Oxidative reactions catalyzed by hydrogen peroxide produced by *Streptococcus pneumoniae* and other Streptococci Cause the Release and Degradation of Heme from Hemoglobin

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Running title: Spn-H_2O_2 oxidizes hemoglobin degrading heme

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Abstract

Streptococcus pneumoniae (Spn) strains cause pneumonia that kills millions every year worldwide. Spn produces Ply, a hemolysin that lyses erythrocytes releasing hemoglobin and also produces the pro-oxidant hydrogen peroxide (Spn-H_2O_2) during growth. The hallmark of the pathophysiology of hemolytic diseases is the oxidation of hemoglobin but oxidative reactions catalyzed by Spn-H_2O_2 has been poorly studied. We characterized the oxidation of hemoglobin by Spn-H_2O_2. We prepared a series of single (Δ*spxB*, or Δ*lctO*), double mutant (Δ*spxB*Δ*lctO*) and complemented strains in TIGR4, D39 and EF3030. We then utilized an *in vitro* model with oxy-hemoglobin to demonstrate that oxy-hemoglobin was oxidized rapidly, within 30 min of incubation, by Spn-H_2O_2 to met-hemoglobin and that the main source of Spn-H_2O_2 was pyruvate oxidase (SpxB). Moreover, extended incubation caused a further oxidation of met-hemoglobin that resulted in the release and the degradation of heme. We then assessed oxidation of hemoglobin and heme degradation by other bacterial species inhabitants of the respiratory tract. All hydrogen peroxide-producing streptococci tested caused the oxidation of hemoglobin and heme degradation whereas those bacterial species that produce <1 μM H_2O_2, neither oxidized hemoglobin nor degraded heme. An *ex vivo* bacteremia model confirmed that oxidation of hemoglobin and heme degradation occurred concurrently with hemoglobin that was released from erythrocytes by Ply. Finally, gene expression studies demonstrated that heme, but not red blood cells or hemoglobin induced an upregulated transcription of the *spxB* gene. Oxidation of hemoglobin may be important for pathogenesis and for the symbiosis of hydrogen peroxide-producing bacteria with other species by providing other with nutrients such as iron.

Introduction.

Streptococcus pneumoniae (Spn) colonizes the lower respiratory epithelium, causing pneumonia that can progress to invasive pneumococcal disease (IPD)(1-4). Spn mainly affects the most vulnerable populations: young children, immunocompromised patients, and the elderly (1, 4, 5). Globally, Spn causes ~15 million cases of illness each year, leading to more than a million deaths due to pneumonia (6-8). Pneumonia is an acute infection of the pulmonary parenchyma that presents with pulmonary hemorrhage, inflammatory congestion, hepatization, suppurative infiltration and lung parenchymal injury (4, 9-13). If left untreated, Spn invades the bloodstream, producing septic shock, multiorgan failure, cardiotoxicity, and death within days from onset (4, 9-12, 14, 15).

Spn strains produce the toxin pneumolysin (Ply) that has cytotoxic activity against lung epithelial and endothelial cells (16-18). Ply is a 53-kDa surface located protein (19, 20) that can also be released through autolysis (21, 22). Besides its toxicity against cells, Ply has hemolytic activity against erythrocytes of several mammals' species and hemolysis is observed whether Ply is released into the supernatant or located on the bacterial membrane (20). Under standard culture conditions, i.e., growing bacteria at 37°C in a 5% CO_2 atmosphere, Ply is responsible for almost all hemolytic activity in cultures of Spn strains (11), causing the release of a large amount of hemoglobin into the supernatant (20, 21, 23). Transcription of the *ply* gene encoding this pneumolysin is upregulated when Spn is cultured under conditions mimicking lung infection (24) and therefore, Ply-induced release of hemoglobin is expected to occur during pneumococcal lung infection.

Release of hemoglobin during hemolytic disease such as sickle cell disease and other hemorrhagic conditions, causes oxidation of hemoglobin (25). Oxidized hemoglobin releases heme, which in turn induces adverse effects including leukocyte activation, cytokine up-regulation, and production of oxidants leading to lung epithelial injury and damage to the vascular system (25-27).

Reactive oxygen species (ROS), such as nitric oxide (NO) (28) hypochlorous acid conjugate base (OCl-)(24), and H_2O_2 (29, 30), drive a catalytic cycle including the oxidation of oxy-hemoglobin to ferryl hemoglobin (Hb-Fe^{+4}), and auto- reduction of the ferryl intermediate to met-hemoglobin (Hb-Fe^{+3}) that is detected by spectroscopy at 405 nm (31, 32). Additional pro-oxidant molecules, such as H_2O_2, produce more ferryl hemoglobin and cause heme degradation (32, 33). Thus, the oxidation of hemoglobin results in highly oxidizing ferryl hemoglobin (Hb-Fe^{4+}), met-hemoglobin (Hb-Fe^{3+}) and the release of toxic heme that disrupts the mitochondria membrane potential of lung epithelial cells and endothelial cells, and caused lipid peroxidation (25, 32-35).

Because Spn causes hemorrhage in the lung and produces a large amount of highly reactive H_2O_2 (36, 37), oxidation of cell-free hemoglobin might occur during pneumococcal disease. H_2O_2 is a by-product of the oxidation of pyruvate, end product of the glycolysis, produced by the enzyme pyruvate oxidase (SpxB) (38, 39). This reaction consumes oxygen and produces H_2O_2. In the absence of oxygen, pyruvate is converted to L-lactate while in the presence of molecular O_2, L-lactate feeds back to pyruvate, via lactate oxidase (LctO), in a reaction that also produces H_2O_2 (38, 39). It has been calculated that ~85% of H_2O_2 released in cultures of Spn occurs through the reaction catalyzed by SpxB, whereas the remaining ~15% is attributed to LctO (40, 41). Experiments using animal models have demonstrated that Spn-H_2O_2 plays a role in lung colonization, and the translocation of Spn from the lungs to the bloodstream (42-45). Details of the mechanism(s) are largely unknown.

We recently discovered that Spn-H_2O_2 oxidizes hemoglobin to met-hemoglobin, a non-oxygen binding form of hemoglobin (23). We also demonstrated that the oxidation of hemoglobin causes the characteristic greenish halo around pneumococcal colonies grown on blood agar plates, known as alpha hemolysis (23). Thus, the phenotype known as alpha hemolysis is not hemolysis but the oxidation of hemoglobin to met-hemoglobin (23). Another alpha-hemolytic streptococci, *S. gordonii*,

oxidizes hemoglobin to met-hemoglobin through catalytic reactions driven by H_2O_2 (46). In this study we comprehensively investigated the oxidation of hemoglobin by Spn. We discovered that the oxidative capacity of Spn cultures, under the culture conditions utilized here, was attributed to H_2O_2 produced through catalytic reactions of SpxB but not to that of LctO. We also found that Spn-H_2O_2 caused the degradation of heme and that a number of H_2O_2-producing, alpha-hemolytic, Streptococci oxidized hemoglobin and degraded heme from hemoglobin. An *ex vivo* model of pneumococcal bacteremia further demonstrated that oxidation of hemoglobin and heme degradation occurred rapidly and that heme, but not hemoglobin or red blood cells, caused an upregulated transcription of *spxB*. Oxidative reactions driven by Spn-H_2O_2 might contribute to the pathogenesis of pneumococcal disease and can also be important for the symbiosis with other non-hydrogen peroxide producing bacteria by providing nutrients such as heme-iron.

Results

***Streptococcus pneumoniae*-produced hydrogen peroxide oxidizes heme-hemoglobin, causing the loss of the Soret peak.** We demonstrated that the alpha and beta chains of oxy-hemoglobin are deoxygenated by *S. pneumoniae* strains but further oxidation of heme-Fe^{+2} was not investigated (23). To assess this, Todd-Hewitt broth (THY) supplemented with sheep oxy-hemoglobin (oxyHb), which contains heme-Fe^{2+}, was inoculated with TIGR4 and incubated at 37°C in a 5% CO_2 and ~20% O_2 atmosphere. As a control, uninfected THY-oxyHb was incubated under the same conditions. After 30 min of incubation, TIGR4 cultures caused the shift of the Soret curve from heme-Fe^{2+}, that peaks at 415 nm, to a Soret curve of heme-Fe^{3+} at 405 nm (Fig. 1A). As expected, this hemoglobin preparation was deoxygenated producing met-hemoglobin (Fig. 1A, inset). Similar oxidation of heme-Fe^{2+} to heme-Fe^{3+} was observed when oxy-hemoglobin was purified from horse erythrocytes and incubated

with Spn strains TIGR4, or D39 (not shown). Cultures of THY-oxyHb inoculated with TIGR4Δ*spxB*Δ*lctO,* or D39Δ*spxB*Δ*lctO*, did not cause the shift of the Soret curve (Fig. 1A and not shown). Hemoglobin in uninfected cultures was not auto-oxidized after this 30 min incubation period (not shown).

Extended incubation of TIGR4 in THY-oxyHb revealed that the Soret curve was loss in a time-dependent manner (Fig. 1B). To assess if Spn-H_2O_2 had caused the loss of the Soret peak, we inoculated THY-oxyHb cultures with TIGR4Δ*spxB*Δ*lctO* and the culture was incubated for 6 h. A Soret curve was observed in this culture but the heme-Fe^{2+} peak (415 nm), observed after 30 min post-inoculation (Fig. 1A), had shifted to 405 nm representing heme-Fe^{3+} (Fig. 1C). The deoxygenation of hemoglobin in THY-oxyHb cultures of TIGR4Δ*spxB*Δ*lctO* was, however, due to autoxidation because uninfected cultures incubated under the same conditions at 37°C, and with a 5% CO_2 and ~20% O_2 atmosphere, had shifted to heme-Fe^{3+} as well (data not shown).

To further confirm that the loss of the Soret peak was due to Spn-H_2O_2, we prepared a complemented strain by cloning back *spxB* and *lctO* (Ω*spxB*Ω*lctO*) between SP_1113 and SP_1114 (Fig. 2). We also complemented TIGR4Δ*spxB* with a single copy of *spxB* to be utilized later (Fig. 2). This chromosomal location is unaffected by exogenous regulatory elements, such that complementing genes were controlled by their own promoters and insertion of genes within SP_1113 and SP_1114 did not affect virulence (47-50). Compared to wt and TIGR4Δ*spxB*Δ*lctO*, complemented strains showed a slight but not significant growth delay (Fig. 2B); however H_2O_2 production was restored to wt levels, as assessed with the Prussian blue-forming reaction and the Amplex Red kit (Fig. 2C and 2D) (51). In THY-oxyHb cultures of Ω*spxB*Ω*lctO* incubated for 6 h, the Soret curve had flattened (Fig. 1C). These results suggest that Spn-H_2O_2 causes heme degradation.

Given that heme-Fe^{2+} in THY-oxyHb cultures was oxidized to heme-Fe^{2+} prior to losing the Soret curve, we next assessed if the oxidation state of heme was important for the subsequent loss

of the Soret curve. To investigate this, we supplemented THY with deoxy-hemoglobin (i.e., met-hemoglobin) containing heme-Fe^{3+} (THY-Hb) and the culture was incubated with Spn. A similar loss of the Soret curve was observed with TIGR4 wt and Ω*spxB*Ω*lctO,* but an intact curve was obtained in cultures of TIGR4Δ*spxB*Δ*lctO* (not shown), indicating that heme-Fe^{3+} was further degraded.

Exogenous hydrogen peroxide supplementation at levels produced in *S. pneumoniae* cultures recapitulates hemoglobin oxidation. We then assessed whether a similar concentration of hydrogen peroxide to that observed in wt cultures of Spn (<1 mM) [Fig. 2 and references (36, 37)] can alter the heme-Fe^{3+} curve. For easy comparison of the multiple concentrations utilized in this experiment, we plotted the maximum absorbance obtained by spectroscopy for THY-Hb (405 nm) and we compared against that of THY-Hb treated with an increasing concentration of hydrogen peroxide. As shown in Fig. 1D, 10 µM hemoglobin in THY-Hb peaks at an absorbance of ~0.8. After 4 h of incubation with hydrogen peroxide, the absorbance was significantly reduced with a concentration of 88 µM and above, including the concentration of hydrogen peroxide observed in Spn cultures (~880 µM) (Fig. 1D). Altogether, these results indicate that the heme moiety in oxidized hemoglobin is degraded by Spn-H_2O_2.

Oxidation of hemoglobin in *S. pneumoniae* cultures is mainly caused by hydrogen peroxide catalyzed through pyruvate oxidase SpxB. Under aerobic conditions, hydrogen peroxide in pneumococcal cultures is produced through catalytic reactions of pyruvate oxidase (SpxB) and lactate oxidase (LctO) (40, 41). To assess the contribution of each enzyme, single Δ*spxB* or Δ*lctO* prepared in the TIGR4, or EF3030, background were assessed in THY-Hb cultures. In cultures of wt strains TIGR4, and EF3030, isogenic mutant TIGR4Δ*lctO*, and EF3030Δ*lctO*, Ω*spxB*Ω*lctO* and TIGR4Δ*spxB*Ω*spxB*

complemented strain the Soret Heme-Hb^{3+} curve was flattened, causing a statistically significant reduction of the 405 nm peak (Fig. 3A). In contrast, in THY-Hb cultures of TIGR4Δ*spxB* or EF3030Δ*spxB,* the Soret curve remained intact, similar to THY-Hb cultures inoculated with the corresponding Δ*spxB*Δ*lctO* mutant (Fig. 3A and 3B). An additional D39Δ*spxB* strain (P878) did not alter the Soret curve while its complemented strain, P1221, caused the loss of the Soret curve (not shown). These experiments demonstrated that oxidation of hemoglobin in cultures of Spn is mainly caused by hydrogen peroxide sourced from reactions catalyzed by SpxB.

Spn-produced H_2O_2 degrades heme-Fe^{3+}. Heme degradation occurs through enzymatic reactions or by nonselective destruction of heme double bonds caused by reactive oxygen species, such as H_2O_2 (31, 52). To investigate whether Spn-H_2O_2 caused degradation of heme, THY-Hb medium was inoculated with TIGR4, EF3030, their hydrogen peroxide mutant derivatives, or complemented strains and heme was assessed in supernatants using an in-gel heme assay (53, 54). Signal of heme, heme-bound to hemoglobin monomers or dimers, was detected in uninfected THY-Hb cultures incubated for 6 h (Fig. 4A). Heme signal in the supernatant of THY-Hb cultures incubated with TIGR4 for 2 h was similar to that of the uninoculated control but it decreased after 4 h post-inoculation and was not detected 6 h post-inoculation (Fig. 4A). In THY-Hb cultures of TIGR4, TIGR4Δ*lctO*, TIGR4Ω*spxB*Ω*lctO*, TIGR4Δ*spxB*Ω*spxB,* EF3030, or EF3030Δ*lctO* the signal of heme had already disappeared after 6 h of incubation (Fig. 4B and 4C). In contrast, in THY-Hb cultures of TIGR4Δ*spxB*Δ*lctO,* TIGR4Δ*spxB*, EF3030Δ*spxB*Δ*lctO*, or EF3030Δ*spxB*, signal of free heme, and heme-bound to hemoglobin was detected, thus confirming that degradation of heme had occurred in these cultures through hydrogen peroxide produced by SpxB.

As a loading control and to additionally investigate if hemoglobin had been degraded, supernatants were probed by Western blot with a polyclonal anti-hemoglobin antibody. Our results revealed a similar concentration of hemoglobin in those supernatants, confirming that the apo-protein was not degraded after the 2, 4 or 6 h incubation period with Spn (Fig. 4A, bottom panel). As expected since heme was degraded by Spn-H_2O_2, the intracellular concentration of iron was 79.19, 18.21, or 108.79 µM of Fe/µg of protein in TIGR4, TIGR4Δ*spxB*Δ*lctO* or TIGR4Ω*spxB*Ω*lctO*, respectively, confirming that iron was released and taken by wt and complemented strain. Altogether, these experiments demonstrated that Spn-H_2O_2 degrades free-heme and heme bound to hemoglobin.

Streptococci that produces hydrogen peroxide, but not other respiratory bacteria, causes the degradation of heme. Non-encapsulated Spn (NESpn) and other alpha-hemolytic streptococci produce hydrogen peroxide (55, 56). We therefore assessed a collection of NESpn and oral streptococci for heme degradation. We included *S. mutans* as an additional control since strains lack production of H_2O_2 (57, 58). As shown in Fig. 5, degradation of heme-Fe^{3+} was observed in THY-Hb cultures of NESpn strains MNZ41, MNZ67 and *S. salivarius*, *S. mitis*, *S. pseudopneumonanie* and *S. oralis* but not in cultures of *S. mutans* (Fig. 5). We also tested cultures of other bacterial species that cohabit the upper airways along with oral streptococci, such as *Haemophilus influenzae, Staphylococcus aureus* and assessed cultures of *Klebsiella pneumoniae*. The incubation of THY-Hb with any of these bacterial species did not cause heme degradation (Fig. 5). Altogether, these experiments demonstrated that Spn and other alpha hemolytic streptococci that produce abundant hydrogen peroxide, oxidizes hemoglobin causing the degradation of heme.

Heme is oxidized and degraded in an *ex-vivo* model of pneumococcal bacteremia. During the course of pneumococcal bacteremia erythrocytes are lysed by the pneumolysin (Ply), released through autolysis (59). Ply is also located on the bacterial membrane where it lyses red blood cells through contact (19, 20). Abundant Spn-H_2O_2 is produce as early as 2 h post-inoculation (Fig. 2D) thereby we sought to assess whether oxidation of hemoglobin and heme degradation occur in an *ex-vivo* model of pneumococcal bacteremia (Fig. 6A). To assess this, we supplemented THY broth with a suspension of red blood cells (THY-RBC), and this medium was infected with TIGR4, TIGR4Δ*ply*, or TIGR4Δ*spxB*Δ*lctO*. The oxidation and concentration of hemoglobin as well as heme degradation were investigated.

As expected, the Soret curve was not observed in supernatants from non-lysed THY-RBC control (not shown) and it was also absent in THY-RBC supernatants infected with TIGR4Δ*ply,* because it does not lyse erythrocytes (Fig. 6B). Given that RBCs contains oxy-hemoglobin, the Soret curve of the positive control, where RBC had been lysed with saponin, peaked at 415 nm with an average absorbance of 1.38 (Fig. 6B). The concentration of cell-free hemoglobin in this control supernatant was 17.35 µM as measured by the Quantichrom assay. The Soret curve from THY-RBC cultures infected with TIGR4 peaked at 405 nm with an average absorbance of 1.39 (Fig. 6B) and concentration of hemoglobin of 12.8 µM. This results indicate that maximum TIGR4-induced release of hemoglobin is achieved within 30 min of incubation, compared with the saponin-induced released control, and that the oxidation of heme-Fe^{2+} to heme-Fe^{3+} had already occurred. Similar to the wt and because TIGR4Δ*spxB*Δ*lctO* produces Ply (21, 23), a Soret curve was observed in THY-RBC cultures infected with this hydrogen peroxide deficient mutant as soon as 30 min post-inoculation (Fig. 5B). This Soret curve peaked at 415 nm with an average absorbance of 1.21 and a concentration of hemoglobin of 16.4

µM, indicating that most hemoglobin had been released but, unlike the wt strain, oxidation of heme-Fe^{2+} did not occur.

After 4 h of incubation, autoxidation of uninoculated, saponin-released THY-RBC cultures and those infected with TIGR4Δ*spxB*Δ*lctO* had occurred (Fig. 5C). THY-RBC medium infected with TIGR4 had already flattened the heme-Fe^{3+} curve and therefore heme had been degraded but not in those of TIGR4Δ*spxB*Δ*lctO* (Fig. 5C). Degradation of heme was not inhibited by supplementing THY-RBC before the infection with protease inhibitors, but it was inhibited when an excess of catalase (1000 U/ml) was added (not shown). Altogether, our results indicate that oxidation of hemoglobin and degradation of heme by Spn-H_2O_2 occur during pneumococcal bacteremia.

Because the release of hemoglobin and its oxidation was observed early during the simulated bacteremia (i.e., 30 min), to gain insights into the mechanism leading to hemoglobin oxidation and heme degradation, we investigated expression of *ply*, *spxB,* and *lctO* during incubation of Spn in THY-RBC, THY-Hb or in THY supplemented with heme. Compared to THY control cultures, the expression of *ply, spxB,* and *lctO* did not significantly change when TIGR4 grew in broth cultures added with RBCs or hemoglobin. However, transcription of *spxB* was upregulated when TIGR4 was incubated in THY containing heme, whereas the transcription *lctO* and *ply* was downregulated (Fig. 7). Together, these experiments indicate that heme was the inducer for Spn-H_2O_2 production.

Discussion

We have demonstrated in this study that H_2O_2 produced by the pneumococcus, Spn-H_2O_2, and other alpha-hemolytic Streptococci, caused the oxidation of hemoglobin to met-hemoglobin with a subsequent degradation of heme. The current study also demonstrated that the oxidation of hemoglobin and heme degradation occurred in a simulated bacteremia infection suggesting that

these oxidative reactions may occur during pneumococcal disease. It has been investigated that nearly 3% of oxy-hemoglobin is auto-oxidized inside erythrocytes, and this auto-oxidation is caused by $2x10^{-7}$ mM of hydrogen peroxide generated in the cytosol of erythrocytes (60). Although auto-oxidation by CO_2 occurred in our *in vitro* system 4 h post-inoculation (i.e., auto-oxidation was not observed when incubated at 37°C with no CO_2), Spn cultures produce 10^7-fold more (~1 mM) hydrogen peroxide within a few hours of incubation and caused the rapid oxidation of hemoglobin leading to the release of heme, a toxic molecule, with its subsequent degradation (61). We demonstrated that heme degradation caused an increased on free-iron in the medium that was taken by pneumococci. These novel observations might have important implications for pathogenesis of hydrogen peroxide-producing Streptococci but also for the symbiosis observed in bacterial communities where some species can benefit from heme and iron release by hydrogen peroxide-producing bacteria.

In vitro oxidation of hemoglobin with hydrogen peroxide produces ferryl hemoglobin [Hb-Fe^{4+}], a highly reactive form of hemoglobin that gets reduced to met-hemoglobin (62). With an increased presence of hydrogen peroxide molecules, met-hemoglobin gets oxidized back to Hb-Fe^{4+}, which in turn causes heme degradation (61). Iron stored in heme-hemoglobin is poorly reactive but once freed up, as a consequence of heme degradation, free iron can react with hydrogen peroxide to produce another highly reactive hydroxyl radical (63, 64). Indeed, we have demonstrated that free iron present in THY medium is enough to generate hydroxyl radicals ($^{\bullet}OH$) (37), killing *S. aureus* strains rapidly (36, 65, 66). The increase of iron in the culture medium due to heme degradation should result in an increased generation of $^{\bullet}OH$ radicals. The potential presence of two highly reactive radicals, Hb-Fe^{4+} and $^{\bullet}OH$, for the cytotoxicity seen during pneumococcal disease is being investigated in our laboratories.

In children with SCD, hemoglobin (Hb)S is oxidized to Hb-Fe^{4+} then reduced to met-hemoglobin and resulting in an acute hemolytic vascular inflammatory process causing acute lung injury (25, 67). Both Hb-Fe^{4+} and met-hemoglobin induce a drop of mitochondrial oxygen consumption and mitochondrial membrane potential in epithelial lung cells (32). Studies of the oxidation of hemoglobin by Spn-H_2O_2, however, have been neglected. We recently demonstrated that the growth of planktonic Spn, and formation of pneumococcal biofilms, is enhanced by supplementing the culture medium with human hemoglobin (68, 69). Besides iron, other nutrients may become accessible to pneumococcus, since the oxidation of hemoglobin by hydrogen peroxide releases globin-derived peptides (26), but also a striking transcriptome remodeling was identified that included upregulated transcription of genes encoding transporters of glyco-conjugated molecules (68, 69).

Besides nutrients, oxidative reactions may lead to unintended consequences. During pneumococcal pneumonia the pulmonary parenchyma presents with hemorrhage, inflammatory congestion, hepatization, suppurative infiltration, and lung parenchymal injury (4, 9-13). Whereas there are a number of virulence factors implicated in the pathophysiology of lung infection (59), the oxidation of hemoglobin through Spn-H_2O_2 may also be an important contributor to pneumococcal disease. For example, mice intranasally inoculated with Spn develop lung hemorrhage, at necropsy, and histological analysis reveals lung consolidation associated with alveolar septal edema and pleomorphic alveolar, interstitial, and perivascular cellular inflammation (70). Spn mutants in the *spxB* gene whether with a pleotropic capsule defect or not, were attenuated for virulence in mouse models of pneumococcal disease (43, 44, 71).

There are other important implications as a result of the oxidation of hemoglobin by hydrogen peroxide produced by streptococci. Heme release and/or heme degradation through, hydrogen peroxide, may be beneficial to other bacteria which do not synthetize large amounts of this pro-

oxidant but that require iron for their metabolism and pathogenesis. For instance, it has been recently demonstrated that dental plaque bacteria such as *S. gordonii*, which produces hydrogen peroxide, oxidizes hemoglobin to release heme, and this free-heme facilitates the colonization of the dental plaque by a Gram-negative strain *Porphyromonas gingivalis* (46). *P. gingivalis* does not produce siderophores but it has a sophisticated mechanism for heme-iron acquisition (72). Conversion of oxy-hemoglobin to met-hemoglobin, by hydrogen peroxide producing bacteria, allows the release of heme that can be taken by other microorganisms.

In summary, we demonstrated that Spn and other hydrogen peroxide-producing Streptococci, oxidizes hemoglobin from human and other species, releasing and degrading heme. These oxidative reactions occurred early during the lag phase of growth indicating that metabolic adaptation for growth in the presence of hemoglobin stimulated production of Spn-H_2O_2. This upregulated metabolic adaption was the result in part of the release of heme since gene expression studies demonstrated an upregulation of *spxB* transcription but not that of *lctO* or the pneumolysin gene *ply*, under the culture conditions tested. Efforts are in place to assess the specific role for these oxidative reactions for pathogenesis and bacterial symbiosis.

Material and Methods

Bacterial strains, material, and growth conditions. All Spn wild type strains and mutant derivatives used in this study are listed in Table 1. Bacterial stocks were prepared in medium containing skim milk-tryptone-glucose-glycerin (STGG) and stored at -80°C (73). Pneumococci were rotinously cultivated at 37°C in a 5% CO_2 and ~20% O_2 atmosphere. Experiments were performed using Todd-Hewitt-broth with 0.5% yeast extract (THY), THY added with 10 µM human met-hemoglobin (Sigma-Aldrich), THY added with 5 µM heme (Sigma-Aldrich), THY added with sheep erythrocytes (Quad Five),

and horse erythrocytes (Lampire). Brain heart infusion (BHI) broth, Bacto agar, Bacto Tryptic Soy Broth, Bact Yeast Extract and Bacto Todd Hewitt Broth were purchased from Becton, Dickinson and Company. Antibiotics utilized were gentamycin, erythromycin, spectinomycin, chloramphenicol, and were all sourced from Sigma-Aldrich. Hydrogen peroxide (Fisher Scientific), Catalase (Sigma-Aldrich), Complete™, Mini Protease Inhibitor Cocktail (Roche), $FeCl_3 \cdot 6H_2O$ and potassium hexacyanoferrate (III) (Sigma-Aldrich), o-dianisidine (Alfa Aesar) /sodium acetate (Sigma-Aldrich), ethanol and methanol (Fisher Scientific).

Preparation of inoculum for experiments. The inoculum was prepared as previously described (37). Briefly, bacteria were inoculated on blood agar plates (BAP) and incubated overnight at 37°C in a 5% CO_2 atmosphere. Bacteria were then harvested from plates by PBS washes and this suspension was used to inoculate THY with or without additives, that was brought to a final OD_{600} of ~0.1. This suspension contained ~5.15×10^8 cfu/ml as confirmed by dilution and platting of aliquots of the suspension.

Bacterial broth culture medium containing oxy-hemoglobin. Sheep or horse erythrocytes were washed three times with sterile PBS (pH=7.4) at $300 \times g$ for 5 min in a refrigerated centrifuge (Eppendorf). These washed erythrocytes suspension was lysed with 0.1% saponin and the concentration of oxy-hemoglobin in this preparation was determined using the QuantiChrom™ Hemoglobin Assay Kit (BioAssay Systems); oxy-hemoglobin was then added to THY broth to a final concentration of ~10 μM. The concentration, and redox state of oxy-hemoglobin was verified by spectroscopy before each experiment. In some experiments, THY containing oxy-hemoglobin was supplemented with an excess of catalase to a final concentration of 1000 U/ml, or with 1X complete protease inhibitors cocktail (Roche).

Studies of the oxidation of oxy-hemoglobin, met-hemoglobin and heme degradation by spectroscopy. Experiments were conducted using six-well microplates (Genesee Scientific) added with THY containing a 1% suspension of washed sheep erythrocytes, 10 µM of oxy-hemoglobin, or 10 µM of met-hemoglobin. These media was inoculated with Spn or the other bacterial species listed in each experiment, and incubated for the indicated time at 37°C in a 5% CO_2 and ~20% O_2 atmosphere. In another set of experiments, THY containing 10 µM of human met-hemoglobin was treated with different concentrations of hydrogen peroxide for 4 h. At the of the incubation time, supernatants containing planktonic bacteria were removed from cultures and bacteria were pelleted down by centrifugation at 13,000 rpm x 5 min in a refrigerated micro-centrifuge (Eppendorf). The oxidation of oxy-hemoglobin to met-hemoglobin and further oxidation and degradation of met-hemoglobin was determined by analyzing the spectra spanning 200 nm through 1000 nm in bacteria-free supernatants, using a spectrophotometer Omega BMG LabTech (ThermoFisher).

Quantification of hydrogen peroxide production by *S. pneumoniae* strains. Hydrogen peroxide production was quantified from Spn cultures inoculated in six-well microplates (Genesee Scientific) that had been added with THY. These cultures were incubated at 37°C in a 5% CO_2 and ~20% O_2 atmosphere for the indicated time. At the end of the incubation, the culture supernatant was collected, centrifuged at 4°C for 5 min at 15,000 rpm and then the supernatant was transferred to a new tube and kept in ice for no more than 30 min. Some supernatants were filtered sterilized using a 0.4 µm syringe filter (Fisher scientific). Collected supernatants were diluted with 1× reaction buffer of the Amplex Red H_2O_2 assay kit (Molecular Probes), and hydrogen peroxide levels were quantified according to the manufacturer's instructions using a spectrophotometer Omega BMG LabTech (ThermoFisher).

Qualitative detection of hydrogen peroxide production by *S. pneumoniae* strains. Qualitative detection of H_2O_2 production by Spn was performed using the Prussian blue agar assay, which limit of detection is 2.5 nm H_2O_2 (51). Prussian blue agar plates were made with 1.0 g/liter of $FeCl_3 \cdot 6H_2O$, 1.0 g/liter of potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$) also known as Prussian blue, 37 g/liter of dehydrated BHI broth, and 15 g/liter of agar. Prior to experiments, detection of H_2O_2 was assessed by dropping 10 µl of a 1 mM H_2O_2 solution that reduced $K_3[Fe(CN)_6]$ and causing its precipitation and then a blue halo within minutes. PBS was used as a negative control. Spn strains or culture supernatants (10 µl) obtained as described above, were inoculated, or spotted, respectively, onto Prussian Blue agar plates. These plates were incubated at 37 °C in a 5% CO_2 and ~20% O_2 atmosphere overnight (Spn) or for 4 h (supernatants) after which plates were photographed with a Canon Rebel EOS T5 camera system and digital pictures were analyzed.

Western blotting. Bacteria were grown in THY with 10 µM hemoglobin at 37°C for 2 h, 4 h, and 6 h. After incubation the cells were harvested by centrifuge at 15,000 rpm for 5 min and the supernatant were transferred to new tube. The collected samples were combined with 4x reducing sample buffer and boiled 5 minutes. The mixture was loaded into 10 well 4-12% Mini-PROTEAN® TGX™ precast gel (Biorad), and run 2 hours at 90 V in running buffer. Gels were transferred to nitrocellulose membranes in transfer buffer supplemented with 20% ethanol with Trans-Blot Turbo Transfer System. Membranes were blocked 1 h at room temperature in 5% non-fat dry milk in TBS supplemented with 0.1% Tween-20 (TBST). Then a membrane was incubated with the Hb polyclonal antibody (Invitrogen) at 1:1000 dilution in 5% non-fat dry milk in TBST overnight at 4°C. Next day, the blot was washed 3x 5 min with TBST, and incubated with donkey anti-goat IgG-HRP (Santa Cruz) secondary antibody 1:5000 in 5% non-fat dry milk in TBST 1 h at room temperature. Blots were washed 3x 5 min in TBST and 2mL each SuperSignal Substrate (Thermo Fisher Scientific) reagent was added to blot and

incubated for 5 min at room temperature. Blots were imaged on a ChemiDoc MP (BioRad) using ImageLab 5.0 software, automatic exposure settings for Chemiluminescence, high specificity, optimizing for bright bands.

In-gel heme staining. Bacteria were grown in THY with 10 µM hemoglobin at 37°C for 2, 4, or 6 h, after which supernatants were collected, spun down and combined with non-reducing loading buffer. The mixtures were loaded into a non-denaturing 12% Mini-PROTEAN® TGX™ precast gel (Biorad), and run for 2 h at 90 V in running buffer lacking SDS. The gel was then stained using a published protocol (31). Briefly, after electrophoresis gels were immersed into a methanol/sodium acetate (pH=5) solution and incubated at room temperature on rocking platform (VWR) at 1 speed for 2 min with constant shaking. After incubation, the gel was added with a solution of o-dianisidine/sodium acetate (pH=5)and incubated for 20 min in the dark. To visualize heme in the gel, 3% hydrogen peroxide was added, and the reaction was stopped by washing with distilled water. The gel was then photographed with a Canon Rebel EOS T5 camera system and digital pictures were analyzed.

RNA extraction and qRT-PCR analysis. Bacteria were inoculated into THY, or THY containing 5 µM heme, 5 µM Hb, or a 1% suspension of RBC and incubated at 37°C for 2 h. After incubation the bacterial suspension was collected, and mixed with RNAprotect Reagent (Qiagen). Then cells were harvested and the total RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's instruction. DNA was removed using the Turbo DNase-free kit (Life Technologies). The RNA concentration was obtained using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and 200 ng of RNA were cDNA transcribed using the iScript™ cDNA Synthesis Kit (Biorad). Gene expression analysis was carried out using the PerfeCTa SYBR® Green SuperMix (Quantabio) and CFX96 Touch Real-Time PCR system (Biorad). Primers used for the qRT-PCR analysis are listed in Table 2. The following conditions were utilized: 1 cycle at 95°C for 3 min and 40 cycles of 95°C for 15 s, 60°C for 30

s and 72°C for 30 s. Melting curves were generated to confirm the absence of primer dimers. The relative quantitation of mRNA expression was normalized to the constitutive expression of the housekeeping 16S rRNA gene and calculated by the comparative CT ($2^{-\Delta\Delta CT}$) method (74).

Preparation of *lctO* mutation in Spn strains EF3030 and D39. To prepare an isogenic *lctO* mutant, we PCR-amplified, using primers lcto-spec1 and lcto-spec2 (Table 2), an insertionally inactivated *lctO*-spectinomycin fragment from strain TIGR4Δ*lctO* (37). This PCR product was purified using the QIAquick PCR purification kit (Qiagen) and then transformed into EF3030 or D39 using an standard transformation procedure (75). Transformants were harvested in BAP with spectinomycin (100 µg/ml) and the insertional inactivation of *lctO* was confirmed by PCR with primers lcto-spec1 / lcto-spec2 and sequencing.

Construction of complemented strains. To complement *spxB*, or *spxB* and *lctO,* into TIGR4Δ*spxB* or TIGR4Δ*spxB*Δ*lctO*, respectively, we chose a 'neutral' chromosomal location within the *hplA* gene (SP1113) in which insertions do not affect virulence (48). A *spxB* complemented fragment was prepared by amplifying an upstream region carrying a fragment of the *hlpA* gene, using as a template DNA purified from TIGR4, and primers hlpA-up1 and hlpA-up2 (Table 2). We then generated a PCR fragment, using DNA purified from TIGR4 as a template, containing the *spxB* gene and its promotor with primers spxB1 and spxB2 (Table 2). A downstream fragment containing the *catP* gene from chloramphenicol resistance and a fragment of ORF SP1114 was amplified using as a template DNA from strain JWV500 using primers hplA-down1 and hlpA-down2. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and ligated by splicing overlap extension PCR with primers hlpA-up1/hlpA-down2. The PCR-ligated product was verified in a 0.8% DNA gel, purified from the gel using QIAquick Gel Extraction Kit (Qiagen) and re-amplified by PCR with primers hlpA-up1 and hlpA-down2. This PCR product was purified as above and used to transform TIGR4Δ*spxB* using a standard

procedure (75). Transformants were harvested in BAP plates containing chloramphenicol (5 µg/ml) and screened by PCR using primers spxB1/spxB2 to confirm the complementation of *spxB*.

To construct a *spxB* and *lctO* complemented strain, an upstream DNA fragment containing *hlpA* and *spxB* was amplified using DNA purified from TIGR4Δ*spxB*Ω*spxB* and primers hlpA1 and spxB2. Then, a DNA fragment was prepared by PCR amplification of the *lctO* gene and its putative promotor region using TIGR4 DNA as a template and primers lctO1 and lctO2. The downstream fragment containing the *catP* gene and a fragment of ORF SP1114 was used as prepared above. After purification with a QIAquick PCR purification kit (Qiagen), these three DNA fragments were ligated by splicing overlap extension PCR with primers hlpA-up1/hlpA-down2 and purified. This construct was re-amplified with primers hlpA-up1 and hlpA-down2, purified and transformed into TIGR4Δ*spxB*Δ*lctO*. Transformants were screened by PCR using primers hlpA-up1/hlpA-down2and selected completed clones were further confirmed by H_2O_2 production.

Metal content analysis. Pneumococcal biofilms ultured in THY-Hb for 4 h were washed 3x with ice-cold PBS, resuspended in 100 µl PBS and sonicated at high intensity for 5 min in 30 s on 30 s off cycles using a Bioruptor (Diagenode, Denville, NJ, USA). Protein was determined in all samples by the Bradford method and a known mass of sample was mineralized in concentrated HNO_3 trace metal grade (Thermo Fisher Scientific) as previously described (76). Iron in each sample was determined by atomic absorbance spectroscopy (AAS) using a flame 55 AA Absorption Spectrometer (Agilent, Santa Clara, CA, USA). Analytical grade standards for iron (Thermo Fischer Scientific) were diluted in 18ΩV water. The iron content on each sample was normalized to the initial mass of protein as previously reported for other metals (77, 78).

Statistical analysis. All statistical analysis was performed using the software Graph Pad Prism (version 8.3.1).

Figure legends

Fig. 1. Hydrogen peroxide produced in *S. pneumoniae* cultures oxidizes human hemoglobin. TIGR4, TIGR4Δ*spxB*Δ*lctO,* or TIGR4Ω*spxB*Ω*lctO* was inoculated in THY containing oxy-hemoglobin [Hb (Fe^{2+})] and cultures were incubated for (A) 30 min, (B) 2 and 6 h or (C) 6 h at 37°C in a 5% CO_2 atmosphere. Supernatants were collected and the spectra were obtained using a spectrophotometer Omega BMG LabTech. The inset in A shows the absorbance peaks of the alpha chain and the beta chain of oxy-hemoglobin, flattened in met-hemoglobin. In (A and C) the dotted lines indicate the Soret peak for oxy-hemoglobin (415 nm) or met-hemoglobin (405 nm). (D) THY supplemented with met-hemoglobin (Hb-Fe^{3+}) was added with the indicated concentration of hydrogen peroxide, or left untreated. These cultures were incubated for 6 h at 37°C in a 5% CO_2 atmosphere after which the supernatants were purified and the spectra were obtained as above. The absorbance at 405 nm obtained for each assessed concentration, was utilized to construct the graphic. Error bars in all panels represent the standard errors of the means calculated using data from at least three independent experiments performed with two technical replicates. In (D) One-way ANOVA with Tukey's test for multiple comparison was performed, *$p<0.05$ compared with untreated THY-Hb-Fe^{3+}.

Fig. 2. Preparation of a TIGR4Δ*spxB*Δ*lctO*-derivative complemented strain carrying *spxB* and *lctO* or *spxB*. (A) Schematic diagram showing the chromosomal region where *spxB* and *lctO* (top) or *spxB* (bottom) was complemented in TIGR4Δ*spxB*Δ*lctO*, to create TIGR4Ω*spxB*Ω*lctO*, TIGR4Ω*spxB*. Promotor regions of each gene (P) are indicated as well as terminators (†); gene IDs were included using TIGR4 nomenclature. The size of the final construct is indicated below each panel. (B-D) TIGR4,

TIGR4Δ*spxB*Δ*lctO*, TIGR4Ω*spxB*Ω*lctO*, or TIGR4Ω*spxB* was inoculated in THY and incubated at 37°C in a 5% CO_2 atmosphere. (B) The OD_{600} of the cultures was obtained at the indicated time. (C) Supernatants from 4 h cultures were sterilized and an aliquot was inoculated onto Prussian-blue agar plates (51) and the plates were incubated 2 h at room temperature after which they were photographed. (D) Hydrogen peroxide was quantified from these supernatants using the Amplex Red assay.

Fig. 3. Oxidation of hemoglobin occurs through hydrogen peroxide produced through SpxB activity but not LctO. (A) TIGR4, TIGR4Δ*spxB*Δ*lctO*, TIGR4Δ*spxB*, TIGR4Δ*lctO*, TIGR4Ω*spxB*Ω*lctO*, or TIGR4Ω*spxB* or (B) EF3030, EF3030Δ*spxB*Δ*lctO*, EF3030Δ*spxB*, or EF3030Δ*lctO*, was inoculated in THY containing met-hemoglobin [Hb (Fe^{3+})] and cultures were incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Supernatants were collected and the spectra were obtained using a spectrophotometer Omega BMG LabTech. The absorbance at 405 nm obtained from each assessed strain, was utilized to construct the graphic. Error bars in panels (A and B) represent the standard errors of the means calculated using data from at least three independent experiments performed with two technical replicates each. One-way ANOVA with Tukey's test for multiple comparison was performed; NS=not significant compared to THY-Hb (Fe^{3+}), *p<0.0001 compared with untreated THY-Hb (Fe^{3+}), or Δ*spxB*Δ*lctO*, or Δ*spxB*; **p<0.003 compared with untreated THY-Hb (Fe^{3+}).

Fig. 4. Oxidation of human hemoglobin by Spn-H_2O_2 leads to heme degradation. (A) TIGR4 wt was inoculated in THY supplemented with met-hemoglobin (THY-Hb) and incubated for 2, 4 or 6 h at 37°C in a 5% CO_2 atmosphere. Supernatant was harvested and run in a 12% polyacrylamide gel under non-denaturing conditions and heme was stained by an in-gel heme staining procedure. Free heme, heme-bound to hemoglobin monomer (Hb-monomer) or to hemoglobin dimers (Hb-dimer), and the observed molecular size in kDa, are indicated. (B) TIGR4, TIGR4Δ*spxB*Δ*lctO*, TIGR4Δ*lctO*, TIGR4Δ*spxB*,

TIGR4Ω*spxB*, TIGR4Ω*spxB*Ω*lctO*, or (C) EF3030 wt strains or EF3030Δ*spxB*Δ*lctO*, EF3030Δ*spxB*, or EF3030Δ*lctO*, was inoculated into THY-Hb and incubated for six hours. Supernatants were harvested and analyzed as in (A).

Fig. 5. Oxidation of hemoglobin occurs in cultures of oral Streptococci that produce hydrogen peroxide. THY supplemented with met-hemoglobin (THY-Hb) was left uninoculated or inoculated with the indicated strain and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. Supernatants were collected and the spectra were obtained using a spectrophotometer Omega BMG LabTech. The absorbance obtained at 405 nm for each assessed strain, was utilized to construct the graphic. Error bars represent the standard errors of the means calculated using data from at least three independent experiments performed with two technical replicates. One-way ANOVA with Tukey's test for multiple comparison was performed, *p<0.05, or NS=not significant, compared with untreated THY-Hb.

Fig. 6. Hemoglobin is oxidized by Spn-H_2O_2 in an *ex vivo* bacteremia model with erythrocytes. (A) Model of *ex vivo* pneumococcal bacteremia showing pneumococci with pneumolysin (Ply) located in the membrane; Ply also gets released through autolysis. Membrane-permeable hydrogen peroxide (H_2O_2) is produced as a by-product of the oxidation of pyruvate. Bottom panel, oxy-hemoglobin (Hb-Fe^{2+}) is then release from erythrocytes by Ply-induced erythrocyte lyses and once released, oxy-hemoglobin gets oxidized by Spn-H_2O_2 to met-hemoglobin (Hb-Fe^{3+}). Alternatively, top panel, Spn-H_2O_2 permeates inside erythrocytes causing the oxidation of hemoglobin that is then released from lysed erythrocytes as met-hemoglobin (Hb-Fe^{3+}). (B) A suspension (1%) of sheep erythrocytes in THY was inoculated with TIGR4, TIGR4Δ*spxB*Δ*lctO*, or TIGR4Δ*ply* and the inoculated cultures were incubated for 30 min (B) or 4 h (C) at 37°C in a 5% CO_2 atmosphere. As a control, erythrocytes were lysed with saponin and oxy-hemoglobin (THY-Hb^{2+}), that was released into the supernatant, was

considered as maximum hemoglobin release. Supernatants were collected and the spectra were obtained using a spectrophotometer Omega BMG LabTech. Inset in B shows the absorbance of the oxygenated, or deoxygenated, alpha and beta chains of hemoglobin.

In panels B and C, error bars represent the standard errors of the means calculated using data from at least three independent experiments.

Fig. 7. Regulation of *ply*, *spxB* and *lctO* genes by incubation with erythrocytes, hemoglobin, or heme. TIGR4 wt was inoculated in THY broth alone, in THY broth containing 1% sheep erythrocytes, THY with 10 µM hemoglobin (Hb) or THY with 5 µM heme. Cultures were incubated for 2 h at 37°C in a 5% CO_2 atmosphere, bacteria were then harvested, and RNA was extracted from these cultures and utilized as template in qRT-PCR reactions with primers that amplified the *ply*, *spxB*, or *lctO* gene. Average C_T values were normalized to the 16S rRNA gene, and the fold differences, relative to THY control, were calculated using the comparative C_T method ($2^{-\Delta\Delta C_T}$)(74).

Acknowledgements

This study was supported in part by a grant from the National Institutes of Health (NIH; 5R21AI144571-03) to JEV and Wesleyan University institutional funds to TP-B. BA was supported by a Fulbright scholarship awarded by the US Department of State. FMF is supported by the Ronald E. McNair Postbaccalaureate Fellowship program. The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH or the US Department of State. The authors thank Jaime Carrazco-Carrillo from Wesleyan University for his assistance in some laboratory assays.

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Table 1. Strains utilized in this study.

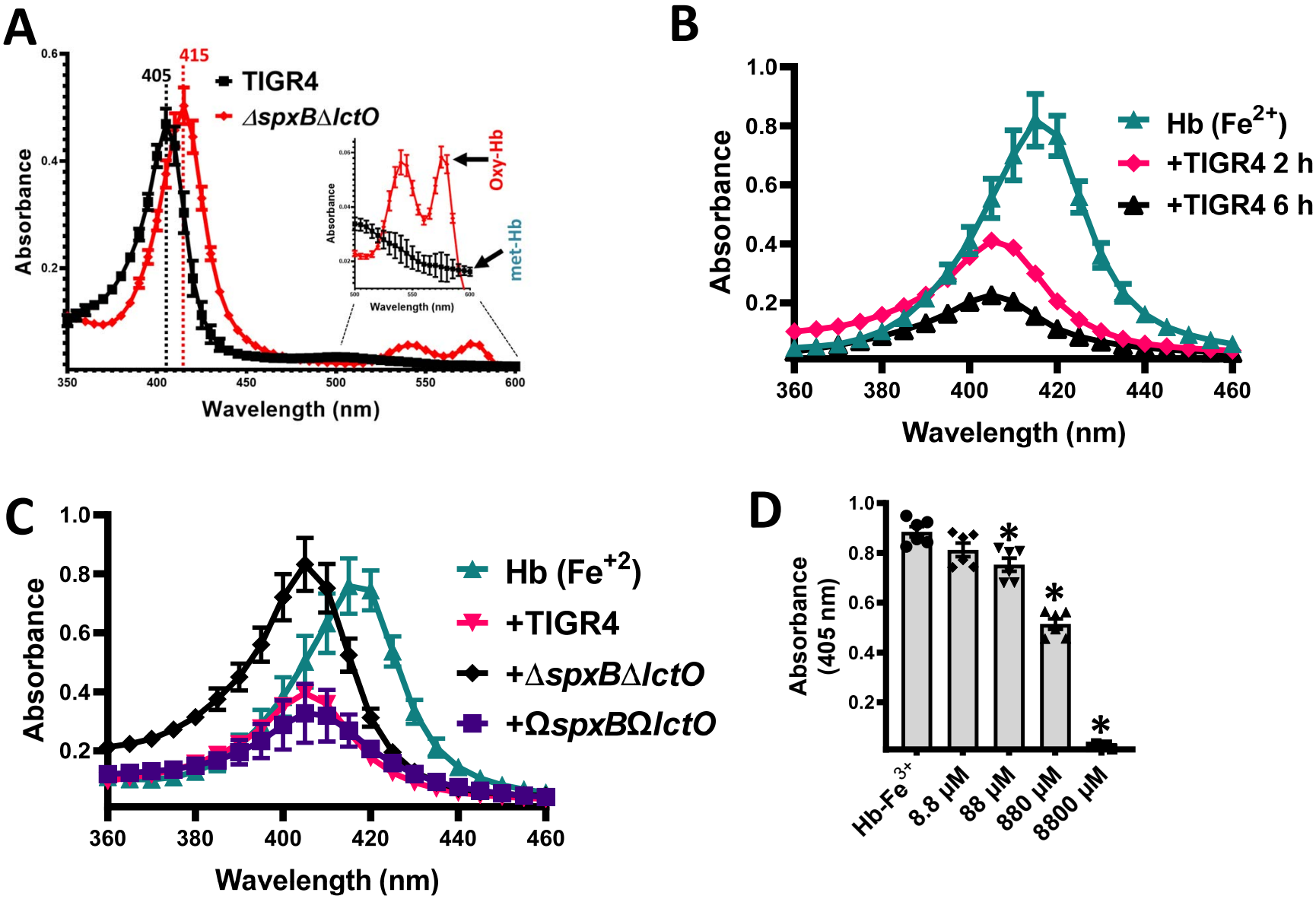
Strain	Characteristics	Reference or source
TIGR4	Reference strain, whole genome sequenced, capsular serotype 4	(79)
SPJV41 (TIGR4Δ*spxB*Δ*lctO*)	TIGR4 with a deleted *spxB* and *lctO* gene by insertion-deletion with an erythromycin and spectinomycin cassette, respectively	(37)
SPJV29 (TIGR4Δ*spxB*)	TIGR4 with an insertionally inactivated *spxB*::*ermB* (Ery^r)	(23)
SPJV42 (TIGR4Δ*lctO*)	TIGR4 with an insertionally inactivated *lctO*:: *aad9* (Spc^r)	(37)
SPJV43 (TIGR4Δ*spxB*Ω*spxB*)	SPJV29 complemented with the gene *spxB* and its promoter	This study
SPJV44 (TIGR4Δ*spxB*Δ*lctO*Ω*spxB-lctO*)	SPJV41 complemented with the gene *spxB* and *lctO*	This study
D39	Avery strain, clinical isolate, capsular serotype 2	(80)
P878	D39 with a deletion in *spxB*	(36)
P1221	P878 complemented with pMU1328::*spxB*	(40)
SPJV45 (D39Δ*spxB*Δ*lctO*)	D39 with a deleted *spxB* and *lctO* gene by insertion-deletion with an erythromycin and spectinomycin cassette, respectively	(23)
EF3030	Clinical isolate, caccine serotype 19F strain	(81, 82)
SPJV49 (EF3030Δ*spxB*Δ*lctO*)	EF3030 with a deleted *spxB* and *lctO* gene by insertion-deletion with an erythromycin and spectinomycin cassette, respectively	(23)
SPJV50 (EF3030Δ*spxB*)	EF3030 with an insertionally inactivated *spxB*::*ermB* (Ery^r)	This study
SPJV51 (EF3030Δ*lctO*)	EF3030 with an insertionally inactivated *lctO*:: *aad9* (Spc^r)	This study
MNZ41	Non-encapsulated S. pneumoniae strain	(83)
MNZ67	Non-encapsulated S. pneumoniae strain	(83)
S. pseudopneumoniae	ATCC BAA-960	
S. salivarius	ATCC 7073	
S. oralis		
K. pneumoniae RGV2	Strain isolated from hospital acquired infection (bacteremia)	(84)
Staphylococcus aureus strain Newman	NCTC 8178, ATCC 13420	(85)

Table 2. Oligonucleotides designed and used in this study

Primer	Sequence (5'→ 3')
hlpA-up1	TCAGCAGGTTCATGAGGGAA
hlpA-up2	TCATTTCTGTTTTATAACAAAGTCCGGATCCTTTAACAGCGT
hlpA-down1	CTCGCCGAAAATCAAATATGATCACTCACGGCATGGATGA
hlpA-down2	CAAAACAACATTGCCCGACG
spxB1	CTTTGTTATAAAACAGAAATGA
spxB2	CATATTTGATTTTCGGCGAG
hlpA-up3	TTTCCCCAATTATTCACCCCACATATTTGATTTTCGGCGAG
lcto1	TGGGGTGAATAATTGGGGAAA
lcto2	CATTCAGTGGAGGCAATCTGT
lcto-spec1	TTTCTGAGTAGGCGGAGTGG
lcto-spec2	GCAATTTTCAGAGCAGCTTGG
JVS109L	TCTACTCAATCGACGTAGGTAACA
JVS110R	TATTGAACGTTTGTGATAACGTCT
JVS59L	TGAGACTAAGGTTACAGCTTACAG
JVS60R	CTAATTTTGACAGAGAGATTACGA
JVS105L	TTTGCAATGTAGAAAATCCAAGTA
JVS106R	AAATCTCTGGAAGGTCAACAGTAG
JVS49L	CTGATGACTGTCAATCGAGATACC
JVS50R	AATGGACGAATCAACTCCATAA
JVS35L	AACCAAGTAACTTTGAAAGAAGAC

JVS36R	AAATTTAGAATCGTGGAATTTTT

Fig. 1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415
405
TIGR4
ΔspxBΔlctO
Oxy-Hb
met-Hb
Absorbance
Wavelength (nm)
B
Hb (Fe2+)
+TIGR4 2 h
+TIGR4 6 h
Absorbance
Wavelength (nm)
C
Hb (Fe+2)
+TIGR4
+ΔspxBΔlctO
+ΩspxBΩlctO
Absorbance
Wavelength (nm)
D
Absorbance (405 nm)
Hb-Fe3+
8.8 μM
88 μM
880 μM
8800 μM

Fig. 2

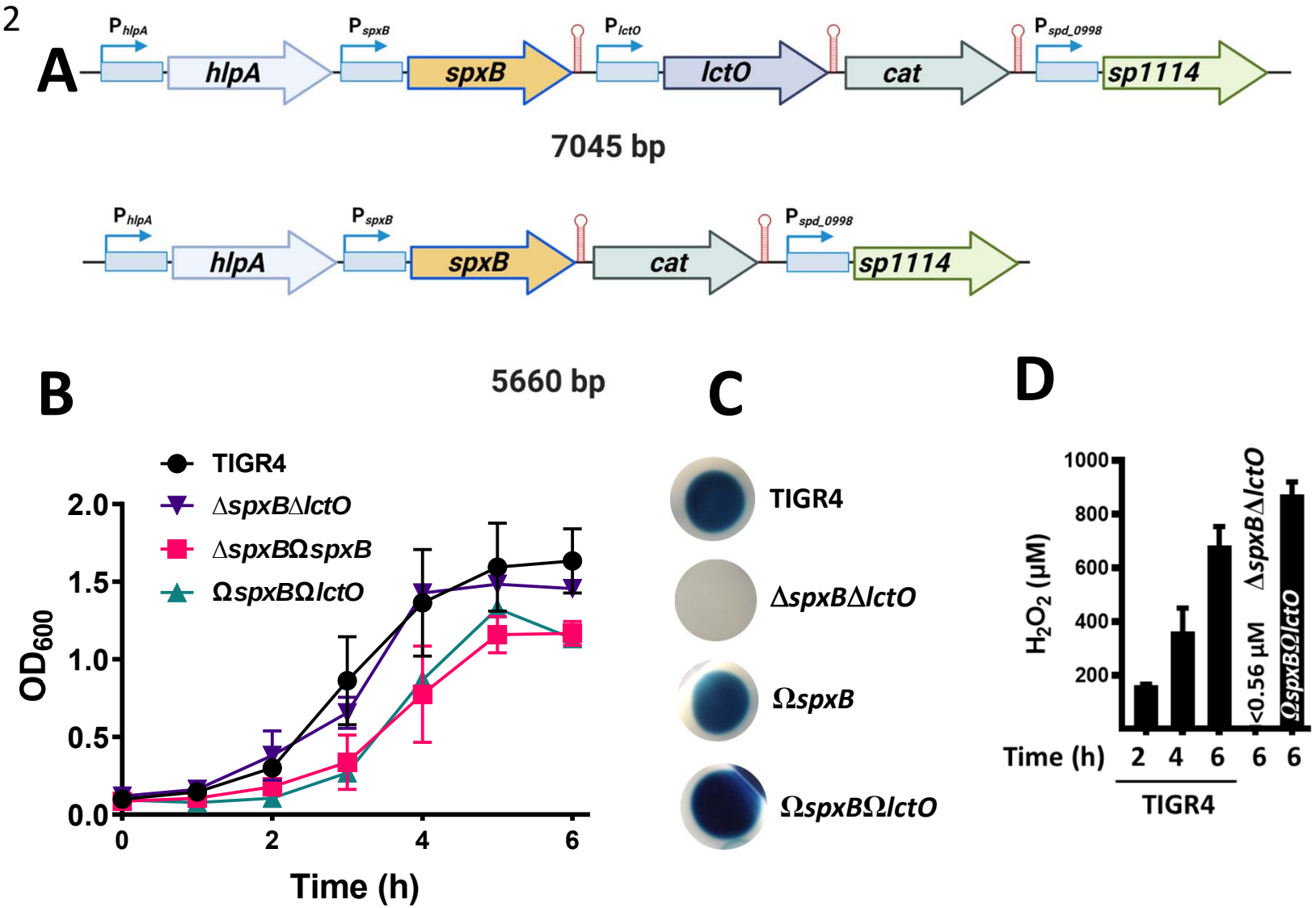

A
P hlpA
hlpA
P spxB
spxB
P lctO
lctO
cat
P spd_0998
sp1114
7045 bp
P hlpA
hlpA
P spxB
spxB
cat
P spd_0998
sp1114
5660 bp
B
TIGR4
ΔspxBΔlctO
ΔspxBΩspxB
ΩspxBΩlctO
OD600
2.0
1.5
1.0
0.5
0.0
0
2
4
6
Time (h)
C
TIGR4
ΔspxBΔlctO
ΩspxB
ΩspxBΩlctO
D
H2O2 (μM)
1000
800
600
400
200
<0.56 μM
ΔspxBΔlctO
ΩspxBΩlctO
Time (h)
2
4
6
6
6
TIGR4

Fig. 3

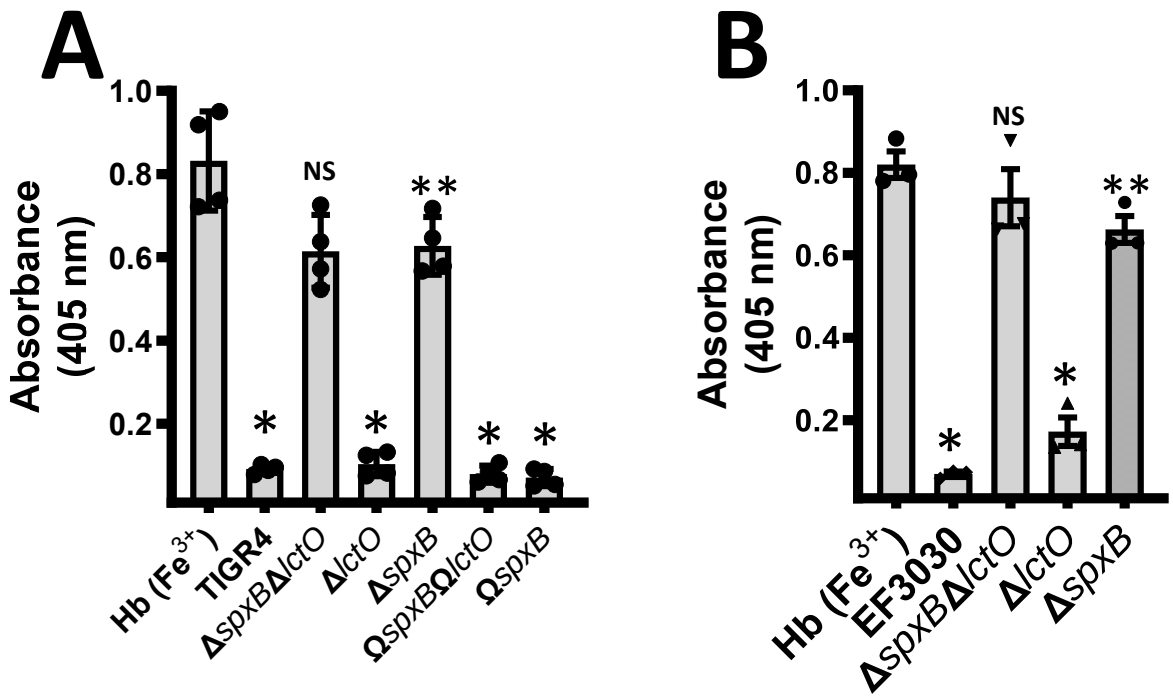

A
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
NS
*
**
Hb (Fe 3+)
TIGR4
ΔspxBΔlctO
ΔlctO
ΔspxB
ΩspxBΩlctO
ΩspxB
B
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
NS
*
**
Hb (Fe 3+)
EF3030
ΔspxBΔlctO
ΔlctO
ΔspxB

Fig. 4

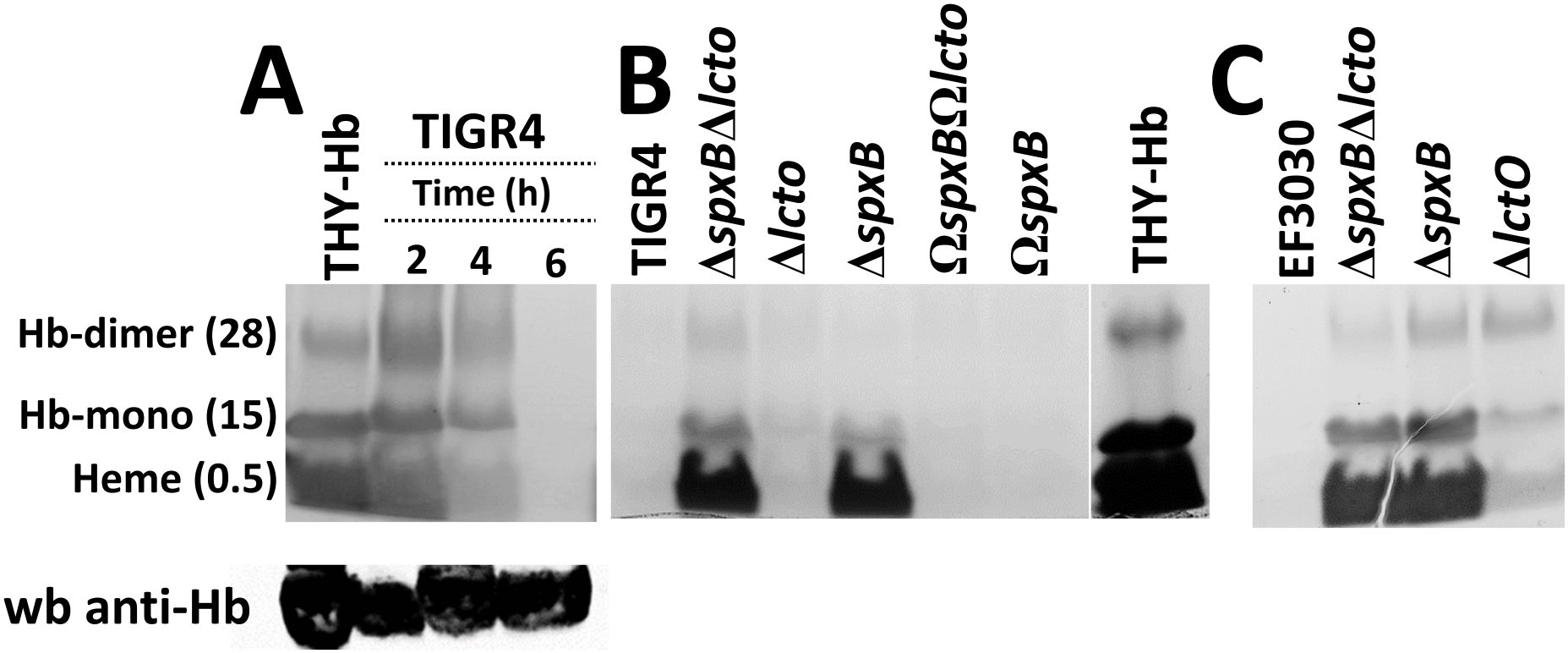

A
THY-Hb
TIGR4
Time (h)
2
4
6
Hb-dimer (28)
Hb-mono (15)
Heme (0.5)
wb anti-Hb
B
TIGR4
ΔspxBΔlcto
Δlcto
ΔspxB
ΩspxBΩlcto
ΩspxB
THY-Hb
C
EF3030
ΔspxBΔlcto
ΔspxB
ΔlctO

Fig. 5

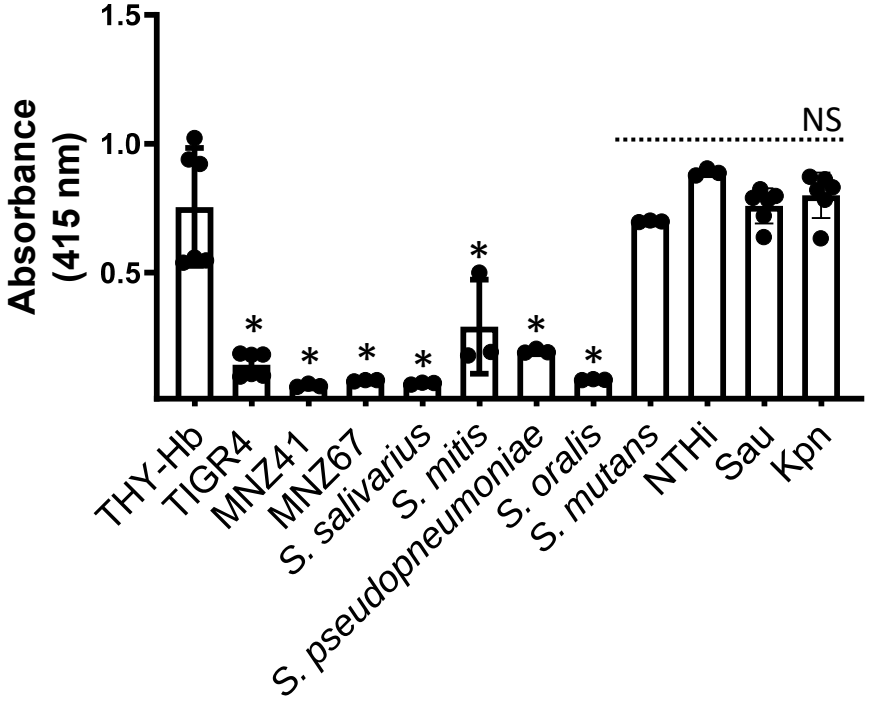

Absorbance (415 nm)
1.5
1.0
0.5
NS
*
THY-Hb
TIGR4
MNZ41
MNZ67
S. salivarius
S. mitis
S. pseudopneumoniae
S. oralis
S. mutans
NTHi
Sau
Kpn

Fig.6

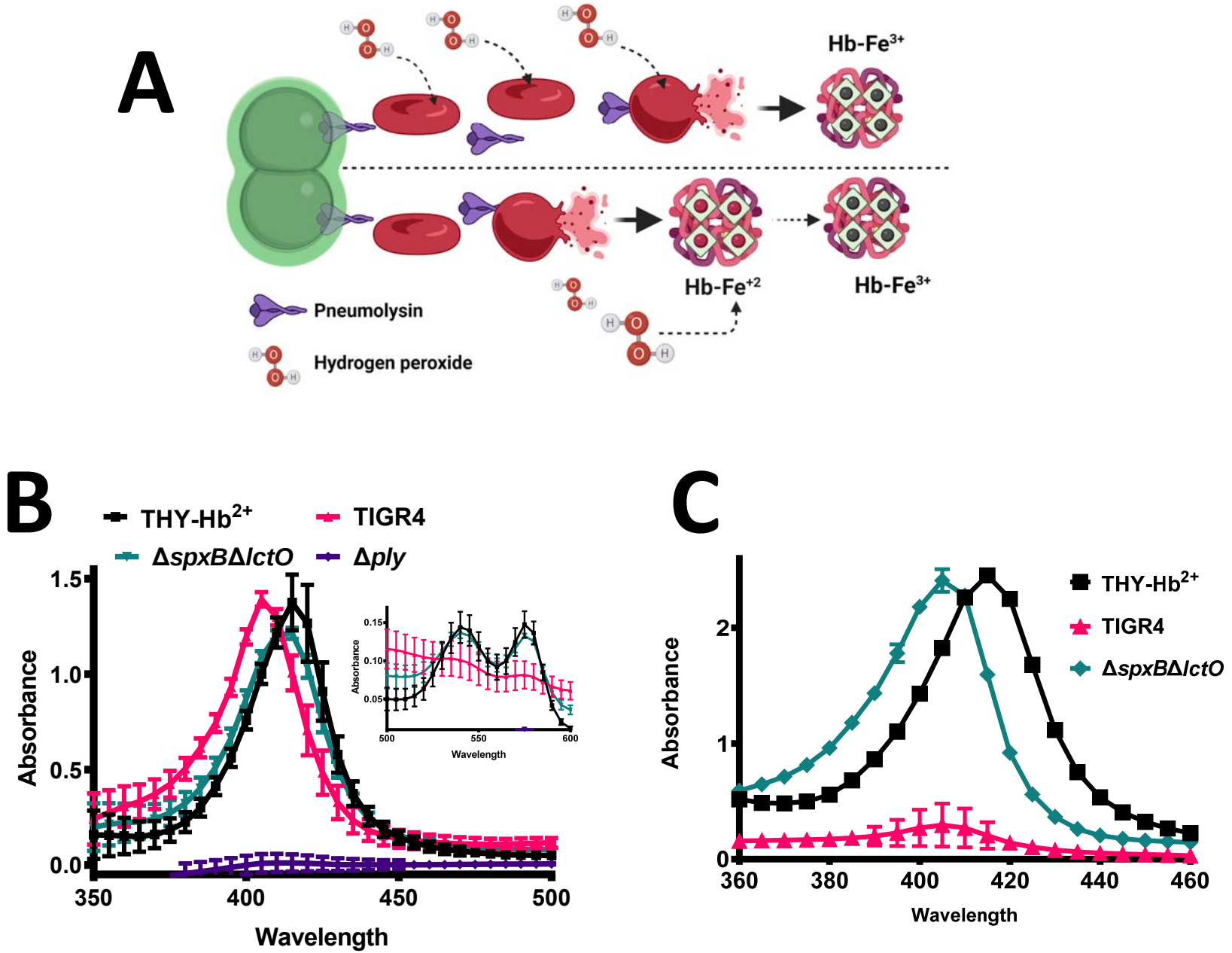
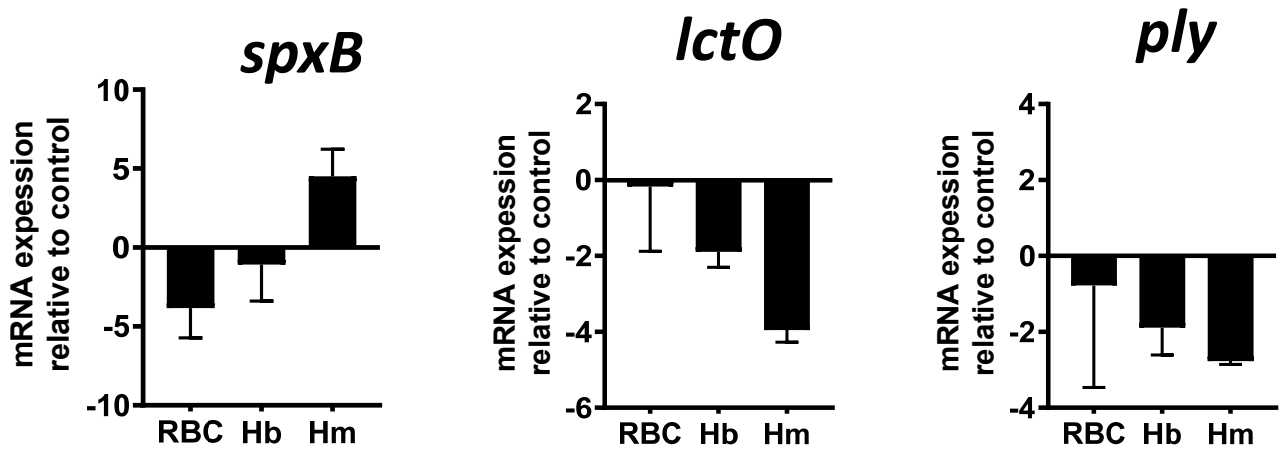

A
Hb-Fe3+
Hb-Fe+2
Hb-Fe3+
Pneumolysin
Hydrogen peroxide
B
THY-Hb2+
TIGR4
ΔspxBΔlctO
Δply
Absorbance
Wavelength
C
THY-Hb2+
TIGR4
ΔspxBΔlctO
Absorbance
Wavelength

Fig. 7


spxB
lctO
ply
mRNA expression relative to control
10
5
0
-5
-10
2
0
-2
-4
-6
4
2
0
-2
-4
RBC
Hb
Hm