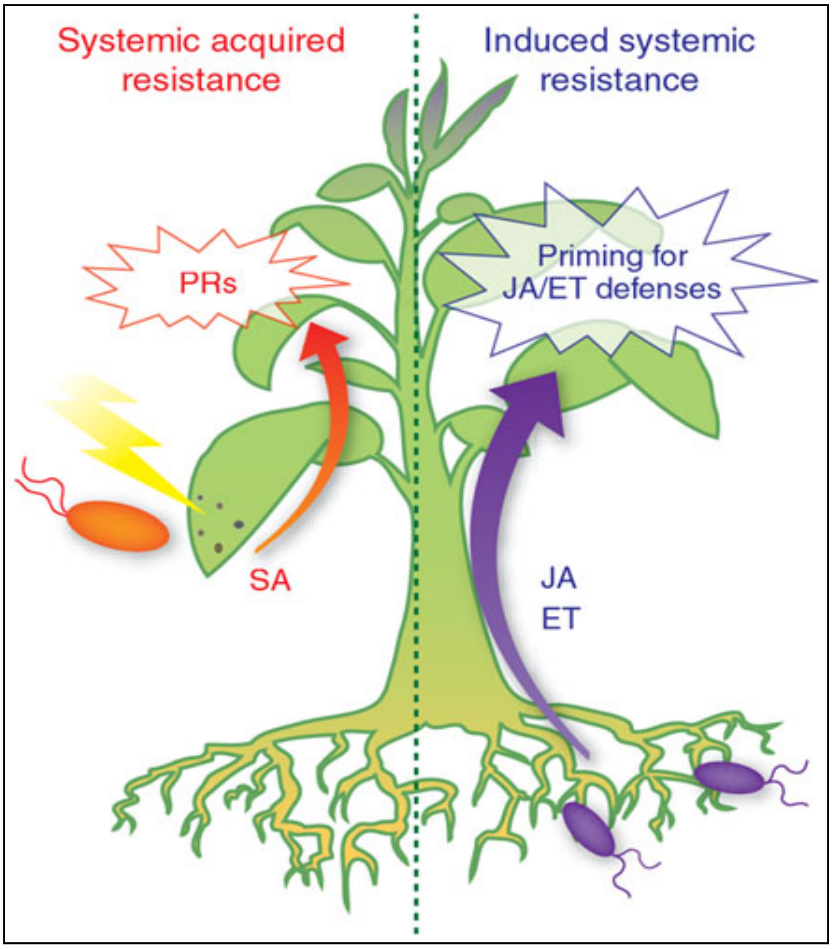

Figure 2-6. Schematic illustration of systemically induced immune responses. Systemic acquired resistance (SAR) is typically activated in healthy tissues of locally infected plants. Induced systemic resistance (ISR) is activated upon colonization of plant roots by beneficial microorganisms. Signaling molecules (SA, JA, ET) travel through the vascular system to activate defense system in distal parts of leaf. *Figure adapted from Pieterse et al. (2009).*

CHAPTER 3

Influence of the Plant Defense Response to *Escherichia coli* O157:H7 Cell Surface Structures on Survival of That Enteric Pathogen on Plant surfaces

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3. 1. ABSTRACT

Consumption of fresh and fresh-cut fruits and vegetables contaminated with *Escherichia coli* O157:H7 has resulted in hundreds of cases of illness and, in some instances, death. In this study, the influence of cell surface structures of *E. coli* O157:H7, such as flagella, curli fimbriae, lipopolysaccharides, or exopolysaccharides, on plant defense responses and on survival or colonization on the plant was investigated. The population of *E. coli* O157:H7 ATCC 43895 wild-type strain was significantly lower on wild-type *Arabidopsis* plants than that of the flagellum-deficient mutant 43895. The population of the *E. coli* O157:H7 flagellum mutant 43895 was greater on both wild-type and *npr1-1* mutant (nonexpressor of pathogenesis-related [PR] genes) plants and resulted in less PR gene induction, estimated based on a weak β-glucuronidase (*GUS*) signal, than did the 43895 wild-type strain. These results suggest that the flagella, among the other pathogen associated molecular patterns (PAMPs), made a substantial contribution to the induction of plant defense response and contributed to the decreased numbers of *E. coli* O157:H7 ATCC 43895 wild-type strain on the wild-type *Arabidopsis* plant. A curli-deficient *E. coli* O157:H7 86-24 strain survived better on wild-type *Arabidopsis* plants than the curli-producing 86-24 wild-type strain did. The curli-deficient *E. coli* O157:H7 86-24 strain exhibited a GUS signal substantially lower than that of the curli-producing wild-type strain. Curli were recognized by plant defense systems, consequently affecting bacterial survival. The cell surface structures of *E. coli* O157:H7 have a significant impact on the induction of differential plant defense responses, thereby impacting persistence or survival of the pathogen on plants.

3. 2. INTRODUCTION

A large number of human disease outbreaks caused by foodborne pathogens have been associated with consumption of contaminated fresh produce, such as alfalfa sprouts, lettuce, spinach, parsley, and cantaloupes. Produce-associated outbreaks accounted for 21% (38/183) of foodborne outbreaks and 34% of (1792 of 5,269) foodborne outbreak-related illnesses from 1982 to 2002 (Rangel et al., 2005). Manure and irrigation water, particularly the method of application, and feral animal feces have been considered as potential sources of pathogenic microorganisms associated with fresh produce (Beuchat and Ryu, 1997). Bacterial surface components, such as curli (fimbriae), flagella, lipopolysaccharides (LPS), and exopolysaccharides (EPS), play an important role in the interactions between bacteria and abiotic or biological surfaces. Curli, also known as thin aggregative fimbriae (Tafi) in *Salmonella,* are found on many pathogenic *E. coli* O157:H7 and *Salmonella* (Olsén et al., 1993; Solomon et al., 2005) strains and are important for bacterial adhesion to plant surfaces (Barak et al., 2005; Lapidot and Yaron, 2009) and biofilm development on inert surfaces (Vidal et al., 1998). Flagella have been reported to mediate bacterial attachment to abiotic or biotic surfaces and biofilm formation on contact surfaces (Kim and Wei, 2009; Xicohtencatl-Cortes et al., 2009). EPS and LPS are involved in bacterial adherence, colonization, and biofilm structure (Barak et al., 2007; Hassan and Frank, 2004). In addition, those bacterial surface structures were demonstrated to activate host defenses, inducing host immune responses (Tukel et al., 2005; Mishina and Zeier, 2007).

Plants have evolved a range of constitutive and inducible mechanisms to protect themselves against microbial pathogens by producing antimicrobial compounds and defense-related proteins, and by induction of local or systemic acquired resistance (SAR) (Durant and Dong, 2004). The molecular mechanism(s) by which enteric pathogens penetrate the physical outer barrier and access the host plasma membrane is unknown. Natural openings such as stomata and wounds are believed to be major potential gateways for pathogen entry. Primary innate immunity is achieved through pattern recognition receptors (PRRs) that recognize conserved pathogen associated molecular patterns (PAMPs). PAMPs that trigger innate immunity include flagellin, the LPS fraction of Gram-negative bacteria, chitins, and glucans from fungi (Nürnberger et al., 2004). Upon recognition by the plant, primary innate defense responses, such as deposition of callose in the cell wall, production of reactive oxygen species and defense-related proteins, which negatively affect bacterial colonization (Nürnberger et al., 2004). are induced. Plant pathogens inject multiple effectors into the host plant through the type III secretion system (TTSS), suppressing the primary innate defense system. To defend against pathogens plants also have evolved secondary defense responses including the activation of resistance proteins (RPs) that inhibit the effectors and subsequently trigger RP-mediated defense responses (de Wit, 2007). Plants are protected also by induced defense responses such as systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is induced by pathogens that cause necrotic lesions, the result of hypersensitive response (HR) or disease symptoms, resulting in protection throughout the plant against a broad spectrum of microorganisms. Activation of SAR is dependent on the signal molecule salicylic acid (SA) and is associated with expression of pathogenesis-

related (PR) genes (Durant and Dong, 2004). ISR is induced by the colonization of roots by rhizosphere bacteria and is activated by jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 1998).

Research on the interactions between foodborne pathogens and plants is limited compared to that on phytopathogens. Previous studies have demonstrated that human enteric pathogens induce plant defense responses upon colonization. Lettuce plants responded at the molecular level to the presence of *Salmonella* Dublin, resulting in increased expression of PR genes (Klarks et al., 2007). *Salmonella* can infect *Arabidopsis* plants, multiply in the plant tissue and cause multiple disease symptoms, such as wilting and chlorosis. Upon infection with *Salmonella* many defense-related genes, regulated by SA- and JA- defense pathways, were expressed (Schikora et al., 2008). Inoculation of *Arabidopsis* with *E. coli* O157:H7 triggered stomatal closure, which is associated with the SA-dependent defense response (Melotto et al., 2006). A recent study showed that live *S.* Typhimurium can actively suppress the defense responses in tobacco plants. Infection with live *Salmonella* did not induce plant defense responses, although heat-killed *Salmonella* and purified LPS did so (Shirron and Yaron, 2011).

Based on previous studies, it is evident that human pathogens are able to colonize plants and induce plant defense responses, although some of their pathogenic mechanisms differ from those of phytopathogens. The induction of plant defense systems by different cell surface structures of *E. coli* O157:H7 is not well understood. Plant defense responses to curli, which are found in many pathogenic *E. coli* O157:H7 strains, have not been reported. The objectives of this study were to investigate whether

E. coli O157:H7 cell surface structures influence plant host defense responses and subsequently affect colonization or survival of the pathogen on plants.

3. 3. MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 3-1. *E. coli* O157:H7 and *Klebsiella pneumoniae* 342 were routinely grown in tryptic soy agar (TSA: Difco, Becton Dickinson, Sparks, Md) at 37°C with appropriate antibiotics. *Pseudomonas syringae* pv tomato (*Pst*) DC3000 and *P. syringae* pv *maculicola* (*Psm*) ES4326 *avrRpt2* were cultured in King's medium B at 30°C. Stock cultures were maintained in tryptic soy broth (TSB: Difco, Becton Dickinson, Sparks, Md) containing 30% glycerol at –70^{o} C. Expression of curli by the wild-type and mutant strains was determined on congo red indicator (CRI) agar. CRI is composed of casamino acids (10 g/L), yeast extract (1g/L), and bacto agar (15 g/L) supplemented with congo red (20 mg/L) and coomassie brilliant blue (10 mg/L) (Hammar et al., 1996). Plates were incubated at 25^{o}C for 48 h before being examined for evidence of curli production. Antibiotics were added to the medium at the following concentrations: 100 µg/mL ampicillin for *E. coli* O157:H7 43895 wild-type and 43895 lab mutant, 25 µg/mL kanamycin for 43895 flagella mutant, 50 µg/mL streptomycin and 30 µg/mL nalidixic acid for *E. coli* O157:H7 86-24 wild type, 50 µg/mL kanamycin and 50 µg/mL streptomycin for *E. coli* 86-24 curli mutant and 86-24 LPS mutant, 50 µg/mL rifampin for *P. syringae* pv. tomato (*Pst*) DC3000 and *K.*

pneumoniae strain 342, and 100 µg/mL streptomycin and 10 µg/mL tetracycline for *P. syringae* pv. *maculicola* (*Psm*) ES4326.

Plants and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) wild-type (CS 70000) and *npr1-1* (CS 3726) were obtained from the Arabidopsis Biological Resource Center, The Ohio State University. Seeds of Col-0 transgenic line (*BGL2*::*GUS*) were kindly provided by Dr. Xinnian Dong (Duke University, NC, USA). The *npr1-1* (nonexpresser of pathogenesis-related [PR] genes) mutant exhibits weak expression of PR genes in response to various systemic acquired resistance (SAR) –inducing treatments, as described previously (Cao et al., 1994). *BGL2-GUS* transgenic plants contain a β-glucuronidase reporter gene (*GUS*) driven by the β-1,3-glucanase (*BGL2*) promoter (Cao et al., 1994). *Arabidopsis* plants were grown in Metromix 360 soil in a climate-controlled greenhouse (22°C ± 2°C, relative humidity 70% ± 5%) under natural light supplemented with 16 h per day of artificial light.

Bacterial challenge of plants

Pseudomonas strains were grown overnight at 30°C in TSB with shaking at 200 rpm. *K. pneumoniae* 342 and *E. coli* O157:H7 strains were grown overnight at 37°C in TSB. Bacterial cells were harvested by centrifugation and resuspended in sterile distilled water to achieve approximately 10^8 CFU/ml. Bacterial levels in water were confirmed by plating serial 1:10 dilutions on TSA. For colonization experiments, 5 week-old wild-type and *npr1-1* mutant plants were dipped in a bacterial suspension for 30 s and the plants

were placed in a growth room under a transparent plastic dome for 1 day to maintain high humidity. Control plants were treated with sterile distilled water and grown as described. At days 0, 1, 3, and 5 postchallenge, three plants from each treatment group were harvested. Weighed plant samples were placed in a sterile stomacher bag with 0.1% peptone water (1:10, wt/vol), macerated by hand, and stomached for 3 min. Appropriate dilutions of the homogenates were plated in duplicate on TSA supplemented with appropriate antibiotics. The populations were calculated and reported as log CFU per g fresh weight of plants. The experiment was conducted twice. For the GUS activity experiment, 4 week-old *BGL2-GUS* transgenic plants were dipped in a bacterial suspension as described above. For the negative and positive controls, plants were dipped in sterile distilled water or 1 mM salicylic acid (SA) for 30 s, respectively. At day 5 postinoculation, plants were harvested and GUS assays were conducted.

GUS histochemical analysis

Pieces of plant leaves were immersed in GUS staining buffer containing 0.1 M Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc; stock solution prepared in dimethylformamide at 50 mM) (Jefferson et al., 1987). Samples in buffer were infiltrated under vacuum for 10 min and then incubated at 37°C for 16 h. After removal of the staining buffer, leaf tissues were soaked for 30 min each in 30, 75, and 95% ethanol solutions to remove chlorophyll. Stained samples were observed using a dissecting microscope.

Quantitative analysis of GUS activity

Whole plants were homogenized using a pre-chilled micro pestle in 200 µL of GUS extraction buffer (50 mM Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 10 mM β–mercaptoethanol) (Jefferson et al., 1987) and centrifuged for 20 min at 16,000 × g at 4°C. Supernatants were assessed for protein concentration and enzyme activity. Protein concentrations were determined by the Bradford method, and GUS reactions were carried out by adding 20 µL of extracts into a reaction tube containing 980 µL of GUS extraction buffer containing 1 mM MUG (4-methylumbelliferyl- β-D-glucuronide) in 37°C. After 0, 10, 20, or 40 min incubation, 200 µL from each reaction tube were added to a tube containing 800 µL 0.2 M Na_2CO_3 to stop the reactions. Fluorescence was measured using a Perkin Elmer LS-50B luminescence spectrometer with excitation at 365 nm, emission at 455 nm, and slit width of 5 nm. The GUS activity was quantified using freshly prepared 4-MU (4-methyl umbelliferone sodium salt) standards of 1-200 nM in 0.2 M Na_2CO_3 stop buffer. The GUS activity was reported as nmol of 4-MU min^{-1} mg $protein^{-1}$.

Statistical analysis

All statistical analyses were conducted using the SPSS software version 19.0 (SPSS Inc, Chicago, IL). Results were analyzed by one way analysis of variance (ANOVA) and followed by Tukey's HSD post hoc analysis with the level of significance set at $P < 0.05$.

4. 4. RESULTS

Bacterial cell surface structures influence bacterial colonization/survival on plant tissue.

To investigate whether bacterial surface structures affect survival of enteric pathogens on plants, wild-type *Arabidopsis* plants were inoculated with two strains of *E. coli* O157:H7 and their mutants, and bacterial populations were monitored over a predetermined time. In addition, to investigate whether plant defense systems influence bacterial colonization, the mutant *Arabidopsis npr1-1*, impaired in SAR defenses, was also used for this colonization study.

The plant pathogen *Pst* DC3000 colonized both wild-type and *npr1-1 Arabidopsis* plants (Figure 3-1). On day 5, *Pst* DC3000 populations (ca. 9 log CFU/g) were similar on wild-type and *npr1-1* mutant plants. The population of *K. pneumoniae* 342 remained constant on both wild-type and *npr1-1* mutant plants during the 5-day period compared with initial numbers on those plants at day 0 (ca. 8 log CFU/g). Numbers of *K. pneumoniae* 342 on *Arabidopsis* wild-type and *npr1-1* mutants plants were not significantly different on day 5 postinoculation. These data may suggest that numbers on wild-type *Arabidopsis* were not controlled by SA-dependent defense responses.

The population of the *E. coli* O157:H7 43895 flagellum mutant on wild-type *Arabidopsis* was significantly ($P<0.05$) greater than that of the wild-type 43895 on day 5 postinoculation (Figure 3-1). The nearly 1.5 log difference in the two populations at day 5 postchallenge suggests that flagella were recognized by the wild-type plants, leading to reduced survival on the plants. *Arabidopsis npr1-1* mutant plants supported a

significantly ($P<0.05$) higher level (1.56 log CFU) of the *E. coli* O157:H7 43895 wild-type (Figure 3-1) than did wild-type *Arabidopsis*, suggesting that survival of the microbe was significantly affected by plant defense responses (PR genes expression) triggered by the perception of bacterial PAMPs. However, populations of the 43895 flagellum mutant on wild-type and *npr1-1* mutant plants were not significantly different ($P>0.05$). This finding suggests that wild-type and *npr1-1* plants failed to recognize the pathogen due to the absence of flagella. Interestingly, the population of the *E. coli* O157:H7 43895 lab mutant strain was greater on wild-type plants than that of the wild-type 43895 strain at day 5 postinoculation, even though the lab mutant strain possesses flagella and other determinants such as curli.

To investigate the roles of curli and lipopolysaccharides (LPS) in bacterial survival on plants, several mutants were used. Survival of the *E. coli* O157:H7 86-24 wild-type strain and its isogenic mutants, curli (*csgD*) and LPS (*waaI*), on wild-type and *npr1-1* mutant *Arabidopsis* were compared (Figure 3-2). At day 5 postchallenge of wild-type plants, the population of the curli deficient strain was significantly greater than that of the 86-24 wild-type strain, which is curli-positive. This finding suggests that curli influenced plant defense responses that negatively affected bacterial survival.

There was no significant difference ($P>0.05$) in populations of the wild-type 86-24 and the LPS mutant 86-24 on wild-type plants, indicating that modification of the LPS structure, specifically in the O antigen region, did not significantly influence plant defense responses. However, numbers of the LPS mutant 86-24 were significantly greater on *npr1-1* mutant plants than on wild-type plants. The presence of other PAMPs in the 86-24 LPS mutant may contribute to lower cell counts on wild-type plants.

Induction of pathogenesis-related genes by *Arabidopsis.*

To investigate whether *E. coli* O157:H7 induce SA-dependent pathogenesis-related (*PR*) genes, a transgenic *Arabidopsis* plant expressing β-glucuronidase (*GUS*) under the control of the *Arabidopsis* β-1,3-glucanase (*BGL2*) promoter was used. The *BGL2* (PR2) gene is regulated by SA and known as a molecular marker of SAR (Cao et al., 1994). Activation of the *BGL2* promoter reflects *PR* gene expression and the onset of SAR. Transgenic plants were inoculated with two *E. coli* O157:H7 isolates and their isogenic mutants, and at 5 days postinoculation, histochemical GUS staining and quantitative GUS enzyme activity assays were conducted. The plant pathogens *Pst* DC3000, the avirulent strain *Psm* ES4326 *avrRpt2*, and salicylic acid (SA) treatment were used as positive controls. A water treatment was included as a negative control. As shown in Figure 3-3, GUS activity in SA treated plants was 130-fold greater than in the water treated negative control. Transgenic plants inoculated with *Pst* DC3000 and *Psm* ES4326 *avrRpt2* exhibited 8-fold and 15-fold increases in GUS activity, respectively, compared to the water-treated control. Psm ES4326 carrying avirulence gene *avrRpt2* induces a rapid gene-for gene resistance and SAR in *Arabidopsis* (Volco et al., 1998). Both the virulent and avirulent phytopathogens induce plant defense response. However, PR gene expression by the avirulent pathogen is generally much stronger and faster than that by virulent pathogens (Jambunathan and McNellis, 2003). When only 43895 strains compared, plants inoculated with the wild-type 43895 showed more than a 2-fold increase in GUS activity compared with that of 43895 flagellum mutant and the 43895 lab mutant. The plants inoculated with the wild-type 43895 exhibited GUS activity comparable to that of those inoculated with the plant pathogen *Pst* DC3000. The GUS

activity observed in the plants inoculated with the lab mutant was 2-fold lower than the level observed in plants challenged with the wild-type 43895. These findings suggest greater induction of SA-mediated PR gene expression by the wild-type strain than by the two mutants, contributing to lower population of the wild-type strain on wild-type *Arabidopsis* plants. In addition, lower GUS activity in plants treated with the 43895 flagellum mutant may imply that the flagellum detection by plants enhances the plant defense responses. In *E. coli* 86-24 strains, the GUS activity of plants inoculated with the 86-24 curli mutant resulted in a 3-fold lower GUS activity compared with that of plants inoculated with the 86-24 wild-type strain, suggesting that recognition of curli by plants induced SA-mediated defense responses (Figure 3-3). In contrast, in plants inoculated with the 86-24 LPS mutant the decrease in GUS activity was only 1.6 fold lower than plants inoculated with wild-type strain.

Histochemical staining showed the pattern of PR gene expression in plants inoculated with different isolates (Figure 3-4). *BGL2* is a SAR marker gene and expression of *GUS* is driven by the *BGL2* promoter. GUS expression can be visualized as blue staining of plant tissue, indicating the induction of SAR. As shown in Figure 3-4, strong GUS expression was observed in transgenic plants treated with 1mM SA and in plants inoculated with *Pst* DC3000 and Psm ES4326 *avrRpt2*, whereas no GUS expression was detected in leaf sample treated with water. SA is an endogenous signal molecule required for the induction of *PR* genes. Application of SA strongly induced the expression of *PR* genes based on the extent of tissue staining. A higher level of GUS expression based on staining was observed in plants inoculated with *E. coli* O157:H7 43895 wild-type than in those inoculated with the flagellum mutant and the lab mutant.

This finding implies that stronger induction of *PR* genes by the 43895 wild-type strain contributed to reduced bacterial survival on wild-type *Arabidopsis* plants. Inoculation of plants with the wild-type *E. coli* O157:H7 86-24 induced greater GUS expression than did inoculation of the isogenic curli and LPS mutants, suggesting that curli and LPS structures were recognized by the plants and positively associated with *PR* gene expression.

3. 5. DISCUSSION

Escherichia coli O157:H7 specific cell surface moieties including flagella, curli fimbriae, and LPS influenced the colonization of plants by the enteric pathogen. The human pathogen triggered plant defense responses, although under the conditions evaluated it did not cause disease in plants. The results of the present study suggest that certain factors associated with *E. coli* O157:H7 may either enhance or mask recognition of the pathogen by plants. Bacterial colonization or survival on plant tissue may be affected by surrounding environmental conditions, such as temperature fluctuation, nutrient limitation, UV, and the physiological state of the plants (Brandl, 2006). These environmental conditions may influence bacterial cell counts, having a subsequent impact on the plant defense response. In this study, all inoculated plants were incubated under controlled growth conditions, such as temperature, humidity, and period of light during the experimental period. The observed differences in enteric bacterial populations on day 5 postinoculation likely resulted from the differences in induced plant defense responses.

Ultimately, this phenomenon may have a significant influence on the microbial safety of crops and human health.

For successful colonization, plant pathogens should suppress the host defense response. Phytopathogens utilize a type III secretion system (TTSS) to deliver effector proteins into the plant host to suppress PAMP-triggered defense responses (Li et al., 2005). The phytotoxin coronatine produced by the virulent pathogen *Pst* DC3000 suppresses SA-dependent defenses, resulting in susceptibility to this pathogen (Melotto et al., 2006). The plant pathogen *Pst* DC3000 colonized, with large numbers, of both wild-type and *npr1-1* plants. Greater populations of *Pst* DC3000 on *npr1-1* mutant than on wild-type plants at day 5 post inoculation indicates that *npr1-1* mutant plant is more susceptible to the plant pathogen due to the impairment in SAR. Visible disease symptoms such as chlorosis and necrosis, caused by *Pst* DC3000, were observed in both plant lines at day 4 postinoculation, but the *npr1-1* mutant plant exhibited more extensive disease symptoms (data not shown).

In contrast to the behavior of the plant pathogen, *E. coli* O157:H7 numbers failed to increase on *Arabidopsis* leaf tissue. During the experimental period, no visible disease symptoms were observed in either wild-type or *npr1-1* mutant *Arabidopsis* plants challenged with *E. coli* O157:H7. Numbers of *E. coli* O157:H7 (wild type and mutants) on plants declined gradually during the experimental period, although colonization patterns differed significantly among isolates. Significant differences in colonization of wild-type and *npr1-1* mutant plants with the *E. coli* O157:H7 wild-type strain 43895 suggests that PAMP-triggered plant defense responses resulted in decreased survival of the microbe on wild-type plants. Structural components of bacteria were considered as

potential inducers of various plant defense responses. Bacterial flagella are known to elicit defense responses in several plants including *Arabidopsis* (Felix et al., 1999), and localized application of flagellin induces SAR in *Arabidopsis* (Mishina and Zeier, 2007). Plants recognize pathogen-associated molecular patterns (PAMPs), which include flagellin, lipopolysaccharides (LPS), and glycoproteins (Nürnberger et al., 2004). Perception of these PAMPs triggers plant innate immunity, which limits bacterial colonization (Chisholm et al., 2006). *E. coli* O157:H7 possesses multiple PAMPs, such as LPS and flagella, so reduced colonization of the *E. coli* O157:H7 wild-type strain 43895 on wild-type *Arabidopsis* may be due to defense responses triggered by perception of multiple PAMPs. *Arabidopsis* plants detected multiple *Salmonella* PAMPs and activated multiple defense pathways of the innate immune system (Schikora et al., 2008). Another study reported that *S.* Typhimurium induced both SA-dependent and SA-independent defenses; recognition of flagella induced the SA-independent pathway (Iniguez et al., 2005). However, the *E. coli* O157:H7 flagellum mutant 43895 exhibited lower GUS activity (Figure 3-3) than the 43895 wild-type strain, suggesting that the flagella made a substantial contribution to the activation of the SA-dependent defensive pathway. Lack of flagella in *K. pneumoniae* 342 resulted in lower GUS induction, implying that colonization of *Klebsiella* on wild-type *Arabidopsis* may be mediated by a SA-independent pathway, consistent with previous reports (Iniguez et al., 2005).

Interestingly, the *E. coli* O157:H7 lab mutant 43895 exhibited a survival pattern on wild-type *Arabidopsis* distinctly different from that of the wild-type strain. The lab strain produced curli (data not shown). Others have shown that an apparent natural mutation in the *csgD* promoter of wild-type 43895 results in curli expression (Uhlich et

al., 2001). The lab strain 43895 is considered a spontaneous mutant originating from frozen stock collections of *E. coli* O157:H7 ATCC 43895. The lab mutant also produces a great amount of exopolysaccharides (EPS), forming a large well defined capsule, while the wild-type strain is a weak EPS producer. The precise roles of EPS in triggering plant defense responses or permitting the bacterium to evade detection have not been elucidated. EPS may mask underlying bacterial outer cell surface components from recognition by a plant host, evading a plant defense response. A strain of *Erwinia amylovora* lacking EPS was recognized by the host plant defense mechanism (Bugert and Geider, 1995). The fact that the 43895 lab mutant induced less GUS also is consistent with a lack of host plant recognition of PAMPs by molecules that trigger SA-dependent defenses.

This study demonstrated that *Arabidopsis* recognized the presence of curli and induced SA-dependent defense responses. Greater numbers of *E. coli* 86-24 curli-deficient mutants and the minimal induction of *BGL2-GUS* on wild-type *Arabidopsis* and also support this finding. However, the finding that numbers of *E. coli* 86-24 curli mutants were lower on wild-type than on *npr1-1* plants may imply that other PAMP-triggered defenses were involved in restricting microbial colonization. From these findings, we propose that curli may function as PAMPs in plant defenses. Curli occur on many pathogenic *E. coli* O157:H7 strains, and similar structures called thin aggregative fimbriae (Tafi) occur in *Salmonella spp*. Curli have not been discussed previously as PAMPs in plant defense studies, and their recognition by plants has been little studied. However, *S.* Typhimurium Tafi, are known to be PAMPs, elicited toll-like receptor 2 (TLR2)-mediated host immune responses (Tukel et al., 2005).

We showed that truncation of LPS structure resulted in reduced GUS induction, suggesting that LPS is involved in SA-mediated defense responses. LPS has been reported to triggered plant defense responses. However, the fact that both 86-24 wild-type and the LPS mutant numbers were similar on wild-type *Arabidopsis*, implies that LPS-triggered defense responses did not have a substantial impact on bacterial survival. *Arabidopsis* exposed to LPS precipitated activation of nitric oxide synthase (NOS), which contributed to the induction of defense genes (Zeidler et al., 2004). Local treatment of *Arabidopsis* with LPS induced SA-mediated *PR* gene expression and SAR (Mishina and Zeier, 2007).

In this study, we used a dip-inoculation method to better equate with the passive infection process of enteric pathogens. There are several methods to inoculate *Arabidopsis* with bacteria, including syringe infiltration and spray and dip inoculation. However, in nature, enteric bacteria would be expected to enter host tissue through the natural openings, such as stomata and wounds. The use of the infiltration method might bypass the natural infection process and SA-mediated stomatal defense responses (Melotto et al., 2006; Zipel et al., 2004).

Several studies have demonstrated that expression of bacterial cell surface components may be influenced by environment conditions. Adverse conditions, such as high temperature, high concentration of salts, carbohydrates, and low molecular weight alcohols resulted in lack of flagellum production by *E. coli* (Li et al., 1993). Loss of flagella in *Rhizobium melilti* was reported to occur during starvation (Wei and Bauer, 1998). EPS and capsular polysaccharides (CPS) production also were influenced by environmental conditions (Duffitt et al., 2011; Ryu and Beuchat, 2005). Curli are

typically expressed under stress conditions, such as low growth temperature (below 30°C) and nutrient limitations (Olsén et al., 1993). These findings suggest that loss or modifications of PAMPs under such conditions may enable human pathogens to evade the plant defense responses, thereby compromising the safety of crops intended for human consumption. In conclusion, differences in bacterial cell surface structure of *E. coli* O157:H7 significantly impacted survival of the human enteric pathogen on plants. Better understanding of the interactions between human pathogens and plants will facilitate development of new intervention strategies for ensuring the microbial safety of produce.

Table 3-1. Bacterial strains used in this study

Strains	Genotype/Phenotype characteristics
E. coli O157:H7 43895	Wild type, curli negative
E. coli O157:H7 43895 Flagella Mut	Δ*fliC,* Flagella mutant, curli negative
E. coli O157:H7 43895 Lab Mut	Spontaneous mutant, curli positive
E. coli O157:H7 86-24	Wild type, curli positive
E. coli O157:H7 86-24 Curli Mut	Δ*csgD*, curli-deficient
E. coli O157:H7 86-24, LPS Mut	Δ*waaI* , truncated LPS, curli-positive
*Klebsiella pneumonia*e 342	Maize endophyte
Pseudomonas syringae pv tomato (Pst) DC3000	Plant pathogen
P. syringae pv *maculicola* (Psm) ES4326 *avrRpt2*	*avrRpt2* avirulent gene

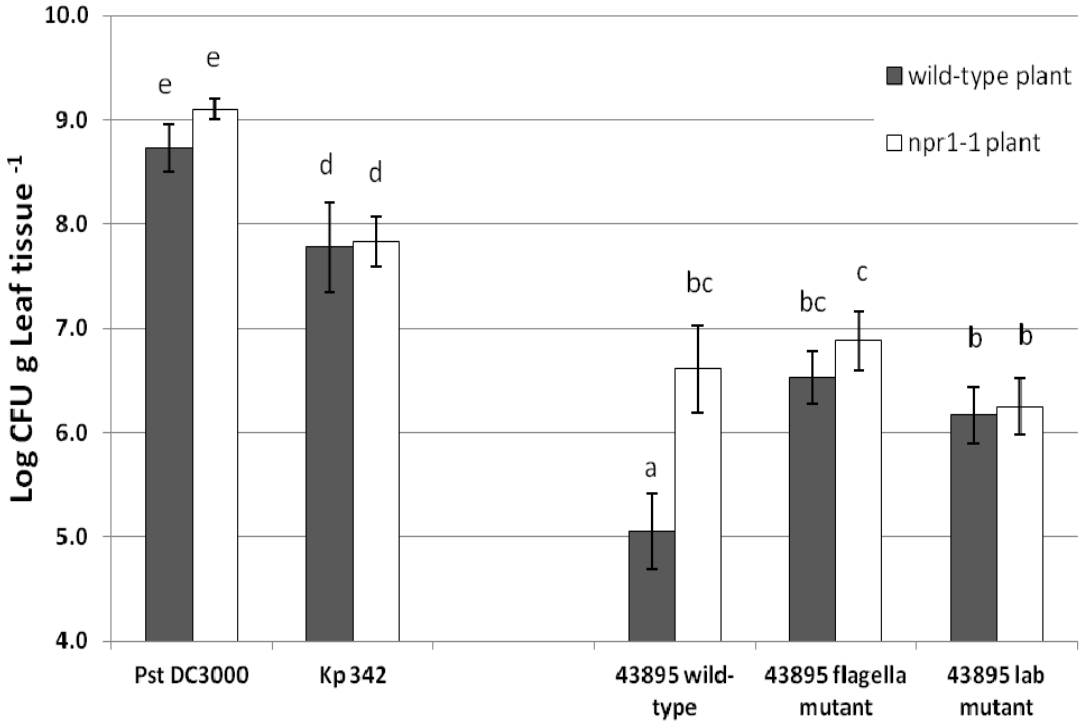

Figure 3-1. Numbers of *E. coli* O157:H7 43895 on wild-type and *npr1-1 Arabidopsis* plants at day 5 post inoculation. Different letters on bar graph indicate that the means are significantly different ($P<0.05$). The error bar indicates the standard deviations from the mean of six replicate plants.

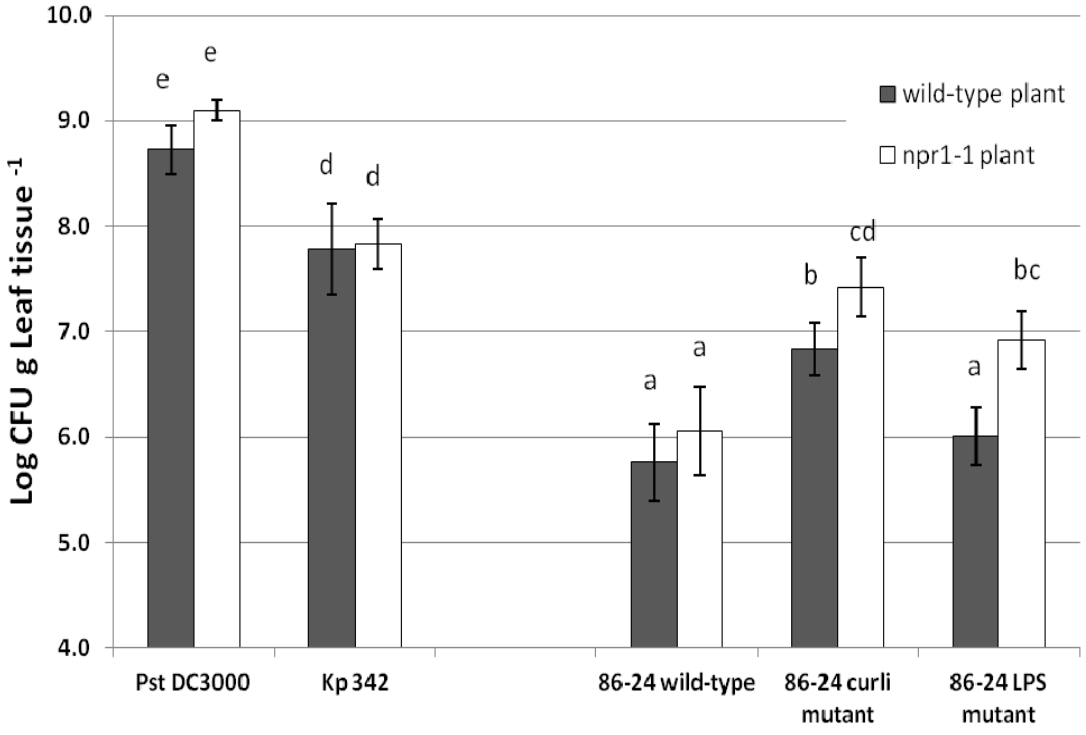

Figure 3-2. Numbers of *E. coli* O157:H7 86-24 on wild-type and *npr1-1 Arabidopsis* plants at day 5 post inoculation. Different letters bar graph indicate that the means are significantly different ($P<0.05$). The error bar indicates the standard deviations from the mean of six replicate plants.

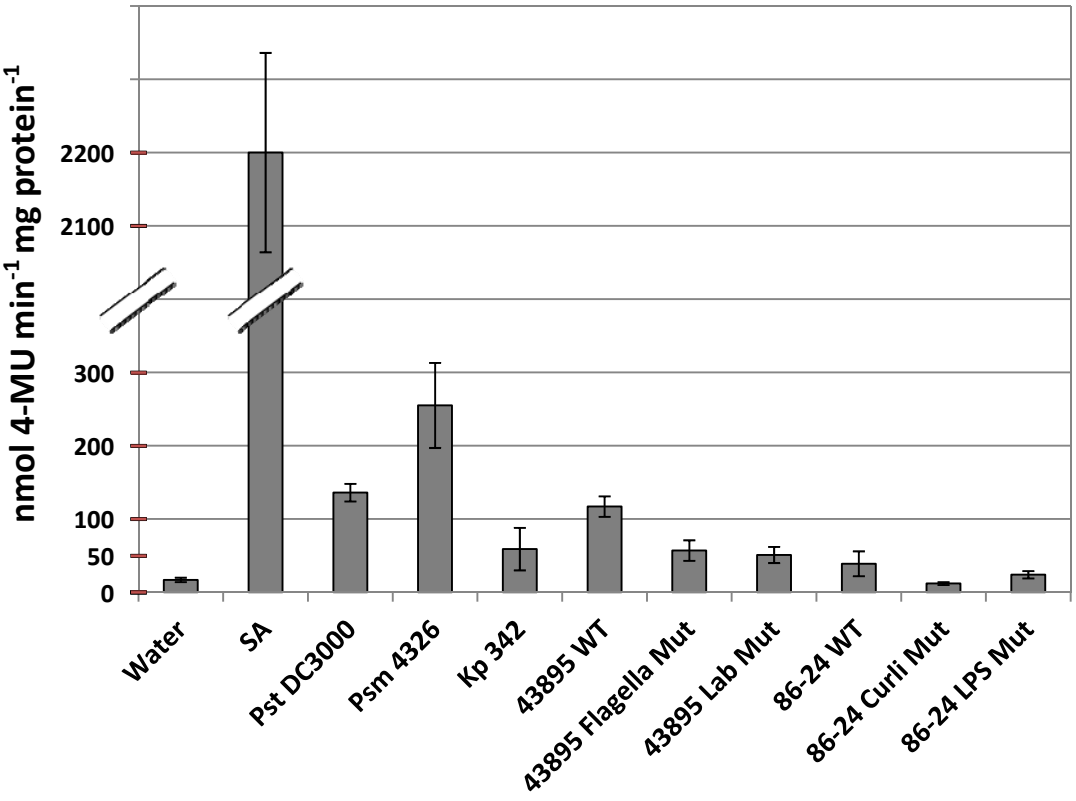

Figure 3-3. Quantitative analysis of GUS activity in transgenic *BGL2-GUS* *Arabidopsis* plants. The error bar indicates the standard deviations from the mean of three individual plants ($P<0.05$).

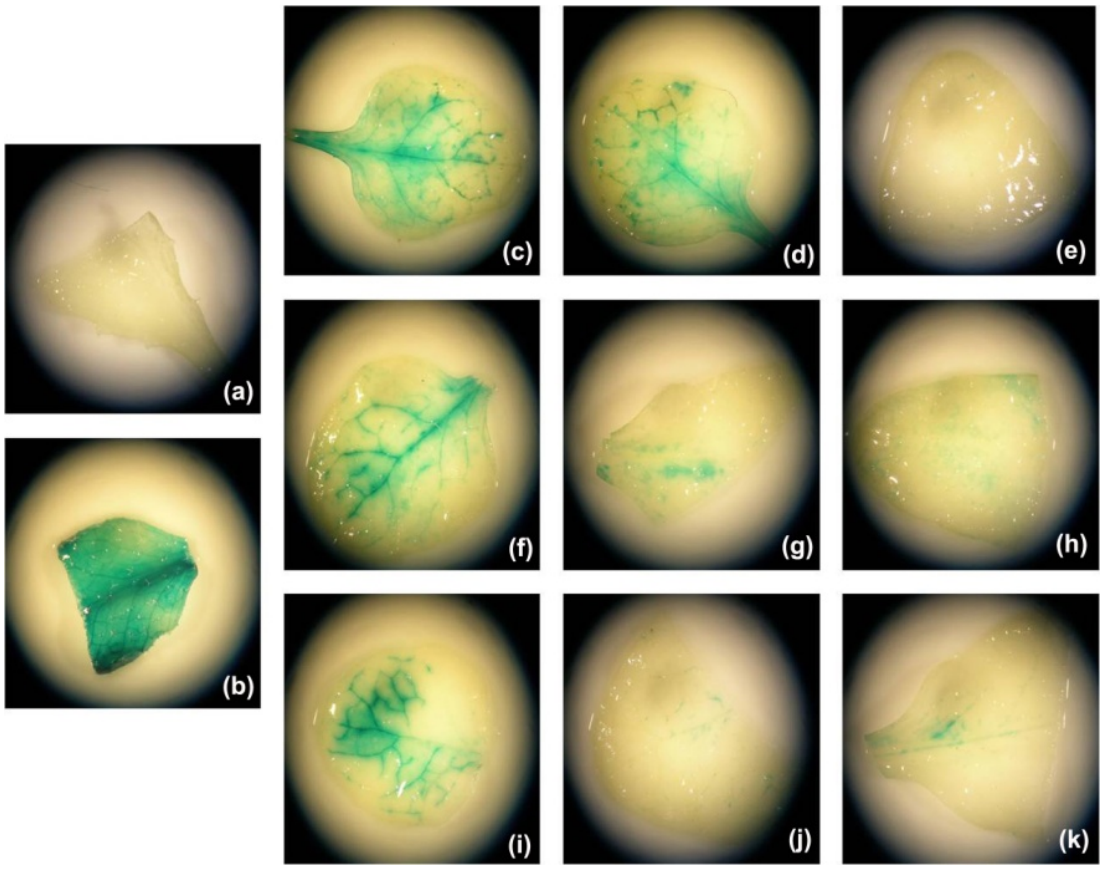

Figure 3-4. Histochemical staining of GUS activity in transgenic *BGL2-GUS* *Arabidopsis* plants, inoculated with water (a), 1 mM salicylic acid (b), Pst DC3000 (c), Psm ES4326 *avrRpt2* (d), Kp 342 (e), wild-type 43895 (f), flagella mutant 43895 (g), lab mutant 43895 (h), wild-type 86-24 (i), curli mutant 86-24 (j), and LPS mutant 86-24 (k).

CHAPTER 4

Exposure of *Escherichia coli* O157:H7 to soil or manure influences capsular polysaccharide production, plant defense response, and persistence of that pathogen on plants

Seungwook Seo and Karl R. Matthews

4.1. ABSTRACT

In this study, we investigated whether growth medium or exposure conditions influence the bacterial capsular polysaccharides (CPS) of *Escherichia coli* O157:H7, and whether changes in CPS impact plant defense responses, consequently affecting its survival on plants. *Escherichia coli* O157:H7 grown in Luria-Bertani (LB) broth supplemented with manure extracts showed approximately 58% increase in CPS production compared to cells grown in LB medium alone. Levels of CPS were significantly higher for *E. coli* O157:H7 cells exposed to soil and manure extracts as compared to the non-exposed LB cultured control. *Arabidopsis thaliana* plants expressing β-glucuronidase (*GUS*) under the control of the β-1,3-glucanase (*BGL2*) promoter were used to investigate whether *E. coli* O157:H7 induces defense-related gene expression. Plants inoculated with *E. coli* O157:H7 grown in LB containing manure extracts or cells exposed to manure extracts exhibited 3-fold and 2-fold lower GUS activity, respectively, suggesting a limited plant defense response compared to plants inoculated with cells grown in LB. On day 5 post inoculation, the population of *E. coli* O157:H7 grown in LB supplemented with manure on plants was significantly greater than the population of *E. coli* O157:H7 grown in LB medium alone. *E. coli* O157:H7 cells exposed to soil or manure exhibited greater survival on plants compared to LB-grown *E. coli* O157:H7. These findings suggest that under conditions tested *E. coli* O157:H7 CPS production influenced by growth medium or exposure environment conditions may enable that human pathogen to evade plant defense responses, increasing its survival on plants.

4.2. INTRODUCTION

During the past decade, fresh produce, such as alfalfa sprout, lettuce, spinach, and parsley have been linked to outbreaks of foodborne illness. Increase in foodborne illness associated with consumption of fresh fruits and vegetables has been attributed to increased consumption of fresh produce as well as changes in agronomic, harvesting, processing, and distribution patterns (Beuchat, 2002). Manure, soil, irrigation water, and method of application have been considered as potential sources of pathogenic microorganisms on fresh produce (Beuchat and Ryu, 1997). Previous studies have shown that human pathogens can survive and persist for a long period of time in the field environment such as soil and animal feces (Islam et al., 2004A; Wang et al., 1996). Healthy cattle are known to be a major reservoir of human pathogens and shedding of the pathogen can result in contamination of soil and water. The survival of enteric pathogens outside the host may be greatly affected by environment conditions where bacteria encounter temperature fluctuation, limited nutrients, and UV radiation (Brandl, 2006).

Bacterial cell surface structures including curli, flagella, and extracellular polysaccharides play an important role in the interactions between enteric pathogens and abiotic and biological surfaces. The expression of bacterial surface components has been shown to be influenced by culture conditions or nutrient composition of the growth medium (Bonet et al., 1993; Hassan and Frank, 2004; Li et al., 1993; Olsén et al., 1993). Those structures were reported to mediate bacterial attachment, colonization, or biofilm formations on plant tissue (Barak et al., 2005; Barak et al., 2007; Xicohtencatl-Cortes et al., 2009). The term, extracellular polysaccharides, refer to capsular polysaccharides

(CPS) and exopolysaccharides (EPS) or slime. EPS are loosely bounded to the cell surface, and easily sloughed from the cell surface, while CPS are tightly associated with the cell surface (Roberts, 1996). CPS protects cells from adverse environments associated with desiccation, osmotic or oxidative stresses, confers resistance to antimicrobial compounds, and evasion of host immune response (Campos et al., 2004; Roberts, 1996).

Plants have evolved defensive mechanisms to protect against pathogenic organisms by producing antimicrobial compounds and defense-related proteins, and by induction of local or systemic acquired resistance (SAR) (Durant and Dong, 2004). Primary innate immunity is achieved through pattern recognition receptors (PRRs) that recognize conserved pathogen associated molecular patterns (PAMPs). PAMPs include flagellin, the LPS fraction of Gram-negative bacteria, chitins, and glucans from fungi. Upon recognition, primary innate defense responses, such as deposition of callose in the cell wall, production of reactive oxygen species and defense-related proteins, which negatively affect bacterial colonization, are induced (Nürnberger et al., 2004). Plants are also protected by systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is induced by pathogens that cause necrotic lesions, the result of hypersensitive response (HR), resulting in protection throughout the plant against a broad spectrum of microorganisms. Bacterial PAMPs are known as major elicitors of SAR (Mishina and Zeier, 2007). Activation of SAR is regulated by the signaling molecule salicylic acid (SA) and is associated with expression of pathogenesis-related (PR) genes (Durant and Dong, 2004). ISR is induced by the colonization of roots by rhizosphere bacteria and is activated by jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 1998).

Research on the plant defense response to human pathogens is limited compared to that on plant pathogens. Recent studies have shown that *Salmonella* and *E. coli* O157:H7 colonization induced SA- or JA-regulated plant defense responses (Moletto et al., 2006; Schikora et al., 2008; Seo and Matthews, 2012; Shirron and Yaron, 2011). In this study, we investigated the effects of growth medium (Luria-Bertani (LB) broth, and LB supplemented with soil or manure extracts) or exposure to carrier environments (soil or manure extracts) on CPS production. Cell surface moieties associated with a bacterium cultured in a laboratory medium (e.g., LB broth) may differ from cell surface moieties expressed by the same bacterium when found in harsh environments. Differences in expression of extracellular components may dramatically impact how the bacterium interacts with plants. Effects of growth medium on cell surface properties, such as EPS, cell size, hydrophobicity, and surface charge were investigated (Hassan and Frank, 2004; Marcus et al., 2012). However, the influence of *E. coli* O157:H7 CPS on plant defense responses and the impact on bacterial survival on a plant were not investigated. The objectives of this study were to investigate whether growth medium or exposure conditions influence the bacterial CPS, and whether that precipitates changes in plant defense responses, consequently affecting bacterial survival on plants.

4.3. MATERIALS AND METHODS

Bacterial strain and preparation of medium

E. coli O157:H7 ATCC 43895 was used in this study. The pathogen was grown in tryptic soy agar (TSA: Difco, Becton Dickinson, Sparks, MD) supplemented with ampicillin at

100 µg/ml at 37°C. Stock cultures were maintained in tryptic soy broth (TSB: Difco, Becton Dickinson, Sparks, MD) containing 30% glycerol at –70^{o} C. Fresh cow manure and soil were obtained from the Rutgers dairy farm and the Rutgers green house, respectively. Upon receipt, manure and soil samples were suspended in sterile distilled water (1:10, wt/vol), and filtered with cheese cloth to remove large particles. The resulting slurry was centrifuged at 3500 × g for 20 min and then the decanted solutions were filtered through a 0.2 micron sterile filter (Nalgene). Filtered soil and manure extracts were stored in at -20°C until needed. Luria-Bertani (LB) media supplemented with soil extract (LB+Soil) or manure extract (LB+Manure) were prepared by mixing a 2× concentrated LB and the soil or manure extracts at a ratio of 1:1 (vol/vol).

Plants and growth conditions

Seed of *Arabidopsis thaliana* ecotype Columbia (Col-0) wild-type (CS 70000) was obtained from the Arabidopsis Biological Resource Center, The Ohio State University. Seeds of Col-0 transgenic line (*BGL2*::*GUS*) were kindly provided by Dr. Xinnian Dong (Duke University, NC, USA). *BGL2-GUS* transgenic plants contain a β-glucuronidase reporter gene (*GUS*) driven by the β-1,3-glucanase (*BGL2*) promoter (Cao et al., 1994). *Arabidopsis* plants were grown in Metromix 360 soil in a climate-controlled greenhouse (22°C ± 2°C, relative humidity 70% ± 5%) under natural light supplemented with artificial light to achieve 16 h per day of light.

Plant exposure

Bacteria was cultured as described above: *E. coli* O157:H7 43895 was grown overnight at 37°C in LB, LB+Soil, and LB+Manure, or exposed to soil or manure extracts as follows. An overnight culture grown in LB medium was centrifuged, cells washed with sterile distilled water, and resuspended in soil or manure extracts. The bacterial suspension in soil extract or manure extract was held at room temperature for 5 days. Bacterial cells (grown or exposed) were harvested by centrifugation and resuspended in sterile distilled water (SDW) to achieve approximately 10^8 CFU/ml. Bacterial levels in water were confirmed by plating serial 1:10 dilutions on TSA.

Plant inoculation; 5 week-old wild-type plants were dipped in a bacterial suspension for 30 s and then placed in a growth room under a transparent plastic dome for 1 day to maintain high humidity. Control plants were treated with sterile distilled water and grown as described. At 0, 3, and 5 days post-challenge three plants from each treatment group were harvested. Weighed plant samples were placed in a sterile stomacher bag with 0.1% peptone water (1:10, wt/vol), macerated by hand, and then homogenized for 3 min using a Stomacher. Appropriate dilutions of the homogenates were plated in duplicate on TSA supplemented with ampicillin (100µg/ml). The colonies were counted and populations were calculated and reported as log CFU per g fresh weight of plants. The experiment was conducted twice. For the GUS activity experiment, 4 week-old *BGL2-GUS* transgenic plants were dipped in a bacterial suspension as described above. For the negative and positive controls, plants were dipped in sterile distilled water or 1 mM salicylic acid (SA) for 30 s, respectively. At 5 day post-inoculation, plants were harvested and GUS assays were conducted.

Quantitative analysis of GUS activity

Whole plants were homogenized using a pre-chilled micro pestle in 200 µL of GUS extraction buffer (50 mM Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 10 mM β–mercaptoethanol) and centrifuged for 20 min at 16,000 × g at 4°C (Jefferson et al., 1987). Supernatants were assessed for protein concentration and enzyme activity. Protein concentrations were determined by the Bradford method, and GUS reactions were carried out by adding 20 µL of extracts into a reaction tube containing 980 µL of GUS extraction buffer containing 1 mM MUG (4-methylumbelliferyl- β-D-glucuronide) in 37°C. After 0, 10, 20, or 40 min incubation, 200 µL from each reaction tube were added to a tube containing 800 µL 0.2 M Na_2CO_3 to stop the reactions. Fluorescence was measured using a Perkin Elmer LS-50B luminescence spectrometer with excitation at 365 nm, emission at 455 nm, and slit width of 5 nm. The GUS activity was quantified using freshly prepared 4-MU (4-methyl umbelliferone sodium salt) standards of 1-200 nM in 0.2 M Na_2CO_3 stop buffer. The GUS activity was reported as nmol of 4-MU min^{-1} mg $protein^{-1}$.

Congo red negative staining

A negative staining technique employing Maneval's stain was used to test for CPS. One loopful (10µl) of each culture was mixed with one drop of 1% Congo red solution on a clean glass slide, spread across the slide using a clean slide, and allowed to air dry. Slides were flooded with Maneval's stain solution (Carolina, Burlington, NC). After 1 min the slide was gently washed with distilled water and air dried. Prepared slides were examined with an oil immersion 100× objective using a light microscope.

Quantification of CPS.

Purification of cell-associated CPS was conducted by a hot phenol-water extraction method (Campos et al., 2004). Bacteria grown in 5 ml of LB, LB+Soil, and LB+Manure media overnight, or cells exposed to 5 ml of soil and manure extracts for 5 days were harvested by centrifugation (5,000 × g, 10 min, 4°C). The cell pellets were washed once with 1 ml sterile distilled water (SDW) and resuspended in 500 µl SDW. Viable cells in suspension were determined by plating serial dilutions on TSA. Phenol (500 µl) was added into a cell suspension tube and incubated at 68°C for 30 min. After the mixture cooled, 500 µl of chloroform was added and vortexed. The aqueous phase was recovered by centrifugation (5,000 × g, 10 min). CPS in aqueous phase was precipitated by adding aqueous solution into a tube containing three volumes of absolute ethanol at -20 °C for 18 hrs. Precipitated CPS was harvested by centrifugation (14,000 × g, 20 min, 4°C) and resuspended in 500 µl SDW. Total carbohydrate content was quantified using the phenol-sulphuric acid method (Dubois et al., 1956). Experiments were conducted twice with three independently grown or exposed batches of bacteria.

Statistical analysis

All statistical analyses were conducted using the SPSS software version 19.0 (SPSS Inc, Chicago, IL). Results were analyzed by one way analysis of variance (ANOVA) and followed by Tukey's HSD post hoc analysis with the level of significance set at $P < 0.05$.

4.4. RESULTS

Influence of soil or manure extracts on extracellular polysaccharide production by *E. coli* O157:H7.

Influence of growth or exposure conditions on production of capsular polysaccharides (CPS), cell surface associated exopolysaccharides (EPS), of *E. coli* O157:H7 was investigated. As shown in Figure 4-1, levels of CPS were significantly higher ($P<0.05$) for cells that were grown in LB+Manure medium as compared to those grown in LB. Notably, bacteria grown in LB+Manure showed approximately 58% increase in CPS production (9.92 μg CPS/10^9 CFU) compared to cells grown in LB alone (4.11 μg CPS/10^9 CFU). Cells grown in LB+Soil produced greater CPS compared to LB-grown cells, however, the influence of soil extracts in LB medium on CPS production were not significant. Exposure of *E. coli* cells to soil or manure extracts also stimulated CPS production. CPS of *E. coli* cells exposed to soil and manure extracts were 6.66 and 7.14 μg CPS/10^9 CFU, respectively, which were significantly greater ($P<0.05$) than the non-exposed LB grown control (4.11 μg CPS/10^9 CFU). The negative staining shows direct visualization of the capsule, appearing as a clear halo surrounding *E. coli* cells (Figigure 4-2). Cells grown in LB+Manure and cells exposed to manure extracts had larger zones of clearing around the cell compared to cell grown in LB. These results suggested that production of CPS, one of the outer cell surface structures, was greatly affected either independently or collectively by the composition of growth media or exposure conditions.

Induction of plant defense responses by *E. coli* O157:H7 grown in supplemented growth medium and exposed to soil or manure extract.

A transgenic *Arabidopsis* plant expressing β-glucuronidase (*GUS*) under the control of the β-1,3-glucanase (*BGL2*) promoter was used to investigate whether *E. coli* O157:H7 induces defense-related *PR* gene expression. The *BGL2* (PR2) gene is regulated by SA, and known as a molecular marker of SAR, and activation of the *BGL2* promoter reflects *PR* gene expression and the onset of SAR (Durant and Dong, 2004). *Arabidopsis* plants (*BGL2-GUS*) were inoculated with *E. coli* O157:H7 grown in different growth media and with cells exposed to soil or manure; at 5 days post inoculation, quantitative GUS enzyme activity assays were conducted. Plants treated with 1mM salicylic acid (SA), a positive control, exhibited approximately 40-fold greater GUS activity compared to those treated with sterile water, a negative control (Figure 4-3). Plants inoculated with *E. coli* O157:H7 grown in LB+Manure and cells exposed to manure extracts exhibited a 3-fold and 2-fold decrease in GUS activity, respectively, compared to those inoculated with *E. coli* O157:H7 grown in LB medium. GUS induction is driven by *BGL2* (PR2) promoter, therefore greater GUS activity is associated with greater expression of defense-related *PR* genes. The low GUS activity observed in plants inoculated with *E. coli* O157:H7 grown in LB+Manure indicates expression of defense related genes was minimal. The GUS activity observed in the plant inoculated with *E. coli* O157:H7 grown in LB+Soil medium was lower than the level observed in plants inoculated with LB-grown cells, and showed comparable GUS activity to those inoculated with soil-exposed *E. coli* O157:H7 cells.

Influence of constituent of soil or manure on survival of enteric pathogens on plant tissue.

To investigate whether constituents of soil or manure influence survival of human pathogens on plants, wild-type *Arabidopsis* plants were inoculated with *E. coli* O157:H7 grown in LB medium, LB+Soil, LB+Manure, or with *E. coli* cells exposed to soil or manure extracts. Bacterial populations were monitored over predetermined time. At day 0 post inoculation, the initial mean population of *E. coli* O157:H7 on *Arabidopsis* plants was approximately 8.34 Log CFU/g plant tissue, and there were no significant differences (P>0.05) between groups of inoculated plants (Data not shown). Thus, growth or exposure conditions showed no effects on initial population of the bacteria onto plant surfaces. The population of bacteria on plants gradually declined during the experimental period but showed different rates of survival depending on the growth or exposure conditions. On day 5 post inoculation the population of *E. coli* O157:H7 43895 grown in LB+Manure on wild-type *Arabidopsis* was significantly (P<0.05) greater than *E. coli* cells grown in LB medium (Figure 4-4). An approximate 3-fold lower induction of plant defenses by LB+Manure-grown *E. coli* O157:H7 cells resulted in greater survival on plants compared to the LB-grown cells. This finding suggests that *E. coli* O157:H7 survival on plants was greatly affected by that pathogens induction of plant defenses. The numbers of *E. coli* O157:H7 cells grown in LB+Soil on plants were not significantly different compared with those grown in LB+Manure and LB alone. At day 5 post inoculation, the numbers of *E. coli* O157:H7 cells exposed to soil and manure on *Arabidopsis* plants was 6.71 and 7.12 Log CFU/g of plant, respectively, levels significantly higher than LB-grown *E. coli* O157:H7 on plants (5.42 Log CFU/g of plant).

The reduced plant defense response, based on GUS activity, in plants inoculated with *E. coli* O157:H7 exposed to soil or manure extracts contributed to the greater survival on plants during 5 days post inoculation.

4.5. DISCUSSIONS

Exposure of *E. coli* O157:H7 to soil and manure extracts influenced CPS production which had a significant impact on survival of that human pathogen on plants by thwarting plant defense responses. CPS functions as a protective barrier against hostile environments, such as desiccation, osmotic or oxidative stresses, and mediates resistance to antimicrobial compounds, and host immune responses (Campos et al., 2004; Roberts, 1996). On day 5 post-inoculation, populations of *E. coli* O157:H7 grown in LB+manure, or exposed to soil or manure extracts, were greater on plant tissue than that pathogen cultured in LB alone. Cells grown in LB alone elicited the greatest GUS activity (Figure 4-3) suggesting greater recognition by the plant defense response system. Collectively, results suggest that the enteric foodborne pathogen, *E. coli* O157:H7 may undergo phenotypic changes when exposed to manure or soil which enhances the ability to survive on plants.

Research suggests that the production of CPS or EPS is influenced by the composition of growth medium. Glucose, potassium phosphate, magnesium, and trace elements are necessary for the CPS production, and increased concentration of carbon and nitrogen sources in growth medium improved capsule production (Bonet et al., 1993). The presence of dextrose in tryptic soy broth (TSB) supported greater capsule production,

while *E. coli* O157:H7 grown in nutrient broth (NB) lacking dextrose did not produce capsule (Hassan and Frank, 2004). Animal manure contains micro- and macro nutrients, such as carbon, nitrogen, potassium, phosphorous, crude proteins, amino acids, and other trace elements (Chen et al., 2003). Supplementation of LB medium with the manure extract nutrients may have contributed to the increased CPS production by *E. coli* O157:H7 compared to cells cultured solely in LB medium. Enhanced CPS on *E. coli* O157:H7 cells exposed for extended periods to soil or manure extracts compared to non-exposed cells may be related to survival tactics. Enteric bacteria no longer associated with a host may encounter unfavorable environmental conditions, including sub-optimal temperature, nutrient limitation, and pH changes. *E. coli* O157:H7 exposed to sterile soil microcosms exhibited increased expression of *rpoS*, general stress regulatory gene, when compared to cells grown in LB. In addition, *wcaL* gene, responsible for the CPS colanic acid biosynthesis, was more highly expressed in cells exposed to soil compared to non-exposed controls (Duffitt et al., 2011).

Bacterial polysaccharides play important roles in plant-microbe interactions; however, the precise roles of those structures in triggering plant defenses or permitting the bacterium to evade detection are not yet known. In this study, *E. coli* O157:H7 cells cultured in or exposed to soil or manure expressed a greater amount of CPS, induced lower GUS activity, and exhibited greater survival on plant tissues (Figure 4-1, 4-3, and 4-4). The greatest effect was associated with cells cultured on or exposed to manure extracts. Previous studies have shown that the human pathogen can trigger plant defense responses upon colonization of plant tissue (Schikora et al., 2008; Seo and Matthews, 2012; Shirron and Yaron, 2011). *E. coli* O157:H7 possesses multiple PAMPs, such as

flagella, lipopolysaccharides (LPS), and glycoproteins. Perception of these structural components triggers plant innate immune response, which limits bacterial colonization or survival on plants. To initiate a plant defense response, enteric bacteria must access plant tissue. The molecular mechanism(s) by which enteric pathogens penetrate the physical outer barrier of plant cells and access the host plasma membrane is unknown. Natural openings such as stomata and wounds are believed to be major potential gateways for pathogen entry. Positive GUS activity (Figure 4-3) suggests that *E. coli* O157:H7 are able to enter the plant host likely through open stomata.

The limited GUS activity observed in plants inoculated with *E. coli* O157:H7 cells expressing greater CPS may be associated with decreased recognition of PAMPs by the plant host. Presence of CPS may mask underlying bacterial cell surface structures from recognition by a plant host, limiting the plant defense response, and consequently resulting in greater survival of bacteria on plants. Several studies have shown that bacterial surface polysaccharides enable bacteria to evade or suppress plant immune responses. Research suggest that EPS expressed by the plant pathogen *Erwinia amylovosa* served to masked cell surface molecules that might be recognized by plants eliciting a defense response since an *E. amylovosa* EPS-lacking mutant was recognized by the host plant defense system (Metzger et al., 1994). Researchers demonstrated that bacterial polysaccharides suppress PAMP-triggered innate immunity by chelating calcium ions, required for activation of the plant defenses (Aslam et al., 2008). However, the precise function of CPS in the evasion or suppression of plant defense is difficult to evaluate. Further studies will be needed to elucidate the roles of CPS in plant defense response at a molecular level.

Environmental conditions, such as temperature fluctuation, nutrient limitation, UV radiation, and the physiological state of the plant significantly affect bacterial colonization or survival on plants (Brandl, 2006). Environmental factors may impact bacteria and plant; ultimately influencing whether the bacteria survives and persist on the plant. In this study, all inoculated plants were incubated under controlled growth conditions, such as temperature, relative humidity, and period of light during the experimental period. Controlling, those variables increases the likelihood that observed differences in *E. coli* O157:H7 populations on day 5 post inoculation likely resulted from the difference in induced plant defenses.

In this study, we used a dip-inoculation method to better equate with the passive infection process of enteric pathogens. In nature, enteric bacteria would be expected to enter host tissue through the natural openings such as stomata and wounds. The use of the infiltration method might bypass the natural infection process and SA-mediated stomatal defense responses (Moletto et al., 2006; Zipfel et al., 2004). Moreover, all inoculated plants were incubated under controlled growth conditions, such as temperature, relative humidity, and period of light during the experimental period. Controlling these conditions permits comparison of plant defense response and influence of *E. coli* O157:H7 CPS.

Healthy cattle are considered reservoirs of *E. coli* O157:H7; cattle feces or soils contaminated with the pathogen could be primary sources for contamination of fresh produce in the field. Survival of enteric pathogens outside their host could be affected by conditions where they encounter unfavorable conditions, i.e. low temperature and nutrient limitation. Research demonstrates that enteric pathogens can survive for an

extended period of time in soil (Islam et al., 2004) and manure (Wang et al., 1996). Adverse conditions including extreme temperature and unbalanced or limited nutrients resulted in lack of flagella production by *E. coli* (Li et al., 1993). In addition, researchers have demonstrated that loss or modifications of PAMPs influenced plant defense responses, and consequently affected the survival of enteric pathogens on plant tissue (Iniguez et al., 2005; Seo and Matthews, 2012). In this study, we showed that capsular polysaccharide production of *E. coli* O157;H7 was significantly affected by the growth medium. These findings suggest that CPS of *E. coli* O157:H7 may enable human pathogens to evade the plant defense responses by possibly masking the PAMPs, and resulting in increased survival of the enteric pathogen on plants. This has important implications in the safety of crops intended for human consumption. Results of this study also underscores how bacterial culture conditions can impact experimental results ultimately leading to conflicting results between two seemingly similar studies. Further research on the mechanism of bacterial CPS in the plant defense response is needed to better understand the human pathogen-plant interaction(s) to enhance the microbial safety of produce.

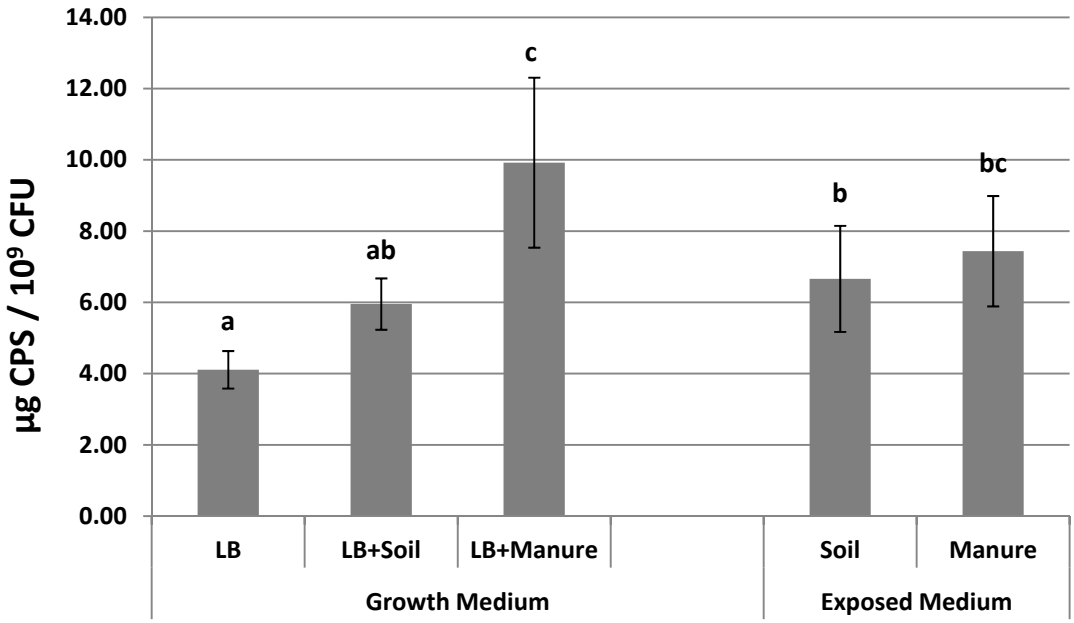

Figure 4-1. Quantitative analysis of capsularpolysaccharide productions by *E. coli* O157:H7. Different letters on bar graph indicate that the means are significantly different ($P<0.05$). The error bar indicates the standard deviations from the mean of six replicate samples.

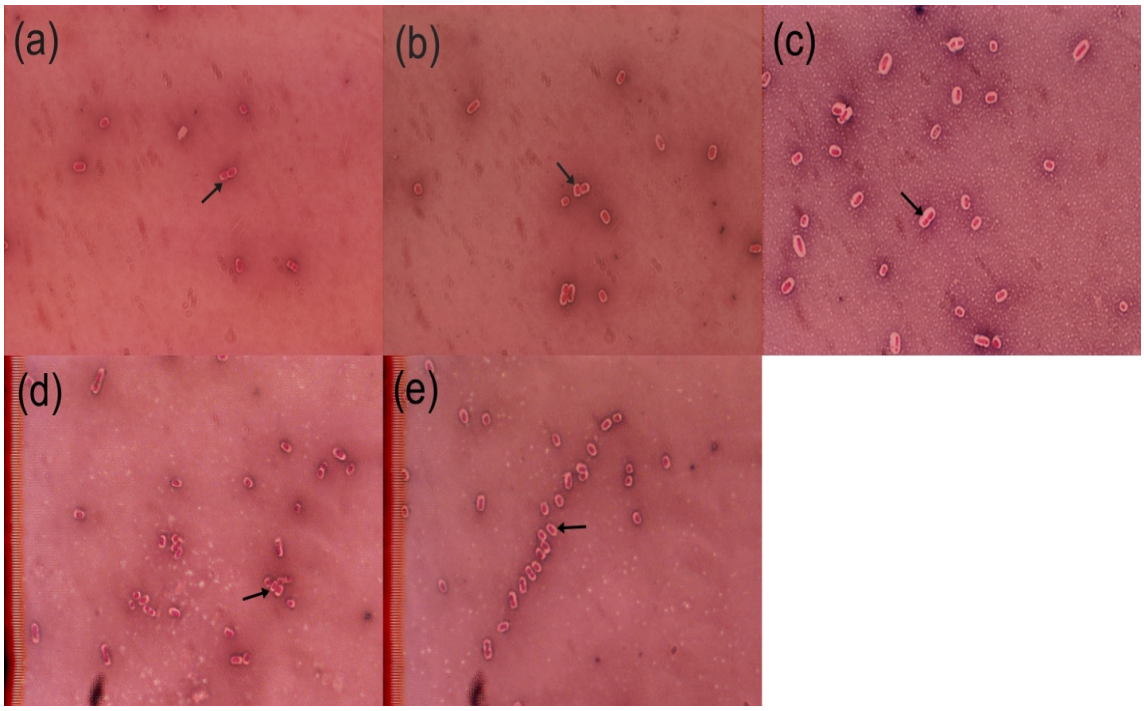

Figure 4-2. Negative capsule staining of *E. coli* O157:H7 cells grown in LB (a), LB+Soil (b), LB+Manure (c), or exposed to soil (d) and manure (e) extracts. Clear haloes (arrow) surrounding cells indicate the presence of capsules.

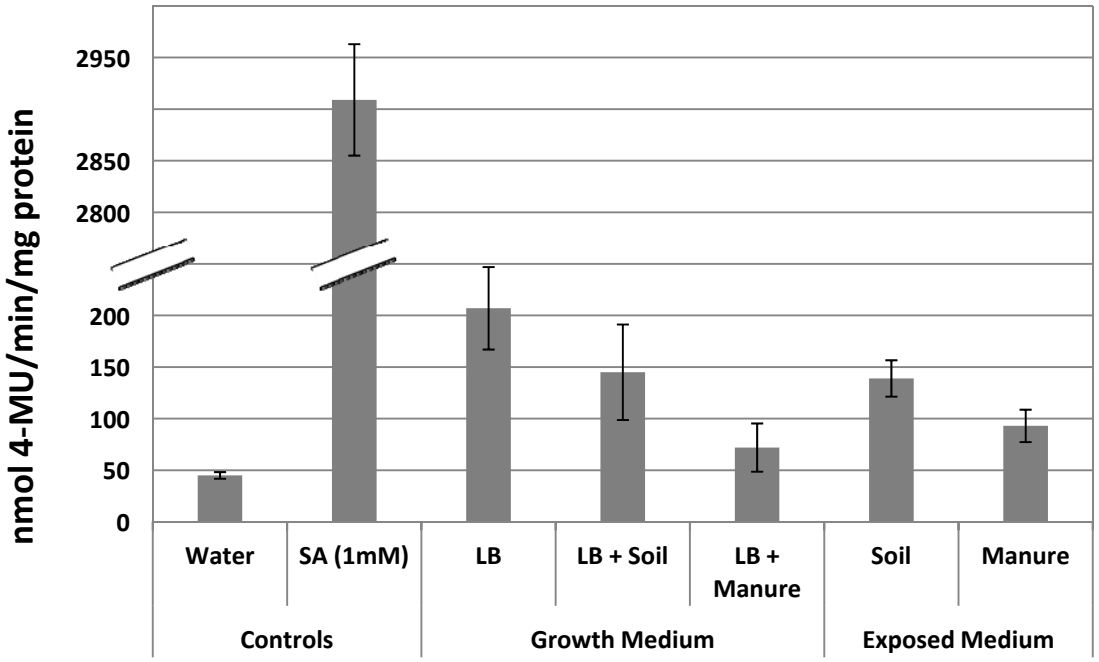

Figure 4-3. Quantitative analysis of GUS activity in transgenic *BGL2-GUS* *Arabidopsis* plants. The error bar indicates the standard deviations from the mean of six individual plants ($P<0.05$).

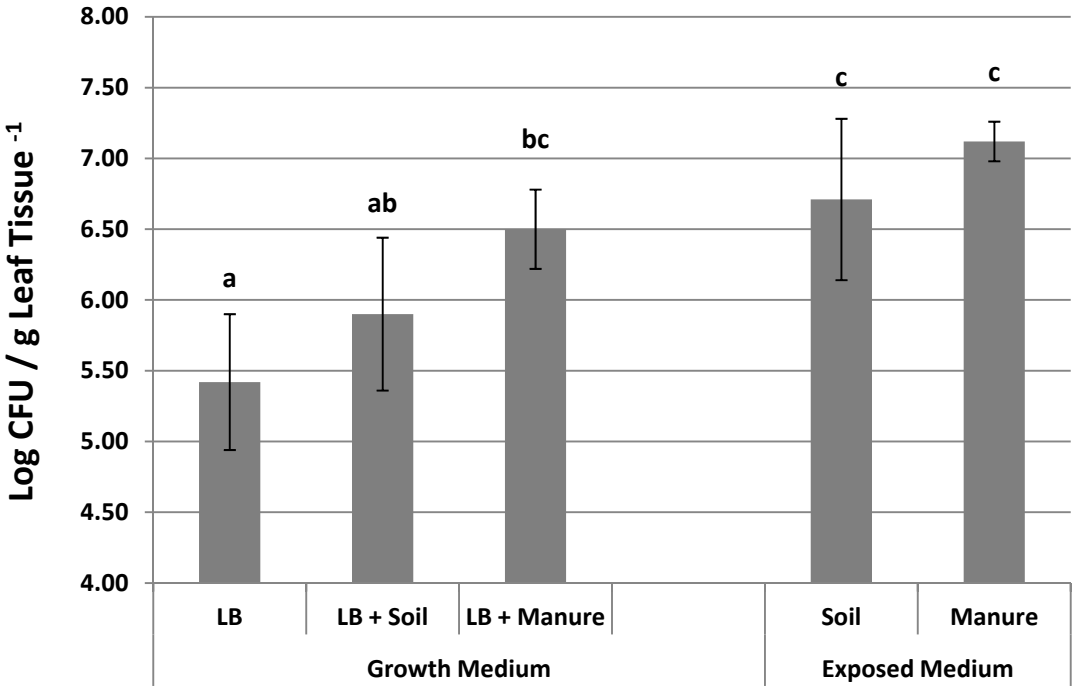

Figure 4-4. Population of *E. coli* O157:H7 on *Arabidopsis* plants at day 5 post inoculation. Different letters on bar graph indicate that the means are significantly different ($P<0.05$). The error bar indicates the standard deviations from the mean of six replicate plants.

CHAPTER 5

General discussion and Conclusions

Contamination of fresh fruits and vegetables with human pathogens has been a great concern to public health since the first appearance of produce-associated outbreaks of *E. coli* O157:H7 in 1991 in the United States. Recent large outbreaks of *E. coli* O157:H7 associated with spinach (CDC, 2006B) in the United States confirmed these concerns, leading to an increased awareness of the potential microbial risks of fresh fruits and vegetables. Persistence and survival of enteric pathogens in/on plants have been extensively studied by numerous researchers. From these studies it is evident that human pathogens are able to colonize plants endophytically and epiphytically and interact at a molecular level with the host plant. In addition, many studies have suggested that plants may serve as a host and reservoir for enteric pathogens. But questions whether enteric pathogens actively invade plants and colonize plants as a plant host microbe, or whether they simply fit in phyllosphere environments and persist have yet to be determined.

The influence of cell surface structures of *E. coli* O157:H7 on plant defense response and their impact on survival of the enteric pathogen on plants was investigated and discussed in chapter 3. Bacterial surface components including flagella, curli (or Tafi in *Salmonella*), LPS, and EPS (CPS), play an important role in bacterial adherence to plant surfaces, which is a prerequisite process in both animal and plant infection. Among those structural components, flagella and curli seem to substantially impact the survival of *E. coli* O157:H7 on plants. *E. coli* O157:H7 mutants lacking flagella induced

lower GUS activity, and showed enhanced survival on wild-type *Arabidopsis* plants than wild-type *E. coli* O157:H7. Flagella are an important structure for bacterial motility and mediate bacterial adherence to plant surfaces as well. Flagella, are PAMPs, are recognized by most plants via the FLS2 receptor and initiate innate defense responses. Curli-deficient *E. coli* O157:H7 86-24 exhibited greater survival on the wild-type *Arabidopsis* plant with lower PR gene expressions (based on lower GUS activity) than the curli-positive *E. coli* O157:H7 86-24 wild-type strain. From those findings, we concluded that curli was recognized by *Arabidopsis* plants and SA-mediated defense responses were responsible for decreased survival of wild-type *E. coli* O157:H7 86-24. However, the fact that lower population of *E. coli* O157:H7 86-24 curli mutant on wild-type *Arabidopsis* plants than on *npr1-1* mutant plants implies that other PAMP-triggered defenses were also involved in restricting microbial population. Curli-producing *E. coli* O157:H7 also possess multiple PAMPs, such as flagella and LPS. Thus, further studies using purified forms of curli to unveil the precise role of curli in plant defense system response are needed. CsgA, a main component of Tafi in *S*. Typhimurium, are recognized by Toll-like receptor 2 (TLR2) and known as PAMPs (Tuket et al., 2005). Receptor proteins that recognize curli or Tafi in the plant system have not been reported. It seems clear that bacteria PAMPs are key factors in the recognition of microbes, leading to downstream signal transduction, and activation of the plant defense system. However, only a few plant PRRs have been identified, and only limited molecular mechanisms underlying PAMP-triggered immunity have been elucidated.

Plant pathogens and symbionts have evolved elaborate strategies to avoid or suppress plant immune systems. Modification or loss of bacterial PAMPs might enable bacteria to avoid being recognized by the plant immune system. Some virulent phytopathogenic bacteria are able to avoid recognition of flagellin by mutating residues within the recognized epitope (Felix et al., 1999; Sun et al., 2006). Lack of flagella in *Klebsiella pneumoniae* 342 strain, a maize endophyte, enables the microbe to avoid triggering the plant defense response, permitting it to colonize plants in very high numbers (Iniguez et al., 2005). LPS, a known PAMPs, is the principal component of the outer membrane of Gram-negative bacteria and induces a defense response in *Arabidopsis* (Zeidler et al., 2004). Modifications of lipid A (Darveau, 1998) and O-antigen of LPS (Lerouge and Vanderleyden, 2002) influence the LPS elicitor activity, resulting in lack of recognition by the host immune system.

Bacterial polysaccharides play important roles in plant-microbe interactions, although the precise role of those structures in regulating the plant defense system is not yet unknown. Bacterial capsules are important virulence factors on the cell surface of many Gram-negative and Gram-positive bacteria. Composition of growth medium and exposure conditions significantly affected the amount of CPS production by *E. coli* O157:H7 (Chapter 4). Addition of manure extracts to a Luria Broth (LB), a standard growth medium, stimulated bacterial CPS production. Moreover, exposure of *E. coli* O157:H7 cells to soil or manure resulted in greater production of CPS compared to non-exposed cells. Nutrient sources from manure extracts necessary for CPS production might positively affect the CPS production by *E. coli* O157:H7. Enhanced CPS production by

E. coli O157:H7 cells exposed for extended periods to soil or manure extracts compared to non-exposed cells may be related to survival tactics of the organism. The influence of capsular polysaccharides (CPS) on plant defense responses and its subsequent effects on the survival of enteric pathogens on plants were discussed in chapter 4. *E. coli* O157:H7 cells with greater CPS exhibited increased survival on plants compared to those with thinner capsules. Moreover, inoculation of *Arabidopsis* plant with highly encapsulated cells resulted in lower plant defense responses (lower PR gene induction) estimated based on lower GUS activity. Several studies provide evidence that bacterial polysaccharides (EPS or CPS) may confer greater virulence to pathogenic bacteria by escaping or suppressing the host immune response, but the information about the precise mechanism is limited. Recent studies have shown that bacterial polysaccharides suppress PAMP-triggered innate immunity by chelating calcium ions, required for activation of the plant defenses (Aslam et al., 2008). Besides the roles of CPS in plant-microbe interactions, results of this study underscores how bacterial culture conditions can impact experimental results ultimately leading to conflicting results between two seemingly similar studies. *E. coli* cells grown in LB medium supplemented with soil or manure extracts produced CPS to a greater extent than LB medium alone. Another study found that tryptic soy broth (TSB) containing dextrose supported greater capsule production of *E. coli* O157:H7, while nutrient broth (NB) lacking dextrose did not produce capsule (Hassan and Frank, 2004). The amount of CPS may influence bacterial cell hydrophobicity (surface free energy) and cell surface charge, which are important factors affecting bacterial adhesion (Hassan and Frank, 2004). Thus, differences in phenotypic

properties of the same strain linked to differences in growth medium might lead to conflicting results.

Enteric pathogens, such as *E. coli* O157:H7 and *Salmonella*, are not phytopathogens. Enteric pathogens in the phyllosphere face great survival challenges because of a number of extreme and fluctuating environmental conditions. The mechanism by which enteric pathogens invade plant tissue following adherence is largely unknown. Plant stomata not only provide a function for efficient gas exchange and water transpiration but also serve as a possible gateway for bacterial entry into intracellular spaces. Like phytopathogens, the enteric pathogens *E. coli* O157:H7 and *Salmonella* aggregate and colonize around and inside stomata, where abundant nutrient sources are available in the apoplastic fluid (Melotto et al., 2006; Seo and Frank, 1999). Melotto et al. (2006) discovered the stomatal function of innate immunity against bacterial invasion. Recognition of bacterial flagellin by FLS2 (flagellin receptor) in stomatal guard cells initiates SA-mediated innate immune responses, resulting in stomatal closure to block bacterial entry. FLS2-mediated defense responses were effective only when plants were contaminated by spray or dip inoculation methods (mimic natural infections), but not when bacteria were artificially passed through the leaf intracellular space (Zipfel et al., 2004). Recognition of microbes by PRRs represents the first line of plant defense and provides the protection against attack by pathogens by initiating innate immune responses. These findings imply that pathogenic bacteria should overcome or suppress the stomatal immune response to invade and establish internal colonization under the natural contamination process.

Recent studies have demonstrated that some enteric pathogens have evolved mechanisms for accessing internal plant tissue, adapting to plant environments, and proliferating in the edible portion of the plant. Shirron and Yaron (2011) found that the human pathogen *Salmonella* Typhimurium actively suppressed early defense responses, such as the production of oxidative burst and pH changes by plant cells in tobacco plants, and the *Salmonella* pathogenicity island 1 (SPI) TTSS was involved in the inhibition of plant responses. They suggested that some bacterial systems evolved to suppress the innate immune responses in the plant host help enteric pathogens to survive in the plant. Similar studies conducted by Schikora et al. (2008) showed that *Salmonella* Typhimurium can overcome plant innate immune responses and cause wilting and chlorosis in infected *Arabidopsis* plants, resulting in death. Prithiviraj et al. (2005) reported that *Staphylococcus aureus* was pathogenic to *Arabidopsis* plant and virulence factors essential for animal pathogenesis were also necessary for plant infection. From these findings, it is evident that some pathogenic enteric bacteria utilize many similar mechanisms as phytopathogens to associate with plants and colonize or invade plants as their alternative host. Components of *Salmonella* TTSS appear to be recognized by host plants. Deletion of genes encoding the structural subunit of the TTSS SPI 1 (*spaS*) and effector protein and translocator (*sipB*) resulted in enhanced endophytic colonization of *Salmonella* on alfalfa roots and wheat seedlings (Iniguez et al., 2005). The role of effector molecules from phytopathogens in suppression of plant host defense responses is well understood, however, the precise role of effectors from enteric pathogens reamins unknown.

In this research, we demonstrated that bacterial cell surface structures of *E. coli* O157:H7, in particular, flagella and curli, significantly impacted the plant defense response, affecting survival of the enteric pathogen on the plant. We also showed that CPS production of *E. coli* O157:H7, influenced by exposure to soil or manure, may regulate plant defense response, and consequently impact survival of that human pathogen. This study was mainly focused on pre-harvest enteric pathogen-plant interactions that influence fate of the enteric pathogen on the plant. Findings from this research will provide a better understanding of the factors involved in intimate association of foodborne pathogens with plants and the plant response to enteric bacteria. We used *Arabidopsis* as a model plant, however, future research on plant defense responses using commercial crops, such as lettuce or spinach, will provide fundamental knowledge about the complexity between human pathogens and plants and facilitate development of new intervention strategies for ensuring the microbial safety of produce.

REFERENCES

Aslam, S.N., M.A. Newman, G. Erbs, K.L. Morrissey, D. Chinchilla, T. Boller, T.T. Jensen, C. De Castro, T. Ierano, A. Molinaro, R.W. Jackson, M.R. Knight, R.M. Cooper. 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. Curr. Biol. 18:1078-1083.

Banatvala, N., P.M. Griffin, K.D. Greene, T.J. Barrett, W.F. Bibb, J.H. Green, and J.G. Wells. 2001. The United States national perspective hemolytic uremic syndrome study: Microbiologic, serologic, clinical, and epidemiologic findings. J. Infect. Dis. 183:1063-1070.

Barak, J.D., L. Gorski, P. Naraghi-Arani, and A.O. Charkowski. 2005. *Salmonella enterica* virulence genes are required for bacterial attachment to plant tissue. Appl. Environ. Microbiol. 71(10): 5685-5691.

Barak, J.D., C.E. Jahn, D.L. Gibson, and A.O. Charkowski. 2007.
The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. Mol. Plant Microbe Interact. 20(9): 1083-1091.

Barnhart, M.M. and M.R. Chapman. 2006. Curli biogenesis and function. Annu. Rev. Microbiol. 60: 131-147.

Beuchat, L.R. 2002. Ecological factors influencing survival and growth of human pathogen on raw fruits and vegetables. Microb. Infect. 4: 413-423.

Bonet, R., M.D. Simon-Pujol, and F. Congregado. 1993. Effects of nutrients on exopolysaccharide production and surface properties of *Aeromonas salmonicida*. Appl. Environ. Microbiol. 59: 2437-2341.

Bower, C.K., J. McGuire, and M.A. Daeschel. 1996. The adhesion and detachment of bacteria and spores on food-contact surfaces. Trends Food Sci. Technol. 7(5): 152-157.

Boyer, R.R., S.S. Sumner, R.C. Williams, M.D. Pierson, D.L. Popham, and K.E. Kniel. 2007. Influence of curli expression by *Escherichia coli* 0157:H7 on the cell's overall hydrophobicity, charge, and ability to attach to lettuce. J. Food Prot. 70(6):1339-1345.

Brandl, M.T. 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annu. Rev. Phytopathol. 44:367-392.

Brandl, M.T. 2008. Plant lesions promote the rapid multiplication of *Escherichia coli* 0157:H7 on postharvest lettuce. Appl. Environ. Microbiol. 74: 5285-5289.

Buchanan, R.L. and M.P. Doyle. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. Food Technol. 51(10): 69-75.

Buchanan, R. 2006. Spinach outbreak as part of broader concerns about produce safety - A FDA perspective. Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, Washington, DC. (www.perishablepundit.com/docs/SpinachSafety-BuchananRobert.pdf)

Bugert, P. and K. Geider. 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Mol. Microbiol. 15(5): 917-933.

Campos, M.A., M.A.Vargas, V. Regueiro, C.M. Llompart, S. Alberti, and J.A. Bengoechea. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. Infect Immun. 72:7107-7114.

Cao, H., S.A. Bowling, A.S. Gordon, and X. Dong. 1994. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6:1583-1592.

Center for Disease Control and Prevention (CDC). 2006. Surveillance for foodborne-disease outbreaks-United States, 1998-2002. MMWR 55 (No. SS-10).

Center for Disease Control and Prevention (CDC). 2011. Investigation announcement: Outbreak of Shiga toxin-producing *E. coli* O104 (STEC O104:H4) infections associated with travel to Germany. Final update (July 8, 2011) http://www.cdc.gov/ecoli/2011/ecoliO104/

Center for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach-United States, September 2006. MMWR Morb. Mortal. Wkly. Rep. 55:1045-1046.

Chen, S., W. Liao, C. Liu, Z. Wen, R.L. Kincaid, J.H. Harrison, D.C. Elliott, M.D. Brown, A.E. Solana**,** and D.J. Stevens. 2003. Value-Added Chemicals from Animal Manure. PNNL-14495, Pacific Northwest National Laboratory, Richland, WA.

Chisholm, S. T., G. Coaker, B. Day, and B. J. Staskawicz. 2006. Host-Microbe interactions: Shaping the evolution of the plant immune response. Cell. 124:803-814.

Cooley, M. B., W.G. Miller, and R.E. Mandrell. 2003. Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. Appl. Environ. Microbiol. 69: 4915-4926.

Cooley, M.B., D. Chao, and R.E. Mandrell. 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. J. Food Prot. 69**:** 2329–2335.

D'Haeze, W. and M. Holsters. 2004. Surface polysaccharides enable bacteria to evade plant immunity. Trends in Microbiol. 12(12): 555-561.

Darveau, R.P. 1998. Lipid A diversity and the innate host response to bacterial infection. Curr. Opin. Microbiol. 1: 36-42.

Deflaun, M.F., B.M. Marshall, E.P. Kulle, and S.B. Levy. 1994. Tn5 insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. 60: 2637-2642.

Dow, M., M.A. Newman, and E. von Roepenack. 2000. The induction and modulation of plant defense responses by bacterial lipopolysaccharides. Annu. Rev. Phytopathol. 38: 241-261.

Duffitt, A.D., R.T. Reber, A. Whipple, and C. Chauret. 2011. Gene expression during survival of *Escherichia coli* O157:H7 in soil and water. Int. J. Microbiol. Article ID 340506.

Dunne, W.M. Jr. 2002. Bacterial adhesion: seen any good biofilms lately?. Clin. Microbiol. Rev. 15:155-166.

Durant, W.E. and X. Dong. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42: 185-209.

Ericson, M.C., C.C. Webb, J.C. Diaz-Perez, S.C. Phatak, J.J. Silvoy, L. Davey, A.S. Payton, J. Liao, L. Ma, and M.P. Doyle. 2010. Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. J. Food Prot. 73**:** 1023-1029.

Felix, G., J. D. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18:265-276.

Food and Drug Administration (FDA). 2007. How the FDA works to keep produce safe. (http://permanent.access.gpo.gov/lps1609/www.fda.gov/fdac/features/2007/207_foodsafety.html)

Frank, J.F. 2001. Microbial attachment to food and food contact surfaces. Adv. Food Nutr. Res. 43:319-370.

Franz, E., A.A. Visser, A.D. Van Diepeningen, M.M. Klerks, A.J. Termorshuizen, and A.H. van Bruggen. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiol. 24(1): 106-112.

Girón, J.A., A.G. Torres, E. Freer, and J.B. Kaper. 2002. The flagella of enteropathogenic Escherichia coli mediate adherence to epithelial cells. Mol. Microbiol. 44: 361-379.

Gophna, U., T.A. Oelschlaeger, J. Hacker, and E.Z. Ron. 2002. Role of fibronectin in curli-mediated internalization. FEMS Microbiol. Lett. 212(1): 55-58.

Hammar, M., Z. Bian, and S. Normark. 1996. Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia* coli. Proc. Natl. Acad. Sci. USA 93:6562-6566.

Hanna, A., M. Berg, V. Stout, and A. Razatos. 2003. Role of capsular colanic acid in adhesion of uropathogenic *Escherichia coli*. Appl. Environ. Microbiol. 69: 4474-4481.

Harapas, D., R. Premier, B. Tomkins, P. Franz, and S. Ajouni. 2010. Persistence of *Escherichia coli* on injured vegetable plants. Int. J. Food Microbiol. 138: 232-237.

Hassan, A.N. and J.F. Frank. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell

hydrophobicity, surface charge, and capsule production. Int. J food Microbiol. 96: 103-109.

Iniguez, A.L., Y. Dong, H.D. Carter, B.M.M. Ahmer, J.M. Stone, and E.W. Triplett. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. Mol. Plant Microbe Interact. 18:169-178.

Islam, M., M.P. Doyle, S.C. Phatak, P. Millner, and X. Jiang. 2004A. Persistence of Enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. J. Food Prot. 67: 1365-1370.

Islam, M., J. Morgan, M.P. Doyle, S.C. Phatak, P. Millner, and X. Jiang. 2004B. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. J. Food Prot. 70: 2497-2502.

Jambunathan, N and T.W. McNellis. 2003. Regulation of *Arabidopsis COPINE 1* gene expression in response to pathogens and abiotic stimuli. Plant Physiol 132:1370-1381.

Jann, B. and K. Jann. 1990. Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Top. Microbiol Immunol. 150: 19-42.

Jefferson, R.A., T.A. Kavanagh, and M.W. Bevan. 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Jeter, C. and A.G. Matthysse. 2005. Characterization of the binding of diarrheagenic strains of *E. coli* to plant surfaces and the roles of curli in the interaction of the bacteria with Alfalfa sprouts. Mol. Plant Microbe Interact. 18(11): 1235-1242.

Jones, J.D.G. and J.L. Dangl. 2006. The plant immune system. Nature 444: 323-329.

Kim, S.H. and C.I. Wei. 2009. Molecular characterization of biofilm formation and attachment of Salmonella enterica serovar typhimurium DT104 on food contact surfaces. J. Food Prot. 72: 1841-1847.

Klerks, M.M., M. van Gent-Pelzer, E. Franz, C. Zijlstra, and A.H.C. van Bruggen. 2007. Physiological and molecular responses of Lactuca sativa to colonization by Salmonella enteric Serovar Dublin. Appl. Environ. Microbiol. 73: 4905-4914.

Kolling, G.L. and K.R. Matthews. 2001. Examination of recovery in vitro and in vivo of nonculturable *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 67: 3928-3933.

Lapidot, A. and S. Yaron. 2009. Transfer of *Salmonella enterica* serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. J. Food Prot. 72: 618-623.

Lerouge, I. and J. Vanderleyden. 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol. Rev. 26(1): 17-47.

Li, C., C.J. Louise, W. Shi, and J. Adler. 1993. Adverse conditions which cause lack of flagella in *Escherichia coli*. J. Bacteriol. 175: 2229-2235.

Li, X., H. Lin, W. Zhang, Y. Zou, J. Zhang, X. Tang, and J. Zhou. 2005. Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. Proc. Natl Acad. Sci. USA 102:12990-12995.

Marcus, I.M., C.H. Bolster, K.L. Cook, S.R. Opot, and S.L. Walker. 2012. Impact of growth conditions on transport behavior of *E. coli*. J. Environ. Monit. 14:984-991.

Melotto, M., W. Underwood, J. Koczan, K. Nomura, and S.Y. He. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell. 126: 969-980.

Metzger, M., P. Bellemann, P. Bugert, and K. Geider . 1994. Genetics of galactose metabolism of Erwinia amylovora and its influence on polysaccharide synthesis and virulence of the fire blight pathogen. J. Bacteriol. 176:450-459.

Meyer, A., A. Pühler, and K. Niehaus. 2001. The lipopolysaccharides of the phytopathogen *Xanthomonas campestris pv. campestris* induce an oxidative burst reaction in cell cultures of Nicotiana tabacum. Planta 213: 214-222.

Mishina, T.E. and J. Zeier. 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis*. Plant J. 50: 500-513.

Mitra, R., E. Cuesta-Alonso, A. Wayadande, J. Talley, S. Gilliland, J. Fletcher. 2009. Effect of Route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. J. Food Prot. 72(7): 1521-1530.

Moreno, M., J.P. Audia, S.M. Bearson, C. Webb, and J.W. Foster. 2000. Regulation of sigma S degradation in *Salmonella enterica* var typhimurium: in vivo interactions between sigma S, the response regulator MviA(RssB) and ClpX. J Mol. Microbiol. Biotechnol. 2: 245-254.

Nicaise,V., M. Roux, and C. Zipfel. 2009. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol. 150: 1638–1647.

Nürnberger, T., F. Brunner, B. Kemmerling, and L. Piater. 2004. Innate immunity in plants and animals, striking similarities and obvious differences. Immunol. Rev. 198: 249-266.

Olsén, A., A. Jonsson, and S. Normark. 1989. Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. Nature 338:652-655.

Olsén, A., A. Arnqvist, M. Hammar, and S. Normark. 1993. Environmental regulation of curli production in *Escherichia coli*. Infect Agents Dis. 2(4): 272-274.

Prigent-Combaret, C., G. Prensier, T.-T. Le Thi, O. Vidal, P. Lejeune, and C. Dorel. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colonic acid. Environ. Microbiol. 2(4): 450-464.

Pieterse, C.M.J., A. Leon-Reys, S. Van der Ent, and S.C.M. Van Wees. 2009. Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5(5): 308-316.

Pieterse, C.M.J., S.C.M. van Wees, J.A. van Pelt, M. Knoester, R. Laan, H. Gerrits, P.J. Weisbeek, and L.C. van Loon. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571-1580.

Prithiviraj, B., H.P. Bais, A.K. Jha, and J.M. Vivanco. 2005. *Staphylococcus aureus* pathogenicity on *Arabidopsis thaliana* is mediated either by direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1-independent hosp responses. Plant J. 42: 417-432.

Rangel, J.M., P.H. Sparling, C. Crowe, P.M. Griffin, and D.L. Swerdlow. 2005. Epidemiology of *Escherichia coli* O157:H7 Outbreaks, United States, 1982–2002. Emerg. Infect. Dis. 11:603-609.

de Rezende, C.E., Y. Anriany, L.E. Carr, S.W. Joseph and, R.M. Weiner. 2005. Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. Appl Environ Microbiol. 71: 7345-7351.

Rice, E.W., C.H. Johnson, D.K. Wild, and D.J. Reasoner. 1992. Survival of *Escherichia coli* O157: H7 in drinking water associated with a waterborne disease outbreak of hemorrhagic colitis. Lett. Appl. Microbiol. 15: 38-40.

Riley, L.W., R.S. Remis, S.D. Helgerson, H.B. McGee, J.G. Wells, B.R. Davis, R.J. Herbert, E.S. Olcott, L.M. Johnson, N.T. Hargrett, P.A. Blake, and M.L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. 308:681-685.

Roberts, I.S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. 50: 285-315.

Schikora, A., A. Carreri, E. Charpentier, and H. Hirt. 2008. The dark side of the salad: *Salmonella typhimurium* overcomes the innate immune responses of *Arabidopsis thaliana* and shows an endopathogenic lifestyle. PLoS One 6:e18855.

Ryu, J-H and L.R. Beuchat. 2005. Factors affecting production of extracellular carbohydrate complexes by *Escherichia coli* O157:H7. Int. J. Food Microbiol. 95:189-204.

Seo, K. and J.F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. J. Food Prot. 62: 3-9.

Seo, S. and K.R. Matthews. 2012. Influence of the plant defense response to *Escherichia coli* O157:H7 cell surface structures on survival of that enteric pathogen on plant surfaces. Appl. Environ. Microbiol. 78(16) 5882-5889.

Sheng, H., J.Y. Lim, M.K. Watkins, S.A. Minnich, and C.J. Hovde. 2008. Characterization of an *Escherichia coli* O157:H7 O-antigen deletion mutant and effect of

the deletion on bacterial persistence in the mouse intestine and colonization at the bovine terminal rectal mucosa. Appl. Environ. Microbiol. 74: 5015-5022.

Shirron, N. and S. Yaron. 2011. Active suppression of early immune response in Tobacco by the human pathogen *Salmonella Typhimurium*. PLoS One 6:e18855.

Sivapalasingam, S., C.R. Friedman, L. Cohen , R.V. Tauxe. 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J. Food Prot. 67:2342-2353.

Solomon, E.B., B.A. Niemira, G.M. Sapers, and B.A. Annous. 2005. Biofilm formation, cellulose production, and curli biosynthesis by Salmonella originating from produce, animal, and clinical sources. J. Food Prot. 68: 906-912.

Solomon, E.B., C.J. Potenski, and K.R. Matthews. 2002. Effect of irrigation method on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. J. Food Prot. 65: 673-676.

Solomon, E.B., S. Yaron, and K.R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68: 397-400.

Sun, W., F.M. Dunning, C. Pfund, R. Weingarten, and A.F. Bent. 2006. Within-Species flagellin polymorphism in *Xanthomonas campestris* pv *campestris* and its impact on elicitation of *Arabidopsis FLAGELLIN SENSING2*-Dependent defenses. Plant Cell. 18: 764-779.

Taguchi, F., R. Shimizu, Y. Inagaki, K. Toyoda, T. Shiraishi, and Y. Ichinose. 2003. Post-translational modification of flagellin determines the specificity of HR induction. Plant Cell Physiol. 44(3): 342-349.

Talley, J.L., A.C. Wayadande, L.P. Wasala, A.C. Gerry, J. Fletcher, U. DeSilva, and S.E. Gilliland. 2009. Association of *Escherichia coli* O157:H7 with filth flies (Muscidae and Calliphoridae) captured in leafy greens fields and experimental transmission of *E. coli* O157:H7 to spinach leaves by house flies (Diptera: Muscidae). J. Food Prot. 72(7): 1547-1552.

Thilmony, R., W. Underwood, and S.Y. He. 2006. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato*DC3000 and the human pathogen *Escherichia coli* O157:H7. Plant J. 46: 34-53.

Tukel, C., M. Raffatellu, A.D. Humphries, R.P. Wilson, H.L. Andrews-Polymenis, T. Gull, J.F. Figueiredo, M.H. Wong, K.S. Michelsen, M. Akcelik, L.G. Adams, and A.J. Baumler. 2005. *CsgA* is a pathogen-associated molecular pattern of *Salmonella enterica* serotype Typhimurium that is recognized by Toll-like receptor 2. Mol. Microbiol. 58: 289-304.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 67:2367-2370.

Van Wees, S.C.M., S. Van der Ent, and C.M.J. Pieterse. 2008. Plant immune responses triggered by beneficial microbes. Curr. Opin. Plant Biol. 11: 443-448.

Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. J. Bacteriol. 180: 2442-2449.

Volko, S.M., T. Boller, and F.M. Ausubel. 1998. Isolation of new *Arabidopsis* mutants with enhanced disease susceptibility to *Pseudomonas syringae* by direct screening. Genetics 149:537-548.

Wachtel, M.R., L.C. Whitehand, and R.T. Mandrell. 2002. Association of *Escherichia coli* O157:H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. J. Food Prot. 65(1):18-25.

Wang, G., T. Zhao, and M.P. Doyle. 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. Appl. Environ. Microbiol. 62: 2567-2570.

Wei, X. and W.D. Bauer. 1998. Starvation-induced changes in motility, chemotaxis, and flagellation of *Rhizobium meliloti*. Appl. Environ. Microbiol. 64:1708-1714.

Wells, J.M. and J.E. Butterfield. 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. Plant Dis. 81: 867-872.

White, A.P., D.L. Gibson, W. Kim, W.W. Kay, and M.G. Surette. 2006. Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. J. Bacteriol. 188(9): 3219-3227.

de Wit, P.J.G.M. 2007. How plants recognize pathogens and defend themselves. Cell. Mol. Life Sci. 64: 2726-2732.

Xicohtencatl-Cortes, J., E.S. Chacón, Z. Saldaña, E. Freer, and J.A. Girón. 2009. Interaction of *Escherichia coli* O157:H7 with leafy green produce. J. Food Prot. 72(7): 1531-1537.

Zeidler, D., U. Zähringer, I. Gerber, I. Dubery, T. Hartung, W. Bors, P. Hutzler, and J. Durner. 2004. Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate

nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. USA 101:15811-15816.

Zipfel, C., S. Robatzek , L. Navarro, E.J. Oakeley, J.D. Jones, G. Felix, and T. Boller. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428:764-767.

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King, J.M., An, H.J., **Seo, S**.-**W**, and Prudente, A. 2012. Ozonation of Hydrocolloids, in Ozone in Food Processing (eds. C. O'Donnell, B.K. Tiwari, P.J. Cullen and R. G. Rice), Wiley Blackwell, Oxford, UK. doi: 10.1002/9781118307472.ch7

S. Seo, J.M. King, W. Prinyawiwatkul, and M. Janes. 2008. Antibacterial activity of ozone-depolymerized crawfish chitosan. Journal of Food Science: 73(8) M400-404.

S. Seo, J.M. King, and W. Prinyawiwatkul. 2007. Simultaneous depolymerization and decolorization of chitosan by ozone treatment. Journal of Food Science: 72(9) 522-526.