JOURNAL OF BACTERIOLOGY, July 2006, p. 4996–5001 Vol. 188, No. 13
0021-9193/06/$08.00+0 doi:10.1128/JB.00317-06
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Interference between *Streptococcus pneumoniae* and *Staphylococcus aureus*: In Vitro Hydrogen Peroxide-Mediated Killing by *Streptococcus pneumoniae*

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Received 3 March 2006/Accepted 21 April 2006

The bactericidal activity of *Streptococcus pneumoniae* toward *Staphylococcus aureus* is mediated by hydrogen peroxide. Catalase eliminated this activity. Pneumococci grown anaerobically or genetically lacking pyruvate oxidase (SpxB) were not bactericidal, nor were nonpneumococcal streptococci. These results provide a possible mechanistic explanation for the interspecies interference observed in epidemiologic studies.

Nasal *Staphylococcus aureus* and nasopharyngeal *Streptococcus pneumoniae* colonization serve as a source for infection of other sites and for transmission between humans (12, 24). Epidemiological studies have suggested that nasopharyngeal carriage of *S. pneumoniae* may inhibit nasal carriage of *S. aureus* (4, 17). Hydrogen peroxide produced by *S. pneumoniae* has been reported to be toxic in vitro to other bacteria (11, 15) and to mammalian cells (2, 5, 9). H_2O_2 is produced by pyruvate oxidase (SpxB) as a by-product of aerobic metabolism (19). We sought to test the hypothesis that *S. pneumoniae* directly inhibits *S. aureus* in vitro by an H_2O_2-dependent mechanism.

(The results of this study were partially presented at the 45th Interscience Conference of Antimicrobial Agents and Chemotherapy, Washington, D.C., October 2005.)

Bacterial strains used in this study are described in detail in Table 1. *S. pneumoniae* strains used were TIGR4 (21), Rx1 (16), and Pn-20. In addition, SpxB-negative variants of TIGR4 and Pn-20 and LytA-negative variants of Rx1 and Pn-20 were created using the Janus cassette (20). Strains of three other *Streptococcus* spp.—*S. gordonii*, group A streptococcus, and group C streptococcus—were also tested. Staphylococcal species used in the study were the following: *S. aureus* strains Newman (NCTC 8178) and ALR and coagulase-negative staphylococci (CNS) (*Staphylococcus epidermidis*, *Staphylococcus xylosus*, and *Staphylococcus sciuri)*. Bovine liver catalase (1,000 U/ml; MP Biomedicals, Inc., Solon, OH) was added to growth medium when specified. Bacterial H_2O_2 production was measured colorimetrically in culture supernatants (8).

To quantify bactericidal activity, we cocultured bacterial strains in brain heart infusion (BHI) and evaluated their survival on selective media. Serial twofold dilutions of the staphylococcal strain were mixed with twofold serial dilutions of the streptococcal strain in a final volume of 100 μl (see legend to Fig. 1 for more details). Cultures with and without catalase were incubated at 37°C in 5% CO_2. At time zero and 6 h, approximately 2 μl of each culture was plated on selective medium using a replica plater (Sigma, Aldrich).

For a given *S. aureus* inoculum, a higher *S. pneumoniae* inoculum led to greater killing (Fig. 1A and B). The diagonal pattern of killing (Fig. 1B) suggests that the inoculum of *S. aureus* that could be killed completely was approximately linearly proportional to the pneumococcal inoculum. Killing was eliminated by the addition of catalase (Fig. 1C to D). No inhibition of *S. pneumoniae* by *S. aureus* was observed (Fig. 1E to F).

To quantify bactericidal efficiency, we report the maximum staphylococcal inoculum "killed" (reduced below the detection limit of 50 CFU/well) by 10^6 CFU of streptococci at 6 h (IK6). Tables 2 and 3 compare the killing efficiencies of different streptococci and the susceptibilities of different staphylococci. SpxB-negative strains did not produce detectable amounts of H_2O_2 and were minimally bactericidal to *S. aureus*. The maximum inoculum of *S. aureus* killed by *S. pneumoniae* Pn-20Δ*spxB* was 1.2 $\log_{10}$ CFU (IK6 = 1.2), 5 orders of magnitude lower than that of the parental strains (Table 2); catalase abolished the residual killing activity. Other streptococcal species that did not produce detectable levels of H_2O_2 demonstrated little or no bactericidal effect (Table 2).

To verify that *S. aureus* is inhibited by a soluble factor produced by *S. pneumoniae* and that this factor is H_2O_2, we evaluated the growth of *S. aureus* (Newman) in filtered pneumococcal supernatant and compared it to growth in fresh BHI and in coculture with *S. pneumoniae* (Pn-20). Figure 2 demonstrates a bactericidal effect of sterile supernatant from aerobic cultures of *S. pneumoniae* to *S. aureus* within 6 h (Fig. 2A). This effect was mitigated either when catalase was added to the medium or with supernatant from anaerobically grown *S. pneumoniae*, which does not produce H_2O_2 (14) (Fig. 2B).

S. pneumoniae variants lacking the major autolytic enzyme LytA exhibited bactericidal activities similar to those of their

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TABLE 1. Bacterial strains and primers used in this study

Strain or primer	Description	Source or reference
S. pneumoniae		
TIGR4		21
TIGR4S	TIGR4 but streptomycin resistant by selection of mutants	22
CP1296	R6 derivative, *cbp3*::*kan-rpsL*$^+$	20
TIGR4Δ*spxB*	TIGR4S but *spxB*::*kan-rpsL*$^+$ by transformation with ligation product of PCR fragments amplified with primer pairs TTM211-TTM212 in TIGR4, DAM406-DAM351 in CP1296, and TTM213-TTM214 in TIGR4, and prior to ligation digested with BamHI, BamHI and ApaI, and ApaI, respectively	This study
Pn-20	Serotype 35B, nasopharyngeal human isolate	This study
Pn-20Δ*spxB*	Pn-20s but *spxB*::*kan-rpsL*$^+$ by transformation with TTM211-TTM214 PCR product of TIGR4Δ*spxB*	This study
Pn-20Δ*lytA*	Pn-20s but *lyt*A::*kan-rpsL*$^+$ by transformation with LAD4-LAD1 PCR product of Rx1Δ*lytA*	This study
Rx1		18
R6		10
Rx1S	Rx1 but streptomycin resistant by selection of mutants	
Rx1Δ*lytA*	Rx1S but *lytA*::*kan-rpsL*$^+$ by transformation with ligation product of PCR fragments amplified with primer pairs LAD4-LAD3 in R6, DAM406-DAM351 in CP1296, and LAD2-LAD1 in R6 and prior to ligation digested with BamHI, BamHI and ApaI, and ApaI, respectively	This study
S. gordonii strain GP251		13
Group A streptococcus strain 771		Kindly supplied by Michael Wessels
Group C streptococcus		This study
S. aureus		
Strain Newman		NCTC 8178
Strain ALR	Nasal isolate from a Wistar rat	Kindly supplied by Jean Lee
S. sciuri	Nasal isolate from an ICR mouse	Kindly supplied by Jean Lee
S. epidermidis strain RP62a		ATCC 35984
S. xylosus	Nasal isolate from a C57BL/6 mouse	This study
Primers		
DAM406	TCTATGCCTATTCCAGAGGAAATGGAT	22
DAM351	CTAGGGCCCCTTTCCTTATGCTTTTGGAC	20
TTM211	CGTTAAGCGAGCGAGTGA corresponds to positions 1002-985 upstream of *spxB* in TIGR4	This study
TTM212	TTTGGATCCGATGCAGTAATTTTCCCTTGAGTC positions 10-34 correspond to positions 26-3 within *spxB* in TIGR4	This study
TTM213	AAAGGGCCCTCTCGCCGAAAATCAAATA positions 8-28 correspond to positions 3-23 downstream of *spxB* in TIGR4	This study
TTM214	CGCCACGTCCAGAACATCC corresponds to positions 1199-1181 downstream of *spxB* in TIGR4	This study
LAD1	CAAGGTATCCATCATTCC corresponds to 1011-993 downstream of *lytA* in R6	This study
LAD2	CGCGGATCCACAGTAGAGCCAGATGGC positions 9-27 correspond to positions 921-939 within *lytA* in R6	This study
LAD3	TTGGGCCCGTTGCACGCCGACTTGAGG positions 10-27 correspond to positions 37-54 within *lytA* in R6	This study
LAD4	CTTTGCTTCTCAGAATCTAGG corresponds to 850-830 upstream of *lytA* in R6	This study

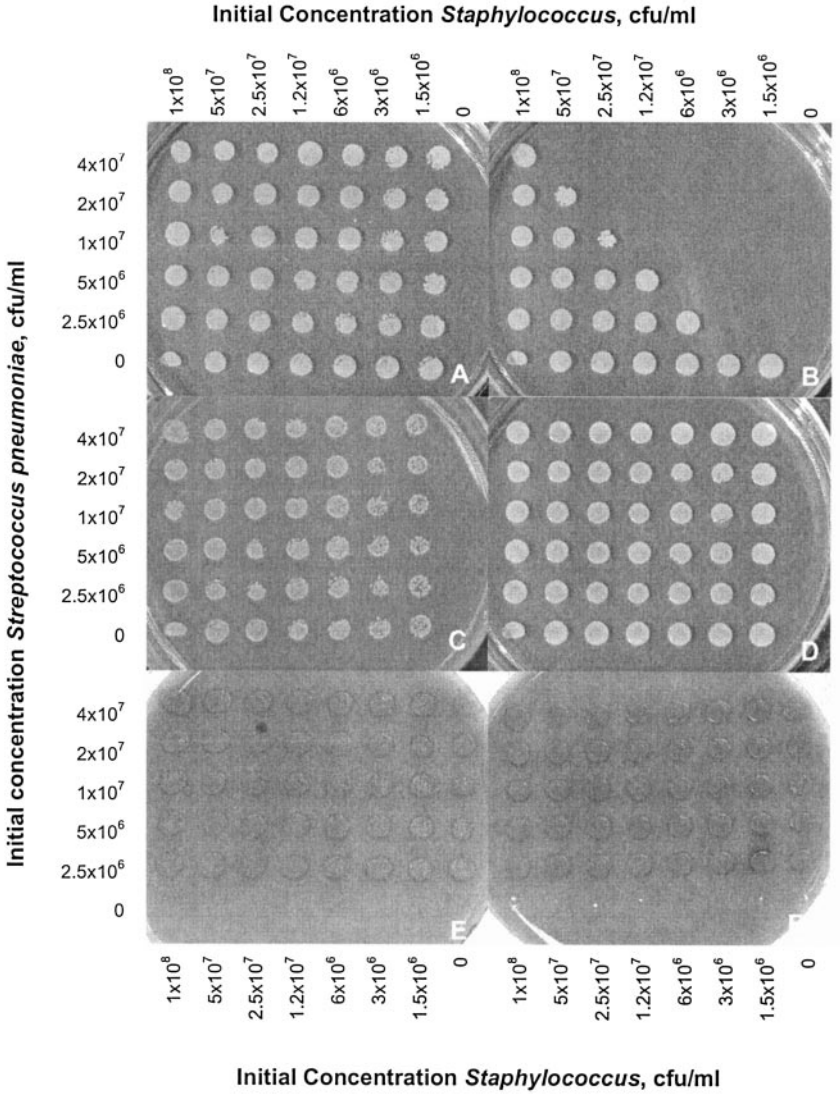

FIG. 1. Growth of *S. aureus* and *S. pneumoniae* on selective media after replica plating from cocultures. Columns of the plate contain twofold dilutions of the staphylococcal strain beginning with 10^8 CFU/ml in the left column. Rows contain twofold dilutions of the streptococcal strain beginning with 4×10^7 CFU/ml. The right column contains the staphylococcal strain only, and the bottom row contains the streptococcal strain only. The bottom right corner well contains uninoculated BHI. A and B—*S. aureus* (Newman) after 0 and 6 h, respectively, grown on selective medium (tryptic soy agar supplemented with 5 μg/ml optochin); C and D—*S. aureus* (Newman) after 0 and 6 h in coculture supplemented with catalase (anaerobic conditions yielded identical results to those shown in panels C to D); E and F—*S. pneumoniae* (Pn-20) after 0 and 6 h, respectively, grown on selective medium (tryptic soy agar supplemented with 5% sheep blood and 8 μg/ml gentamicin).

TABLE 2. Various bactericidal efficiencies of different streptococcus strains against *S. aureus* strain Newman[a]

Streptococcus strain	Bactericidal efficiency (IK6) ($\log_{10}$ CFU ± SD)	Concn of H_2O_2 (mM) in supernatant of 7.5 $\log_{10}$ CFU of *S. pneumoniae* culture
S. pneumoniae (Pn-20)	6.4 ± 0.3	1.60 ± 0.58
S. pneumoniae (Pn-20Δ*spxB*)	1.2 ± 0.8	<0.05
S. pneumoniae (Pn-20Δ*lytA*)	6.2 ± 0.1	1.60 ± 0.87
S. pneumoniae (TIGR4)	6.3 ± 0.3	0.12 ± 0.07
S. pneumoniae (TIGR4Δ*spxB*)	1.4 ± 0.2	<0.05
S. pneumoniae (Rx1)	5.8 ± 0.5	0.20 ± 0.17
S. pneumoniae (Rx1Δ*lytA*)	5.8 ± 0.6	0.43 ± 0.05
S. gordonii	3.8 ± 0.6	<0.05†
Group A streptococcus	0*	<0.05†
Group C streptococcus	0*	<0.05†

[a] IK6, maximal *S. aureus* Newman inoculum killed by 10^6 CFU of streptococci at 6 h. IK values are averages from at least three independent experiments ± SD. *, detection level = 50 CFU/well; †, detection level = 0.05 mM.

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TABLE 3. Susceptibilities of various staphylococci to *S. pneumoniae* strain Pn-20 bactericidal effect and to external H_2O_2 and their catalase activities

Staphylococcus strain	Susceptibility to *S. pneumoniae* killing IK6 (log_{10} CFU ± SD)[a]	H_2O_2 susceptibility (Δlog_{10} CFU)[b]	Catalase activity (mU per 10^8 CFU)
S. aureus (Newman)	6.4 ± 0.3	−2.5	161
S. aureus (Newman) in medium supplemented with catalase	0*	+1.5	ND
S. aureus (Newman) under anaerobic conditions	0*	ND[c]	ND
S. aureus (ALR)	5.35 ± 0.93	−1.5	ND
S. epidermidis	<2	+0.3	524
S. xylosus	<2	+0.2	1,050
S. sciurii	<2	+0.4	7,334

[a] IK6 = maximal staphylococcal inoculum killed by 10^6 CFU of *S. pneumoniae* (Pn-20) at 6 h. *, detection level = 50 CFU/well. IK values are averages from at least three independent experiments ± SD.
[b] Net reduction in log_{10} CFU at 6 h compared to the initial 7.7 log_{10} CFU of *S. aureus* in medium supplemented with H_2O_2 periodically.
[c] ND, not done.

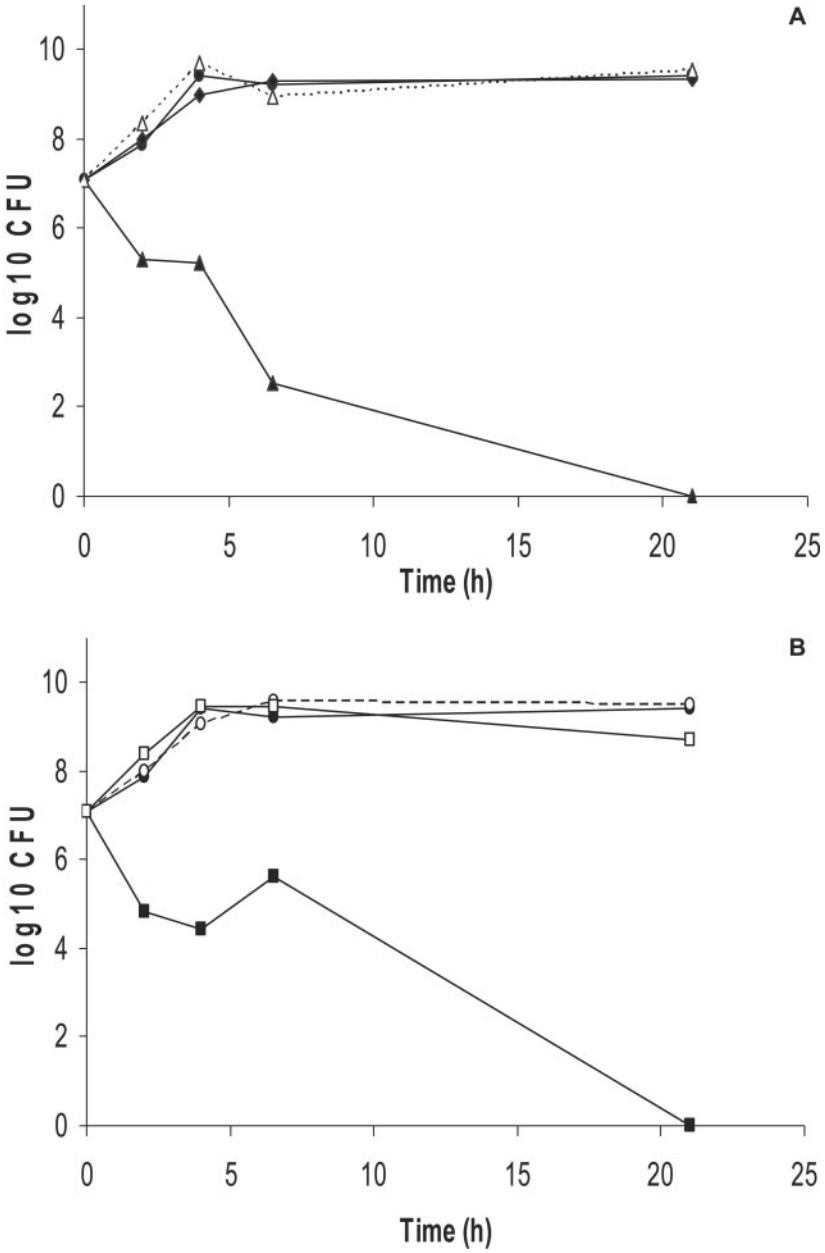

FIG. 2. *S. aureus* growth (A) in coculture with *S. pneumoniae* under aerobic conditions (▲), coculture in anaerobic conditions (◆), coculture with periodic addition of catalase (△), or plain BHI (●). (B) In sterile supernatant from *S. pneumoniae* cultures (■), in supernatant with catalase supplementation (□), in plain BHI (●), or in BHI supplemented with catalase (○).

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parent strains (Table 2). This finding excludes a potential accessory role for this toxic enzyme, while also serving as a control to demonstrate that the significantly reduced bactericidal activity of Δ*spxB* strains was a direct result of deficient SpxB activity and not by the genetic manipulation itself.

S. aureus Newman was more susceptible to *S. pneumoniae*'s bactericidal effect than strain ALR. CNS strains showed undetectable inhibition by *S. pneumoniae* (Pn-20) in cocultures (IK6 < 2). These results correlated (Table 3) with the susceptibilities of the staphylococcal strains to H_2O_2 added to the medium (1 μmol every 90 min) as well as to the catalase activities of these strains measured with the Amplex Red catalase assay kit (Molecular Probes, Eugene, OR). External H_2O_2 added periodically reduced CFU/ml of *S. aureus* strains by 1.5 to 2.5 $\log_{10}$, while the neutralization of this external H_2O_2 by exogenous catalase permitted an increase of 1.5 $\log_{10}$ CFU/ml. In contrast, under the same conditions (with no addition of catalase), the numbers of CFU/ml for CNS strains increased slightly (0.5 $\log_{10}$) (Table 3).

It is striking that *S. aureus*, a catalase-positive species, is so susceptible to a H_2O_2-producing bacteria. The two different *S. aureus* strains tested were highly susceptible to *S. pneumoniae*, while CNS species were highly resistant to *S. pneumoniae*. All staphylococci elaborate catalase, with variation between species in the number and expression of catalase enzymes (1, 3). Indeed the staphylococcal species that were resistant to *S. pneumoniae* secreted higher concentrations of catalase than *S. aureus*; however, the concentrations detected may not linearly reflect differences in intracellular catalase, and the greater antioxidant capacity of these species might be due to additional factors not measured in this study.

Our impetus for this study was the epidemiologic data showing an inverse association between *S. aureus* and *S. pneumoniae* colonization (4, 17). H_2O_2-mediated, unidirectional antagonism as observed here in vitro provides one possible mechanism for this inverse association. If the association is causal and acquisition of *S. pneumoniae* eradicates *S. aureus* carriage, then use of pneumococcal vaccines may eliminate the "protective" effect of *S. pneumoniae* against *S. aureus* carriage and an increase in *S. aureus* carriage will follow. Increased *S. aureus* otitis media has been observed among vaccinees in a pneumococcal conjugate vaccine randomized trial (23). Whether the current increase in severe community-acquired *S. aureus* infections, including methicillin-resistant *S. aureus* (6), is partially caused by the recent introduction of the pneumococcal conjugate vaccine is yet to be determined.

This study provides only the first step towards elucidating the mechanism of *S. pneumoniae*-*S. aureus* interference in vivo. In vivo interference could be caused by a direct bactericidal effect similar to that observed here. Alternatively, or in addition, it could occur via a host cell signaling pathway. Interestingly, such an indirect pathway might also involve H_2O_2, which plays key roles in cell signaling, particularly in regulation of the production of cytokines (7, 25). Future in vivo studies are required to address these important remaining questions.

We thank Michael Wessels for providing *Streptococcus* group A, Jean Lee for providing the staphylococcus strains used in the study and for critical review of the manuscript, and Amit Srivastava for thoughtful advice.

This study was supported by NIH grant 1R01AI48935 to M.L. and 1R01AI067737-01 and the Pamela and Jack Egan Fund to R.M.

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