Research Note

Migration of *Salmonella* Enteritidis Phage Type 30 through Almond Hulls and Shells

MICHELLE D. DANYLUK,1* MARIA T. BRANDL,2 AND LINDA J. HARRIS1

1Department of Food Science and Technology, University of California, Davis, One Shields Avenue, Davis, California 95616; and 2Produce Safety and Microbiology Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710, USA

MS 07-415: Received 5 August 2007/Accepted 20 September 2007

ABSTRACT

The ability of *Salmonella* to migrate from an external aqueous environment through the almond hull and shell, and to colonize the kernel, was evaluated in two ways. First, the outer surface of shell halves from five varieties of almonds that differed in shell hardness were placed in contact with a suspension of *Salmonella enterica* serovar Enteritidis phage type 30 for 24 h at 24°C. *Salmonella* Enteritidis was isolated from the inside of these almond shells in 46 and 100% of the samples, by direct swabbing of the inner surface of the shell and by enrichment from the swab, respectively. These findings suggested that hardness of the shell is not a significant factor in the migration of the pathogen through that tissue. In addition, both motile and nonmotile strains of *S. enterica* serovar Typhimurium migrated through the almond shells to the same extent under the conditions of this assay, indicating that bacterial migration through the wet shell may be a passive process. Second, whole almonds (intact hull, shell, and kernel) were soaked for 24 to 72 h at 24°C in a suspension of *Salmonella* Enteritidis phage type 30 labeled with the green fluorescent protein. Green fluorescent protein–labeled *Salmonella* cells were observed on the outer and inner surfaces of both the almond hull and shell, and on the kernel, by confocal laser scanning microscopy. Our data provide direct evidence that wet conditions allow for *Salmonella* migration through the hull and shell and onto the almond kernel, thus providing a means by which almond kernels may become contaminated in the field.

Recent outbreaks of salmonellosis from consumption of raw almond kernels (5, 12, 16) and peanut butter (6) have raised awareness of nuts or nut products being potential vehicles for foodborne illness. Little is known about the original source of the *Salmonella* in the outbreaks; however, whole-almond contact with soil during harvest is a possible route of contamination.

The almond harvest occurs after the kernels have reached maturation and the hulls begin to dry and split (2, 20). The surface of a dry, intact almond kernel within an intact shell is thought to be virtually sterile when the whole almond is picked from the tree (7, 13, 17, 18). However, mature almonds (kernels surrounded by the shell and hull) are harvested by shaking the nuts off the tree onto the ground, where they are allowed to dry for 1 to 2 weeks, and then the almonds are collected from the orchard floor by mechanical sweepers. Contamination of almond kernels with *Salmonella* is assumed to occur by contact with soil during harvest and the subsequent mixing of soil, hulls, shells, and kernels during the process of removing the hull and shell.

Although uncommon, rain may fall during the harvest when almonds are drying on the orchard floor, resulting in various degrees of almond rehydration. In addition, some almonds may prematurely drop from trees and become soaked with irrigation water. Uesugi and Harris (26) found that a slurry prepared from almond hulls and shells supported the rapid growth of *Salmonella* at both 15 and 24°C. Also, moisture was observed on the inner shell surface of apparently intact shells, after soaking in water. These observations lead to the hypothesis that wetting of almonds during harvest could increase the risk of kernel contamination.

Internalization of *Escherichia coli* O157:H7 and *Salmonella* into plant tissues has been demonstrated both pre- (8, 22, 27) and postharvest (3, 10, 19, 21, 23, 30). In preharvest plant tissues, motility has been established as an important parameter for invasion and movement of *Salmonella* in *Arabidopsis thaliana* (8); nonmotile mutants showed a decreased ability to invade plant tissues, and did not move within the plants. In postharvest plant tissues, infiltration is believed to be due to the wicking action of these tissues; the influence of motility has not been explored.

The objectives of this study were to determine (i) if *Salmonella* can migrate through the hull and shell of mature almonds and contaminate the kernel under conditions in which water soaking of the whole almonds may occur, and (ii) if *Salmonella* migration into almond shells is influenced by its motility.
MATERIALS AND METHODS

Strains, inoculum, and growth media. The primary strain used for these studies was *S. enterica* serovar Enteritidis phage type (PT) 30 (ATCC BAA1045), which was isolated from recalled 2000 to 2001 outbreak-associated almonds, and was provided by Silliker Laboratories (Modesto, Calif.). A spontaneous nalidixic acid–resistant strain of this bacterium was obtained in our laboratory and transformed with plasmid pGT-KAN for intrinsic labeling with green fluorescent protein (4). The plasmid was stably maintained in *Salmonella* Enteritidis PT 30. The green fluorescent protein–labeled strain, named MB323, was used in microscopy studies to determine the localization of *Salmonella* Enteritidis PT 30 on inoculated almonds.

Bacterial strains used to determine the influence of motility on migration were obtained from Dr. Robert Mandrell (U.S. Department of Agriculture—Agricultural Research Service), and included *S. enterica* serovar Typhimurium (SIJW1103) and two non-motile mutants of this parent (SIJW1368, ΔflhDC, and SIJW1809, fian) (15, 28).

The inoculum was prepared as described by Uesugi et al. (25), with minor modification. All media were obtained from Difco, Becton Dickinson (Sparks, Md.), unless otherwise specified. Overnight cultures in tryptic soy broth (TSB) were used to inoculate plates of tryptic soy agar (TSA) to produce a bacterial lawn after incubation at 35 ± 2°C for 24 ± 2 h. TSB and TSA were supplemented with either nalidixic acid at 50 μg/ml or with both gentamicin at 15 μg/ml and nalidixic acid, as appropriate. The cells were collected in 25 ml of 0.1% peptone, using a sterile swab wetted with the same solution. Inoculum concentrations were adjusted based on absorbance at 600 nm, and confirmed by serial dilution in 0.1% peptone or Buttefield’s phosphate buffer (BPB; Hardy Diagnostics, Santa Maria, Calif.) and plating onto TSA, or TSA and bismuth sulfite agar (BSA) (both supplemented with nalidixic acid) for strain MB323. Inoculum suspensions were prepared in BPB.

Almond shell preparation, inoculation, and incubation. In-shell almonds, provided by the Almond Board of California, included two hard-shell varieties (Padre and Peerless) and three soft-shell varieties (Carmel, Monterey, and Nonpareil). Almond kernels were removed from shells, leaving the almond shell halves intact. Hot glue (Aleene’s Ultimate Glue Gun, Duncan Enterprises, Fresno, Calif.) was applied to two opposite points on the inside edge of each shell half, and six shell halves were then glued into the lid of a petri dish (150 by 15 mm). Each lid with attached shells was placed on the bottom half of a petri dish that contained approximately 100 ml of a 10^6 CFU/ml suspension of *Salmonella* Enteritidis PT 30. The final volume of inoculum used was determined visually to ensure that only the rounded bottom of each shell was in contact with the inoculum, and that the inoculum did not reach the upper attached edge of the shell. These almond shells were incubated at 23 ± 3°C for 24 ± 2 h.

Recovery of *Salmonella*. Almond shells were aseptically removed from the petri dish lid. The presence of *Salmonella* on the inner surface of the shells was determined in two ways. First, a sterile cotton swab was wetted with lactose broth and then run along the inner surface of the almond shell. Care was taken not to touch the edges or outer surface of the almond shell with the swab. The swab was streaked onto xylose lysine deoxycholate agar (XLD) for isolation by direct plating; XLD plates were then incubated at 35 ± 2°C for 24 h. Presumptive *Salmonella* colonies were confirmed by triple sugar iron agar, and lysine iron agar slants. Second, the same swabs that were used for direct plating were placed into 25 ml of lactose broth and incubated at 35 ± 2°C for 24 h to enrich for *Salmonella*. After incubation, 0.1 and 1 ml of the enrichment culture were transferred to 10 ml of Rappaport-Vassiliadis R 10 broth and to 10 ml of tetrahionate broth, respectively. Rappaport-Vassiliadis R 10 broth and tetrahionate broth cultures were incubated for 24 h at 42 and 37°C, respectively. After incubation, samples from both broths were streaked onto BSA, XLD, and Hektoen Enteric agar. Hektoen Enteric agar and XLD plates were incubated at 35 ± 2°C for 24 ± 2 h, and BSA plates were incubated at 35 ± 2°C for 48 ± 2 h. Two typical *Salmonella* colonies were selected from each enrichment and confirmed by culture on LIA and triple sugar iron agar slants. Cultures on the slants that were characteristic of *Salmonella* were confirmed with specific group *Salmonella* O Antiserum Group D1 factors 1, 9, and 12.

Whole-almond preparation, inoculation, and incubation. Whole almonds (hull, shell, and kernel) of the Carmel variety were provided by the Almond Board of California. Three whole almonds with intact hulls (i.e., the hull had not split open, and the shell was not exposed) were placed into 100 ml of a suspension of 10^3, 10^6, or 10^9 CFU/ml of *Salmonella* strain MB323 in BPB. Containers were sealed, the almonds were briefly swirled in the suspension, and the suspensions were incubated at 24°C for 24 to 72 h. Following incubation, the suspension in which the almonds were soaked was serially diluted in 9 ml of BPB, and the strain MB323 colonies were enumerated on nalidixic acid–supplemented TSA and BSA after incubation at 35 ± 2°C for 24 ± 2 or 48 ± 2 h, respectively.

Confocal laser scanning microscopy. Localization of *Salmonella* Enteritidis PT 30 MB323 on inoculated and soaked whole almonds was determined by preparing thin sections of the interior and exterior surfaces of the hull and the shell, and of the brown pellicle (kernel skin), using sterile razor blades. Care was taken not to contaminate the razor blade or the tissue section by contact with almond surfaces other than the one being examined. Sections were mounted in Aquapolymount (Polysciences, Inc., Warrington, Pa.) and observed under a Leica TCS-NT confocal microscope (Leica Microsystems, Wetzlar, Germany) using argon (488 nm) and krypton (568 nm) lasers. Signals from the green fluorescence of *Salmonella* strain MB323 cells and from the red autofluorescence of the almond tissue were acquired with BPS950 and LP590 emission filters, respectively.

Statistics. A linear regression comparing the presence of *Salmonella* as determined by direct plating with the visual detection of water droplets on the inner surface of almond shells was performed using Excel (Microsoft, Redmond, Wash.).

RESULTS

Migration of water and *Salmonella* through almond shells. Within 1 h of placing almond shells in contact with the *Salmonella* suspension, the inner smooth surface of the shells of all varieties had visibly darkened, presumably because of water soaking of the shell tissue. Almond shells that appeared cracked after exposure to the bacterial suspension were excluded from further study.

Water droplets were visible on the inner surface of a number of shells of each variety after incubation and prior to swabbing (Table 1). The number and size of water droplets varied greatly among almond varieties, including those grouped as hard or soft shell. The inner surfaces of all Mon-
TABLE 1. Detection of water droplets and *Salmonella Enteritidis* PT 30, by direct plating and enrichment, on the inside of almond shells following 24 h of incubation

<table>
<thead>
<tr>
<th>Almond variety</th>
<th>Visible water droplets</th>
<th>Salmonella detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct plating</td>
</tr>
<tr>
<td>Hard shell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Padre</td>
<td>0/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Peerless</td>
<td>4/6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/6</td>
</tr>
<tr>
<td>Soft shell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carmel</td>
<td>1/6</td>
<td>3/11</td>
</tr>
<tr>
<td>Monterey</td>
<td>6/6</td>
<td>6/12</td>
</tr>
<tr>
<td>Nonpareil</td>
<td>6/6</td>
<td>3/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of positive tests per total number tested.

terey and Nonpareil shells were almost completely covered with water droplets of diverse sizes. Fewer of the Peerless and Carmel shells had droplets on the inner surface, and these were small in size. Although the inner shell surface of the Padre variety was obviously darker after contact with the bacterial suspension, no water droplets were visible.

*Salmonella* was detected on the inner shell surface of all varieties of almonds by direct plating (Table 1). The frequency of direct *Salmonella* isolation varied from 27% (Carmel) to 80% (Peerless). The presence of *Salmonella* did not correlate ($R^2 < 0.80$) with the visible detection of water droplets on the inner shell surface and was not directly related to the type of shell variety (hard or soft). Following enrichment of the swabs, *Salmonella* was isolated from 100% of almond shells tested (Table 1). The parental strain of *Salmonella* Typhimurium and two nonmotile mutants were all detected by enrichment in at least five of six shells of both Carmel and Peerless almonds tested (data not shown).

**Migration of *Salmonella* through intact whole almonds.** *Salmonella* concentrations in the suspensions in which the whole almonds were submerged increased to $10^7$ CFU/ml after incubation for 24 h, when the initial inoculum concentration was $10^3$ or $10^6$ CFU/ml. The concentration of *Salmonella* in the suspension remained stable when the initial inoculum was $10^9$ CFU/ml.

Whole Carmel almonds placed in water quickly absorbed moisture. The rapid uptake of water by the almond hull led to a swelling in its size and a darkening in color. Under confocal laser scanning microscopy (CLSM), individual cells of *Salmonella* MB323 moving in the apparent aqueous environment between trichomes (hairs) of the surface of the hull (exocarp) were observed after incubation of the whole almonds in the suspension for 24 h (not shown). Cells of *Salmonella* MB323 were also observed on the inner surface of the hull (mesocarp), and were apparent as chains at some locations (Fig. 1A).

When the almond hull was carefully removed to expose the almond shell (endocarp), the shell appeared watersaturated. Microcolonies and individual cells of *Salmonella* MB323 were observed on thin sections of the outer surface of the shell (not shown). *Salmonella* MB323 cells were observed in abundance on the inner surface of the shell, which appeared smooth macroscopically, but has a complex structure under the microscope (Fig. 1B). On this inner surface, the pathogen was present mostly as individual cells rather than as microcolonies.

*Salmonella* MB323 cells were observed on, and in the cracks between, the large cells of the epidermal layer of the pellicle (Fig. 1C). *Salmonella* cells that had migrated through intact almond tissue to the kernel were best observed on the various tissue layers under CLSM, when a high concentration of the pathogen ($10^9$ CFU/ml) was used in the soaking suspension. *Salmonella* cells were also observed throughout the tissue and on the kernel of almonds that were soaked in lower concentrations of inoculum; however, figures of *Salmonella* cells on the almond tissue at these lower densities are not presented.

**DISCUSSION**

Almond kernels at harvest are surrounded by closed or open shells and by closed or open hulls. Our observations under the confocal microscope demonstrate that the presence of a closed hull and shell did not hinder the movement of *Salmonella* onto the kernel under water-saturated conditions. The presence of *Salmonella* on the inner surface of the hull, where it formed chains of cells, suggests that the pathogen moved either actively or passively with the water infiltrating the hull, and multiplied on this almond tissue. Sufficient nutrients to support extensive growth of *Salmonella* have been reported in water-soaked almond hulls (26). *Salmonella* was mostly observed as single cells on the inner surface of the almond shells, suggesting that little growth occurs on this tissue, and that contamination results from migration of single cells through the shell tissue. The presence of *Salmonella* on the kernel pellicle (brown skin) was observed by exposing dry, whole almonds in closed hulls to inoculum concentrations as low as $10^3$ cells per ml. Figure 1C, showing pellicle tissue contaminated by soaking intact whole almonds in a much higher inoculum concentration of $10^9$ cells per ml, provides proof for the hypothesis that migration of *Salmonella* can occur through an intact (closed), whole almond onto the kernel surface. The pellicle has an outer epidermis that consists of relatively large thinned-out, cup-shaped cells ranging from 100 to 300 μm in width (29). Figure 1C also reveals the complex morphology of the kernel skin and shows the many microsites where *Salmonella* cells may be located on contaminated kernels.

Both motile and nonmotile strains of *Salmonella* moved through the shells of five varieties of almonds, as determined in our qualitative assay. Quantitative measurements of the population sizes of the motile and nonmotile strains on the inner shell surface might reveal subtle differences in the ability to migrate through the shell tissue. During harvest, when almonds are drying on the orchard floor, the dehydration of the hull and shell may lead to structural damage. Upon the addition of water, dehydrated hulls can rapidly rehydrate, absorbing 250 to 300% of their weight in water (24). This movement of water into the dehydrated tissue may facilitate the transfer of *Salmonella* through intact hulls and shells in the absence of motility.
Almond varieties such as Carmel, Monterey, and Nonpareil have very thin, soft shells, which can undergo significant damage in the field during harvest, providing a direct means for contamination of the kernel (14, 18). After harvest, as many as 70% of Nonpareil shells may split along the suture (11). Other varieties such as Padre, Peerless, and Mission are often sold unshelled and have thicker, harder shells, which have a much lower tendency to split. This characteristic may protect the kernel from dry contamination but, as demonstrated in the current study, wet conditions provide an additional route of contamination through the intact shell. Shell hardness did not have any significant effect on the migration of *Salmonella* to the inner shell surface via water movement.

During a multiyear survey of raw almond kernels (not including almonds grown in the outbreak-associated orchards), a large number of *Salmonella* serovars were isolated from raw almond kernels at a frequency of about 0.9% of 100-g samples, thus providing evidence of the presence of *Salmonella* in the almond production environment (9). Levels of *Salmonella* were only 1 to 3 most probable number (MPN)/100 g of kernels or less. However, wetting of almond hulls provides a means for levels of *Salmonella* to increase substantially. In the present study, increases of $10^4$ CFU/ml were observed within 24 h on soaked almonds with relatively low initial contamination rates. Thus, low levels of *Salmonella*, if present in the orchard environment, may increase to sufficient population sizes on the almond hull to migrate through the tissues onto the kernel when the intact mature almonds are exposed to moisture. In addition, increased levels of *Salmonella* in the hulls may increase the likelihood of contamination of the kernel during harvest or hulling and shelling. Although almonds that become wet in the field are re-dried prior to hulling and shelling, this drying may occur several days after a rain event, thus allowing sufficient time for *Salmonella* colonization of the hull and subsequent migration to the kernel. Drying has been shown to reduce *Salmonella* on hulls by only 1 to 3 log CFU/g (26).

The wetting of harvested almonds should be considered a risk factor for *Salmonella* contamination of almond kernels. When whole almonds on the ground are exposed to water sources such as irrigation or rainwater, *Salmonella*, if present, can both multiply in the almond hull and migrate through the various layers of the wet almond tissue to the kernel. The recent development of almond-specific “good
agricultural practices” has begun to address this potential risk. The good agricultural practices document recommends segregating rained-upon almonds and increasing sanitation procedures after handling these almonds to avoid potential cross-contamination (1). In addition, it is recommended that nuts exposed to moisture be processed with a validated kill step for Salmonella.

ACKNOWLEDGMENTS

The financial support of the U.S. Department of Food and Agriculture Cooperative State Research, Education, and Extension Service grant 2002-03886, and of the Almond Board of California is gratefully acknowledged. The technical support of A. Uesugi and the editorial skills of S. Yada are also appreciated.

REFERENCES


