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Substratum Topography Influences Susceptibility of *Salmonella enteritidis* Biofilms to Trisodium Phosphate

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Established (48- and 72-h) *Salmonella enteritidis* biofilms grown in glass flow cells with or without artificial crevices (0.5-, 0.3-, and 0.15-mm widths) were subjected to a 10% trisodium phosphate (TSP) solution under different flow regimens (0.3, 0.6, 1.2, and 1.8 cm s^{-1}). The abundance of biofilm remaining after TSP treatment, the biocidal efficacy of TSP, and the factors which contributed to bacterial survival were then evaluated by using confocal laser microscopy and a fluorescent viability probe. Biofilm age affected the amount of biofilm which remained following a 15-s exposure to TSP. After TSP treatment of 48-h biofilms, 29% of the original biofilm remained at the biofilm-liquid interface, whereas 75% of the biofilm remained at the base (the attachment surface). Following TSP treatment of 72-h biofilms, 27% of the biofilm material remained at the biofilm-liquid interface, 73% remained at the 5-μm depth, and 91% remained at the biofilm base. Results obtained using the *Bac*Light viability probe indicated that TSP exposure killed all the cells in 48-h biofilms, whereas in the thicker 72-h biofilms, surviving bacteria (~2% of the total) were found near the 5- and 0-μm depths. In the presence of artificially constructed crevices, an inverse relationship was shown to exist between bacterial survival (ranging from ~13 to 83% of total biofilm material) and crevice width. This relationship was further influenced by the velocity of TSP flow; high TSP flow velocities (1.8 cm s^{-1}) resulted in the lowest number of surviving bacteria at the base of crevices (~42% survival). Extended time courses demonstrated that after TSP stress was relieved, biofilms continued to grow within crevices but not in systems without crevices. It is suggested that advective TSP flux into crevices and through the biofilm matrix was enhanced under conditions of high flow. These results suggest that the inherent roughness of the substratum on which the biofilm was grown and the timing of TSP application are important factors controlling the efficacy of TSP treatment.

Meat carcasses are typically exposed to bacterial contamination at some point following the slaughter of the animal and subsequent processing. While enteric bacterial pathogens usually originate from the animal itself (e.g., feces or digesta) or from various surfaces, liquids, or treatments associated with processing, the end result is that some meat products contaminated with bacteria may be rendered unfit for human consumption or for sale or may lead to the outbreak of disease (8). It has been estimated that of the yearly reported cases of salmonellosis, approximately 50% are the consequence of surface-contaminated chicken products. Overall, the economic and social losses due to bacterial fouling of meat products have been estimated at 1 to 4 billion dollars in terms of medical care, absent workers, and fatalities (27).

Typically, processes for reducing microbial loads on meat products include spraying carcasses with high-pressure jets of water or dipping carcasses in solutions containing various antimicrobial agents (e.g., hydrogen peroxide, ozone, chlorine dioxide, acetic acid, lactic acid, or trisodium phosphate [TSP], etc.) (19, 20, 23, 24, 29). A wide range of chemicals have been evaluated, as treatment or rinse solutions, for their abilities to both remove bacteria and inhibit subsequent bacterial regrowth. However, an industry-wide standard which satisfies both sensory and health-related concerns has yet to be developed.

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Organisms which survive sanitary steps may potentially proliferate and develop into microbial biofilms on meat surfaces before consumption if the correct conditions are encountered. Thomson et al. (26) suggested that recontamination of chicken carcasses after antimicrobial treatment is a significant cause of meat spoilage. While improved sanitary practices within the confines of the processing plant will invariably improve the quality of the final product, it is generally recognized that some contact with microorganisms during processing is inevitable. The sources for recontamination vary, but include water distribution systems, cutting boards, knifes, tables, hooks, conveyors, and employees, etc. It is consequently clear that more effective control of food pathogens is dependent upon a better understanding of the factors which lead to the survival of surface- and carcass-borne bacteria after antimicrobial treatment.

Understanding why surface-borne pathogens are so difficult to eradicate from spoilable meat products requires an improved knowledge of the nature of the microbial microenvironment. Bacteria living within biofilms are inherently more resistant to antimicrobial treatments than bacteria suspended in solution or grown in batch culture (24). Unfortunately, the majority of antimicrobial agents are tested against laboratory-cultured pathogens grown in batch culture. Consequently, it remains unclear how well many antimicrobial compounds will perform against food-borne pathogens under industrial conditions. Possible explanations for the increased resistance of biofilm bacteria include (i) limitations to the free diffusion of antimicrobial agents through the biofilm matrix, (ii) interaction of the antimicrobial agents with the biofilm matrix (cells and

polymer), (iii) variability in the physical and chemical environments associated with individual biofilm bacteria or regions of the biofilm (e.g., varied conditions of pH, osmotic strength, or nutrients), and (iv) varied levels of metabolic activity within the biofilm milieu (1, 9, 11). It is not yet clear how the physical and chemical factors associated with food products impact the above scenarios. During the present study, TSP was applied to established (48- and 72-h) *Salmonella enteritidis* biofilms in an attempt to elucidate how substratum roughness, TSP flow velocity, and biofilm age affected TSP efficacy. These analyses were performed nondestructively on fully hydrated living biofilms by using scanning confocal laser microscopy (SCLM), image analyses, and fluorescent molecular probes.

MATERIALS AND METHODS

Organism, culture conditions, and flow cell construction. *S. enteritidis* ATCC 4931 was routinely cultivated in 250-ml Erlenmeyer flasks containing 50 ml of 10% Trypticase soy broth (TSB; Difco Media Co., Detroit, Mich.). Cells were incubated on a flask shaker at room temperature (21 ± 2°C) until mid-log phase and were thereafter used to inoculate flow cells. Modified flow cells were required to achieve Köhler illumination during phase-contrast microscopic analysis as well as to permit SCLM observation. Flow cells (chamber dimensions, 1.3 by 5 by 80 mm) were constructed by using glass coverslips (no. 1 thickness; Corning Glassware), a silicone gasket, polycarbonate plastic blocks (Lexan; General Electric), and S/P medical-grade silicone tubing (1.5-mm inside diameter; 3.18-mm outside diameter; Dow Corning Co., Baxter, Ill.). Pourable silicone adhesive (translucent RTV 118; G. E. Silicones, Waterford, N.Y.) was used to assemble the flow cell components.

Modified flow cells incorporating microcrevices were constructed by using two silicone blocks (3 by 3 mm by 0.5 mm) positioned on the upper flow cell surface (the coverslip) and oriented perpendicular to the direction of flow. Crevices with widths of 0.15, 0.30, and 0.50 mm (0.5 mm deep) were constructed and used in separate flow cells. Except for the addition of crevices, these flow cells were identical in design and construction to those described above. Flow cells were then mounted on a base plate which was custom milled to fit the stage mounts of the Nikon FXA microscope.

Culture apparatus. Two reservoirs were used to provide a once-through supply of either growth medium or test solution containing TSP (10% TSP [wt/vol], pH 12.5; AvGuard; Rhône-Poulenc) to the flow cell. The growth medium reservoir contained 10% TSB and was used for routine *S. enteritidis* biofilm cultivation. The second reservoir contained TSP, which was applied as an antimicrobial agent to established (48- or 72-h) biofilms. Both reservoirs were positioned upstream from the flow cell and were connected with 3.18-mm-outside-diameter silicone tubing, Teflon tubing connectors and adapters, and silicone adhesive. A "Y" connector positioned between the two reservoirs permitted rapid switching between the TSB and TSP.

Prior to initiating experiments, the glass flow cell surfaces were conditioned by pumping TSB through the system overnight. An ~1-ml pulse of log-phase *S. enteritidis* cells was used to inoculate flow cells, as previously described (13). During biofilm cultivation, growth medium flow (0.3 cm s^{-1}) was maintained for 48 or 72 h with a Watson Marlow 201Z peristaltic pump. Inoculated chambers were then mounted on a Nikon microscope stage for high-magnification phase-contrast or laser microscopic analysis while a continuous nutrient flow was maintained. All flow cell experiments were performed in triplicate.

Microscopy. Optical thin sections were obtained by using an MRC-600 Lasersharp fluorescence confocal laser system (Bio-Rad Microscience, Mississauga, Ontario, Canada) mounted on a Nikon FXA microscope equipped with a ×60, 1.4 N.A. objective (12). Phase-contrast microscopy was performed by using the Nikon FXA microscope equipped with a ×100, 1.3-numerical aperture (N.A.) phase-contrast objective lens.

Determining the effect of TSP on *S. enteritidis* biofilms. The TSP solution was applied to 48- and 72-h biofilms for 15 s to determine the effect on biofilm material (cells and polymers). TSP was applied by clamping off the flow of the growth medium and unclamping the tubing connecting the TSP reservoir to the flow cell chamber, while maintaining a constant flow velocity of 0.3 cm s^{-1}. Following the 15-s treatment interval, the TSP flow supply was halted and the flow of the TSB growth medium was resumed for an additional 30 min. In separate flow cells, distilled water was applied to biofilms for a 15-s interval in place of TSP as a control.

The effect of the TSP and distilled-water treatments was then examined by using the area occupied by biofilm materials and cellular viability at defined biofilm depths (see below) as indices of antimicrobial efficacy. The imaging of biofilms was performed using fluorescein as a negative stain and SCLM (2). Fluorescein solution was prepared as a sterile 0.1% (wt/vol) solution (pH 7.8; molecular weight, 289; Sigma, St. Louis, Mo.) and was aseptically injected into the flow cell prior to SCLM analysis of the biofilms. Horizontal (*xy*) optical thin sections ($n = 5$) were acquired at the base (glass-liquid interface; 0-μm section depth), at the 5-μm section depth, and at the surface (biofilm-liquid interface; either the 5- or 7.5-μm section depth) of the biofilms. *xy* thin sections were then used to determine percent cellular area occupied by *S. enteritidis* biofilms before and after TSP treatment by using the Bio-Rad MRC 600 host computer and software. Sagittal (*xz*) optical thin sections of biofilms were also randomly acquired and used to determine the average biofilm thickness ($n = 7$). Statistical analyses (determination of means, standard deviations, and paired Student's *t* tests) were performed with Systat statistical software (Evanston, Ill.). Following SCLM imaging, fluorescein was washed from the system with sterile, fluor-free medium.

Determining the effect of TSP on bacterial viability. The Live/Dead *Bac*Light viability assay (Molecular Probes Inc., Eugene, Oreg.) was used to assess the survival of biofilm bacteria in TSP-treated and untreated biofilms. The two components of the *Bac*Light fluorescent probe were prepared as described previously (10). Approximately 0.3 ml of this solution was aseptically injected into the flow cell chamber (with flow off) in darkness for a 15-min reaction period. Unbound probe was then washed from the flow cell with fluor-free medium. The *Bac*Light probe discriminates between cells as follows: cells which emit green fluorescence are viable (have an intact cell membrane), whereas cells which emit red fluorescence are nonviable (have a compromised cell membrane). Previous studies conducted by using the *Bac*Light probe in soil system biofilms have shown that the green viability fluor does not always penetrate bacterial exopolymers (EPS), resulting in an underestimation of viable counts (3). Korber et al. (10), using plasmolysis as a viability control, demonstrated that the *Bac*Light probe effectively penetrated *S. enteritidis* biofilm EPS and thus did not underestimate viable counts. During the present study, untreated biofilms were used as a positive (viable) control for the staining procedure.

After the biofilm was stained with the viability probe, *xy* optical thin sections of the TSP-treated and untreated biofilms were acquired by using dual-channel SCLM (providing green and red fluorescence emission images from the same microscopic field). Following correction for spectral overlap of green fluorescence in the red channel, images were transferred to a MacIntosh PowerPC 8100 computer and analyzed with Ultimage image analysis software (GTFS Inc., Santa Rosa, Calif.). Images were digitally merged to determine the total number of cells present within each optical thin section. The red channel was analyzed to quantify the numbers of dead cells. The percent viability of the cells present within each optical thin section was then calculated as the difference between the total number of cells present and the number of dead cells. Determination of means and standard deviations and analyses of variance were performed with SYSTAT statistical software.

Determining the effect of substratum roughness on TSP efficacy. The effect of substratum roughness on the efficacy of TSP treatments was examined by using biofilms cultivated in the presence of crevices 0.5 mm deep and either 0.15, 0.30, or 0.50 mm in width. Biofilms were cultivated in crevices for 72 h with 10% TSB medium at a flow velocity of 0.3 cm s^{-1} before treatment with 10% TSP for 15 s (flow rate = 0.3 cm s^{-1}). The impact of TSP treatment, as well as the protection provided by different crevice widths, was quantified by SCLM and image analysis (i.e., the change in biofilm material remaining and cellular viability following TSP treatment at defined biofilm depths), as described above. Control biofilms without crevices were exposed to the same TSP treatment regimen for comparison.

To determine whether TSP treatment resulted in subsequent loss of biofilm material or a decrease in biofilm thickness, extended flow cell experiments were conducted. These experiments involved monitoring TSB-cultivated *Salmonella* biofilms for 3 h prior to, and for 17 h following, TSP treatment (performed as described above). At the time of TSP treatment, control and crevice-protected biofilms had already been cultivated for a 72-h period under flowing conditions.

Determining the effect of laminar flow velocity on TSP efficacy. TSP solutions applied at different laminar flow velocities were used to further study the protective effect of surface heterogeneities. Biofilms (72 h) grown in 10% TSB at a flow velocity of 0.3 cm s^{-1} within crevices (width = 0.15 mm; depth = 0.5 mm) were subjected to TSP (as described above) at flow rates of either 0.3, 0.6, 1.2, or 1.8 cm s^{-1}. The effect of TSP under different flow regimens, as well as the protection provided by crevices, was then quantified by SCLM and image analysis (i.e., the change in biofilm material remaining and cellular viability following TSP treatment), as described above. Control biofilms without crevices were exposed to the same TSP treatment regimen for comparison.

RESULTS AND DISCUSSION

Architecture of *S. enteritidis* biofilms. Previous studies have demonstrated that both pure-culture and mixed-species biofilms embody a significant degree of heterogeneity. Documented in terms of channels, pores, detectable microcolonies, distinctive cellular arrangements, regions of high cell or polymer density, bacterial metabolic condition, or chemical gradients (6, 9, 11, 14, 25), the heterogeneity in biofilms provides a source of hypotheses to account for bacterial resistance to a variety of control strategies (4, 5).

During the present study, *S. enteritidis* biofilms were initially believed to be relatively homogeneous, as no obvious micro-

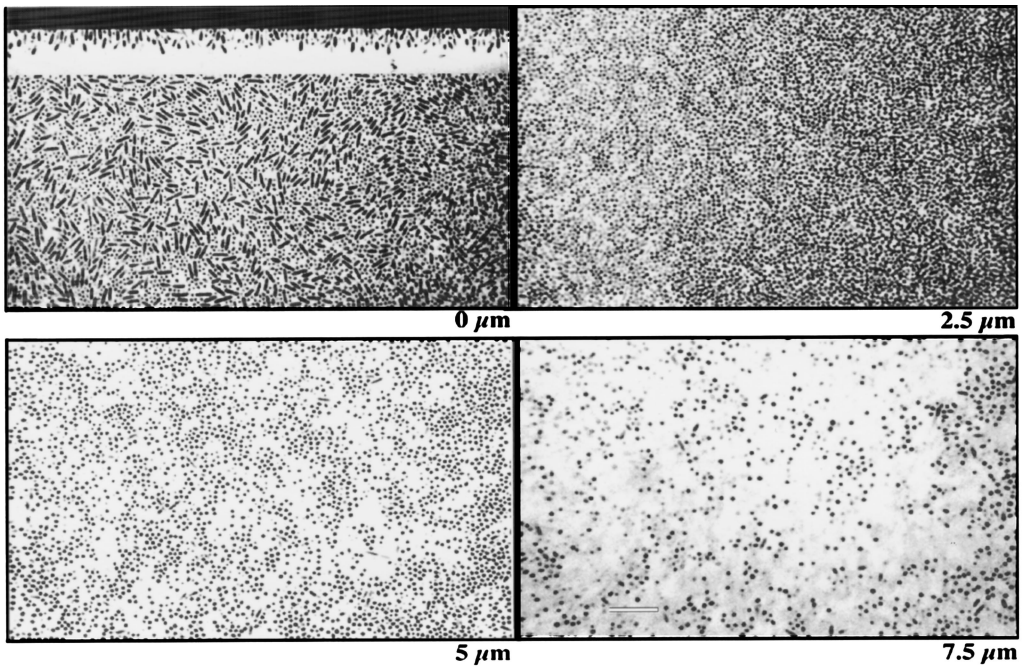

FIG. 1. Horizontal (*xy*) and vertical (*xz*) optical thin sections (0 to 7.5 μm) of a negatively stained *S. enteritidis* biofilm grown in flow cells (0.3-cm s^{-1} flow velocity) for 72 h. The basal region of the biofilm (0 μm) consisted of a dense arrangement of longitudinally and polarly attached cells. At the 2.5-, 5.0-, and 7.5-μm section depths, nearly all cells were oriented polarly. An *xz* optical thin section, included at the top of the 0-μm section shows the cross-sectional orientation of individual cells. In this *xz* presentation, the substratum (the glass coverslip) to which the biofilm is attached is located above the cells (the black region), with the biofilm-liquid interface positioned below the biofilm. Bar = 10 μm.

colonies or channel structures were observed. However, following image analysis of these biofilms it was revealed that the highest concentration of cellular material was observed at the biofilm attachment surface and less was observed at the biofilm-liquid interface (Fig. 1), similar to what was documented for *Pseudomonas fluorescens* (12, 14). *S. enteritidis* biofilms were relatively thin and consistent in thickness (mean thickness at 48 h ± standard deviation = 4.5 ± 1.3 μm; $n = 50$); however, thickness did increase marginally over time (mean thickness at 72 h = 7.7 ± 1.6 μm; $n = 60$). While these values are low compared with the average thickness of *P. fluorescens* biofilms grown on the same medium (mean thickness at 48 h = 32 ± 14 μm) (11), the density of *S. enteritidis* cells at the attachment surface makes this apparent difference in total biomass less significant. The bases of *S. enteritidis* biofilms were densely colonized (~50% of the available surface area) by longitudinally and polarly attached cells (Fig. 1); the 50% value was more than two times as large as those reported for *Pseudomonas aeruginosa* and *P. fluorescens* biofilms (~16 and 21%, respectively) (14). Polarly attached biofilm bacteria, previously documented for both *P. fluorescens* and *Pseudomonas fragi* systems (12, 15) and seen to be quite prevalent in *S. enteritidis* biofilms (Fig. 1), may favor the diffusion of nutrients and wastes into and out of the biofilm. This would facilitate the development of the high cell densities presently observed near the bases of *S. enteritidis* biofilms.

One explanation for *S. enteritidis* not forming thicker biofilms (<10 μm after a 72-h incubation) may be the use of TSB as the carbon source in this study. Wolfaardt et al. (28) previously demonstrated the effect of growth medium on biofilm development with a diclofop methyl-degrading microbial community. When diclofop methyl biofilms were cultivated in TSB, a labile carbon source, they formed thinner (4.5 ± 0.7 μm) biofilms than when the same system was cultivated in diclofop (23.6 ± 15.1 μm), a recalcitrant, halo-organic compound. Moreover, the TSB-grown biofilms were less variable in terms of thickness, spatial orientation of cells, cell density, and cell morphology than were the diclofop methyl-grown biofilms. Dewanti and Wong (7) also studied the impact of culture media on biofilm formation by *Escherichia coli* O157:H7. They reported that the use of TSB resulted in poor initial attachment and promoted eventual detachment of biofilm cells. In contrast, the authors found that the use of minimum salts medium amended with glucose as the carbon source resulted in extensive biofilms.

Effect of TSP on *S. enteritidis* biofilms. *S. enteritidis* biofilms were subjected to pulse treatment of TSP at a laminar flow velocity of 0.3 cm s^{-1}. For comparative purposes, an identical treatment regimen was also performed with distilled water to differentiate the impact of liquid flow from that of the antimicrobial agent. The effects (i.e., changes in percent cellular area and cellular viability) of these treatments at different depths are shown in Fig. 2. Compared with no treatment (control biofilms), the application of distilled water did not significantly impact the amount of cellular material at the biofilm base (the 0-μm sampling depth). Similarly, the viability of water-treated *S. enteritidis* biofilms, determined with the *Bac*Light viability probe, remained unchanged ($P < 0.05$) (i.e., 98 to 99% of the cells remained viable). In contrast, TSP application killed all cells in the 48-h biofilms, while concurrently reducing the amount of biofilm material remaining (75 and 29% at the 0- and 5-μm section depths, respectively) (Fig. 2A). TSP treatment also had a significant effect on 72-h biofilms; 27% of the biofilm material remained at the biofilm-liquid interface, whereas the amount of cellular material at the attachment surface was not significantly altered after treatment ($P < 0.05$; Fig. 2B). This resilient cellular material positioned near the glass surface may have contributed to the survival of a small

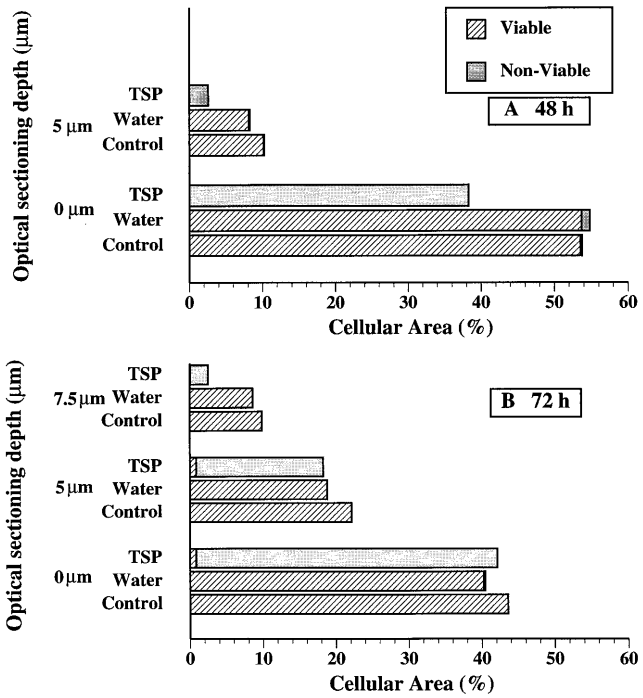

FIG. 2. Protection of *S. enteritidis* cells embedded within biofilms following a 15-s exposure to 10% TSP (TSP flow rate = 0.3 cm s^{-1}). Prior to TSP treatment, biofilms were cultivated in flow cells for either 48 h (A) or 72 h (B) with 10% TSB as the carbon source and a medium laminar flow velocity of 0.3 cm s^{-1}. The percent cellular areas and percent cell viabilities from the biofilm base (0-μm section depth) to the biofilm-liquid interface (the 5-μm section depth for the 48-h biofilm; the 5- and 7.5-μm section depths for the 72-h biofilms) are shown. For comparative purposes, an identical treatment regimen was also performed with distilled water to differentiate the impact of liquid flow from that of the antimicrobial agent. Note that after 72 h, some embedded cells were protected from TSP killing, while cells at the surface of the biofilm were not.

number of cells (1 to 2%). SCLM analysis revealed (data not shown) that despite reductions in cellular material at the biofilm-liquid interface and significant changes in cellular viability, TSP treatment had little effect on the basic appearances of biofilms.

The survival of a small number of cells in the 72-h *S. enteritidis* biofilms is clearly of significance from a food-processing perspective. These viable cells, located near the biofilm attachment surface, were covered by a layer of dead cells and their EPS. Previously, Somers et al. (24) determined that *Listeria monocytogenes* and *Salmonella typhimurium* biofilms grown on stainless steel and buna-N rubber were more resistant to TSP than planktonic cells. The biofilm-EPS matrix has repeatedly been cited as a protective barrier which may buffer cells from the influence of a range of stress factors including antimicrobial agents (4, 21), and while there are a number of possible mechanisms, the specific process remains unclear. Regardless of the active mechanism, surviving cells may act as a focus of bacterial regrowth in the case of spoilable food products, possibly resulting in enteric disease.

In food-processing environments, surface-associated pathogens and food spoilage bacteria are likely to be protected by other EPS-producing microorganisms, by the substantial accumulation of organic materials of food-processing origins, and by surface irregularities of processing equipment and meats. Consequently, it is clear that longer TSP treatment regimens would be required to achieve similar killing efficiency of food biofilms, as these films would likely differ from the relatively homogeneous, <10-μm-thick *S. enteritidis* biofilms grown on labile TSB carbon described in the present study. It should also be noted that some bacteria have demonstrated resistance to alkaline conditions. Somers et al. (24) reported that *L. monocytogenes* was highly resistant to TSP treatment; a 12% TSP treatment for 5 min or an 8% TSP treatment for 10 min was required to obtain significant reduction in *Listeria* cell numbers.

It is not yet clear whether the presence of surviving bacteria in 72-h biofilms was the consequence of a thicker microbial film or whether the age of the biofilm bacteria (and state of metabolic activity) and associated polymers played a role. However, less biofilm was removed following TSP treatment of 72-h biofilms than of 48-h biofilms (Fig. 2B). Studies by LeChevallier et al. (16) as well as by Lee and Frank (18) similarly demonstrated that biofilm age had a significant effect on increased cell resistance to chlorination. Further applied study is required to delineate the effect of biofilm age on the efficacy of TSP treatment under industrial conditions.

Effect of substratum roughness and laminar flow velocity on efficacy of TSP treatment. Surface roughness may hinder the penetration of antimicrobial agents or high-pressure washes, thereby protecting bacteria. Bacteria may survive within these sites and subsequently proliferate, further limiting advective transport of antimicrobial agents through the formation of biofilm biomass and associated EPS and culminating in the spoilage or contamination of the food product. The importance of crevices as protective structures was examined by using TSP inhibition experiments and specialized flow cells having crevices of defined width. Generally, biofilms grown inside crevices were less susceptible to TSP treatment (delivery rate = 0.3 cm s^{-1}) than those cultivated in the absence of crevices. While TSP treatment resulted in nearly 100% killing of control biofilm bacteria (no crevices), a significant ($P < 0.05$), inverse relationship existed between TSP efficacy and crevice width (for crevice widths of 0.5, 0.3, and 0.15 mm, the viabilities of remaining cells at the biofilm base were 13 ± 12, 35 ± 19, and 83 ± 12%, respectively [mean ± standard deviation]) (Table 1). The smallest crevices tested during this study (0.15 mm in width) provided the best protection against TSP in terms of the number of surviving bacteria. It is likely that a combination of physical and microbial barriers acted in concert to hinder advective transport of TSP when *S. enteritidis* biofilms were grown in 0.15-mm crevices. It is noteworthy that the sizes of crevices on food and industrial equipment surfaces commonly span this range. Interestingly, no clear relationship be-

TABLE 1. Effect of crevice width on percent cellular area and viability of 72-h *S. enteritidis* biofilm bacteria[a] following treatment with 10% TSP for 15 s at 0.3 cm s^{-1}

Approx crevice width (mm)	% Cellular area remaining[b,c] ± SD	% Viability[b,d] ± SD
No crevice	91 ± 2	0
0.5	84 ± 11	13 ± 12
0.3	95 ± 4	35 ± 19
0.15	97 ± 4	83 ± 12

[a] Determined from horizontal optical thin sections obtained at the base of the biofilm (0-μm section depth)
[b] Values are the means of triplicate TSP treatments.
[c] Values are not significantly different.
[d] Values for 0.5- and 0.3-mm crevice widths are significantly different ($P < 0.05$; analysis of variance) from 0.15-mm and no-crevice values but are not significantly different from each other.

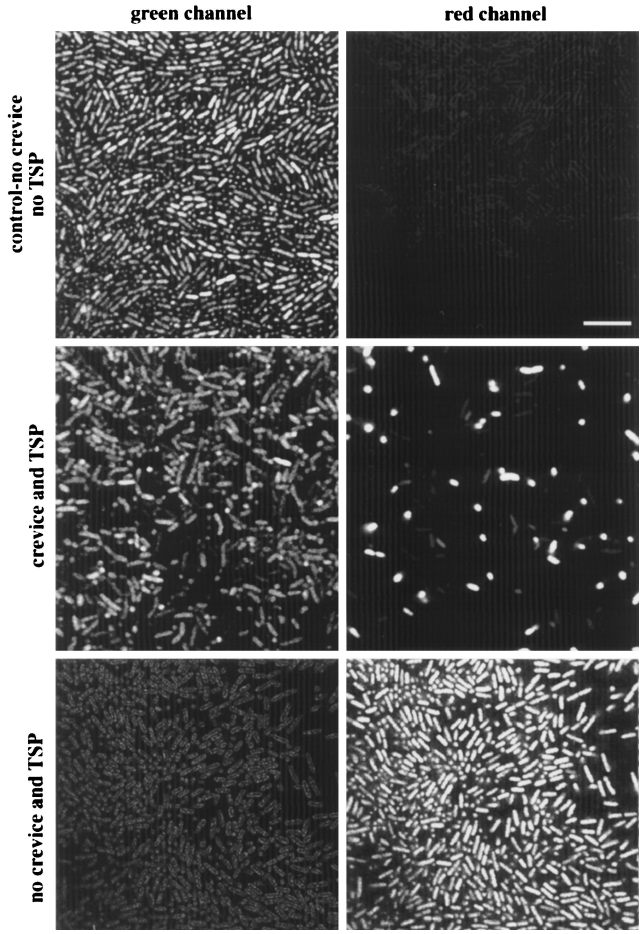

FIG. 3. Representative dual-channel *xy* optical thin sections showing the differential fluorescence emission resulting from *Bac*Light staining of 72-h *S. enteritidis* biofilms (0-μm depth). Corresponding fluorescent images detected in the green (viable cells) and red (dead cells) channels of (from top to bottom) control biofilms (no crevices, no TSP treatment), TSP-treated biofilms in crevices (0.15-mm width), and TSP-treated biofilms without crevices are shown. Note that all control biofilm cells remained viable and hence fluoresced in the green wavelengths. In the crevice-protected TSP-treated biofilm, some cells were killed (as indicated by the strong red fluorescence emitted by some cells); however, most cells remained viable. In the case of the unprotected (no crevice) TSP-treated biofilm, all the cells were killed, as indicated by the bright fluorescence emitted by cells in the red channel, and weak fluorescence emitted by the same cells in the green channel. Bar = 10 μm.

tween crevice width and the percent area occupied by cell material was found when a 0.3-cm sec^{-1} flow velocity was used (Table 1).

Figure 3 demonstrates how the *Bac*Light viability probe and dual-channel SCLM imaging was utilized to provide information on the viabilities of treated and untreated *S. enteritidis* biofilm bacteria. In these images, live cells stained green (green channel), whereas dead cells are shown in the red channel. Note the impact of the 0.15-mm-wide crevices on the survival of *S. enteritidis* bacteria after TSP treatment.

The 0.15-mm crevice width was subsequently used to examine the protective effect of physical barriers against TSP treatment applied at various flow velocities to 72-h *S. enteritidis* biofilms. TSP solutions (flow velocity range, 0.3 to 1.8 cm s^{-1}) were delivered for a 15-s interval, and the changes in percent cellular area and bacterial viability were monitored at the biofilm base as well as at the biofilm surface. The rate of TSP flow clearly affected the efficacy of TSP killing and biofilm removal (Fig. 4). When TSP was applied at higher flow velocities, increased killing and decreased amounts of remaining biofilm material resulted, presumably as a consequence of either increased advective transport of TSP under high-flow conditions or shear removal. After treatment of biofilm bacteria grown in crevices with 1.8-cm s^{-1} TSP, 42% at the biofilm base and 19% at the biofilm-liquid interface remained viable. Control biofilms grown without crevices were completely killed when TSP was applied at laminar flow velocities that were ≥0.6 cm s^{-1}. Crevices also favored the retention of *S. enteritidis* biofilm bacteria, primarily at the biofilm-liquid interface, at higher TSP flow velocities (≥0.6 cm sec^{-1}) (Fig. 4). When directly compared to biofilms without crevices, crevice-protected biofilms all had higher percentages of remaining biofilm (except at the base of biofilms treated at a flow velocity of 1.8 cm s^{-1}) for all flow velocities of TSP application. Le Chevalier et al. (17) reported that *E. coli* and enteric pathogens such as *S. typhimurium* attached to granular activated carbon particles were not susceptible to chlorine treatment at 1.4 to 1.6 mg $liter^{-1}$ for 1 h. An examination of the activated carbon particles by scanning electron microscopy revealed that bacteria colonized cracks and crevices, and were coated with EPS (17). Similarly, the physical protection provided by surfaces which microor-

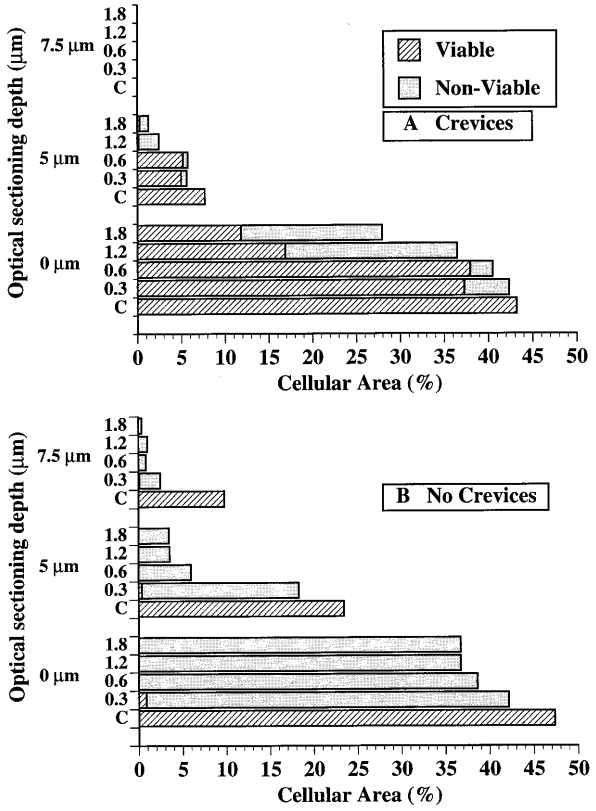

FIG. 4. The effect of TSP flow velocity on the efficacy of TSP treatment on *S. enteritidis* biofilms grown with (A) and without (B) crevices (width = 0.15 mm; depth = 0.5 mm). Biofilms were initially grown under flowing conditions (0.3 cm s^{-1}) for 72 h with 10% TSB as the growth medium. A 10% TSP solution was then applied for 15 s at either 0.3, 0.6, 1.2, or 1.8 cm s^{-1}. The effect of TSP flow velocity, in terms of the abundance of biofilm material and cellular viability, was determined at the base (0 μm) and at the biofilm-liquid interface (5 μm in panel A; 5 and 7.5 μm in panel B) of control (C) and TSP-treated biofilms. For each sectioning depth, the results of applying TSP at different flow rates are shown. Note that even though crevice-associated biofilms were less thick than biofilms grown without crevices, a higher percentage of surviving bacteria remained in crevices.

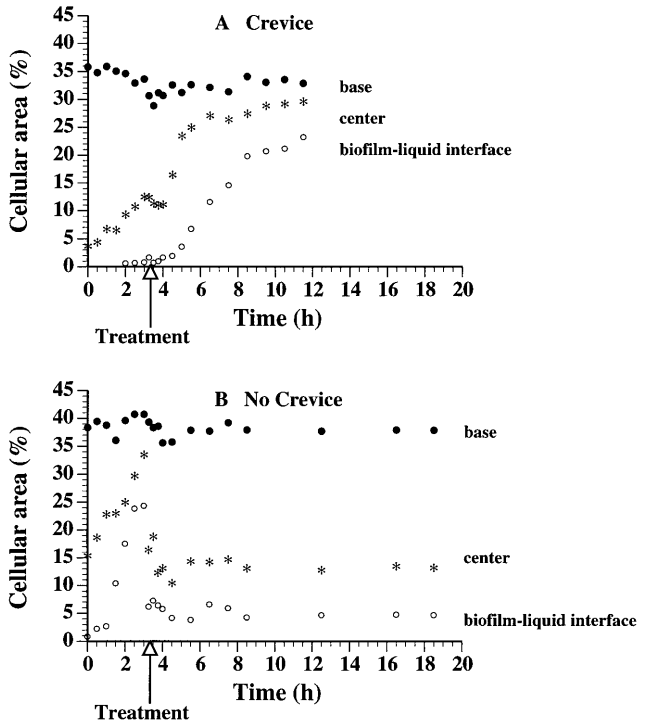

FIG. 5. The effect of TSP treatment (10% TSP delivered at 0.3 cm s^{-1} for 15 s) on the percent cellular area ($n = 7$) of *S. enteritidis* biofilms grown in flow cells with (A) or without (B) crevices (width = 0.15 mm; depth = 0.5 mm). Flow cells were inoculated and irrigated with sterile medium for 69 h (time zero on *x* axis) before cell area measurements were made. Biofilm measurements were determined at three locations (the base, the biofilm-liquid interface, and the center of the biofilm) by analyzing horizontal optical thin sections before (time zero to 3.25 h) and after (3.5 h onward) TSP treatment. The crevice-protected biofilm (A) continued to grow (percent cellular area values increased at the center and biofilm-liquid interface) following TSP treatment (after which flow of sterile growth medium was resumed). In contrast, the unprotected biofilm (B) exhibited a reduction in biofilm material at the center and biofilm-liquid interface following TSP treatment and did not show subsequent increases in biofilm measurements over time.

ganisms colonize is believed to be a contributing factor for problems encountered in biofilm control (22).

Notably, the *S. enteritidis* cells inside crevices required longer incubation periods to form confluent biofilms. The 72-h biofilms inside crevices had a mean biofilm thickness ± standard deviation of 5.0 ± 0.7 μm and were thinner ($P < 0.05$) than biofilms (7.7 ± 1.6 μm) grown without crevices. The patterns of cellular distribution of the biofilms inside crevices, as revealed by the horizontal thin sections (*xy*) (data not shown), were also similar to those of the biofilms grown without crevices. Despite slight differences in biofilm thickness (i.e., thinner biofilms within crevices), the crevices still resulted in greater cell survival and retention of cellular material than biofilms in the absence of crevices.

The results reported in the previous sections were obtained by comparing biofilms before and immediately following TSP treatment. Consequently, it was not clear whether TSP treatment led to the eventual deterioration (sloughing) of the biofilm under flowing conditions. Therefore, *S. enteritidis* biofilms were monitored for 3 h prior and for 17 h following TSP treatment to delineate the physical impact of TSP treatment. Image analysis of *xy* optical thin sections revealed that the percent cellular biomass at the biofilm-liquid interface was increasing (Fig. 5) before treatment. Following treatment, biofilms grown without crevices lost 3, 51, and 71% of their cellular material at the 0- (base), 5- (center), and 7.5-μm (surface) depths, respectively, and showed no evidence of growth even though TSP stress was removed and substrate (TSB) was provided for an additional 17 h. No further cell detachment was detected during this observation period. In contrast, crevice-protected biofilms lost 8, 9, and 50% of their cellular material at the base, center, and surface, respectively, but continued to grow, as evidenced by an increase in cellular material at the biofilm-liquid interface over time (Fig. 5).

Changes in biofilm thickness (obtained by measuring *xz* optical sections) were also monitored over time (Fig. 6). Biofilms grown in the absence of crevices became markedly reduced in thickness (~46%) following TSP treatment, with biofilm thickness remaining constant over the subsequent monitoring period. In contrast, crevice-protected biofilms were only slightly impacted (<5% decrease in biofilm thickness) by TSP application, with subsequent growth leading to an eventual increase in average biofilm thickness from 5 to 10 μm over the 8-h period following treatment (Fig. 6).

The results obtained during this study indicate that bacteria present within cracks or crevices likely acquire a significant degree of physical protection against antimicrobial treatments. Consequently, surface roughness likely favors bacterial survival

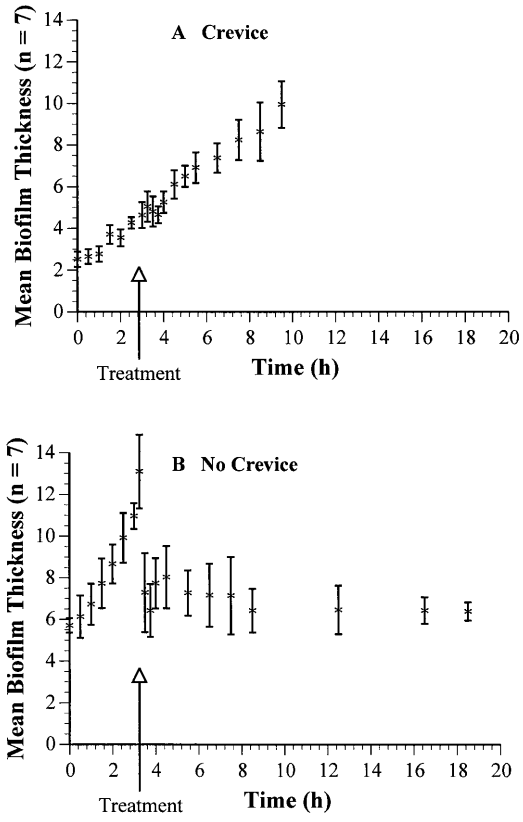

FIG. 6. The effect of TSP treatment (10% TSP delivered at 0.3 cm s^{-1} for 15 s) on the mean thickness ($n = 7$) of *S. enteritidis* biofilms grown in flow cells with (A) or without (B) crevices (width = 0.15 mm; depth = 0.5 mm). Flow cells were inoculated and subsequently irrigated with sterile medium for 69 h (time zero on *x* axis) before the biofilm thickness was monitored. The mean thicknesses of biofilms were determined by analyzing *xz* thin sections before (time zero to 3.25 h) and after (3.5 h onward) TSP treatment. The crevice-protected biofilm (A) continued to grow (biofilm thickness increased) after TSP treatment (after which flow of sterile growth medium was resumed). In contrast, the unprotected biofilm (B) exhibited a significant reduction in biofilm thickness following TSP treatment and did not resume growth.

under conditions of stress. While little may be done to decrease or limit the inherent surface roughness associated with food products, other surfaces within the processing plant environment should be kept in good condition (i.e., old cutting boards or steel surfaces with scratches should periodically be replaced or polished, as the finest cracks or scratches may potentially lead to significant increases in cell survival). It has also been demonstrated that physiological and physical parameters, such as biofilm age and the velocity of antimicrobial flow, may also impact process efficacy. More research in this area is needed to delineate factors which promote the survival of bacteria during cleansing procedures.

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