Research Note

Survival of *Salmonella* on a Polypropylene Surface under Dry Conditions in Relation to Biofilm-Formation Capability

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ABSTRACT

This study was conducted to gain insights into the survival of *Salmonella* on a polypropylene surface in relation to the ability of these bacteria to form a biofilm. We selected *Salmonella* strains known for the relative ease or difficulty with which they formed biofilms based on microtiter plate assays and studied the survival of these strains on polypropylene discs in a desiccation chamber by sequentially counting CFUs. The biofilm-forming strains survived longer on the plastic disc surface than did biofilm-deficient strains. The biofilm-forming strains remained at over 10⁵ CFU per plate up to day 175, whereas the biofilm-deficient strains decreased to below 10⁵ CFU per plate on day 20 or below 10⁴ CFU per plate on day 108. Extracellular materials on the polypropylene surface were observed by scanning electron microscopy and crystal violet staining for the biofilm-forming strains but not for the biofilm-deficient strains. The extracellular polymeric materials on the polypropylene surface may have protected the bacterial cells from dryness, although the possibility of some inherent resistance to environmental stresses linked to biofilm formation could not be excluded. These results indicate that *Salmonella* strains with high biofilm productivity may be a greater risk to human health via food contamination by surviving for longer periods compared with strains with low biofilm productivity.

To gain insights into the survival of *Salmonella* on a plastic surface in relation to its ability to form biofilm, we selected strains with a relatively high or low ability to produce biofilm. Crystal violet was used to stain bacteria adhered to polypropylene plates. We grew these strains on polypropylene discs in a desiccated chamber and counted the number of surviving cells over time.

MATERIALS AND METHODS

**Bacteria.** *Salmonella* strains used in this study were isolated in Japan (Table 1).

**Microtiter plate assay.** A microtiter plate assay was performed according to methods used widely (2, 5, 18, 21, 27). Bacterial strains were cultured overnight in triptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) at 37°C. The culture was diluted 40 times with 1:5 TSB solution (TSB diluted five times with distilled water), and 0.1 ml of the diluted bacterial suspension was poured into wells of a 96-well flat-bottom polypropylene microplate (Greiner Labortechnik, Kremsmuenster, Germany). The plate was sealed with Parafilm M (Structure Probe, Inc., West Chester, PA) and incubated at 28°C for 48 h. After the plate was washed twice with distilled water and dried at room temperature, the inner surface of the wells was stained with 0.1% crystal violet (Sigma-Aldrich Co., St. Louis, MO) for 20 min. The dye solution was removed, and the wells were washed three times with distilled water. The remaining dye adhering to the inner surface of the wells was dissolved with 0.2 ml of 95% ethanol and transferred into the wells of a 96-well polystyrene microplate (Nunc A/S, Roskilde, Denmark) to measure the optical density at 595 nm (OD₅⁹₅) with a microplate reader (model 2550, EIA Reader, Bio-Rad Laboratories,

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Survival of bacteria on plastic discs. Salmonella strains were grown overnight in 1:5 TSB at 37°C. The culture was centrifuged at 2,300 × g for 15 min, and the cells were suspended in 1:5 TSB. The cell suspension (0.1 ml of biofilm cells plus planktonic cells) was dropped onto a polypropylene square disc (10 mm × 10 mm and 0.5 mm thick; Tokyu Hands Inc., Tokyo, Japan), which had been disinfected with 70% ethanol before use. The disc was placed in a culture dish (35 mm diameter by 10 mm thick; Tokyu Hands Inc., Tokyo, Japan), mounted on stubs, and then coated with an E-1030 ion sputter (Hitachi) for subsequent examination in an S-4000 scanning electron microscope (Hitachi). The cells on plastic discs in a desiccated chamber were examined by scanning electron microscopy (SEM). The cells on plastic discs were removed from the discs in the same manner as described above. After washing twice by centrifugation with PBS at 3,000 × g for 10 min, the cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS for more than 1 h. One drop of the cell suspension in the glutaraldehyde solution was placed on a glass slide precoated with 0.01% poly-L-lysine, kept for 1 h at room temperature, fixed with 0.1% OsO4 in PBS for 30 min, and then dehydrated through graded ethanol solutions to absolute ethanol. The dehydrated cells were then transferred to absolute isooamyl acetate, critical point dried with CO2 in an HCP-2 critical point drier (Hitachi, Tokyo, Japan), mounted on stubs, and then coated with an E-1030 ion sputter (Hitachi) for subsequent examination in an S-4000 scanning electron microscope (Hitachi).

Statistical analysis. Significant differences were determined with an unpaired Student’s t test (StatView 5, SAS Institute Inc., Cary, NC).

RESULTS

From 410 Salmonella strains, 17 were selected as high and low producers of biofilms, based on the microtiter plate assay (Table 1), and used in the experiments for determining bacterial survival on plastic discs. The survival

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<tr>
<th>Table 1. Serotypes and origin of Salmonella strains used in this study and their titers in the microplate assay</th>
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<td>SEC 307</td>
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* Overnight culture of each strain diluted with 1:5 triptic soy broth was poured into wells of a 96-well flat-bottom polypropylene microplate and incubated at 28°C for 48 h. After the plate was washed with distilled water and dried at room temperature, the inner surface of the wells was stained with 0.1% crystal violet. The dye solution was removed, and the wells were washed with distilled water. The remaining dye adhering to the inner surface of the wells was dissolved with 0.2 ml of 95% ethanol, and the OD95 was determined with a microplate reader.

Hercules, CA). For blanks, diluted TSB was processed in the same manner as the cultured bacteria.
experiment included three biofilm-forming strains and three biofilm-deficient strains. All three biofilm-forming strains maintained their populations greater than $10^{4}$ CFU per plate until day 175, whereas the two biofilm-deficient strains decreased below $10^{2}$ CFU per plate by day 20 (Fig. 1). Strain SEC 307, a biofilm-deficient strain, also decreased more rapidly than did the biofilm-forming strains, although more slowly than the other two biofilm-deficient strains (Fig. 1).

The tendency toward longer survival for biofilm-forming strains than for biofilm-deficient strains was confirmed by a short experiment with the other six biofilm-forming and five biofilm-deficient strains. Populations decreased more slowly in biofilm-forming strains than in biofilm-deficient strains until day 30 (Fig. 2), and the reduction in the population of biofilm-deficient strains was variable (Fig. 2).

The formation of biofilm on the disc in the drying chamber was assessed by the crystal violet staining method for strains SEC 54, 64, 280, and 282, and the $OD_{595}$ values of the dye containing ethanol were $1.21 \pm 0.043 (n = 3)$, $1.73 \pm 0.094 (n = 3)$, $0.093 \pm 0.019 (n = 3)$, and $0.789 \pm 0.046 (n = 3)$, respectively.

The biofilm-forming strains (SEC 54 and 64) and the biofilm-deficient strains (SEC 280 and 282) were examined by SEM. The formation of an extracellular matrix by biofilm-forming strains was confirmed by SEM (SEC 64, Fig. 3) but was not noted in the other strains (SEC 282, Fig. 3; SEC 54 and 280, data not shown).

**DISCUSSION**

Seventeen strains selected by microtiter plate assay had relatively high or low $OD_{595}$ values compared with the other strains. The human isolates produced a relatively large amount of extracellular material, but many isolates from chicken feed or eggs produced a relatively small amount of these materials. However, the linkage between biofilm-forming strains and foodborne infection is uncertain because only a small number (13) of human-derived strains were used for this screening experiment.

The experiments concerning survival of selected strains on polypropylene discs under desiccation conditions clearly indicated that *Salmonella* strains with relatively high biofilm productivity can survive under dry conditions much longer than those strains with low biofilm productivity. The biofilm-forming strains maintained populations greater than $10^{4}$ CFU per disc until day 175. The formation of an exopolymeric matrix was found by SEM and crystal violet staining in the biofilm-forming strains but not in the biofilm-deficient strains, suggesting that the exopolymeric matrix on the polypropylene disc directly protects the bacterial cells from desiccation.

Increased resistance against antibiotics has been observed in bacterial cells embedded in a biofilm in flow cells (20, 33). These findings suggest that the extracellular matrix may protect bacterial cells from various external stresses by blocking the penetration of harmful chemicals (11, 19, 31, 35). The barrier function of the extracellular matrix may also help maintain moisture in viable cells, possibly with dead cells surrounding viable cells (1).

During the course of drying in the desiccator, the bacterial suspension on the polypropylene discs lost water, which may have caused hyperosmotic stress. Thus, both the barrier function of the biofilm and inherent resistance of bacterial cells to environmental stresses might be involved in the long-term survival of strains with high biofilm productivity. This hypothesis is consistent with a relatively large variation in the survival of the biofilm-deficient strains (Figs. 1 and 2). The production of major biofilm compo-
In conclusion, Salmonella strains with high biofilm productivity can survive longer on polypropylene surfaces under dry conditions than can strains with low productivity. In this study, survival for more than 200 days was observed at 28°C, but the survival period may be prolonged at lower temperatures. Hiramatsu et al. (7) observed that Salmonella in a desiccated condition can survive longer at 4°C than at 25 or 35°C. The importance of survival of pathogenic bacteria and subsequent cross-contamination of foods in foodborne outbreaks has been indicated by the results of some model experiments (9, 11, 14, 17). Because plastic materials are widely used in food production and cooking, contamination of plastic surfaces with Salmonella strains with high biofilm productivity may pose high risks to human health.

REFERENCES


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