JOURNAL OF BACTERIOLOGY, Apr. 2008, p. 2275–2278 Vol. 190, No. 7
0021-9193/08/$08.00+0 doi:10.1128/JB.00006-08
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Role of *Staphylococcus aureus* Catalase in Niche Competition against *Streptococcus pneumoniae*▽

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Received 2 January 2008/Accepted 15 January 2008

Nasal colonization by *Staphylococcus aureus* is a major predisposing factor for subsequent infection. Recent reports of increased *S. aureus* colonization among children receiving pneumococcal vaccine implicate *Streptococcus pneumoniae* as an important competitor for the same niche. Since *S. pneumoniae* uses H_2O_2 to kill competing bacteria, we hypothesized that oxidant defense could play a significant role in promoting *S. aureus* colonization of the nasal mucosa. Using targeted mutagenesis, we showed that *S. aureus* expression of catalase contributes significantly to the survival of this pathogen in the presence of *S. pneumoniae* both in vitro and in a murine model of nasal cocolonization.

Staphylococcus aureus causes a wide range of infections ranging from minor skin infections to life-threatening invasive diseases. The emergence of methicillin-resistant strains with high virulence potential in both hospital and community settings is contributing to a current public health crisis (9, 12, 13).

A major risk factor for *S. aureus* infection is antecedent colonization of the nasal mucosa (19). Successful colonization depends not only on the ability of *S. aureus* to survive host factors (4, 6) but also on coexistence with other bacteria (16, 21).

The latter concept has been underscored by two recent reports that implicate *Streptococcus pneumoniae* as a primary competitor for niche colonization (3, 15).

Specifically, one surveillance study performed in an area where pneumococcal vaccination was not practiced showed that the *S. pneumoniae* carriage rate in children was negatively associated with *S. aureus* nasal carriage (15). The other study showed that children with recurrent otitis media vaccinated with the 7-valent pneumococcal vaccine had an increased incidence of *S. aureus*-related acute otitis media and *S. aureus* colonization after vaccination (3), suggesting that there is a natural competition for colonization between *S. aureus* and *S. pneumoniae*.

S. pneumoniae produces H_2O_2 as an antimicrobial factor to reduce competition by other upper respiratory pathogens, such as *Haemophilus influenzae*, *Neisseria meningitides*, *Moraxella catarrhalis*, and *S. aureus* (14, 16). Since *S. aureus* is a natural colonizer of the human nares, we hypothesized that its success derives in part from a relative resistance to H_2O_2 killing by other microflora. Here we tested this hypothesis by generating a catalase knockout mutant strain of *S. aureus* and examining the role of enzymatic H_2O_2 inactivation in niche competition with *S. pneumoniae*.

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▽ Published ahead of print on 25 January 2008.

MATERIALS AND METHODS

Bacterial strains, media, and mice. *S. aureus* strains were cultured at 37°C in Todd-Hewitt broth (THB) or on Todd-Hewitt agar (THA) (Difco). *S. pneumoniae* TIGR4 was cultured in THB with 0.5% yeast extract (THY) at 37°C in a 5% CO_2 incubator. Eight- to 10-week old female CD1 mice were purchased from Charles River Laboratories, Wilmington, MA. When included, antibiotics were added at the following concentrations: 100 μg ampicillin/ml, 50 μg erythromycin/ml, and 100 μg spectinomycin/ml.

Generation of catalase-deficient *S. aureus* ΔKatA mutant. In-frame allelic replacement of the *S. aureus katA* gene with a spectinomycin adenyltransferase (*spec*) cassette was performed using PCR-based methods as described previously (11), with minor modifications. Primers were designed based on the previously published *S. aureus katA* sequence cross-referenced to the genome of *S. aureus* strain N315 (10). PCR was used to amplify 500 bp upstream of *katA* with primers *kat*AupF (5′-ATGGTCGACTATGACATCAACACTTGTAAC-3′) and *kat*AupR (5′-TCA AATATATCCTCCTCATCCCTCCACAATTTATAATAAT-3′) along with 500 bp of sequence immediately downstream of *katA* with primers *kat*AdownF (5′-AA TAACAGATTAAAAAAATTATAAATTTGATATGTAGTTTCTATA-3′) and *kat*AdownR (5′-ATCGGATCCTACCCAGAATTACTTCGTACT-3′). The *kat*AupR and *kat*AdownF primers were constructed with 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the *spec* gene, respectively. The upstream and downstream PCR products were then combined with a 650-bp amplicon of the complete *spec* gene for use as templates in a second round of PCR using primers *kat*AupF and *kat*AdownR. The resultant PCR amplicon, containing an in-frame substitution of *katA* with *spec*, was subcloned into temperature-sensitive vector pMAD (1) to create the knockout plasmid. This vector was transformed initially into permissive *S. aureus* strain RN4220 and then into *S. aureus* strain Newman by electroporation. Transformants were grown at 30°C and shifted to the nonpermissive temperature for plasmid replication (40°C), and differential antibiotic selection and blue-white color selection with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used to identify candidate mutants. Allelic replacement of the *katA* allele was confirmed unambiguously by PCRs that documented targeted insertion of *spec* and the absence of *katA* in chromosomal DNA isolated from the final mutant, which was designated the ΔKatA mutant.

Complementation studies. Primers katAF_KpnI (5′-ATAGGTACCTCCCAT GGTAAAGCCAAGAG-3′) and katAR_BamHI (5′-ATAGGATCCTTTACGC GCACGTTAAACAC-3′) were used to amplify the *katA* gene from the chromosome of wild-type (WT) *S. aureus* strain Newman. The fragment was directionally cloned into the shuttle expression vector pDCerm (8), and the recombinant plasmid (pKatA) was used to transform the *S. aureus* ΔKatA mutant by electroporation. For the complementation studies, the isogenic WT and ΔKatA *S. aureus* strains were transformed with the control pDCerm plasmid. Strains containing the pDCerm or pKatA plasmid were maintained in THB or on THA containing erythromycin.

H_2O_2 susceptibility assay. H_2O_2 susceptibility assays were performed using overnight *S. aureus* cultures grown at 37°C with shaking. Bacteria were harvested by centrifugation, suspended in phosphate-buffered saline (PBS) at a concentra-

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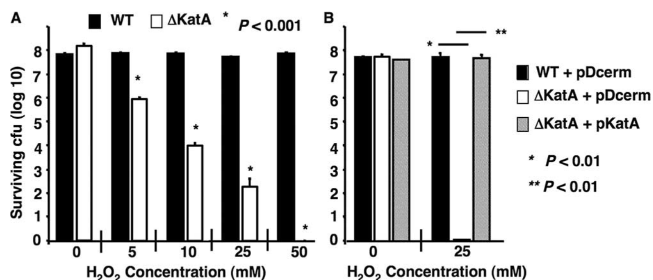

FIG. 1. *S. aureus* catalase confers resistance to H_2O_2 killing. (A) Susceptibility of WT and ΔKatA *S. aureus* strains to different concentrations of H_2O_2. (B) Restoration of resistance to H_2O_2 killing upon complementation of the ΔKatA mutant with pKatA. All experiments were performed at least three times, and similar results were obtained.

tion of 5×10^7 CFU/ml, and mixed with various concentrations of H_2O_2. The killing assay was terminated after 2 h of incubation at 37°C by addition of 5,000 U/ml of catalase (Sigma), which was followed by enumeration of surviving bacterial CFU on THA.

Susceptibility of *S. aureus* to *S. pneumoniae* killing in vitro. (i) Plate assay. Overnight *S. aureus* cultures were centrifuged, washed, and suspended in PBS at a concentration of 5×10^8 CFU/ml. Two hundred microliters was plated on THY plates, and a paper disk impregnated with 1.5×10^9 log-phase *S. pneumoniae* cells was placed in the center of each plate. The zone of *S. aureus* growth inhibition was measured after 24 h of incubation at 37°C in the presence of 5% CO_2.

(ii) Liquid culture-based assay. Overnight *S. aureus* cultures were centrifuged, washed in PBS, diluted to obtain a concentration of 1×10^9 CFU/ml, and mixed with log-phase *S. pneumoniae* at a ratio of 1:1, 1:5, or 1:10 in THY. After 4 h of incubation at 37°C in the presence of 5% CO_2, the remaining H_2O_2 was quenched with 50 μl of a 5,000-U/ml exogenous catalase solution, and the surviving *S. aureus* cells were diluted in PBS and plated on THA plates. As a control, parallel experiments were performed in an identical fashion in the presence of 1,000 U/ml catalase.

Murine nasal cocolonization studies. Mice were inoculated intranasally with a 10 μl of a mixture containing 10^8 WT cells and 10^8 *S. aureus* ΔKatA cells. After 30 min, the mice were divided into two groups and given either 10 μl of THY or 3×10^8 early-stationary-phase *S. pneumoniae* cells in THY. After 3 days, the mice were sacrificed, the nasal tissue was homogenized and vortexed for 5 min in PBS, and the CFU were enumerated on THA with or without spectinomycin after appropriate dilution. Occasional contaminants were excluded during counting of the CFU by the morphology or color of the bacterial colonies. Animal experimentation guidelines were followed in the animal studies.

Statistical analysis. The significance of experimental differences in H_2O_2 sensitivity and *S. pneumoniae* killing in vitro was evaluated by using the unpaired Student *t* test. The results of the mouse in vivo challenge studies were evaluated by using the nonparametric two-tailed Wilcoxon and Mann-Whitney tests.

RESULTS

To address the role of catalase in niche competition, a KatA deletion mutant of *S. aureus* strain Newman was generated by allelic replacement of the *katA* gene with a spectinomycin acetyltransferase cassette. Deletion of the *katA* gene was confirmed by PCR and by the absence of effervescence upon exposure of the ΔKatA mutant to H_2O_2 (data not shown).

To assess the effect of *katA* deletion on *S. aureus* susceptibility to H_2O_2, the WT and ΔKatA strains were exposed to a range of H_2O_2 concentrations in PBS. In the absence of catalase, *S. aureus* was highly susceptible to H_2O_2 killing (Fig. 1A). Complementation with pKatA restored the ability of the ΔKatA mutant to resist H_2O_2 killing (Fig. 1B). The pDCerm vector used for complementation was also placed into the ΔKatA mutant, and it had no impact on H_2O_2 susceptibility.

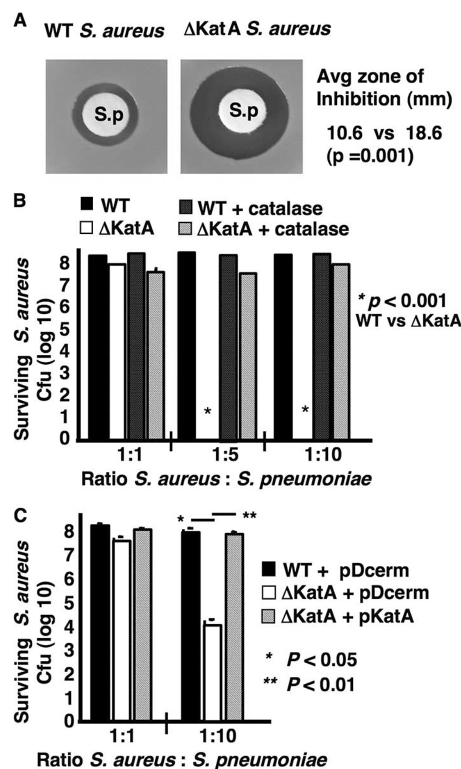

FIG. 2. Catalase protects *S. aureus* against *S. pneumoniae* killing in vitro. (A) Effect of a disk impregnated with *S. pneumoniae* on growth of the WT or ΔKatA *S. aureus* strain. (B) Survival of the WT or ΔKatA *S. aureus* strain upon coculture with *S. pneumoniae* at ratios of 1:1, 1:5, and 1:10. (C) Restoration of resistance to *S. pneumoniae* killing upon complementation of the ΔKatA mutant with pKatA. All experiments were repeated at least three times, and similar results were obtained.

Since *S. pneumoniae* produces H_2O_2 in quantities sufficient to kill other bacterial species (14), we tested whether catalase has an important survival function for *S. aureus* when it is cultured in the presence of *S. pneumoniae*. As shown in Fig. 2A, a disk impregnated with log-phase *S. pneumoniae* cells partially inhibited growth of the WT *S. aureus* strain on a THY plate but had a much more profound effect on the growth of the isogenic ΔKatA mutant.

In a more quantitative liquid culture-based assay, at a ratio of *S. aureus* to *S. pneumoniae* of 1:1, minimal killing of WT or mutant *S. aureus* was noted (Fig. 2B). However, at a ratio of 1:5 or 1:10, the survival of the ΔKatA mutant in the presence of *S. pneumoniae* was reduced by as much as 8 logs compared to the survival of the parent strain (Fig. 2B). The differential killing was most likely a result of H_2O_2 production by *S. pneumoniae*, since no killing of *S. aureus* was observed if an exogenous source of catalase was added to the culture at the start of the assay (Fig. 2B). Complementation with pKatA restored the ability of the ΔKatA mutant to resist *S. pneumoniae* killing (Fig. 2C).

Next, to extend the biological relevance of these findings, the role of *S. aureus* catalase was assessed using a murine model of nasal colonization. In this study, mice were inoculated intranasally with equal numbers of WT and ΔKatA *S. aureus* cells with or without *S. pneumoniae*. After 3 days, the surviving WT

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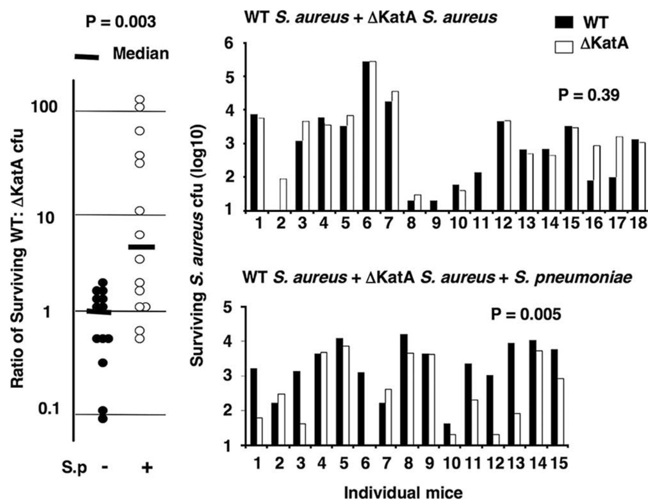

FIG. 3. Catalase protects *S. aureus* against *S. pneumoniae* killing in a murine model of nasal colonization. Mice were inoculated intranasally with a 1:1 mixture of the WT and ΔKatA *S. aureus* strains. After 30 min, the mice were inoculated in the same nostrils with either buffer or *S. pneumoniae* at a ratio of *S. pneumoniae* to *S. aureus* of 3:1. Surviving bacteria from the nostrils were quantitated after 3 days. The graph on the left shows the ratios of the surviving WT *S. aureus* strain to the surviving the ΔKatA *S. aureus* mutant for individual mice. The numbers of surviving WT and ΔKatA *S. aureus* CFU recovered from each mouse are plotted on the right. Mice that were poorly colonized (≤5 WT CFU and ≤5 ΔKatA CFU as enumerated on THA plates) were excluded from the surviving ratio plot (left) but were included in the survival graphs on the right. The data were compiled from three experiments performed in the same way. The minimum detection level of the assay is 20 CFU. S.p, *S. pneumoniae*.

and ΔKatA cells were harvested from the noses of the mice. As shown in Fig. 3, the survival of the WT strain and the survival of the ΔKatA strain in noses of mice did not differ significantly at day 3 when they were inoculated alone, but a notable difference in the levels of survival in favor of WT *S. aureus* was apparent in mice given *S. pneumoniae* as a competitor for the same niche.

DISCUSSION

Multiple studies have shown that colonization of the upper airway with *S. pneumoniae* is negatively correlated with *S. aureus* colonization, and introduction of the *S. pneumoniae* vaccine has increased the rate of *S. aureus* nasal colonization (3, 15). By eradicating carriage of *S. pneumoniae* vaccine strains, immunization removes an important niche competitor that utilizes H_2O_2 to restrict colonization by other bacteria. In this study, we showed that the *S. aureus* catalase is a major factor in *S. aureus* defense against *S. pneumoniae* killing due to neutralization of secreted H_2O_2. H_2O_2 is used as an antimicrobial factor by many other microbes (2, 18), including *Streptococcus sanguinis* in the oral cavity and lactobacilli in the vagina, two sites frequently cocolonized by *S. aureus*. Thus, it could be speculated that in *S. aureus* catalase is an important tool for securing a niche on multiple mucosal surfaces in the human host. The presence of catalase may also explain the preferential survival of WT *S. aureus* compared to the ΔKatA mutant in the cotton rat model of nasal colonization previously reported by Cosgrove and coworkers (5).

S. aureus encodes a number antioxidants, including, alkyl hydroperoxide reductase, and staphyloxanthin, which may supplement catalase in defense against H_2O_2-producing organisms, such as *S. pneumoniae*.

Although catalase is a factor produced by many bacteria, several studies have failed to establish a function for catalase in systemic virulence (7, 17, 20). Our finding that catalase plays an important role in *S. aureus* in mucosal niche competition points to an alternative role that catalase could play in the most proximal steps of disease pathogenesis. The *S. aureus* catalase could thus be a novel pharmacologic target for decolonization strategies, a desirable therapeutic endpoint in many clinical scenarios.

ACKNOWLEDGMENTS

We thank Terence Doherty for critical reading of the manuscript.

This work was supported by a Burroughs-Wellcome Career Award and by National Institutes of Health grant AI074832 to G. Y. Liu.

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