Involvement of NADH Oxidase in Competition and Endocarditis Virulence in Streptococcus sanguinis

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Here, we report for the first time that the Streptococcus sanguinis nox gene encoding NADH oxidase is involved in both competition with Streptococcus mutans and virulence for infective endocarditis. An S. sanguinis nox mutant was found to fail to inhibit the growth of Streptococcus mutans under microaerobic conditions. In the presence of oxygen, the recombinant Nox protein of S. sanguinis could reduce oxygen to water and oxidize NADH to NAD\(^+\). The oxidation of NADH to NAD\(^+\) was diminished in the nox mutant. The nox mutant exhibited decreased levels of extracellular H\(_2\)O\(_2\); however, the intracellular level of H\(_2\)O\(_2\) in the mutant was increased. Furthermore, the virulence of the nox mutant was attenuated in a rabbit endocarditis model. The nox mutant also was shown to be more sensitive to blood killing, oxidative and acid stresses, and reduced growth in serum. Thus, NADH oxidase contributes to multiple phenotypes related to competitiveness in the oral cavity and systemic virulence.

NADH oxidase can catalyze the reduction of oxygen to H\(_2\)O\(_2\) or H\(_2\)O with concomitant oxidation of NADH to NAD\(^+\) in bacteria. During glycolysis, bacterial cells produce NADH from NAD\(^+\). To balance the NAD\(^+\)/NADH ratio for maintaining glycolysis, NADH oxidase has been recognized as playing an important role in producing NAD\(^+\) from NADH. Yamamoto et al. (1) have proposed that NADH oxidase is involved in converting pyruvate to acetyl-coenzyme A (CoA) under aerobic conditions in Streptococcus agalactiae. There are two genes, nox-1 and nox-2, encoding NADH oxidases in Streptococcus mutans (2, 3). The nox-1 gene encodes an H\(_2\)O\(_2\)-forming NADH oxidase (3), whereas nox-2 has been proposed to encode an H\(_2\)O-forming NADH oxidase (2). However, most streptococci, including Streptococcus pneumoniae and S. agalactiae, possess orthologs of only nox-2. In S. agalactiae, the inactivation of nox was shown to reduce or eliminate growth under aerobic conditions (1), while growth was not affected by nox inactivation in S. pneumoniae under aerobic or anaerobic conditions (4). In S. pneumoniae and S. agalactiae, nox inactivation attenuates virulence in animal models (1, 4). In addition, the efficiency of competence for genetic transformation was significantly altered in a S. pneumoniae nox mutant (4). These data imply NADH oxidase is important for multiple biological functions in streptococci.

Infective endocarditis (IE) is a dangerous disease with a mortality rate of approximately 30% at 1 year (5). Between 2000 and 2011, the incidence of IE in the United States increased from 11 to 15 cases per 100,000 persons (6). Treatment of endocarditis is complicated. Medical treatment can involve prolonged hospitalization and often fails, necessitating the surgical replacement of infected heart valves (7–9). Antibiotic prophylaxis generally has not been recommended for invasive dental procedures for many years, and IE prophylaxis for dental procedures has been restricted to a smaller number of cardiac conditions with very high risk for adverse outcomes from IE (10). IE may be complicated by an increasing frequency of antibiotic resistance (11). The oral streptococci are common causes of IE (6, 12). The incidence of streptococcal IE in the United States rose significantly, from 26 to 42 cases per million persons, between 2000 and 2011 (6).

Streptococcus sanguinis is a normal inhabitant in the oral cavity but one of the most common pathogens of IE (13–15). It can inhibit the growth of S. mutans and is regarded as an antagonistic bacterium against S. mutans in the oral cavity (16). The production of H\(_2\)O\(_2\) has been demonstrated to be responsible for the inhibition by S. sanguinis of the growth of S. mutans (16). In our previous studies, we identified several genes that are related to both competition and H\(_2\)O\(_2\) production, including spxB, ackA, spxR, spxA1, and tpx (17, 18).

In S. sanguinis, an ortholog of nox-2 (named nox in this study; SSA_1127) is present, but there is no nox-1 ortholog. In this study, we found the nox gene was involved in competition with S. mutans as well as virulence for IE and examined the possible mechanisms by which the nox gene could affect the competition and IE.

MATERIALS AND METHODS

Ethics statement. All animal experiments were handled in compliance with the U.S. Office of Laboratory Animal Welfare and U.S. Department of Agriculture guidelines, as well as institutional policies. All procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (protocol AM10030).

Bacterial strains, growth, and antibiotics. S. sanguinis strain SK36 and its mutants and S. mutans UA159 (Table 1) were grown in brain heart infusion (BHI) broth or agar (BD, San Jose, CA) at 37°C under microaerobic conditions (6% O\(_2\), 7.2% CO\(_2\), 7.2% H\(_2\), and 79.6% N\(_2\)) as described in the present study.
TABLE 1 Strains and primers in this study

| Strain or primer | Description or sequencea | Source |
|-----------------|--------------------------|--------|
| S. sanguinis strains | SK36 Human plaque isolate | Kilian et al. (37) |
| | Δnox | Kan; ΔSSA_1127::aphA-3 | This study |
| | Δnox_compl | Erm; ΔSSA_1127::erm | This study |
| | ΔspxB | Kan; ΔSSA_0391::aphA-3 | Chen et al. (17) |
| | JFP36 | Erm; ΔS6_0169::pSerm | Turner et al. (25) |

a Kan, kanamycin; Erm, erythromycin.

Preceding (19). Antibiotics, including 500 μg/ml kanamycin and 10 μg/ml erythromycin (Fisher scientific, Pittsburgh, PA), were used for mutant construction and culture.

Deletion and complementation of the nox gene. The open reading frame (ORF) of the nox gene in S. sanguinis SK36 was replaced by a promoterless kanamycin cassette (aphA-3) as described previously (19). Briefly, three pairs of primers, nox_F1 and nox_R1, nox_F3 and nox_R3, and kan_F2 and Kan_R2 (Table 1), were used for PCR amplification of 1-kb upstream and downstream flanking regions of the nox gene and for the promoterless aphA-3, respectively. The three PCR-amplified fragments were combined by second-round PCR amplification using primers nox_F1 and nox_R3. The final linear recombinant PCR amplicon was transformed into S. sanguinis SK36 to obtain the nox-deleted mutant using kanamycin for selection.

The nox mutant was complemented by a similar strategy. Upstream sequence (1 kb) plus the ORF of the nox gene, the promoterless erythromycin cassette (erm), and 1 kb of sequence downstream of the nox gene were PCR amplified and then combined to obtain the recombinant PCR amplicon in which the nox ORF was followed by the erm cassette. The recombinant amplicon was transformed into the nox mutant to obtain a complemented strain of the nox mutant using erythromycin for selection. The primers used are listed in Table 1.

Determination of S. mutans inhibition by S. sanguinis. The inhibition of S. mutans by S. sanguinis was determined as described previously (17). Briefly, cultures of S. sanguinis strains were dropped onto BHI agar plates to form spots and incubated microaerobically at 37°C. After overnight growth, S. mutans UA159 cultures were dropped near S. sanguinis spots and incubated microaerobically at 37°C for 1 day. No growth of S. mutans in the contact zone on an agar plate was viewed as the inhibition of S. mutans by S. sanguinis; otherwise, S. sanguinis was judged to have failed to inhibit S. mutans.

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up to exponential growth phase (optical density at 450 nm [OD450] of ~0.8) under microaerobic conditions, were harvested and washed by centrifugation and mechanically disrupted in cold 50 mM potassium phosphate buffer (pH 7.0) with 1 mM phenylmethylsulfonyl fluoride (PMSF) using FastPrep lysis matrix B (MP Biomedicals, Solon, OH). The disrupted cell suspensions were centrifuged at 16,000 × g for 15 min at 4°C, and the supernatant was harvested to use for NADH oxidase activity assays. The activity was measured in the reaction mixture composed of oxygen-saturated potassium phosphate buffer, 0.1 mM β-NADH, and cell extract at room temperature by monitoring the OD340.

In all assays, the recombinant SAA_0375 protein, annotated as a lipoprotein transporter and prepared the same way as rNox, was used as a negative control, and protein concentrations were determined by the Bradford method (22) using bovine serum albumin as a standard. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Determination of extracellular and intracellular H2O2 production.** To determine whether extracellular H2O2 was changed in the S. sanguinis nox mutant, the Amplex red assay was performed as described previously (17). Exponential cultures of S. sanguinis SK36, the nox mutant, and its complemented strain grown under microaerobic conditions were diluted 10-fold in prewarmed BHI with Amplex red (50 μM) and 1 U/ml horseradish peroxidase (Life Technologies, Grand Island, NY). The diluted cultures were incubated at 37°C in a FLUOstar microplate reader (BMG Technologies), and OD560 values were measured at 10-min intervals for 4 h.

To examine whether intracellular H2O2 was changed in the S. sanguinis nox mutant, the cells were isolated and disrupted for H2O2 determination. Exponential cultures of SK36, the nox mutant, and its complemented strain grown under microaerobic conditions were washed with ice-cold potassium phosphate buffer (50 mM, pH 7.0) with 1 mM PMSF by 4,000-rpm centrifugation at 4°C and resuspended in potassium phosphate-cold potassium phosphate buffer (50 mM, pH 7.0), 0.05 mM thiamine pyrophosphate, 0.01 mM FAD, 0.97 mM MgSO4, 1.5 mM sodium pyruvate, Amplex red (50 μM), and 0.1 U/ml horseradish peroxidase. The reaction mixture without sodium pyruvate was set as the baseline. All reactions in the same plate were monitored at a wavelength of 560 nm at 37°C in a FLUOstar microplate reader.

**Blood killing.** Overnight microaerobically cultured nox mutant and JFP36 cells were diluted 10-fold in BHI and incubated at 37°C for 3 h. Equal volumes of nox mutant and JFP36 were mixed together, washed with Hanks’ balanced salt solution (HBSS) buffer, and resuspended in HBSS buffer to approximately 2 × 10⁶ CFU/ml. The suspension was mixed 1:9 with human fresh blood (Virginia Blood Service) and incubated at 37°C with rotary shaking at 250 rpm. After 0, 45, and 90 min of incubation, the mixture was serially diluted in sterile distilled H2O and spread on erythromycin- or kanamycin-containing BHI agar plates for CFU counting. The bacterial survival was expressed as the Δnox/JFP36 ratio of CFU at treatment time divided by the Δnox/JFP36 ratio of CFU at time zero.

**qRT-PCR.** To analyze the expression of spaB by quantitative RT-PCR (qRT-PCR), S. sanguinis SK36, the nox mutant, and the complemented strain cells were cultured microaerobically at 37°C in BHI broth. At the exponential growth phase (OD₅₆₀ of ~0.8), RNAprotect bacterial reagent (Qiagen, Valencia, CA) was added to the cultures (2:1) to stabilize the RNA, and then cells were harvested by centrifugation at 4,000 × g for 15 min. RNA from the sample cells was isolated through lysosome lysis, mechanical disruption with FastPrep lysing matrix B, and purification with an RNasy minikit (Qiagen, Valencia, CA) as described in the manufacturer’s protocol. DNA was removed by treatment in columns with DNase I during purification. In the reverse transcription reaction, first-strand cDNA was synthesized in a 20 μl-reaction mixture containing 4 μl of 5× first-strand buffer, 100 ng RNA, 1.5 μg random primers, 1 μl of 10 mM deoxyxucleoside triphosphate (dNTP) mix, 1 μl of 0.1 M diithiothreitol (DTT), 1 μl RNaseOUT recombinant RNase inhibitor (40 U/μl), and 1 μl of SuperScript III reverse transcriptase (200 U/μl) by following the manufacturer’s protocol (Life Technologies, Grand Island, NY). The reaction without reverse transcriptase was conducted in parallel as a control for possible DNA contamination. The qRT-PCR was composed of 5 μl SYBR green PCR master mix (Life Technologies, Grand Island, NY), 10 pmol each of paired primers spaB_L and spaB_R (Table 1), and 1 μl of 50-fold-diluted cDNA template. The reaction was performed at 95°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min using an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies, Grand Island, NY), followed by dissociation curve analysis. The housekeeping gene gyrA, with primers gyrA_L and gyrA_R (Table 1), was used as a normalization control. The specificity of the primers for the genes was determined by melting profiles from the dissociation curve analyses and agarose gel electrophoreses of the qRT-PCR products (23).

**Statistical analysis.** All data were obtained in at least biological triplicates. Student’s t test was used for NADH oxidase activity analysis of rNox. For data on qRT-PCR and CI, one-sample t test was applied to analyze the...
values of mutant or complemented mutant compared to a value of 1. Other data were statistically analyzed by analysis of variance (ANOVA) with post hoc Tukey’s honestly significant different (HSD) test. The significance was set as a P value of <0.05.

RESULTS

Diminishment of competition in the *S. sanguinis* nox mutant.

To examine whether the nox gene is involved in competition, the effect of the nox deletion on the inhibition of *S. mutans* was assessed on BHI agar plates under microaerobic conditions. The result showed the wild-type and nox-complemented strains inhibited *S. mutans* at the contact zone on agar plates, but the nox mutant failed to inhibit *S. mutans* (Fig. 1). The mutation of the nox gene in SK36 produced an effect that was similar to that of catalase addition (Fig. 1). These data suggest that the nox gene is involved in the competition of *S. sanguinis* with *S. mutans*, and that the decreased H2O2 secretion is responsible for the diminished inhibition of *S. mutans*. To confirm the complementary result, we also examined the competition of nox gene neighboring mutants from SSA_1114 to SSA_1126 and downstream gene mutants from SSA_1128 to SSA_1130. We did not find the loss of competition in any of these mutants, suggesting a lack of polar effect (data not shown).

H2O-forming NADH oxidase activity of the rNox protein.

To determine whether Nox directly generates H2O2, we prepared rNox protein in *E. coli* by cloning the *S. sanguinis* nox gene into vector pET-46 EK/LIC and introducing the subsequent plasmid into *E. coli* BL21(DE3)pLysS for expression. The NADH oxidase activity of the purified rNox protein was assayed under aerobic conditions. The result showed that the rNox protein oxidized NADH to NAD+, unlike the negative-control recombinant SSA_0375 protein (Fig. 2A). However, this protein did not generate H2O2, unlike the positive-control *B. licheniformis* NADH oxidase protein, which produced abundant H2O2 from oxygen (Fig. 2B). These data indicate that the rNox protein has the activity of an H2O-forming NADH oxidase under aerobic conditions, which is consistent with the annotation of the *S. sanguinis* nox gene as encoding an H2O-forming NADH dehydrogenase.

Diminishment of NADH oxidase activity in the nox mutant.

To confirm the NADH oxidase activity of Nox in *S. sanguinis* cells, NADH oxidase activities were compared among the wild-type strain (SK36), the nox deletion mutant, and its complemented strain. Compared to that in the wild-type strain, NADH oxidase activity was dramatically decreased in lysates of the nox mutant and restored after complementation of the mutant (Fig. 3). This result suggests that the Nox protein possesses NADH oxidase activity and, moreover, is the primary NADH oxidase in *S. sanguinis* cells.

Extracellular decrease and intracellular increase of H2O2 in the nox mutant.

To examine whether the nox gene was involved in H2O2 production in *S. sanguinis* cells, extracellular and intracellular H2O2 levels in the wild-type, nox mutant, and complemented strain were determined. The results showed the level of extracellular H2O2, detected using Amplex red, decreased in the nox mutant; the level of intracellular H2O2 increased in the nox mutant, suggesting a lack of polar effect (data not shown).
tant to the same level as that in a mutant deleted for the gene encoding the H$_2$O$_2$-producing pyruvate oxidase, SpxB. H$_2$O$_2$ levels in the nox complemented strain were the same as those of the wild type (Fig. 4A). Surprisingly, the level of H$_2$O$_2$ in an intracellular extract of nox mutant cells was significantly greater than that in the wild-type and complemented strains (Fig. 4B). Our previous study confirmed that SpxB is a major producer of H$_2$O$_2$ in S. sanguinis (17). However, the transcription of the spxB gene, assayed using qRT-PCR, and the SpxB activity in the nox mutant were not changed compared to that of the wild type and the complemented mutant (see Fig. S1 in the supplemental material).

**Involvement of the NADH oxidase gene in endocarditis virulence.** S. sanguinis is one of most common causes of bacterial endocarditis. A number of virulence factors for endocarditis have been identified in S. sanguinis (26–29). After the inoculation of precatheterized rabbits with a 1:1 mixture of the nox mutant and the wild type, followed by overnight incubation, the CI of the nox mutant relative to that of the wild type in the infected vegetation was measured. As shown in Fig. 5, the CI in the vegetation was 0.016, which was significantly less than 1 ($P = 0.0003$), indicating the reduced fitness of the mutant. To confirm the reduced fitness of the mutant was not due to a general growth deficiency, we also performed competitions with the mutant and the wild type in BHI medium (i.e., in vitro). The CI of the nox mutant compared to that of the wild type was not significantly different from 1 after the incubation of the mixed inoculum in BHI medium overnight (Fig. 5). This suggested that there was no growth difference between the mutant and the wild type. These data indicated that the virulence of the nox mutant was impaired and that the nox gene played a role in IE.

**Blood killing of the nox mutant.** Survival of the nox mutant compared to that of the wild type was examined in human blood. As shown in Fig. 6A, the CI of the nox mutant compared to that of the wild type was significantly less than 1 after 45 min and 90 min.
of incubation in blood. The complementation of the nox mutant restored survival to wild-type levels. These data indicated that the deletion of the nox gene gave rise to the decreased survival of S. sanguinis in human blood.

Reduced growth of the nox mutant in serum. The growth of the nox mutant in human serum was compared to that of the wild type. The results showed that the nox mutant was recovered in lower numbers than the wild type from human serum after overnight growth under microaerobic conditions (Fig. 6B). Thus, nox is required for the normal growth of S. sanguinis under in vivo-like conditions.

Sensitivity of the nox mutant to exogenous H$_2$O$_2$ and acid. Neutrophil oxidative burst and acidification of phagosomes have been implicated in the bactericidal function of phagocytes (30); therefore, the sensitivity of the nox mutant to exogenous H$_2$O$_2$ and acid was examined. With H$_2$O$_2$ treatment, the survival of the nox mutant was markedly reduced after 1 h compared to that of the wild type. Complementation of the nox mutant restored survival to the same level as that of the wild type (Fig. 7A). Upon acid treatment, survival of the nox mutant also exhibited a significant decrease compared to that of the wild type (Fig. 7B). These data indicated that the nox mutant was more sensitive to H$_2$O$_2$ and acid stresses than the wild type.

DISCUSSION
The nox orthologs have been demonstrated to encode NADH oxidase in S. pneumoniae (4), S. mutans (2), and S. agalactiae (1). This NADH oxidase is proposed to produce H$_2$O from O$_2$ (1, 2, 4), but this has not been confirmed. In this study, the S. sanguinis nox mutant exhibited a dramatic reduction in NADH oxidase activity compared to that of the wild type (Fig. 3), and rNox also exhibited NADH oxidase activity (Fig. 2A). Furthermore, the lack of H$_2$O$_2$ formation from O$_2$ by rNox also was demonstrated (Fig. 2B). These data indicate that S. sanguinis Nox does indeed function as an H$_2$O-forming NADH oxidase in the presence of oxygen.

It is interesting that the nox deletion decreased the extracellular H$_2$O$_2$ level (Fig. 4A) but increased the intracellular H$_2$O$_2$ level (Fig. 4B). The expression of the spxB gene and H$_2$O$_2$-producing activity of its gene product did not change in the nox mutant compared to that of the wild type (see Fig. S1 in the supplemental material), suggesting the increase in intracellular H$_2$O$_2$ level is not caused by enhancing the activity of the H$_2$O$_2$ producer. In E. coli, two scavenging enzymes, alkyl hydroperoxide reductase and catalase, were responsible for scavenging intracellular H$_2$O$_2$ (31). The mutation of either one could cause intracellular H$_2$O$_2$ to be elevated. Although intracellular H$_2$O$_2$ could penetrate the membrane to exit the cell, no H$_2$O$_2$ escaped from E. coli cells in the presence of these two enzymes. However, there are no homologs of these genes in the S. sanguinis genome, and S. sanguinis has been demonstrated to secrete H$_2$O$_2$ to inhibit S. mutans growth (16, 17). Here, we also showed the similar antagonistic results for S. sanguinis wild-type strain SK36 against S. mutans (Fig. 1 and 4A). These findings suggest S. sanguinis keeps the endogenous H$_2$O$_2$ level balanced through efflux instead of scavenging enzymes. In addition, we found the nox mutant failed to inhibit the growth of S. mutans (Fig. 1), further indicating extracellular H$_2$O$_2$ was decreased. In another study, we report a reduction in membrane fluidity in the nox mutant and do not find peroxidase-like genes up- or downregulated in the expression profiling of the mutant (38). It has been demonstrated that the permeation of H$_2$O$_2$ across biomembranes is rapid but limited (32), and that the E. coli membrane is semipermeable to H$_2$O$_2$ in cells (31). Therefore, it is possible that the decrease in membrane fluidity influences the diffusion of H$_2$O$_2$ across cell membranes, which leads to a decrease in extracellular H$_2$O$_2$ and increase in intracellular H$_2$O$_2$ in the nox mutant.

NADH oxidase has been documented to be involved in virulence in other streptococci. A nox insertion or deletion mutant was found to be significantly attenuated for the virulence of S. pneumoniae in an intraperitoneal model of sepsis in BALB/c mice (4, 33), a murine respiratory tract infection model, and a Mongolian gerbil otitis media infection model (34). Yamamoto et al. found significant attenuation in the virulence of S. agalactiae in lung, intraperitoneal, and intravenous murine infection models in the nox mutant (1). In this study, we found that the virulence of S. sanguinis in the rabbit endocarditis model was attenuated by the deletion of nox (Fig. 5), implicating nox in the virulence of S. sanguinis in IE.

Our study found the survival of the S. sanguinis nox mutant was significantly decreased in human blood (Fig. 6A) as well as human serum (Fig. 6B). The survival of the nox mutant also was diminished upon exposure to exogenous H$_2$O$_2$ or acid (Fig. 7). The neutrophil oxidative burst, which generates reactive oxygen species, and acidification of phagosomes have been proposed to play pivotal roles in the bactericidal function of phagocytes (35, 36). Therefore, these results suggest that the decreased survival of the nox mutant in blood is one of the reasons for attenuation in the virulence of S. sanguinis for IE, and that the decreased ability of
the nox mutant to survive in human blood may be caused by both the growth reduction in human serum and greater sensitivity to acid and oxidative stresses. Since the nox gene is widespread in other species of streptococci, continuation of this work may lead to a comprehensive elucidation of the underlying mechanisms of competition and virulence for streptococci.

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