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Inhibitory and Bactericidal Effects of Hydrogen Peroxide Production by *Streptococcus pneumoniae* on Other Inhabitants of the Upper Respiratory Tract

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An inverse correlation between colonization of the human nasopharynx by *Streptococcus pneumoniae* and *Haemophilus influenzae*, both common upper respiratory pathogens, has been reported. Studies were undertaken to determine if either of these organisms produces substances which inhibit growth of the other. Culture supernatants from *S. pneumoniae* inhibited growth of *H. influenzae*, whereas culture supernatants from *H. influenzae* had no effect on the growth of *S. pneumoniae*. Moreover, coculture of *S. pneumoniae* and *H. influenzae* led to a rapid decrease in viable counts of *H. influenzae*. The addition of purified catalase prevented killing of *H. influenzae* in coculture experiments, suggesting that hydrogen peroxide may be responsible for this bactericidal activity. *H. influenzae* was killed by concentrations of hydrogen peroxide similar to that produced by *S. pneumoniae*. Hydrogen peroxide is produced by the pneumococcus through the action of pyruvate oxidase (SpxB) under conditions of aerobic growth. Both an *spxB* mutant and a naturally occurring variant of *S. pneumoniae*, which is downregulated in SpxB expression, were unable to kill *H. influenzae*. A catalase-reversible inhibitory effect of *S. pneumoniae* on the growth of the respiratory tract pathogens *Moraxella catarrhalis* and *Neisseria meningitidis* was also observed. Elevated hydrogen peroxide production, therefore, may be a means by which *S. pneumoniae* is able to inhibit a variety of competing organisms in the aerobic environment of the upper respiratory tract.

Bacterial pathogens are generally studied individually, although in their natural environment they often coexist or compete with multiple other microbial species. The focus of this report is bacterial pathogens that commonly colonize and infect the respiratory tract of humans. The results of clinical studies that surveyed the etiologic agents in cases of otitis media in children and chronic bronchitis in adults showed that *Streptococcus pneumoniae* and *Haemophilus influenzae* are the most prevalent bacterial pathogens (14, 23). The frequency with which these two species are isolated from the same specimen, however, is significantly less than would be predicted based on their relative prevalence (25, 30). This suggests that there may be inhibitory effects of one species on the other in vivo. This would not be an unexpected finding considering our current understanding of the pathogenesis of colonization and infection by these species. For instance, since both *S. pneumoniae* and *H. influenzae* express cell surface phosphorylcholine, which mediates adherence to the receptor for platelet-activating factor, there may be competition for the same host cell receptor (12, 35, 46). In addition, phosphorylcholine is immunogenic, and antibody generated against phosphorylcholine from one species may promote clearance of a heterologous species bearing the same epitope (9, 31, 45). However, the presence of phosphorylcholine is required for viability in the case of the pneumococcus, while *H. influenzae* is able to switch off expression of this antigen (44, 51). Another example is the neuraminidase secreted by the pneumococcus, which has the potential to remove sialic acid residues from bacterial competitors known to express this as a cell surface structure (6, 10). The lipopolysaccharide of the respiratory tract pathogen, *Neisseria meningitidis*, and at least some strains of *H. influenzae* are sialylated and, in the case of the former, this modification acts to increase resistance to clearance mediated by complement (17, 21, 28, 29).

In order to begin to examine the interactions of the coinhabitants of the heavily colonized mucosal surface of the human upper respiratory tract, we tested the effect of coculture in vitro on growth and viability. These studies revealed that the pneumococcus produces an inhibitory substance that was shown to be hydrogen peroxide. This suggests that the production of H_2O_2 by *S. pneumoniae*, previously shown to be cytotoxic for cultured alveolar epithelial cells, may also be an effective mechanism for limiting or eliminating competitive flora, including common pathogens such as *H. influenzae* and *N. meningitidis*, which share the same microenvironment (15). These species, furthermore, are sensitive to levels of peroxide generated by the pneumococcus despite their production of catalase, an enzyme that acts to eliminate hydrogen peroxide (7, 8, 37).

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strains used in this study are described in Table 1. All strains were cultured in brain heart infusion broth (BHI) with or without 1.5% agar (Difco Laboratories, Detroit, Mich.). *H. influenzae* was grown in BHI medium supplemented with hemin and L-histidine (dissolved in 1% triethanolamine, each at a final concentration of 2.5 μg/ml) (sBHI) plus NAD (2.0 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). All organisms were grown at 37°C with aeration except streptococci, which were grown without shaking. Plates containing streptococci and neisseriae were incubated in the presence of supplemental carbon dioxide using candle extinction jars. Pneumo-

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TABLE 1. Hydrogen peroxide sensitivity and production by various bacterial pathogens

Species	MIC (mM)[a]	MBC (mM)[b]	H_2O_2 generated (mM)[c]	Source or reference[d]
Gram-negative				
H. influenzae Rd	0.4	0.5	<0.1	26
H. influenzae Eagan	0.4	0.5	<0.1	26
N. meningitidis MC58C3	0.4	5	<0.1	32
M. catarrhalis Bc1	1.1	160	<0.1	Clinical isolate
E. coli RS218	ND	15	<0.1	1
S. enterica serovar Typhimurium LT2	ND	20	<0.1	Collection of K. Sanderson
K. pneumoniae Kp1	ND	20	<0.1	Clinical isolate
P. aeruginosa PA01	ND	60	<0.1	ATCC 15692
Gram-positive				
S. pyogenes P87	ND	40	<0.1	Clinical isolate
S. agalactiae P60	ND	80	<0.1	Clinical isolate
S. equisimilis P107	ND	20	<0.1	Clinical isolate
E. faecium P119	ND	80	<0.1	Clinical isolate
S. aureus A1	ND	10	<0.1	Clinical isolate
S. pneumoniae strains				
P394 (type 4)	1.6	80		TIGR genome strain
D39 (type 2)	1.2	80	0.44 ± 0.08	4
P383 (type 6B)	ND	ND	0.53 ± 0.08	22
P384 (type 6A)	ND	ND	0.71 ± 0.13	22
P878 D39 (*spxB*::Tn*phoA*)	1.6	80	<0.1	38
P62 (type 9V opaque variant)	ND	ND	<0.1	22
P64 (type 9V transparent variant)	ND	ND	0.43 ± 0.13	22

[a] The MIC was determined as the minimum concentration of H_2O_2 necessary to prevent turbid growth of a 1-in-50 inoculum of a stationary-phase culture following overnight incubation at 37°C. ND, not determined.
[b] The MBC was determined as the minimum concentration of H_2O_2 necessary for >99.9% killing of washed, log-phase cells in BHI medium after 30 min at 37°C.
[c] H_2O_2 concentration present in culture supernatants after incubating approximately 5×10^7 washed, log-phase cells for 1 h in BHI medium at 37°C.
[d] TIGR, The Institute for Genome Research.

cocci were plated on BHI containing 200 U of bovine liver catalase per ml (Worthington Biochemical, Freehold, N.J.).

Supernatant inhibition assays. Cultures of *S. pneumoniae* P394 were grown in liquid BHI medium at 37°C under atmospheric conditions. After reaching mid-log phase (optical density at 620 nm [OD_{620}] = 0.3 to 0.4), the cultures were harvested and spun at 10,000 × *g* for 2 min, and the supernatant was filtered through 0.2-μm (pore-size) filters. The target organism was grown in liquid BHI or sBHI medium to mid-log phase (OD_{620} = 0.3 to 0.4) and then diluted 10-fold in phosphate-buffered saline (PBS). Bacterial lawns were obtained by spreading 50 μl of diluted culture on BHI or sBHI agar with or without 200 U of catalase per ml. Then, 10-μl aliquots of supernatant were spotted onto these plates and allowed to dry prior to incubation at 37°C for 16 h. In some experiments, aliquots of supernatant were treated with proteinase K (final concentration, 50 μg/ml; Sigma) at 37°C for 1 h or heated to 65°C for 20 min prior to adding them to plates containing target organisms.

Coculture experiments. Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 × *g* and 4°C, washed in ice-cold Hanks balanced saline solution (HBSS; Gibco BRL, Gaithersburg, Md.), and then resuspended in BHI at the original culture volume. Equal volumes of *S. pneumoniae* and the target strain were then mixed and incubated at 37°C in 96-well polystyrene microtiter plates (Dynex Technologies, Inc., Chantilly, Va.). As a negative control, each strain was mixed with an equal amount of BHI alone. Where indicated, individual wells were supplemented with catalase (final concentration, 1,000 U/ml). Serial dilutions were then prepared in HBSS, and an aliquot was plated on BHI agar plates containing catalase (final concentration, 200 U/ml) for viable counts. Dilutions of mixed cultures were spread on BHI plates supplemented with 2.0% Fildes enrichment (Difco) and grown under atmospheric conditions which selectively inhibited the growth of *S. pneumoniae* and allowed enumeration of the target species. Removal of the Fildes enrichment, which provides a source of hemin and NAD, provided selective conditions preventing the growth of *H. influenzae*.

Hydrogen peroxide sensitivity assays. Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 × *g* and 4°C, washed in ice-cold HBSS, and resuspended in fresh BHI medium. Resuspended bacteria were added to microtiter plate wells in duplicate containing twofold dilutions of H_2O_2 (Sigma) in BHI medium and incubated at 37°C for 30 min. Aliquots from each well were applied to BHI agar plates containing 200 U of catalase per ml for viable counts. The concentration of H_2O_2 required to cause a 99.9% decrease in the number of colonies compared to the negative control without peroxide was recorded as the minimum bactericidal concentration (MBC). For MIC determination, 50-fold dilutions of stationary-phase cultures in BHI containing twofold dilutions of H_2O_2 were incubated at 37°C overnight. The minimum concentration necessary to prevent turbid growth was considered the MIC.

Hydrogen peroxide production assays. Hydrogen peroxide production was measured in an assay developed by Pick and Keisari and modified by Duane and coworkers (15, 36). Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 × *g* and 4°C, washed in ice-cold HBSS, and resuspended in BHI medium to twice the original culture volume. Wells for negative controls contained 1,000 U of catalase per ml. After 1 h of incubation under atmospheric conditions at 37°C, the cultures were harvested, spun at 10,000 × *g* for 2 min, and filtered through a 0.2-μm (pore-size) membrane. Immediately prior to the assay, phenol red and horseradish peroxidase were added to peroxide assay buffer (5.0 mM K_2HPO_4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose; pH 7.4) at final concentrations of 0.46 mM and 0.046 U/ml, respectively. Aliquots of filtered supernatant were added to the assay mixture at a ratio of 1 to 4 and incubated for 30 min at 37°C in duplicate. After the reactions were stopped by the addition of NaOH (final concentration, 0.004 N) the absorbance was recorded at a wavelength of 610 nm. Concentrations were calculated in comparison to a standard curve with known amounts of H_2O_2 added to control supernatant from wells containing catalase which had been heated to 100°C for 20 min to eliminate catalase activity.

Two-dimensional protein gel electrophoresis. Two-dimensional protein gel electrophoresis followed by staining, computerized comparison, and mass spectrometric analysis of the proteins, was done as described elsewhere (K. Overweg, C. D. Pericone, L. G. C. Verhouf, J. N. Weiser, H. D. Meiring, A. D. P. J. M. De Jong, R. De Groot, and P. W. M. Hermans, submitted for publication).

Western transfer and immunoblotting. P878 containing an in-frame fusion of Tn*phoA* to the gene for pyruvate oxidase (*spxB*) was grown on tryptic soy agar plates containing catalase (200 U/ml) (38). Bacteria were grown for 16 h at 37°C under atmospheric conditions (20% O_2, 0.03% CO_2), in a candle extinction jar (17% O_2, 3% CO_2), or in the GasPak anaerobic system (<0.01% O_2, 10% CO_2) (Becton Dickinson, Cockeysville, Md.). Cells were harvested from plates, adjusted to equal density based on absorbance at 620 nm, washed in cold PBS, and treated at 100°C for 5 min in gel loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM β-mercaptoethanol; 10% glycerol; 2% sodium dodecyl sulfate [SDS], 1% bromophenol blue) prior to separation in SDS–10% polyacrylamide gel electrophoresis (PAGE) gels. Equal loading was confirmed by measurement of total protein in whole-cell sonicates using the Micro BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.). After transfer to Immobilon P membranes (Millipore Co., Bedford, Mass.), immunoblotting was carried out with an antibody raised against PhoA and detected with an antiserum to rabbit immunoglobulin G conjugated to alkaline phosphatase as described previously (43).

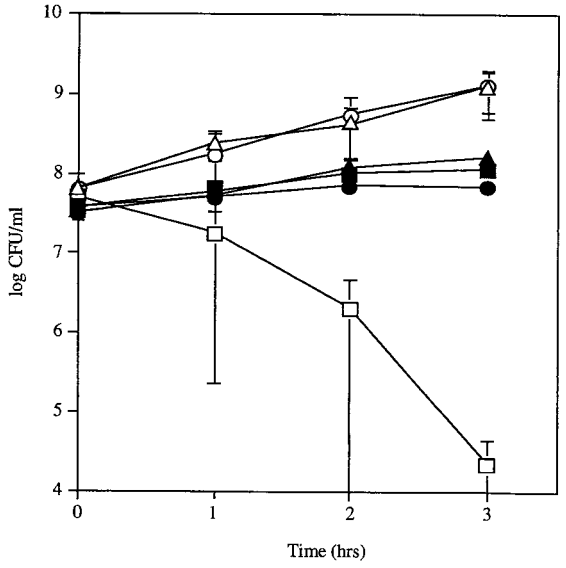

FIG. 1. Effect of coculture of *S. pneumoniae* P394 and *H. influenzae* Rd. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI containing heat-inactivated catalase either with (□) or without (○) *S. pneumoniae* for the time indicated, and viable counts were determined in duplicate on selective media. Viable counts of *S. pneumoniae* incubated in coculture with (■) or without (●) *H. influenzae* were determined in duplicate by plating on selective media. The same amount of active catalase (1,000 U/ml) was included during coculture of *S. pneumoniae* (▲) and *H. influenzae* (△). Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations.

RESULTS

Bactericidal effect of *S. pneumoniae* on *H. influenzae*. The hypothesis that pathogens inhabiting the same host environment might generate growth-inhibitory substances was examined. Initial experiments tested the effect of culture supernatant of *S. pneumoniae* P394 and *H. influenzae* Rd on the growth of the other species. Aliquots of culture supernatant filtrates from one organism were added to a lawn of the other organism which had been spread on solidified medium which supports the growth of only that species. A zone of completely inhibited growth was observed when supernatants from *S. pneumoniae* were added to lawns of *H. influenzae*, while the inverse showed no observable effect on growth (data not shown). This demonstrated that *S. pneumoniae* produced a substance that inhibited the growth of *H. influenzae*. Similar results were obtained using three nontypeable clinical isolates of *H. influenzae*, as well as the type b isolate, Eagan. Likewise, unrelated *S. pneumoniae* clinical isolates of types 2, 6A, and 6B were all capable of inhibiting the above-mentioned strains of *H. influenzae*, demonstrating that the observed effect was not strain specific.

To test whether this growth-inhibitory effect was also bactericidal, both species were grown to mid-log phase and cocultured in liquid medium. When 10^8 CFU of *H. influenzae* Rd per ml were cocultured with 5×10^7 CFU of *S. pneumoniae* P394 per ml, the viable count of *H. influenzae* decreased to below detectable levels (10^4 CFU/ml) within 3 h, whereas the viable count of *H. influenzae* cultured in the absence of *S. pneumoniae* under the same conditions increased to 10^9 CFU/ml (Fig. 1). In contrast, the viable count of *S. pneumoniae* increased to 10^8 CFU/ml, whether cultured with *H. influenzae* or in the absence of *H. influenzae*. These observations showed that the substance produced by *S. pneumoniae* was not only inhibitory but also bactericidal against *H. influenzae*.

In similar dose-response experiments, 10^7 CFU of *S. pneumoniae* per ml reduced the number of *H. influenzae* from 10^8 to $<10^4$ CFU/ml within 3 h (Fig. 2). *S. pneumoniae* at 10^6 CFU/ml reduced the equivalent number of *H. influenzae* approximately 10-fold within 3 h. The growth of the equivalent number of *H. influenzae* with 10^5 CFU of *S. pneumoniae* per ml was comparable to that of *H. influenzae* grown in the absence of *S. pneumoniae*.

The bactericidal effect of *S. pneumoniae* is due to hydrogen peroxide production. Supernatants from cultures of *S. pneumoniae* treated with proteinase K or heated to 65°C for 20 min retained inhibitory activity, suggesting that the inhibitory substance was not likely to be a protein (data not shown). In addition, the inhibitory effect was diminished when *S. pneumoniae* was grown under less-than-atmospheric levels of environmental oxygen (data not shown). It had previously been shown that *S. pneumoniae* makes substantial amounts of H_2O_2 when grown aerobically (2, 34). It was therefore suspected that the inhibitory effect of *S. pneumoniae* supernatant might be due to H_2O_2 production. Further support for this possibility came from the observation that the inhibitory effect was inversely proportional to the level of hemin in the growth medium (data not shown). Hemin had previously been shown to mitigate the effects of oxidative stress on *H. influenzae*, presumably because of its ability to decompose hydrogen peroxide (24, 27). Catalase, a heme-containing enzyme which specifically degrades H_2O_2, was then added to BHI plates at a concentration of 200 U/ml. This eliminated the inhibitory effect of *S. pneumoniae* culture supernatants on *H. influenzae* (data not shown).

The effect of catalase on the bactericidal activity of *S. pneumoniae* was then explored using quantitative coculture experiments with bacteria grown in liquid medium. *H. influenzae* cultured with *S. pneumoniae* in the presence of 1,000 U of catalase per ml grew at the same rate as *H. influenzae* cultured alone, whereas heat-inactivated catalase (100°C for 20 min) was unable to eliminate the bactericidal activity of *S. pneumoniae* (Fig. 1). To confirm that hydrogen peroxide was re-

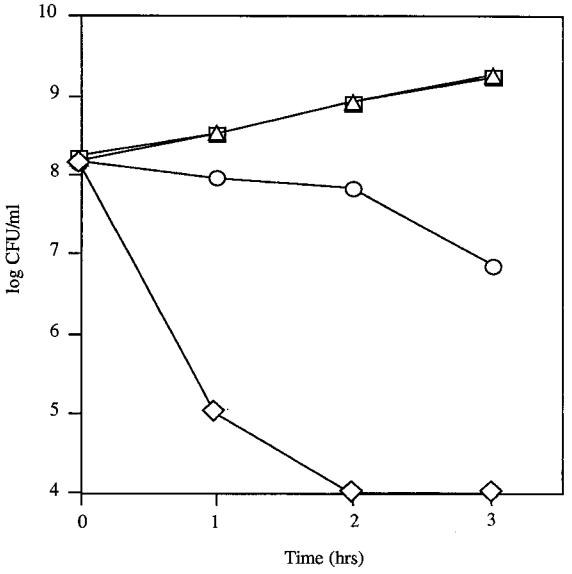

FIG. 2. Dose-dependent killing of *H. influenzae* Rd by *S. pneumoniae* P394. Following growth to mid-log phase, *H. influenzae* was washed and cultured alone (triangles) or with 10^5 (squares), 10^6 (circles), or 10^7 (diamonds) CFU of *S. pneumoniae* per ml and incubated in sBHI for the times indicated; viable counts were determined on selective media. Values represent the average of two independent determinations in duplicate.

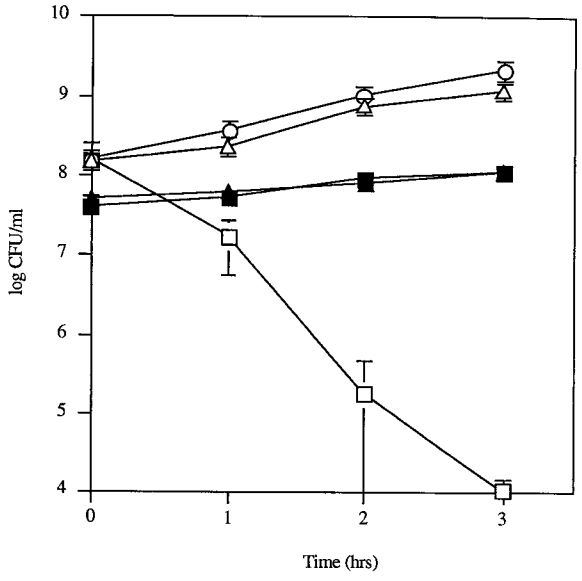

FIG. 3. Effect of coculture of *H. influenzae* Rd with *S. pneumoniae* D39 and its *spxB* mutant, P878. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI alone (○), with D39 (□), or with P878 (△) for the times indicated, and viable counts were determined in duplicate on selective media. Viable counts of D39 (■) or P878 (▲) incubated in coculture with *H. influenzae* were determined in duplicate by plating on selective media. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations.

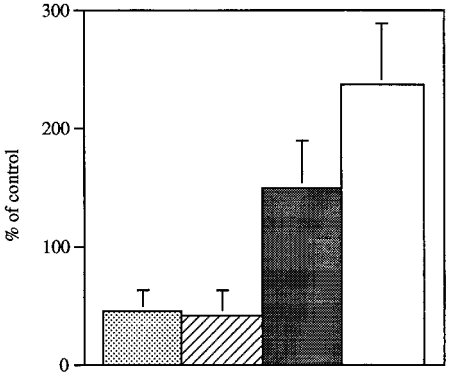

FIG. 4. Effect of coculture of *S. pneumoniae* P394 with either *M. catarrhalis* (Bc1) or *N. meningitidis* (MC58C3). Following growth to mid-log phase, *S. pneumoniae* (P394) was washed and incubated in BHI alone, with *N. meningitidis* (MC58C3) for 1.5 h, or with *M. catarrhalis* (Bc1) for 3 h. Viable counts of *N. meningitidis* (stippled bar) or *M. catarrhalis* (hatched bar) incubated in coculture with *S. pneumoniae* were determined in duplicate by plating on selective media. Viable counts of *S. pneumoniae* in coculture with *N. meningitidis* (black bar) or *M. catarrhalis* (white bar) were determined in duplicate on selective media. Values represent the change in viable count expressed as a percentage of a control culture containing that organism alone. Values are the average of three experiments, and error bars represent the standard deviations.

sponsible for the bactericidal activity of the pneumococcus, 10^8 CFU of *H. influenzae* per ml were cocultured with 5×10^7 CFU of an *S. pneumoniae* strain per ml in which the pyruvate oxidase gene (*spxB*) was insertionally inactivated. This mutant has previously been shown to produce <1% of the H_2O_2 of its parent strain, D39 (38). As expected, the *spxB* mutant was unable to kill *H. influenzae* in coculture experiments, in contrast to its parent strain D39 (Fig. 3). The growth of D39 and that of the *spxB* mutant were indistinguishable under these conditions.

Bactericidal effect of *S. pneumoniae* on other respiratory tract pathogens. The inhibitory effect of *S. pneumoniae* was tested on two other common inhabitants of the human respiratory tract: a clinical isolate of *Moraxella catarrhalis* and an unencapsulated mutant of a type b *N. meningitidis* strain (MC58C3). Catalase-reversible inhibition of *N. meningitidis* by supernatants from *S. pneumoniae* culture was observed on BHI agar. While an inhibitory effect of pneumococcal supernatant was not seen on lawns of *M. catarrhalis*, cross-streaking of *S. pneumoniae* and *M. catarrhalis* on BHI agar revealed a catalase-reversible inhibitory effect on *M. catarrhalis* only in the immediate vicinity of *S. pneumoniae*. Coculture experiments to examine the bactericidal effect on these species showed that 10^8 CFU of *N. meningitidis* per ml incubated with 5×10^7 CFU/ml *S. pneumoniae* for 1.5 h resulted in a catalase-reversible 45 ± 19% decrease in viable count compared to *N. meningitidis* cultured in the absence of *S. pneumoniae* (Fig. 4). *M. catarrhalis* grown at 10^8 CFU/ml in the presence of 5×10^7 CFU of *S. pneumoniae* per ml for 3 h resulted in a catalase-reversible 43 ± 21% decrease in viable counts compared to *M. catarrhalis* grown alone (Fig. 4). In contrast, the viable count of *S. pneumoniae* increased substantially when grown with either *N. meningitidis* or *M. catarrhalis* compared to *S. pneumoniae* grown alone (Fig. 4).

Hydrogen peroxide production and sensitivity to hydrogen peroxide. The relative sensitivities of *S. pneumoniae* P394 and the three other respiratory tract pathogens to hydrogen peroxide were examined using quantitative H_2O_2 challenge assays (Fig. 5). After a 30-min exposure to 0.1 mM H_2O_2, the survival of *S. pneumoniae*, *M. catarrhalis*, and *N. meningitidis* was unaffected, whereas the number of viable *H. influenzae* decreased by approximately twofold. At a concentration of 1.0 mM H_2O_2, the survival of *S. pneumoniae* and *M. catarrhalis* was unaffected, whereas the number of *H. influenzae* decreased approximately 2,000-fold, and the number of *N. meningitidis* decreased approximately 20-fold. At a concentration of 10 mM H_2O_2, *H. influenzae* and *N. meningitidis* decreased to undetectable levels (<100 CFU/ml), whereas the number of *S. pneumoniae* decreased only threefold, and *M. catarrhalis* was unaffected.

A survey of bacterial species was made to determine if the levels of hydrogen peroxide production and resistance exhibited by *S. pneumoniae* are unusual among human pathogens. Of the species tested for peroxide generation, only *S. pneu-*

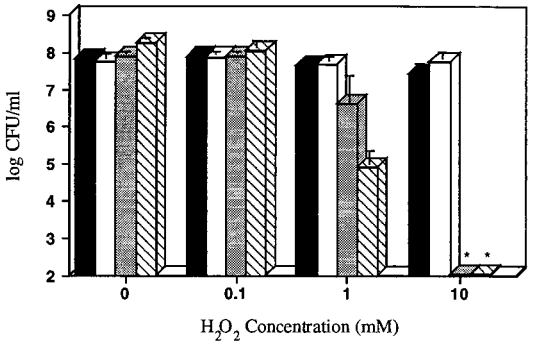

FIG. 5. Effect of H_2O_2 on the survival of *S. pneumoniae* (P394), *M. catarrhalis* (Bc1), *N. meningitidis* (MC58C3), and *H. influenzae* (Rd). Following growth to mid-log phase, *S. pneumoniae* (black bars), *M. catarrhalis* (white bars), *N. meningitidis* (stippled bars), or *H. influenzae* (hatched bars) were washed and incubated at 37°C in BHI or sBHI containing the indicated concentration of H_2O_2. After 30 min, viable counts were determined on BHI or sBHI plates containing 200 U of catalase per ml. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations. *, Below the limit of detection.

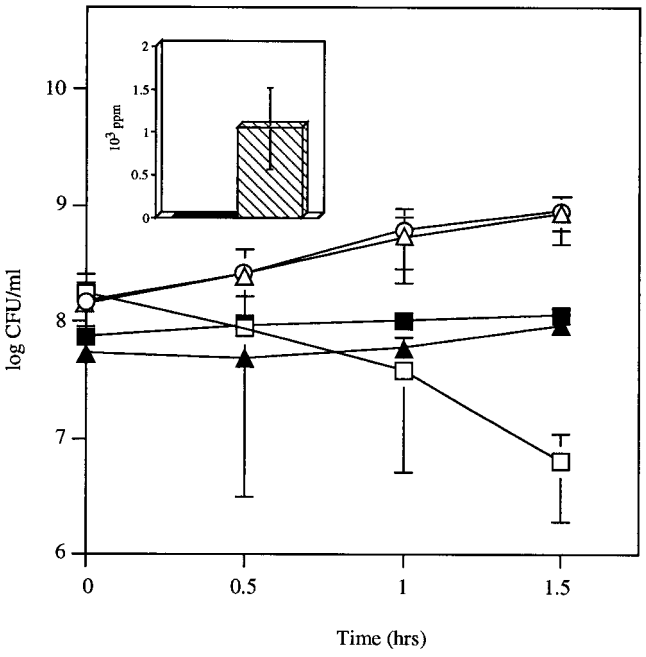

FIG. 6. Effect of coculture of *H. influenzae* Rd with *S. pneumoniae* opaque (P62) or transparent (P64) variants of a type 9V isolate. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI either alone (○), with P62 (△), or with P64 (□) for the times indicated, and viable counts were determined in duplicate on selective media. Viable counts of P62 (▲) or P64 (■) incubated in coculture with *H. influenzae* were determined in duplicate by plating on selective media. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations. (Inset) Relative expression of SpxB in *S. pneumoniae* variants P62 (black bar) and P64 (hatched bar) as determined by two-dimensional gel electrophoresis followed by mass spectrometric analysis. Results represent the average of four independent experiments, with error bars representing the standard deviations.

moniae isolates exhibited production of detectable levels (>0.1 mM) of hydrogen peroxide using a horseradish peroxidase-phenol red assay (Table 1). Survival in different concentrations of exogenously added H_2O_2 varied widely among the species of gram-negative and gram-positive bacteria tested. The species most susceptible to growth inhibition and killing by H_2O_2 was *H. influenzae* (MIC, 0.4 mM; MBC, 0.5 mM). *N. meningitidis* was also relatively sensitive (MIC, 0.4 mM; MBC, 5.0 mM). *M. catarrhalis* was relatively insensitive to the effects of hydrogen peroxide (MIC, 1.1 mM; MBC, 160 mM). The pneumococcus was also relatively insensitive (MIC, 1.6 mM; MBC, 80 mM), thus explaining its ability to survive endogenously produced hydrogen peroxide.

Factors affecting hydrogen peroxide production by *S. pneumoniae*. Strains P62 and P64, two naturally occurring phase variants of the same strain, were tested for H_2O_2 production after it was determined by comparison of two-dimensional gel electrophoresis of whole bacterial proteins followed by microsequencing that the major difference in whole-cell protein expression was in the higher SpxB expression in P64 compared to P62 (Fig. 6, insert) (Overweg et al., submitted). Phase variation in SpxB expression correlated with difference in H_2O_2 generation, with P64 producing significant amounts of H_2O_2, whereas production by P62 was undetectable (Table 1). The bactericidal effect of these variants on *H. influenzae* was then compared in coculture experiments (Fig. 6). After 1.5 h of coculture, the decrease in the viable counts of *H. influenzae* in the presence of P64 was approximately 100-fold, whereas P62 had no effect.

The production of H_2O_2 by the pneumococcus correlated with the concentration of O_2 in the environment, being decreased in microaerobic conditions (data not shown). In order to determine the effect of environmental oxygen on SpxB expression, Western blots were performed on lysates from strain P878, which contains an in-frame fusion of PhoA to SpxB, using an antibody to bacterial alkaline phosphatase. Equal amounts of whole-cell lysates of P878 cultured under various concentrations of O_2 and CO_2 were separated by SDS-PAGE, transferred to a membrane, and immunoblotted. A band corresponding to the SpxB-PhoA fusion protein was detected in samples grown aerobically but was almost completely absent from samples grown anaerobically (Fig. 7). The highest level of expression of SpxB was noted in the conditions of high oxygen and increased carbon dioxide, which correspond to the conditions expected of the mucosal surface of the respiratory tract.

DISCUSSION

This study documents the production of a soluble antimicrobial substance by *S. pneumoniae*. Several lines of evidence demonstrate that this substance is hydrogen peroxide. The effect of the pneumococcus in coculture experiments was completely eliminated by the addition of active but not inactivated catalase. A similar antimicrobial effect was reproduced by the addition of exogenous H_2O_2 at concentrations shown to be generated by the pneumococcus in liquid culture. This effect, furthermore, was absent in a pyruvate oxidase (*spxB*) mutant that synthesizes <1% of parental levels of H_2O_2 as well as a spontaneous variant that is downregulated in expression of SpxB (Overweg et al., submitted). Anaerobic growth conditions also lead to a diminished expression of SpxB which correlated with a loss of antimicrobial effect (data not shown). Finally, the degree of antimicrobial effect against three species was proportional to their sensitivity to both growth inhibition and killing mediated by exogenous hydrogen peroxide.

Among the gram-positive ($n = 6$) and gram-negative ($n = 7$) species tested, the pneumococcus was the only species that generated concentrations of H_2O_2 that were >0.1 mM in liquid culture when at mid-log phase growth in aerobic conditions. For one of the *S. pneumoniae* strains tested, the average H_2O_2 concentration after 1 h of culture was 1.1 mM. This is consistent with the observation that *S. pneumoniae* produce approximately the same amount of H_2O_2 per gram of total cellular protein as neutrophils during the oxidative burst (15). The calculated concentrations of H_2O_2 produced by *S. pneu-*

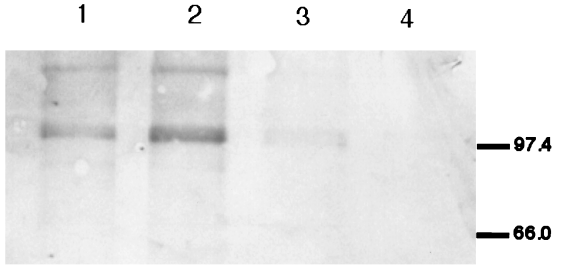

FIG. 7. Western blot showing the effect of environmental oxygen and carbon dioxide tension on pyruvate oxidase (SpxB) expression in *S. pneumoniae* P878, which contains an in-frame fusion to PhoA. Cell lysates of *spxB*::*phoA* mutant (P878) grown under 20% O_2–0.03% CO_2 (lane 1), 17% O_2–3% CO_2 (lane 2), or <0.01% O_2–10% CO_2 (lane 3) were electrophoresed on an SDS–10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and immunoblotted with an antibody to PhoA. As a negative control, cell lysates from the parent strain (D39) grown under 17% O_2–3% CO_2 (lane 4) were included. Size markers are in kilodaltons.

moniae in the present study agree with those previously reported for this species (2, 5, 38). Our results, furthermore, confirmed that the *spxB* mutant was deficient in H_2O_2 production (38). The mechanism that allows for the survival and growth of the pneumococcus, a catalase-negative organism, in substantial concentrations of hydrogen peroxide is unknown. *S. pneumoniae* contains NADH oxidase but lacks other systems involved in the oxidative stress response, such as OxyR (3). It was noted in this study that the mutant deficient in pyruvate oxidase activity often grew to a higher density in liquid culture. A similar effect on pneumococcal growth in liquid culture was observed in the presence of exogenous catalase and in coculture with *M. catarrhalis* or *N. meningitidis*, species that both produce high levels of catalase (37). Furthermore, the pneumococcus requires catalase for optimal growth on solid surfaces where the density of organisms is high (42). These observations support previous findings that endogenous production of hydrogen peroxide is permissive for growth but may have an adverse effect on its rate (2, 20, 34). This negative effect of hydrogen peroxide on growth raises the question as to why the pneumococcus, an organism that does not express catalase activity, synthesizes copious amounts of this highly toxic substance. It has been suggested that H_2O_2 generated by *S. pneumoniae* contributes to the pathogenesis of disease in the respiratory tract by its cytotoxic effects on the epithelial barrier of the host (15, 19). This effect, however, required $\geq 10^8$ CFU/ml, a density of bacteria unlikely to occur in the commensal state for this organism. In contrast, the antimicrobial effect was evident in coculture experiments with as few as 10^6 CFU/ml. Data presented here support the hypothesis that the pneumococcus generates unusually high amounts of hydrogen peroxide as a means of inhibiting and/or killing other species that may compete for the same environmental niche in the heavily colonized human nasopharynx.

Many lactic acid bacteria produce significant amounts of hydrogen peroxide during aerobic growth (50). In fact, several species of lactobacilli and oral streptococci have been shown to produce levels of H_2O_2 in liquid culture similar to that of *S. pneumoniae* (1 to 10 mM) (5, 16, 18, 47). Organisms shown to be killed or inhibited in vitro due to peroxide production by lactic acid bacteria include *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and various other members of the oral flora (13, 16, 41, 50, 52). In the case of the pneumococcus, Colebrook was the first to describe its inhibitory activity by cross-streaking it with *N. meningitidis* and *M. catarrhalis* on solid medium (11). Similarly, McLeod and Gordon reported in 1922 the inhibition of growth of *S. aureus* due to *S. pneumoniae* culture supernatants, an effect they attributed to the presence of hydrogen peroxide (34). Our own study was able to take advantage of a genetically defined mutant that is essentially deficient in H_2O_2 production to confirm this hypothesis about the nature of the inhibitory substance generated by *S. pneumoniae* (38). Moreover, we demonstrate here that this antimicrobial effect may be a factor in the ability of the pneumococcus to compete against the other major pathogens residing in the upper respiratory tract of humans. The antimicrobial effect of the pneumococcus against three gram-negative, catalase-positive species that also colonize the mucosal surface of the human nasopharynx was assessed. The most dramatic effect was seen in coculture experiments with *H. influenzae*, where there was a 4-log decline in viable counts over 3 h due to the presence of 5×10^7 CFU of *S. pneumoniae* per ml. This was the most sensitive bacterial species among those tested to both the inhibitory and the bactericidal effects of the pneumococcus. If a similar effect occurs in vivo, this could at least in part account for the previously noted lower-than-expected rates of coinfection with *S. pneumoniae* and *H. influenzae* in otitis media and chronic bronchitis (25, 30). The inhibitory and bactericidal effects of H_2O_2 on *H. influenzae* occur despite the measurable expression of catalase by this species (8). In other words, a catalase-negative species, *S. pneumoniae*, is able to efficiently kill a catalase-positive species, *H. influenzae*, using H_2O_2. The level of catalase activity as measured by the ability to catalyze the decomposition of hydrogen peroxide, however, varies widely from species to species, and *H. influenzae* seems to be an example of a catalase-positive organism with relatively low catalase activity as measured in vitro (7, 33). *H. influenzae* possesses only one gene for catalase, unlike the other gram-negative species *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella flexneri*, which produce two catalases (8). A previously reported catalase-deficient mutant of *H. influenzae*, strain AB2593 (Rd::*hktE*$^-$) was not significantly more sensitive to the antimicrobial effect of the pneumococcus compared to its parent strain, implying that catalase does little to protect *H. influenzae* under these conditions (data not shown) (8). *H. influenzae* may possess an impaired ability to upregulate catalase production in response to elevated levels of H_2O_2, possibly as a result of *H. influenzae*'s inability to synthesize protoporphyrin IX, the biosynthetic precursor of heme, a required component of catalase (48). This finding is consistent with the observation that 10^8 CFU of exponentially growing *H. influenzae* produce only 5.7 U of catalase, and this expression level is induced only threefold by oxidative stress (8). Furthermore, the addition of *H. influenzae* to cultures of *S. pneumoniae* had only a small effect on the hydrogen peroxide concentration, suggesting that the endogenous production of catalase by *H. influenzae* was insufficient for these levels of H_2O_2 (data not shown). The effect of the pneumococcus was less dramatic against the meningococcus, where growth inhibition and minimal killing were observed after 1.5 h of coculture. When *M. catarrhalis*, a target species with markedly greater catalase activity, was tested, only a slight inhibitory bactericidal effect was evident after 3 h of coculture, although a catalase-reversible effect was noted with a higher density of pneumococci when the two organisms were cross-streaked on BHI agar.

In considering the contribution of hydrogen peroxide production to pneumococcal carriage, it should be noted that the studies presented here are based exclusively on in vitro effects. The synthesis of H_2O_2 by the pneumococcus in vivo has not been determined, although maximal expression of SpxB was noted in an oxygen and carbon dioxide rich environment, as would be expected on the surface of the upper respiratory tract. In addition, the antimicrobial effect correlated with variability in the expression of SpxB and was present in a variant with a transparent colony phenotype but not the opaque variant of the same isolate (Overweg et al., submitted). Only the transparent form has been shown to persistently colonize the nasopharynx in an animal model of carriage (42). This suggests that the increased production of H_2O_2 associated with this phenotype may contribute to its ability to efficiently colonize a host, whereas the opaque phenotype may be outcompeted by the other flora. Another consideration in extrapolating these results to the situation in vivo is that host factors on the mucosal surface may act to inactivate bacterial hydrogen peroxide. In this regard, viridans streptococci, which may generate concentrations of hydrogen peroxide similar to that of *S. pneumoniae*, have been suggested to prevent colonization of gram-negative bacilli, including *H. influenzae*, in the human oropharynx by a mechanism that may be mediated in part by H_2O_2 production (39, 40). In addition, the *spxB* mutant of *S. pneumoniae* does not persist within the airway in an animal

model of colonization in rabbits (38). Although the mechanism for this defect in carriage is unknown and there are several plausible explanations, it is possible that it results from an inability of the mutant to suppress local competitors. Future studies will address the significance of these observations to pneumococcal carriage and the maintenance of the normal microflora of the upper respiratory tract.

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