Investigation of Possible Situation of Internalization of *Salmonella* Enteritidis in Tomato Fruits and Bacterial Survival during Tomato Plant Cultivation

Tomoko MISHIMA^{1*}, Nozomi KIDO¹, Satoko NAKASHIMA¹, Mami YAMAKAWA¹, Natsumi MIYAJI¹, Kevin Webby SOLI¹, Ken-ichi HONJOH¹, Md. Latiful BARI² and Takahisa MIYAMOTO¹

¹ Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi, 812-8581, Japan

² Food Analysis and Research Laboratory Center for Advanced Research in Sciences, University of Dhaka, Dhaka-1000, Bangladesh

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Tomatoes have recently been implicated as an important vehicle in outbreaks of produce-associated salmonellosis. Traceback reports suggested that pre-harvest contamination of *Salmonella enterica* might be the main reason for these outbreaks; however, the site of pathogen attachment remains unclear. Therefore, it is important to investigate the mechanisms of *Salmonella* contamination of fresh produce. To trace the presence of *Salmonella* in soil and plants, *Salmonella* Enteritidis transformed with a pEGFP plasmid vector (*S. Enteritidis*-EGFP) was used. Soil was artificially contaminated with *S. Enteritidis*-EGFP at 10⁴, 10⁶ or 10⁸ CFU/g, followed by cultivation of tomato plants in the contaminated soil. Samples of the soil and each organ of the tomato (fruit, stems/leaves, and root) were assayed for *Salmonella* by plating onto Tryptic Soy Agar and using the MPN method. *Salmonella* levels in the soil gradually decreased over time, and soil persistence was dependent on the initial inoculation level. *Salmonella* levels were below the detection limit (< 100 CFU/g) in fruits and stems/leaves, regardless of the level of soil contamination. Moreover, *S. Enteritidis*-EGFP was not detected in the tomato fruits after root injury. These results indicate that the internalization of *Salmonella* in tomato fruits might not occur after cultivation in contaminated soil.

Keywords: *Salmonella*, fresh-produce, soil, contamination

Introduction

Outbreaks of foodborne illnesses associated with fresh produce, including minimally processed fruits and vegetables, have been increasing in developed countries (Beuchat *et al.*, 1996; De Rover, 1998; Sivapalasingam *et al.*, 2004). Sivapalasingam *et al.* (2004) reported that the number of outbreaks associated with fresh produce increased from two outbreaks in 1973 to 16 in 1997. Moreover, recent outbreaks in Germany were reported to be caused by the unusual enteroaggregative verocytotoxin-producing *Escherichia coli* (EAggEC VTEC) O104:H4 bacterium, and bean and seed sprouts (including fenugreek, mung beans, lentils, adzuki beans and alfalfa) were implicated in the outbreaks (WHO., 2011, iii). In 2011, a *Salmonella agona* outbreak linked to papayas from Mexico (USDA., 2011, i) and *Listeria monocy-*

togenes infection associated with cantaloupes (USDA., 2011, ii) were also reported in the United States. In particular, consumption of raw and minimally processed fruits and vegetables, the main reason for these outbreaks, has increased substantially in the United States during the past two decades (Sivapalasingam *et al.*, 2004). In Japan, there has been an increase in the consumption of ready-to-eat vegetables, packaged salads and pre-cut salads, and a concomitant increase in associated foodborne illnesses, according to reports of the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) (2007). According to the Ministry of Health, Labour and Welfare-2010 report, approximately 4% of cases of foodborne illnesses were due to vegetables in Japan; whereas, in the United States, this number was approximately 20% (MAFF, 2007). Moreover, other reports revealed that sprouts and green leafy vegetables in the market might have been contaminated with *E. coli* and *Salmonella* spp (Kobayashi *et al.*, 2008).

*To whom correspondence should be addressed.

E-mail: tmishima@agr.kyushu-u.ac.jp

In the farm-to-table steps, including production, processing, and distribution, there are several possible points of contamination of fresh produce with foodborne pathogens. These include the irrigation water, improperly composted manure, wild and domestic animals, wash water, handling by workers (Cieslak *et al.*, 1993; Beuchat *et al.*, 1997; Tauxe *et al.*, 1997). Pathogens are able to survive in improperly composted manure and contaminated soil, thereby contaminating the fresh produce. Water used for irrigation, pesticide mixing, produce washing and rinsing, or making processed foods were highlighted as potential contamination sources (Jacobsen and Bech, 2012; Berger *et al.*, 2010).

Tomatoes are one of the most common vehicles of produce-associated Salmonellosis. Since 1998, 12 outbreaks caused by *S. enterica* were linked to tomato consumption in the United States (CDC, 2005; Cummings *et al.*, 2001). Tomato contamination was also reported to originate from the fields where the tomatoes were grown (CDC, 2007). However, the route of contamination remains vague. It was shown that *S. enterica* can contaminate entire tomato plants following direct root inoculations in a hydroponic system (Guo *et al.*, 2002), and *S. enterica* was observed to contaminate tomato fruits by direct inoculation of flowers (Guo *et al.*, 2001). These results suggest that *Salmonella* contamination of tomatoes can occur during cultivation.

Additionally, *S. enterica* has been shown to contaminate carrots, radish, lettuce and parsley in field studies (Islam *et al.*, 2004). Beuchat *et al.* (2003) investigated the potential role of an endoparasitic nematode in facilitating the internalization of *Salmonella* into tomato tissues. They reported that the internalization of *Salmonella* into tomato tissues via nematode-facilitated root entrance appears to be remote. Investigation of contamination or internalization of *S. enterica* into tomato fruits through artificially injured roots has not been reported. Moreover, the contamination mechanisms of fresh produce in soil remain unclear. It is important to control the contamination factors of fresh produce during cultivation. Therefore, the objective of this study was to investigate the contamination mechanisms of tomato fruits grown in soil contaminated with *S. Enteritidis*.

Materials and Methods

Bacterial strain and culture conditions *Salmonella* Enteritidis IFO3313 was purchased from the Institute for Fermentation, Osaka (IFO), Japan and was electrophoretically transformed with a plasmid vector, *pEGFP* (BD Biosciences, San Jose, California, USA.), carrying a gene for an enhanced green fluorescent protein (EGFP). The transformed strain was designated *S. Enteritidis*-EGFP. One loopful of *S. Enteritidis*-EGFP was inoculated into 10 mL of Tryptic Soy

Broth (Difco Laboratories Inc, Cockeysville, MD, USA) supplemented with 50 µg/mL of ampicillin (Nacalai Co. Ltd, Tokyo, Japan) and incubated with shaking at 37°C overnight. Cells were harvested by centrifugation at $5,000 \times g$ for 5 min and resuspended in sterile water at concentrations of approximately 10^5 , 10^7 , and 10^9 CFU/mL.

Preparation of S. Enteritidis-EGFP-contaminated soil

Commercially available soil (peat-moss/perlite/vermiculite; pH 5.5-6.5, water content 10%) was purchased from Oishi Bussan Co. Ltd., Tokyo, Japan. Since the soil showed excellent permeability, drainage, and water retention, and was stable for plant growth, the soil was used in experiments. The soil was sterilized by autoclaving before inoculation. Approximately 500 g of soil was mixed with 250 mL of a 10^5 , 10^7 , or 10^9 CFU/mL *S. Enteritidis*-EGFP suspension. The concentrations of *S. Enteritidis*-EGFP in contaminated soil were approximately 10^4 , 10^6 , or 10^8 CFU/g after mixing. The contaminated soils were then placed in individual plastic pots (diameter: 7.5 cm). Each contaminated soil was divided into six pots, only one of which was used for cultivation of tomato, and the others were used for sampling of soils during the cultivation periods (70 days). To determine the time variation of *S. Enteritidis*-EGFP counts in the soils, one pot of the soil sample was used and analyzed at each contaminated level (10^4 , 10^6 , or 10^8 CFU/g) every two weeks during tomato cultivation.

Sterilization of seeds Seeds of *Solanum lycopersicum* cv. Micro-Tom were provided by Gene Research Center, University of Tsukuba (Ibaraki, Japan). The seeds were surface disinfected with 70% ethanol for 10–15 s, and then dipped in 0.2% sodium hypochlorite solution for 10 min. After sterilization, the seeds were rinsed three times with sterile water for germination.

Growth condition of tomato plants Tomato seeds were planted in the contaminated soil and grown in a growth chamber (TAITEC Co., Ltd, Saitama, Japan) at 25°C with a 16:8 h light:dark photoperiod. Sterile tap water (20–30 mL) was added directly to the soil daily, without leaf or stem contact. Furthermore, 20–30 mL of nutrient solution (0.2% Hypoex; Hypoex, Osaka, Japan) was applied to tomato plants once per week.

Sampling methods of tomato plants During cultivation, *S. Enteritidis*-EGFP levels in soil samples (as shown in Fig. 1a) were determined every two weeks. Contaminated soil without tomato seeds served as the control sample. After 10 weeks of cultivation, tomato fruits (29 fruits with calyces) were harvested to determine viable bacterial and *S. Enteritidis*-EGFP counts on fruits (Fig. 1b), stems/leaves (Fig. 1c), and root (Fig. 1d). Tomato plants (Fig. 1e) were harvested by cutting the stem with sterilized scissors at 2 cm above the soil.

Fig. 1. Photos of tomato plant parts and soil used for cultivation. (a), tomato plant (whole); (b), tomato fruit; (c), tomato stems/leaves; (d), tomato root; (e), soil.

*Irrigation with contaminated water to maintain high *S. Enteritidis*-EGFP soil levels* To examine the effect of higher contamination levels of *S. Enteritidis*-EGFP in the soil, bacterial culture was diluted in sterile water at a concentration of approximately 10^9 CFU/mL and the solution was used as irrigation water. Twenty to 30 mL of contaminated water (10^9 CFU/mL) was applied directly to the soil, without leaf or stem contact. To maintain the concentration (10^7 – 10^8 CFU/g) of *S. Enteritidis*-EGFP in soil, 20 – 30 mL of the *S. Enteritidis*-EGFP suspension (approximately 10^9 CFU/mL) was applied every two weeks to the soil. On the day following the irrigation date, the contaminated soils (approximately 5 g) were harvested and viable and *S. Enteritidis*-EGFP counts were determined, except for at time 0 day; the soil at time 0 day was harvested just after *S. Enteritidis*-EGFP contamination. Tomato plants were cultivated in the soil for 10

weeks, and fruits were harvested (with calyx) and analyzed for *S. Enteritidis*-EGFP content.

*Detection methods for *S. Enteritidis*-EGFP in plants and soil* Samples of tomato fruits (4 – 8 g) and stems/leaves (5 – 9 g) were transferred to separate Stomacher bags, diluted ten-fold (one part tomato to 9 parts buffered peptone water) with buffered peptone water (BPW; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), and homogenized for 30 sec using a Masticator (IUL instruments, Barcelona, Spain). Aliquots (1 mL) of the homogenized samples was serially diluted with sterile water, and then spread (100 μ L) onto Tryptic Soy agar (TSA, Difco), Desoxycholate-hydrogen sulfide-lactose agar (DHL, Nissui) and TSA containing 50 μ g/mL ampicillin (TSA/amp) in duplicate and incubated at 37°C for 48 h. The colonies on TSA were counted as total viable bacterial counts, colonies expressing green-fluorescence on TSA/amp were counted as *S. Enteritidis*-EGFP, red colonies on DHL were counted as Enterobacteriaceae, while black colonies on DHL were counted as *S. Enteritidis*-EGFP. For the determination of viable counts less than the lower detection limit of the plating method, the most probable number (MPN) method was performed. The MPN test was performed as follows: Three test tubes containing 10 mL of BPW were labeled as “0.1”, another three tubes containing 10 mL of BPW were labeled as “1”, while three tubes containing 10 mL of double strength BPW were labeled as “10”. MPN tubes labeled as “10”, “1” and “0.1” were aseptically inoculated with 10 mL, 1 mL and 0.1 mL of sample, respectively.

The test tubes were incubated at 37°C for 20 ± 2 h. An aliquot (100 μ L) of the culture was inoculated into 10 mL of Rappaport Vassiliadis broth (Merck KGaA, Darmstadt, Germany), and incubated at 42°C for 22 ± 2 h. Finally, one loopful of the culture was streaked over DHL and incubated at 37°C for 24 ± 2 h. MPN of *Salmonella* was determined using the most probable number table (3 tubes). In order to confirm the identity of black colonies without green fluorescence as *Salmonella* colonies, several black colonies were randomly chosen and investigated for the *invA* gene using PCR assay (Rahn *et al.*, 1992).

*Contamination of tomato fruits with *S. Enteritidis*-EGFP via artificial root injury* To investigate whether root injury promotes the invasion of *S. Enteritidis*-EGFP, via vessels, into tomato fruits, the main root of the tomato plant, which had been cultivated in *S. Enteritidis*-EGFP contaminated soil (10^8 CFU/g), was cut off at flowering time. The root was cut off 1 cm below the soil surface after 8 weeks, and the tomato plant was subsequently replanted in *S. Enteritidis*-EGFP contaminated soil at approximately 10^7 CFU/g. Two weeks after replantation, five fruits were harvested. To investigate *S. Enteritidis*-EGFP invasion into tomato fruits, fruits without

calyces were sterilized with 200 ppm sodium hypochlorite solution for 5 min, and washed with sterile water. Sterilized fruits and non-sterilized calyces were each analyzed for viable bacterial counts.

Flower inoculation with *S. Enteritidis*-EGFP To investigate the survival of *S. Enteritidis*-EGFP in fruits, 25 flowers were inoculated with 100 μ L of *S. Enteritidis*-EGFP (approximately 10^7 CFU/mL) at 9 weeks of cultivation. Of the inoculated flowers, 16 flowers become fruits and all the fruits were used for the *Salmonella* assay.

Results

Survival of *S. Enteritidis*-EGFP in soil To investigate the survival of *Salmonella* in soil, *S. Enteritidis*-EGFP was added to the soil at an initial contamination level of 10^4 , 10^6 , or 10^8 CFU/g. TSA/Amp plates were used for *S. Enteritidis*-EGFP counts, while DHL plates were used for Enterobacteriaceae counts. On the DHL plates, approximately 60% of colonies were black, with green fluorescence and without green fluorescence (data not shown). Several black colonies without green fluorescence were randomly chosen and used for colony PCR, as described by Rahn *et al.* (1992), to confirm the identity of colonies as *Salmonella* spp (data not shown). *S. Enteritidis*-EGFP counts in the 10^4 CFU/g sample increased to 10^6 CFU/g within the first two weeks and decreased to 10^1 CFU/g after 10 weeks, and viable bacterial counts increased to 10^8 CFU/g within the first two weeks and remained at 10^8 CFU/g after 10 weeks (Fig. 2a). *S. Enteritidis*-EGFP counts in the 10^6 CFU/g sample also increased to 10^8 CFU/g and decreased to 10^6 CFU/g after 10 weeks (Fig. 2b). On the other hand, viable bacterial counts increased to 10^8 CFU/g and remained at that level for 10 weeks. At an initial contamination level of 10^8 CFU/g in soil, *S. Enteritidis*-EGFP counts decreased to 10^5 CFU/g within 4 weeks and was maintained for 10 weeks (Fig. 2c). Similar experimental results were obtained with the control samples on which tomato seeds were not sown (data not shown).

Relationship between contamination levels in soil and tomato parts To investigate contamination of tomato plants with *Salmonella*, the effect of the initial concentration of *S. Enteritidis*-EGFP in the soil was studied. Initial concentrations of *S. Enteritidis*-EGFP were 10^4 , 10^6 , or 10^8 CFU/g. After 10 weeks cultivation, fruits, stems/leaves, and roots were harvested for use in the *Salmonella* assay. Although bacteria from fruits and stems/leaves of plants were countable on TSA/Amp, the colonies did not show green fluorescence and were not similar to colonies of *Salmonella* in morphology. Based on these results, we determined that *S. Enteritidis*-EGFP was not detected in the fruits and stems/leaves of plants, regardless of the initial contamination level (Fig. 3).

Fig. 2. Change in viable, Enterobacteriaceae, and *S. Enteritidis*-EGFP counts in soil initially contaminated with *S. Enteritidis*-EGFP at (a) 10^4 CFU/g, (b) 10^6 CFU/g, and (c) 10^8 CFU/g during tomato plant cultivation. $\circ$, Viable cell counts on TSA; $\square$, Enterobacteriaceae counts on DHL; $\blacktriangle$, *S. Enteritidis*-EGFP, expressing green fluorescence, counts on TSA/amp. Data are means $\pm$ standard deviations (SD) (n = 3).

On the other hand, *S. Enteritidis*-EGFP was detected in the roots and soil. Viable bacterial counts of fruits and stems/leaves were $10^2 - 10^3$ and $10^4 - 10^6$ CFU/g, respectively. Viable bacterial counts were $10^7 - 10^8$ CFU/g, irrespective of

Fig. 3. Viable, Enterobacteriaceae and *S. Enteritidis*-EGFP counts from parts of tomato plants cultivated in contaminated soil. Initial concentration of *S. Enteritidis*-EGFP was (a) 10^4 CFU/g, (b) 10^6 CFU/g, or (c) 10^8 CFU/g during tomato plant cultivation. Black bar, viable cell counts on TSA; Gray bar, viable counts on TSA/Amp; White bar, Enterobacteriaceae counts on DHL (n = 1).

initial concentration.

Possibility of contamination of tomato cultivated in highly contaminated soil Internalization of *S. Enteritidis*-EGFP was investigated in tomato plants that were cultivated

Fig. 4. Changes in viable, Enterobacteriaceae, and *S. Enteritidis*-EGFP counts in continually-contaminated soil. The soils were harvested as described in the Materials and Methods. ○, Viable cell counts on TSA; □, Enterobacteriaceae counts on DHL; ▲, *S. Enteritidis*-EGFP, expressing green fluorescence, counts on TSA/amp. Data are means ± SD (n = 3).

in highly contaminated soil. For this purpose, highly contaminated water (10^9 CFU/mL) was applied to the soil every two weeks and the soil contamination level was determined (Fig. 4). Although viable and Enterobacteriaceae counts were maintained at relatively high levels ($10^7 - 10^8$ CFU/g), unexpectedly, *S. Enteritidis*-EGFP counts were not high (10^6 CFU/g). During 10 weeks of cultivation, no *S. Enteritidis*-EGFP was detected in tomato fruits with calyces on either selective or nonselective agar (Table 1). Furthermore, *S. Enteritidis*-EGFP counts were lower than the detection limit of *Salmonella* by the MPN method (< 0.3 MPN/g). Viable bacterial counts of 20 harvested fruits from 3 plants were 6.1 ± 1.9 log CFU/g. Contamination of tomato plants with *S. Enteritidis*-EGFP was not confirmed.

Possibility of internalization of *S. Enteritidis*-EGFP into fruits via root injury To analyze the effect of root injury on *S. Enteritidis*-EGFP internalization into tomato plants, the main root of one tomato plant was cut and replanted in contaminated soil. Two weeks after replantation, the edible parts of 5 tomato fruits, without calyces, were prepared and surface-sterilized for microbial analysis.

As shown in Table 1, fruit contamination was not observed in tomato plants grown in soil maintained at a high level of *S. Enteritidis*-EGFP contamination (approximately 10^7 CFU/g). Furthermore, even if the main root of the tomato plant was cut and replanted in highly contaminated soil, no *S. Enteritidis*-EGFP was detected by the agar plate and MPN methods (the detection limit is < 0.3 MPN/g). Although viable bacterial counts in calyces were detected (2.5 ± 1.4 log CFU/g), the *S. Enteritidis*-EGFP level was below

Table 1. Viable cell counts and MPN values of non-sterile tomato fruits (with calyces) cultivated in soil contaminated with *S. Enteritidis*-EGFP.

Tomato fruits	Population (log CFU/g)			MPN value (per g)
	Viable cell counts	<i>S. Enteritidis</i> -EGFP	Enterobacteriaceae	MPN
	6.1 ± 1.9*	ND	ND	< 0.3

* The value is mean ± SD (n = 20). ND, not detected.

Table 2. Viable counts and MPN of edible parts and calyces of tomato after root damage and cultivation in soil contaminated with *S. Enteritidis*-EGFP. Edible parts were surface-sterilized before microbial analysis.

	Population (log CFU/g)			MPN value (per g)
	Viable cell counts	<i>S. Enteritidis</i> -EGFP	Enterobacteriaceae	MPN
	1.6 ± 1.0*	ND	ND	< 0.3
Calyces	2.5 ± 1.4*	ND	ND	< 0.3

*The value is mean ± SD (n = 5). ND, not detected.

Table 3. Viable counts and MPN of edible parts and calyces of tomato after inoculation of flowers with *S. Enteritidis*-EGFP. Edible parts were surface-sterilized before microbial analysis.

	Population (log CFU/g)			MPN value (per g)
	Viable cell counts	<i>S. Enteritidis</i> -EGFP	Enterobacteriaceae	MPN
	2.6 ± 1.1*	ND	ND	< 0.3
Calyces	2.1 ± 1.2*	ND	ND	< 0.3

*The value is mean ± SD (n = 16). ND, not detected.

the detection limit (< 0.3 MPN/g) (Table 2).

Survival of *S. Enteritidis*-EGFP in fruits after inoculation to flowers After direct inoculation of *S. Enteritidis*-EGFP to 25 flowers of one tomato plant, 16 fruits were harvested and viable bacterial counts and *S. Enteritidis*-EGFP counts determined. The viable bacterial counts of the edible parts and calyces (non-sterilized) were $10^2 - 10^3$ CFU/g by the agar plate method. However, *S. Enteritidis*-EGFP levels were below the detection limit (< 0.3 MPN/g) (Table 3).

Discussion

We first investigated the survival of *S. Enteritidis*-EGFP in soil. As shown in Fig. 2, changes in *S. Enteritidis*-EGFP cell numbers in the soil during experiments were dependent on the initial concentration of *S. Enteritidis*-EGFP. *S. Enteritidis*-EGFP levels in the soil increased once within two weeks and then decreased gradually with time. *Salmonella* spp. have been reported to survive from a few days up to 332 days in manure-amended soils (Holley *et al.*, 2006; Islam *et al.*, 2004; You *et al.*, 2006) and *S. enterica* is known to survive for five weeks in fallow soil (Nicholson *et al.*, 2005; Jensen *et al.*, 2006). In addition, bacteria, which are often found in vegetables and are associated with foodborne out-

breaks, were reportedly able to survive in manure for long periods (Cieslak *et al.*, 1993; Beuchat *et al.*, 1996; Hattori *et al.*, 2008). The present study revealed that *S. Enteritidis*-EGFP could survive in soil for three months in a concentration dependent manner.

To investigate the internalization of *S. Enteritidis*-EGFP into tomato plants, *S. Enteritidis*-EGFP was added to soil at an initial concentration of 10^4 , 10^6 , or 10^8 CFU/g before cultivation. After 10-week cultivation, the fruits, stems/leaves, and roots were harvested for use in the *Salmonella* assay. As shown in Fig. 3, *S. Enteritidis*-EGFP was not detected in the fruits and stems/leaves of plants, regardless of initial contamination level. Although it was suggested that *S. enterica* may have the capacity to contaminate plants in contaminated soil (Barak and Liang, 2008) and *S. enterica* was capable of attaching to and contaminating the root and stems/leaves of tomato plants in contaminated soil, no relationship between *S. Enteritidis*-EGFP levels in the soil and that in tomato organs (fruits and stems/leaves) was found in the present study.

As shown in Fig. 3, *S. Enteritidis*-EGFP was not detected in the fruits and stems/leaves of tomato plants cultivated in contaminated soil, regardless of initial contamination level. Guo *et al.* (2002) reported that internalization of *S. enterica*

into the hypocotyls and cotyledons (stems/leaves) of tomato plants occurred after 1, 5, 9 days of hydroponic growth in contaminated nutrient solution. Thus, we assumed that internalization or contamination of *Salmonella* to tomato plants would be affected by the bacterial level in the soil during tomato cultivation. To investigate possible bacterial internalization into tomato cultivated in highly contaminated soil, *S. Enteritidis*-EGFP suspension (approximately 10^9 CFU/mL) was applied every two weeks. The *S. Enteritidis*-EGFP contamination level in edible parts was below the detection limit (< 0.3 MPN/g) (Table 1). Jablasone *et al.* (2004) reported that *S. Enteritidis* was not detected in plant tissue when water contaminated with the pathogen was irrigated directly onto the soil. Additionally, Miles *et al.* (2009) reported that all tomatoes cultivated with irrigation water containing 10^7 CFU/mL of *S. Montevideo* every two weeks were negative for *S. Montevideo* contamination. However, Barak and Liang (2008) reported the ability of *S. Enteritidis* to contaminate both the root and stems/leaves of tomato plants following irrigation with contaminated water. Hintz *et al.* (2010) reported that *S. Newport* may contaminate tomato plant tissues when the pathogen is consistently applied at the root site. Although we investigated the possibility of contamination under similar conditions for cultivation or contamination as they reported, we could not confirm the internalization of *Salmonella* or its contamination of tomato fruits. Furthermore, because irrigation water might act as a source of contamination for tomato plants, the risk of bounded water from contaminated soil was minimized during irrigation. Under this growth condition, *S. Enteritidis*-EGFP levels in the contaminated soil were high (10^8 CFU/g), however no *S. Enteritidis*-EGFP was detected in the plants and fruits. On the other hand, in the field, water exposed to contaminated soil might contact the stems/leaves and fruits, leading to the contamination of tomato plants and fruits.

Next, root injury was considered as a possible route for internalization or contamination of *Salmonella* to tomato fruits. For this purpose, the root of a tomato plant grown in contaminated soil was cut and the plant were replanted in contaminated soil. *S. Enteritidis*-EGFP counts in fruits of the plant with root injury were determined. As shown in Table 2, *S. Enteritidis*-EGFP levels in fruits was below the detection limit (< 0.3 MPN/g). In general, plant pathogens infect plants by invading its tissues via a wound area or a natural open stoma, subsequently colonizing the interior of the plant (Hammond-Kosack *et al.*, 2000). Miles *et al.* (2009) reported that *S. Montevideo* might have initially colonized the root and entered via cracks. Therefore, we investigated whether *Salmonella* could invade tomato fruit via root injury. Although the tomato plant with the injured root was replant-

ed in soil highly contaminated with *S. Enteritidis*-EGFP, *S. Enteritidis*-EGFP was not detected in the edible part (Table 2). Because the number of tested plants was one, this might be insufficient to estimate the effect of root injury on the movement of *S. Enteritidis*-EGFP into tomato fruits. However, our results suggest that the possibility of *S. Enteritidis*-EGFP contamination of tomato fruits via root injury and vessels is relatively low. As was reported by Barak and Liang (2008), *Salmonella* likely colonizes the rhizosphere. Furthermore, Schikora *et al.* (2008) demonstrated the invasion of *S. Typhimurium* into the roots of *Arabidopsis* by microscopic observation of GFP expressed by the bacteria. Additionally, *S. enterica* was observed to concentrate at the root tip and lateral branches of tomato plants and *Arabidopsis*, and has the potential to invade the root at lateral root junctions (Cooley *et al.*, 2003). Although we also attempted to observe the invasion of *S. Enteritidis*-EGFP into the roots, stem/leaves, and fruits of tomato plants with intact roots, we did not observe EGFP fluorescence, suggesting that *S. Enteritidis*-EGFP was not internalized (data not show). If it is possible for *S. Enteritidis*-EGFP to be transported from an injured root to the fruit, it is necessary to investigate *S. Enteritidis*-EGFP invasion into the interior of injured roots and its colonization of the interior of stems, which are possible routes to tomato fruits.

Plants harbor defense mechanisms against infection by plant pathogens, which detect specific components of plant pathogens, and thereby initiate a defense response (Blumwald *et al.*, 1998). *Salmonella enterica* serovar Typhimurium colonizes the roots and leaves of various plants and induces a defense response in plants (Iniguez *et al.*, 2005; Schikora *et al.*, 2008). However, the defense response induced by *S. Typhimurium* is much lower than that induced by the plant pathogen *Pseudomonas syringae* (Schikora *et al.*, 2008). Moreover, as plants do not recognize *S. Typhimurium* as a potentially harmful pathogen, they do not initiate a defense response against *Salmonella* (Berger *et al.*, 2010). Thus, *Salmonella* might have the capacity to colonize, although we could not confirm this in tomato plants.

Furthermore, as another possible route for *Salmonella* internalization into tomato, we investigated the possibility of contamination by attachment to tomato flowers. Guo *et al.* (2001) reported that brushing tomato flowers with a five-strain cocktail of *S. enterica* (serovar Enteritidis, Harford, Michigan, Montevideo, and Poona) resulted in contamination of 25% of ripe fruits. The present result, however, showed that *S. Enteritidis* counts were below the detection limit (< 0.3 MPN/g) in the edible parts of tomato and calyces (Table 3). Differences between our results and that of Guo *et al.* (2001) could be attributable to differences in the tomato species and

inoculation methods employed. Although such differences in strain numbers and methods for inoculating *Salmonella* might lead to differences in infection of *Salmonella* to tomato, the present result showed that *S. Enteritidis*-EGFP could not survive on/in tomato fruits when inoculated onto flowers.

It is well established that the incidence of *S. Enteritidis* on fresh produce is significantly higher on injured produce (Wells *et al.*, 1997; Wells *et al.*, 1999). This study did not show the internalization and existence of *Salmonella* in the fruits of tomato plants cultivated in soil contaminated with *Salmonella*. However, as shown in Fig. 2, if soil was contaminated with pathogens such as *Salmonella*, the pathogen could survive during cultivation, leading to the contamination of fruits and vegetables by direct and/or indirect attachment of the pathogens. Thus, produce contact with soil, water, and/or fertilizers should be avoided in the production of safe fresh produce.

Further research is necessary to support or amend these hypotheses governing the interaction of bacterial plant pathogens, such as *S. Enteritidis*, or normal inhabitants, with plants in the absence of plant disease. These data provide useful information for the development of guidelines for the management of manures, to minimize the risk of pathogen transfer from animal manures to the human food chain.

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References

- Barak, J.D. and Liang, A.S. (2008). Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. *PLoS ONE*, **3**, 1657:1-5.
- Berger, C.N., Sodha, S.V., Shaw, R.K., Griffin, P.M., Pink, D., Hand, P. and Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environ. Microbiol.*, **12**, 2385-2397.
- Beuchat, L.R. (1996). Pathogenic microorganisms associated with fresh produce. *J. Food Prot.*, **59**, 204-216.
- Beuchat, L.R. and J.-H. Ryu. (1997). Produce handling and processing practices. *Emerging Infect. Dis.*, **3**, 439-465.
- Beuchat, L.R. (2003). Potential of a plant-parasitic nematode to facilitate internal contamination of tomato plants by *Salmonella*. *J. Food Prot.*, **66**, 1459-1461.
- Centers for Disease Control and Prevention (2005). Outbreaks of *Salmonella* infections associated with eating Roma tomatoes – United States and Canada, 2004. *MMWR Morb Mortal Wkly Rep.*, **54**, 325-328.
- Centers for Disease Control and Prevention (2007). Multistate outbreaks of *Salmonella* infections associated with raw tomatoes eaten in restaurants – United States, 2005-2006. *MMWR Morb Mortal Wkly Rep.*, **56**, 909-911.
- Cieslak, P.R., Barrett, T.J., Griffin, P.M., Gensheimer, K.F., Beckett, G., Buffington, J. and Smith, M.G. (1993). *Escherichia coli* O157:H7 infection from a manured garden [7]. *The Lancet*, **342**, 367.
- Cooley, M.B., Miller, W.G. and Mandrell, R.E. (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.
- Cummings, K., Barrett, E., Mohle-Boetani, J.C., Brooks, J.T., Farrar, J., Hunt, T., Fiore, A., Komatsu, K., Werner, S.B. and Slutsker, L. (2001). A multistate outbreak of *Salmonella enterica* serotype Baildon associated with domestic raw tomatoes. *Emerging Infect. Dis.*, **7**, 1046-1048.
- Guo, X., Chen, J., Brackett, R.E. and Beuchat, L.R. (2001). Survival of *Salmonellae* on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening. *Appl. Environ. Microbiol.*, **67**, 4760-4764.
- Guo, X., Chen, J., Brackett, R.E. and Beuchat, L.R. (2002). Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. *J. Food Prot.*, **65**, 274-279.
- Guo, X., van Iersel, M.W., Chen, J., Brackett, R.E. and Beuchat, L.R. (2002). Evidence of association of *Salmonellae* with tomato plants grown hydroponically in inoculated nutrient solution. *Appl. Environ. Microbiol.*, **68**, 3639-3643.
- Hammond-Kosack, K. and Jones, J.D.G. (2000). Responses to plant pathogens. In "Biochemistry and Molecular Biology of Plants" ed. by Buchanan, B. B., Gruissem, W., Jones, R. L. pp. 1102-1109.
- Hattori, T., Miyashita, K. and Saitoh, A. (2008). "Microbiology of soil." Yokendo, Tokyo. (in Japanese).
- Hintz, L.D., Boyer, R.R., Ponder, M.A., Williams, R.C. and Rideout, S.L. (2010). Recovery of *Salmonella enterica* Newport introduced through irrigation water from tomato (*Lycopersicon esculentum*) fruit, roots, stems, and leaves. *HortScience*, **45**, 675-678.
- Holley, R.A., Arrus, K.M., Ominski, K.H., Tenuta, M. and Blank, G. (2006). *Salmonella* survival in manure-treated soils during simulated seasonal temperature exposure. *J. Environ. Qual.*, **35**, 1170-1180.
- Iniguez, A.L., Dong, Y., Carter, H.D., Ahmer, B.M.M., Stone, J.M. and Triplett E.W. (2005). Regulation of enteric endophytic bacterial colonization by plant defenses. *Mol Plant-Microbe Interact.*, **18**, 169-178.
- Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P. and

- Jiang, X. (2004). Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Appl. Environ. Microbiol.*, **70**, 2497-2502.
- Islam, M., Morgan, J., Doyle, M.P., Phatak, S.C., Millner, P. and Jiang, X. (2004). Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. *J. Food Prot.*, **67**, 1365-1370.
- Jablasone, J., Brovko, L.Y. and Griffiths, M.W. (2004). A research note: The potential for transfer of *Salmonella* from irrigation water to tomatoes. *J. Sci. Food Agric.*, **84**, 287-289.
- Jacobsen, C.S. and Bech, T.B., (2012). Soil survival of *Salmonella* and transfer to freshwater and fresh produce. *Food Res. Int.*, **45**, 557-566.
- Jensen, A.N., Dalsgaard, A., Stockmarr, A., Nielsen, E.M. and Baggesen, D.L. (2006). Survival and transmission of *Salmonella enterica* serovar Typhimurium in an outdoor organic pig farming environment. *Appl. Environ. Microbiol.*, **72**, 1833-1842.
- Kobayashi, T., Takahashi, E., Sasaki, H. (2008). The researches of microbial contamination of sprout vegetable (2006-2007). *Miyagiken Hoken Kankyou Senta Nenpou*, **26**, 103-104 (in Japanese).
- Miles J.M., Sumner, S.S., Boyer, R.R., Williams, R.C., Latimer, J.G. and McKinney, J.M. (2009). Internalization of *Salmonella enterica* serovar Montevideo into greenhouse tomato plants through contaminated irrigation water or seed stock. *J. Food Prot.*, **72**, 849-52.
- Ministry of Agriculture, Forestry and Fisheries of Japan (2007). The research trends for prevention techniques of microbiological hazards from ready-to-eat vegetables in United States of America. 50. Tokyo. (in Japanese).
- Nicholson, F.A., Groves, S.J. and Chambers, B.J. (2005). Pathogen survival during livestock manure storage and following land application. *Bioresour. Technol.*, **96**, 135-143.
- Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galán, J.E., Ginocchio, C., Curtiss III, R. and Gyles, C.L. (1992). Amplification of an *invA* gene sequence of *Salmonella* typhimurium by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probe.*, **6**, 271-279.
- Schikora, A., Carreri, A., Charpentier, E. and Hirt, H. (2008) The dark side of the salad: *Salmonella* typhimurium overcomes the innate immune response of *Arabidopsis thaliana* and shows an endopathogenic lifestyle. *PLoS ONE*, **5**, art. no. e2279.
- Sivapalasingam, S., Friedman, C.R., Cohen, L. and Tauxe, R.V. (2004). Fresh produce: A growing cause of outbreaks of food-borne illness in the United States, 1973 through 1997. *J. Food Prot.*, **67**, 2342-2353.
- Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M. and Breznak, J.A. (2004). New strategies for cultivation and detection of previously uncultured microbes. *Appl. Environ. Microbiol.*, **70**, 4748-4755.
- Tauxe, R., Kruse, H., Hedberg, C., Potter, M., Madden, J., Wachsmuth, K. (1997). Microbial hazards and emerging issues associated with produce: a preliminary report to the national advisory committee on microbiologic criteria for foods. *J. Food Prot.*, **60**, 1400-1408.
- U.S. Food and Drug Administration. Center for Food Safety and Applied Nutrition. 2001e. Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce.
- U.S. Food and Drug Administration. (2011). FDA working with Mexico to protect safety of imported papayas.
- U.S. Food and Drug Administration. (2011). FDA confirms *Listeria monocytogenes* on Jensen Farms' Rocky Ford-brand cantaloupes.
- Wells, J.M. and Butterfield, J.E. (1997). *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. *Plant Dis.*, **81**, 867-872.
- Wells, J.M. and Butterfield, J.E. (1999). Incidence of *Salmonella* on fresh fruits and vegetables affected by fungal rots or physical injury. *Plant Dis.*, **83**, 722-726.
- World Health Organization. (2011). EHEC outbreak.
- You, Y., Rankin, S.C., Aceto, H.W., Benson, C.E. and Toth, J.D. (2006). Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl. Environ. Microbiol.*, **72**, 5777-5783.

URL Cited

- i) <http://www.cfsan.fda.gov/~comm/ift3-toc.html> (September 30, 2011)
- ii) <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm269495.htm> (August. 25, 2011)
- iii) <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm272527.htm> (September 19, 2011)
- iv) <http://www.euro.who.int/en/what-we-do/health-topics/communicable-diseases/sections/news/2011/06/ehec-outbreak-update-16> (September 16, 2011)