

Hydrogen Peroxide-Mediated Interference Competition by *Streptococcus pneumoniae* Has No Significant Effect on *Staphylococcus aureus* Nasal Colonization of Neonatal Rats[∇]

Elisa Margolis*

Department of Biology, Emory University, Atlanta, Georgia 30322

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It has been proposed that the relative scarcity of *Staphylococcus aureus* and *Streptococcus pneumoniae* cocolonization in the nasopharynxes of humans can be attributed to hydrogen peroxide-mediated interference competition. Previously it has been shown in vitro that H₂O₂ produced by *S. pneumoniae* is bactericidal to *S. aureus*. To ascertain whether H₂O₂ has this inhibitory effect in the nasal passages of neonatal rats, colonization experiments were performed with *S. aureus* and *S. pneumoniae*. The results of these experiments with neonatal rats are inconsistent with the hypothesis that hydrogen peroxide-mediated killing of *S. aureus* by *S. pneumoniae* is responsible for the relative scarcity of cocolonization by these bacteria. In mixed-inoculum colonization experiments and experiments where *S. aureus* invaded the nasopharynxes of rats with established *S. pneumoniae* populations, the density of *S. aureus* did not differ whether the *S. pneumoniae* strain was H₂O₂ secreting or non-H₂O₂ secreting (SpxB). Moreover, the advantage of catalase production by *S. aureus* in competition with a non-catalase-producing strain (KatA) during nasal colonization was no greater in the presence of H₂O₂-producing *S. pneumoniae* than in the presence of non-H₂O₂-producing *S. pneumoniae*.

Recent epidemiological investigations of the carriage of *Staphylococcus aureus* and *Streptococcus pneumoniae* suggest that cocolonization by these two commensal (and occasionally invasive) bacteria is negatively correlated (1, 7, 11, 16, 24). One corollary of this observation is that a reduction in the frequency of colonization by one of these species would lead to a corresponding increase in the frequency of colonization by the other. In fact, it has been proposed that the reduction of *S. pneumoniae* colonization due to the pneumococcal conjugate vaccine has played a role in the increase in *S. aureus* acute otitis media and bacteremia (5, 23). The mechanism proposed to account for this cocolonization pattern is interference competition, or allelopathy, mediated by the killing of *S. aureus* by *S. pneumoniae*-produced hydrogen peroxide (H₂O₂) (19). In broth, high densities of *S. pneumoniae* mixed with *S. aureus* result in the demise of *S. aureus*, and killing does not occur if the pneumococci genetically lack pyruvate oxidase (SpxB) or if catalase is present to neutralize the H₂O₂ (19). While much attention has been given to the appealing hypothesis that loss of H₂O₂-mediated interference competition by *S. pneumoniae* is responsible for the increase in invasive *S. aureus* infections, there has been only indirect confirmation (14) that this allelopathic mechanism operates during nasal colonization.

In this study, I tested the effect of H₂O₂ production by *S. pneumoniae* on nasal colonization of *S. aureus* in two scenarios where interspecies interference may occur in a neonatal rat model: the invasion of an established population of *S. pneumoniae* by *S. aureus* and the use of mixed inocula of both

species. By using isogenic strains of *S. pneumoniae* that either produce or do not produce H₂O₂ (SpxB), I demonstrated that H₂O₂ production by *S. pneumoniae* has no effect on *S. aureus* colonization. Furthermore, the advantage of catalase-producing *S. aureus* in the presence of *S. pneumoniae* is manifest whether *S. pneumoniae* produces or does not produce H₂O₂. I discuss some potential reasons why *S. pneumoniae*-mediated H₂O₂ allelopathy is effective against *S. aureus* in vitro but not in vivo.

MATERIALS AND METHODS

Bacterial strains, media, and inoculum preparation. *S. pneumoniae* TIGR4 (21) and an SpxB-negative variant of TIGR4 (designated SpxB) (19) were provided by Marc Lipsitch. *S. aureus* PS80 (serotype 8, ATCC 27700) and Newman (NCTC 8178) were obtained from the American Type Culture Collection. A catalase-deficient, KatA-negative variant of Newman (designated KatA) (14) was provided by George Liu. *S. aureus* strains were cultivated in Luria-Bertani (LB) broth cultures and agar plates incubated at 37°C. *S. pneumoniae* strains were grown in Todd-Hewitt broth supplemented with 5 g of yeast extract (THY), and plates were supplemented with 40 ml of sheep blood (BBL). Broth cultures and agar plates of *S. pneumoniae* were incubated at 37°C with 5% CO₂.

Inocula for all the infant rat experiments were prepared by initially growing strains to late logarithmic phase (optical density of 0.35 to 0.8). These were stored at –80°C and then thawed on the day of the experiment before being suspended in 2 ml of either LB or THY. Cultures that reached mid-exponential phase were centrifuged (5,000 × g, 3 min) and resuspended in phosphate-buffered saline with 0.1% gelatin (PBSG). Inocula and animal specimen densities were estimated by plating dilutions on LB agar plates for Newman or PS80, on LB plates supplemented with spectinomycin (100 mg/liter) for KatA, and on THY blood plates supplemented with streptomycin (40 mg/liter) for TIGR4 or kanamycin (75 mg/liter) for SpxB.

Infant rat model. All in vivo experiments were performed under the guidelines approved by the Emory University Institutional Animal Care and Use Committee. Three-day-old pups, born of timed-pregnant Sprague-Dawley rats (Charles River Laboratories), were pooled, randomly reassigned to dams, and maintained in microisolator cages in a biocontainment facility. At 3 or 5 days of age, rats were intranasally inoculated by touching a drop of 10⁶ to 10⁷ bacteria of either

* Mailing address: Department of Biology, Emory University, Atlanta, GA 30322. Phone: (404) 727-2956. Fax: (404) 727-2880. E-mail: emargol@emory.edu.

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a mixture of *S. aureus* and *S. pneumoniae* strains or one strain alone suspended in 5 μ l PBSG to the right and nares then another such drop to the left external nares (9, 12, 13).

Two days after the inoculation, nasal wash was collected from 200 μ l of PBSG instilled into a 5-cm intramedic polyethylene tubing (PE50; Clay Adams) placed into the trachea, and nasal epithelium was scraped from the nasal passages after a second wash with 200 μ l of PBSG and removal of the frontal bones. The nasal epithelium, which has been suggested to represent a distinct population (2), was homogenized in 1 ml of PBSG.

In all experiments, 100 μ l of the nasal wash and nasal epithelium samples were plated directly and serially diluted onto selective plates. Plates were incubated overnight at 37°C, and all colonies were counted. The limit for detection at any site was 20 CFU/ml.

Experimental design. In the mixed-inoculum experiments, 5-day-old rats were intranasally inoculated with 10^6 to 10^7 bacteria of a mixture of an *S. aureus* strain(s) (Newman, PS80, KatA, or a mixture of Newman and KatA) and an *S. pneumoniae* strain (TIGR4 or SpxB) at a ratio of 1:5 (*S. aureus* to *S. pneumoniae*). For each pairing between an *S. aureus* strain and an *S. pneumoniae* strain, this experiment was replicated in three different groups of 6 to 12 rats; results from a single replicate are shown (all others are available at www.ecf.net).

For the experiments testing whether *S. aureus* can invade when *S. pneumoniae* is established, groups of six 3-day-old rats were inoculated in both nostrils with 10^6 *S. pneumoniae* bacteria (TIGR4 or SpxB) or with PBS. All of these rats were then inoculated 48 h later with 10^6 to 10^7 *S. aureus* bacteria (Newman, PS80, KatA, or a mixture of Newman and KatA). For each pairing between an *S. aureus* strain and an *S. pneumoniae* strain, this experiment was replicated in two different groups of 6 to 12 rats; results from a single replicate are shown (all others are available on www.ecf.net).

Statistical analysis. Welch's *t* test was used to evaluate the statistical significance of H_2O_2 production by *S. pneumoniae* for the bacterial density of *S. aureus* following growth on agar surfaces or during nasal colonization. To ascertain whether catalase-producing *S. aureus* was being selected during nasal colonization, the selection rate constant (r_{nk}) was calculated. The selection rate is a measure of the relative recoveries of the catalase-producing *S. aureus* strain (Newman) and the catalase-deficient *S. aureus* strain (KatA) inoculated into the nasal passages over the 48 h and is given by $r_{nk} = [N_n(48)/N_n(0)] - [N_k(48)/N_k(0)]$, where $N_n(0)$ and $N_k(0)$ are the initial densities in the inoculum of Newman and KatA, respectively, and $N_n(48)$ and $N_k(48)$ are the densities detected at the specific site after 48 h in the rat (22). A selection rate of 0 indicates that there is no selection for catalase production, and a positive rate indicates an advantage for the catalase-producing strain. The *P* values given for the selection rates were determined using the two-tailed probability (from the *t* distribution with $n - 1$ degrees of freedom) of rejecting by chance the null hypothesis that the selection rate constant equals zero, indicating equal fitness for the catalase-producing (Newman) and catalase-deficient (KatA) strains.

RESULTS

Previously it has been shown that in vitro H_2O_2 production by *S. pneumoniae* limits the growth of *S. aureus* in liquid culture (19). To determine whether this occurs in vivo, neonatal rats were used, because both *S. aureus* and *S. pneumoniae* readily colonize the nasal passages of neonatal rats in single-clone and mixed cultures (with low inoculum densities). Within 48 h after a single species is inoculated alone, both *S. aureus* and *S. pneumoniae* reach a population of 10^3 CFU/ml in the nasal wash and epithelium, and this population is maintained for at least 5 days (data not shown). Despite large individual variation in bacterial densities with either species, the average bacterial densities in neonatal rats are similar to the bacterial loads reported for humans (20). To determine whether H_2O_2 plays a role in competition during colonization when both *S. pneumoniae* and *S. aureus* are introduced in a mixed inoculum, 5-day-old neonatal rats were challenged with a mixture of *S. pneumoniae* and *S. aureus*. The three strains of *S. aureus* were separately mixed with either an H_2O_2 -producing *S. pneumoniae* strain (TIGR4) or a non- H_2O_2 -producing *S. pneu-*

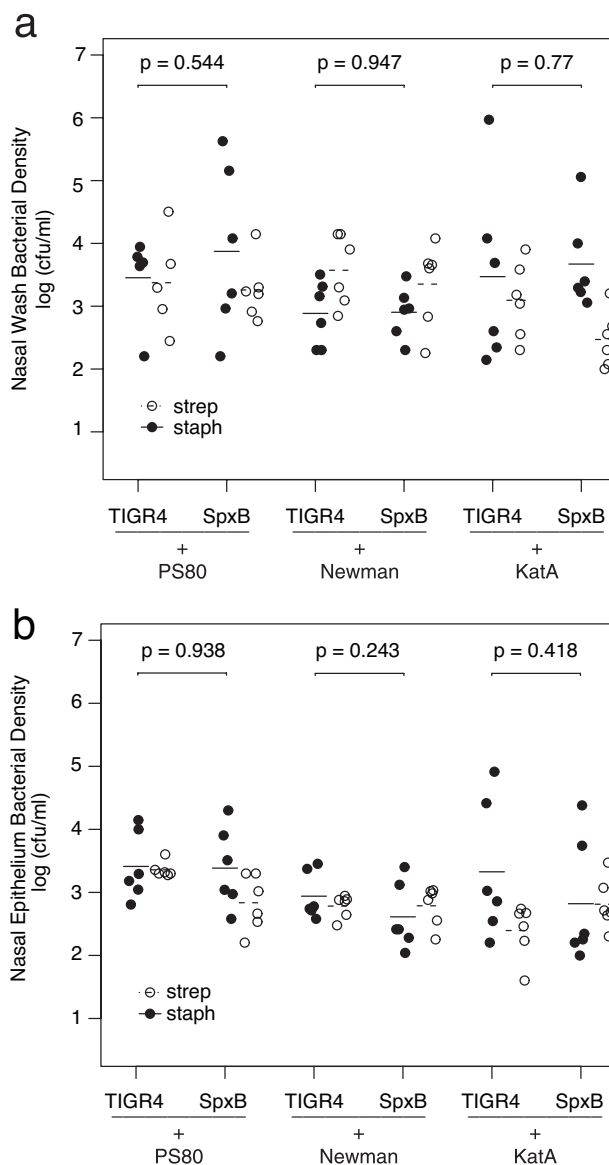


FIG. 1. Forty-eight-hour densities of *S. aureus* and *S. pneumoniae* from nasal washes (a) and epithelia (b) of colonized rats. Five-day-old neonatal rats were colonized with 5×10^6 CFU of an *S. pneumoniae* strain which produces H_2O_2 (TIGR4) or one that does not (SpxB) in the left nostril and with 1×10^6 CFU of either the *S. aureus* PS80, Newman, or catalase-deficient Newman (KatA) strain.

moniae strain (SpxB). If H_2O_2 produced by *S. pneumoniae* killed the *S. aureus*, one would expect that *S. aureus* would reach a statistically significantly lower density in the presence of the H_2O_2 -producing strain (TIGR4) than when inoculation was with SpxB. This was not observed (Fig. 1).

Since the amount of H_2O_2 produced is proportional to the number of bacteria, the maximum amount of H_2O_2 would be present when the population of the producing strain was at a high density. Consequently, one would expect that H_2O_2 production would be most effective in preventing invasion in habitats that are already colonized by the H_2O_2 -producing population. To ascertain whether this is the case for *S. pneumoniae* H_2O_2 production, *S. aureus* was inoculated intranasally into

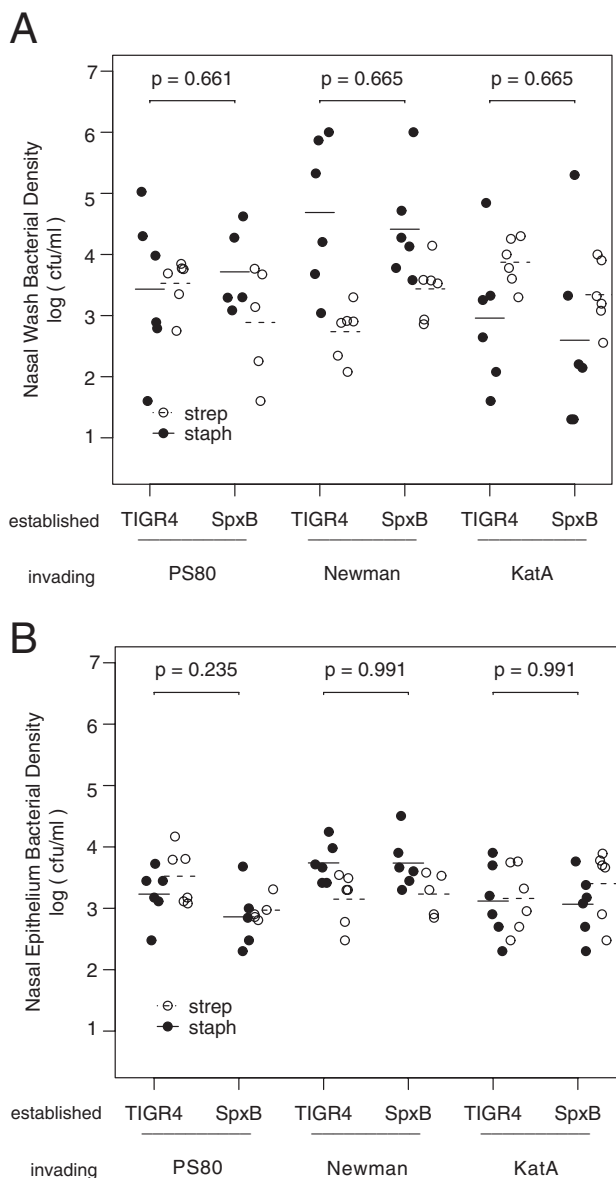


FIG. 2. Forty-eight-hour densities of *S. aureus* and *S. pneumoniae* from nasal washes (a) and epithelia (b) of colonized rats. Three-day-old neonatal rats were colonized with 10^7 CFU of an *S. pneumoniae* strain which produces H_2O_2 (TIGR4) or one that does not (SpxB). Five-day-old neonatal rats were colonized with 10^6 CFU of either the *S. aureus* PS80, Newman, or catalase-deficient Newman (KatA) strain.

neonatal rats with established populations (inoculated 48 h earlier) of either H_2O_2 -producing (TIGR4) or non- H_2O_2 -producing (SpxB) *S. pneumoniae*. At 48 h, the nasal wash and epithelium were sampled and the densities of both *S. pneumoniae* and *S. aureus* estimated. The results of this experiment suggest that *S. aureus* is equally able to invade populations of *S. pneumoniae* in the nasal passages of rats whether the *S. pneumoniae* strain is capable of producing H_2O_2 or not and whether the *S. aureus* produces catalase or not (Fig. 2).

One explanation for why *S. aureus* is uninfected by *S. pneumoniae*-produced H_2O_2 during cocolonization and is able to invade established populations of H_2O_2 -producing *S. pneu-*

moniae is that wild-type *S. aureus* produces a sufficient amount of catalase to neutralize the H_2O_2 . Recent results by Park and colleagues (14) suggest that this may be the case. In their experiments with a mouse cocolonization model, *S. pneumoniae* producing H_2O_2 selected for catalase-producing *S. aureus* in mixtures with otherwise isogenic KatA mutants that do not produce catalase.

To discern whether H_2O_2 production by *S. pneumoniae* selects for catalase-producing *S. aureus* in the neonatal rat model, mixtures of catalase-producing (Newman) and non-catalase-producing (KatA) *S. aureus* were inoculated either in coinoculation with or invading on an established population of either H_2O_2 -producing (TIGR4) or non- H_2O_2 -producing (SpxB) *S. pneumoniae* or a PBS buffer control. The selection rate, which compares the relative recoveries of the two strains in the nasal epithelium, measured the competitive performance of the catalase-producing *S. aureus* strain and the non-catalase-producing *S. aureus* strain. If the selection rate constant is 0, there is no advantage for the catalase producer, while a value in excess of 0 would mean that catalase production is favored. The results of this experiment for the nasal epithelium are presented in Table 1.

The catalase-producing strain had a marked advantage over the nonproducer when coinoculated with a non- H_2O_2 -producing strain (SpxB); however, there was no detectable selection for catalase production without *S. pneumoniae* present (PBS control) or with coinoculation with the H_2O_2 -producing strain (TIGR4). The selective advantage for catalase production was even more pronounced when the mixture of *S. aureus* strains was invading neonatal rats with established populations of *S. pneumoniae*. In this case, the fitness advantage of the catalase-producing strain was significant regardless of whether the established *S. pneumoniae* strain produced H_2O_2 . Similar results were obtained when fitness was estimated from densities determined from the nasal wash (results not shown).

DISCUSSION

The hypothesis that hydrogen peroxide-mediated killing of *S. aureus* by *S. pneumoniae* is responsible for the relative scarcity of cocolonization of these two species of bacteria is appealing. It provides an explanation for why the increase in invasive *S. aureus* infections could be due to the vaccine-associated decrease in pneumococcal colonization. This hypothesis is supported not only by epidemiological studies (1, 7, 11, 16,

TABLE 1. Selection for catalase production in *S. aureus* in the nasal epithelium

Established population or control	Mixed inoculum		Established <i>S. pneumoniae</i> inoculum	
	Selection rate (mean \pm SEM) ^a	P value ^b	Selection rate (mean \pm SEM)	P value
PBS	0.48 \pm 0.83	0.583		
TIGR4	0.48 \pm 0.42	0.296	5.52 \pm 0.59	0.006
SpxB	1.63 \pm 0.39	0.006	3.40 \pm 0.64	0.002

^a KatA⁺ relative to KatA⁻.

^b Two-tailed probability from the *t* distribution of rejecting by chance the null hypothesis that the selection rate constant equals 0, indicating equal fitness for the catalase-producing (Newman) and catalase-deficient (KatA) strains.

24) but also by in vitro experiments (19) that show that H₂O₂ produced by *S. pneumoniae* is bactericidal to *S. aureus* and limits the density which *S. aureus* reaches in liquid culture. The neonatal rat nasal colonization results of this study are inconsistent with this hypothesis. Hydrogen peroxide production provided no advantage to *S. pneumoniae* in competing with *S. aureus* either in colonizing the nasal mucosa of these rats or preventing established populations of *S. pneumoniae* from being colonized by *S. aureus*. Although the results of our experiments indicated that the production of catalase (which neutralizes H₂O₂) provides a competitive advantage to *S. aureus* in the presence of *S. pneumoniae*, that advantage occurred whether *S. pneumoniae* was H₂O₂ producing or not.

I propose three classes of explanations for why *S. pneumoniae*-mediated H₂O₂ killing of *S. aureus* is effective in vitro but fails to prevent colonization of *S. aureus* in the nasal passages of neonatal rats: (i) *S. pneumoniae* does not produce H₂O₂ in sufficient quantities in vivo, (ii) H₂O₂ produced in the nasal passages is inactivated either by the host or by other members of the nasal flora, or (iii) the rate of replication of *S. aureus* more than makes up for killing by *S. pneumoniae*-produced H₂O₂. In support of the first explanation is the observation that in broth when *S. pneumoniae* is at low densities relative to *S. aureus*, there are insufficient amounts of H₂O₂ to inhibit the growth of *S. aureus* (16). The same would be expected on surfaces (plates or nasal epithelium) if the *S. aureus* and *S. pneumoniae* colonies are too far apart for *S. aureus* to come into contact with the zone of inhibition formed by *S. pneumoniae* (3). This may have been the case in the nasal passages of neonatal rats if *S. aureus* and *S. pneumoniae* were not colocalized in the nasal passages or if their densities were too low. It should be noted that in the neonatal rat, at least the recovered density of *S. aureus* or *S. pneumoniae* was never greater than 10⁵ CFU/nose. It would be of interest to ascertain whether the densities of *S. pneumoniae* in the nasal passages of humans are greater than that observed in these rats. In support of the second explanation is the observation that the nasal epithelium produces both catalase and glutathione peroxidase (a scavenger of H₂O₂) (4). As for the third explanation, I am unaware of estimates of the exponential growth rates of *S. aureus* in the nasal passage, much less the extent to which that growth rate is reduced by H₂O₂ killing.

Although the results of these experiments are consistent with earlier observations that catalase production provides a fitness advantage to *S. aureus* when it is coinoculated with *S. pneumoniae* in a mouse model (14), they suggest that this advantage is not due to the production of H₂O₂ by *S. pneumoniae*. In my experiments, this advantage of catalase-producing *S. aureus* over otherwise isogenic strains that did not produce this enzyme occurred whether the *S. pneumoniae* in the nasal epithelia of the rats can produce H₂O₂ or not. Why, then, would catalase production provide a fitness advantage to *S. aureus* only when *S. pneumoniae* is present? Perhaps in this habitat catalase production by *S. aureus* increases the survival rate in neutrophils by reducing oxidative stress (6, 8), or *S. pneumoniae* may indirectly select for catalase production in *S. aureus*, as the presence of both species could synergistically elicit a stronger innate immune response (particularly neutrophil infiltration) as has recently been observed for *Haemophilus influenzae* and *S. pneumoniae* (10, 15).

The results of the study can be seen as a cautionary tale, with the moral being that what occurs in a flask may not predict what occurs in a bacterium's natural habitat. They also support the recent epidemiological evidence reported by Regev-Yochay and colleagues (17) that H₂O₂ is not the major determinant to explain the pattern of cocolonization. Why, then, is *S. pneumoniae*-*S. aureus* cocolonization rarer than expected? One can speculate that the scarcity of cocolonization may be due either to different host preferences by these bacterial species or to competitive interactions other than H₂O₂ allelopathy, perhaps resource or immune-mediated competition (18). In support of the possibility of immune-mediated competition, there was a negative association between *S. pneumoniae* and *S. aureus* colonization only in human immunodeficiency virus-negative children and not in human immunodeficiency virus-positive carriers (11). Distinguishing between bacterial interactions and host preferences is especially important for vaccination efforts; reducing the incidence of one species with a vaccine may have the undesired consequence of increasing the incidence of a competing commensal or pathogenic species.

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