Streptococcus pneumoniae Is Desiccation Tolerant and Infectious upon Rehydration

Rebecca L. Walsh and Andrew Camilli
Graduate Program in Molecular Microbiology, Sackler School of Graduate Biomedical Science, Howard Hughes Medical Institute and Department of Molecular Biology and Microbiology, School of Medicine, Tufts University, Boston, Massachusetts, USA

ABSTRACT Streptococcus pneumoniae (pneumococcus) is a frequent colonizer of the nasopharynx and one of the leading causative agents of otitis media, pneumonia, and meningitis. The current literature asserts that S. pneumoniae is transmitted person to person via respiratory droplets; however, environmental surfaces (fomites) have been linked to the spread of other respiratory pathogens. Desiccation tolerance has been shown to be essential for long-term survival on dry surfaces. This study investigated the survival and infectivity of S. pneumoniae following desiccation under ambient conditions. We recovered viable bacteria after all desiccation periods tested, ranging from 1 h to 4 weeks. Experiments conducted under nutrient limitation indicate that desiccation is a condition separate from starvation. Desiccation of an acapsular mutant and 15 different clinical isolates shows that S. pneumoniae desiccation tolerance is independent of the polysaccharide capsule and is a species-wide phenomenon, respectively. Experiments demonstrating that nondesiccated and desiccated S. pneumoniae strains colonize the nasopharynx at comparable levels, combined with their ability to survive long-term desiccation, suggest that fomites may serve as alternate sources of pneumococcal infection.

IMPORTANCE Even with the advent of multivalent capsular polysaccharide conjugate vaccines, S. pneumoniae continues to be a major cause of morbidity and mortality worldwide. Every year, there are approximately 7 million cases of pneumococcus-based otitis media in the United States alone, while pneumococcal invasive diseases are responsible for more than 1 million deaths globally. It is believed that the human upper respiratory tract is the sole niche of S. pneumoniae and, thus, that spread occurs via close contact with an infected individual. In this study, we characterized the desiccation tolerance of S. pneumoniae and found that it can survive for many weeks postdehydration and retain infectivity. Our results suggest that desiccation tolerance is an inherent trait of this genetically variable species and that fomites may be a source of transmission.

The Gram-positive encapsulated bacterium Streptococcus pneumoniae is a common inhabitant of the human nasopharynx but can shift from commensal to pathogen, causing invasive diseases, including otitis media, pneumonia, bacteremia, and meningitis (1, 2). Based on polysaccharide capsule differences, more than 90 serotypes of S. pneumoniae have been identified (3, 4). Even with the recent introduction of capsular polysaccharide-protein conjugate vaccines directed at multiple serotypes, S. pneumoniae remains a devastating pathogen worldwide (1, 5). The current medical view is that transmission of this pathogen occurs through direct contact with respiratory secretions from infected individuals (2).

Commonplace activities such as talking, coughing, and sneezing disseminate large amounts of bacteria into the external environment (6, 7), and research on respiratory tract pathogens has implicated dust and disintegrating sputum as reservoirs of bacterial transmission (8). Fomites, or environmental surfaces, have been described as probable or confirmed sources of infections caused by Staphylococcus aureus, Chlamydia trachomatis, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, various enterococci, and other bacteria (9–15). Recent comprehensive literature searches on microbial persistence on dry surfaces identified more than 30 types of clinically relevant bacteria, including S. pneumoniae, that survive for anywhere from 30 minutes to over 30 months (8, 16).

Environmental survival often hinges on an organism’s ability to withstand periods of desiccation. “Animalcule” recovery from desiccation was first described by Antonie van Leeuwenhoek over three centuries ago (17–19). At present, the best-known and most-studied microbes that tolerate extended periods of desiccation are the cyanobacteria, extremophiles, and sporeformers (19–23). Desiccation tolerance is linked to a switch to a metabolically inactive state as well as the ability to repair protein oxidation and DNA damage upon rehydration (20, 22, 24, 25). If S. pneumoniae were shown to survive desiccation, this bacterial durability could change our understanding of its transmission. As the only known reservoir of S. pneumoniae is the human upper respiratory tract, few studies have examined its capacity to persist in the environment. Confounding issues in interpreting these prior data include the following: (i) different dissemination strategies were used be-
We developed a desiccation protocol and studied the phenomenon of S. pneumoniae survival periods of desiccation under ambient conditions, but also it retains its infectivity, as assessed by murine nasopharyngeal colonization.

RESULTS

S. pneumoniae can survive long periods of desiccation. To examine the ability of S. pneumoniae to withstand dehydration, we developed a desiccation protocol. Encapsulated S. pneumoniae strain D39 (serotype 2) was grown overnight on blood agar, scraped off the plate, evenly divided, and spread thinly onto four polystyrene petri dish lids. The bacteria from one lid were immediately resuspended in Todd-Hewitt broth supplemented with yeast extract (THY; initial time point \( T_0 \)), and the other three lids were desiccated in the dark under ambient conditions for predetermined times prior to rehydration. The number of viable cells per lid was determined by plating serial dilutions on blood agar and counting the resultant colonies, and the percent survival was calculated by dividing the time point by the \( T_0 \) viable count. Our results show that S. pneumoniae survives desiccation periods of at least 4 weeks at ambient temperature and humidity (Fig. 1).

In the desiccation experiment described above using colonies scraped from blood agar plates, the starting population was likely to be very heterogeneous with respect to growth rate and, thus, physiological state. To examine a more homogeneous starting population, we tested exponentially growing S. pneumoniae cells from broth culture for desiccation over a 7-day period. We observed the same level of bacterial viability at 4- and 7-day time points as that found when using plate-grown bacteria (data not shown), suggesting that the physiological state does not dictate an ability to survive longer periods of desiccation.

Desiccation and starvation are separable stresses. Numerous forces that contribute to bacterial cell death over time are at work during desiccation (21, 22, 27). It is possible that cell death of S. pneumoniae is due primarily to nutrient deprivation, regardless of other factors. To test this, we conducted simultaneous experiments under two conditions, desiccation and starvation with maintenance of hydration. The desiccated samples were treated as described above, while the starved samples were spread onto phosphate-buffered saline (PBS) agar plates rather than polystyrene petri dishes. All samples were placed in the dark under ambient conditions for 6, 24, or 48 h prior to collection for comparison to the \( T_0 \) viable count. We saw a significant difference in bacterial recovery between the desiccated and starved samples (Fig. 2), with the starved samples losing viability at a much higher rate than the desiccated samples. The starved samples underwent a shift from slightly higher survival after 6 h \( (P < 0.05) \) to lower survival at 24 h \( (P < 0.05) \) and 48 h \( (P < 0.001) \), confirming that nutrient deprivation is a different phenomenon than dehydration.

S. pneumoniae desiccation tolerance is not dependent on the polysaccharide capsule. As research has indicated that Gram-positive bacteria survive desiccation better than their Gram-negative counterparts (28–30), we wondered if the polysaccharide capsule of S. pneumoniae contributes to its ability to withstand desiccation. A capsule is present in essentially all S. pneumoniae clinical isolates and is required for efficient host colonization as well as invasive disease (4, 31, 32). To assess the role of the capsule in desiccation tolerance, we compared the survival of D39 to that of an acapsular derivative, AC326. We saw no significant difference in bacterial viability at any time point at up to 1 week of desiccation (Fig. 3), leading us to conclude that the capsule is not an important factor in surviving desiccation. An anti-type 2 capsule Western blot demonstrated the presence of the capsule in cells desiccated for 1, 2, or 7 days, confirming that the lack of phenotypic difference between D39 and the acapsular strain is not due to
D39 downregulating production of the capsule, and thus appearing acapsular, during desiccation (data not shown).

Desiccation tolerance is a property shared by diverse pneumococcal strains. *S. pneumoniae* has a highly plastic genome, with up to 10% of variation between strains (2, 33–35). Since we eliminated the capsule as the basis for desiccation tolerance, it is possible that some genetic specificity of the D39 strain enhances its ability to survive desiccation. To determine if desiccation tolerance is shared or not by other strains, we performed 48-h desiccation experiments on 17 strains representing 14 different serotypes. All 17 strains tested survived desiccation at viabilities ranging from 0.1 to 10% (Fig. 4). For three of the serotypes (6A, 6B, and 18C), we tested both opaque- and transparent-colony-phase variants and saw no correlation between phase and desiccation tolerance. As capsular polysaccharide expression differs greatly between these two phase variants and is related to virulence in a mouse model (36), this supports our previous conclusion that the capsule is not a key factor in *S. pneumoniae* desiccation tolerance.

Additionally, our experiments indicate that the ability to withstand the stresses of desiccation is a trait shared by numerous strains of *S. pneumoniae*. Desiccated *S. pneumoniae* retains infectivity. To extrapolate the importance of fomites as a source of *S. pneumoniae* transmission, it first must be established that, upon rehydration, desiccated bacteria are capable of colonization. Because *S. pneumoniae* is naturally virulent in mice and can colonize the nasopharynx asymptptomatically, as in humans, we used this murine model to test whether desiccation affects colonization. We intranasally inoculated 8- to 12-week-old female Swiss-Webster mice with 5 µl/nare of PBS (mock) or PBS-suspended nondesiccated *S. pneumoniae* D39 (grown overnight on blood agar plates) or desiccated *S. pneumoniae* D39 (for 48 h). Three days postinoculation, the mice were killed, and the nasal lavage fluid was plated on blood agar containing 3 µg/ml gentamicin, to which *S. pneumoniae* is naturally resistant. The mock group had no detectable *S. pneumoniae* colonies, but the bacteria desiccated for 48 h colonized the murine nasopharynx well (Fig. 5). In the nondesiccated wild-type group, 80% of mice (4/5) had detectable levels of colonization, while 75% of mice (6/8) in the desiccated group were measurably colonized. The relatively low dose of inoculum used (~1.5 × 10^4 CFU/mouse) likely increased the spread seen in the data, as the mice had a better chance of clearing the bacteria than if the dose used was higher. Although the median load of bacteria recovered was higher for the nondesiccated challenge group, there was no significant difference in colonization levels between it and the desiccated group (P = 0.27), indicating that desiccation does not exert a major negative impact on the ability of *S. pneumoniae* to colonize hosts.

**DISCUSSION**

To our knowledge, this is the first study to describe both the desiccation tolerance of *S. pneumoniae* under ambient conditions and the ability of those desiccated bacteria to be infectious upon rehydration. Using a polystyrene surface as a fomite model, we demonstrate the environmental survival of *S. pneumoniae* over a period of 4 weeks. Although there was a decline in bacterial survival...
over time, the median total concentration of viable cells even after 14 days of desiccation fell within a previously suggested 50% infective dose (ID_{50}) range for invasive pneumococcal disease (IPD) for this pathogen (37, 38). Since more bacteria are generally needed for invasive disease than for intranasal inoculation, this number may be sufficient for nasopharyngeal colonization, the first step toward infection (39). However, numerous variables will likely impact the frequency of transmission via fomites in natural settings.

It is important to make a distinction between the process of desiccation tolerance and its intrinsic nutrient limitation. It could be argued that the death of \textit{S. pneumoniae} over time on fomites is simply a result of starvation and not due to stresses associated with desiccation and rehydration. However, two points argue against this hypothesis. First, we saw significantly less longer-term recovery of starved but hydrated cells versus that of desiccated cells, which indicates that nutrient deprivation and desiccation are distinct stresses. Our data are further supported by results from a previous study, in which \textit{S. pneumoniae} mixed with dust, pus, and blood was viable approximately twice as long under dry versus moist conditions (26). Second, despite the absence of nutrients on fomites, we were able to recover viable cells after long time periods (up to 28 days) of desiccation. This suggests that the bacteria enter into some kind of stasis, enabling them to tolerate the stresses of desiccation. Desiccation may trigger an evolved survival pathway of \textit{S. pneumoniae}, or survival may simply be attributed to a fortuitously protective cellular architecture. An investigation of the bacterial factors that mediate desiccation tolerance should be able to distinguish between these possibilities.

Numerous species of Gram-negative and Gram-positive bacteria have been shown to persist under desiccating conditions, although the literature suggests that Gram-positive bacteria exhibit enhanced tolerance to dry conditions (29, 30). One factor shared by many Gram-negative and Gram-positive bacteria is the presence of an extracellular polysaccharide. In \textit{S. pneumoniae}, the polysaccharide capsule is a virulence factor, helping cells penetrate the mucus layer overlaying mucosal epithelia and escape phagocytosis (32, 36, 39). Using an acapsular mutant strain, we demonstrated that the capsule is not required for \textit{S. pneumoniae} D39 desiccation tolerance. This corresponds with the results from our desiccation experiment on serotypes 6A, 6B, and 18C, in which we observed no clear trend associated with capsular phase variance. The variants of serotype 6A demonstrated near-identical recovery, while the other two serotypes displayed opposite recovery patterns between opaque and transparent variants.

Similar variation in desiccation tolerance was seen across other \textit{S. pneumoniae} serotypes, including those seven previously identified as the most common in cases of IPD globally (40). We observed recovery across all 17 tested strains, accounting for 14 unique serotypes. Thus, a resistance to the stresses of desiccation appears to be a phenomenon intrinsic to the \textit{S. pneumoniae} species. This is especially interesting, considering the wide genetic diversity among \textit{S. pneumoniae} strains. Its natural transformable ability, combined with its high rate of recombination, enables \textit{S. pneumoniae} to adapt readily to both antibiotic and vaccine selective pressures (1, 5, 41). Several years after the introduction and widespread administration of the heptavalent polysaccharide capsule vaccine (PCV7), IPD caused by the seven vaccine serotypes markedly decreased (42, 43). Simultaneous with this decrease was the emergence of multidrug-resistant nonvaccine serotypes, especially multidrug-resistant 19A, as leading causes of IPD (5, 41, 44, 45). Our data support the hypothesis that desiccation tolerance is a species-wide phenomenon; therefore, this ability is vertically transmitted in \textit{S. pneumoniae} and likely would not be gained or lost as a result of its frequent horizontal transfer events.

Fomites have been linked to the transmission of other Gram-positive, nontuberculous pathogens. Methicillin-resistant \textit{S. aureus} was shown to survive 1 to 90 days on common hospital materials (12) and has been proposed to cause infections via direct fomite-person transmission (26). Twenty weeks after desiccation of blood containing group A streptococci, growth in fresh blood indicated that the bacteria retained both viability and virulence (46). The hypothesis that \textit{S. pneumoniae} may survive in the environment and use fomites as sources of transmission is not unprecedented. In fact, Walther and Ewald’s “sit and wait” hypothesis, which predicts that virulence correlates with durability in the external environment, identified a high-virulence, high-survival group of human respiratory tract pathogens that includes variola (smallpox) virus, \textit{Bordetella pertussis}, \textit{Mycobacterium tuberculosis}, \textit{Corynebacterium diphtheriae}, and \textit{S. pneumoniae} (8). Here we tested this hypothesis by investigating the capacity of desiccated \textit{S. pneumoniae} to colonize the murine nasopharynx. Colonization at levels not significantly different from those of nondesiccated \textit{S. pneumoniae} argues in favor of the hypothesis that fomites serve as an alternate source of spread of \textit{S. pneumoniae}.

It has been noted that increased postdesiccation recovery rates are seen when bacterial cells are more concentrated at the time of dissemination (16, 22, 47, 48), and so it is conceivable that the bacterial viability in our experiments is artificially high based on the density of bacteria spread on the polystyrene surface (2.2 × 10^8 CFU over an area of ~600 mm²). In experiments testing the number of beta-hemolytic streptococci expelled by respiratory activities, Hamburger and Green showed that nose blowing, more than coughing or sneezing, forces out the most bacteria (6). In patients with beta-hemolytic streptococci-positive nose cultures, nose blowing resulted in an average expulsion of 1.1 × 10^7 CFU and a maximum of over 1 × 10^8 CFU (6); therefore, it is possible that an \textit{S. pneumoniae} (alpha-hemolytic streptococcus) carrier could expel concentrations approximating those used in our experiments. Additionally, they recovered an average of 7.9 × 10^4 CFU from the hands of nasal carriers 3 h after they were last washed and found that these individuals transferred beta-hemolytic streptococci to other surfaces they touched (6). These surfaces, as well as dried handkerchiefs used during nose blowing, served as major sources of airborne beta-hemolytic streptococci (6). We propose, then, that fomites may serve as reservoirs for \textit{S. pneumoniae}, which we show can survive for weeks in a desiccated state and after which are still capable of colonizing the nasopharynx of susceptible hosts.

**MATERIALS AND METHODS**

**Bacterial strains.** The \textit{S. pneumoniae} strains used in this study are listed in Table 1. Mid-exponential-growth-phase \textit{S. pneumoniae} bacteria were stored as “starter cultures” in microcentrifuge tubes at −80°C in THY plus 12% glycerol in 0.25- to 0.4-ml aliquots.

**Desiccation.** An \textit{S. pneumoniae} starter culture containing ~10^7 CFU was thawed, the entire contents were plated on Trypticase soy agar plus 5% sheep’s blood (Northeast Laboratory) (blood agar), and after 16 h of growth at 37°C in a 5% CO₂ incubator, the bacterial lawn was scraped off the plate surface and pooled in the center of the plate using a plastic straightedge (catalog no. 165-3320, Bio-Rad Gel Releaser). The bacteria
TABLE 1 Strains used in this study

| Strain       | Genotype or description | Serotype (type of variant) | Reference or source |
|--------------|-------------------------|---------------------------|---------------------|
| AC326        | Acapsular D39           |                           |                     |
| AC353        | TIGR4, Sm derivative    |                           |                     |
| AC1356       | Clinical isolate 1      | 1                         | J. Weiser           |
| AC1357       | Clinical isolate 6A (opaque) | 4A                      | J. Weiser           |
| AC1358       | Clinical isolate 6A (translucent) | 6A                      | J. Weiser           |
| AC1359       | Clinical isolate 6B (opaque) | 6B                      | J. Weiser           |
| AC1360       | Clinical isolate 6B (translucent) | 6B                      | J. Weiser           |
| AC1361       | Clinical isolate 5      | 5                         | J. Weiser           |
| AC1362       | Clinical isolate 23F    | F                         | J. Weiser           |
| AC1363       | Clinical isolate 8      | 8                         | J. Weiser           |
| AC1364       | Clinical isolate 14     | 14                        | J. Weiser           |
| AC1365       | Clinical isolate 9V     | V                         | J. Weiser           |
| AC1366       | Clinical isolate 18C (opaque) | 18C                      | J. Weiser           |
| AC1367       | Clinical isolate 18C (translucent) | 18C                      | J. Weiser           |
| AC1368       | Clinical isolate 3      | 3                         | J. Weiser           |
| AC1369       | Clinical isolate 19F    | F                         | J. Weiser           |
| AC1371       | Clinical isolate 15     | 15                        | J. Weiser           |
| AC1770       | D39                     | 2                         | 51                  |

were then split evenly by eye into four pools, and each of which was spread thinly and evenly using the plastic straightedge onto the inner side of a 100-mm by 15-mm polystyrene petri dish lid (Fisher). There was approximately 10⁶ CFU spread on each lid. Immediately after spreading, the bacteria on one lid were resuspended with 1.5 ml THY, serially diluted in THY, and plated to determine the number of CFU, constituting the initial time point (T₀). The remaining three lids were closed over the petri dish bottoms and placed in the dark at room temperature for specific times (1 h to 28 days) until being similarly resuspended and serially diluted to determine the number of CFU. Desiccations were conducted in the dark based on previous research implicating phototoxic damage as a cause of enzyme, protein, and DNA damage during desiccations under light conditions (22). The percent remaining viable bacteria was calculated by dividing the time point number of CFU by the T₀ number of CFU. Ambient temperature and relative humidity were monitored throughout the duration of each experiment. The range for all experiments was 21 to 25°C and 18 to 45% relative humidity. Neither the temperature nor humidity variation observed impacted the degree of desiccation tolerance.

Mid-exponential-growth-phase desiccation. An S. pneumoniae starter culture containing ~10⁶ CFU was thawed and used to seed a 10-ml starter culture of THY plus Oxylase (Oxylase, Inc.). After 4 h of growth to mid-exponential phase (optical density at 600 nm [OD₆₀₀] = 0.4) at 37°C in a 5% CO₂ incubator, the culture was split evenly into 20 aliquots (480 μl each) that were spun for 7 minutes at 1500 × g at room temperature. The supernatants were removed except for 50 μl, and each pellet was resuspended and transferred to the inner side of a 100-mm by 15-mm polystyrene petri dish lid, onto which it was spread thinly and evenly using the plastic straightedge. Approximately 2 × 10⁶ CFU were spread on each lid. Immediately after the pellet was spread, the petri dishes were dried open in the biosafety cabinet with airflow on for 30 minutes. Next, the bacteria on one lid were resuspended with 1.5 ml THY, serially diluted in THY, and plated to determine the number of CFU while the remaining three lids were closed over the petri dish bottoms and desiccated in the dark under ambient conditions for 1, 2, or 7 days, at which time they were similarly resuspended, serially diluted, plated for the number of CFU, and spun to freeze the cell pellets.

The cell pellets were thawed and resuspended in 20 μl 10 mM Tris (pH 7.5), 10 μl protease K buffer (50 mM EDTA, 0.5% Tween 20, 0.5% Triton X-100, 50 mM Tris [pH 8]), and 2 μl protease K (20 mg/ml). After 30 minutes of incubation at 37°C, each sample was mixed with 38 μl 10 mM Tris (pH 7.5) and 17.5 μl sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris [pH 6.8], 12.5 mM EDTA, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue) and heated for 10 minutes at 99°C. The samples were loaded (20 μl each) onto a 10% SDS-PAGE gel, which was run for 75 minutes at 120 V before being transferred onto a nitrocellulose membrane (Invitrogen) using a semidry transfer apparatus (Bio-Rad) for 70 minutes at 25 V. Ponceau staining was used for 30 s to confirm effective protease K treatment before being washed off with distilled water (dH₂O). Using the Snap i.d. protein detection system (Millipore), the membrane was blocked with 30 ml 1× NapBlock (G Biosciences) in 1× Tris-buffered saline (TBS). The membrane was incubated for 10 minutes with polyclonal rabbit anti-type 2 capsule antiserum (Statens Serum Institut) at 1:1,000 in TBS. After being washed with 90 ml 1× TBS, the membrane was incubated for 10 minutes with Cy5 goat anti-rabbit IgG (Invitrogen) at 1:7,1,000 in 1× TBS while protected from light and then washed with 90 ml 1× TBS and visualized using FLA-9000 Starion (Fujifilm).

Mouse model of nasopharyngeal colonization. In all animal experiments, 8- to 12-week-old female outbred Swiss-Webster mice (Charles River Laboratories or Taconic Laboratories) were used. Mice were mock infected with 1× PBS or infected with either nondonedicated or 48-h desiccated S. pneumoniae D39. The nondonedicated S. pneumoniae was prepared by plating one starter culture on blood agar. After 16 h of growth, the colonies were resuspended in 1× PBS and adjusted to ~1 × 10⁶ CFU/ml. The desiccated S. pneumoniae was prepared as described in a desiccation experiment, except at 48 h postdesiccation, the bacteria were resuspended in 1× PBS and adjusted to ~1 × 10⁶ CFU/ml by OD₆₀₀ measurement.

Mice were lightly anesthetized by inhalation of 2.5% isoflurane and inhaled (5 μl per nare) with 1× PBS, nondonedicated S. pneumoniae D39, or desiccated S. pneumoniae D39. Mice were killed by CO₂ asphyxiation 3 days later. Bacteria colonizing the nasopharynx were recovered by lavage with 0.5 ml sterile 1× PBS through an opening made in the trachea. The lavage fluid was serially diluted and plated on blood agar containing gentamicin (3 μg/ml).

Statistical analysis. Differences in survival between starved and desiccated samples were tested using two-way analysis of variance (ANOVA) with Bonferroni posttest correction. Survival data of 48-h desiccated acapsular (AC326) and encapsulated (AC1770) strain D39 were shown to be normally distributed by the D’Agostino-Pearson omnibus normality test, and differences were then tested using the unpaired Student t test. Differences in colonization of mice were tested using the Mann-Whitney two-tailed U test.

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