

Biofilm Formation Enhances Fomite Survival of *Streptococcus pneumoniae* and *Streptococcus pyogenes*

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Both *Streptococcus pyogenes* and *Streptococcus pneumoniae* are widely thought to rapidly die outside the human host, losing infectivity following desiccation in the environment. However, to date, all literature investigating the infectivity of desiccated streptococci has used broth-grown, planktonic populations. In this study, we examined the impact of biofilm formation on environmental survival of clinical and laboratory isolates of *S. pyogenes* and *S. pneumoniae* as both organisms are thought to colonize the human host as biofilms. Results clearly demonstrate that while planktonic cells that are desiccated rapidly lose viability both on hands and abiotic surfaces, such as plastic, biofilm bacteria remain viable over extended periods of time outside the host and remain infectious in a murine colonization model. To explore the level and extent of streptococcal fomite contamination that children might be exposed to naturally, direct bacteriologic cultures of items in a day care center were conducted, which demonstrated high levels of viable streptococci of both species. These findings raise the possibility that streptococci may survive in the environment and be transferred from person to person via fomites contaminated with oropharyngeal secretions containing biofilm streptococci.

Streptococcus pneumoniae and *Streptococcus pyogenes* are obligate human pathogens often carried asymptotically in the nasopharynx. Streptococcal transmission can be the result of the inhalation of large airborne droplets (produced by coughing or sneezing) from infected individuals (1); however, the risk of transmission from contaminated environmental surfaces is unknown. Interestingly, neither streptococcal pneumonia nor pneumococcal pneumonia is generally regarded as contagious, and hospitalized patients with pneumonia are not routinely isolated (2). The reason is that numerous steps intervene between transmission of the organism and development of disease (3, 4).

In contrast, studies have shown that when a person is colonized with *S. pyogenes*, then between 10% and 25% of close contacts become colonized, with asymptomatic nasal carriers expelling on average 100 times as many streptococci into the environment as do actively sick individuals (5–7). Studies of *S. pneumoniae* have led to the same conclusions, with a recent study by Tigoï et al. estimating that the rate of transmission probability per 30-day duration of contact with a nasopharyngeal carrier is 0.23 (8). While most longitudinal studies have focused on the individual host and not on the particular environmental setting, several studies have documented the isolation of streptococci from objects (9, 10) and shown that streptococci have the ability to persist for long periods on environmental surfaces (11, 12).

Biofilm formation has been recently recognized as an important growth form contributing to nasopharyngeal colonization and persistence by many bacterial species, including streptococci. Biofilms are complex multicellular communities surrounded by a mature matrix composed of extracellular DNA, proteins, and polysaccharides that link bacterial cells (13). Importantly, this biofilm lifestyle is inherently more resistant to antimicrobial agents and provides a protected mode of growth that allows cells to survive under conditions of hostile environments (14).

In this study, we hypothesized that biofilm formation during colonization may contribute to the spread of streptococci by in-

creasing its survival during desiccation and contributing to transmission from fomites. We investigated the environmental survival of *S. pneumoniae* and *S. pyogenes* and determined whether biofilm physiology of streptococci enhanced survival following environmental desiccation and fomite formation.

MATERIALS AND METHODS

Ethics statement. Human exposure experiments were approved by the Human Subjects Institutional Review Board at the University at Buffalo, Buffalo, NY. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University at Buffalo, Buffalo, NY. All bacterial inoculations and treatments were performed under conditions to minimize any potential suffering of the animals.

Reagents. Cell culture reagents were from Invitrogen, Carlsbad, CA. Bacterial and cell culture media and reagents were from VWR Inc., Radnor, PA. Chemically defined bacterial growth medium (CDM) was obtained from JRH Biosciences, Lexera, KS. Sheep blood was purchased from BioLink, Inc., Liverpool, NY. All remaining reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Cells and bacterial strains. NCI-H292 bronchial carcinoma cells (ATCC CCL-1848) were grown on cell culture-treated polystyrene plates as described previously (15), and SCC13 keratinocytes were grown in keratinocyte serum-free medium supplemented with 50 µg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor,

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and 0.3 mM calcium. Bacterial strains were grown in a synthetic medium (CDM) as described previously (16). The term “planktonic cells” uniformly refers to cells grown either to mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.5) or into stationary phase, defined here as cultures grown 2 h after the OD₆₀₀ had stopped increasing. The study used the *S. pneumoniae* serotype 19F otitis media EF3030 isolate (17) and the classical serotype 2 Avery D39 strain (18). The group A streptococcal strains used were the M3 clinical 771 isolate and the *S. pyogenes* JRS4 strain that is a spontaneous streptomycin-resistant derivative of a clinical M6 isolate (strain D471) from the Rockefeller University collection (19).

Static biofilm model on prefixed epithelia. Both *S. pyogenes* and *S. pneumoniae* biofilms on prefixed epithelia were produced from bacteria grown in CDM to mid-logarithmic phase (OD₆₀₀ = 0.5) that were then washed and resuspended in fresh prewarmed medium to a density of 2×10^4 CFU in a 500- μ l volume and seeded into sterile round glass coverslips in the bottom of polystyrene 24-well plates with confluent NCI-H292 cells for *S. pneumoniae* or SCC13 cells for *S. pyogenes* that were prefixed in 4% paraformaldehyde as described previously (20). Biofilms were cultured at 34°C in 5% CO₂ for the indicated times with change of culture media every 12 h.

Desiccation. Desiccation of bacterial populations was performed essentially as described previously (12). In brief, planktonic cultures either grown to mid-exponential phase or grown 2 h into the stationary phase were pelleted by centrifugation for 4 min at $9,000 \times g$ at room temperature. The supernatants were removed, and cells were resuspended in phosphate-buffered saline (PBS). Approximately 1×10^8 CFU of bacteria was pipetted onto each well of a 24-well polystyrene plate, and then the plates were dried open in a biosafety cabinet with airflow for 30 min. Biofilm-derived fomites were likewise washed once with PBS before being dried down onto the plastic surface in the biosafety cabinet for 30 min. Fomites were stored with lids closed over the polystyrene wells in the dark at room temperature to the indicated time points. To determine the number of remaining viable bacteria, at indicated time points, with time zero (T0) for the initial inoculum being determined 30 min after initial desiccation, 1 ml of PBS was added to desiccated samples for 5 min to allow rehydration. Samples were then scraped, pipetted to thoroughly resuspend the sample before application of the seal, and floated on a sonicator water bath for 2 s to disperse any larger bacterial aggregates. Cells were then collected and processed in a vortex apparatus twice for 20 s at high speed to ensure a homogenous solution and then used to determine viable CFU per ml by viable plate counts on tryptic soy agar (TSA)–5% blood agar plates.

Fomite survival on hands. Studies were performed essentially as described previously (21). Hands, which were clear of visible lesions, were cleaned using chlorhexidine for 3 min, rinsed, and dried. The palmar surface of the left hand was inoculated with *S. pneumoniae* strain EF3030 or *S. pyogenes* strain 771 suspended in chemically defined medium. Each of the strains was pipetted onto the skin of the hand in 16 swab sites on a pre-drawn grid, and at indicated times a cotton swab was moistened in sterile PBS and used to sample one grid location (approximately 1 cm²) by rolling over the site for 5 s. Swabs were then placed in 1 ml of sterile PBS for 10 s and sonicated, and then the number of viable CFU in the PBS was determined by enumerating the viable colonies from serial dilutions added directly onto blood agar plates. Throughout the study period, volunteers remained in the laboratory and their hands did not make contact with any objects. After the final time point, hands were decontaminated with chlorhexidine and pressed onto sterile blood agar plates to confirm decontamination.

Mouse colonization model. Six-week-old female BALB/cByJ mice from Jackson Laboratories (Bar Harbor, ME) were maintained in filter-top cages on standard laboratory chow and water *ad libitum* until use. For colonization experiments, 1-month-old desiccated fomites were resuspended in 200 μ l of PBS and then 20 μ l suspension was pipetted into the nares of nonanesthetized mice. For all experiments, aliquots of the bacte-

ria prepared for inoculation were plated on TSA blood agar plates to verify quantities. Mice were monitored at least twice daily for illness and mortality by the presence of huddling; ruffled fur; lethargy; anorexia (leading to weight loss); and body temperature. Mice were euthanized by cervical dislocation. Nasopharyngeal tissue (16) and nasopharynx-associated lymphoid tissue (NALT) were collected as described previously (22). Harvested tissue was homogenized in 1 ml of PBS and sonicated, and the homogenate was serially diluted on TSA–5% blood agar plates. Pneumococci were verified by their sensitivity to optochin, using an optochin-diffusion assay on blood agar, resulting in a clearing zone around the optochin disc (from Fluka Analytical/Sigma-Aldrich) of at least 15 mm in diameter (20). Group A streptococci were verified by the presence of a beta-hemolytic zone on blood agar (19). Noncolonized mice did not show any alpha- or beta-hemolytic colonies.

Day-care screening. During a 1-hour time period when children were not present in the facility, samples were taken from 5 of each of the following objects: books, stuffed toys, hard toys, hard surfaces, and ribbed linens. For each sample, sterile cotton swabs were moistened in sterile PBS and then used to rub a small 10-cm² area before being replaced in labeled sterile conical tubes for transport to the laboratory and immediately streaked onto blood agar plates. *S. pneumoniae* isolates were identified based on alpha-hemolytic colony morphology and optochin sensitivity. *S. pyogenes* isolates were identified based on beta-hemolytic colony morphology and confirmed by the use of group A streptococcus (GAS)-specific antibodies.

Statistical analysis. The data were analyzed for statistical significance by a two-tailed Student's *t* test or analysis of variance (ANOVA) with correction for multiple groups using Prism 5 software (GraphPad, La Jolla, CA). A *P* value of <0.05 was considered significant.

RESULTS

Biofilm bacteria are highly tolerant to desiccation. Both laboratory strains and clinical isolates were examined. Under the experimental conditions, where bacteria were desiccated on a plastic surface, the viable counts of both planktonic *S. pneumoniae* and *S. pyogenes* decreased drastically (~ 3 to ~ 6 log₁₀) during the first 24 h and *S. pneumoniae* fomites were no longer viable by 3 days postdesiccation (Fig. 1A and C). It did not matter whether the bacteria had been grown to mid-logarithmic phase or into stationary phase before desiccation, as the rates of death were very similar (Fig. 1A to D). For both streptococcal species, biofilm-derived fomites were significantly more tolerant to desiccation than their planktonic counterparts (Fig. 1E and F). Viable cells could be recovered from biofilm-derived pneumococci for up to 1 month postdesiccation compared to a survival time of less than 3 days when planktonic cultures were used. Biofilm-derived *S. pyogenes* were also significantly more tolerant to desiccation than planktonic cells and showed a minimal-to-medium decrease in viability of between 1 and 5 log₁₀ by 4 months postdesiccation. Our experiments indicate that biofilm-derived streptococci are better able to withstand desiccation than broth-grown planktonic cultures.

Desiccated biofilm streptococci retain infectivity. To determine if fomites could represent a source of *S. pneumoniae* transmission, we determined whether biofilm-derived fomites were capable of colonization in a murine model. *S. pneumoniae* and *S. pyogenes* biofilm-derived fomites that were 1 month old were resuspended in PBS and inoculated intranasally into 8-week-old female BALB/cByJ mice. Two days postinoculation, mice were euthanized, and nasal tissue homogenates (for recovery of *S. pneumoniae*) and nasopharynx-associated lymphoid tissue homogenates (for recovery of *S. pyogenes*) were removed and plated on

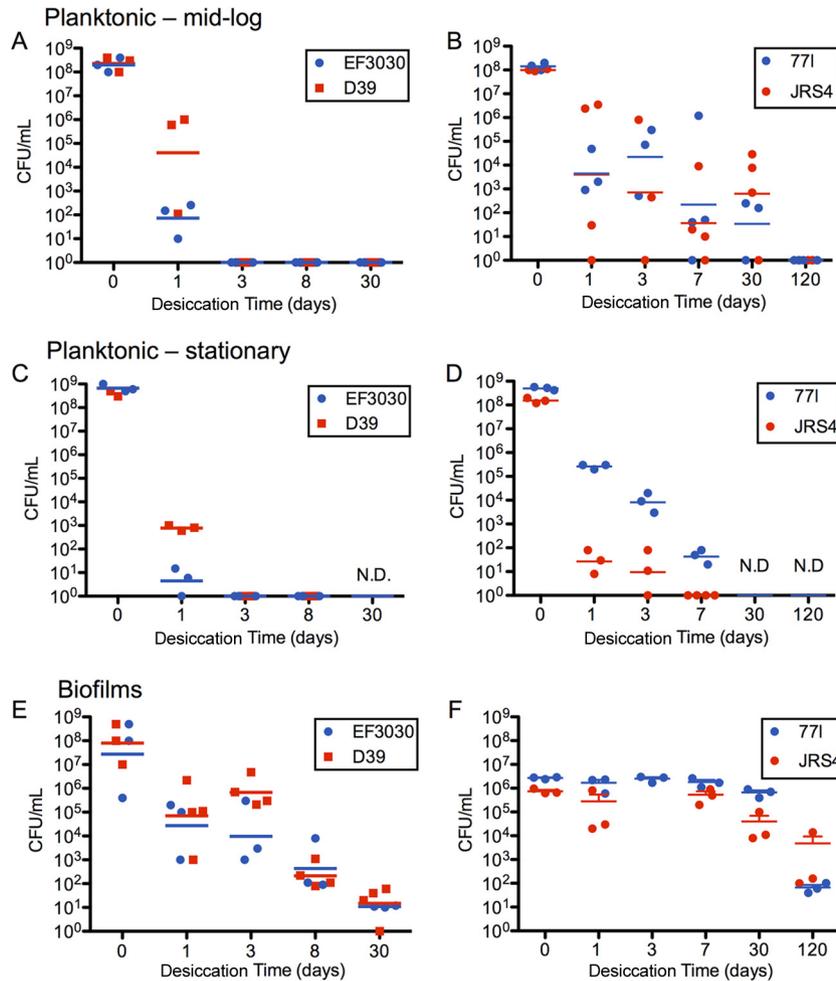


FIG 1 Survival of desiccated streptococcal fomites on plastic. Data represent the viability of desiccated pneumococci grown to mid-log phase (A) or 2 h into stationary phase (C), GAS grown to mid-log phase (B) or 2 h into stationary phase (C), or biofilm-derived pneumococcal (C) and GAS (D) fomites over 30 to 120 days postdesiccation. Biofilm bacteria showed significantly increased survival for both bacterial species from day 3 and on ($P < 0.001$ for days 3 and 8 and $P < 0.01$ for day 30 for pneumococci compared with both sets of broth-grown conditions and $P < 0.01$ for days 3, 7, and 30 and $P < 0.001$ for day 120 compared with both sets of broth-grown conditions). N.D. or N.D. in the figure indicates that results were not determined.

blood agar. Despite relatively low inocula being used, $\sim 10^4$ CFU of *S. pyogenes* and $\sim 10^2$ CFU of *S. pneumoniae*, both species were able to efficiently colonize the nasopharynx, with all mice showing stable asymptomatic colonization with no spread to the lungs or bloodstream at 2 days postinoculation. These results suggest that biofilm streptococci are desiccation tolerant and retain the ability to colonize tissues *in vivo* (Fig. 2).

Survival on hands. Whether pneumococci or *S. pyogenes* survive long periods on hands following inoculation from nasal secretions could be important in informing our understanding of transmission. *S. pneumoniae* has been shown to be frequently recovered from the hands of school-age children; however, previous work has found that planktonic cells survive on hands for an average of only 3 min (21). We tested the viability of biofilm pneumococci and biofilm *S. pyogenes* (as would be found in nasal secretions) on hands, and while planktonic pneumococcal cells rapidly lost viability, biofilm-derived cells from both *S. pneumoniae* and *S. pyogenes* could be recovered at high densities after 3 h (the end of the study time frame) (Fig. 3). Neither *S. pyogenes* nor *S. pneumoniae* was found in hand cultures either before the onset of the study or after decontamination at the end of the study.

Environmental recovery of *S. pyogenes* and *S. pneumoniae*. Having found that biofilm-derived cells of both *S. pyogenes* and *S. pneumoniae* could survive on environmental surfaces and hands, we were interested in whether these pathogens could be recovered

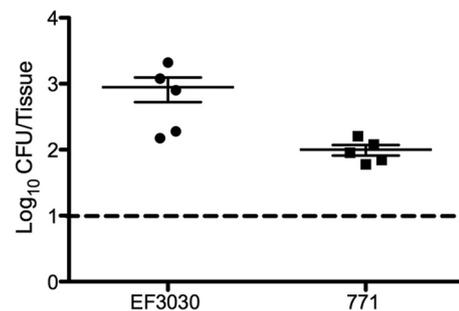


FIG 2 Colonizing capacity of streptococcal fomites. Data represent 48-h colonization of the nasopharyngeal tissue (pneumococci) and nasopharynx-associated lymphoid tissue (NALT; GAS) of 1-month-old biofilm-derived EF3030 pneumococcal fomites and GAS 771 fomites. The dashed line represents the detection limit.

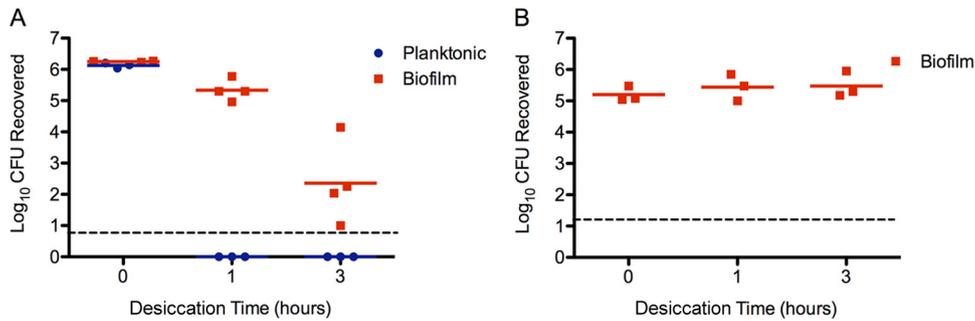


FIG 3 Survival of streptococcal populations on hands. Data represent the viability of broth-grown planktonic-derived and biofilm-derived pneumococcal (A) and GAS (B) fomites on hands over 3 h. The dashed line represents the detection limit. Survival of biofilm bacteria was significantly increased for pneumococci at both 1 and 3 h ($P < 0.01$ for both time points).

from day care centers. Immediately before the morning opening of a day care center, soft and hard toys along with books, hard surfaces, and crib linens were swabbed. The results presented in Table 1 demonstrate that *S. pneumoniae* colonies could be identified on 4/5 soft toys (stuffed animals) sampled in the facility and that *S. pyogenes* was also found on a number of surfaces within the day care center.

DISCUSSION

Several studies of the ability of pathogens to persist on various surfaces have been published (11, 21, 23), and it is now widely recognized that many common bacterial species are capable of prolonged survival outside the human host (12, 24, 25). Still, the traditional tenet of pathogenesis in the literature maintains that streptococcal infection is exclusively the result of the inhalation of respiratory droplets (1). However, to date, all literature investigating the infectivity of desiccated streptococci has used planktonic populations (12, 24, 26). In contrast, our results clearly show that while planktonic cells grown either to mid-log phase or into stationary phase rapidly lose viability when desiccated, biofilm bacteria formed under conditions similar to those found in secretions from chronic carriers remain viable and infectious in a murine colonization model. Stationary-phase bacteria were included for comparison as there is an increasing literature to suggest that bacteria can become more resistant to stress conditions and antimicrobial agents during stationary-phase growth and that a specific population, referred to as persisters, that is dormant may be associated with this phenotype (27, 28). However, for these two streptococcal species, bacteria in a stationary-growth phase did not have a survival advantage on abiotic surfaces over bacteria grown to the mid-logarithmic phase.

Instead, our findings suggest that biofilm formation by strep-

tococcal strains may contribute to its success in persisting as fomites. While it is challenging to relate *in vitro* observations to pathogenesis *in vivo*, the discovery that *S. pneumoniae* and *S. pyogenes* fomites are capable of causing colonization in a murine infection model suggests that contact with environmental surfaces may be an important vehicle for the spread of streptococci between individuals. Commonly handled objects that are microbially contaminated could serve as reservoirs of bacteria that can easily transfer to the hands through direct contact and can in turn be easily transferred to the nares and oropharynx. Children in particular may have increased contact with fluids and fomites on objects that may come into direct contact with damaged barriers or mucus membranes (29). For example, in a daycare setting one could envision the transmission of bacteria through desiccation-resistant mucosal secretions that are present as fomites on shared toys and other objects (23, 30).

While the level and extent of streptococcal fomite contamination have not been well explored in the literature, the few existing studies provide an alarming perspective. Direct bacteriologic cultures have found that between 4% and 11% of toys are often positive for streptococci (31–33), and culture-independent methods have identified a high abundance of streptococcus-related species in toddler day care rooms (34). In this study, we found that both *S. pneumoniae* and *S. pyogenes* could be cultured from soft toys found in a day care center. Consistent with these observations, epidemiologic surveys of children enrolled in child-care centers have found that pneumococci can be cultured from the hands of between 22% and 37% of children, suggesting that indirect transmission of bacteria may play an underappreciated role in the spread of infection (21, 30, 35). Investigating whether pneumococcal hand contamination represented frequent inoculation or prolonged bacterial survival, Smith-Vaughn and colleagues observed that planktonic cells were unable to survive for significant lengths of time on hands (21). Our results support these findings but add the complementary and important viewpoint that biofilm-derived cells found in nasopharyngeal secretions can survive for substantially longer periods of time.

In summary, our results suggest that although direct inhalation of aerosolized respiratory droplets is undoubtedly a major driver of bacterial transmission, it is not unlikely that in some cases fomites found on environmental surfaces may harbor viable streptococci and facilitate transmission of infection. This is supported by an elegant study from 1946 by Hodges and MacLeod, investigating a pneumococcal outbreak in a military

TABLE 1 Isolation of *S. pneumoniae* and *S. pyogenes* from items in a day care center

Item category ^a	No. of CFU per swab ^b									
	<i>Streptococcus pneumoniae</i>					<i>Streptococcus pyogenes</i>				
Soft toys	15	4	3	3	ND	22	ND	1	ND	3
Hard toys	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Books	ND	ND	ND	ND	ND	1	1	ND	ND	ND
Hard surfaces	ND	ND	ND	ND	ND	ND	ND	ND	2	>200
Cribs	ND	ND	ND	ND	2	1	2	3	ND	ND

^a A total of 5 items in each category were swabbed for potential bacterial growth.

^b ND, not detected.

academy (36). The study showed that when winter arrived, the levels of disease and the serotype distributions in two squadrons were almost identical despite an absence of personal contact between individuals in the different units. As the lack of contact between individuals in the two squadrons did not enable droplet spread, the authors explored the possibility that bacteria could survive on surfaces in the classrooms that the squadrons shared and found from culturing floor dust that 16% to 39% of cultures contained viable pneumococci. They suggested this to be a highly possible mode of transmission, which is information that has since been forgotten and overlooked. Recognizing the potential of environmental surfaces as reservoirs for streptococcal colonization underscores the importance of proper hygiene and provides a way to disrupt the spread of infection by simple measures such as hand washing and disinfection of commonly handled objects.

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