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SHORT COMMUNICATION

Interaction of *Salmonella enterica* with basil and other salad leaves

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Contaminated salad leaves have emerged as important vehicles for the transmission of enteric pathogens to humans. A recent outbreak of *Salmonella enterica* serovar Senftenberg (*S.* Senftenberg) in the United Kingdom has been traced to the consumption of contaminated basil. Using the outbreak strain of *S.* Senftenberg, we found that it binds to basil, lettuce, rocket and spinach leaves showing a pattern of diffuse adhesion. Flagella were seen linking *S.* Senftenberg to the leaf epidermis, and the deletion of *fliC* (encoding phase-1 flagella) resulted in a significantly reduced level of adhesion. In contrast, although flagella linking *S. enterica* serovar Typhimurium to the basil leaf epidermis were widespread, deletion of *fliC* did not affect leaf attachment levels. These results implicate the role of flagella in *Salmonella* leaf attachment and suggest that different *Salmonella* serovars use strain-specific mechanisms to attach to salad leaves.
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Salmonella enterica has long been recognized as an important source of food-borne infections and has commonly been associated with the consumption of contaminated poultry and poultry products (Olsen *et al.*, 1992; Baggesen and Wegener, 1994). The global increase in human infections with serovar Enteritidis observed in the late 1980s and early 1990s (Rodrigue *et al.*, 1990) was almost entirely attributable to the presence of this organism within the poultry production industry. However, implementation of control measures including vaccination has resulted in recent years in a reduction in cases of human salmonellosis associated with the consumption of poultry and egg products (Kessel *et al.*, 2001).

In recent years, there has been an increase in consumer demand for fruit, salad and vegetables. Such ready-to-eat foods require little or no cooking before consumption. In a study of general outbreaks of infectious intestinal diseases in England and Wales, carried out between 1992 and 2006, 23% of outbreaks were of food-borne origin and 4% of food-borne outbreaks were linked to prepared salad (reviewed in Little and Gillespie, 2008). *Salmonella* was the most commonly identified pathogen acquired from fresh produce in the United States, being isolated in 48% of cases between 1973 and 1997 (Sivaplasingham *et al.*, 2004).

The newsworthy international outbreaks of *S. enterica* that have been linked to ready-to-eat plant produce include a Scandinavian/UK outbreak of *S. enterica* serovar Thompson associated with consumption of rucola lettuce (Nygård *et al.*, 2008), a Danish outbreak of *S. enterica* serovar Anatum infection linked to imported basil (Pakalniskiene *et al.*, 2006), a tomato-associated outbreak of *S. enterica* serovar Braenderup infection in the United States (Gupta *et al.*, 2007), an outbreak of *S. enterica* serovar Typhimurium (*S.* Typhimurium) DT204b in several European countries associated with the consumption of lettuce (Crook *et al.*, 2003) and an outbreak of *S. enterica* serovar Senftenberg (*S.* Senftenberg) infection associated with imported Israeli basil affecting the United Kingdom, Denmark, the Netherlands and the United States (Pezzoli *et al.*, 2007).

A recent study has shown differential binding of *S. enterica* to different lettuce cultivars (Klerks *et al.*, 2007). Adhesion of *Salmonella* to alfalfa sprouts was shown to be associated with thin aggregative

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fimbriae (Tafi), the extracellular cellulose matrix involved in biofilm formation and the O-antigen capsule (Barak *et al.*, 2007). The aim of this study was to investigate the mechanism used by *S. enterica* to attach to salad leaves.

Leaf adhesion assays were initially performed using the UK basil outbreak *S.* Senftenberg strain 070885. Leaves were inoculated and processed as we described before for *Escherichia coli* O157 (Shaw *et al.*, 2008). Briefly, freshly excised leaves of 10 mm width from supermarket-sourced basil, lettuce, rocket and spinach plants were trimmed, affixed to petri dish base and immersed in 4 ml of bacterial cultures or bacterial culture pellets re-suspended in H_2O ($OD_{600} = 1.00$, equivalent to $6.5 \pm 0.7 \times 10^8$ colony-forming unit (CFU) ml^{-1}) and incubated statically at 20 or 37 °C for 1 h; non-adherent bacteria were removed by washing. Inoculated leaves were processed for scanning electron microscopy (SEM) and immunofluorescence as described (Shaw *et al.*, 2008).

Diffusely adherent *S.* Senftenberg, covering large areas of basil (Figure 1a), lettuce, rocket and spinach (data not shown) leaf surfaces, were observed by SEM and immunofluorescence. Similar attachment levels and patterns were seen after incubations at 20 or 37 °C. No adherent epiphyte microorganisms were seen on unwashed or mock-inoculated leaves (data not shown). High-resolution SEM revealed peritrichous filaments linking *S.* Senftenberg to the leaf epidermis (Figure 1c). We used monoclonal JIM5 pectin antibodies (Plant Probes) (together with anti-mouse Alexa Fluor 594), anti-FliCg,s,t antiserum (together with goat anti-rabbit Alexa Fluor 488) and immunofluorescence (Shaw *et al.*, 2008) to determine if the filaments are flagella. The depicted figures, representative of randomly selected fields, show abundance of flagella (green) linking *S.* Senftenberg (blue) to the leaf epidermis (red; Figure 1d).

To determine the role flagella play in leaf attachment, we deleted the phase-1 flagella *fliC* gene from *S.* Senftenberg 070885. Deletion of *fliC* and insertion of a Kn cassette were performed using the lambda red system (Datsenko and Wanner, 2000), generating strain ICC301. Primer pair Flic/Kn Forward (5′-ATGGCACAAGTCATTAATACCAACAGCCTCTCGCTGATCACTCAAAATAATATCTGTGTAGGCTGGAGCTGCTTCG) and Flic/Kn Reverse (5′-TTAACGCAGTAAAGAGAGGACGTTTTGCGGAACCTGGTTCGCCTGCGCCAGCATATGAATATCCTCCTTAG) was used to amplify the kanamycin cassette in pKD4. Control *fliC* Forward and Reverse primers (5′-gtgccgataacaagggttacggtgag and 5′-GGAATTAAAAAAGGCAGCTTTCGCTG, respectively) and SEM verified the deletion and lack of flagellar expression (data not shown). Basil leaves were inoculated with *fliC* mutant bacterial cultures ($OD_{600} = 1.00$, equivalent to $6.5 \pm 0.7 \times 10^8$ CFU ml^{-1}) and incubated statically at 20 or 37 °C for 1 h as above. The number of leaf-adherent bacteria was quantified by excising 30-mm^2 washed leaf disks into sterile water. The leaf disks were homogenized and serial dilutions were plated onto MacConkey agar plates. This revealed that at both 20 and 37 °C, wild-type *S.* Senftenberg adhered at significantly higher levels than the Δ*fliC* mutant (Figure 1e); the low-level-attached Δ*fliC* mutant bacteria formed aggregates on the leaf surface (Figure 1b). No bacterial growth was seen after plating gently washed and homogenized uninfected leaf extracts (data not shown).

We next investigated the ability of other *Salmonella* serovars ($OD_{600} = 1.00$, equivalent to $6.5 \pm 0.7 \times 10^8$ CFU ml^{-1}) to bind basil leaves as above. Using SEM we found that *S.* Typhimurium (strain SL1344) (Figure 2a) and *S. enterica* serovar Enteritidis strain PT4 (data not shown) adhered efficiently through long filamentous structures (Figure 2b), whereas no adhesion was seen, after scanning 1.2 mm^2 (in two independent experiments), on leaves inoculated with *S. enterica* serovar Arizona (strain SARC5), *S. enterica* serovar Heidelberg (strain SARB23) and *S. enterica* serovar Agona (strain SARB1) (data not shown). The *S.* Typhimurium filaments were identified as phase-1 flagella by staining with mouse monoclonal anti-FliCi antibodies and goat anti-mouse Alexa Fluor 488 (Figure 2c). No phase-1 flagella were seen by immunofluorescence on the *S.* Typhimurium Δ*fliC* mutant (Figure 2d). In contrast to *S.* Senftenberg, no significant difference was observed in adhesion levels (leaves inoculated with $6.5 \pm 0.7 \times 10^8$ CFU ml^{-1}) between the wild-type strain and *S.* Typhimurium Δ*fliC* mutant when incubations were carried out at either 20 or 37 °C (Figures 2d and e). These results suggest the existence of serovar-specific attachment mechanisms, which is in agreement with a report by Klerks *et al.* (2007).

Concluding remarks

Although the mechanisms used by enteric pathogens to colonize the mammalian gut mucosa have been extensively studied, those involved in attachment to vegetables and salad leaves are not well known. In this study, we have shown that the outbreak *S.* Senftenberg isolate attached to a variety of salad leaves (including basil, lettuce rocket and spinach) and that flagella played a major role in bacterial leaf interaction. In contrast, although abundant flagella were seen linking *S.* Typhimurium to basil leaf surface, deletion of the gene for the phase-1 flagellin *fliC* had no measurable effect on the level of leaf association.

The incidence of human infection by enteric bacteria through the consumption of contaminated salad leaves has increased in the last few years (Little and Gillespie, 2008). Leaf contamination can occur during crop growth (for example, through contaminated water, wild or domesticated animals, flies or birds), harvest, distribution, processing and

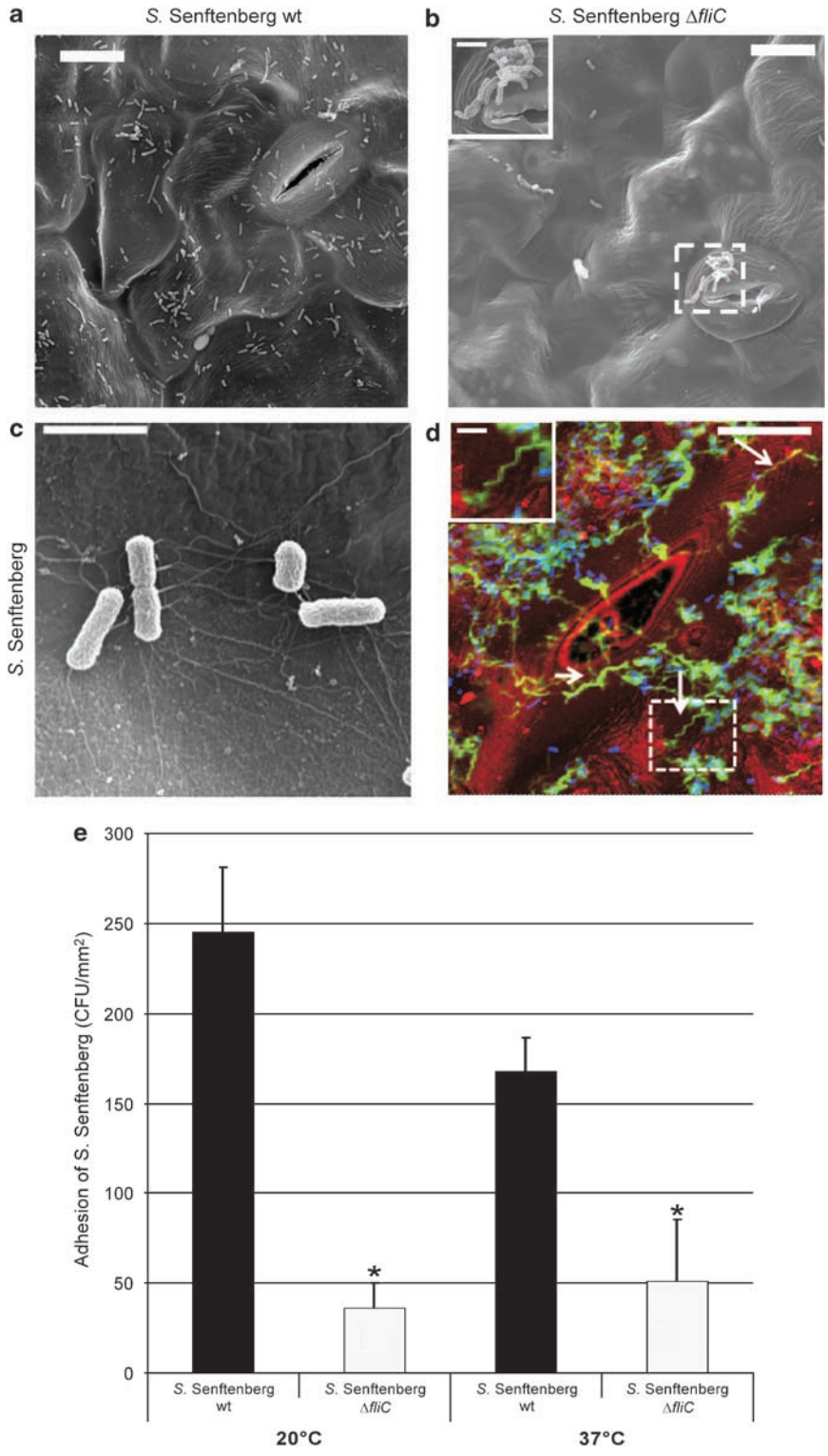

Figure 1 Attachment of *Salmonella enterica* serovar Senftenberg to basil leaves. Wild-type *S.* Senftenberg attaches in a diffuse pattern (**a**) whereas clumps of *S.* Senftenberg Δ*fliC* were seen at a low frequency (**b**). Peritrichous flagella-like structures bound laterally to the leaf surface were observed linking *S.* Senftenberg to the leaf epidermis (**c**). These structures were shown to be flagella (green) (arrows and inset) using FliC antibodies (**d**). *S.* Senftenberg and the leaf epidermis are shown in blue and red, respectively. Flagella-like structures were not seen on *S.* Senftenberg Δ*fliC* (**b**). Bars = 10 μm (**a**, **b** and **d**), 1 μm (**c**) and 0.5 μm (**b** and **d,** inset). Quantification of leaf attachment by wild-type and *S.* Senftenberg Δ*fliC* to basil leaves at 20 or 37 °C (**e**). Deletion of *fliC* resulted in a significant reduction in leaf attachment. Results are presented as mean ± s.d. Significant differences are indicated by asterisks (*$P<0.05$).

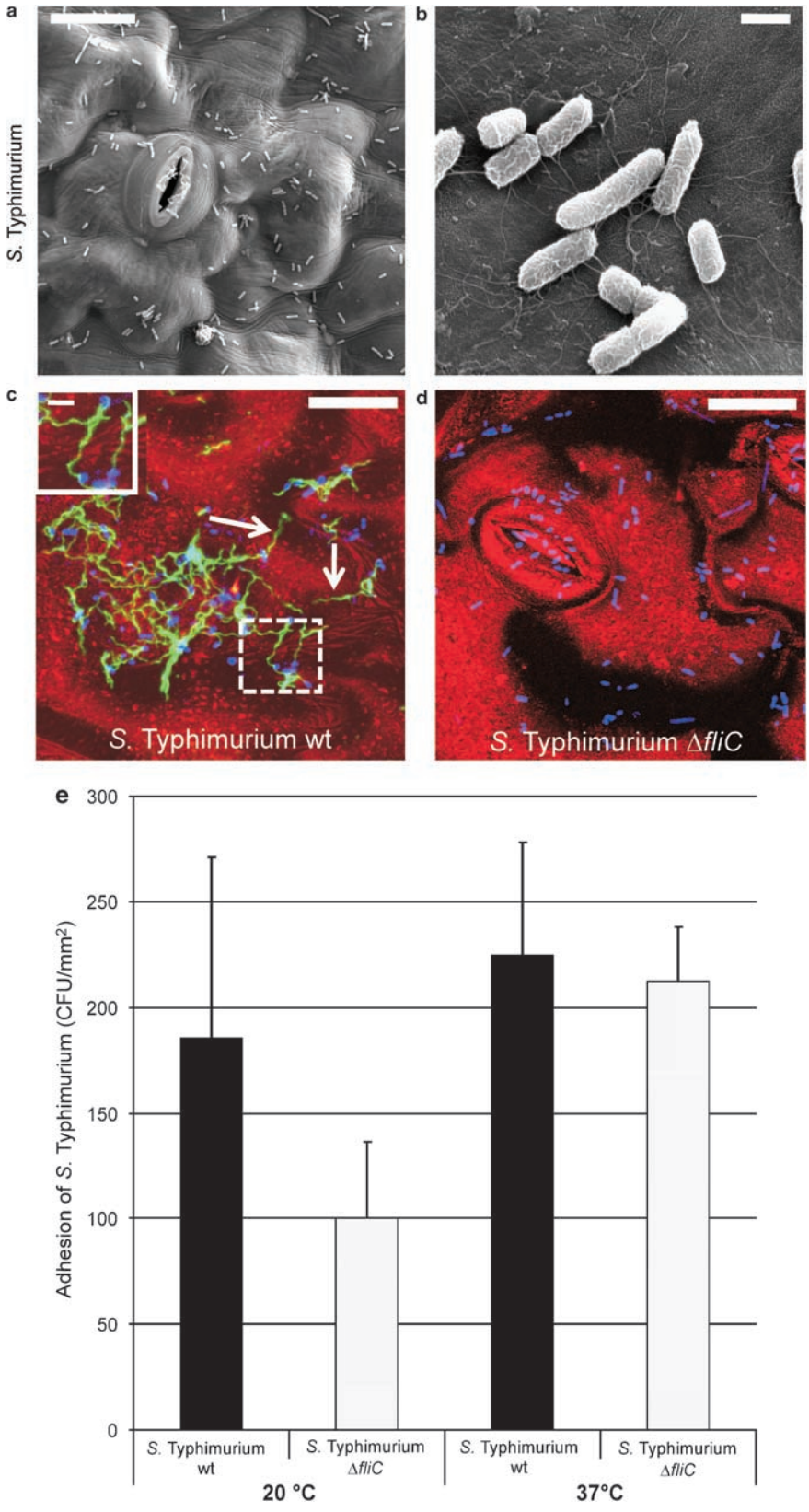

Figure 2 Attachment of *Salmonella enterica* serovar Typhimurium to basil leaves. Wild-type (**a**) and Δ*fliC S.* Typhimurium (**d**) attach to leaves in a diffuse pattern. Peritrichous flagella-like structures bound laterally to the leaf surface were observed linking *S.* Typhimurium to the leaf epidermis (**b**). These structures were shown to be flagella (green) (arrows and inset) using FliC antibodies (**c**). *S.* Typhimurium and the leaf epidermis are shown in blue and red, respectively. No FliC staining was seen on *S.* Typhimurium Δ*fliC* (**d**). Bars = 10 μm (**a**, **c** and **d**), 0.1 μm (**b**) and 0.2 μm (**c**, inset). Quantification of leaf attachment of wild-type and *S.* Typhimurium Δ*fliC* to basil leaves at 20 or 37 °C (**e**). Deletion of *fliC* did not affect leaf attachment levels. Results are presented as mean ± s.d.

packing or cooking. A better understanding of the mechanism involved in the attachment of *S. enterica* to salad leaves would be useful in developing interventions to minimize contamination and transmission and in the development of accurate risk assessments.

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