**Streptococcus pneumoniae** Forms Surface-Attached Communities in the Middle Ear of Experimentally Infected Chinchillas

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*(See the editorial commentary by Apicella, on pages 774–5.)*

**Background.** *Streptococcus pneumoniae* (pneumococcus) causes respiratory and systemic infections that are a major public health problem worldwide. It has been postulated that pneumococci persist in vivo in biofilm communities.

**Methods.** In this study, we analyzed whether pneumococci form biofilms in vivo, and if so, whether biofilms correlated with bacterial persistence. Chinchillas were infected with *S. pneumoniae* TIGR4 and euthanized at varying times after infection, after which the superior ear bullae were excised and examined by culture and microscopy.

**Results.** Dense material, resembling the biofilms of other otitis media pathogens, was visible in the middle ear as late as 12 days after infection. Scanning electron microscopy revealed bacteria within an electron-dense matrix, similar to pneumococcal biofilms formed in vitro. Viability staining revealed groups of viable diplococci, as well as viable and nonviable host cells, attached to a fibrous matrix that was positive when stained with propidium iodide. Cryosections of biofilms were treated with polyclonal antibodies against the pneumococcal surface components pneumococcal surface protein A family 2, pneumococcal surface protein C, choline-binding protein, and neuraminidase, coupled with appropriate secondary antibody conjugates. Immunofluorescent staining showed the presence of pneumococcal communities within the material recovered from the middle ear chamber.

**Conclusions.** On the basis of these data, we conclude that pneumococci form biofilms in vivo and that this process may be intertwined with the formation of neutrophil extracellular traps. These findings provide new insights into the potential causes of antibiotic treatment failure and bacterial persistence in chronic pneumococcal otitis media.

Otitis media (OM) is one of the most common pediatric infections, with most children experiencing ≥1 episode by age 3 [1, 2]. OM has a tremendous socioeconomic impact. It is a leading cause of pediatric office visits, antibiotic prescriptions, and surgical intervention, as well as sequelae that include speech and language problems [3]. The clinical definition of OM includes acute OM (AOM) and chronic OM with effusion (OME), which may present separately or can represent different stages of the same disease [1, 4]. AOM is characterized by middle ear effusion and inflammation (fever, otorrhea, and/or otalgia), while OME is defined by the presence of effusion without signs or symptoms of overt infection [5, 6]. If a patient develops 3 episodes of AOM in 6 months or 4 episodes in 12 months, the disease is referred to as recurrent AOM [7]. Persistent AOM is characterized by the continuation of middle ear infection symptoms during the receipt of antibiotic therapy or the recurrence of AOM within 1 month after the receipt of antibiotic therapy [7]. It has been estimated that 20%-30% of children have recurrent AOM, and there are indications that the incidence of recurrent AOM is in-
Bacterial persistence in biofilm communities represents one hypothesis about how pathogens cause recurrent or persistent OM in the face of host immune responses and/or antibiotic therapy [10].

A biofilm is a sessile bacterial community attached to a substratum or interface, which is encased in an extracellular matrix material [11]. Compared with planktonic cultures, biofilms display altered phenotypes with respect to growth rate and gene transcription, and the bacteria in these biofilm communities are inherently resistant to host defenses and antibiotic therapies [12]. It is estimated that >60% of all bacterial infections may involve biofilms; these include a majority of chronic infections that involve long-term microbial persistence [11, 12]. Direct microscopic evidence for the presence of biofilms in OM has recently been obtained by analysis of middle ear tissue from patients with OM who underwent surgical installation of drain tubes, and it included the observation of surface-attached communities of many of the bacterial species commonly associated with OM [13].

*Streptococcus pneumoniae* (pneumococcus) is one of the organisms most frequently isolated from patients with recurrent and persistent AOM [4]. In addition to OM, *S. pneumoniae* is a leading cause of community-acquired pneumonia and meningitis, especially in children and the elderly. It is especially noteworthy that while the introduction of the heptavalent pneumococcal capsular conjugate vaccine has clearly reduced in the incidence of pneumococcal OM [14], there are also indications of an increased incidence of OM caused by pneumococcal serotypes not included in the vaccine [15]. Thus, understanding how pneumococci cause persistent and/or recurrent OM remains an important topic for study.

Pneumococci can form biofilm communities, and there has been much recent progress in defining the bacterial components or variants involved in the formation of pneumococcal biofilms in vitro [16–21]. In this study, we used the chinchilla model for OM to investigate whether pneumococci formed biofilms in vivo. All animals exhibited symptoms of OM within 48 h after infection, and viable bacteria were cultured from samples from the middle ear chambers up to 12 days after inoculation. In many of the infected animals, a dense, surface-attached material was observed in the middle ear chambers after euthanasia. Electron microscopy revealed coccolid forms enclosed in an electron-dense matrix. These communities were in many ways comparable with pneumococcal biofilms formed in vitro, although there were also notable differences, including the presence of far more matrix surrounding the in vivo communities. By using other microscopic methodology, the communities were shown to contain extracellular fibrous DNA and host cells. Viable bacteria were present, as demonstrated by viability staining, and aggregates of cocci that were reactive with antisera specific for pneumococcal surface factors were observed by use of immunofluorescent microscopy. These data are consistent with the interpretation that pneumococci form biofilm communities during experimentally induced OM and further indicate that the formation of these communities involves both bacterial and host factors.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *S. pneumoniae* TIGR4 is a serotype 4 clinical isolate strain for which a complete genomic sequence is available [22]. Bacteria were grown on brain-heart infusion agar (Difco) with 5% sheep blood, brain-heart infusion broth with 10% added horse serum, or Todd-Hewitt yeast extract (THY) broth at 37°C and 5% CO₂, as indicated.

**Continuous-flow biofilm assays.** Stovall glass flow cells were inoculated with 10 mL of exponentially growing *S. pneumoniae* culture in THY medium and incubated without flow for 3 h at 37°C to allow for attachment of the bacteria. Media flow was then initiated (5 mL/min) with a peristaltic pump and maintained for 24 h.

**Chinchilla infections.** Healthy adult chinchillas (*Chinchilla lanigera*) were purchased from Rauscher’s Chinchilla Ranch and allowed to acclimate to the vivarium for 1 week prior to infection. All animals were examined by otoscopy prior to infection, and none had any visible signs of middle ear infection or other overt disease. *S. pneumoniae* TIGR4 was diluted with pyrogen-free PBS, and the bacterial density was confirmed by plate counts. Chinchillas (3 animals per group for each experimental time period) were anesthetized with isoflurane and inoculated via transbular injection with 0.1 mL of bacterial suspension containing either ~40 cfu, 4 cfu, or 1 cfu. All animals were monitored by otoscopic examination for clinical signs of OM. Groups of animals were euthanized at 1, 3, 7, or 12 days after infection, and the superior bullae were opened to expose the middle ear cavity, as described elsewhere [23]. The presence of a surface-attached material consistent in appearance with biofilms formed by nontypeable *H. influenzae* [23, 24] was assessed by visual inspection and photographed. Where present, this material was removed and fixed for 15 min in 2% paraformaldehyde-PBS and processed for microscopic analyses. Middle ear effusion fluids were collected and serially diluted, and bacterial load was assessed by plate count. If no effusion was present, the middle ear cavity was washed with 0.5 mL of sterile PBS and serial dilutions were plated. Plate counts were used to determine the number of colony-forming units per ear. All infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

**Antibodies.** The generation of rabbit antisera used against pneumococcal surface protein C (PspC) [25] and pneumococcal surface protein A family 2 (PspA-FAM2) [26] have been described elsewhere. In a similar manner, choline-binding protein (PcpA) was overexpressed, purified, and used to vaccinate rab-
bits, as described elsewhere [27]. Antisera reactive with neuraminidase (NanA) were generated by immunization of New Zealand white rabbits with peptide FLNanA571, which corresponds to aa 230–746 of the NanA protein, a region containing the catalytic domain of the NanA enzyme. The rabbits were immunized with 1/100 g FLNanA571 in complete Freund’s adjuvant, followed by 2 monthly boosting doses of 100 μg FLNanA571 in complete Freund’s adjuvant and, 1 month later, with a final boosting dose of 100 μg FLNanA571. The vaccination protocols were approved by the University of Alabama at Birmingham Animal Care and Use Committee.

Microscopy. The excised biofilms and middle ear tissue were fixed, and portions were processed for analysis by scanning electron microscopy and immunofluorescent microscopy. For scanning electron microscopy, the samples were fixed for 60 min with 2.5% glutaraldehyde–PBS and then rinsed twice (10 min per wash) in PBS prior to dehydration in a graded ethanol series. The samples were then subjected to critical point drying, mounted onto stubs, and sputter coated with palladium prior to viewing with a Phillips SEM-515 scanning electron microscope. For immunofluorescent microscopy, samples were embedded in OCT resin (Sakura Finetek), cut into sections (5 μm) using a cryotome, and placed on adhesive slides. The sections were stored at -20°C. For immunofluorescent staining, the slides were brought to room temperature, and then fixed briefly with 2% paraformaldehyde–PBS prior to antibody staining, as indicated above. Individual sections were treated with polyclonal antibodies against the pneumococcal surface components PspC, PspAFAM2, PcpA, and NanA, followed with goat anti–rabbit Texas Red antibody conjugate (Jackson Laboratories). Analysis was performed by using a Zeiss LSM 510 confocal laser scanning microscope. Cryosections were also stained with hematoxylin and eosin, in accordance with standard protocols that we have described elsewhere [28].

Live-dead staining. Bacterial viability in the communities found within the middle ear was assessed by use of BacLight Live/Dead staining (Molecular Probes). Portions of unfixed bacterial communities were cut into small pieces (2–3 mm) and incubated with 0.5 mL of PBS that contained a mixture of SYTO 9 and propidium iodide for 15 min, and then washed 3 times with PBS buffer. Samples were visualized by using a Zeiss LSM510 confocal scanning laser microscope.

RESULTS

Surface-attached pneumococci communities in vivo. The primary hypotheses addressed in our infection studies were that pneumococci form biofilms during experimentally induced OM and that these biofilms promote bacterial persistence in the middle ear. After euthanasia, the superior bullae were excised, revealing macroscopically visible material (figure 1), which was similar in appearance to the biofilms formed by nontypeable Haemophilus influenzae that we have observed previously [23, 24]. The frequency of infection, presence of “biofilm” material, presence of effusion, and clearance varied for each of the infectious doses examined (table 1), but visible material and effusion were evident as early as 3 days after infection for each dose tested. Moreover, S. pneumoniae was cultured from middle ear fluid or saline lavage fluid obtained from all 3 animals in the 12-day group. Bacterial counts recovered from effusion or saline lavage fluid ranged from 101–107 per ear with 8 × 104 cfu recovered from the lone animal found to be infected 7 days after challenge. The lower frequency of infection found at 7 days after challenge, compared with 12 days after challenge, is potentially interesting.
It is not clear whether animals in the 7-day group had inapparent infections that were below the level of detection, the disease persisted in the 12-day group, or the disease reemerged in the 12-day group. These possibilities will be discussed further below.

**Comparison of pneumococcal communities in vitro and in vivo by electron microscopy.** As a first step in determining whether the material observed in the infected animals was, in fact, a biofilm, we used scanning electron microscopy to analyze the matter excised from the middle ear chamber. As a basis for comparison, we also conducted microscopic analysis of a 24-h in vitro *S. pneumoniae* biofilm generated under of continuous flow. In both cases, clear 3-dimensional structures extending >10 μm above the surface were observed (figure 2). Pneumococcal cells, approximately 0.5 –1.0 μm in diameter, predominate the in vi-

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**NOTE.** Data are the no. of ears for which a given result was obtained/total no. of ears in the group. ND, not done.

* a Recovery of *S. pneumoniae* colony-forming units from effusion or saline lavage fluid. Counts ranged from 10¹–10⁷ per ear.

b Presence or absence of a macroscopically visible, surface-attached bacterial community in the middle ear chamber.

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**Figure 2.** Scanning electron microscopy of surface-attached pneumococcal communities in vitro and in vivo. *A*, *Streptococcus pneumoniae* TIGR4 was cultured under continuous flow conditions and the biofilm was allowed to mature for 24 h. White arrows indicate pneumococcal cells of various sizes. *B*, Micrograph of the macroscopic structure recovered from the chinchilla middle ear chamber. White arrows indicate coccoid shapes consistent in size with pneumococci. Note the seemingly complex matrix and other cellular structures present. *C* and *D*, Hematoxylin-eosin (HE)–stained cryosections of pneumococcal biofilm material recovered from the chinchilla middle ear chamber 3 days (*C*) or 7 days (*D*) after infection. The apical surfaces of the biofilms are labeled to provide orientation.
tro biofilm, although a fibrous material was also observed in some fields of view (figure 2A). In contrast, the material observed in vivo was visibly denser and there was an abundance of opaque matrix material across the exposed surface (figure 2B). Coccoid forms that—based on size and morphology—were consistent with pneumococcal cells were visible on the surface. Other structures that were consistent in size with host cells appeared to be present on the biofilm surface. To better establish the composition of the biofilm structure, portions of the biofilms were sectioned, stained with hematoxylin and eosin, and examined by light microscopy. Sections of biofilms obtained at 3 days and 7 days after infection contained a high proportion of neutrophils and other host cells both on the biofilm surface and embedded throughout the structure (figure 2C–2D). The distribution of neutrophils in the biofilm changed over time; at 3 days after infection, they were mostly found on the apical surface (figure 2C), whereas neutrophils were present throughout the entire biofilm structure at 7 days after infection (figure 2D).

**Fibrous structures near the bullar surface revealed by electron microscopy.** To see how the bacterial community might differ at the surface interface with the host, whole bullae were removed and examined by scanning electron microscopy. Electron microscopy of the bullae from a mock-infected animal revealed an undisturbed epithelial surface (figure 3A). In bullar samples harvested 3 days after infection, we observed web- or net-like structures reminiscent of neutrophil extracellular traps (NET) (figures 3B–3D) [29, 30]. Coccoid forms that were consistent in size with pneumococci were observed, as were 3-dimensional formations of matrix material. Larger structures that were consistent in size with host phagocytes or other cells were also visible. Staining with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) showed a fibrous matrix that could be stained with propidium iodide, consis-
Living cells were visualized at 3 days (figures 4A–4C) and 7 days (figures 4D–4F) after infection. Vertical stacked Z-slice images (figures 4C and 4F, margins) of cryosections indicated that living cells were present throughout the thickness of the matrix. Zeiss LSM Image Browser software 3-dimensional distance analysis predicted the thickness of this structure to be ≥250 μm (figure 4G). Taken together, the data indicated that the relative distribution of living cells (i.e., SYTO 9–stained cells) was approximately equal to that of structures stained with propidium iodide.

**Immunohistochemical staining of pneumococci in bacterial communities.** To further confirm the presence and distribution of *S. pneumoniae* in the surface-attached bacterial community, material excised from the middle ear 12 days after infection was stained with antipneumococcal antibodies and fluorescent antibody conjugate. Polyclonal antibodies against PspC, PcpA, NanA, and PspA-FAM2, coupled with appropriate secondary antibody conjugates, were used for immunofluorescent staining. Low- and high-magnification images are provided (figure 5). Each of the labeled proteins appeared to be randomly distributed across the surface of the sample. These data clearly demonstrate the presence of pneumococcal communities in the material recovered from the middle ear chamber, which have on their surface all 3 choline-bound proteins and neuraminidase.

**DISCUSSION**

Pneumococci have long been thought to form biofilm communities during chronic infections. One of the more useful animal models for studying in vivo biofilms, especially as they pertain to OM, has been the chinchilla [11, 16, 23, 24, 26–28, 35–37]. Much of this work has focused on nontypeable *H. influenzae* and has included study of biofilm architecture, the role of specific genes in biofilm formation, the makeup of the biofilm matrix, and the effect of biofilms on virulence and inflammation [23, 24, 31–33]. While pneumococcal biofilms have not been directly studied in this model, there is a considerable body of work showing that pneumococci can establish OM in the chinchilla [34–43]. More recent work also shows that pneumococcal strains differ in localized and systemic disease in the chinchilla [44], which is consistent with prior reports showing disparate gene content among pneumococcal strains [45]. Notably, in the former study [44], pneumococcal strain TIGR4 had a moderate to low propensity to cause systemic disease, which is consistent with our data. These data provided validation for our initial hypothesis that pneumococci establish biofilm communities in the chinchilla middle ear. In several cases, the existing data based on biofilm formation in vitro are consistent with the findings in this study. Two of the surface proteins (PcpA and PspA) that were stained in the pneumococcal communities (figure 5) have been previously linked to biofilm formation in vitro [21]. Each of these (and the related PspC, which was also stained in the in vivo communities) are choline-bound pneumococcal surface proteins. The growth of pneumococci in media containing excess...
choline, which would result in the release of these proteins, also significantly reduced biofilm formation in static assays in vitro [21]. NanA is also present in the biofilm communities, which is consistent with microarray data obtained from biofilms in vitro [46]. There have also been indications that neuraminidase expression promotes biofilm formation for other bacterial species [47]. It should be pointed out that the sequence of the TIGR4 nanA gene lacks the predicted sortase attachment site, and thus the enzyme would be predicted to be released, rather than attached to the bacterial surface [22].

We have intentionally been quite selective in the use of the term “biofilm” in this study. In our view, a biofilm community is defined by the presence of a matrix material and by resistance to host clearance. There were clearly viable pneumococcal communities present in the surface-attached material excised from the middle ear chamber following infection. The structures observed in this study have many features in common with NET [48]. The DNA fibers in NET provide a scaffold for the entrapment of microbes, wherein they may be killed either by antimicrobial factors or by incoming neutrophils. Pneumococci have been shown to survive in NET formed in vitro by means of capsule production, alanylation of lipoteichoic acids in the cell wall, and by endonuclease production that may degrade the DNA matrix material [29, 30]. The data from our study are consistent with a key role for neutrophils and other host components in promoting biofilm formation in the middle ear chamber and are consistent with prior work showing that neutrophils and neutrophil components promote biofilm formation by Pseudomonas aeruginosa [49]. In this context, it is conceivable that the biofilm matrix material includes host-derived factors, such as NET material, in addition to polysaccharides or other material of bacterial origin. The key question is whether the structure of the biofilm affects host clearance and antimicrobial efficacy. Despite the presence of many neutrophils in the biofilm structure (especially at 7 days after infection), the data showed a lack of complete clearance in all of the infected animals.

Even a small number of bacteria that remained viable in the biofilm structure would be sufficient to cause the recurrent and/or persistent cases of AOM observed in the pediatric population. In our results, we only identified 1 macroscopically visible structure at 7 days after infection, yet macroscopically visible S. pneumoniae communities were observed in both ears of the 3 chinchillas examined at 12 days after infection. These results are consistent with the persistence of a pneumococcal community in the middle ear chamber, along with episodic waves of neutrophilic influx and severe inflammation. The current clinical definition of recurrent AOM includes a new episode of AOM, often following antibiotic treatment and an asymptomatic period during which bacterial numbers are reduced to low or nonculturable levels [7]. In at least 1 study that compared isolates from sequential culture samples obtained from patients with recurrent AOM, the subsequent infections appeared to be caused by the original infecting strain [50]. Because increased resistance to both antimicrobials and host clearance are hallmarks of biofilms, it is plausible that the formation of pneumococcal biofilm communities contributes to these chronic infections.

Figure 5. Fluorescent antibody staining reveals pneumococci in the material recovered from the chinchilla middle ear chamber 12 days after infection. The material was embedded and cryosectioned, as detailed in Methods, and stained with sera from rabbits immunized with pneumococcal surface protein C (PspC), choline-binding protein (PcpA), neuraminidase (NanA), or pneumococcal surface protein A family 2 (PspA-FAM2). Low- (column 1) and high-magnification (column 2) images are provided.
In summary, in this study we have defined pneumococcal biofilm communities in the context of experimentally induced OM that appear to be formed as a result of a convergence between both host and pathogen properties. Our ongoing efforts are devoted to defining the host and pneumococcal components involved in the formation and maintenance of this biofilm structure and understanding how this microenvironment might facilitate pneumococcal carriage or disease recurrence.

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References


