

Divergent Mechanisms for Passive Pneumococcal Resistance to β -Lactam Antibiotics in the Presence of *Haemophilus influenzae*

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Background. Otitis media, for which antibiotic treatment failure is increasingly common, is a leading pediatric public health problem.

Methods. In vitro and in vivo studies using the chinchilla model of otitis media were performed using a β -lactamase-producing strain of nontypeable *Haemophilus influenzae* (NTHi 86-028NP) and an isogenic mutant deficient in β -lactamase production (NTHi 86-028NP *bla*) to define the roles of biofilm formation and β -lactamase production in antibiotic resistance. Coinfection studies were done with *Streptococcus pneumoniae* to determine if NTHi provides passive protection by means of β -lactamase production, biofilm formation, or both.

Results. NTHi 86-028NP *bla* was resistant to amoxicillin killing in biofilm studies in vitro; however, it was cleared by amoxicillin treatment in vivo, whereas NTHi 86-028NP was unaffected in either system. NTHi 86-028NP protected pneumococcus in vivo in both the effusion fluid and bullar homogenate. NTHi 86-028NP *bla* and pneumococcus were both recovered from the surface-associated bacteria of amoxicillin-treated animals; only NTHi 86-028NP *bla* was recovered from effusion.

Conclusions. Based on these studies, we conclude that NTHi provides passive protection for *S. pneumoniae* in vivo through 2 distinct mechanisms: production of β -lactamase and formation of biofilm communities.

Otitis media (OM) is one of the leading public health problems in pediatrics, with most children experiencing at least 1 episode by 3 years of age and 40% of older children having 6 or more total episodes [1]. Common causes of OM include nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* (pneumococcus), and *Moraxella catarrhalis*; NTHi and pneumococcus account for \sim 75% of infections [2]. In recent years, epidemiologic studies have shown that co-infections with multiple bacterial species are an important part of both acute and

chronic OM, particularly co-infections with NTHi and pneumococcus [3–5].

As with many upper airway infections, treatment failure and antibiotic-resistant organisms are common problems in OM, with failure rates as high as 50% in some populations [6–9]. The majority of strains of both NTHi and *M. catarrhalis* produce a β -lactamase [10–12]. β -Lactam resistance among pneumococcal strains is much less common, although some strains (\sim 20%) have developed resistance through mutation of the penicillin-binding proteins [10].

Common theories proposed to explain OM treatment failure involving antibiotic-susceptible bacterial strains are the formation of biofilm communities and passive protection by other bacterial pathogens that produce a β -lactamase [13–16]. Bacteria in biofilms are known to be more resistant to antibiotic killing than are planktonic bacteria [17–20]. Previous studies have also shown that *M. catarrhalis* can passively protect pneumococcus from antibiotic killing in a mouse pneumonia model and in biofilms in vitro, which is likely mediated by

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β -lactamase as protection was abolished by β -lactamase inhibitor [21, 22].

In this study, we used a β -lactamase-producing strain of *NTHi* and an isogenic mutant deficient in β -lactamase to distinguish between the roles of biofilm formation and β -lactamase production in *NTHi* antibiotic resistance. In addition, these strains were utilized to determine whether *NTHi* provides passive protection for pneumococcus and, if so, the mechanism behind this protection.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Nontypeable *H. influenzae* 86-028NP (*NTHi* 86-028NP) is an OM isolate that has been fully sequenced and is known to cause OM featuring biofilms in the chinchilla infection model [23–28]. Bacteria were grown on brain heart infusion (BHI) agar supplemented with hemin (ICN Biochemicals) and nicotinamide adenine dinucleotide (Sigma) and 3 μ g/mL of vancomycin (Sigma). *S. pneumoniae* TIGR4 is a well-studied clinical isolate for which a complete genomic sequence is available, and which we have recently shown to form biofilms during experimental OM infection [29, 30]. Pneumococci were grown on trypticase soy agar (BD) with 5% sheep blood (Hemostat Laboratories) or in supplemented BHI (sBHI) medium with 10% horse serum.

Generation of β -Lactamase-Deficient Mutant (*NTHi* 86-028NP *bla*)

A \sim 2-kb DNA fragment containing the β -lactamase triethylene melamine open reading frame (allele designated *NTHi* 2055) was amplified from *H. influenzae* 86-028NP genomic DNA using β lac forward (TGG TTA CGC TCG GGT CTC AA) and β lac reverse (ATG GCA CAA GTT ACA CGA TTC AA) primers with an annealing temperature of 55.9°C and extension time of 2.5 min. The fragment was ligated into pCR2.1 (Invitrogen) and transformed into *Escherichia coli* according to the manufacturer's instructions to generate plasmid pCR- β lac. The pCR- β lac was digested with *HincII*, a unique site within the β -lactamase gene, then dephosphorylated with calf intestinal phosphatase (New England Biolabs). This fragment was then ligated with a chloramphenicol resistance cassette [31] cut with *SmaI*, and transformed into *E. coli*, generating pCR- β lacCm. The pCR- β lacCm was linearized with *NotI* and introduced into *NTHi* 86-028NP via natural transformation, as described previously [23, 24], to generate *NTHi* 86-028NP *bla::Cm* (*NTHi* 86-028NP *bla*). Colonies that grew on sBHI containing chloramphenicol (1.5 μ g/mL) were screened using primers flanking the site of insertion, forward (GAT GCT GAA GAT CAG TTG GG) and reverse (GTA TGG CTT CAT TCA GCT CC) with an annealing temperature of 52°C and extension time of 2.5 min.

Amoxicillin Susceptibility

Bacteria were harvested from overnight sBHI plates and resuspended in phosphate-buffered saline (PBS) to a density of \sim 10⁸ colony-forming units (CFUs)/mL as determined by optical density. To determine planktonic minimum inhibitory concentrations (MICs), \sim 10⁶ CFUs of *NTHi* 86-028NP or *NTHi* 86-028NP *bla* were suspended in 5 mL of sBHI with varying concentrations of amoxicillin (Sigma). Cultures were grown for 16–20 h at 37°C at 150 rpm in a shaking incubator (New Brunswick C24 Incubator Shaker). The turbidity of the medium was used to determine bacterial growth and survival. To determine biofilm susceptibility to amoxicillin, \sim 10⁷ CFUs of *NTHi* 86-028NP or *NTHi* 86-028NP *bla* were plated in 1.5 mL of sBHI in 24-well plates (Falcon) and incubated at 37°C and 5% CO₂. After 24 h, the supernatant was removed from each well and replaced with 1.5 mL of sBHI containing various concentrations of amoxicillin (Sigma). After 48 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping, resuspended in .2 mL of sterile PBS, diluted, and plated for bacterial counts.

Biofilm Protection Assay

NTHi 86-028NP or *NTHi* 86-028NP *bla* (\sim 10⁷ CFUs) was plated in each well of a 24-well plate (Falcon) in a total volume of 1.5 mL of sBHI and incubated at 37°C and 5% CO₂. After 24 h, supernatants were removed and replaced with 1.5 mL of sBHI plus 10% horse serum. *S. pneumoniae* TIGR4 (\sim 10⁷ CFUs) was then added to co-infection and *S. pneumoniae*-alone wells. After 48 h, the supernatant was removed and replaced with sBHI with or without amoxicillin. After 72 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping, resuspended in .2 mL of PBS, diluted, and plated for bacterial counts.

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 clinical signs of middle ear infection or other overt disease. The chinchilla infection protocols were performed essentially as described elsewhere [23, 24, 28, 29]. On day 0, *NTHi* 86-028NP and *NTHi* 86-028NP *bla* were harvested from a plate, and a freezer stock of known CFUs/mL of *S. pneumoniae* TIGR4 was thawed; both were diluted using sterile PBS, and bacterial density was confirmed by plate count. Chinchillas (3–5 animals per group per time point) were anesthetized with isoflurane and inoculated via transbullar injection with .1 mL of bacterial suspension containing *NTHi* 86-028NP, *NTHi* 86-028NP *bla*, *S. pneumoniae* TIGR4, *NTHi* 86-028NP and *S. pneumoniae* TIGR4 or *NTHi* 86-028NP *bla* and *S. pneumoniae* TIGR4, as indicated for each experimental group. Infectious doses ranged from 10² to 10⁴ CFUs, as indicated for each experiment. On days

4, 5, and 6, groups of animals were injected with either 20 µg of amoxicillin (.1 mL of 200 µg/mL; Sigma) or .1 mL of sterile PBS directly in the middle ear. Oral administration of the antibiotic was contraindicated due to the risk of developing a *Clostridium difficile* infection [32]. All animals were euthanized 7 d after infection. Animals exhibiting overt symptoms of systemic disease were euthanized prior to day 7. After euthanasia, the superior bullae were opened to expose the middle ear cavity as described elsewhere [24], and the presence of visible biofilm formation was assessed. If present, middle ear effusion fluids were collected. The middle ear cavity was lavaged with 1 mL of sterile PBS. Effusion and lavage fluids were combined, serially diluted, and assessed by plate count. For animals that received both bacteria, fluid was plated on 2 separate plates, 1 selective for *NTHi* (sBHI plus vancomycin) and 1 selective for *S. pneumoniae* (TSA plus sheep blood plus gentamicin). Middle ear bullae were aseptically removed and homogenized using a PowerGen 1000 homogenizer (Fisher Scientific); the bullar homogenates were plated to assess tissue-associated bacterial load. All of the chinchilla infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

RESULTS

Generation and Confirmation of *NTHi* β-Lactamase Mutant

To define the role of β-lactamase in *NTHi* antibiotic resistance, an isogenic β-lactamase-deficient mutant of *NTHi* 86-028NP was generated (*NTHi* 86-028NP *bla*) by insertional disruption of the *NTHi* 2055 gene (National Center for Biotechnology Information Reference Sequence NC_007146) with a chloramphenicol resistance cassette. No difference in growth was observed between *NTHi* 86-028NP and *NTHi* 86-028NP *bla* in the absence of antibiotic (data not shown). In addition, no difference in *in vitro* biofilm formation was observed between the 2 strains in terms of biomass, mean thickness, maximum thickness, or surface-to-biovolume ratio, as determined by COMSTAT (MATLAB, Version 5.1) analysis of confocal laser scanning microscopy Z-series of 24-h biofilms (Supplementary Figure 1). To confirm the β-lactamase-negative phenotype of the mutant, the MIC of amoxicillin (a β-lactam antibiotic) for both *NTHi* 86-028NP and *NTHi* 86-028NP *bla* was determined. The MIC for *NTHi* 86-028NP was <32.0 µg/mL of amoxicillin (data not shown). The MIC of *NTHi* 86-028NP *bla* was <1.0 µg/mL, <32-fold that of the parental strain (data not shown). The inactivation of the β-lactamase gene rendered an amoxicillin-resistant strain of *NTHi* amoxicillin susceptible, confirming the phenotype of *NTHi* 86-028NP *bla*.

Role of β-Lactamase in *NTHi* Biofilm Antibiotic Resistance *In Vitro*

To define the role of β-lactamase in *NTHi* antibiotic resistance in biofilm, the susceptibility of *NTHi* 86-028NP and *NTHi* 86-

028NP *bla* biofilms to amoxicillin was assessed. Various concentrations of amoxicillin were added for 24 h to preformed *NTHi* biofilms of each strain (0–2 mg/mL). *NTHi* 86-028NP was resistant to killing at all concentrations of amoxicillin tested (Figure 1). Interestingly, although *NTHi* 86-028NP *bla* was much more susceptible than *NTHi* 86-028NP to amoxicillin killing in planktonic culture (MIC 32-fold less), biofilms formed by this strain were also resistant to all concentrations of amoxicillin tested (Figure 1). There was a significant decrease in the recovered CFUs of *NTHi* 86-028NP *bla* from biofilms compared with the parental strain at all antibiotic concentrations tested; however, this difference was <1 log and ~10⁸ CFUs were still recovered at all concentrations tested. Based on these data, the production of a β-lactamase is necessary for resistance of *NTHi* to amoxicillin in planktonic culture, but it does not appear to be required for biofilm resistance *in vitro*.

Role of β-Lactamase in *NTHi* Amoxicillin Resistance in Chinchilla Model of Otitis Media

Whereas the previous experiment showed that β-lactamase production was not necessary for biofilm resistance to amoxicillin *in vitro*, we wished to confirm this result *in vivo* using the chinchilla model of experimental OM. *NTHi* 86-028NP establishes a chronic infection in the middle ears of chinchillas, with biofilm formation occurring by day 3 in most animals [23, 28]. On day 0, ~10³ CFUs of *NTHi* 86-028NP or *NTHi* 86-028NP *bla* were inoculated directly into the middle ears of chinchillas via transbullar injection. On days 4, 5, and 6 after infection, 20 µg of amoxicillin or sterile PBS was injected directly into the middle ear. The concentration of amoxicillin in the middle ear

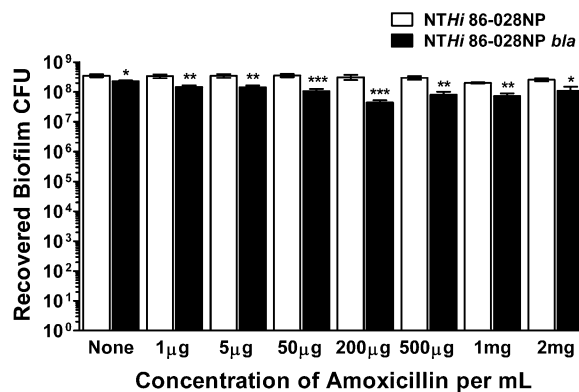


Figure 1. Antibiotic susceptibility of nontypeable *Haemophilus influenzae* (*NTHi*) *in vitro* biofilms. Varying concentrations of amoxicillin (0–2 mg/mL) were added to preformed *NTHi* 86-028NP (white bars) and *NTHi* 86-028NP *bla* (black bars) biofilms. The amounts of amoxicillin noted on the x-axis indicate the concentration per milliliter added to each well. After 24 h, biofilms were scraped, serially diluted, and plated for bacterial counts. Bars represent the mean (± standard error of the mean). The graph represents data combined from 3 experiments (8–9 replicates total). Statistical significance was assessed by Mann-Whitney nonparametric analysis. **P* < .05; ***P* < .005; ****P* < .001. CFU, colony-forming unit.

following oral antibiotic treatment in children is 1–8 µg/mL [6]. The dose in our experiment is a higher concentration than these bacteria would normally experience, allowing for a stringent test of NTHi resistance in vivo. On day 7, all of the animals were euthanized, and the effusion and bullae were harvested and plated for bacterial counts.

In animals infected with NTHi 86-028NP, no difference was shown in recovered CFUs from animals treated with amoxicillin or PBS in either the effusion (planktonic bacteria) or bullar homogenate (tissue-associated/biofilm bacteria) (Figure 2). This confirms the in vitro results, where a β-lactamase-producing strain of NTHi was resistant to amoxicillin killing. However, in animals infected with the β-lactamase-deficient NTHi 86-028NP *bla*, no bacteria were detectable in either the effusion or the bullar homogenate of the group treated with amoxicillin, whereas the group treated with PBS had bacterial counts equivalent to those of animals infected with the parental strain (Figure 2). These results demonstrate that β-lactamase production is required for NTHi resistance to amoxicillin in the chinchilla model of OM. Biofilm formation in the PBS-treated animals was within the normal range seen at this time post-infection for animals infected with either the parental strain (5 of 6) or the β-lactamase-deficient mutant (3 of 6) of NTHi. Based on these results, we conclude that either biofilm formation by NTHi is not protective in this model, or the biofilms formed by NTHi 86-028NP *bla* were not sufficient to provide protection against this concentration of amoxicillin.

NTHi Passive Protection of *S. pneumoniae* In Vitro

Because NTHi and *S. pneumoniae* are frequently found together in the middle ear, we wanted to determine whether NTHi could

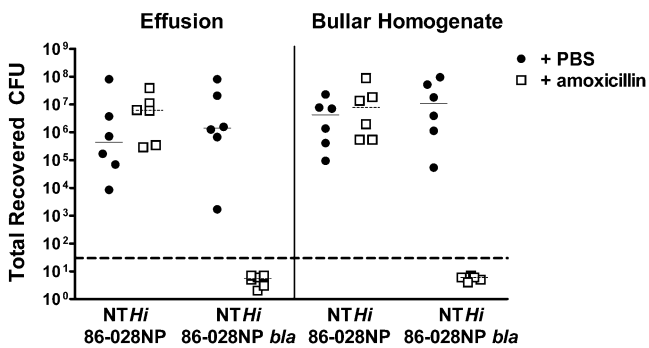


Figure 2. Nontypeable *Haemophilus influenzae* (NTHi) amoxicillin resistance in chinchilla model of otitis media. Chinchillas were infected with either $\sim 10^3$ colony-forming units (CFUs) of NTHi 86-028NP or NTHi 86-028NP *bla* on day 0. On days 4, 5, and 6, animals were treated with either 20 µg of amoxicillin (white squares) or sterile phosphate-buffered saline (PBS) (black circles) directly into the middle ear. All animals were harvested on day 7 after infection. The left half of the graph represents counts from the effusion (planktonic bacteria); the right half is from bullar homogenates (biofilm/tissue-associated bacteria). The long dashed line represents the limit of detection. The short lines represent the mean CFUs for each group.

provide passive protection for pneumococcus against amoxicillin. *S. pneumoniae* is extremely susceptible to killing by β-lactam antibiotics [33]. Both *S. pneumoniae* planktonic and biofilm bacteria were completely killed by as little as .064 µg/mL of amoxicillin in vitro (data not shown and Figure 3). To determine whether NTHi provides passive protection for *S. pneumoniae* within a biofilm in vitro, biofilms containing NTHi 86-028NP and *S. pneumoniae* were treated with amoxicillin. NTHi 86-028NP completely protected pneumococcus from amoxicillin killing, with no difference in recovered pneumococcal CFUs between wells treated with amoxicillin and those treated with medium alone (Figure 3). This passive protection extended to at least 500 µg/mL of amoxicillin (data not shown). Interestingly, although NTHi 86-028NP *bla* viability was not affected by treatment with amoxicillin and its biofilms have a similar structure to that of the parental strain (Supplementary Figure 1), NTHi 86-028NP *bla* provided no protection for pneumococcus with as little as .064 µg/mL of amoxicillin (Figure 3). These data clearly demonstrate that NTHi provides passive protection for pneumococcus in vitro within a biofilm through the production of a β-lactamase.

NTHi Passive Protection of *S. pneumoniae* in the Chinchilla Model of Experimental Otitis Media

To determine whether NTHi provides protection against amoxicillin killing for pneumococcus in vivo, and whether this protection was dependent on the production of β-lactamase, a co-infection was performed using the chinchilla model. It has been previously established by our laboratory that NTHi and pneumococcus establish a chronic co-infection in this model with the formation of very large biofilm communities (larger

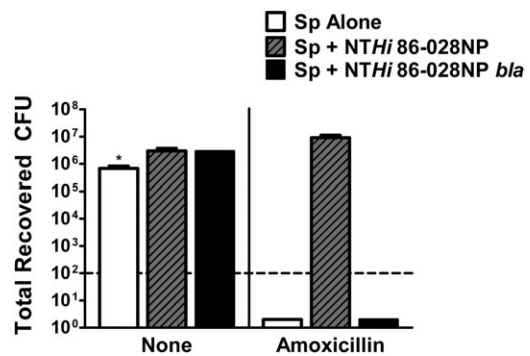


Figure 3. Nontypeable *Haemophilus influenzae* (NTHi) passive protection of *Streptococcus pneumoniae* in vitro. Medium alone or .064 µg/mL of amoxicillin was added to preformed biofilms containing *S. pneumoniae* (Sp) alone (white bars), *S. pneumoniae* and NTHi 86-028NP (gray bars with black lines), or *S. pneumoniae* and NTHi 86-028NP *bla* (black bars). Twenty-four hours after the addition of antibiotics, the biofilms were scraped, serially diluted, and plated for *S. pneumoniae* bacterial counts. Bars are the mean (\pm standard error of the mean) of quadruplicate wells. The graph is representative of 3 independent experiments.

than with either bacterial species alone) that contain both bacterial species [34]. Chinchillas were infected with either *NTHi* 86-028NP and pneumococcus, *NTHi* 86-028NP *bla* and pneumococcus, or pneumococcus alone directly into the middle ear. The same infection/treatment timeline and doses were used as in the previous chinchilla experiment (Figure 2). In brief, animals were infected on day 0, treated with 20 µg of amoxicillin or PBS on days 4, 5, and 6, and euthanized and processed on day 7.

In the animals infected with pneumococcus alone, $\sim 10^8$ CFUs were recovered from both the effusion (planktonic bacteria) and bullar homogenates (biofilm/tissue-associated bacteria) of PBS-treated animals (Figures 4A and 4B). In contrast, no bacteria

were detectable in the effusion, and recovered CFUs were right at the limit of detection in bullar homogenates of animals infected with pneumococcus alone and treated with amoxicillin (Figures 4A and 4B). This confirms that the dose of amoxicillin given essentially clears the infection in animals given pneumococcus alone.

In animals that were co-infected with *NTHi* 86-028NP and pneumococcus and treated with PBS, equivalent numbers of both bacterial species were recovered from both the effusion and bullar homogenate, as expected (Figures 4A and 4B). Interestingly, in animals in this group that were treated with amoxicillin, pneumococcus was recovered from all 4 effusions and 3 of 4 bullar homogenates, with a mean of $\sim 10^7$ CFUs recovered (Figures 4A and 4B). The recovered CFUs of *NTHi* were equivalent to those of PBS-treated animals (Figures 4A and 4B). These results are similar to what was seen in vitro (Figure 3) and clearly demonstrate that *NTHi* passively protects pneumococcus in the chinchilla OM model.

In animals that were co-infected with the β -lactamase-deficient mutant of *NTHi* and pneumococcus and treated with PBS, equivalent numbers of both species were recovered from the effusion and bullar homogenate, as seen with the parental strain (Figures 4A and 4B). In the animals in this group that were treated with amoxicillin, no pneumococcus was detected in the effusion; however, *NTHi* 86-028NP *bla* was recovered from the effusion in 5 of 6 ears, with a mean of $\sim 10^7$ CFUs (Figures 4A and 4B). In addition, *NTHi* 86-028NP *bla* was recovered from the bullar homogenate of 6 of 6 ears and pneumococcus was recovered from the bullar homogenate of 5 of 6 ears in the amoxicillin-treated animals (Figures 4A and 4B). This is in stark contrast to the results in Figure 2, where animals infected with *NTHi* 86-028NP *bla* alone and treated with amoxicillin had no detectable bacteria in either the effusion or bullar homogenate. This is also contrary to what was seen in the in vitro passive protection assay (Figure 3), where *NTHi* 86-028NP *bla* provided no protection for pneumococcus. These data indicate that although β -lactamase production may be necessary for passive protection in vitro, it is not required for *NTHi* passive protection of pneumococcus against amoxicillin killing in vivo. In addition, these studies indicate that the presence of pneumococcus in the middle ear may afford protection for *NTHi*, even though pneumococcus is very susceptible to amoxicillin killing.

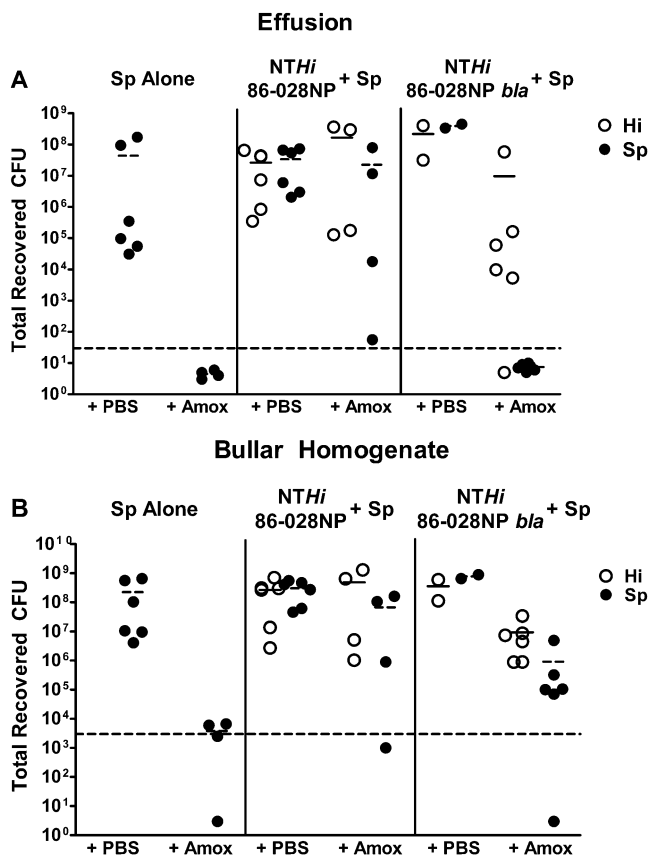


Figure 4. Nontypeable *Haemophilus influenzae* (*NTHi*) passive protection of *Streptococcus pneumoniae* in chinchilla model of otitis media. Chinchillas were infected with either $\sim 10^3$ colony-forming units (CFUs) of *NTHi* 86-028NP and $\sim 10^3$ CFUs of *S. pneumoniae* (*NTHi* 86-028NP + Sp), $\sim 10^3$ CFUs of *NTHi* 86-028NP *bla* and $\sim 10^3$ CFUs of *S. pneumoniae* (*NTHi* 86-028NP *bla* + Sp), or 10^2 CFUs of *S. pneumoniae* alone (Sp alone) on day 0. On days 4, 5, and 6, animals were treated with either 20 µg of amoxicillin (amox) or sterile phosphate-buffered saline (PBS) directly into the middle ear. All animals were harvested on day 7 after infection. The effusion (A) and bullar homogenate (B) were serially diluted and plated for bacterial counts. The white circles represent the recovered CFUs of *NTHi*, and the black circles represent the recovered CFUs of *S. pneumoniae*. The long dashed line represents the limit of detection. The short solid lines represent the mean CFUs of *NTHi* in each group, and the short dashed lines represent the mean CFUs of pneumococcus in each group.

DISCUSSION

As antibiotic treatment failure becomes increasingly common, it is important to understand bacterial mechanisms of antibiotic resistance so that this knowledge can be applied to improve treatment. It is known that biofilm formation and the production of β -lactamase both contribute to *NTHi* antibiotic resistance; however, the contribution of each during an infection

and the role in passive protection of other bacterial species had not been elucidated.

We have shown that, in vitro, *NTHi* 86-028NP and *NTHi* 86-028NP *bla* biofilms are both resistant to amoxicillin killing at all concentrations tested, 0–2 mg/mL (Figure 1). Despite this, only *NTHi* 86-028NP was able to passively protect pneumococcus from amoxicillin killing in biofilms in vitro (Figure 3). When the antibiotic resistance of *NTHi* 86-028NP was tested in the chinchilla model, equal numbers of CFUs were recovered from the effusion (planktonic bacteria) and bullar homogenate (biofilm/tissue-associated bacteria) regardless of whether the animal was treated with PBS or amoxicillin (Figure 2). However, *NTHi* 86-028NP *bla* was cleared from both the effusion and bullar homogenate of every animal. It is possible that *NTHi* 86-028NP *bla* did not form sufficient biofilms to protect against the continuous, large doses of amoxicillin given. If this were the case, *NTHi* 86-028NP *bla* might persist if a lower antibiotic concentration was given, or if biofilm formation was increased. Alternatively, biofilm formation may not be fully protective in vivo. Taken together, these data indicate that although the production of β -lactamase is not required for *NTHi* antibiotic resistance in vitro, it is necessary for in vivo *NTHi* resistance and in vitro passive protection of pneumococcus.

In this study, we also demonstrated that *NTHi* provides passive protection for pneumococcus in the chinchilla model (Figure 4). When co-infected with *NTHi* 86-028NP, pneumococcus was recovered from both the bullar homogenate and effusion of the majority of animals. Interestingly, when co-infected with *NTHi* 86-028NP *bla*, both *NTHi* and pneumococcus were recovered from the bullar homogenate, but only *NTHi* was recovered from the effusion (Figure 4). These data point toward 2 separate mechanisms of *NTHi* protection of pneumococcus from amoxicillin killing in vivo. The first is through the production of β -lactamase, which provides strong protection in both the effusion and bullar homogenate. The second mechanism is through the formation of biofilm communities. As mentioned previously, *NTHi* and pneumococcus form a much larger biofilm together than either bacterium does on its own [34]. This larger mixed biofilm appears to have provided protection against amoxicillin killing for both pneumococcus and *NTHi* 86-028NP *bla*, both of which were cleared when inoculated on their own and treated with amoxicillin. In contrast, prior work has shown a lack of protection by a β -lactamase-producing strain of *NTHi* in a rat experimental OM model [35]. Potential differences in the 2 studies that could explain the differences seen are the route and dose of amoxicillin and the animal model used. In the previous study, amoxicillin was added to the animals' water for oral ingestion and reached a mean serum concentration of ~ 4 μ g/mL. Although that is more clinically relevant than our method of delivery, giving the antibiotic directly in the middle ear allowed for a consistent concentration of drug in the middle ear. Additionally, the

concentration of amoxicillin in our study was much higher, 20 μ g, and should provide a more stringent test of protection than the lower dose achieved in the above-mentioned study. The most important difference in the studies is that biofilm formation occurs in the majority of chinchillas with OM, whereas it is unclear if biofilms are formed in the rat model of OM. This could explain the differences in protection seen between the 2 studies and would indicate an even larger role for biofilm formation in passive protection.

Supplementary Data

Supplementary data are available at <http://jid.oxfordjournals.org/> online.

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