Characterization of a Staphylococcus aureus Surface Virulence Factor That Promotes Resistance to Oxidative Killing and Infectious Endocarditis

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Staphylococcus aureus is a prominent human pathogen and a leading cause of community- and hospital-acquired bacterial infections worldwide. Herein, we describe the identification and characterization of the S. aureus 67.6-kDa hypothetical protein, named for the surface factor promoting resistance to oxidative killing (SOK) in this study. Sequence analysis showed that the SOK gene is conserved in all sequenced S. aureus strains and homologous to the myosin cross-reactive antigen of Streptococcus pyogenes. Immunoblotting and immunofluorescence analysis showed that SOK was copurified with membrane fractions and was exposed on the surface of S. aureus Newman and RN4220. Comparative analysis of wild-type S. aureus and an isoegenic deletion strain indicated that SOK contributes to both resistance to killing by human neutrophils and to oxidative stress. In addition, the S. aureus sok deletion strain showed dramatically reduced aortic valve vegetation and bacterial cell number in a rabbit endocarditis model. These results, plus the suspected role of the streptococcal homologue in certain diseases such as acute rheumatic fever, suggest that SOK plays an important role in cardiovascular and other staphylococcal infections.

Staphylococcus aureus is a commensal that often colonizes skin and mucosal membranes (11, 28). This species is usually benign in healthy individuals, but it is a high-risk pathogen for immunocompromised individuals. As a consequence of its numerous virulence factors and its adaptability, S. aureus is one of the most significant human pathogens for both nosocomial- and community-associated infections (20). Moreover, an increasing resistance to antibacterial agents and the adaptation and emergence of methicillin- and vancomycin-resistant S. aureus (MRSA and VRSA, respectively) strains is alarming (2, 13).

S. aureus is the causative agent of diverse human and animal maladies, including, but not limited to, abscesses, food poisoning, toxic shock syndrome, septicemia, and endocarditis (3, 46, 49). This cadre of diseases results from S. aureus strain heterogeneity. Although numerous, most S. aureus virulence factors are categorized into one of the following groups according to their functions: (i) surface proteins that promote adhesion, internalization, and colonization; (ii) toxins and enzymes that promote tissue damage, inflammation, and invasion and dissemination; (iii) surface factors that affect phagocytosis by leukocytes; (iv) factors that enhance survival in phagocytes; or (v) superantigens and other molecules that modulate the immune system by altering the function of lymphocytes and antigen-presenting cells (1, 12, 44).

Our bioinformatics analysis of 13 S. aureus genomic sequences in search of potential virulence factors for staphylococcosis-induced cardiovascular diseases revealed a conserved open reading frame (ORF) 96 to 100% identity among all S. aureus sequences. The predicted translation products from these ORFs share 59% identity with the 67-kDa myosin cross-reactive antigen (MCRA) of Streptococcus pyogenes (19). The S. aureus homologue (ORF SA0102) was reported initially by Kuroda et al. (25) in reference to the N315 strain genome sequence. They described SA0102 as one of two major histocompatibility complex class II (MHC-II) β-chain homologues in the N315 genome. The 67-kDa S. pyogenes protein and the SA0102 predicted translation product share 62% and 34% similarity (19% and 21.2% identity), respectively, to the murine β1 domain of the mouse I-Aa chain (19; also our unpublished results).

The 67-kDa streptococcal homologue is a putative virulence factor, and hybridization studies suggested that related proteins exist in streptococcal groups A, C, and G (19). This protein is a member of extensive MCRA protein family. It reacts with sera of patients with acute rheumatic fever (ARF), acute glomerulonephritis, and active streptococcal infections (19). It also reacts with anti-myosin antibody in sera of patients with ARF. Recently, this streptococcal protein was described as fatty acid double bond hydratase (47). Although members of the MCRA protein family are widely distributed among bacteria, only three proteins from this family have been...
biochemically characterized (6, 47), and the exact role of the vast majority of proteins and homologues belonging to this family remains unknown. The objective of this study is to characterize the 67-kDa myosin cross-reactive homologue of S. aureus. To address this goal, we constructed deletion mutants in two well-characterized S. aureus strains, RN4220 and Newman, compared the properties of the parental and mutant strains, and investigated the molecular relatedness of the structural gene in a number of clinical isolates. The data suggest that this protein is ubiquitous among S. aureus clinical isolates and could contribute to infectious diseases such as endocarditis by promoting survival in phagocytes and resistance to oxidative killing. Due to the latter property, we tentatively designated the S. aureus protein SOK, a surface factor promoting resistance to oxidative killing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used are described in Table 1. Escherichia coli was grown using Luria-Bertani (Difco Laboratories, Detroit, MI) medium that was supplemented with ampicillin (Am; 100 μg/ml) or chloramphenicol (Cm; 34 μg/ml) when necessary. Exception where indicated, S. aureus was propagated using tryptic soy (TS) medium (Difco Laboratories, Detroit, MI). Plasmid selection for S. aureus was conducted with erythromycin (Em), Cm, or tetracycline (Tc) (5, 10, or 10 μg/ml, respectively).

Bioinformatics analysis. The search for homologues was performed using BLAST on the NCBI web server (http://www.ncbi.nlm.nih.gov/). Sequences were aligned by the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html). The following tools on the Expasy Proteomic Server were used to analyze various properties of the SOK protein: Compute pi/Mw was used to calculate isoelectric point and molecular weight, SignalP, version 3.0, was used to calculate the signal peptide cleavage site by artificial neural networks (NN) and hidden Markov models (HMM), ProtScale was used to determine hydrophobicity, and PSIPred was used to determine transmembrane topology (using MEMSAT on the PSIPred Protein Structure Prediction Server). The BPROM program (Softberry, Inc., Mount Kisco, NY) was used to predict bacterial promoter.

DNA isolation. Genomic DNA was isolated by using Genomic DNA Prep Plus kits (A&A Biotechnology, Gdynia, Poland) facilitated by a method to promote S. aureus lysis (41). Plasmids were isolated with a MiniPrep isolation kit (Qiagen GmbH, Hilden, Germany) using the manufacturer’s protocol. S. aureus cells were treated with lysozyme in the kit resuspension buffer. DNA was quantified using a NanoDrop ND-1000 instrument (Nanodrop Technologies, Wilmington, DE).

RFLP analysis. A 1,789-bp fragment containing predicted sok coding region plus flanking promoter and ribosome binding sites was amplified using primers 12631 and 12632 (Table 2). PCR products were purified, quantified (see above), and digested with Csp6I, Hin6I, or TaqI. These enzymes were selected based on two criteria: (i) that at least three cleavage sites exist within the PCR-amplified sok gene and (ii) that predicted digestion patterns are readily discernible by agarose gel electrophoresis. Fragments were separated by electrophoresis in 2% agarose gels to compare sok genotypes according to digestion profiles. At least a one-band difference was used as the criterion for designating unique restriction fragment length polymorphism (RFLP) profiles. Concordance between sp gene and predicted Sok RFLP was calculated as described previously (30).

RNA isolation and RT-PCR. RNA was isolated with an RNeasy Mini Kit (Qiagen Science, Germantown, MD) according to the manufacturer’s protocol. Bacteria were disrupted using a FastPrep FP120 homogenizer (ThermoSavant, Holbrook, NY) (45 s at 6.0 m/sec). DNA was removed using RNase-free DNase I (Ambion, Austin, TX), and RNA was further purified by treatment again with the same RNA isolation protocol. RNA samples with a ratio of the optical density at 260 nm (OD260)/OD280 of ≥2.0 were used. First-strand cDNA was synthesized from 1 μg of RNA using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA), and 5 μl of a 100-fold diluted aliquot was used as the template (final sample volume, 25 μl; MicroAmp Optical 96-Well Reaction Plate; Applied Biosystems). Real-time PCR (RT-PCR) was performed in an ABI Prism 7500 system using SYBR green mix as recommended (Applied Biosystems). Results and PCR-RFLP were calculated as described previously (30).

Cloning and purification of SOK. Recombinant SOK (rSOK) was expressed in Escherichia coli BL21(DE3) pLysS using pGEX-5T, which expresses proteins with an N-terminal His tag and a glutathione S-transferase (GST) tag (5). The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lithuania); the 1,827-bp product was digested with BamHI and XhoI. The predicted SOK ORF was amplified with Pfu DNA polymerase (Fermentas, Lith
Waltham, MA). rSOK was purified on the glutathione-Sepharose 4B resin, according to the manufacturer's recommendation (Amersham, Piscataway, NJ), and GST label was cleaved with thrombin (Sigma-Aldrich, St. Louis, MO). The rSOK was purified on the glutathione-Sepharose 4B resin, Waltham, MA. The plasmid was then electroporated into S. aureus titer, 15,000, was determined by immunoblotting (9), and diluted (1/10,000) antiserum was typically used for experiments.

**Immunofluorescence analysis of S. aureus.** Cells samples were prepared by the method of Hiraga et al. (17). Slides were visualized using a Zeiss LSM 5 Pascal instrument and software (version 4.0, service pack 2; Carl Zeiss MicroImaging GmbH, Heidelberg, Germany) following treatment with anti-rSOK rat serum (see above) and Alexa Fluor 488-conjugated goat anti-rat IgG(H+L) (2 mg/ml) secondary antibodies (Molecular Probes, Inc., Eugene, OR).

**SOK subcellular localization.** Cells and supernatants from overnight (on) cultures (25 ml) were separated by centrifugation (at 8,000 × g for 10 min). Culture supernatants were precipitated (2 h at −20°C) with 9 volumes of trichloroacetic acid-actone (1:8, vol/vol), pelleted, and resuspended in distilled water (1 ml). The cell pellet was washed with deionized water and treated (18,000 × g for 30 min). The obtained supernatant containing soluble cytoplasmic proteins was stored at −80°C until needed. The resulting cell pellet, containing crude membrane and wall fractions, was resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% amidosulfobetaine-14 [ASB-14], and 5% Triton X-100, 2 mM tributylphosphine, 1% b-mercaptoehanol), and incubated at room temperature for 2 h. The sample was clarified by centrifugation (75,000 × g for 20 min), and supernatant fluids were recovered for analysis of integral membrane proteins. Cell wall proteins in the pellet were released by incubation for 3 h in TE buffer (50 mM Tris-HCl and 10 mM EDTA, pH 8.0) with lysostaphin (40 μg/ml). Proteins were analyzed by SDS-PAGE and immunoblotting (see above).

**SOK deletion mutagenesis.** DNA fragments, 918 (5′) and 698 (3′) bp in length, were amplified by PCR from within sok through external flanking regions. The fragment included nucleotides (nt) −154 through 764; the 3′ fragment encompassed nt 11584 through an additional 59 nt after the predicted stop codon (nucleotide numbering is relative to the predicted ATG initiation codon) (see Fig. S1A in the supplemental material). PCR products were digested with appropriate enzymes (Table 2) and ligated on opposite ends of the Tc cassette in pDG1515 (50), resulting in sOK1 vector. This construct was propagated in E. coli, DH5α and digested with BamHI and KpnI. The Tc cassette in pDG1515 (50) was cloned into the BamHI and SalI sites of pMIN164 (18), resulting in sOK3. This plasmid was electroporated into the E. coli DH5α and digested with BamHI and KpnI. This Tc cassette plus flanking sOK fragments were cloned into the pCL10 shuttle vector, resulting in sOK2.

The plasmid was then electroporated into S. aureus RN4220 and Newman as recommended (ECM 600 electroporator; BTX Molecular Delivery Systems). Transformed cells were grown in TS broth (TSB) with Tc (24 h at 43°C) and plated on TS agar (TSA) containing Tc to select colonies with the first recombination. One colony was transferred to TSB and grown (at 30°C for 5 days) with daily transfer to fresh TSB. After 5 days, an aliquot was grown on TSA with Tc. Colonies were screened for a Tc Cm− phenotype indicating that the second recombination resulted in deletion of a 404-nt sok sequence and insertion of the Tc cassette. To complement the mutation, a fragment representing the sok ORF with 260 nt upstream was amplified using MEXP primer and ligated into the BamHI and SalI sites of pMIN164 (18), resulting in sOK3. This plasmid was electroporated into the S. aureus RN4220 and Newman strains (selection was accomplished on TSA with Tc and Em).

**PMN killing assay.** Human polymorphonuclear leukocytes (PMNs) were isolated from heparinized venous blood of four different donors in accordance with a human subject protocol approved by the University of Idaho Institutional Review Board for Human Subjects (approval number 05-056). Donors were informed of the procedure risks and provided a written consent prior to participation. Killing of bacteria by human PMNs was determined as described previously (22), with the following modifications. PMNs (106) were combined with opsonized bacteria (108) in 96-well plates which were centrifuged at 400 × g for 5 min, followed by incubation at 37°C for 15 min. PMNs were treated with 400 μM gentamicin (Sigma-Aldrich Co.) for 10 min to remove any remaining extracellular bacteria (time [T] zero). Cultures were further maintained for time points up to 180 min. At the times indicated in Fig. 3A, gentamicin was removed by aspiration, and cells were gently washed with PBS and lysed in sterile water, and the bacteria were plated on TSA. CFU were enumerated following overnight incubation, and the percentage of bacteria killed was calculated using the following equation: (CFU/CFU0) × 100, where CFU0 = , indicates number of enumerated colonies for time point zero and CFU/CFU0 is the number of CFU for each analyzed time point. The assay measures the percentage of the total number of viable ingested bacteria compared to the number at time point zero.

**ROS analysis.** Human PMNs (see above) were mixed with 10 mM 2,7-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes Inc., Eugene, OR) and incubated for 30 min at room temperature in the dark (8). Opsonized bacteria (above) were mixed with the PMNs at a 1:10 ratio, and transferred to precoated wells of a 96-well plate. The plate was centrifuged (for 5 min at 700 × g at 4°C) to synchronize phagocytosis. Reactive oxygen species (ROS) production was monitored during incubation (at 37°C) using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) with 485-nm excitation and 538-nm emission wavelengths. Data were analyzed by SoftMax Pro software, version 5.0.1 (Molecular Devices), and are presented as the rate of change (ΔF/F0) in fluorescence over time.

**H2O2 and O2− susceptibility assays.** S. aureus cells (mid-exponential growth phase) were harvested by centrifugation, washed once with PBS, and adjusted to 1 × 106 and 2 × 106 CFU/ml, respectively, for H2O2 and O2− assays (27). To assess the effect of H2O2 on S. aureus viability, the bacteria were incubated with various concentrations of H2O2 (Sigma-Aldrich Co., St. Louis, MO) in glass tubes for 1 h at 37°C, followed by addition of Micrococcus luteus catalase (1,000 U/ml) (Sigma-Aldrich Co.) to quench the remaining H2O2. The percentage of surviving bacteria was calculated by using the following equation: (CFU/CFU0) × 100, where CFU0 = , indicates number of enumerated
TABLE 3. Comparison of sok PCR-RFLP results with spa typing

<table>
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<tr>
<th>Isolate no. or name (country/yr)</th>
<th>sok cluster</th>
<th>sok PCR-RFLP pattern</th>
<th>Type</th>
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<th>Hint6</th>
<th>TaqI</th>
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* a spa typing results used in this table were previously published by Malachowa et al. (30).

b Countries are abbreviated as follows: BG, Bulgaria; CZ, Czech Republic; LT, Lithuania; PL, Poland; RU, Russia; SL, Slovenia; TR, Turkey; UK, United Kingdom.

c Years are abbreviated by the last two digits (e.g., 97 is 1997 and 00 is 2000).

d The BN4 strain does not belong to any spa cluster; the spa type is t159.

e sok typing results used in this table were previously published by Malachowa et al. (30).

f Countries are abbreviated as follows: BG, Bulgaria; CZ, Czech Republic; LT, Lithuania; PL, Poland; RU, Russia; SL, Slovenia; TR, Turkey; UK, United Kingdom.

g The BN4 strain does not belong to any spa cluster; the spa type is t159.
with a Tc' cassette introduced by allelic replacement, as described previously, to generate strain NM10 (Δsok). Strain NM10 was transformed with a plasmid harboring the promoter region plus the coding region of sok to produce the complemented strain designated NM20. RT-PCR confirmed the lack and restoration of detectable sok expression in the mutant and complemented strains, respectively, plus the absence of any detectable effect on expression of the adjacent downstream gene (results not shown).

Gene expression studies were consistent with immunofluorescence microscopy using rat antiserum prepared against rSOK. The Newman, NM10, and NM20 strains and a strain lacking protein A (DU5875) were examined for SOK by immunofluorescence microscopy (Fig. 1). The S. aureus parental and complemented strains expressed detectable levels of SOK on the cell surface. SOK was also detectable on the surface of the strain lacking protein A. In contrast, SOK was not detected on the surface of the sok deletion strain (Fig. 1).

Proteins in culture supernatants and subcellular fractions were resolved by SDS-PAGE (Fig. 2A) and analyzed by immunoblotting (Fig. 2B). The SOK protein was detected in integral membrane fractions but not in fractions containing cytoplasmic proteins or proteins covalently bound to the cell wall (Fig. 2). The membrane fraction contained an immunoreactive band with a migration consistent with a ~55-kDa protein, which is smaller than predicted (67.6 kDa) based on bioinformatic analysis (see above). Internal controls for an extracellular protein (ScpA, 44 kDa), an integral membrane protein (AgrC, 42 kDa), and a covalently bound membrane protein (SrtA, 24 kDa) were appropriately detected from the corresponding protein fractions (Fig. 2C).

SOK affects S. aureus survival and ROS production in PMNs. Human PMNs were cultured under conditions to promote synchronized phagocytosis of opsonized S. aureus. After 15 min, extracellular bacteria were killed with gentamicin, and the percentages of viable intracellular bacteria were quantified after incubation for additional time periods. The parental strain displayed nearly linear killing kinetics throughout the 3-h incubation period following addition of gentamicin, after

FIG. 1. Surface localization of SOK. S. aureus bacterial strains DU5875 (A and B), Newman (C and D), NM10 (E and F), and NM20 (G and H) (magnification of ×227) were observed by immunofluorescence using SOK-specific rat antiserum, followed by Alexa Fluor 488-conjugated goat anti-rat Ig(H+L) secondary antibody (A, C, E, and G) or combined fluorescence with differential interference contrast (B, D, F, and H).

FIG. 2. Subcellular localization of SOK in S. aureus Newman using SDS-PAGE (A), immunoblotting with anti-rSOK rat antiserum (B), and immunoblotting with control antibodies (for extracellular protein, anti-ScpA; for integral membrane protein, anti-AgrC; and for covalently bound protein, anti-SrtA) (C). Subcellular localization of SOK was assessed using various bacterial cellular proteins. Lanes M, Page-Ruler Prestained Protein Ladder (Fermentas); lanes 1, extracellular proteins; lanes 2, cytoplasmic fraction; lanes 3, integral membrane fraction; lanes 4, covalently bound proteins. α, anti.
which 43.7% of intracellular bacteria remained viable (Fig. 3A). The isogenic sok deletion strain (NM10) was more sensitive to PMN killing: 49.5% of bacteria were killed after 65 min, and after 3 h, only 12.7% remained viable. The complemented strain (NM20) showed killing kinetics similar to that of the parental strain.

Disruption of the sok gene affected the PMN ROS production following phagocytosis of S. aureus Newman. Phagocytosis of the parental Newman isolate rapidly induced PMN ROS production compared to resting PMNs (Fig. 3B). Interestingly, ROS levels in cultures harboring phagocytosed S. aureus bacteria lacking SOK were dramatically different from levels in cultures containing the parental strain (Fig. 3B). Despite nearly identical levels of ROS for the first 40 min, levels rose dramatically for the next 30 min until they eventually declined to levels at or below those of PMN cultures harboring the phagocytosed Newman parental strain. The pattern of ROS production was restored to the parental strain by SOK complementation.

SOK affects S. aureus sensitivity to 1O2 killing but not H2O2.

Since SOK expression influenced ROS levels and survival in PMNs harboring intracellular staphylococci, we assessed whether SOK expression affects susceptibility to the ROS molecules H2O2 or 1O2. Analysis of the bacterial cell viability of the Newman and NM10 strains after 1 h of incubation with various concentrations of H2O2, indicated that SOK expression did not affect the viability of S. aureus cells when they were exposed to H2O2. All strains showed dose-dependent killing effects with respect to increasing concentrations of H2O2. Following exposure to 10 mM H2O2, all strains showed approximately 50% survival (49.5 ± 10.3%, 53.8 ± 4.3%, and 53.1 ± 6.1% for Newman, NM10, and NM20, respectively). Following exposure to 100 mM H2O2, all strains showed approximately 5 to 6% survival (5.6% ± 0.68%, 6.6% ± 1.2%, and 5.5% ± 0.95% for Newman, NM10, and NM20, respectively) (Fig. 4A).

Although sok did not affect survival of bacteria during incubation with H2O2, additional experiments indicated that the sok deletion strain was more sensitive to 1O2 than the parental strain (Fig. 4B and C). Methylene blue releases singlet oxygen (1O2) species when exposed to light and was therefore used to measure the susceptibility of S. aureus strains to 1O2. In the presence of 0.5 and 1 μg/ml methylene blue for a 60-min incubation time, the survival rates of both strains dropped significantly, compared to the same concentration of methylene blue for a 30-min incubation. The strain lacking SOK (NM10) was 4 and 24 times more sensitive to 1O2 than the parental strain after incubation for 30 min with 0.5 and 1 μg of methylene blue, respectively. The highest level (498 times) of difference in susceptibilities to 1O2 between mutant and wild-type strains was observed when bacteria were incubated with 1 μg of methylene blue for 60 min. Longer incubation times (2 and 3 h) as well as higher concentrations of methylene blue (3 and 6 μg/ml) were tested for the Newman and NM10 strains, but no bacteria were able to survive under such conditions (results not shown).

SOK enhances virulence in a model of staphylococcal endocarditis. To investigate the effect of sok, and thus SOK, on virulence of S. aureus, an infective endocarditis model was used. New Zealand White rabbits with aortic valve leaflets previously damaged by cardiac catheterization were challenged with the parental S. aureus strain RN4220 or Newman or with sok deletion derivatives of the parent strains (NM1 or NM10, respectively). Animals were also challenged with sok deletion strains complemented with a plasmid encoding sok (NM2 or NM20). Rabbits were challenged intravenously with 1 × 109 CFU of Newman or Newman derivatives. RN4220 and its derivatives were used to infect rabbits at a dose of 2 × 109 CFU per animal. Hearts were harvested immediately from animals that died and from survivors that were euthanized after infection. Heart tissues were examined, and vegetations on aortic valves were removed, weighed, and homogenized to
enumerate the bacteria contained in the vegetations. If vegetations were not observed, the aortic valves were removed from hearts and homogenized to enumerate bacteria adhering to host tissue.

Infection with parent S. aureus RN4220 caused vegetations in all animals (45.2 ± 5.1 mg). In contrast, vegetations were observed in only one of six rabbits infected with NM1 (a sok deletion strain) (6.0 mg) (Fig. 5A). Animals infected with the parental RN4220 strain showed greater weight loss than animals infected with NM1 and had diarrhea and mottled faces (Fig. 5B). Vegetations were also observed in animals infected with the complemented strain (NM2) (40.0 ± 7.9 mg). Statistical analysis of the vegetation sizes by an unpaired t test showed that the parental RN4220 strain and NM2 strain produced vegetation sizes that were significantly larger (P < 0.0001 and P < 0.0003, respectively) than the vegetations produced by the sok deletion mutant but not significantly different (P = 0.58) from each other. Consistent with vegetation sizes, S. aureus RN4220 and NM2 also produced vegetations with larger bacterial loads (log_{10} CFU of 4.90 ± 0.35 and 3.71 ± 0.71/heart, respectively) than the sok mutant (log_{10} CFU of 1.49 ± 0.18/heart). As measured by an unpaired t test, the bacterial loads of vegetations produced by RN4220 and NM2 were significantly different from the bacterial loads of vegetations produced by the sok deletion mutant NM1 (P < 0.00001 and P < 0.007, respectively).

Infection with parent strain Newman and the sok deletion mutant NM10 produced vegetations in all infected animals, but vegetations produced by the Newman strain were significantly larger than vegetations produced by NM10 as measured by a t test (87.0 ± 24.0 mg and 7.0 ± 6.6 mg, respectively; P < 0.01). Statistical analysis by an unpaired t test showed that the bacterial load of vegetations produced by the Newman strain (log_{10} CFU of 7.31 ± 0.74/heart) was significantly higher than the bacterial load of vegetations produced by strain NM10 (log_{10} CFU of 4.02 ± 1.24/heart; P < 0.05). Infection with the complemented strain, NM20, caused vegetations with bacterial loads statistically similar to those of the parental Newman strain (log_{10} CFU of 6.71 ± 0.80/heart; P < 0.6).

**DISCUSSION**

This study characterized the molecular and biological properties of an S. aureus protein tentatively designated SOK. The protein was designated SOK to highlight its cell surface location and enhancement of resistance to oxidative killing. *In silico* sequence analysis for potential staphylococcal virulence factors involved in systemic or cardiovascular infections identified SOK, partly because of its relatedness to the *S. pyogenes* 67.7-kDa myosin cross-reactive protein. SOK has not been extensively studied but was previously noted during the report of the *S. aureus* N315 genome sequence (25). The N315 SOK is encoded by ORF SA0102 and was described as an MHC-II β-chain homologue. SOK and its *S. aureus* homologues share

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**FIG. 4.** *sok* affects susceptibility to \(^1\)O\(_2\) but not H\(_2\)O\(_2\). (A) The susceptibility of various strains (Newman, NM10, and NM20) to treatment with various concentrations of H\(_2\)O\(_2\) was measured by enumeration of CFU after incubation with H\(_2\)O\(_2\). (B and C) The susceptibility of various strains (Newman, NM10, and NM20) to methylene blue-producing \(^1\)O\(_2\) by photoactivation was measured by enumeration of CFU after 30 min or 60 min. Two controls (L, incubation under a direct light source without methylene blue; D, incubation in darkness without methylene blue) were included for each experiment. Data shown are the means of four experiments which were performed in triplicate (n = 12). Statistical significance (P < 0.01) between the Newman and NM10 strains is indicated by asterisks.
20 to 21% identity and 34% similarity to murine HLA class II β-chain homologues (RefSeq NP_996988) on the almost 300-residue-long fragment (unpublished data).

SOK has homologues in other bacteria and shares 49.8 to 62.3% sequence homology with proteins in *Bacillus*, *Bradyrhizobium*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Rhodopseudomonas* genera and less, but significant, relatedness to numerous homologues in other Gram-positive and Gram-negative bacteria species (see Fig. S1B in the supplemental material). SOK and its relatives have several conserved sequences which, in SOK, encompass residues 75 to 102, 174 to 211, and 495 to 539, showing >90% homologies. Previously, the most well-characterized protein in this family was the *S. pyogenes* 67-kDa myosin cross-reactive antigen (19, 47), which reacts with sera from patients with streptococcal infections and post-streptococcal sequelae (19). Considering the numerous SOK homologues and various degrees of relatedness, plus the unique association of *S. pyogenes* with immunopathologies, the ability to cross-react with myosin antibodies may be unique to the streptococcal homologue. Studies to determine whether other SOK homologues possess this property are ongoing in our laboratories.

This high level of sequence conservation is also reflected in PCR-RFLP results, which generated only eight different pattern types among a collection of 59 clinical isolates digested with three different restriction endonucleases. The PCR-RFLP results for *sok* were compared with *spa* typing results, which were previously reported for these same isolates (30). The *spa* typing method was chosen here as one of the most suitable techniques for evaluation of long-term evolutionary changes (15, 40). The differences between RFLP patterns reflect the
variability of the sok gene among the 13 sequenced S. aureus genomes analyzed, where this variability is caused by point mutations in sok (data not shown). Nearly 97% concordance between deduced spa typing clusters and sok PCR-RFLP groups illustrates the significance of the sok gene for staphylococcal epidemiology. It is possible that the high concordance between spa typing and sok PCR-RFLP is the result of the close genetic linkage of these two genes. In the majority of sequenced S. aureus genomes, these two genes are separated by less than 7.5 kb, including four ORFs, and in some genomes, such as those of the MW2 and MSSA476 strain, these two genes are separated by less than 3 kb, which include only two ORFs (4, 25).

Consistent with immunoblotting results showing colocalization of SOK in the membrane fractions, immunofluorescence experiments indicated that SOK is exposed on the S. aureus cell surface. However, sequence analysis did not reveal the presence of a typical LPXTG cell wall-anchoring motif although SOK has one predicted N-terminal transmembrane domain encompassing residues 25 through 42 within a potential signal peptide sequence (10). Repeated attempts to obtain a pure or partially purified mature form of SOK for the purpose of N-terminal sequencing to help explain the size discrepancy between bioinformatic analysis (~67.6 kDa) and immunoblotting results (~55 kDa) were unsuccessful. Thus, whether SOK has a signal peptide which is removed during maturation and localization is still to be resolved. It has been shown that staphylococcal proteases such as metalloprotease and metallo-cysteine protease are involved in the maturation process for staphylococcal virulence factors such as lipase and serine protease (35, 38). It is possible that SOK might be processed by staphylococcal protease in the maturation and localization process. This possibility is currently under investigation.

One striking effect of SOK is its ability to confer resistance to killing by PMNs following phagocytosis. The mechanism by which SOK could contribute to virulence is not completely understood but likely is due at least partly to its role in promoting survival in phagocytes, mediated by the resistance to PMN oxidative killing. The human innate immune response is an essential first line of defense against bacterial pathogens (16, 21, 39, 44). PMNs are recruited early to sites of infection and typically efficiently recognize and ingest invading S. aureus. The ability of PMNs to kill S. aureus is based on the bactericidal activity of ROS produced by the NADPH-dependent oxidase and antimicrobial compounds contained within granules. The importance of ROS in protection against S. aureus is exemplified by the increased susceptibility of NADPH oxidase-deficient chronic granulomatous disease patients to severe staphylococcal infections. To test the involvement of SOK in resistance to the innate immune response, PMN bactericidal activity was assessed following phagocytosis of a serum-opsonized S. aureus Newman wild-type strain and an isogenic sok mutant derivative. Our data indicate that survival of the S. aureus sok mutant strain in human PMNs was decreased compared to survival of the isogenic parent strain at all times tested. In addition, PMN ROS production was increased following interactions with the S. aureus sok deletion strain compared to that induced by the wild-type strain. Further examination of the role of SOK in ROS resistance indicates that SOK is involved in resistance to singlet oxygen and not hydrogen peroxide. Inasmuch as our study demonstrates that SOK is localized to the cell surface of S. aureus and that the mutant strain elicits altered PMN ROS production, it is possible that SOK participates in pathogen recognition by neutrophils. However, efficient PMN ROS production and killing in response to wild-type strains of S. aureus have been demonstrated previously (36, 48). It is also possible that the apparent increase in the PMN oxidative burst following interactions with the sok mutant strain is due to alterations in metabolic pathways controlling the S. aureus oxidative stress response. S. aureus has been shown to produce both catalase and superoxide dismutase in response to oxidative stress (29). Increased production of either enzyme following ingestion by PMNs would result in a decrease in oxidation of the DCF substrate in the in vitro ROS assay. Alternatively, the differential effects in ROS levels in cells following the parental versus deletion mutant could be the result of more efficient quenching of singlet oxygen by the parental strain. There are a number of additional reactive oxygen species that are present in the phagosome of polymorphonuclear phagocytes. Activation of the NADPH oxidase generates superoxide, a relatively unstable molecule that rapidly dismutates itself to form hydrogen peroxide and oxygen (or is catalyzed by superoxide dismutase). The diversity of ROS present in the phagosome, in addition to relative instability, confounds simulation of this environment through use of independent compounds. In addition, the direct role of ROS in microbicidal activity has been intensely debated (34). At the very least, PMN microbicidal activity is likely the result of integrated activities of both oxygen-dependent and -independent molecules. In our study we tested directly the ability of the S. aureus Δsok strain to survive following neutrophil phagocytosis through in vitro killing assays. It is unclear whether the ability of the S. aureus Δsok strain to alter PMN ROS production kinetics and the increased sensitivity to singlet oxygen/PMN killing are linked.

With use of deletion mutagenesis, we demonstrated that SOK affects in vivo staphylococcal virulence. Under the conditions tested, the lack of the SOK protein severely debilitated the virulence of two S. aureus strains tested in the rabbit endocarditis model. Vegetation formation was completely abrogated or reduced to minimal levels, and the number of cultivable bacteria in the hearts was reduced by several logs. We hypothesize that the inability of the SOK-deficient strains to cause vegetations as significant as those formed by the parent and complemented strains is due to their increased elimination before vegetations can form. This is consistent with our prior studies with Enterococcus faecalis, related to the surface protein enterococcal aggregation substance, where it has been shown that the aggregation substance also interferes with phagocyte killing (37) and contributes significantly to vegetation formation (43). Our prior studies also suggest that once vegetations begin to form in E. faecalis endocarditis, the enterococci become trapped within host- and bacterium-derived matrices that significantly reduce phagocytic removal (32). Interestingly, McAleese et al. (31) demonstrated that sok expression was elevated 2.6-fold in isolates from a heart valve compared to bloodstream isolates of the same patient. The reduced ability of sok deletion strains to form vegetations suggests that sok expression may be important as S. aureus interacts with host cells in heart tissues.
Collectively, this study resulted in partial biochemical and biological characterization of the SOK protein. Certainly, to get a full characterization of the SOK protein and its complete role in staphylococcal infections further investigation is required. SOK protects bacteria during the infection process from neutrophil killing, as well as promoting vegetation formation during heart infections. The presence of sok homologues in a diverse group of organisms that includes both pathogens and environmental bacteria suggests that SOK and its homologues may function to protect bacteria from ROS encountered both in animal hosts and in the environment.

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REFERENCES


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