Hydrogen Peroxide Production by *Streptococcus pneumoniae* Results in Alpha-hemolysis by Oxidation of Oxy-hemoglobin to Met-hemoglobin

Erin McDevitt, Faidad Khan, Anna Scasny, Courtney D. Thompson, Zehava Eichenbaum, Larry S. McDaniel, Jorge E. Vidal

Department of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, Mississippi, USA

Department of Biology, Georgia State University, Atlanta, Georgia, USA

ABSTRACT *Streptococcus pneumoniae* and other streptococci produce a greenish halo on blood agar plates referred to as alpha-hemolysis. This phenotype is utilized by clinical microbiology laboratories to report culture findings of alpha-hemolytic streptococci, including *S. pneumoniae*, and other bacteria. The alpha-hemolysis halo on blood agar plates has been related to the hemolytic activity of pneumococcal pneumolysin (Ply) or, to a lesser extent, to lysis of erythrocytes by *S. pneumoniae*-produced hydrogen peroxide. We investigated the molecular basis of the alpha-hemolysis halo produced by *S. pneumoniae*. Wild-type strains TIGR4, D39, R6, and EF3030 and isogenic derivative Δply mutants produced similar alpha-hemolytic halos on blood agar plates, while cultures of hydrogen peroxide knockout ΔspxB ΔlctO mutants lacked this characteristic halo. Moreover, in the presence of catalase, the alpha-hemolysis halo was absent in cultures of the wild-type (wt) and Δply mutant strains. Spectroscopic studies demonstrated that culture supernatants of TIGR4 released hemoglobin-bound heme (heme-hemoglobin) from erythrocytes and oxidized oxy-hemoglobin to met-hemoglobin within 30 min of incubation. As expected, given Ply hemolytic activity and that hydrogen peroxide contributes to the release of Ply, TIGR4Δply and ΔspxB ΔlctO isogenic mutants had significantly decreased release of heme-hemoglobin from erythrocytes. However, TIGR4Δply that produces hydrogen peroxide oxidized oxy-hemoglobin to met-hemoglobin, whereas TIGR4ΔspxB ΔlctO failed to produce oxidation of oxy-hemoglobin. Studies conducted with all other wt strains and isogenic mutants resulted in similar findings. We demonstrated that the so-called alpha-hemolysis halo is caused by the oxidation of oxy-hemoglobin (Fe^{2+}) to a non-oxygen-binding met-hemoglobin (Fe^{3+}) by *S. pneumoniae*-produced hydrogen peroxide.

IMPORTANCE There is a misconception that alpha-hemolysis observed on blood agar plate cultures of *Streptococcus pneumoniae* and other alpha-hemolytic streptococci is produced by a hemolysin or, alternatively, by lysis of erythrocytes caused by hydrogen peroxide. We noticed in the course of our investigations that wild-type *S. pneumoniae* strains and hemolysin (e.g., pneumolysin) knockout mutants produced the alpha-hemolytic halo on blood agar plates. In contrast, hydrogen peroxide-defective mutants prepared in four different strains lacked the characteristic alpha-hemolysis halo. We also demonstrated that wild-type strains and pneumolysin mutants oxidized oxy-hemoglobin to met-hemoglobin. Hydrogen peroxide knockout mutants, however, failed to oxidize oxy-hemoglobin. Therefore, the greenish halo formed on cultures of *S. pneumoniae* and other so-called alpha-hemolytic streptococci is caused by the oxidation of oxy-hemoglobin produced by hydrogen peroxide. Oxidation of oxy-hemoglobin to the nonbinding oxygen form, met-hemoglobin, might occur in the lungs during pneumococcal pneumonia.

Citation McDevitt E, Khan F, Scasny A, Thompson CD, Eichenbaum Z, McDaniel LS, Vidal JE. 2020. Hydrogen peroxide production by *Streptococcus pneumoniae* results in alpha-hemolysis by oxidation of oxy-hemoglobin to met-hemoglobin. mSphere 5:e01117-20. https://doi.org/10.1128/mSphere.01117-20.

Editor Michael David Leslie Johnson, University of Arizona

Copyright © 2020 McDevitt et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to Jorge E. Vidal, jvidal@umc.edu.

Received 30 October 2020
Accepted 13 November 2020
Published 9 December 2020
Historically, *Streptococcus pneumoniae* and other streptococci of the viridians group are classified as alpha-hemolytic bacteria on the basis of a greenish halo that surrounds colonies when grown aerobically on blood agar plates (1, 2). This alpha-hemolytic activity has been related to the production of a hemolysin, which, in the case of *S. pneumoniae* strains, is referred to as pneumolysin (Ply) (3). An observation made that anaerobic cultures of alpha-hemolytic streptococci lack this greenish discoloration has linked the phenotype also to the lysis of erythrocytes by hydrogen peroxide that streptococci produce as a metabolic by-product (1). In this study, we investigated whether Ply or *S. pneumoniae*-produced hydrogen peroxide was responsible for alpha-hemolysis and identified the molecular basis of the phenotype.

Pneumolysin is encoded by *ply* (4), while hydrogen peroxide released in cultures of *S. pneumoniae* strains and alpha-hemolytic streptococci is a by-product of the metabolism of the enzymes pyruvate oxidase (*SpxB*) and lactate dehydrogenase (*LctO*) (5). We utilized different Δ*poly* mutants and *S. pneumoniae* TIGR4Δ*spxB* Δ*lctO* from previous publications (6–8) and prepared Δ*spxB* Δ*lctO* double mutants in three other backgrounds, vaccine serotype 19F strain EF3030 (9, 10), serotype 2 strain D39, and strain R6, and a new EF3030Δ*poly* mutant (11). We previously demonstrated by Western blotting and a hemoglobin release assay that the Ply knockout mutants do not produce Ply (6) and that TIGR4Δ*spxB* Δ*lctO* does not produce detectable levels of hydrogen peroxide in supernatants from Todd-Hewitt broth supplemented with yeast extract (THY broth) cultures incubated for 4 h (7).

*S. pneumoniae* strains were inoculated on blood agar plates containing 5% sheep blood, and plates were incubated at 37°C under aerobic conditions and a 5% CO₂ atmosphere. Overnight cultures of strains TIGR4, D39, R6, and EF3030 showed the classic alpha-hemolytic halo surrounding colonies, whereas D39Δ*poly*, R6Δ*poly*, TIGR4Δ*poly*, and EF3030Δ*poly* mutant strains also showed an indistinguishable alpha-hemolysis halo (Fig. 1 and not shown). The median diameters of alpha-hemolysis halos produced by the D39 wild type (wt), TIGR4 wt, and EF3030 wt were very similar although statistically different from those produced by the respective *ply* mutant strains (Fig. 1, inset, and not shown). Blood agar plates with cultures of isogenic D39Δ*poly* produced alpha-hemolysis halos with a diameter of 2.26 mm (Fig. 1). An additional TIGR4Δ*poly* mutant (AC4037) yielded a similar alpha-hemolysis halo (not shown) (12). In contrast, blood agar plates inoculated with TIGR4Δ*spxB* Δ*lctO* and three additional double Δ*spxB* Δ*lctO* mutants, R6Δ*spxB* Δ*lctO*, D39Δ*spxB* Δ*lctO*, and EF3030Δ*spxB* Δ*lctO*, completely lacked the alpha-hemolytic halo (Fig. 1). Blood agar plates of TIGR4Δ*spxB* Δ*lctO*, D39Δ*spxB* Δ*lctO*, and EF3030Δ*spxB* Δ*lctO* did not produce the alpha-hemolytic halo even after 72 h of incubation (not shown).

To confirm that hydrogen peroxide was responsible for the alpha-hemolytic halo, TIGR4 wt or its isogenic TIGR4Δ*poly* was inoculated on blood agar plates containing catalase (400 U/ml). As shown in Fig. 1, catalase inhibited alpha-hemolysis. Moreover, adding pure hydrogen peroxide to blood agar plates spanning the concentration produced by *S. pneumoniae* strains (i.e., 800, 80, and 8 μM) produced similar alpha-hemolytic halos on plates made of sheep or horse blood (not shown). Other streptococci, including *S. mutans*, are not hemolytic when grown on blood agar plates (1, 2).

Colonies of *S. mutans* strain ATCC 25175 on blood agar plates resembled those of the *S. pneumoniae* isogenic hydrogen peroxide knockout mutants (Fig. 1). Similarly to *S. pneumoniae* and other streptococci, *S. mutans* harbors a putative α-hemolysin (13). *S. mutans* lacks production of detectable hydrogen peroxide in the supernatant when grown in aerobic cultures, and it is highly susceptible to hydrogen peroxide produced by alpha-hemolytic oral streptococci (14, 15). Altogether, this evidence indicates that hydrogen peroxide but not the hemolysin Ply caused the alpha-hemolytic phenotype observed in aerobic cultures of *S. pneumoniae* strains.

**KEYWORDS** *Streptococcus pneumoniae*, alpha-hemolysis, hemoglobin, met-hemoglobin, oxidation
Erythrocytes carry hemoglobin that reversibly binds oxygen through a penta-coordinate heme molecule containing ferrous iron (Fe^{2+}), known as oxy-hemoglobin (16).

When hemoglobin is released from erythrocytes, heme-hemoglobin can be observed by optical spectroscopy at \(415\) nm (16–18). This region is known as the Soret region peak and represents heme-hemoglobin, while oxy-hemoglobin is characterized by two absorption peaks of \(540\) and \(570\) nm (17, 18). Oxy-hemoglobin (Fe^{2+}) is autoxidized to met-hemoglobin (Fe^{3+}) or oxidized by radicals such as hydrogen peroxide (17, 18), inducing spectral changes, i.e., flattening the oxy-hemoglobin absorbance peaks. *S. pneumoniae* produces and releases an abundance of hydrogen peroxide into the culture supernatant that intoxicates human cells (19) or that rapidly kills *Staphylococcus aureus* strains and other bacterial species (7, 20). Hydrogen peroxide is a by-product of the metabolism of two different enzymes, pyruvate oxidase (SpxB) and lactate dehydrogenase (LctO) (5).

To further investigate the molecular basis of the alpha-hemolytic phenotype, we utilized a modified hemoglobin release assay that, when coupled with optical spectroscopy, allowed us to quantify the release of heme-hemoglobin and to observe the oxidation of oxy-hemoglobin to met-hemoglobin. As a control of heme-hemoglobin release and the presence of oxy-hemoglobin, we obtained the UV-visible absorption spectra of a 3% suspension of sheep erythrocytes that had been lysed with an equal volume of water or lysed with 0.1% final concentration of saponin (Fig. 2A and not shown). After centrifugation of the lysed erythrocyte suspension at 300 \(\times\) g for 5 min, no red blood cells were visible in the bottom; therefore, this was considered the maximum heme-hemoglobin released. As expected, three characteristic peaks were observed. The Soret peak, for which the wavelength of maximum absorption was 415 nm and its absorbance was set as 100% hemoglobin release (Fig. 2A), and two oxy-hemoglobin peaks at 540 and 570 nm (Fig. 2A). Similar peaks were observed when hemoglobin was released from sheep or horse erythrocytes with saponin (not shown). To investigate the release of heme-hemoglobin, *S. pneumoniae* strains were inoculated at similar densities of \(5 \times 10^{9}\) CFU/ml in THY broth (pH 7) and incubated at 37°C in a 5% CO\(_2\) atmosphere for 1, 2, 3, or 4 h. Bacterium-free supernatants were harvested and

---

**FIG 1**  Hydrogen peroxide but not pneumolysin causes the alpha-hemolytic phenotype on blood agar plates. *S. pneumoniae* wt strains TIGR4 (T), D39 (D), and EF3030 (E) or Δply and ΔspxB ΔlctO mutant derivatives, or *S. mutans* strain ATCC 25175, were inoculated onto blood agar plates and incubated for 24 h at 37°C in a 5% CO\(_2\) atmosphere. Another set of plates were added with 400 U/ml of catalase (Cat) and then inoculated as described above. Plates were photographed with a Canon Rebel EOS T5 camera system, and digital pictures were analyzed. Phenotypes were confirmed at least three times. Bar, 2 mm. (Right) Hemolytic halos measured with ImageJ software for at least 25 colonies from images obtained from cultures on blood agar plates of D39 wt, D39Δply, TIGR4 (T4), or TIGR4Δply. unpaired Student’s t test was performed to assess significance. *\(P<0.05\).
then incubated with equal volumes of a 3% suspension of sheep erythrocyte at 37°C in a 5% CO₂ atmosphere for 30 min, after which, the treated erythrocyte suspensions were centrifuged at 300 × g for 5 min to collect supernatants. Experiments presented below were conducted with TIGR4 wt and its isogenic mutants. We also performed similar experiments using D39, R6, and EF3030 wt strains and their isogenic mutants, with essentially similar results (not shown).

The Soret peak of heme-hemoglobin released in the control (Fig. 2B) represented 100% of heme-hemoglobin released (Fig. 2C). A time course study demonstrated that TIGR4 released ~60% of heme-hemoglobin as soon as 1 h postinoculation (Fig. 2B and C) and produced, after 4 h of incubation, a Soret peak representing ~85% of heme-hemoglobin released compared to the maximum heme-hemoglobin released in the control (Fig. 2C). As expected given that hydrogen peroxide contributes to release of Ply into the supernatant (21), hemoglobin released by TIGR4ΔspxB ΔlctO after 4 h of

**FIG 2** Heme-hemoglobin release by *S. pneumoniae* strains. (A) Suspension (3%) of sheep erythrocytes was lysed, centrifuged at 300 × g for 5 min, and incubated for 30 min at 37°C in a 5% CO₂ atmosphere; the spectrum was obtained using a spectrophotometer Omega BMG LabTech (Thermo Fisher). (B and C) TIGR4 wt, TIGR4Δply, or TIGR4ΔspxB ΔlctO was inoculated in THY broth (pH 7.0) and incubated for 4 h at 37°C in a 5% CO₂ atmosphere. (D) TIGR4Δply was incubated in THY broth alone or with catalase (200 U/ml) for 4 h. Bacterium-free supernatants were harvested by centrifugation at 13,000 × g for 5 min, and equal volumes were incubated with a 3% suspension of sheep erythrocytes for 30 min at 37°C. After pelleting down the erythrocytes as described above, the hemoglobin-containing supernatant was collected. (B) The UV-visible absorption spectrum was obtained at the 4-h time point. Maximum heme-hemoglobin release (C) or that released by an untreated control (D) was set to 100%, and the percent release by culture supernatants was calculated. Error bars represent the standard errors of the means calculated using data from at least three independent experiments. Student t test (*, *P < 0.05) analysis was performed to compare Soret absorbances at 415 nm generated by the wt and isogenic mutant at the same time point.
Oxy-hemoglobin is oxidized to met-hemoglobin by *S. pneumoniae*-produced hydrogen peroxide.

(A) Suspension (3%) of sheep erythrocytes was mixed with equal volumes of cell-free culture supernatants of strain TIGR4 wt that had been grown as described for Fig. 2B for the indicated times. The mixture was incubated for 30 min at 37°C in a 5% CO₂ atmosphere. As a control, erythrocytes were lysed and incubated under the same conditions. The absorbance spectra were then obtained using a spectrophotometer Omega BMG LabTech (Thermo Fisher). (B) TIGR4 wt, TIGR4 Δply, or TIGR4 ΔspxB ΔlctO was inoculated in THY broth (pH 7.0) and incubated for 4 h at 37°C in a 5% CO₂ atmosphere. Bacterium-free supernatants were harvested by centrifugation at 13,000 × g for 5 min, and equal volumes were incubated for 30 min at 37°C with hemoglobin-containing erythrocytes lysates. Hemoglobin-containing supernatants were collected, and the UV-visible absorption spectra were obtained. (C) Oxy-hemoglobin-containing lysates (control) were incubated for 30 min at 37°C with H₂O₂ (880 μM), TIGR4 wt supernatant harvested as described above, or TIGR4 wt supernatant and catalase (200 U/ml). Error bars

(Continued on next page)
incubation was ~50% of that released by the wt strain (Fig. 2B and C). In contrast to the TIGR4 strain and TIGR4ΔspxB ΔlctO, the isogenic Ply knockout mutant TIGR4Δply induced the release of <30% heme-hemoglobin compared to that by the control. This residual release of hemoglobin was in part caused by hydrogen peroxide activity in the supernatants, since it was significantly reduced in cultures of TIGR4Δply incubated with catalase (200 U/ml) (Fig. 2D). Because a mutation in ply renders the strain unable to lyse erythrocytes, all but Δply mutant strains produce the so-called alpha-hemolytic phenotype; these results further support that Ply-associated hemolytic activity is not responsible for the alpha-hemolysis phenotype observed in blood agar plates.

Oxy-hemoglobin can react with reactive oxygen species, including hydrogen peroxide, to produce met-hemoglobin, the non-oxygen-binding form of hemoglobin (22, 23). To assess the presence of met-hemoglobin, we evaluated the oxy-hemoglobin peaks in hemoglobin preparations incubated with S. pneumoniae supernatants. Oxy-hemoglobin peaks were clearly observed in the control preparation (Fig. 3A) but were completely flattened when culture supernatants of the TIGR4 strain obtained after 3 or 4 h of incubation were incubated with the suspension of erythrocytes for an additional 30-min period. This change in the absorption pattern of oxy-hemoglobin was compatible with the oxidation of oxy-hemoglobin to met-hemoglobin (22). Note that autooxidation of oxy-hemoglobin to met-hemoglobin did not occur within the 30-min incubation of the assay, since the oxy-hemoglobin peaks were observed. Because culture supernatants from TIGR4Δply or TIGR4ΔspxB ΔlctO did not contain heme-hemoglobin at the same level as those from TIGR4, we could not evaluate the oxidation of oxy-hemoglobin in these isogenic mutant strains using the modified hemoglobin release assay.

To further confirm whether oxy-hemoglobin is oxidized to met-hemoglobin by hydrogen peroxide produced in culture supernatants of TIGR4Δply but not in supernatants of hydrogen peroxide knockout mutant TIGR4ΔspxB ΔlctO, we incubated preparations of oxy-hemoglobin that had been previously released from erythrocytes, as mentioned earlier, with culture supernatants of TIGR4 or isogenic mutants. We reasoned that if hydrogen peroxide present in culture supernatants was responsible for the oxidation of oxy-hemoglobin, then having oxy-hemoglobin already as a substrate would allow us to observe such a reaction. As expected, supernatants from 4-h cultures of the wt strain that were incubated for 30 min with the oxy-hemoglobin preparation converted oxy-hemoglobin to met-hemoglobin (Fig. 3B). Supernatants from the isogenic TIGR4Δply significantly oxidized oxy-hemoglobin to met-hemoglobin, indicating that oxidation occurred due to the hydrogen peroxide activity retained by the ply knockout mutant. Confirming this hypothesis, oxy-hemoglobin was observed almost intact after a 30-min incubation with supernatants of the isogenic TIGR4ΔspxB ΔlctO, indicating that met-hemoglobin was produced by hydrogen peroxide secreted into the culture supernatant. Oxidation of oxy-hemoglobin did not occur in supernatants of the wt strain treated with catalase (200 U/ml), but oxy-hemoglobin was oxidized to met-hemoglobin when the oxy-hemoglobin preparations were treated with H2O2 (880 μM) at a similar concentration to that produced in culture supernatants of S. pneumoniae strains (7, 21, 24) (Fig. 3C). Oxidation of hemoglobin to met-hemoglobin was observed using horse erythrocytes and when releasing hemoglobin from erythrocytes using water or saponin (not shown).

In conclusion, we demonstrated in this study that the so-called alpha-hemolysis phenotype observed on blood agar plates when incubated under aerobic conditions is
an oxidative reaction caused by *S. pneumoniae*-produced hydrogen peroxide that converts oxy-hemoglobin to met-hemoglobin.

**ACKNOWLEDGMENTS**

This study was supported in part by a grant from the National Institutes of Health (NIH; 1R21AI144571-01 to J.E.V.) and generous start-up funds from the University of Mississippi Medical Center (UMMC).

The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH or UMMC.

We thank Andrew Camilli from Tufts University and James C. Paton from University of Adelaide for the kind gifts of strains AC4037 and D39Δply, respectively. We also thank Bernard Beall and Lesley McGee from the Centers for Disease Control and Prevention for providing *S. mutans* as well as David Stephens and Emilio Rodriguez from Emory University School of Medicine for earlier discussions of preliminary findings and assistance on some procedures, respectively.

**REFERENCES**

1. Facklam R. 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev 15:613–630. https://doi.org/10.1128/cmr.15.4.613-630.2002.

2. Coykendall AL. 1989. Classification and identification of the viridans streptococci. Clin Microbiol Rev 2:315–328. https://doi.org/10.1128/cmr.2.3.315.3.

3. Mitchell TJ, Dalziel CE. 2014. The biology of pneumolysin. Subcell Biochem 80:145–160. https://doi.org/10.1007/978-94-017-8881-6_8.

4. Walker JA, Allen RL, Falmagne P, Johnson MK, Boulnois GJ. 1967. Molecular cloning, characterization, and complete nucleotide sequence of the gene for *pneumolysin*, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect Immun 55:1184–1189. https://doi.org/10.1128/iai.55.4.1184-1189.1987.

5. Lisher JP, Tsui HT, Ramos-Montanez S, Hentchel KL, Martin JE, Trinidad JC, Winkler ME, Gedroc DP. 2017. Biological and chemical adaptation to endogenous hydrogen peroxide production in *Streptococcus pneumoniae* D39. mSphere 2:e00291-16. https://doi.org/10.1128/mSphere.00291-16.

6. Shak JR, Ludewick HP, Howery KE, Sakai F, Yi H, Harvey RM, Paton JC, Klugman KP, Vidal JE. 2013. Novel role for the *Streptococcus pneumoniae* toxin pneumolysin in the assembly of biofilms. mBio 4:e00655-13. https://doi.org/10.1128/mBio.00655-13.

7. Wu X, Gordon O, Jiangle W, Antezana BS, Angulo-Zamudio UA, Del Rio C, Moller A, Brissac T, Tierney ARP, Warncke K, Orihuela CJ, Read TD, Vidal JE. 2019. Interaction between *Streptococcus pneumoniae* and *Staphylococcus aureus* generates -OH radicals that rapidly kill *Staphylococcus aureus* strains. J Bacteriol 201:e00474-19. https://doi.org/10.1128/JB.00474-19.

8. Berry AM, Yoither J, Briles DE, Hansman D, Paton JC. 1989. Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. Infect Immun 57:2037–2042. https://doi.org/10.1128/IAI.57.7.2037-2042.1989.

9. Keller LE, Luo X, Thornton JA, Seo KS, Moon BY, Robinson DA, McDaniel LS. 2015. Immunization with pneumococcal surface protein K of nonencapsulated *Streptococcus pneumoniae* provides protection in a mouse model of colonization. Clin Vaccine Immunol 22:1146–1153. https://doi.org/10.1128/CVI.00456-15.

10. Junges R, Mainschein-Cline M, Morrison DA, Petersen FC. 2019. Complete genome sequence of *Streptococcus pneumoniae* serotype 19F strain EF3030. Microbiol Resour Announc 8:e00198-19. https://doi.org/10.1128/MRA.00198-19.

11. Lanie JA, Ng WL, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, Tettelin H, Glass JI, Winkler ME. 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J Bacteriol 189:38–51. https://doi.org/10.1128/JB.01148-06.

12. Price KE, Greene NG, Camilli A. 2012. Export requirements of pneumolysin in *Streptococcus pneumoniae*. J Bacteriol 194:3651–3660. https://doi.org/10.1128/JB.00114-12.

13. Adijic O, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najar F, Lai H, White J, Roe BA, Ferretti JJ. 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci U S A 99:14434–14439. https://doi.org/10.1073/pnas.172501299.

14. Kaftsz JK, Rivera-Ramos I, Abrahants J, Martinez AR, Rosalen PL, Derr AM, Quivey RG, Lemos JA. 2010. Two Spa proteins modulate stress tolerance, survival, and virulence in *Streptococcus mutans*. J Bacteriol 192:2546–2556. https://doi.org/10.1128/JB.00028-10.

15. van der Hoeven JS, Hoogendoom H. 1990. Uptake of oxygen, release and degradation of hydrogen peroxide by *Streptococcus mutans* NCTC 10449. Antonie Van Leeuwenhoek 57:91–95. https://doi.org/10.1007/BF00403160.

16. Kakar S, Hoffman FG, Storz JF, Fabian M, Hargrove MS. 2010. Structure and reactivity of hexameric hemoglobin. Biophys Chem 152:1–14. https://doi.org/10.1016/j.bpc.2010.08.008.

17. Ludlow JT, Wilkerson RG, Sahu KK, Nappe TM. 2020. Methemoglobinemia. StatPearls, Treasure Island, FL.

18. Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM. 1993. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci U S A 90:9285–9289. https://doi.org/10.1073/pnas.90.20.9285.

19. Brissac T, Shenoy AT, Patterson LA, Orihuela CJ. 2017. Cell invasion and pyruvate oxidase-derived H₂O₂ are critical for *Streptococcus pneumoniae*-mediated cardiomyocyte killing. Infect Immun 86:e00569-17. https://doi.org/10.1128/IAI.00569-17.

20. Khan F, Wu X, Matzkin GL, Khan MA, Sakai F, Vidal JE. 2016. *Streptococcus pneumoniae* eradicates preformed Staphylococcus aureus biofilms through a mechanism requiring physical contact. Front Cell Infect Microbiol 6:104. https://doi.org/10.3389/fcimb.2016.00104.

21. Bryant JC, Dabbs RC, Oswalt KL, Brown LR, Rosch MW, Seo KS, Donaldson JR, McDaniel LS, Thornton JA. 2016. Pyruvate oxidase of *Streptococcus pneumoniae* contributes to pneumolysin release. BMC Microbiol 16:271. https://doi.org/10.1186/s12866-016-0881-6.

22. Smalley JW, Olczak T. 2017. Heme acquisition mechanisms of *Staphylococcus aureus* - strategies used in a polymicrobial community in a heme-limited host environment. Mol Oral Microbiol 32:1–23. https://doi.org/10.1111/momi.12149.

23. Alyash AI, Patel RP, Cashon RE. 2001. Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. Antioxid Redox Signal 3:313–327. https://doi.org/10.1089/152306001300185250.

24. Pericone CD, Overweg K, Hermans PW, Weiser JN. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 68:3990–3997. https://doi.org/10.1128/iai.68.7.3990-3997.2000.