Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Interactions of *Salmonella enterica* with lettuce leaves

Y. Kroupitski[1,2], R. Pinto[1], M.T. Brandl[3], E. Belausov[4] and S. Sela[1]

1 Microbial Food-Safety Research Unit, Department of Food Science, Institute for Technology and Storage of Fresh Produce, Agricultural Research Organization (ARO), The Volcani Center, Beth-Dagan, Israel
2 Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
3 Produce Safety and Microbiology Research Unit, US Department of Agriculture, Agriculture Research Center, Albany, CA, USA
4 Confocal Microscopy Unit, Agricultural Research Organization (ARO), The Volcani Center, Beth-Dagan, Israel

Keywords
acid, biofilm, chlorine, disinfection, fresh-cut, leaf, persistence, produce, Romaine lettuce, *Salmonella*, stress, survival.

Correspondence
Shlomo Sela, Microbial Food-Safety Research Unit, Department of Food Science, Agricultural Research Organization (ARO), The Volcani Center, POB 6, Beth-Dagan 50250, Israel. E-mail: shlomos@volcani.agri.gov.il

2008/1153: received 6 July 2008, revised and accepted 22 October 2008

doi:10.1111/j.1365-2672.2009.04152.x

Abstract

Aims: To investigate the interactions of *Salmonella enterica* with abiotic and plant surfaces and their effect on the tolerance of the pathogen to various stressors.

Methods and Results: *Salmonella* strains were tested for their ability to form biofilm in various growth media using a polystyrene plate model. Strong biofilm producers were found to attach better to intact Romaine lettuce leaf tissue compared to weak producers. Confocal microscopy and viable count studies revealed preferential attachment of *Salmonella* to cut-regions of the leaf after 2 h at 25°C, but not for 18 h at 4°C. Storage of intact lettuce pieces contaminated with *Salmonella* for 9 days at 4°C resulted only in small changes in population size. Exposure of lettuce-associated *Salmonella* cells to acidic conditions (pH 3·0) revealed increased tolerance of the attached *vs* planktonic bacteria.

Conclusions: Biofilm formation on polystyrene may provide a suitable model to predict the initial interaction of *Salmonella* with cut Romaine lettuce leaves. Association of the pathogen with lettuce leaves facilitates its persistence during storage and enhances its acid tolerance.

Significance and Impact of the Study: Understanding the interactions between foodborne pathogens and lettuce might be useful in developing new approaches to prevent fresh produce-associated outbreaks.

Introduction

Outbreaks of salmonellosis are associated traditionally with consumption of food of animal origin. However, in recent years, outbreaks have increasingly been linked to raw and minimally processed fruits and vegetables (Sivapalasingam *et al.* 2004). *Salmonella* may contaminate fresh product(s) in a pre- and post-harvest fashion. Sources for *Salmonella* contamination may include irrigation and wash (processing) water, handling by workers, and contact with contaminated surfaces (Beuchat and Ryu 1997; Tauxe *et al.* 1997). The ability of foodborne pathogens to survive and grow on produce is affected by their ability to adapt to the new ecological environments outside of their hosts (Beuchat 2002; Brandl 2006). Thus, attachment and persistence of salmonellae on raw or minimally processed fresh produce is critical to their contamination cycle.

A major trait that may be associated with the adaptation of foodborne pathogens to life on produce is biofilm development (Heaton and Jones 2008). *Salmonella enterica* was shown to form biofilms on several surfaces, including food contact surfaces, food and leaves (Sinde and Carballo 2000; Joseph *et al.* 2001; Lapidot *et al.* 2006). As complete removal or killing of salmonellae on fresh or fresh-cut produce is currently a difficult task, a key factor in the development of new strategies to prevent outbreaks associated with produce is to increase our knowledge about the behaviour of pathogens in association with specific crops. Most studies on the biology of foodborne pathogens on lettuce have been performed with iceberg or crisp-head lettuce. In the present study,

we have investigated the ability of several *S. enterica* serovars with different biofilm formation capabilities to attach to and colonize Romaine lettuce leaves. Additionally, we have studied the persistence of these bacteria on intact leaves during storage at 4°C and their tolerance to acid-challenge.

Materials and methods

Bacterial strains, media and growth conditions

S. enterica strains used in this study are listed in Table 1. The majority of the strains were clinical isolates obtained from the National *Salmonella* Reference Laboratory (NSRL), Ministry of Health, Israel. *Salmonella* serotype Enteritidis, *Salmonella* Typhimurium, *Salmonella* Hadar and *Salmonella* Virchow are the most frequently isolated serotypes in clinical settings in Israel during the last decade (Ministry of Health, Israel). *Salmonella* serotype Amager is a very rare serotype in Israel that was isolated from a food item. The *S.* Newport strain was associated with a multi-state outbreak of salmonellosis epidemiologically linked to alfalfa sprouts (Inami and Moler 1999). *Salmonella* Typhimurium SL1344 is a laboratory strain that has been used previously in many studies and its complete genome sequence is available on the Sanger Institute web site. For each experiment, bacteria were grown in LB broth for 18–20 h at 37°C with shaking (150 rev min^{-1}) to obtain stationary phase cultures. For bacterial enumeration, cultures and leaf washings were dilution plated in duplicate on LB agar (HyLabs, Rehovot, Israel) and the number of colony-forming units (CFU) was determined by plate counts after incubation at 37°C for 24 h.

Biofilm formation in 96-wells polystyrene plate

Biofilm formation was tested in 96-wells flat-bottomed polystyrene microtiter plates (Greiner, Germany) as previously described (Danese *et al.* 2001; Sela *et al.* 2006). Briefly, 10 μl of stationary-phase culture (~9 $\log_{10}$ CFU ml^{-1}) of the tested strain was added to a well containing 90 μl of growth medium. Growth media that were tested included LB broth, LB without NaCl (LBNS), diluted LB (1 : 10), trypticase soy broth (TSB) and diluted TSB (1 : 20). In some experiments, glucose was added to LBNS at a final concentration of 2%. Negative control wells contained growth medium without bacteria. The plates were incubated under static conditions at 30°C for 3 days, then washed, fixed and stained with 0·1% Crystal violet, as described before (Sela *et al.* 2006). The optical density (OD) of the eluted crystal violet was measured at 595 nm using an ELISA Reader (ELx 800 U.V; BioTek Instruments, Inc., Winooski, VT). Evaluation of biofilm formation was performed as described before (Stepanovic *et al.* 2000). The cut-off OD_{595} (ODc) was defined as three standard deviations above the mean OD_{595} of the negative control. Biofilm producing strains were classified as follows: no biofilm producer ($OD \leq ODc$), weak producer ($ODc < OD \leq 2 \times ODc$), moderate producer $2 \times ODc < OD \leq 4 \times ODc$) and strong biofilm producer ($OD > 4 \times ODc$).

Table 1 *Salmonella* strains used in this study

Strain no.	*S. enterica* serovar	Origin/source of strain
303	Enteritidis	Clinical isolate, NSRL*
305	Hadar	Clinical isolate, NSRL
306	Virchow	Clinical isolate, NSRL
308	Thompson	Clinical isolate, NSRL
309	Poona	Clinical isolate, NSRL
310	Amager	Food isolate, NSRL
311	Typhimurium SL1344	Wray and Sojka 1978
312	Newport	Alfalfa sprouts/ M. Brandl, USDA

*NSRL, National *Salmonella* Reference Laboratory, Ministry of Health, Central Laboratories, Israel

Attachment of *S. enterica* serovars and colonization of cut-lettuce pieces

Romaine lettuce (*Lactuca sativa*) was purchased from a local retail store and kept at 4°C for a maximum of 2 days before the onset of the experiments. The outermost leaves of the lettuce head were aseptically removed and the inner 4–5 leaves that were longer than 15 cm were taken for the experiments. The leaves were washed under running water for 30 s, dried at room temperature and each leaf was cut into three pieces of 2 × 2 cm with a sterile scalpel. *Salmonella enterica* serovars grown for 18 h in LB were centrifuged at 1900 ***g*** for 15 min and the pellet was washed twice with sterile deionized water (SDW). Inocula were prepared by resuspending the pellet in 10 ml SDW. Lettuce pieces were submerged in the *Salmonella* suspension (7 $\log_{10}$ CFU ml^{-1}) for 2 h at 25°C. The lettuce pieces were then rinsed twice by gentle immersion for 1 min in 25 ml sterile double-distilled water (SDDW) to remove unattached bacteria. In parallel, following the 2 h incubation period and the rinsing step, some of the lettuce pieces were air-dried and incubated for another 3 days at 30°C under 98 % relative-humidity to investigate colonization and biofilm formation by salmonellae. Following the 3 day incubation, the lettuce pieces were rinsed again as before to remove cells that were not strongly associated with the surface and the bacteria were released as described above. Each lettuce piece was transferred into 100 ml of sterile 0·1% peptone water in sterile polyethylene bags and homogenized for 2 min

at high speed to release attached cells. Samples were serially diluted in 0·1% peptone water and the number of viable *Salmonella* was determined by dilution plating in duplicate on xylose lysine deoxycholate (XLD) selective agar (Acumedia, Baltimore, MD, USA) and incubation at 37°C for up to 72 h. Black colonies were considered to be *Salmonella*. Each serovar was tested on three lettuce pieces processed individually. The experiments were repeated three times on different days.

Comparative attachment of *Salmonella* Typhimurium to intact and cut-edges of lettuce leaf

To distinguish between attachment to the intact surface and to the edges of cut lettuce, attachment assays were performed with a single serovar, *S.* Typhimurium, as described by Takeuchi *et al.* (2000) with the following modifications. Five 2 × 2 cm lettuce pieces prepared as described above were submerged in a *S.* Typhimurium suspension (8 $\log_{10}$ CFU ml^{-1}) for 2 min or 2 h at 25°C and for 18 h at 4°C. At the end of the incubation period, the lettuce pieces were rinsed as described above and 0·3 cm edges of each square tissue sample were cut. The samples of each type of tissue (edges and intact) were combined resulting in two composite samples. To evaluate potential differences in the susceptibility to sanitizer of the attached bacteria at the two sites, inoculated intact and cut-edge leaf tissues (following attachment of *S.* Typhimurium for 2 h) were submerged in 30 ml of a commercial sanitizer (Sterosept, Johnson Diversey, Yavne, Israel) at a concentration of 200 ppm free-chlorine (active compound) for 2 min. Free chlorine was determined with a 'Free chlorine test kit' (HI 383 1F; Hanna Instruments, Padova, Italy). The pieces were then washed immediately twice for 1 min with SDDW. The number of *S.* Typhimurium cells associated with each type of tissue was assessed following pummeling of five pieces (per sample) in 100 ml 0·1% peptone water and dilution plating of the resulting suspension, as described above. Each experiment was repeated three times on different days.

Survival of *Salmonella* on intact lettuce leaf surfaces during cold storage

Leaf squares (*c.* 4 × 4 cm) were inoculated with 100 μl of ∼7 $\log_{10}$ *Salmonella* CFU by spot inoculation of a 2 × 2 cm area on the upper intact surface, air dried for 1 h at ambient room-temperature and stored at 4°C inside Petri dishes. The number of viable *Salmonella* cells on the contaminated lettuce pieces was measured immediately after the 2 h incubation and 3 and 9 days later as follows: each lettuce piece was transferred into a single 50 ml sterile polypropylene tube (Labcon, Petaluma, CA, USA) containing 40 ml of SDDW and vortexed vigorously for 1 min at maximum speed (Mini-Gennie Vortex; Biofan, Latvia) to release the leaf-associated bacteria. This method was comparable to a stomacher technique (data not shown) and allowed for a more rapid processing of multiple samples. Each experiment was performed with three replicate lettuce pieces per serovar and repeated independently twice.

Confocal microscopy

To visualize *Salmonella* on lettuce leaves, we have chromosomally tagged *S.* Typhimurium SL1344 with the cyan fluorescent protein (CFP) by incorporating the *cfp* gene into the *attB* site, as described by Choi and Schweizer (2006). Fluorescent bacteria were visualized under an Olympus IX81 confocal laser scanning microscope (CLSM) (Olympus Co. Ltd., Tokyo, Japan). CFP was excited at 458 nm and its signal gathered with a BA 480–495 emission filter. Chlorophyll autofluorescence was detected using a BA 660 IF emission filter. The images were colour-coded: blue for the CFP signal and red for chlorophyll autofluorescence. The transmitted light images were obtained using Nomarski differential interference contrast (DIC). Three dimensional images were obtained using the FLUOVIEW 500 software supplied with the CLSM (Olympus, Tokyo, Japan) and 3D analysis was made using the IMARIS software (Bitplane, Zurich, Switzerland).

Acid challenge

Lettuce leaf pieces were spot-contaminated with ∼7 $\log_{10}$ *Salmonella* CFU on their upper intact surface only, as described for the cold-storage assays. Exposure to acidic conditions was performed by submerging lettuce pieces for 30 min at 25°C in 50 ml acidic solution (pH 3·0) composed of freshly-squeezed 'Grand' apple juice. The juice was cleared by centrifugation (4000 ***g***, 10 min at 4°C) and filtered through a 0·22 μm membrane to remove suspended material. The pH of the final solution was typically 3·0 and was adjusted to this value with citric acid if required. Following acid challenge, the bacterial cells were washed off the lettuce pieces in 25 ml SDDW and the number of viable *Salmonella* cells was enumerated as described above. In control experiment, contaminated lettuce samples were submerged in an equal volume of SDDW (pH 6·5) instead of an acidic solution. Un-inoculated samples were also examined for the presence of *Salmonella*. For acid challenge of planktonic bacteria, 10 μl of washed stationary phase bacteria (∼7 $\log_{10}$ CFU) was added to 1 ml acidic solution for 30 min at 25°C. The cells were washed twice by centrifugation, resuspended in 1 ml of SDDW and dilution plated onto XLD

agar. The number of surviving cells was determined as described above.

Statistics

Data were analysed using statistical analysis software (Statistical Analysis Systems Institute, Version 8·2, Cary, NC, USA). Statistically significant differences ($P \leq 0{\cdot}05$) between mean values were determined by the Tukey–Kramer multiple range test using One Way Analysis of Variance (ANOVA).

Results

Biofilm formation on 96-well polystyrene plate

Quantification of biofilm mass in the eight *S. enterica* serovars is shown in Fig. 1. Maximal biofilm mass was produced in LBNS medium, except for serovar Poona, which formed more biofilm in LB. The different serovars were classified based on their biofilm production in LBNS, according to the criteria proposed by Stepanovic *et al.* (2000). Five serovars (Enteritidis, Virchow, Thompson, Typhimurium and Newport) out of eight were classified as strong biofilm producers, while the other three (Hadar, Poona and Amager) were classified as weak biofilm producers. Interestingly, all three 'weak-producers' except *S.* Poona, displayed no apparent difference in the amount of biofilm mass formed in LB or LBNS. Dilution of LB (1 : 10) resulted in a significant reduction of biofilm mass in all strains. Addition of glucose to LB had no apparent effect on biofilm production, except in serovar Newport, where biofilm formation was significantly repressed ($P < 0{\cdot}05$) (data not shown). However, glucose had a pronounced effect on biofilm formation in LBNS-grown bacteria. It significantly inhibited biofilm formation by the strong biofilm producers ($P < 0{\cdot}05$), but promoted biofilm formation in two (Hadar and Amager) of the three weak biofilm producers ($P < 0{\cdot}05$). Biofilm formation in TSB was significantly higher than in LB in four serovars, Enteritidis, Thompson, Typhimurium and Newport ($P < 0{\cdot}05$), whereas an opposite effect was observed in serovars Hadar, Poona and Amager ($P < 0{\cdot}05$) and no significant difference was observed in *S.* Virchow. Growth in diluted TSB (1 : 20) had no significant effect on biofilm mass compared to growth in TSB for all strains, except for *S.* Enteritidis, *S.* Virchow and *S.* Newport ($P < 0{\cdot}05$).

Attachment and colonization of *Salmonella* serovars on leaf pieces

The capacity of *Salmonella* serovars to attach to lettuce was determined by immersion of cut lettuce pieces in a *Salmonella* suspension (Fig. 2). After incubation with lettuce tissue for 2 h at 25°C, all serovars that were previously categorized as strong biofilm producers on polystyrene displayed high levels of attachment to lettuce tissue, while the three weak biofilm producers (Hadar, Poona and Amager) exhibited relatively low levels of attachment. For most strains, post-attachment incubation of the lettuce pieces for 3 days at 30°C under humid conditions did not result in any further substantial change in the number of

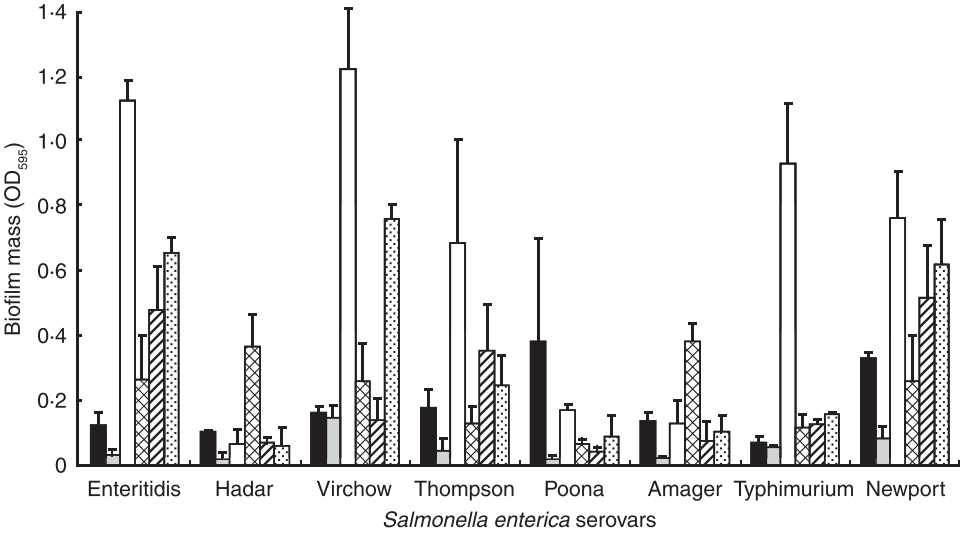

Figure 1 Quantification of *Salmonella* biofilms. Formation of biofilm was tested in 96-wells microtitre polystyrene plates in the presence of various growth media. Biofilm mass was determined using the crystal violet method. The data are presented as the average OD_{595} and standard deviation of three independent experiments each with three replicate wells. (■ LB, ▩ 1/10 LB, □ LBNS, ▨ LBNS + GLU, ▧ TSB, ▩ 1/20× TSB).

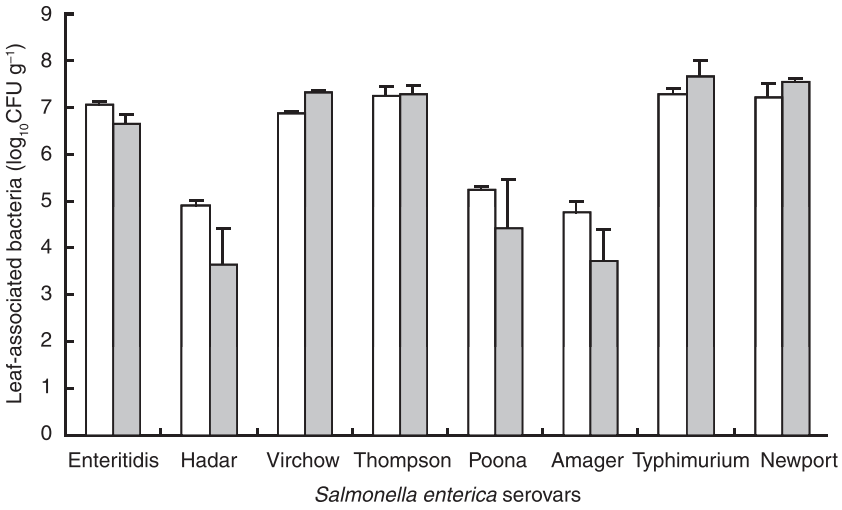

Figure 2 Viable counts of *S. enterica* serovars associated with leaf pieces after incubation for 2 h (□) at 25°C and following storage for 3 days (▨) at 30°C. The data represent the average log CFU g^{-1} and standard deviation of three independent experiments with 3 replicates each.

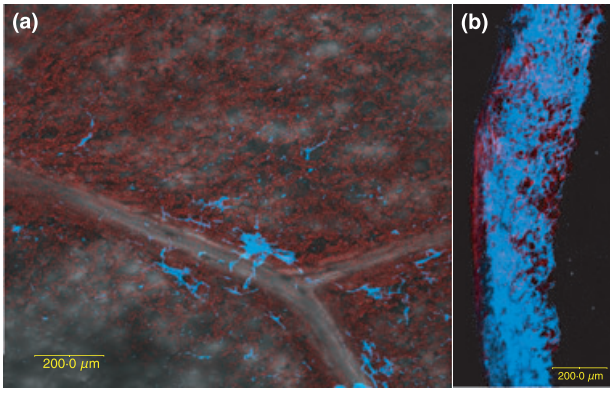

Figure 3 Confocal microscopy image of biofilms of CFP-tagged *S.* Typhimurium (pseudocolour blue) associated with an intact leaf surface (a) and with cut leaf tissue (b) following storage at 30°C for 3 days. The pseudo-3D image was obtained by stacking of optical slices of the leaf. The autofluorescence of the chloroplasts was assigned the pseudocolour red.

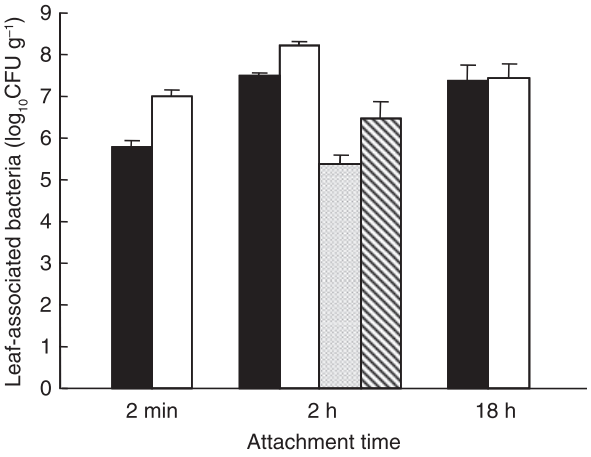

Figure 4 Attachment of *S.* Typhimurium to intact and cut edges of lettuce pieces. Lettuce squares were incubated for 2 or 120 min at 25°C and for 18 h at 4°C. Lettuce pieces (after 2 h attachment) were treated with 200 ppm free-chlorine for 2 min, washed and the number of surviving bacteria measured. The number of leaf-associated *Salmonella* cells under each tested condition is presented as the average number in three independent experiments. (■ intact, □ cut-edge, ▨ disinfected intact, ▧ disinfected cut-edge).

viable cells on the lettuce tissue, although a small but significant reduction ($P < 0{\cdot}05$) in the number of bacteria was observed in serovar Amager. Using a CFP-tagged *S.* Typhimurium strain, we observed the presence of bacterial aggregates/biofilms on both intact and cut surface of lettuce leaf (Fig. 3). While massive bacterial growth occurred on virtually all cut surfaces 3 days after inoculation and incubation at 30°C, bacterial aggregates on the intact surface had a more scattered pattern than on the cut surface.

Attachment of *Salmonella* Typhimurium to intact and injured leaf tissues

To examine the attachment pattern of *S.* Typhimurium to Romaine lettuce leaves, we quantified attachment of the pathogen on intact and cut surfaces of leaves after contact with the plant tissue for 2 min and 2 h at 25°C and for 18 h at 4°C (Fig. 4). More attachment was observed after 2 h than after 2 min, with preferential binding to the cut edges at both incubation times ($P < 0{\cdot}05$). In contrast, comparable attachment levels were observed following incubation for 18 h at 4°C. Treatment of *S.* Typhimurium-contaminated lettuce pieces with 200 ppm free chlorine for 2 min after 2 h-attachment, resulted in 2·5 log reduction of the pathogen population sizes on the intact surface and 1·7log reduction on the cut

edges of leaves. The effect of the sanitizer was significantly greater on intact than on cut tissue ($P < 0{\cdot}05$).

Localization of the lettuce-attached bacteria was investigated by confocal microscopy using CFP-tagged *S.* Typhimurium. The number of cells that had attached to lettuce pieces after 2 and 18 h were comparable for strain 311 and its CFP-derivative (data not shown). Representative images of bacterial attachment to different regions of the cut leaf after 2 and 18 h of incubation are shown in Figs 5 and 6. After 2 h at 25°C, few *S.* Typhimurium cells had attached to the cuticle of the intact leaf surface, while the majority of cells were located in the cut-edge regions, with clear preference for the wounded tissue (Fig. 6). *Salmonella* Typhimurium cells were particularly localized in the regions between adjacent cells of the vascular bundle and in the mesophyll tissue. Microscopy of the lettuce

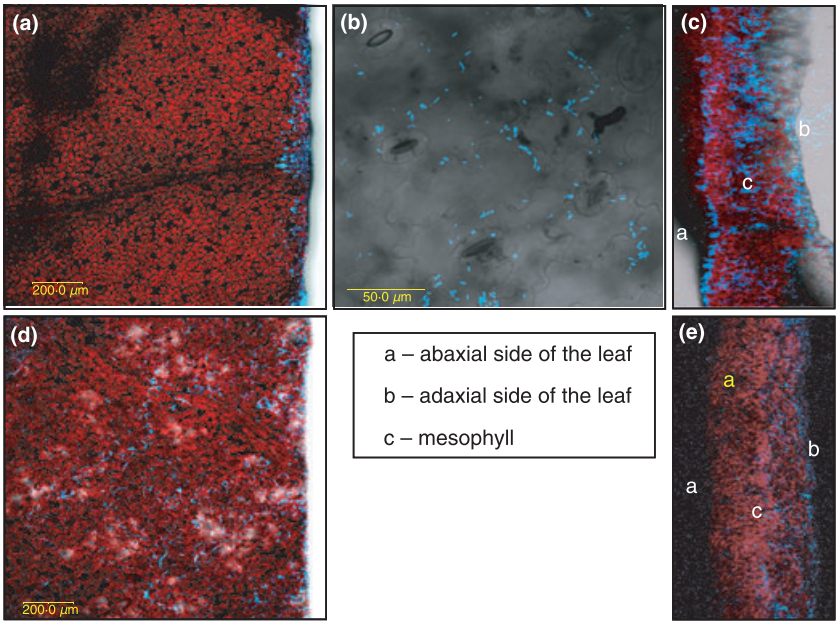

Figure 5 Confocal microscopy images of CFP-tagged *S.* Typhimurium cells attached to lettuce leaf tissue after 2 h at 25°C (a, b and c) and 18 h at 4°C (d and e). (a) Upper surface of leaf with intact and cut edge areas. Numerous *Salmonella* cells (pseudocolour blue) are present in the cut region, with some cells having penetrated in the vascular system; (b) Few *Salmonella* cells are present on the intact cuticle surface; (c) Side view of the cut area of the leaf with multiple bacterial cells attached to both the adaxial and abaxial sides; (d) Upper surface of a leaf with intact and cut edge areas. Similar numbers of attached bacteria are seen on the intact regions and on the cut edge; (e) Side view of the cut area of the leaf showing bacterial attachment. The autofluorescence of the chloroplasts is shown in red.

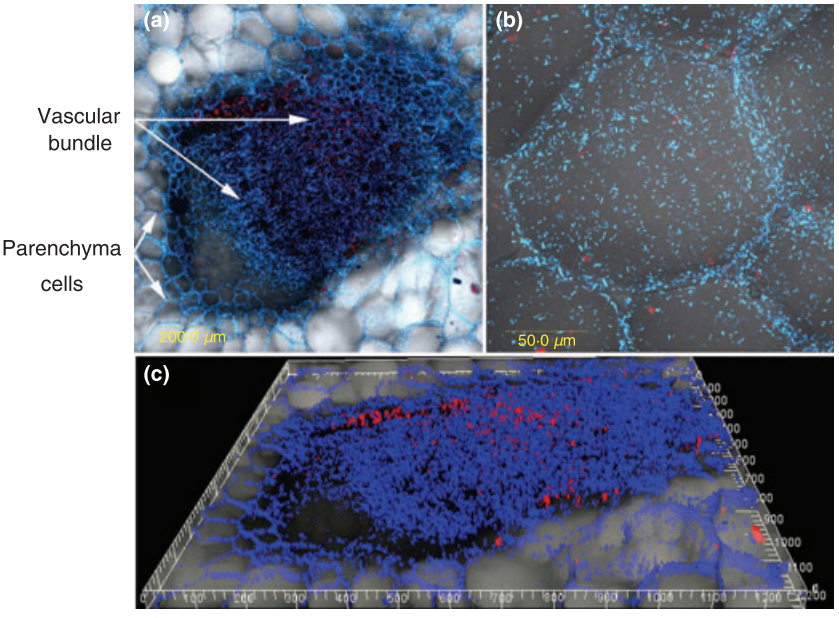

Figure 6 Confocal microscopy images of CFP-tagged *S.* Typhimurium cells attached to a cut area of the leaf near a vein after 2 h of incubation at 25°C. (a) Side view of xylem and phloem cells colonized by CFP-tagged bacteria at high density. Preferential affinity of *S.* Typhimurium for the cell wall of parenchyma cells surrounding the vascular bundle was also observed; (b) Close-up view of a parenchyma cell showing attraction of the bacteria to cell walls; and (c) pseudo 3-D image of the region shown in panel A constructed by the Imaris software, revealing preferential attachment along the cell wall axis.

pieces after 18 h of attachment at 4°C showed comparable numbers of CFP-bacteria on both intact and injured tissues, as was revealed also by bacterial plate counts. In general, fewer bacteria were observed on the cut edges after 18 h at 4°C than after 2 h at 25°C, in accordance with the bacterial plate count data.

Survival of *Salmonella* on lettuce leaves during cold-storage.

To investigate the fate of *Salmonella* serovars on lettuce leaves during cold storage, the intact surface of leaves was spot-inoculated and pathogen population sizes were measured immediately after inoculation and then after 3 and 9 days of storage at 4°C (Table 2). The population size of the majority of the tested serovars changed by <0·5 log CFU per sample during storage suggesting that the *Salmonella* population remained rather constant under these conditions.

Tolerance of lettuce-associated *Salmonella* to acid challenge.

Exposure of planktonic *Salmonella* cells to acidic conditions (pH 3·0) for 30 min resulted in a substantial decrease of 3·9 to 7·16 $\log_{10}$ CFU ml^{-1} (Table 3). In contrast, in six of the eight lettuce-associated *Salmonella* serovars, the average decrease in population size under acidic conditions ranged from 1·67 to 5·0 log CFU ml^{-1}. Two serovars, Amager and Typhimurium, were significantly ($P < 0·05$) more susceptible to acid challenge, both when attached to lettuce and in the planktonic state. In all serovars, acid tolerance was significantly enhanced in lettuce-associated compared to planktonic cells.

Table 2 Changes in population size of *S. enterica* serovars on intact lettuce surface stored at 4°C

S. enterica serovar	Biofilm producer*	Log_{10} difference† of *salmonellae* CFU per sample	
		3 days	9 days
Enteritidis	Strong	0·05 (±0·17)	0·05 (±0·10)
Hadar	Weak	−0·44 (±0·17)	0·48 (±0·09)
Virchow	Strong	−0·22 (±0·18)	−0·61 (±0·18)
Thompson	Strong	0·33 (±0·14)	−0·16 (±0·10)
Poona	Weak	−0·13 (±0·08)	0·37 (±0·20)
Amager	Weak	0·47 (±0·08)	0·51 (±0·11)
Typhimurium	Strong	0·05 (±0·24)	0·01 (±0·15)
Newport	Strong	0·31 (±0·10)	−0·38 (±0·12)

*Biofilm formation was rated on 96-well polyethylene plate, as detailed in the Materials and Methods.

†The data represent the average Log_{10} CFU per sample difference in two independent experiments, each with three replicates. Log_{10} difference/g = $\log_{10}$ CFU g^{-1} at time (t)–$\log_{10}$ CFU g^{-1} at time ($t = 0$). Sample denotes lettuce leaf square of 4 × 4 cm^2.

Table 3 Reduction in *Salmonella* population size following exposure to acidic conditions (pH-3·0)

S. enterica serovar	Biofilm producer*	Log_{10} CFU reduction†	
		Lettuce-associated	Planktonic
Enteritidis	Strong	2·91 (±0·12)	4·16 (±0·17)
Hadar	Weak	2·36 (±0·10)	>6·60‡
Virchow	Strong	1·82 (±0·10)	>6·51‡
Thompson	Strong	2·03 (±0·16)	>6·90‡
Poona	Weak	1·67 (±0·25)	>7·16‡
Amager	Weak	5·01 (±2·03)	>6·86‡
Typhimurium	Strong	4·56 (±0·09)	5·65 (±0·17)
Newport	Strong	2·04 (±0·09)	3·90 (±0·13)

The data represent the average of two experiments, each with three replicates. For each serovar, log CFU reduction of lettuce-associated bacteria was significantly lower than that for planktonic bacteria ($P < 0·05$).

*Biofilm formation was rated on 96-well polyethylene plate, as detailed in the Materials and Methods.

†Log_{10} CFU reduction = $\log_{10}$ CFU in SDDW treated sample–$\log_{10}$ CFU in acid-treated sample.

‡Bacteria were not detected at the minimum level of sensitivity (<10 CFU ml^{-1}).

Discussion

The ability of *Salmonella* to persist outside its host is a critical trait that enables this pathogen to occasionally contaminate fresh-produce and cause food-borne outbreaks. Fresh-cut vegetables and fruits differ in their surface morphology, tissue composition and metabolic activities and thus provide a wide range of diverse ecological niches (Beuchat 2002; Heaton and Jones 2008). An important feature critical for survival of foodborne pathogens outside the host is their capacity to form and live in sessile communities as biofilms. Biofilm populations are highly resistant to various stresses, including antimicrobial agents used in the food industry (Sinde and Carballo 2000; Joseph *et al.* 2001; Scher *et al.* 2005; Lapidot *et al.* 2006).

In most strains examined in our study, low biofilm formation was found in LB medium, while maximal biofilm growth was evident in LBNS, a rich medium devoid of NaCl. Rich medium with low salt is known to be optimal for rdar morphotype expression (Römling 2005). Dilution of LB (1 : 10) resulted in a significant inhibition of biofilm formation, compared to LB, in all tested strains. Others had reported that biofilm formation was more pronounced in diluted medium (1 : 20 TSB) and argue that this feature is consistent with induction of biofilm formation under starvation stress (Stepanovic *et al.* 2004;

Solomon *et al.* 2005). While similar findings were observed in our study with TSB and a diluted TSB in one strain (*S.* Virchow), no such a trend was detected in the other strains and in strains grown in LB *vs* diluted LB (1 : 10) medium. These observations might imply that other factors besides starvation, such as the genetic background of each strain as well as the chemical composition of the medium (LB *vs* TSB), may influence the establishment of a *Salmonella* biofilm. Addition of glucose to LBNS inhibited biofilm formation by the strong biofilm formers, which differs from the report by Bonafonte *et al.* (2000) who showed that pre-incubation of *S.* Enteritidis in TSB+2% glucose enhances biofilm production in a minimal medium containing the same glucose concentration. However, glucose enhanced biofilm production in two out of the three poor biofilm formers (Hadar and Amager), suggesting the presence of opposing biofilm control mechanisms in different *S. enterica* isolates.

Our study demonstrates that *Salmonella* strains that were capable of forming extensive biofilms on polystyrene plates also attached to cut lettuce significantly better compared to strains categorized as poor biofilm formers. It has been shown that cellulose and curli have a major role in biofilm formation in *Salmonella* on polystyrene (Römling 2005), in the attachment of enteric pathogens to alfalfa sprouts (Barak *et al.* 2005, 2007; Jeter and Matthysse 2005; Matthysse *et al.* 2008) and in survival on parsley after disinfection (Lapidot *et al.* 2006). Therefore, a likely correlation exists between biofilm formation *in vitro* and attachment to plant tissue. This hypothesis is worthy of further investigation with a larger number and variety of strains than was used in our study.

Biofilm formation on polystyrene may provide a suitable model to predict not only the initial interaction of *S. enterica* cells with cut Romaine lettuce leaves but also the biofilm production during colonization of leaves. *Salmonella* has the ability to form biofilm on various plant tissues, such as on the rind of cantaloupes and on tomato fruit (Iturriaga *et al.* 2007). Also, large homogeneous (Brandl *et al.* 2005) and heterogeneous (Brandl and Mandrell 2002) aggregates of *S.* Thompson were observed on inoculated cilantro leaves. Our observation of the presence of large biofilms of *S.* Typhimurium on intact and injured lettuce leaf tissue corroborates these findings. Our inability to observe concomitant increase in *S.* Typhimurium population during storage at 30°C for 3 days, may result from the inability to completely disintegrate the bacterial aggregates present on the leaves, thus leading to an underestimation of the *Salmonella* population size. Studies to investigate the possible correlation between *in-vitro* and *in-planta* biofilm development by *Salmonella* using weak and strong biofilm producers are underway in our laboratory.

Our attachment assays performed over short time periods (2 and 120 min) and at 25°C, demonstrated preferential attachment to injured lettuce leaf tissue. It was reported that attachment of *S.* Chester to green pepper disks occurs mainly on the surface of injured tissue, but rarely on the unbroken skin (Liao and Cooke 2001). Further more, the population of *Salmonella* Chester retained after two rinses on apple disks that contained no skin was 13% to 19% higher than that retained on disks that contained skin (Liao and Sapers 2000). On the other hand, we observed comparable attachment of *S.* Typhimurium cells to intact and injured lettuce tissue when the assay was performed over 18 h and at 4°C. This was also reported under similar assay conditions for *S.* Typhimurium on iceberg lettuce (Takeuchi *et al.* 2000). These results indicate that the incubation time and temperature affect the attachment pattern of *S.* Typhimurium to plant tissue. As fresh produce is generally not immersed in water for 18 h, the attachment pattern associated with the short exposure time represents a more relevant scenario. The increased attachment to damaged tissue represents an even greater problem considering that disinfection of injured leaf tissue was less efficacious than that of intact leaf tissue, a result that is consistent with previous reports about disinfection of other produce items (Liao and Sapers 2000; Liao and Cooke 2001). These findings underscore one of the main problems associated with processing lettuce and other fresh-cut items.

Our studies on the storage of artificially contaminated lettuce leaves at 4°C demonstrated that all *Salmonella* strains tested in this study persisted on intact lettuce leaf surfaces throughout the 9-day storage period with rather small variations in the CFU numbers among the serovars. The observed variations in persistence among the serovars are likely related to experimental and/or strain variations, rather than to a specific serovar, since examination of 10 *S.* Typhimurium isolates of different origins showed a comparable range of variations (data not shown). Others have reported similar persistence rates of *Salmonella* on shredded iceberg lettuce during 12 days (Weissinger *et al.* 2000) and 14 days of storage at 4°C (Chang and Fang 2007). Thus, survival of *Salmonella* appears to be similar at 4°C on both Romaine and Iceberg lettuce.

Salmonella Typhimurium and *S.* Typhi can survive acid stress when associated with solid foods, such as ground beef and boiled egg white (Waterman and Small 1998). Surface contact was shown to mediate acid protection in *S.* Typhimurium when the pathogen was associated also with fresh produce, such as apple, tomato and cucumber (Gawande and Bhagwat 2002a,b). It was proposed that this phenomenon could explain the low infectious dose observed in some food-borne salmonellosis outbreaks.

Surface-mediated acid tolerance was reported to occur in several *S. enterica* serovars, but not in *S.* Pullorum and *S.* Paratyphi A (Gawande and Bhagwat 2002b). Our study is the first to examine acid tolerance in various *S. enterica* serovars following attachment to intact Romaine-lettuce leaf. All the tested strains displayed enhanced tolerance to high acidity upon attachment to lettuce tissue compared with planktonic cells. There was no significant correlation between the susceptibility of the strains to acid and their ability to produce biofilm *in vitro*. Stomach acidity is considered to be an important first line barrier against *Salmonella* infections. Therefore, it is possible that the enhanced ability of lettuce-associated *Salmonella* cells to withstand low pH lowers the infectious dose of the pathogen when present on this food matrix.

Finally, our results contribute to the knowledge regarding the interactions of *S. enterica* serovars with biotic surfaces and especially with Romaine lettuce leaf tissues. The currently limited efficacy of commercial disinfectants used in the fresh-cut industry emphasizes the need to understand the behaviour of food-borne pathogens on produce to identify critical factors that may contribute to the ability of this pathogen to persist and retain its infectivity when residing outside the host.

Acknowledgements

We thank Herbert P. Schweizer (Colorado State University, Fort Collins, CO) for his gift of pUC18T-mini-Tn7T-Gm-*ecfpb* and I. Ofek for encouragement and discussions. This work was supported in part by a BARD grant (US-3949-06) awarded to M.T.B. and S.S.

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