Attachment of Salmonella typhimurium to Skins of Turkey that had been Defeathered through Three Different Systems: Scanning Electron Microscopic Examination

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ABSTRACT

Attachment of Salmonella typhimurium to the skin of turkeys that had been processed through three different defeathering systems (conventional, kosher, and steam-spray) was examined at varying incubation times (10–60 min) using scanning electron microscopy. The extent of attachment varied with the type of defeathering method and increased with incubation time. Conventional and kosher skins showed slow rate of attachment, whereas steam-spray skin allowed a sharp increase of attachment, due to the collagenous connective tissue of dermis exposed during processing. Exposed dermis in kosher skin revealed the very adhesive properties of dermis. Attachment fibrils appeared as incubation time increased. Cells on steam-spray skin produced a considerable amount of long, thin fibrils connecting them to each other and to the skin after 30 min of incubation. While a few thick and short attachment fibrils were observed in conventional skin, no fibrils were detected in kosher skin. The high number of attached cells and the greatest amount of fibril formation on the surface of steam-spray skin suggest the positive relationship between bacterial attachment and fibril formation. Depressions produced by attached cells were observed in conventional skin surface, but not in kosher skin, probably due to the fatty and pliable properties of conventional skin. Pits, observed only on the surface of steam-spray skin, reflected the weak property of basal membrane covering the dermis. The above results suggest that exposing dermis is undesirable for reducing bacterial attachment during poultry processing.

Bacterial attachment is a complex phenomenon involving many disparate factors. Whether a particular microorganism can adhere to a certain substratum depends on the properties of the microbial strain itself and on characteristics of the substratum and of the environment. When biological surfaces are involved, many additional, complex physiological factors can come into play (19).

In poultry processing, the intact skin which excludes most microorganisms is disrupted during the defeathering step. This damaged skin surface is easily attacked by microorganisms, such that the bruised skin is very susceptible to microbial infection. In previous studies (10,11), different skin microtopographies were produced by three different poultry defeathering systems (conventional, kosher, and steam-spray). These microtopographies appeared to influence the adhesion and penetration of Salmonella typhimurium.

The mechanism of bacterial attachment is generally accepted as a two-step process. The initial step involves retention of bacteria in a liquid film on the surface of skin and meat (24). Bacteria are believed to be closely associated and entrapped in inaccessible sites mainly by physical forces (6). Reversible adhesion in this stage is associated with the energy balance between attractive van der Waals force and electrostatic repulsion (14). The second step is characterized by a time-dependent, irreversible exopolymer formation by bacteria. At this point, bacteria multiply and form a microcolony which can lead to formation of a biofilm. Formation of these extracellular polysaccharides or attachment fibrils has been observed on the surface of meat or skin (3,12,20). However, there has been a controversy on the nature of those fibrils. Recent introduction of cryo-scanning electron microscopy (SEM) allows the observation of materials with a high water content such as that found in the natural state. Therefore, the artificial appearance which may occur during the dehydration stage of conventional SEM technique can be eliminated (15,25). Fraser and Gilmour (7) even claimed that the fibril formation of Pseudomonas fragi ATCC 4973 was not an attachment feature of these cells but was associated with the ethanol dehydration stage of conventional method.

Previous studies (10,11) have discussed the influence of three different poultry defeathering systems on morphology and consequent influence on attachment and penetration of S. typhimurium. In this study, the attachment of S. typhimurium on each skin was examined using SEM by varying the time that skin was exposed to S. typhimurium.

MATERIALS AND METHODS

Turkey breast skin and irradiation

Whole breast skins were removed from turkey carcasses immediately after the defeathering step at a conventional, kosher, and steam-spray processing plant and stored at -15°C (3 replications x 4 birds x 3 processing plants). Before inoculation, skin...
samples were sterilized with 5 kGy of gamma irradiation to kill all indigenous microflora on the skin (10). No cells were observed on the surface of irradiated control skins by SEM.

*S. typhimurium inoculum*

*S. typhimurium* (ATCC 13311), which had been maintained on a slant of tryptic soy agar (Difco) at 4°C, was cultured in tryptic soy broth for 24 h at 35°C. It was centrifuged at 3,000 x g for 10 min at 4°C, and resuspended in phosphate buffered physiological saline (PBPS, 0.85% NaCl, pH 7.2) at a cell density of 1 x 10^8 CFU/ml (10).

**Inoculation and incubation of turkey skins**

The inoculation procedure described previously (10) was followed except for different incubation times. A set of sterile skin pieces (1 cm^2) was stretched and pinned on sterile cork sheets. Then, the skin surface was inoculated with *S. typhimurium* (10^6 CFU/ml) by evenly spreading the inoculum with a micropipette (0.1 ml/cm^2) and incubated in a moist chamber at room temperature (20°C). Skins were removed after each 10, 20, 30, 45, and 60 min of incubation, and the inoculated surface was rinsed with PBPS (1.0 ml/cm^2 of skin surface) to remove unattached cells by gently applying PBPS and draining off remaining fluid. After draining for 30 s, each skin was directly immersed into 3% glutaraldehyde fixative for SEM observation.

**Scanning electron microscopy (SEM)**

Samples were prepared for SEM as described by Kim and Doores (10). The fixed, inoculated skins were cut into smaller pieces, dehydrated with graded ethanols and washed with hexamethyldisilazane (HMDS) (8,16). After complete evaporation of HMDS, skin pieces were sputter-coated with gold on a cold stage and viewed with an ISI 60 scanning electron microscope at 10 kV (Pleasanton, CA).

The number of attached cells on each skin was directly counted from the screen of the SEM at 3,000 X. Sixteen different areas were randomly selected and counted. Data were statistically analyzed by analysis of variance of a completely randomized design. Each mean was separated by using Fisher’s least significant difference procedure (18).

**RESULTS AND DISCUSSION**

**Incubation study**

The number of *S. typhimurium* attaching to skin appeared to be dependent on the skin microtopography produced by different defeathering method as well as incubation time (Fig. 1). As incubation time increased, all three skins (conventional, kosher, and steam-spray processed skin) showed gradual increase of attachment, but with different rates. At 10 min of incubation, no significant difference in the extent of bacterial attachment was observed among the skins receiving different treatments. After 10 min, however, significant differences in the rate of attachment appeared between steam-spray skin, and conventional and kosher skin (p < 0.01). There was no significant difference between conventional and kosher skin. If the data in Fig. 1 are converted to the number of cells attached per sq cm, attachment of *S. typhimurium* increased from ~1 x 10^3 (at 10 min) to 6.0 x 10^5 (at 60 min) in conventional and kosher skins, and to 1.7 x 10^6 (at 60 min) in steam-spray skin. Almost three times more cells were attached to steam-spray skin than to the others.

The above data are based on direct counting of surface-attached cells by SEM. The problem of using this method is that attached cells might be removed during serial washing steps (10). However, a significantly high attachment rate on steam-spray skin suggests the presence of specific salmonelae receptors on this skin which could be the collagenous connective tissue in exposed dermal surface (10). Specific adhesion of *S. typhimurium* to a network of collagen fibers on the muscle fascia has been reported (4,23). These reports claimed that prior immersion of tissue in water for 20 min of extended immersion in bacterial suspension is required for adhesion to collagen fibers. In this study, a significant increase in attachment was observed on steam-spray skin after 10 min of incubation.

After 30 min of incubation, cells attached to each other began to appear on the surface of steam-spray skin. This may reflect multiplication of attached cells or attachment of cells to those already attached. The secondary stage of bacterial attachment, which is characterized by an increased strength of attachment due to polysaccharide formation by attached bacteria, appeared to have started at 30 min of incubation in steam-spray skin. Butler et al. (2) reported that continued attachment occurs over a 30-min period on meat surfaces. The increased S value of *S. typhimurium* at 30 min incubation agrees with previous findings (17).

The adhesive property of dermal surface was clearly demonstrated by the exposure of dermis to kosher skin. In one micrograph, the entire epidermal layer of kosher skin was partly separated from the dermis and folded back. Therefore, it was possible to observe both sides of epidermis and the surface of dermis simultaneously in one piece of kosher skin (Fig. 2a). Whereas few cells were observed on the *stratum corneum* layers (Fig. 2c), which is a normal surface of kosher skin, cells were densely colonized on the surface of dermis as well as the bottom side of epidermis (Fig. 2b). This high attachment might be caused by collagenous basal lamina, which connects epidermis and dermis. Basal lamina seemed to be separated on both sides of skin and formed a collagenous network entrapping *S. typhimurium* on the surface of dermis (Fig. 2d), whereas it appeared as round spots evenly spread on the bottom side epidermis (Fig. 2e).

**Attachment fibril formation**

Attachment fibrils connecting the cells to each other and to the skin surface could be observed during the incubation...
Figure 2. Scanning electron micrographs of kosher skin showing exposed dermis: a) dermal surface and both sides of epidermis, b) densely colonized exposed dermis and bottom side of epidermis, c) a few cells attached to the stratum corneum surface, d) cells entrapped in the network of basal lamina, e) basal lamina remaining on the bottom side of epidermis. d, dermis; be, bottom side of epidermis; se, surface of epidermis; sc, stratum corneum; bl, basal lamina.

study (Fig. 3). The characteristics and extent of fibrils formed by *S. typhimurium* varied with skin microtopography and/or incubation time (Table 1). The cells attached to steam-spray skin produced a considerable number of long, thin fibrils connecting to each other or to the skin after 30 min of incubation (Fig. 3a). While a few thick and short attachment fibrils were observed in conventional skin, their emergence was independent of incubation time (Fig. 3b). Actually no fibrils were detected in kosher skin (Fig. 3c). These results reflect the characteristic surface properties of each skin influencing the adhesion mechanism of bacteria. The fact that *S. typhimurium* attached to steam-spray skin in much higher numbers and produced the greatest number and mass of fibrils suggests the positive relationship between attachment and fibril formation.

There are still controversies on the nature of attachment fibrils. Formation of attachment fibrils was frequently observed during extended contact of several bacteria onto surfaces (3,5,6,13,26). Although each study was conducted under different conditions, the formation of attachment fibrils generally appeared to be dependent on bacterial strains and contact time (20,26). Temperature dependency was also reported by Stone and Zottola (21). For the skin surfaces used in this study, only the cells attached to steam-spray skin showed the contact time-dependency for the formation of fibrils. Little bacterial adhesion to conventional skin in this study can be related to the report of Thomas and McMeekin (22). From electron microscopic observation of spoiled chicken skin, they mentioned that spoilage bacteria were not attached to the skin by extracellular bridging substances but were free within a stable nutritious film where specific or nonspecific attachment mechanisms apparently have no survival value. If this is true, cells do not need to adhere to the surface in the nutritious film on conventional skin. However, considering the initial contamination of pathogenic bacteria during rearing and processing, the persistence of bacteria on poultry skin should be at least based on real attachment via ligand forma-
Figure 3. Production of attachment fibrils by S. typhimurium on the surface of three types of turkey skins: a) long and thin fibrils in steam-spray skin, b) short and thick fibrils on conventional skin, c) no fibril formation on the surface of kosher skin.

The reduced attachment of S. typhimurium to kosher skin surface cannot be explained by the nutritious surface film described by Thomas and McMeekin (22). Rather, the very hydrophobic stratum corneum layers seemed to contribute to low attachment.

Most of the previous reports on attachment fibrils are based on scanning electron microscopic observation of artificially inoculated surfaces such as the findings in this study. Recently, the use of cryo-scanning electron microscopy (cryo-SEM) appears to overcome the artificial aspects of the conventional scanning electron microscopic technique. Fraser and Gilmour (7) claimed that fibril formation of P. fragi (ATCC 4973) was associated with the ethanol dehydration stage of the chemical preparation method for SEM and was not an attachment feature of these cells, because cryo-treated cells did not show the formation of fibrils. Fibrils seen on cells prepared by the conventional chemical method were believed to be the shrunken residue of formerly hydrated extracellular polymer layer. Similarly, van Doom et al. (25) reported that a layer of extracellular polysaccharide produced by Pseudomonas aeruginosa was not found when conventional SEM preparation technique was used, but cryo-SEM allowed the observation of this layer. These researchers stated that the polysaccharide layer disappeared partially as a result of glutaraldehyde fixation but mainly during alcohol dehydration and critical point drying. Herald and Zottola (9) did not deny the possibility of looking at collapsed exopolymers in their electron micrographs of Yersinia enterocolitica attached to stainless steel and showed the dependence of fibril formation on pH and temperature.

On the contrary, Lee Wing et al. (12) observed bleb-like evaginations and coiled glycocalyx when P. fragi was inoculated on beef surface and incubated. They suggested those were the actual outgrowth of a long fibrous material bridging microorganisms to each other or to the substrate and may not be the result of stretching of extracellular polymers when microorganisms are adjacent to each other. Their electron micrographs showed the preference of polar sites for formation of these fibers.

There seems to be no doubt of the origin of attachment fibrils as exopolysaccharide material produced by bacteria in irreversible phase of attachment. Even if they are artifacts of conventional chemical preparation method for SEM, the different extent of fibril formation depending on the time of contact would still reflect the increase of exopolymer formation during attachment by S. typhimurium (Fig. 1).

Depression formation
Depressions on the skin surface by attached cells were sometimes observed in conventional skin (Fig. 4). These depressions often appeared to be empty and supported the possibility of cells being washed away on the fatty and pliable conventional skin which has the remainder of stratum corneum layers on top (70). The possibility cannot be excluded that the HMDS treatment, which is an alternative of

TABLE 1. The characteristics of attachment fibrils produced by S. typhimurium on the surface of turkey skins from three different defeathering systems.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Defeathering system</th>
<th>Incubation time (min) at 20°C</th>
<th>Characteristics of fibrils</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Conventional</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Kosher</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Steam-spray</td>
<td>0</td>
<td>1</td>
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\textsuperscript{a} Observations were made on six samples/each system.
\textsuperscript{b} These numbers indicate the relative mass of fibrils based on a subjective scale of 0 to 3, where 0 = none, 1 = low, 2 = medium, 3 = high.
SALMONELLA ATTACHMENT TO TURKEY SKIN

Figure 4. Depressions produced by previously attached cells on the surface of conventional skin: a) attached cell plus a few depressions, b) many depressions with no attached cells.

Critical point drying, might cause the removal of attached cells on conventional skin and left the empty depression, although HMDS was reported to be a suitable alternative (1,8). If the cells were in loose attachment in a fatty film on the surface of conventional skin, HMDS might act as a solvent washing the cells away with fat. Fraser and Gilmour (7) explained the clear space around some chemical preparations of surface grown organisms as the extra area occupied by the extracellular polymer (encasing each bacterium) prior to treatment. This layer was believed to be flattened or spread out to afford a greater degree of contact with the substrate rather than cylindrical shape of the enclosed bacterium. Basically, no depression was observed on the surface of kosher skin. Keratinized epidermal layers (stratum corneum) left on kosher skin must be resistant to water penetration and other chemical treatments, i.e., alcohol dehydration and HMDS washing; therefore, no shrinkage or depression was observed on its surface.

Pits were rarely observed on the surface of steam-spray skin (Fig. 5). Void zones were formed around the cells, which were considered to be different from the depressions observed in conventional skin. Therefore, it looked like a certain kind of digestion had occurred by the cells. Similar phenomena were observed by other authors (3,22). Butler et al. (3) described these pits as 'erosion trough' and suggested they probably occurred via enzymatic degradation of the host surface by bacteria. However, from cryo-SEM observation of microorganisms in a liquid film on spoiled chicken surface, McMeekin et al. (15) concluded that the pits are dehydration artifacts caused by the chemical preparative methods employed rather than the result of degradation of film proteins by extracellular enzymes or microbial origin. Although cryo-SEM allows us to observe the natural attachment state with hydrated water, the details of adhesion between cells and surface which are available in conventional SEM technique are obscured in a sense. The appearance of these pits only on the steam-spray skin may suggest the ease of digesting membrane, presumably basal lamina, covering the collagen fibers of dermal layers.

This study demonstrated the diverse attachment characteristics of S. typhimurium on the skin of turkeys that had been defeathered through three different systems. Both the skin microtopography and the physicochemical properties of each skin surface were responsible for the results. The possibility of reducing bacterial attachment by manipulating skin microtopography has already been mentioned (10). In this study, steam-spray processed skin showed a higher rate of salmonellae attachment than others, due to specific adhesion of cells to collagenous dermal surface. It strongly suggests that exposing dermis should be avoided to allow less bacterial attachment or contamination during poultry processing. Once all epidermis was removed, salmonellae could penetrate deep into the skin, also (11). Microtopography of poultry skin may be easily controlled by changing scalding/picking conditions. Further research is necessary to find out the optimum poultry processing conditions which minimize the microbial attachment/contamination of carcasses.

ACKNOWLEDGMENT

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REFERENCES