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Investigation into Metabolism and Signaling of Hemoglobin in *Streptococcus pneumoniae*

by

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Under the Direction of Zehava Eichenbaum, Ph.D.

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Doctor of Philosophy

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ABSTRACT

Pathogenic bacteria require iron during growth and have evolved elaborated machinery to obtain iron during infection. Most of the host iron is found in heme bound to hemoglobin[1, 2]. This dissertation uncovers novel mechanisms used by the Gram-positive pathogen, *Streptococcus pneumoniae*, to leverage the host hemoglobin as an iron source. Chapter 1 describes my contributions to the discovery that hemoglobin provides *S. pneumoniae* with nutritional iron and serves as a host cue that promotes pneumococcal growth, biofilm development, and adaptation to the host mucosal surfaces. In Chapter 2, I characterize the role of the endogenously produced hydrogen peroxide in iron metabolism, uncovering extracellular heme degradation by *S. pneumoniae*. In Chapter 3, I investigate the function of Spd_0739 (aka PnrA and Spbhp-37), a ligand-binding protein previously linked to the uptake of nucleosides and heme. I demonstrate that Spd_0739 inhibits iron and heme uptake in *S. pneumoniae* and that its absence renders the expression of the heme transporter PiuBCDA growth inhibitory. These studies revealed a new connection between iron and nucleotide metabolism in *S. pneumoniae* and implicate Spd_0739 as a mediator of this association.

INDEX WORDS: Heme, Heme binding, Hydrogen peroxide, Heme degradation, Hemoglobin, Iron, *Streptococcus pneumoniae*, Spbhp-37, PnrA, PiuBCDA, SpxB, LctO, CSP-1, ComC

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DEDICATION

To my wife, Sarah Marie Womack, for being my rock and standing by me throughout this journey. To my mother, Vivian Robinson-Womack, who has always supported by goals and taught me the value of hard work and perseverance. To my late Father, Edroyal Womack Jr., who taught me to have confidence in myself and instilled a don't quit attitude. Lastly, to my unborn child who I haven't met yet, but I already love and work hard to create a future for.

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I would like to thank all my previous members of the Eichenbaum Lab, especially Dr. Fahmina Akhter, Dr. Nila Chatterjee, and Dr. Kristin Lyles each of whom acted as great mentors and I will never forget the time and the skills you invested with me.

I would also like to acknowledge Corbett Ouellette for being a great colleague and friend. Thank you for reading and discussing my work time and again. Thank you also to Mercy Coleman for her contributions to the project.

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1 INTRODUCTION

1.1 Iron and the host pathogen interphase.

Iron is an essential metal for most life forms. In mammals, iron has a role in cellular processes ranging from energy generation and DNA replication to oxygen transport and protection against oxidative stress. Similarly, iron is vital for the majority of bacteria due to its involvement in multiple enzymatic activities and key metabolic processes. For example, many of the enzymes involved in heterotrophic metabolism contain iron or an iron complex. These include heme-containing cytochromes, [Fe–S]-type ferredoxins, and [Fe–S] enzymes, such as fumarase and aconitase of the TCA cycle. Iron exists mostly in one of two readily inter-convertible redox states: the reduced Fe^{2+} ferrous form and the oxidized Fe^{3+} ferric form[3]. The wide redox range allows iron to act as highly versatile co-factor or prosthetic component for the catalysis of many electron-transfer and acid-base reactions. While required for diverse biological processes, iron redox can also cause a serious hazard because of the Fe^{2+}-triggered Fenton/Haber-Weiss reaction, which produces reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the highly destructive hydroxyl radical (•OH). An abundance of ROS can damage [Fe–S] clusters, protein carbonylation, Cys/Met-residue oxidation, membrane lipid peroxidation, and contribute to DNA damage [4]. To manage the harm, iron homeostasis is carefully regulated, and the metal is kept sequestered in both mammals and microorganisms.

Under physiological conditions, 70% of the iron in the human body is in heme complexed with hemoglobin and found in the intracellular components of erythrocytes [5]. Hemoglobin and heme released to the serum due to hemolysis are removed by specialized

proteins such as haptoglobin and hemopexin. Specialized iron-sequestering proteins called transferrin and lactoferrin add additional protection by taking away ferric iron from the circulation and secretion, respectively. Decreasing the availability of free iron in the host is also important to resisting infections. This is evident in patients who have conditions that cause systemic iron overload such as hemochromatosis and thalassemia, and are thus more susceptible to communicable diseases [6]. Invading microorganisms trigger a response termed nutritional immunity, which boosts the host iron sequestration mechanisms. Bacteria require 10^{-5} to 10^{-7} M iron, but the concentrations of bioavailable iron in the host can be as low as 10^{-24} M due to nutritional immunity[7]. Invading pathogens have evolved a multitude of mechanisms to compete for the host iron they deploy during infection depending on the niche they occupy and iron preference. These include the production of soluble molecules (i.e., siderophores) or proteins (i.e., hemophores) to capture iron or heme respectively; surface receptors for heme, hemoproteins, transferrin or lactoferrin; and cytoplasmic membrane transporters for ferric or ferrous iron, heme, or other iron complexes [5, 8-15]. Some pathogens, such as *Streptococcus pneumoniae*, have evolved mechanisms to induce erythrocytes lysis facilitating hemoglobin release [10, 16].

Many types of bacteria produce and export siderophores. These ferric iron chelators can out compete the host transferrin and lactoferrin proteins for the iron due to their high affinity (10^{50} M in enterobactin for example, compared with 10^{36} M in transferrin and lactoferrin)[6]. Gram-negative bacteria express outer membrane receptors to capture and import iron-loaded siderophores into the periplasm. The siderophore-iron complexes are then taken up across the cytoplasmic membrane by dedicated transporters. Heme is a lipophilic and redox active molecule that can cause oxidative damage to the cellular constituents. In Gram-negative bacteria, heme

import mechanisms are similar to those used for siderophores uptake and consists of homologous outer membrane receptors and inner membrane transporters. Gram-positive bacteria, which do not have an outer membrane, typically use receptors to obtain heme from host hemoproteins and a surface protein-relay system that shuttles the heme across the thick cell wall. Heme is escorted to the binding protein component of a membrane transporter for delivery into the cytoplasm [1, 14, 17, 18].

1.2 IRON UPTAKE MECHANISMS IN *STREPTOCOCCUS PNEUMONIAE*.

Streptococcus pneumoniae (pneumococcus) is an obligate human pathogen often carried asymptomatically in the nasal pharynx. In susceptible individuals, *S. pneumonia*e can spread and produce a range of mild to life-threatening ailments such as otitis media, bacterial pneumonia, bacteremia, and meningitis. Pneumococcus is the leading ethological agent of bacterial pneumonia and a serious pathogen for children under the age of 5 and adults over the age of 60. Globally, pneumococcus accounts for ~15 million annual cases worldwide and 800,000 deaths per year [19, 20]. In developed countries, pneumococcal infection is responsible for approximately 30% of all adult pneumonia cases and has a mortality rate of 11% to 40% [21]. Due to the heavy toll on human health inflicted by pneumococcus, vaccine efforts are a high priority. Current pneumococcal vaccines target the serotype-specific capsule that varies in polysaccharide subunits. The 23-polyvalent vaccine offers the most coverage with protection from 23 of the over 90 serotypes of pneumococcus. However, this vaccine does not protect children under the age of 2 [21-24]. Additionally, serotype replacement of non-vaccine serotypes has been reported following the introduction of vaccines [25].

S. pneumoniae requires iron for growth and can obtain the metal from heme and various host hemoproteins [26-28]. Hemoglobin is the largest reservoir of iron in the host and therefore serves as a key iron source for pneumococcus during infection, where the concentration of free iron or heme are extremely low under normal physiological conditions. The containment of hemoglobin in erythrocytes creates a barrier that separates heme iron from potential pneumococcal invaders. The means to lyse erythrocytes and acquire heme have been described in *S. pneumoniae* [29-32]; however, the understanding of the molecular mechanisms that mediate heme acquisition is lacking.

Bacteria typically import heme and degrade it intracellularly to liberate the iron. Surface heme relay proteins (similar to those identified in other Gram-positive pathogens) have not been identified in pneumococcus up to date, suggesting pneumococci may use novel mechanisms acquire heme from the host. Several ABC (ATP-binding cassettes) transporters were implicated in the import of heme across the cytoplasmic membrane in this pathogen (Figure 1). The PiaABC system is encoded on one of pneumococcal pathogenicity islands. Growth phenotype on hemoglobin iron linked the PiaABC proteins to heme import. Yet, the ligand binding component PiaA was crystalized with ferrochrome, suggesting this system import siderophores (likely those produce by neighboring organisms) [33]. *S. pneumoniae* also express the *piuBCDA* proteins, which include of a substrate-binding lipoprotein (PiuA), a heterodimer membrane permease (PiuBC), and cytoplasmic ATPase (PiuD). Interestingly, PiuA, which binds heme *in vitro* with high affinity, also binds norepinephrine and enterobactin in a even higher affinity [34-36]. Hence the role of the PiuBCDA in iron metabolism in pneumococcus remains unclear. Recently, another putative ABC transporter named Spd_0088-0090 was linked to heme import; while these proteins were annotated as components of a carbohydrate import system (and don’t share

significant homology to characterized heme uptake proteins), the substrate-binding component, Spd_0090, binds heme in *vitro*. Interestingly, a knockout Δ*spd_0090* mutant enhanced virulence[37]. Lastly, Spd_0739 (aka *pnrA* or *spbhp-37*) is highly expressed gene located near the *pnrBCD* gene cluster, which encodes nucleosides scavenging proteins [38]. PnrA, a substrate-binding protein was crystalized with several nucleosides and biochemical and mutant studies suggested it functions in guanosine import [39]. The same protein, referred to as Spbhp-37), however, was eluted from pneumococcal lysate using a hemin affinity column and was demonstrated to bind hemoglobin *in vitro.* The addition of PnrA antiserum to the growth medium undermined pneumococcal growth on hemoglobin iron[40]. Further characterization of this system's role in heme metabolism is explored in this dissertation. In addition to the iron complex transporters described above, two pneumococcal ABC systems are suggested to take up metal iron. These are the *pitABC1(spd_0223-0227)* and *pitABC2* (*spd_1607-1609*) transporters [41-43]. Findings of this dissertation indicated the *pit* transporters are important for pneumococcal growth on heme iron, since the bacterium can degrade heme extracellularly, releasing the metal iron from the protoporphyrin ring and making it available for import.

In this dissertation, I demonstrate that hemoglobin is a major iron metabolite to *S. pneumoniae*, which promotes robust growth and signaling of colonization and biofilm. Here, I aim to understand the mechanisms utilized by *S. pneumoniae* to acquire iron from hemoglobin. I also further characterize the role of the lipoprotein Spd_0739 in the transport of heme from hemoglobin.

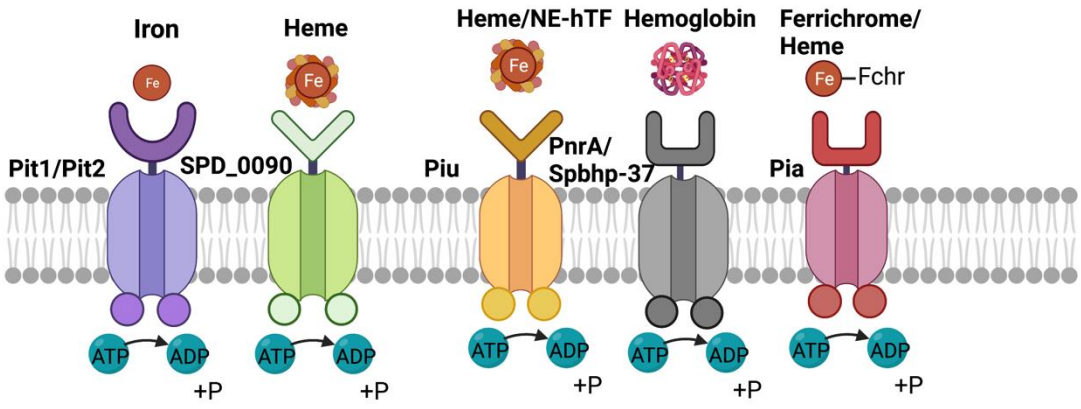

Figure 1: Schematic illustration of reported iron uptake mechanisms in Streptococcus pneumoniae.

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2 CHAPTER 1: HEMOGLOBIN HAS A SUBSTANTIAL IMPACT ON PNEUMOCOCCAL PHYSIOLOGY, GROWTH AND HOST ADAPTATION

2.1 Background

Streptococcus pneumoniae colonize the human upper respiratory track and can persist asymptomatically. The rate of asymptomatic carriage is influenced by factors such as age and viral co-infections, with 20-60% of children at school age are colonized by *S. pneumoniae*. Pneumococcal colonization precedes the transition into a disease state and spread within the body and is required for transmission to other individuals. The pathogen establishment in the upper respiratory tract involves the formation of bacterial aggregates, which develop into biofilms. Pneumococci in biofilms consists of hyper-adhesive cells that are more resistant to desiccation, environmental stressors, host immune responses. Biofilms also shield the pathogen from antimicrobials in addition to creating an ideal environment for genetic exchange; collectively leading to antibiotic resistance. Changes in the nasopharyngeal environment by coinfection by respiratory viruses, changes in host microflora, inflammation, and tissue trauma can trigger the dispersal of pneumococcal biofilms into planktonic cells that are more invasive [44]. The dispersal of biofilms can lead to local spread and broncho-aspiration which allow *S. pneumoniae* to breach the epithelial and endothelial barriers and penetrate tissues, providing access to the bloodstream. Circulating *S. pneumoniae* can penetrate heart tissue, creating microscopic lesions within the myocardium that contain intracellular biofilms[45].

While a formidable pathogen human pathogen, culturing pneumococcus *in vitro* can be difficult. It is a fastidious pathogen that requires enriched medium, and *in vitro* cultivation often includes media replenishment or the use of bioreactors that allow for continuous growth. When grown *in vitro* in batch cultures, the pathogen produces a limited biomass and exhibits a dramatic

reduction in viability after entering the stationary phase of growth [46, 47]. *S. pneumoniae* produces and releases to the growth medium hydrogen peroxide, who accumulation plays a significant role in the cultures diminished viability. [48]. Like with *in vitro* models for planktonic growth, *S. pneumoniae* exhibits limited only biofilm formation in *in vitro* systems with a biotic surface. To enhance pneumococcal biofilm production, long incubation with replenished growth medium, an inoculum with cell at the logarithmic phase of growth, or supplementation with a synthetic competence-stimulating peptide (CSP) are often required in these systems[49, 50]. Several *ex vivo* models for pneumococcal biofilms were develop, which include static or continuous flow bioreactors with live cultures of respiratory or mucosal epithelial cells [51]. Such models allow for more extensive biofilms compared with simpler *in vivo* models, still, pneumococcal biofilms in these systems are not fully developed [29].

The difficulty in mimicking pneumococcal infections using *ex vivo* and *in vivo* models highlight the significant knowledge gaps we have in the understanding pneumococcal physiology and pathogenesis. These difficulties also imply that current infection models are missing host factors that influence the pathophysiology of this important human pathogen. Studies done by the research team of Dr. Zehava Eichenbaum and in collaboration with Dr. Jorge E Vidal identified hemoglobin as such host element that has dramatic influence on pneumococcal planktonic and biofilm growth and host adaptation. This work resulted in two publications, which I coauthored [29, 30].

The first publication revealed that hemoglobin triggers prolonged logarithmic growth, higher biomass, and extended viability in both iron-deplete and standard medium and that hemoglobin induces significant changes in the pneumococcal transcriptome [30]. Transcriptome

remodeling under the influence of hemoglobin includes virulence genes and metabolism pathways, specifically those that allow for the use of the host glycoconjugates. Moreover, it was demonstrated pneumococci cultivated in the presence of hemoglobin, become more adapted to use human α − 1 acid glycoprotein as a sugar source. Lastly, inactivation of a pneumococcal hemoglobin receptor (Spbhp-37) resulted in reduced growth enhancement by hemoglobin [30].

The second paper demonstrated that hemoglobin induces early and vigorous biofilm growth on abiotic surfaces and under *in vitro* conditions that do not otherwise allow for biofilm formation [29]. This biofilm response is specific for hemoglobin and is not observed with iron salts or free heme. The addition of whole human blood to the growth medium also triggers biofilm development but only in Ply-producing streptococci, suggesting the hemolysin role in biofilm enhancement is to release hemoglobin from the erythrocytes. The shift into an adherent state in the presence of hemoglobin involved significant transcriptome shift in *S. pneumoniae* that included the activation of multiple regulatory pathways. Additional experiments revealed the involvement of a regulatory circuit that requires CSP-1 signaling but not the two-component system, ComDE, which typically mediates CSP signaling [29].

In the following sections of this chapter, I elaborate on the experiments from our published work conducted by myself.

2.2 Hemoglobin stimulates a robust planktonic growth of *S. pneumoniae in vitro*

To test the influence of the host hemoglobin on *S. pneumoniae* cultivated in a regular laboratory medium (Todd-Hewitt with Yeast extract Broth, THYB), we used a kinetic growth assay in 96-well microtiter plates developed in our laboratory. Testing the addition of hemoglobin in increasing concentrations to THYB revealed *S. pneumoniae* grown in the

presence of hemoglobin to a much higher biomass compared with un supplemented THYB and that this growth response was dependent on the hemoglobin dose (Figure 2A). Heat-inactivated hemoglobin did not have the growth inducing properties. Likewise, the addition of bovine serum albumin (which has a similar size as hemoglobin, (~60 Kda)) instead of hemoglobin did enhanced growth to the same extend as hemoglobin. To rule out the possibility that a contaminant in the hemoglobin preparation was responsible for the observed phenotype, I filtered the hemoglobin solution (using a 10,000 MW cutoff filter) and tested both fractions for growth impact. Filtered hemoglobin retained activity while the flow-through was somewhat growth-inhibitory (Figure 2B). *S. pneumoniae* produces hydrogen peroxide in millimolar concentrations during aerobic growth, which can be growth inhibitory. Since hemoglobin has a peroxide-like activities it seemed possible that this intrinsic activity of hemoglobin is responsible for the observed growth enhancement. To test this hypothesis, I compared the impact of externally added catalase on pneumococcal growth but observed only a minimal effect.

Each of the four subunits of hemoglobin has one iron bound heme. Adding iron salt ($FeNO_3$) in equimolar concentrations did not provide the growth benefits that the addition of hemoglobin had (Figure 2D). Supplementing THYB with free heme did not improve growth at the low µM range and was inhibitory above ten µM (Figure 2E). To evaluate whether the improved bacterial growth resulted from an increased uptake of iron, I used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine the intracellular iron levels in pneumococci grown in THYB with and without hemoglobin. The equal amounts of iron found in cells grown in THYB or THYB with 20 µM hemoglobin did not support this suggestion (Figure 2F).

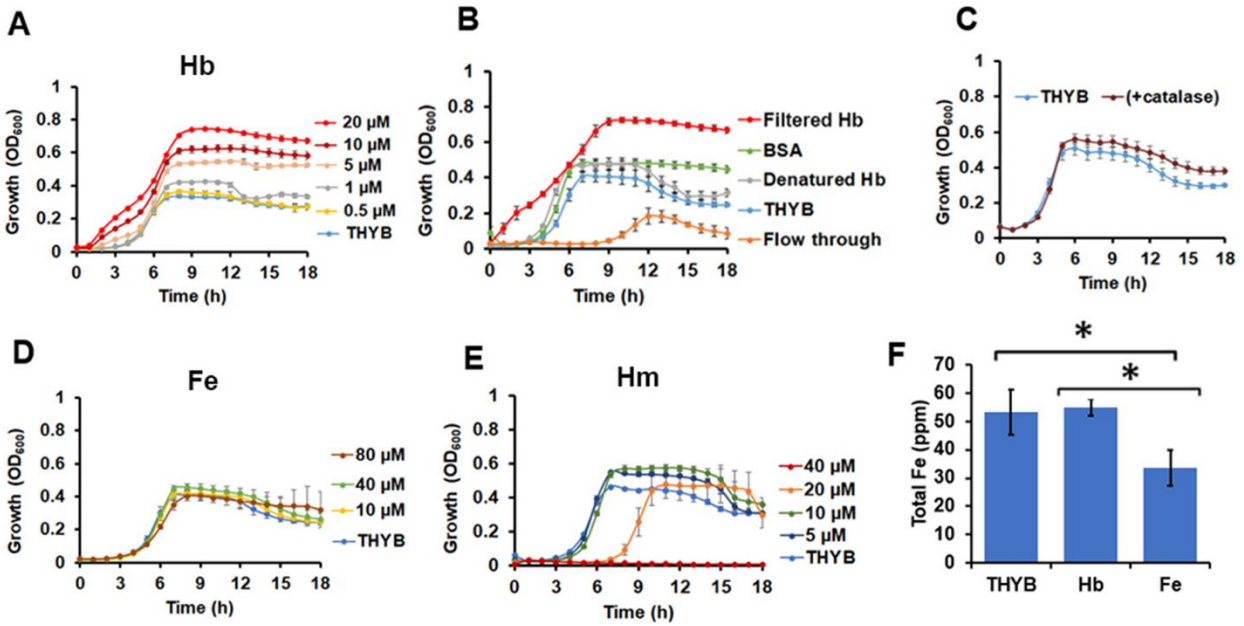


Figure 2: Hemoglobin stimulates a robust growth of S. pneumoniae D39 in laboratory (iron-complete) medium.

THYB was inoculated with D39 cells grown on BAPs (18 h, starting $OD_{600} = 0.05$. Shown is growth in (A) THYB with 0–20 µM hemoglobin (Hb); (B) THYB with 20 µM BSA, denatured hemoglobin, filtered hemoglobin, or the flow-through; (C) THYB with or without catalase. (D) THYB supplemented with 0–80 µM $FeNO_3$ (Fe); (E) THYB with 0–40 µM heme (Hm). The data are representative of three independent experiments performed in triplicates; error bars indicate SD. (F) Total intracellular iron content measured by ICP-MS in culture samples (normalized to optical density) grown in THYB, THYB with 20 µM hemoglobin (Hb), or THYB with 80 µM FeNO3 (Fe). The data represents the average of three independent biological replicates; error bars indicate SD. The asterisks denote statistical significance, $P \leq 0.05$ (THYB vs. Fe, and Hemoglobin vs. Fe, Student's t-test).

2.3 Hemoproteins promote growth more than free iron or heme

To better understand the growth supported by the addition of hemoglobin, I tested whether other heme sources found in the host impact *S. pneumoniae* growth. THYB containing the iron chelator 2,2′-di-pyridyl ($THYB_{DP}$), as well as $THYB_{DP}$ supplemented with free heme, iron, or hemoglobin was inoculated with *S. pneumoniae* D39 (serotype 2). The cultures were grown in microtiter plates for 18 hours in the presence of equine myoglobin or human serum. (Figure 3A). The addition of 20 µM myoglobin or serum (5 to 40% [vol/vol]) reinstated growth

in the iron-deprived medium that surpassed the one observed in a medium complemented with iron or heme. As with hemoglobin, the addition of myoglobin and serum improved growth also in standard THYB (Figure 3B). Thus, *S. pneumoniae in vitro* cultivation is stimulated by myoglobin and serum when they are added as the sole iron source (i.e., in $THYB_{DP}$) or as an added nutrient (i.e., THYB), although not to the same extent as with hemoglobin.

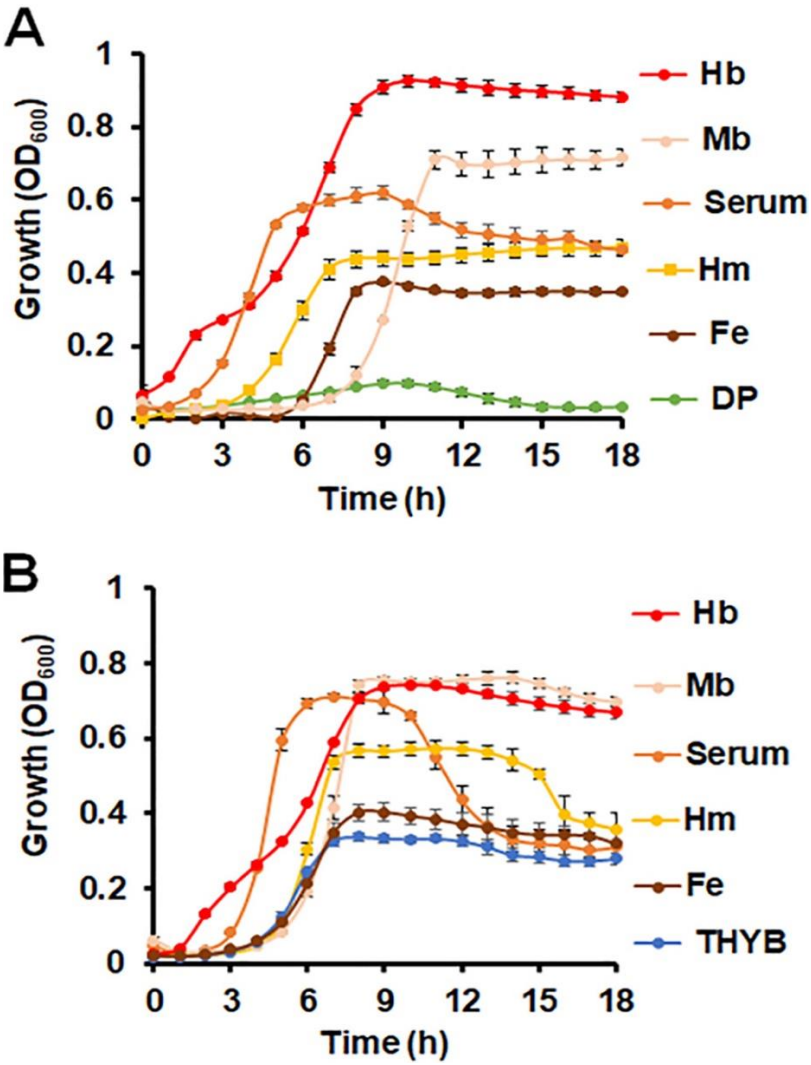


Figure 3: Hemoproteins promote greater growth than equimolar free heme or iron.

Myoglobin and serum promote pneumococcal growth in iron-depleted and iron complete medium. THYB was inoculated with D39 cells grown on BAPs (18 h, starting OD600 of 0.05). Cultures were grown in microtiter plates. Growth is shown as follows. (A) THYB with 3 mM 2,2-dipyridyl ($THYB_{DP}$), $THYB_{DP}$ and 2 mM FeNO3, 10 μM heme, 10% human serum, 20 μM equine myoglobin, or 20 μM hemoglobin. (B) THYB, THYB supplemented 80 μM FeNO3,

10 μM heme, 10% human serum, 20 μM equine myoglobin, or 20 μM hemoglobin. The data are representative of three independent experiments performed in triplicates; error bars indicate the standard deviations (SD).

2.4 THE HEMOGLOBIN RECEPTOR, SPD_0739 (SPBHP-37), CONTRIBUTES TO THE GROWTH PHENOTYPE OBSERVED WITH HEMOGLOBIN.

This section describes my investigation of Spbhp-37, a ligand-binding proteins reported to bind heme and promote pneumococcal growth on hemoglobin [30, 40]. Analyzing the literature and examining the published genome of *S. pneumoniae* allowed me to conclude that the pneumococcal gene encoding *spbhp-37* is the same as the one encoding *pnrA*, which was described in previous studies as a guanosine-binding protein *(spd_0739 & sp_0845)* [39]. I therefore refer to this gene in this dissertation as Spd_0739, although in the literature they are described as two separate proteins (aka PnrA or Spbhp-37).

At the time of the study, the ligand-binding proteins, PiuA and Spd_0739, were the only pneumococcal transport proteins reported to bind hemoglobin (and heme) [34, 36, 37, 40]. Transcriptome studies revealed that the addition of hemoglobin to the medium results in the repression of the *piuBCDA* genes but had no impact on that of *spd_0739*, whose expression remained very high (more than 30-fold above that of *piuA*) *[30]*. The high expression under our experimental conditions and binding to hemoglobin raised to possibility that Spd_0739 is a mediator of the hemoglobin effect on *S. pneumoniae*. To test this possibility, I constructed a non-polar deletion mutant in *spd_0739* and compared it to the wildtype parent during growth on hemoglobin or heme as the iron source. Wild type *S. pneumoniae* cannot grow in THYB that contain 3 mM of the iron chelator, DP, but the addition of 20 μM hemoglobin to THYB with DP

promotes robust pneumococcal growth that exceeds the growth seen in regulator THYB (blue symbols, Figure 4A and 4B respectively). Hence, hemoglobin enhances the growth of wild type *S. pneumoniae* both iron-depleted medium (i.e., $THYB_{DP}$, Fig. 4A and regular THYB, Figure 2A and 4B). In the presence of 10 μM heme the pneumococci grew to the same level as they did in regular THYB (I did not use higher concentration of heme since it was growth inhibitory[52]). Unlike the wildtype strain, the growth of the Δ*spd_0739* mutant in $THYB_{DP}$ was restored with the addition of hemoglobin but not with heme. Moreover, the mutant growth in media supplemented with hemoglobin was impaired compared with the WT strain (orange symbols, Figure 4A and 4B). Lastly, the mutant exhibited some growth phenotype also in regular (un supplemented) THYB. I next examined if the Δ*spd_0739* mutant was impaired in its transcriptome response to hemoglobin. For this, I used RT-PCR to compare the expression of the *aliA* and *argG* genes between the wildtype and mutant strains. The *aliA* gene encode an oligopeptide-binding component of the Ami system, and *argG* is an arginine biosynthesis gene, both genes are important for pneumococcal colonization and were induced by hemoglobin in the wildtype strain [53, 54] (blue symbols, Figure 4D). This analysis revealed that unlike with the wildtype strain, the *aliA* and *argG* expression was not changed significantly in Δ*spd_0739.* Together, these observations support the notion that Spd_0739 helps mediate the hemoglobin effect on *S. pneumoniae*.

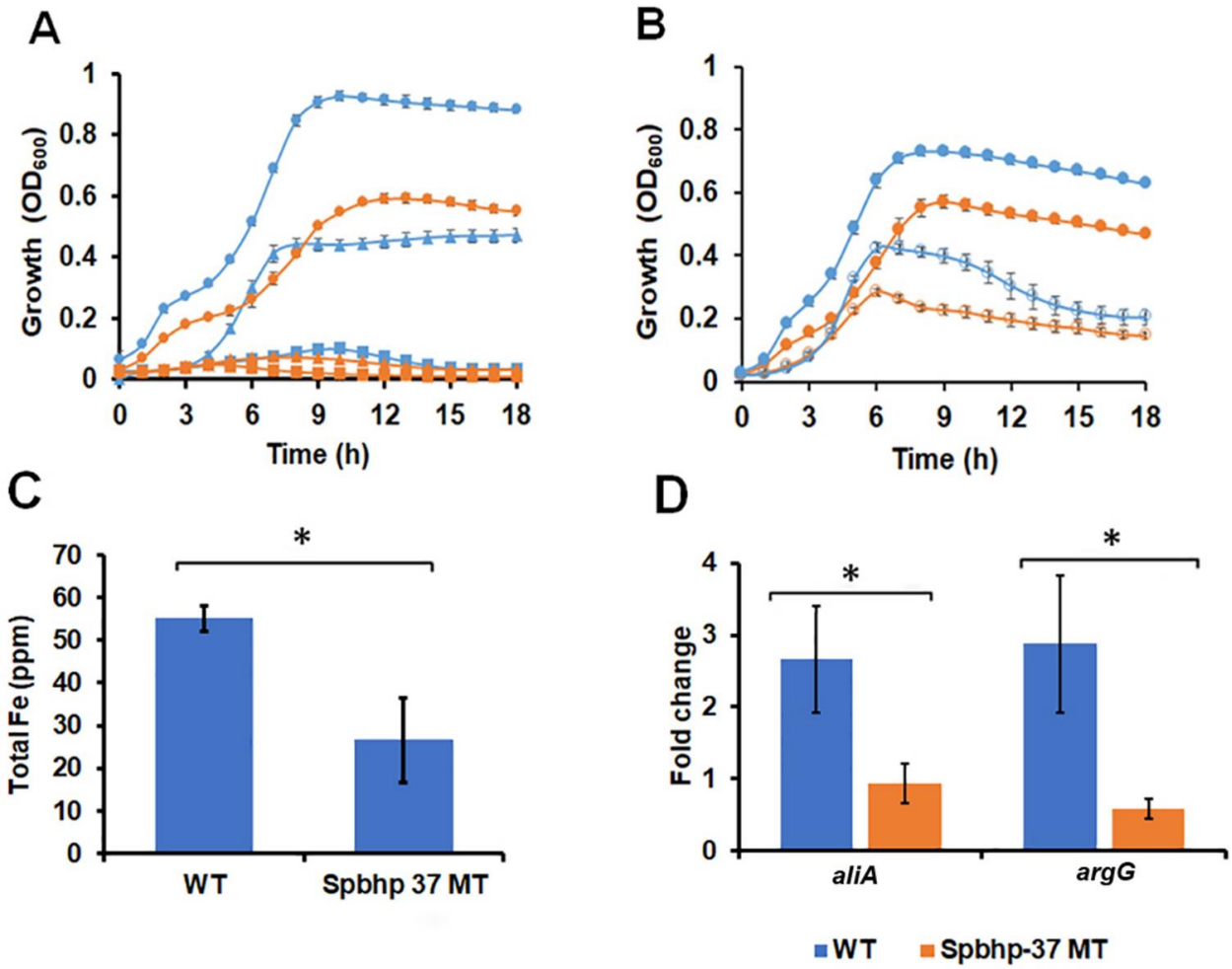


Figure 4: A spd_0739* (spbhp-37) *deletion mutant exhibits a lessened growth response to hemoglobin.

The heme/hemoglobin binding protein, Spd_0739 (Spbhp-37), plays a role in mediating the positive impact of hemoglobin in S. pneumoniae. Fresh medium was inoculated with D39 (blue) and the isogenic *Δspd_0739 (spbhp-37)* strain (orange) grown on BAPs (18 h, starting O.D.600 = 0.05). Shown is growth in (A) THYB with 3 mM 2, 2-Dipyridyl (DP), $THYB_{DP}$ and 10 µM heme (triangles) or 20 µM hemoglobin (circles), or (B) THYB (empty symbols) or THYB with 20 µM hemoglobin (full symbol). The data are representative of three independent experiments performed in triplicates; error bars indicate SD. (C) Total intracellular iron content (ppm) determined by ICP-MS in D39 wild type and Δ*spd_0739 (spbhp-37)* mutant cultures samples (normalized to optical density) grown in THYB with 20 µM hemoglobin. The data represents the average of three independent biological replicates; error bars indicate SD. (D) Fold change in gene expression in the wild type and *Δspd_0739 (spbhp-37)* strains 2 h post hemoglobin treatment relative to the control (saline) as determined by qRT-PCR. The experiments were performed in duplicates with at least two biological replicates. The replicates data are shown as the mean ± SD. The asterisks denote statistical significance, $P \leq 0.05$ (WT vs. MT, Student's t-test).

2.5 Hemoglobin induces an early formation of *S. pneumoniae* biofilms *in vitro*

Hemoglobin promotes biofilm in pneumococcus. We investigated this with the addition of human hemoglobin to iron-depleted $THYB_{DP}$ or regular THYB (iron complete) and found that hemoglobin facilitates vigorous pneumococcal growth that exceeded the one observed when the media were supplemented with free iron or heme [30]. We noticed that *S. pneumoniae* cultures grown for 18 hours in THYB with hemoglobin formed a film that coated the wells, suggesting biofilm formation. Crystal violet staining confirmed that in the presence of hemoglobin, *S. pneumoniae* produced biofilms in both standard THYB (Fig. 5A) and iron-depleted THYB (Fig. 5B). Biofilm induction by hemoglobin occurred in a dose-dependent manner, starting with 0.5 μM hemoglobin in THYB (Fig. 5A) or 5 μM hemoglobin in $THYB_{DP}$ (Fig. 5B). We did not observe biofilm formation with heme or free iron. Importantly, myoglobin or serum supports pneumococcal growth without induction of biofilm. The addition of a control protein (bovine serum albumin [BSA], which has a molecular weight [MW] similar to that of hemoglobin) or heat-denatured hemoglobin also did not stimulate biofilms. To consider the possible involvement of contaminants in the hemoglobin preparation, we filtered the hemoglobin solution using a 10,000-MW cutoff and tested both fractions. Although filtered hemoglobin-induced biofilms, the flowthrough did not (Fig. 5C). Finally, hemoglobin-induced biofilms were also tested in different *S. pneumoniae* strains (Fig. 5D), including the reference strain TIGR4 (serotype 4) and two clinical isolates (both serotype 6B). Therefore, biofilm induction is dependent on hemoglobin in its native form and conserved in multiple serotypes.

The hemoglobin/heme-binding protein, Spd_0739, contributes to growth on hemoglobin iron [40] and a D39 *Δspd_0739* mutant exhibits an attenuated growth compared to the parental strain also in regular THYB supplemented with hemoglobin [30]. Examining the *Δspd_0739*

mutant for biofilm formation revealed that it produced similar or slightly higher biofilm biomass compared to the wild-type strain (Fig. 5E), suggesting biofilm formation is independent of this heme uptake protein.

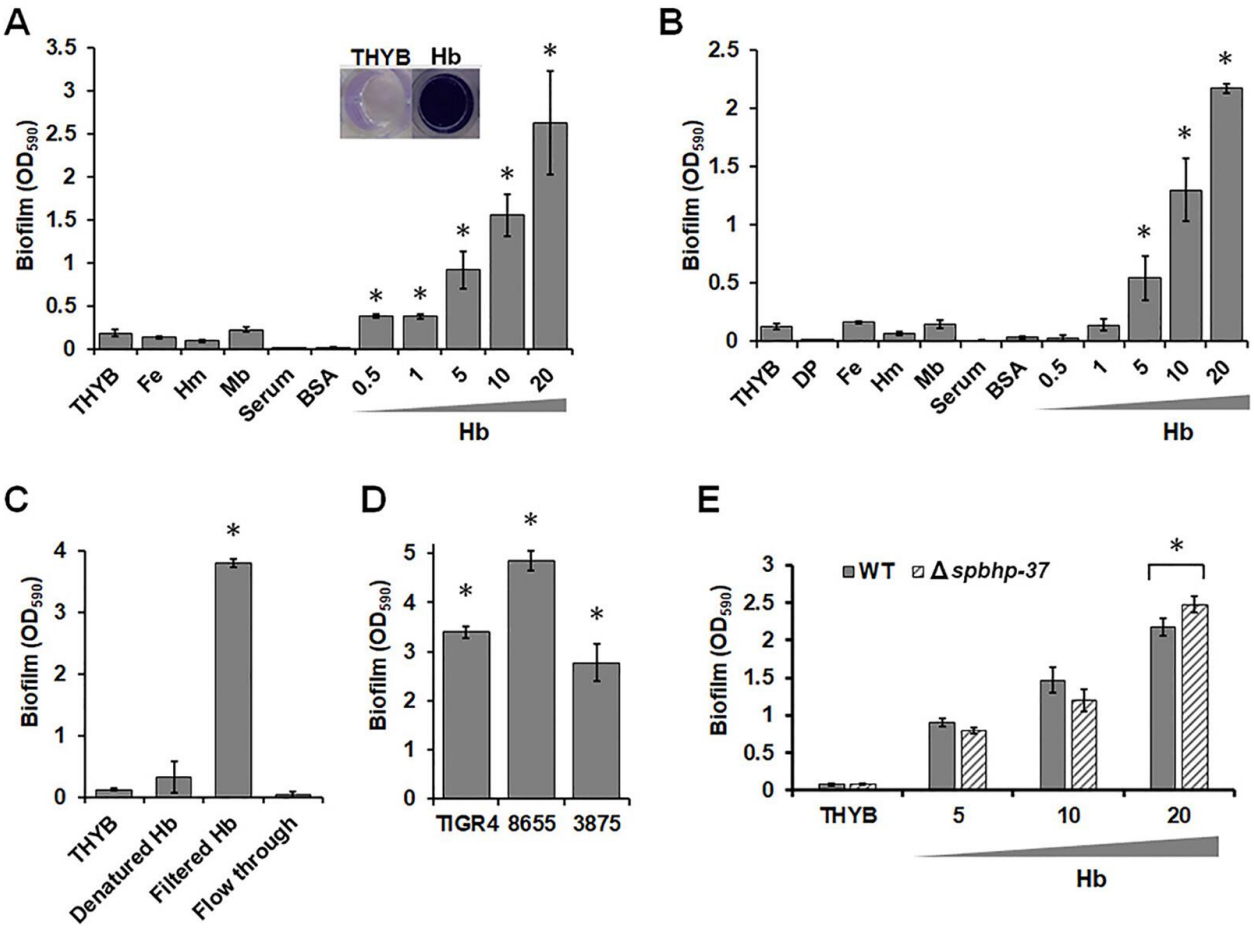


Figure 5: Hemoglobin induces early robust biofilm in vitro.

Hemoglobin induces biofilm formation in *S. pneumoniae*. THYB was inoculated with *S. pneumoniae* cells grown on BAPs (OD600 = 0.05). Cultures were grown in microtiter plates; biofilms (18 h) produced by D39 (serotype 2) are shown. (A) THYB, THYB with 80 μM FeNO3, 10 μM heme, 20 μM equine myoglobin, 10% human serum, 20 μM BSA, or 0.5 to 20 μM hemoglobin. (B) THYB with 3 mM DP, $THYB_{DP}$ and 2 mM FeNO3, 10 μM heme, 20 μM equine myoglobin, 10% human serum, 20 μM BSA, or 0.5 to 20 μM hemoglobin. (C) THYB with 20 μM denatured hemoglobin, filtered hemoglobin, or the flowthrough after hemoglobin filtration. (D) Biofilm formation by TIGR4 (serotype 4) and the clinical isolates 8655 and 3875 (serotype 6B) grown in THYB with 20 μM hemoglobin. The inset in panel A shows crystal violet staining following growth in THYB or THYB with hemoglobin (18 h). (E) Biofilm formation by D39 (solids) and isogenic *Δspd_0739 (spbhp-37)* (stripes) strains grown in THYB or THYB with 5 to 20 μM hemoglobin. The data show means ± the SD of at least two independent experiments, each performed in triplicates. The asterisks denote statistically significant differences: $P \leq 0.05$ (THYB-Hb versus THYB in panels A to D and THYB-Hb WT versus *Δspd_0739 (spbhp-37)* in panel E [Student t test]).

2.6 Human blood cells trigger biofilm formation in a Ply-dependent manner.

The vast majority of the host hemoglobin is within erythrocytes; we thus examined the impact of supplementing the growth medium with blood. The addition of washed red blood cells (RBCs) (0.1 to 0.5%) to THYB triggered biofilm formation in the two test strains (D39 and TIGR4), although to a lower level than hemoglobin (Figure 6A). Since Ply releases hemoglobin from RBCs *in vitro* [31, 32, 55], we sought to determine whether pneumolysin is needed for the induction of biofilms by blood. Previously described *ply* knockouts in both D39 and TIGR4 backgrounds [32] did not produce biofilms when grown in THYB supplemented with blood (Figure 6B), suggesting that the absence of hemolytic activity prevents biofilm induction by erythrocytes in the *ply* mutants. The *ply* mutants grown with hemoglobin produced 10-fold more biofilm biomass than cells grown in THYB; hence, signaling of biofilms by hemoglobin remains. Still, the total biofilm biomass was lower compared to the wild-type strains grown with hemoglobin (Figure 6C). Therefore, similar to our previous findings, the data suggest that Ply contributes to biofilm development also independently of its hemolytic activities.

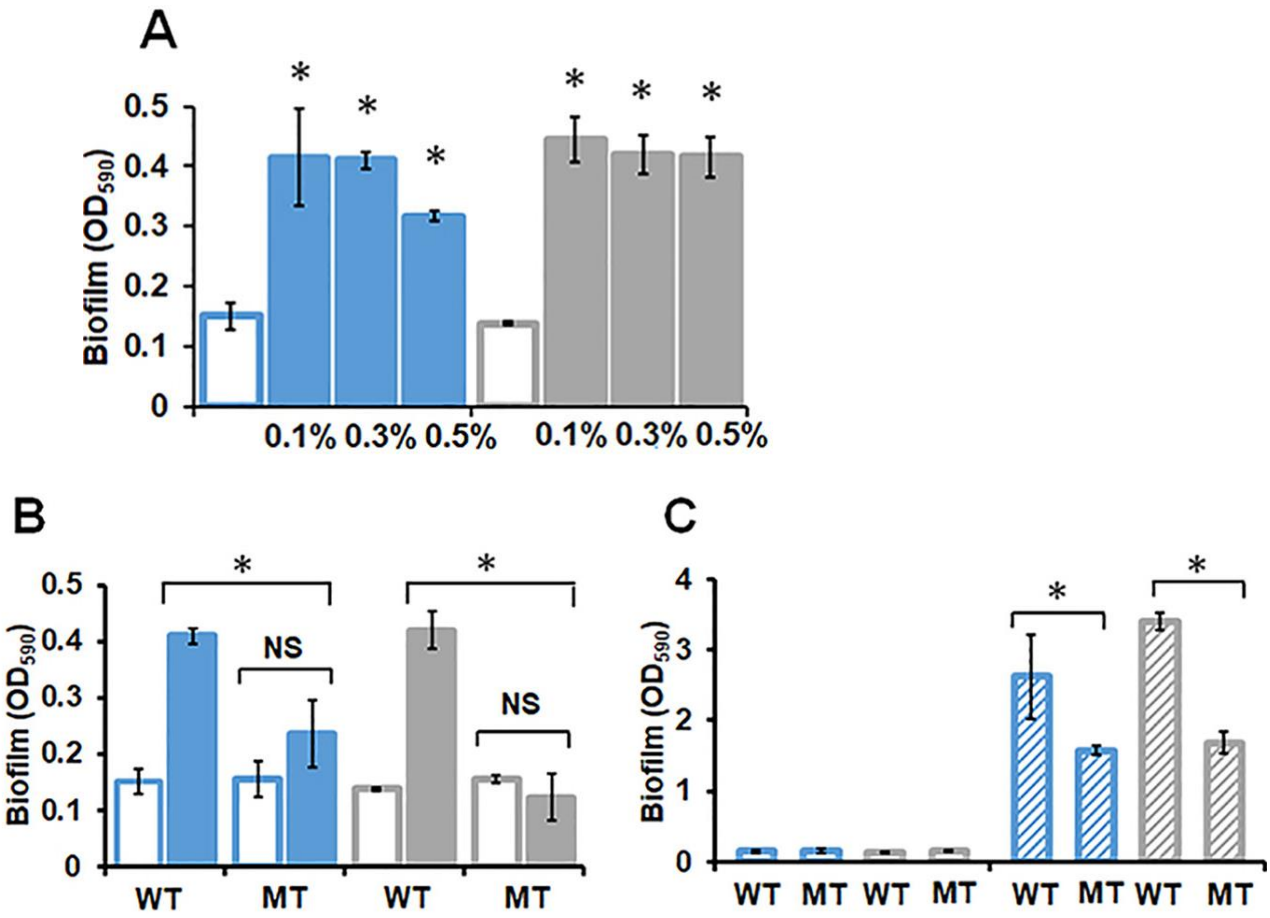


Figure 6: Human red bloods cells trigger biofilm formation in a Ply-dependent manner.

Blood cells activate biofilm formation in a *ply*-dependent manner. Fresh medium was inoculated (starting OD_{600} = 0.05) with wild-type (WT) or *Δply* mutant (MT) isogenic pairs in D39 (blue) or TIGR4 (gray) strains grown on BAPs. The cultures were grown in microtiter plates, and biofilm formation at 18 h is shown. (A) Wild-type strains grown in THYB (empty) or THYB supplemented with 0.1 to 0.5% (vol/vol) washed human blood cells (filled). (B and C) Wild-type and mutant strains were grown in THYB (empty) or THYB with 0.3% washed human blood cells (filled) (B) and in THYB (empty) or THYB with 20 μM hemoglobin (stripes) (C). The data are expressed as means ± the SD of at least two independent experiments, each performed in triplicates. The asterisks denote statistical significance: $P \leq 0.05$ (THYB versus THYB-blood in panels A and B [Student t test; NS, not significant] or THYB versus THYB-blood WT versus MT in panels B and C [two-way analysis of variance [ANOVA]).

2.7 BIOFILM FORMATION IN THE PRESENCE OF HEMOGLOBIN INVOLVES THE *COMC* GENE BUT NOT THE RELATED TWO COMPONENT SYSTEM C*OMDE*.

Various sensory and signaling two-component systems (TCS) regulate bacterial attributes such as virulence, biofilms, sporulation, and toxin production, in response to external stimuli. *S.*

pneumoniae encodes 13 TCS pairs and one orphan regulator, RitR [56]. Our RNA-seq revealed that *S. pneumoniae* transitioning into sessile growth induced the TCSs genes, *liaRS*, *vicRK*, and *ciaRH*. To explore how the early transition in lifestyle *S. pneumoniae* exhibits in the presence of hemoglobin is coordinated, we tested mutants in regulatory genes that were upregulated in newly adherent pneumococci compared to planktonic cells (both grown with hemoglobin). Deletion mutants in *comC*, the TCS *ciaRH*, and *yesMN* and in the histidine kinase *vicK* (*vicR* is an essential gene) were tested for growth and biofilm formation with or without hemoglobin (Figure 7A-B). Other than the *ΔciaRH* mutant, the mutants grew similarly to the wild type in THYB and created minimal but similar biomass on the plate surface after 18 hours of incubation in THYB (Figure 8). The *ciaRH* mutant exhibited severely attenuated growth and reduced biofilm biomass in THYB. Hemoglobin enhanced the growth of all strains, including that of the *ciaRH* mutant (see Figure 9A). Compared to the wild-type strain, we did not observe a significant difference in biofilm generated by the *ΔyesMN* and *ΔvicK* mutants after 18 hours of incubation (Figure 9A). However, inactivation of *ciaRH* or *comC* resulted in a 37% ($P = 0.01$) or a 67% ($P = 0.001$) reduction, respectively, demonstrating that both systems contribute to the biofilm response to hemoglobin. During competence activation, CSP is produced by *comC* and exported to the extracellular milieu where it binds and activates the histidine kinase, ComD. ComD is autophosphorylated and phosphorylates the response regulator, ComE, which in turn induces the *com* regulon. Interestingly, we found that deleting *comD* or *comE* genes had only a small impact on biofilm formation in the presence of hemoglobin (Figure 9B)**.** This suggests the presence of a yet-to-be-described non-canonical regulatory circuit that depends on CSP but not on *comDE* in biofilm formation and that other sensory proteins are involved. The *ciaRH* genes respectively encode a response regulator and membrane-bound histidine kinase. These proteins function together as a

TCS that contributes to pneumococcal cell stability during environmental stress. Like *comCDE*, the *ciaRH* genes are also necessary for nasopharyngeal colonization and biofilm formation by *S. pneumoniae*, although they negatively impact CSP levels [57].

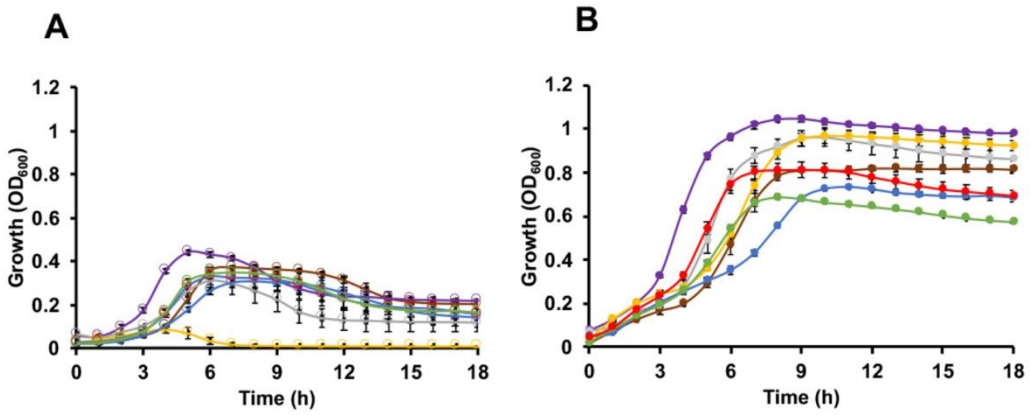


Figure 7: Growth of S. pneumoniae D39 wildtype and isogenic mutants in THYB and THYB supplemented with hemoglobin.

THYB was inoculated with D39 wildtype (red) and the isogenic mutant strains Δ*vicK* (blue), Δ*yesMN* (green), Δ*comC* (brown), Δ*comD* (purple), Δ*comE* (grey), and Δ*ciaRH* (yellow), grown on blood agar plates (18 h, starting OD_{600} = 0.05). Growth is shown as follows **(A)** THYB; **(B)** THYB supplemented with 20 μM hemoglobin. The data are representative of at least two independent experiments performed in triplicates; error bars indicate SD.

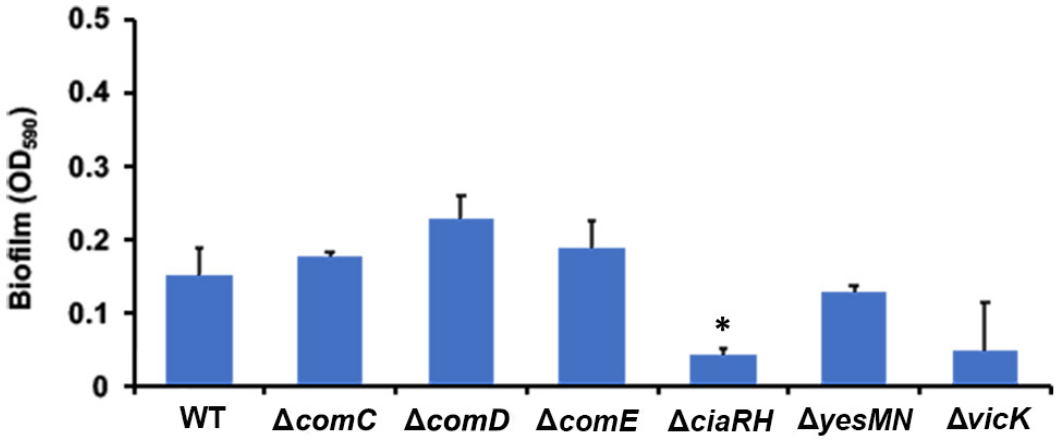


Figure 8: Sessile growth of S. pneumoniae D39 wildtype and isogenic mutants in THYB.

Biofilms were visualized and quantified after 18 hours. The data are expressed as the mean ± SD of at least two independent experiments each done in triplicates. The asterisks denote statistical significance, $P \leq 0.05$ (wild type vs. mutants, Student's t-test).

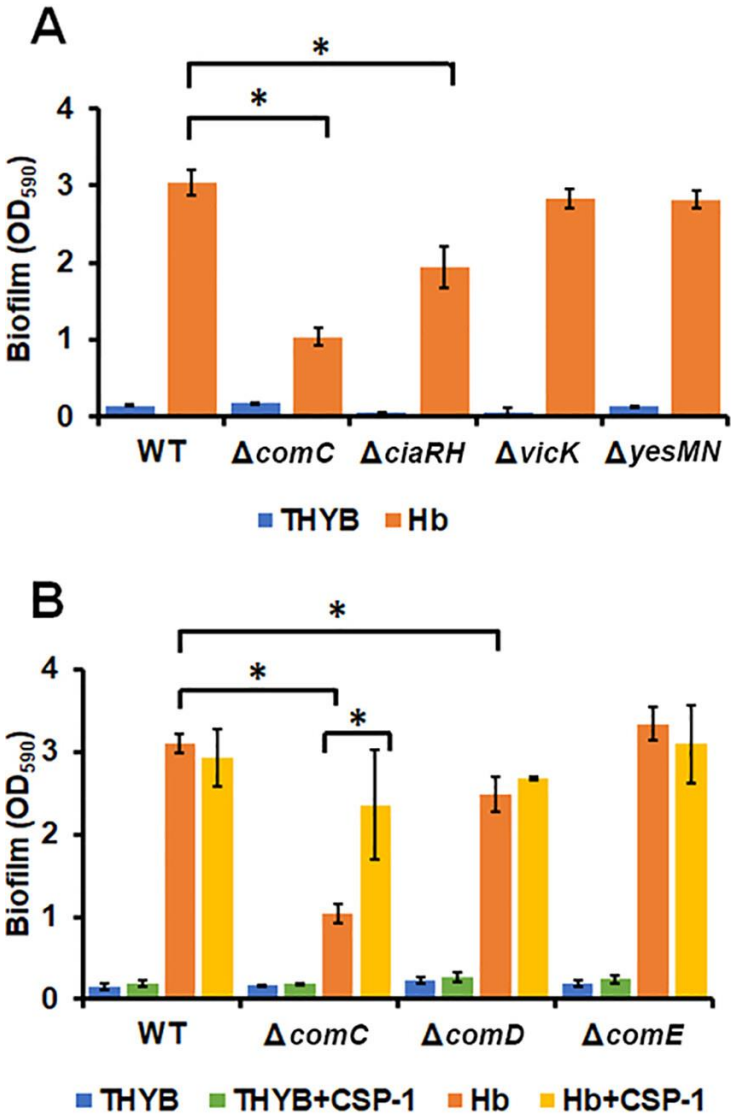


***Figure 9: Biofilm formation in the presence of hemoglobin involves the* comC *gene but not the related TCS* comDE.**

Biofilm formation in the presence of hemoglobin involves *comC* and is mostly independent of the *comDE* genes. THYB was inoculated with D39 wild-type and isogenic mutant strains grown on BAPs (starting $OD_{600} = 0.05$). (A and B) Biofilm biomass produced by *S. pneumoniae* grown after 18 h in THYB or THYB with 20 μM hemoglobin (Hb) (A and B), 200 ng/ml CSP1 (B), or Hb and CSP1 (B). The data are expressed as the means ± the SD of at least two independent experiments, each performed in triplicates. Asterisks denote statistical significance: $P \leq 0.05$ (wild type versus mutant for each comparison of +Hemoglobin versus –Hemoglobin and of Hemoglobin versus Hemoglobin+CSP-1 [two-way ANOVA]).

2.8 Conclusion

In these reports, we demonstrate that hemoglobin promotes growth and the production of biofilm in a dose-dependent manner. Supplementation with hemoglobin enhanced growth in concentrations that were growth inhibitory with equal molar concentrations of free heme. Furthermore, supplementation with other hemoproteins also stimulates robust growth but without signaling biofilm production. The signaling of hemoglobin-induced biofilms is conserved among serotypes and requires hemoglobin in its native state. Additionally, hemoglobin remodels the transcriptome of planktonic cells promoting the expression of alternative carbohydrate metabolism for carbon sources found in the host nasal pharynx [30].

Spd_0739 (Spbhp-37) is reported to bind heme and hemoglobin and inactivation by knockout or antibodies are attenuated in the ability to grow on hemoglobin and heme sources[40]. *S. pneumoniae* has reductant mechanisms to acquire heme and other iron sources and typically requires multiple knockouts in these systems to notice a growth phenotype. Knockout of *spd_0739* has a large phenotype without knockout of other iron transporter which requires further investigation on the hierarchy of heme transport in *S. pneumoniae*. Additionally, the studies implicating Spd_0739 as a nucleoside binding lipoprotein of a nucleoside scavenging system [39, 58] bring to question if Spd_0739 is acting directly in heme transport or causing pleiotropic changes in expression that impact heme import.

Hemoglobin promotes biofilm in iron-complete and iron-depleted media in a dose-dependent manner. This biofilm is stimulated with the inclusion of erythrocytes, however, requires the expression of *ply*, which codes for pore-forming pneumotoxin. The induction of

several TCSs accompanied the pneumococcal transition into biofilms. Mutant analysis of selected genes demonstrated that only *comC* and *ciaRH* play a major role in biofilm development under our experimental conditions. The observation that the *ciaRH* mutant is also impacted in growth and biofilm development in regular THYB raises the possibility that the reduction in biofilm development in the presence of hemoglobin is a reflection of a general growth/biofilm phenotype. The CiaRH TCS orchestrates the pneumococcal cell wall's integrity and retention by preventing the lysis that is otherwise induced by stress and antibiotic treatment [48, 59-62]. Curiously, the addition of hemoglobin improved the mutant's planktonic growth in THYB significantly raising the possibility that hemoglobin activates signaling downstream of the *ciaRH* signaling pathway.

The c*omC* mutant exhibited a significant defect in biofilm growth in the presence of hemoglobin. Moreover, while exogenically added CSP-1 complemented the *comC* phenotype in THYB containing hemoglobin, it had no impact on biofilm development by the wild type or the *comC* mutant in regular THYB. Therefore, CSP-1 is not sufficient for triggering early biofilm development in *S. pneumoniae* grown in THYB. Still, this signaling peptide promotes the development of biofilm growth induced by hemoglobin. The *comC*-encoded CSP is exported and orchestrates competence development by binding and activating the surface histidine kinase, ComD, which in turn relays the signal to the ComE response regulator. Interestingly, the data suggest that CSP-1 function in biofilm development under the influence of hemoglobin is mostly independent of the ComDE pathway (Fig. 8B). The *comC*-dependent but *comDE*-independent biofilm phenotype constituted a significant deviation from the canonical pathway for competence activation and, to the best of our knowledge, was not previously described. These intriguing

findings suggest the presence of a new regulatory route for CSP in the hemoglobin induction of biofilms.

3 CHAPTER 2: ENDOGENOUSLY PRODUCED H_2O_2 IS INTIMATELY INVOLVED IN IRON METABOLISM IN *STREPTOCOCCUS PNEUMONIAE*

3.1 Abstract

Streptococcus pneumoniae, the ethological agent of serious human infections in humans, requires iron and readily uses hemoglobin and heme to obtain the metal. During growth in aerobic environments, pneumococci produce and secretes large amounts of H_2O_2, which can interact with free, and heme bound iron, leading to reactive species formation and heme degradation in some cases. Here, we investigated the impact of endogenously produced H_2O_2 on the use of hemoglobin as an iron source in *S. pneumoniae*. Cultivation of wild type pneumococci with met-hemoglobin (hemoglobin with ferric iron) resulted in the consumption of H_2O_2 and enhanced spending of the heme and hemoglobin in the medium. H_2O_2 degrades only free heme or hemoglobin-heme, which is at the ferrous state. Surprisingly, I found that iron was released from met hemoglobin in pneumococcal cultures by a new process that required both H_2O_2 and growing cells. I analyzed bacterial growth, iron and heme cellular content, in wildtype and mutant strain defective in H_2O_2 production (Δ*spxB*Δ*lctO)* during cultivation on met hemoglobin iron. All test strains grew and accumulated heme under our experimental conditions. The lower heme content recovered from a knockout mutant of the *piuBCDA* transporter, suggest heme is accumulated by an active process and established PiuBCDA as a heme importer. The Δ*spxB*Δ*lctO* mutant accrued substantially more heme than the wild type, but both strains contained equal amounts of iron. Notably, all strains exhibited a higher amount of cellular heme when cultivated in the presence of the iron chelator, nitrilotriacetic acid (NTA) compared with cells growing in Chelex treated medium. RT-PCR analysis revealed that compare with the wild

type, Δ*spxB*Δ*lctO* strain upregulated the expression of multiple heme and iron transporters. Moreover, cultivating pneumococci in medium containing NTA leads to activation of iron transporters compared to cells grown in Chelex-treated medium, although both cultures were supplemented with equal amount of met hemoglobin. Hence, Streptococci can import and degrade heme intracellularly independently of H_2O_2. Nevertheless, in the presence of H_2O_2 production, pneumococci benefit from extracellular degradation of the met-hemoglobin heme, which release metal iron for import. In summary, this study strongly links H_2O_2 production to iron metabolism and uncovered extracellular heme degradation by H_2O_2 as a key iron acquisition mechanism that is likely to play a significant role during upper respiratory tract colonization and in bacterial pneumonia, where the pathogen.

3.2 Introduction

Streptococcus pneumoniae is a catalase-negative facultative anaerobe that continues to cause severe illness and mortality despite vaccine availability[23, 63]. Pneumococcus is carried asymptomatically in the nasopharynx of up to 65% of the population. In some carriers, the bacterium translocates through mucosal, epithelial and endothelial barriers and proliferate at otherwise sterile sites such as the middle ear, lungs, blood, meninges, and the endocardium [64]. Each year pneumococcus accounts for ~10.6 million invasive infections, causing ~2 million deaths, of which children and the elderly are most impacted. With the emergence of SARS-CoV-2, bacterial co-infections were prevalent in COVID-19 patients with *S. pneumoniae* the most common infection partner [65].

The total iron pool in human adults is around 3-4g of which about 75% is in the form of heme mostly bound to hemoglobin [8]. To avoid toxicity and overcome solubility problems the host sequesters virtually all the iron in the extracellular and intracellular milieus using

specialized proteins that transport, bind, or store iron or heme. During infection, iron availability is further decreased due to a coordinated host response named nutritional immunity. Hence, invading bacteria that require iron, such as *S. pneumoniae,* rely on dedicated mechanisms to capture the metal from host proteins. We recently showed that heme, hemoglobin, and several other host heme sources restore pneumococcal growth in an iron-deplete medium. Notably, hemoglobin promotes growth *in vitro* to a greater capacity than other iron or heme sources and supports growth in concentrations that were toxic with equivalent amounts of free heme. In addition to facilitating vigorous planktonic proliferations, hemoglobin induces early and robust pneumococcal biofilms *in vitro*. These and other observations established hemoglobin as a principal nutrient and host signal for pneumococci [29, 30].

Gram-positive pathogens often employ a protein-relay machinery consisting of surface proteins that capture the heme from the host and deliver it across the thick cell wall and eventually to the substrate-binding component of a membrane-bound ABC transporter [1, 10, 11, 14]. The molecular mechanisms for heme or iron acquisition are not fully explained in *S. pneumoniae*. Surface receptors such as those that shuttle heme across the cell wall in other Gram-positive bacteria have not been described in this human pathogen. Several ABC transporters involved in iron uptake were identified, but the *in vivo* ligand of some transporters remained undetermined. The two pneumococcal ABC systems proposed to import ferric iron are the *pit1ABCD (spd_0223-0227)* and *pit2ABC* (*spd_1607-1609*) transporters [42, 43]. In the D39 strain, the substrate binding component, *pitA1* is truncated but the permease and ATP binding proteins are intact. *S. pneumoniae* also encodes the *pia* transporter of which, PiaA, the ligand-binding protein was crystalized with ferrochrome [66]. Previous studies also, demonstrated that the Pia proteins support pneumococcal growth on heme iron[41]. Similarly, the PiuBCDA

transporter promotes streptococcal growth using hemoglobin as a single source of iron [36], but the substrate-binding component, PiuA, binds *in vitro* norepinephrine and enterobactin in higher affinity than heme [36]. A recent study implicated an additional pneumococcal ABC transporter, *spd_0088-0090*, in heme uptake [37]. Due to the redundancy of iron uptake mechanisms, genetic knockouts in multiple transport systems are required before attenuated pneumococcal growth is observed [43].

Several studies reported the presence of free hemoglobin in the mucosa of the upper respiratory tract due to bleeding and erythrocyte lysis[67]. In intact erythrocytes, hemoglobin iron is found in its reduced form (ferrous, Fe^{+2}) bound to oxygen (oxy-hemoglobin) or carbon dioxide. Upon erythrocytes lysis, the iron in oxy-hemoglobin is autoxidized to ferric (Fe^{+3}) forming met-hemoglobin (met-hemoglobin), which cannot bind oxygen [67]. Oxy-hemoglobin interactions with hydrogen peroxide (H_2O_2) leads to heme degradation; these reactions start with the formation of ferryl hemoglobin (Hemoglobin-Fe^{+4}=O), which in turn mediates one electron oxidation of H_2O_2 creating a superoxide radical in the heme binding pocket. Subsequent reactions with the porphyrin ring degrade the heme and release free iron and two fluorescent products [68, 69]. On the other hand, met-hemoglobin interactions with H_2O_2 do not lead to heme degradation but catalytically consume the H_2O_2 [68]. In these catalase-like reactions one H_2O_2 molecule serves as a two-electron acceptor leading to the formation of oxo-ferryl complex (Fe^{+4}=O) and transient apo-protein radicals (e.g., *Hemoglobin-Fe^{+4} =O) while a second H_2O_2 molecule serves as a two-electrons donor that reduces the ferryl hemoglobin back to met-hemoglobin, while producing molecular oxygen (O_2).

In the human host, pneumococcus is exposed to oxygen levels that varies from 20% to 5% in the upper to lower respiratory tracts to virtually no free oxygen in the blood [70]. In the presence

of oxygen, *S. pneumoniae* produce H_2O_2 as a byproduct of the enzymatic reactions of the pyruvate oxidase (*spxB*) and lactate oxidase (*lctO*) [31, 55, 70-72]. The SpxB and LctO enzymes are responsible for ~80% and ~20% of the pneumococcal H_2O_2 production, respectively. SpxB activity augments the pneumococcal release of Ply, a pore-forming toxin, which promotes erythrocyte lysis, likely increasing the availability of free hemoglobin in the immediate bacterial environment [73]. Our group demonstrated that *spxB* is highly expressed during growth with hemoglobin [30] and that the pneumococci-produced H_2O_2 catalyzes the oxidation of oxy-Hemoglobin from rapturing erythrocytes to met-hemoglobin [31]. In this study, we were prompted to investigate the possible role of H_2O_2 production in iron acquisition from hemoglobin during pneumococcal planktonic growth.

3.3 MATERIALS AND METHODS

Bacterial growth and media

Frozen stocks of *S. pneumoniae* were preserved in Skim milk-Tryptone-Glucose-Glycerin (STGG) as described and stored at -80°C [74]. *S. pneumoniae* cells from STGG stock, were plated on Trypic Soy blood agar plates (BAPs) and incubated at 37°C. Cells collected from BAPs following overnight incubation were used to inoculate fresh medium in a starting OD_{600} of 0.05. Pneumococci were grown in Todd-Hewitt both containing 0.5% (w/vol) yeast extract (THYB), iron-depleted THYB ($THYB_{Chx}$ or $THYB_{NTA}$) or iron-depleted THYB supplemented with hemoglobin. $THYB_{Chx}$ was prepared by adding 5% (w/vol) Chelex-100 resin (Sigma-Aldrich) to THYB and incubating with continued mixing overnight. The resin was removed using a 0.45-micron filter and the medium was supplemented with 2mM $MgCl_2$ and 100µM $CaCl_2$. $THYB_{NTA}$ was prepared by adding 3mM NTA, 0.55mM $ZnSO_4$ and 0.55 mM $MnCl_2$ to fresh THYB followed by filter sterilization (0.45-micron filters). Hemoglobin stock solutions

were prepared fresh by resuspending lyophilized power of human hemoglobin (Sigma Aldrich) in 1 x PBS (1mM). Hemoglobin was then added at the indicated concentration and the medium was then filter sterilized (0.45-micron filters). Pneumococci were grown in 96-well flat bottom tissue culture plates at 37°C in a Multiscan Skyhigh spectrophotometer (Thermo Scientific). Optical density was measured hourly after brief shaking.

Hemoglobin Spectroscopic Measurements

Absorbance spectra (250 to 700 nm) of hemoglobin samples in PBS or THYB were determined using a DU730 LifeScience UV/VIS or Beckman-Coulter DU 730 UV/Vis spectrophotometer in a quartz cell with an optical path length of 10mm. In some experiments, 10µM hemoglobin was incubated with 10mM DTT (Sigma Aldrich) or 30µM potassium ferricyanide (Thermofisher) for 1 hour in PBS prior to spectroscopic measurements.

Bacterial Strains and Mutant Construction

The bacterial strains and plasmids used in the study are listed in **Table 1** and the primers in **Table 2**. The Δ*spxB*Δ*lctO* knockout and its isogenic *ΩspxBΩlctO* complemented strain were engineered *S. pneumoniae* D39 strain as described for the construction of this mutation and complementation in TIGR4 strain [72].

Quantification of Hydrogen Peroxide Concentration in Pneumococcal Growth Media

Pneumococcal cells collected from BAP following overnight growth at 37ºC were used to inoculate fresh THYB or THYB supplemented with 20µM met-hemoglobin at OD_{600} 0.05 (6 mL in 15 mL falcon tubes). Supernatant was prepared from culture samples by centrifugation and

filtration (0.45-micron) and the hemoglobin was removed by filtration with Ultra-15 centricon filters (molecular wight cutoff of 30,000, Amicon). The sample H_2O_2 content was measured using the Quantitative Peroxide Assay Kit (ThermoFisher) per manufacturer's instructions. A serial dilution of 30% H_2O_2 in THYB was used to generate a standard curve, from which we a derived the H_2O_2 concentration in media samples.

Measurement Of Free Iron Concentration in Pneumococcal Growth Media

We determined the concentration of free iron in the medium samples using an optimized ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate, Milli Sigma) based assay as described [75]. Briefly, hemoglobin was incubated in 2 ml THYB or in spent THYB (collected from overnight pneumococcal cultures) in 12 well flat bottom microtiter plates, at 37°C. Hemoglobin-free samples collected at different time points were treated with 50 mM ascorbic acid and incubated with the 50mg ferrozine/ml and 500mM potassium acetate (pH 5.5) in 96-well flat bottom plates for 2.5 hours prior to reading. Absorbance at 562 nm was determined after 135 min incubation at 37°C, and iron concentration in the medium was calculated using a standard curve. The same was done with the cell- and hemoglobin- free supernatants samples collected from of pneumococcal cultures grown in THYB with hemoglobin with or without 200 U/µL catalase (Sigma Aldrich). Net iron released into the culture during growth was calculated by subtracting the iron values determined in uninoculated THYB containing hemoglobin.

Determination of Heme Content

Fresh $THYB_{Chx}$ or $THYB_{NTA}$ was supplemented with 20μM hemoglobin, inoculated with *S. pneumoniae* OD_{600} 0.05 in 12 well flat bottom microtiter plates and incubated at 37°C. 6 ml culture samples (at OD_{600} 1.0) were collected at different time points. The cells were harvested by centrifugation, washed three times within PBS, resuspended in 2 ml DMSO and sonicated (20% amplitude for 30s). Heme was extracted by acidified chloroform and its concentration determined using a standard curve made with hemin solutions in DMSO as described [76]. Briefly, 2 mL of 50 mM glycine buffer, pH 2.0, 0.1 mL of 4 N HCl (pH 2.0), 0.2 mL of 5 M NaCl (pH 2.0) and 2 mL of chloroform:isopropanol was added to the cell lysates. The reactions were mixed vigorously and were allowed to incubate at room temperature for 1 min. Absorbance of the organic phase at 388, 450 and 330 nm were recorded and fed into the correction equation $Ac = 2 \times A388 - (A450 + A330)$. Hemoglobin (if was present in the culture medium) was removed by filtration prior to heme extractions.

Total Iron by ICP-MS

Fresh $THYB_{Chx}$ or $THYB_{NTA}$ supplemented with 20 μM hemoglobin was inoculated with *S. pneumoniae* grown on BAPs (starting OD_{600} 0.05) and allowed the culture to grow in 12 well microtiter plates at 37 °C for 6 hours. 6 mL culture samples (at OD_{600} 1.0) were harvested, washed three times with phosphate-buffered saline and sent for ICP analysis (Center for Applied Isotope Studies, University of Georgia, Athens, GA) as described [30].

qRT-PCR Analysis

Quantitative reverse transcription PCR (qRT-PCR) analysis was carried out using the Power SYBR Green RNA-to-Ct 1-Step Kit (Applied Biosystems) and StepOne DNA PCR machine (Applied Biosystems) according to the manufacturer's specifications. A total of 25 ng RNA was used per qRT-PCR reactions and each reaction was done in duplicates. Primers used for qRT-PCR are listed in **Table 2**. The relative expression was normalized to the endogenous control *gyrB* gene and fold changes were calculated using the comparative 2−ΔΔCT method.

3.4 Strains and primers

Table 1: Chapter 2 Strains and plasmids

S. pneumoniae	**Description**	**Source or references**
D39	Avery strain, clinical isolate, WT (capsular serotype 2), CSP1	[77, 78]
D39 Δ*spxB*Δ*lctO*	D39 derivative with double knockout of *spxB* and *lctO* genes	This study
D39 ΩspxBΩlctO	D39 Δ*spxB*Δ*lctO* mutant expressing the *spxB* and *lctO* genes from a heterologous chromosomal location	This study
D39 Δ*piuBCDA*	D39 derivative with Δ*piuBCDA*::*ermC* mutation (Erm^R)	This study
E. coli		
One shot Top10	Cloning strain	Invitrogen
Plasmids		
pAF103	Plasmid for allelic replacement of Δ*piuBCDE::ermC* with pUC19 backbone, Amp^r	This study

Table 2: Chapter 2 Primers

Target	**Primer name**	**Sequence (5'-3')**	**Comments**
Puc19L	ZE 730-FW	GTCTGGAAGGCATGCAAGCTTGGCGTAATCAT	Sense primer for cloning of the pUC19 segment of pAF103
pUC19L	ZE 731-RV	GAACATGAGTACCGAGCTCGAATTCACTGGCC	Antisense primer for cloning of the pUC19 segment of pAF103
5'piuB & upstream region (D39)	ZE 732 FW	CTCGGTACTCATGTTCTCTTCGACTGCTTCTC	Sense primer for cloning of the left arm of *piu* segment of pAF103
5'piuB & upstream region (D39)	ZE 733 RV	CACACGGTGGTAATAGTCTGCATGAGAAGGCC	Antisense primer for cloning of the left arm *piu* segment of pAF103
ermC (pJRS233)	ZE 734-FW	CTATTACCACCGTGTGCTCTACGACCAAAACT	Sense primer for cloning of the *ermC* segment of pAF103
ermC (pJSR233)	ZE 735-RV	TTGCTTGCCAAAGCTGCCGACAACACGGGAGC	Antisense primer for cloning of the *ermC* segment of pAF103
3' *piuA* & downstream region (D39)	ZE 736-FW	CAGCTTTGGCAAGCAAGGATGACTACTGGACT	Sense primer for cloning of the right arm of *piu* segment of pAF103

3' *piuA* & downstream region (D39)	ZE 737-RV	TGCATGCCTTCCAGACCACTTTTCCCTTAAAC	Antisense primer for cloning of the right arm *piu* segment of pAF103
gyrB (D39)	ZE 661-FW	GGCACTGTATGGTATCACACAAG	qRT-PCR
gyrB RV	ZE 662	TCTCTAAATTGGGAGCGAATGTC	qRT-PCR
piuB FW	ZE 864	TGATTTCGACCAGCAGACCTG	qRT-PCR
piuB RV	ZE 865	CTGTACTCGGTGCAGCAAACTG	qRT-PCR
Pit 2	Spd_160 FW	ATTGCCCGTCCTGTACCACC	qRT-PCR
Pit 2	(Spd_1609) RV	TGCTGCTTGCTCTGGAGGTT	qRT-PCR
piaA FW	ZE 151-FW	AAAAATGGTGCCGTTGCTGT	qRT-PCR
piaA RV	ZE 152-RV	AAAGTGGTGTTGGAGTGCATGA	qRT-PCR
SPD_0225 (pit1) FW	ZE 153-FW	CCAAGCGAAGTTTGGCCTCC	qRT-PCR
SPD_0225 (pit1)	ZE 153-RV	CACCTTGGCATGCGCCTTAG	qRT-PCR
SPD_0310 FW	ZE 155-FW	TGATCAAGGCAGCGGCTGTA	qRT-PCR
SPD_0310 RV	ZE 156-RV	GAAACTGGTGGACCAGCCCT	qRT-PCR
SPD_0090 FW	ZE 171-FW	ACGGTCCAGAAGGCAAGAACT	qRT-PCR
SPD_0090 RV	ZE 172-RV	ACCAGTGTTCCATCCACCCA	qRT-PCR

3.4.1 Plasmid Construction

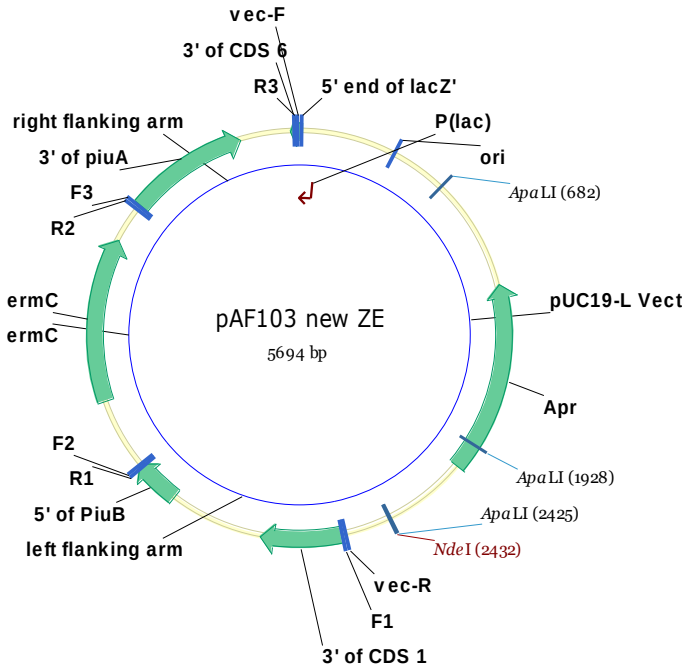


Figure 10: Illustration of pAF103 plasmid

The Δ*piuBCDA* mutant was created by replacing the *piuBCDA* gene cluster with the *ermC* gene from plasmid pJRS233[79]. A chimeric fragment consisting of the *ermC* gene, expressed from its the native promoter, preceding with a 1013 bp fragment of D39 *piuB* upstream region (including the first 213 bp of *piuB* ORF) and proceeding with an 818 bp fragment with *piuA* downstream area (including the last 534 bp *piuA* ORF) was cloned into pAF103 by Gibson cloning using the GeneArt Seamless Cloning kit (Invitrogen) and transformed into One Shot TOP10 *E. coli* stain. A linear fragment (1252 bp) containing *ermC* fluked with *S. pneumoniae* chromosomal fragments was amplified from pAF103 and introduced into competent D39 WT cells[80]. Pneumococcal clones harboring the Δ*piuBCDA* mutation were selected on erythromycin (0.5 µg/mL) and the chromosomal deletion of *piuABCD* genes was confirmed by PCR analysis.

3.5 Results

Interactions between hydrogen peroxide and met-hemoglobin promote their removal from the medium during pneumococcal growth.

Since free oxy-hemoglobin is rapidly oxidized by H_2O_2, I rationalized that *S. pneumoniae* encounters mostly met-hemoglobin in the respiratory mucosa [72]. To begin investigating the impact of H_2O_2 production on pneumococcal interactions with met-hemoglobin and iron uptake we constructed a double Δ*spxB*Δ*lctO* mutant and a complemented strain (expressing *spxB* and *lctO* genes from heterologous location in the chromosome) in the background of the D39 strain. I monitored the H_2O_2 levels in the culture media during growth and confirmed that the Δ*spxB*Δ*lctO* mutant did not produce H_2O_2 at detectable levels, while I found 5-7 mM of H_2O_2 in the culture supernatant of WT and the complemented strains (Fig. 11A).

I next examined the H_2O_2 amount in pneumococcal cultures growing in the presence of met-hemoglobin (Fig. 11B). I solubilized lyophilized hemoglobin (Sigma) in PBS and determined the absorbance spectrum following incubation with oxidative (potassium ferricyanide) or reducing (DTT) agents. The Soret peak of the hemoglobin incubated with or without potassium ferricyanide overlapped. In the presence of DTT, however, the absorbance maximum shifted right from 408 nm to 418 nm, indicating iron reduction (Fig. 12.). These spectral characteristics indicate the hemoglobin in our stock solution is in the oxidized (met-hemoglobin) state. Analysis of the H_2O_2 levels in culture medium revealed that when the WT and complemented strains were grown in THYB supplemented with 20 µM met-hemoglobin, H_2O_2 was not detectable at the 3- and 6-hour time points (Fig. 11B). Hence, the H_2O_2 produced by pneumococci is removed, likely due to reactions with the medium met-hemoglobin. Low

amounts of H_2O_2 (10-25% compared to THYB) were detected after 18 H suggested met-hemoglobin depletion during incubation allows for some accumulation of H_2O_2 at the later time point.

While the hemoglobin remains soluble in un-inoculated THYB, visual inspection suggested that some of the met-hemoglobin is falling out of solution, with more precipitation occurring in the WT cultures compared to the Δ*spxB*Δ*lctO* mutant. Hemoglobin precipitation was confirmed by centrifugation and washes of the cell pellet. We used spectroscopic measurements to follow the levels of the hemoglobin that remained in solution during growth. The hemoglobin displayed a somewhat broad Soret peak, suggesting that some of the heme iron in the met-hemoglobin was reduced by the medium. Nevertheless, we did see distinct changes in the Soret maximum during incubation in regular THYB even after 18 H of incubation. On the other hand, in medium that was inoculated with the WT *S. pneumoniae*, the hemoglobin spectrum maximum decreased and left-shifted in the first 3 H forming the narrow peak at 408 nm, that is typical of oxidized hemoglobin (met-hemoglobin). The Soret peak decreased dramatically within the next 3 H of incubation, and eventually was below the detection level by the 18 H time point (Fig. 10C). In the Δ*spxB*Δ*lctO* culture, the changes in the Soret happened in a much lower rate; no changes in absorption were observed in the first 3 H of incubation. While the Soret peak decreased during longer incubation, it was significantly higher compared to the Soret Maximum with the WT strain at the 6 H time point. Altogether, the data suggest H_2O_2 produced by *S. pneumoniae* interact with the met-hemoglobin in the medium via reactions that remove both H_2O_2 and the protein [72]. Still, mechanisms that are independent of H_2O_2 also contribute to the decrease in the levels of soluble met-hemoglobin in the pneumococcal growth medium.

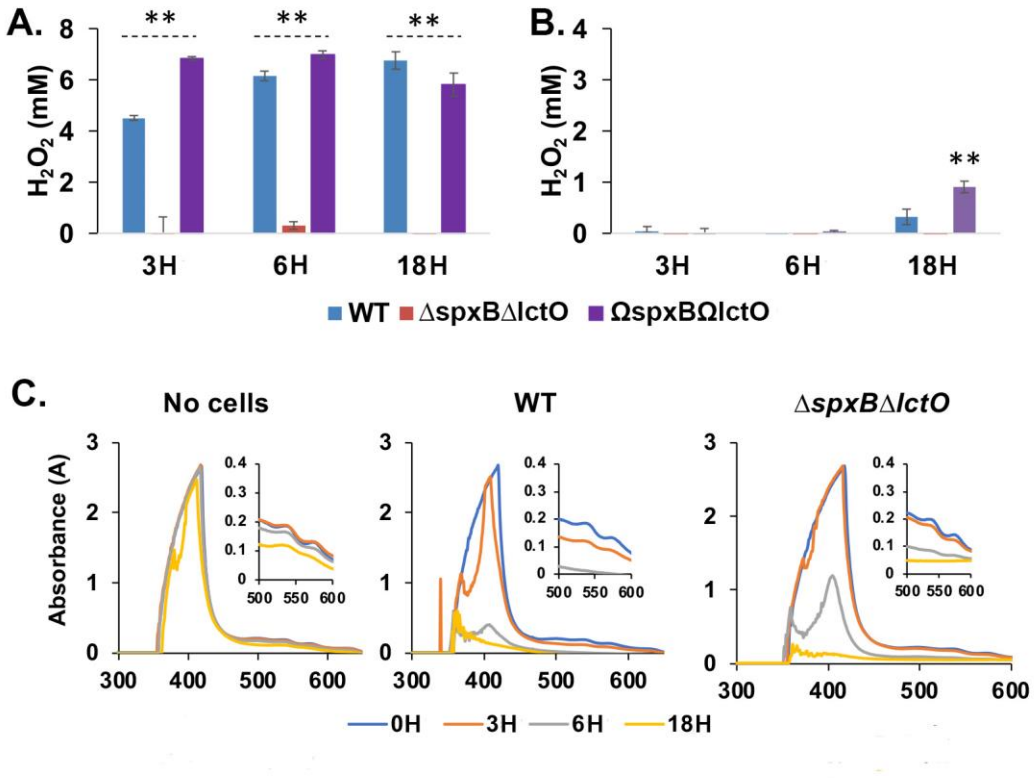


Figure 11: Hydrogen peroxide levels in the growth medium of S. pneumoniae grown in THYB with and without met-hemoglobin.

Shown are hydrogen peroxide concentration in the culture supernatant of D39 WT, Δ*spxB*Δ*lctO* mutant, and complemented (Ω*spxB*Ω*lctO*) strains grown in THYB (**A**) or in THYB supplemented with 20 µM met-hemoglobin (**B**). **C**) The absorbance spectra of uninoculated THYB containing 10µM hemoglobin, THYB-10µM hemoglobin inoculated with the D39 WT or the Δ*spxB*Δ*lctO* mutant incubated at 37ºC for up to 18 H (spent THYB medium used as blank) Cells were removed by centrifugation prior to absorbance reading. The data was derived from experiments done in duplicates and repeated twice and analyzed by ANOVA, where * indictates a $P \leq 0.05$ and ** ≤ 0.01.

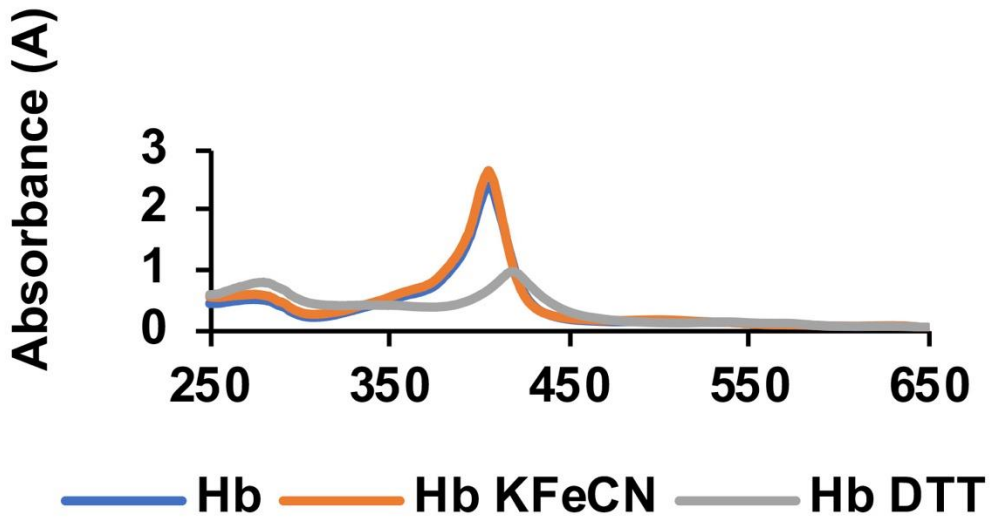


Figure 12: Confirmation of the redox state of hemoglobin (Sigma)

Insert shows the absorbance spectra of 10 µM hemoglobin stock solutions in PBS (Hb), PBS with 30µM potassium ferricyanide (Hb KFeCN) or 10mM dithiothreitol (Hb DTT).

H_2O_2 reaction mediates iron release from met-hemoglobin in a cell dependent manner.

I next tested if the interactions between the endogenously produced H_2O_2 and met-Hemoglobin release iron into the culture supernatant. THYB supplemented with 20 µM met-hemoglobin was allowed to incubate at 37ºC. The hemoglobin was then removed by filtration and the concentration of free iron in the medium was determined at different time points using the chromophore ferrozine [75]. The addition of met-hemoglobin to THYB led to an immediate release of small amounts of iron (~20 nM), but no significant change in iron levels was observed from that point during incubation (insert in Fig. 13A). Hence, it seems possible that a minute fraction of the hemoglobin solution contains free iron. Still, the hemoglobin was generally stable in THYB and did not spontaneously release iron during incubation in THYB at 37ºC. Notably, when THYB supplemented with 20 µM met-hemoglobin and was inoculated the WT D39 cells, the free iron concentration continued to rise above the baseline observed with un-inoculated

medium. The addition of catalase prevented this rise in iron level in the early time points, and miniscule amounts of additional iron were detected after 18 H (Fig. 13A). Moreover, we did not observe iron release from met-hemoglobin into the medium in the Δ*spxB*Δ*lctO* culture, while the complemented strain released iron in higher amounts compared with the WT strain (Fig. 13A). Hence, H_2O_2 produced by the growing pneumococci acted to release iron from met-hemoglobin into the extracellular environment.

To examine if H_2O_2 was sufficient to cause iron release in the pneumococcal culture, we added met-hemoglobin into the supernatant samples collected from overnight cultures of the WT (which contains ~7 mM H_2O_2, Fig. 11A). Spent medium collected from Δ*spxB*Δ*lctO* culture served as a negative control. Interestingly, we did not see an increase in free iron during incubation of met-hemoglobin in either spent media (Fig. 13B). Hence, endogenously produced H_2O_2 is necessary but it is not sufficient to release iron from the externally added met-hemoglobin, and this process also requires the bacterial cells.

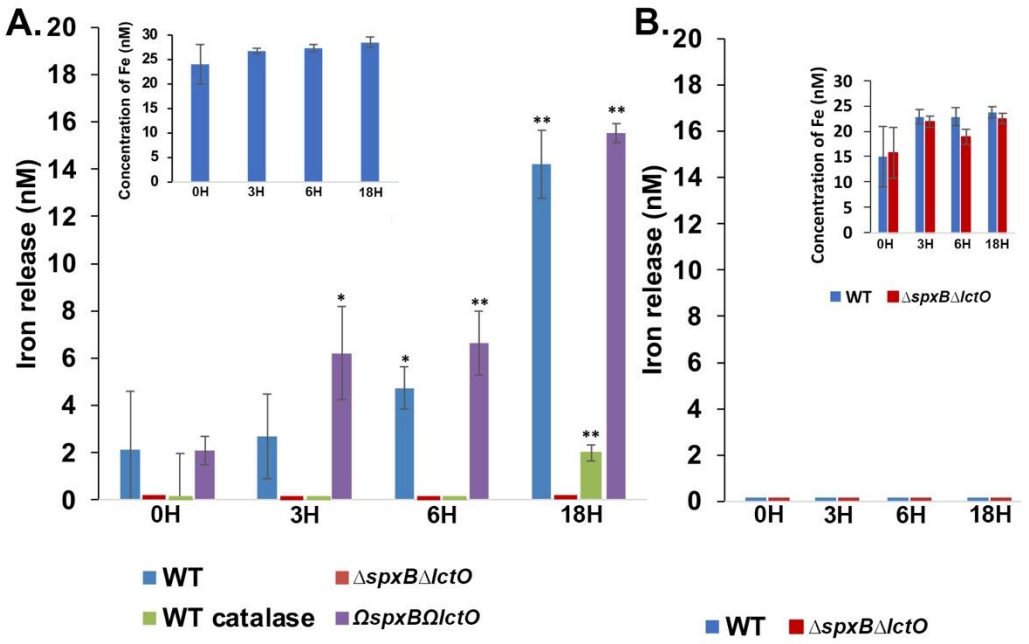


Figure 13: Endogenously produced H_2O_2 releases iron from hemoglobin into the culture supernatant in a cell dependent manner.

A) Un-inoculated THYB supplemented with 20 µM met-hemoglobin or medium inoculated with D39 WT, Δ*spxB*Δ*lctO* mutant, or the complemented strain (Ω*spxB*Ω*lctO*) were incubated at 37 C. The WT cells were also used to inoculate THYB with 20 mM met-Hemoglobin containing catalase (200U/µL) (WT catalase). Free iron levels in media samples collected at different time points were determined after removing the cells by centrifugation and the hemoglobin by filtration. Insert shows iron concentrations in un-inoculated THYB incubated with with 20 µM met-hemoglobin. Net iron released into the culture media was calculated by subtracting the iron concentration found in un inoculated medium at the same time point (see insert). B) 20 µM met-hemoglobin was added to the supernatant samples collected from overnight cultures of the D39 WT or Δ*spxB*Δ*lctO* mutant and allowed to incubate at 37ºC. The iron concentration in the samples was determined after the hemoglobin was removed by filtration (as in A). Insert shows the concentration of free iron in the reactions. Subtracting the iron concentration found in un-

inoculated THYB incubated with 20 µM met-hemoglobin, see insert in A) from those found in spent media incubated with hemoglobin shown (see the the insert) revealed no net iron release. Data was derived from experiments done in duplicates and repeated twice and analyzed by Student T-test, where * indictates a $P \leq 0.05$ and ** ≤ 0.01.

***S. pneumoniae* grown on met-hemoglobin as an iron source imports free iron and not just heme.**

To better understand how H_2O_2 production impact *S. pneumoniae* use of met-hemoglobin as an iron source, I aimed to determine the heme and iron content in cells growing on met-hemoglobin iron. For this we explored two separate growth assays; depleted the free iron from the medium by either using THYB pretreated with Chelex-100 resin ($THYB_{Chx,}$ Fig. 14A) or in THYB containing the iron chelator, nitrilotriacetic acid ($THYB_{NTA}$, Fig 14B). The main difference between these two growth assays is that with $THYB_{Chx,}$ the iron is removed ahead of inoculation from the medium. Hence when met-hemoglobin is added (Fig. 14C and 14D), the bacteria can use both the heme and free iron (if it is freed into the supernatant during growth). In $THYB_{NTA}$, however, the only source of iron that is available for pneumococcus is the met-hemoglobin heme (Fig. 14E and 14F), since NTA is in excess and will chelate any iron released into the medium.

Chelating the iron in THYB either by Chelex or NTA impaired pneumococcal growth (Fig. 13, empty symbols). The addition of 5 and 20 µM met-hemoglobin to the media restored the growth of both the WT and the Δ*spxB*Δ*lctO* strains in a dose-dependent manner (Fig. 14). Therefore, growth arrest in $THYB_{chx}$ or THY_{NTA} resulted from iron depletion. Moreover, Δ*spxB*Δ*lctO* growth in iron-depleted medium supplemented with met-hemoglobin demonstrates

that H_2O_2 production is not essential for pneumococcus to use met-hemoglobin as an iron source and the pathogen has other means to liberate iron from heme.

We next measured the heme content in cells collected at different time points during growth in $THYB_{Chx}$ or $THYB_{NTA}$ supplemented with 20 µM met-hemoglobin (Fig. 15A and 15B). All tested strains accumulated heme with the heme levels peaking at the logarithmic phase of growth (6 H) and then reduced by the 18 H time point. The WT strain imported more heme when grown in $THYB_{NTA}$ compared with $THYB_{Chx,}$ (exhibiting 4-times more heme at the late logarithmic phase of growth, Fig. 15A and 15B). We also tested heme accumulation by a knockout of the *piuBCDA* system, previously implicated in heme import. The Δ*piuBCDA* strain imported significantly less heme than the WT, when grown in $THYB_{NTA}$ (Fig. 15B, 6H), confirming for the first time, that the Piu proteins contributes to heme uptake *in vivo*. Interestingly, cellular heme levels in the WT and Δ*piuBCDA* were comparable during growth in $THYB_{Chx}$ (Fig15A) and significantly lower than those found in the WT when cultivated in $THYB_{NTA}$ supplemented with met-hemoglobin.

The most noticeable difference in heme content was exhibited by the Δ*spxB*Δ*lctO* mutant, which accumulated 5-fold more than the WT in $THYB_{Chx}$ and 2-fold more in $THYB_{NTA}$ more heme than the other strains (Fig. 15A and 15B). Moreover, the cellular levels of heme in this strain remained high even after 18 H of incubation. The D*spx*D*lctO* mutant also imported 2-fold more heme when grown in $THYB_{NTA}$ compared with $THYB_{Chx,}$

Despite the big difference in heme content between the WT and the Δ*spxB*Δ*lctO* strains when grown on met-hemoglobin iron in either $THYB_{Chx}$ or in $THYB_{NTA}$, ICP-MS analysis (done at the 6 H time point) did not reveal meaningful difference in cellular iron content between these two strains (Fig. 15C). I observed an upward trend in total iron in the NTA chelated sample

compared to chelex-100 depleted samples, but the difference was not statistically significant. Therefore, the WT strain imports mostly free iron when grown on met-hemoglobin iron, while in the absence of extracellular heme degradation, the Δ*spxB*Δ*lctO* is importing heme to fulfill its needs for iron. Importantly, the two stains balance iron and heme uptake and keep total cellular iron levels at a steady state regardless of the iron source available to them.

I also evaluated the total heme concentration (i.e., bound to met-hemoglobin and free) in the medium during growth of the WT, Δ*piuBCDA*, or Δ*spxB*Δ*lctO* strains in $THYB_{NTA}$ supplemented with met-hemoglobin (Fig. 16A). In these experiments I removed the cells and then extracted the heme from the medium using acidified chloroform as described. In the cultures of the WT and Δ*piuBCDA* strains, about 30% of the heme found in the medium after 3 H of growth was spent by the 6 H time point and was almost completely diminished after 18 H of incubations. The heme was also removed during growth of the Δ*spxB*Δ*lctO* cells, but at a lower rate. The culture supernatant of this strain contained about ~33% more heme than those of the WT and Δ*piuBCDA* strains even after 18 H (Fig. 16A). To determine the fraction of free heme (unlike heme that is bound to met-hemoglobin), I repeated the assay after removing the met-hemoglobin by filtration (Fig. 16B). I found only minute amounts (~4% of the total heme) of free heme in the medium of all cultures, indicating most of the heme remaining in the supernatant at any time point is still bound to met-hemoglobin.

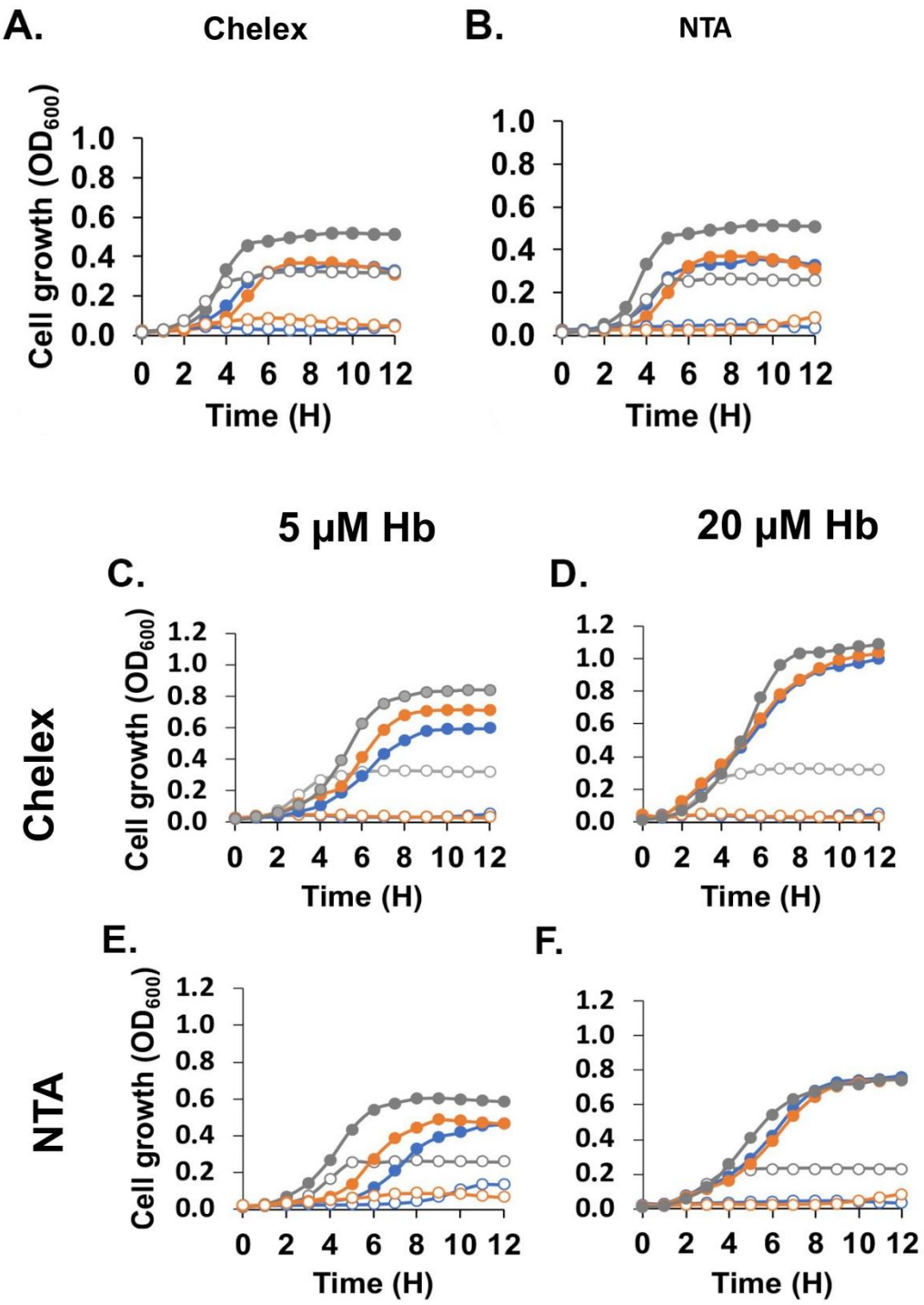


Figure 14: S. pneumoniae can use hemoglobin as an iron source independently of H_2O_2 production.

Cells of D39 WT (blue), Δ*piuBCDA* (orange), and Δ*spxB*Δ*lctO* (gray) isogenic strains collected from blood agar plastes were used to inoculate fresh media in microtiter plates at OD_{600} 0.05. The cultures were incubated at 37ºC and optical density was recorded. **A.** *S. pneumonaie* growth in regular THYB or in THYB treated with Chelex-100, $THYB_{Chx}$. **B.** *S. pneumoniae* growth in THYB containing 3mM NTA, $THYB_{NTA}$. **C** and **D** show *S. pneumoniae* growth in $THYB_{Chx}$ supplemented with 5 or 20 µM met-hemoglobin, respectively. **E** and **F** show *S. pneumoniae* growth in $THYB_{NTA}$ supplemented with 5 or 20 µM met-hemoglobin, respectively. Experiements were done in triplicates and repeated twice. In all panels, empty symbols represent growth in iron-deplete and full symbols in iron-replete medium. All experiments were done in triplicates and repeated twice. Each curve shown is derived from an average of three bioreplicates from representative experiments.

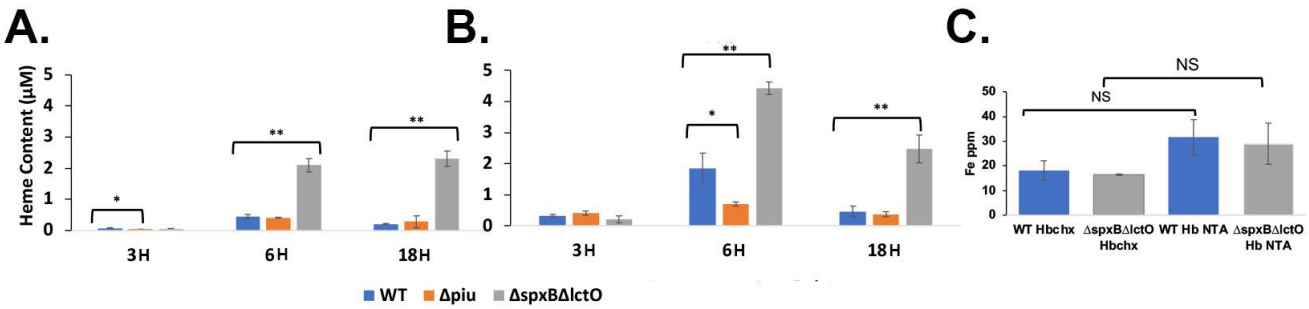


Figure 15: H_2O_2 production and access to free iron influence heme uptake from S. pneumoniae grown on hemoglobin as an iron source.

Cells of D39 WT (blue), Δ*piuBCDA* (orange), and Δ*spxB*Δ*lctO* (gray) isogenic were allowed to grow in $THYB_{Chx}$ or $THYB_{NTA}$ supplimented with 20 µM met-hemoglobin (as described in Fig. 3). Cells harvested at different time points were washed and the heme content in the cell lysate was determined by the acidified chloroform method. Shown is the cellular heme content in pneumococci grown in $THYB_{Chx.}$ (A) or $THYB_{NTA}$ (B). Intracellular iron content measured by ICP-MS in cell samples collected at the 6 H time point is shown in C. The data (normalized to optical density) is derived from experiments done in duplicates and repeated twice. The Student T-test was used to determine statistical significance, where * indicates a $P \leq 0.05$ and ** ≤ 0.01, NS indicates not significant.

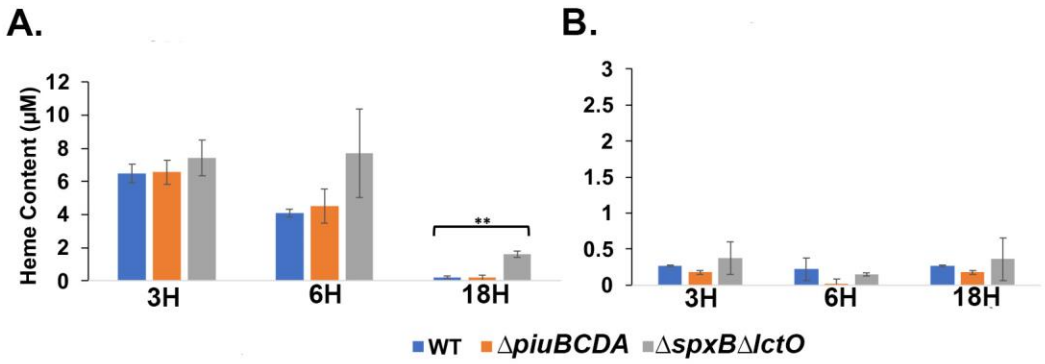


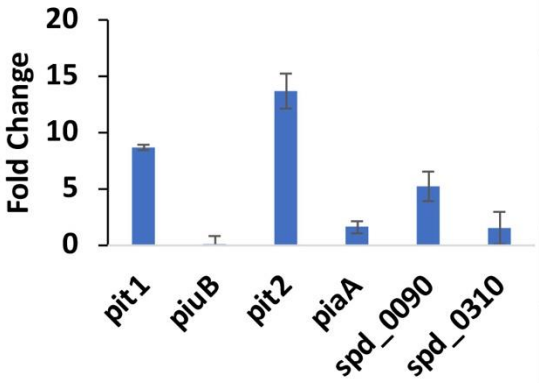
Figure 16: The heme in the hemoglobin medium is spent faster by hydrogen peroxide producing S. pneumoniae.

Cells of D39 WT (blue), Δ*piuBCDA* (orange), and Δ*spxB*Δ*lctO* (gray) isogenic were allowed to grow in $THYB_{NTA}$ supplemented with 20 μM met-hemoglobin (as described in Fig. 3). Culture samples (2mL) were collected along the growth, the cells were removed by centrifugation, and total heme content was determined by the acidified chloroform method[76] (A) or the hemoglobin was removed by filtration prior to determination of free heme concentration (B). The data was derived from experiments done duplicates and repeated twice. The Student T-test was used to determine statistical significance, where * indicates a $P \leq 0.05$.

Inactivation of H_2O_2 production in pneumococcus drives overexpression of iron and iron-complex importers.

The growth of the Δ*spxB*Δ*lctO* mutant was significantly impaired in $THYB_{Chx}$ and in $THYB_{NTA}$ compared with regular THYB (Fig. 13A and 13B, gray symbols,). Still, the Δ*spxB*Δ*lctO* mutant exhibited limited growth in these iron-deplete media (gray empty symbols), where the growth of the WT strain was completely diminished (blue empty symbols), indicating the mutant is more resistant to iron depletion. To compare iron metabolism in these two strains we compared the expression of iron related genes during growth in regular THYB. RNA was prepared from cell samples collected at the mid logarithmic phase, and genes expression was analyzed by RT-PCR. I observed strong activation of several iron and heme importers in the Δ*spxB*Δ*lctO strain* (Fig. 17). This includes the activation of *pitA2* (14-fold), *pitB* (8.7-fold),

spd_0090 (5.2-fold), and *pia*A (1.6-fold). The expression of *spd_0310*, encoding a putative heme shuttling protein was also induced 1.6-fold. [25]. Curiously, the *piuB* gene was strongly downregulated (0.067-fold) in the Δ*spxB*Δ*lctO* mutant. Hence, inactivation of the *spxB and lctO* has significant impact on iron hemostasis in *S. pneumoniae.*



Genes	Fold Change
pitB (spd_0225)	8.7±0.24
piuB	0.067±0.76
pitA2 (spd_1609)	14.0±1.5
piaA	1.6±0.49
spd_0090	5.2±1.3
spd_0310	1.6±1.4

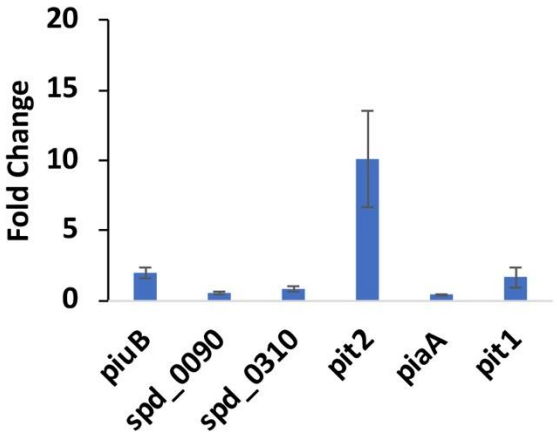
Figure 17: Knockout of H_2O_2 production activates the expression of multiple iron-uptake systems.

Fold change in the expression in of iron metabolism genes in Δ*spxB*Δ*lctO* mutant compared to the isogenic WT strain as determined by qRT-PCR. RNA was prepared from cells grown in THYB up to the mid log phase of growth**.** Data derived from two bioreplicates and processed in duplicates is shown in a bar graph and table format.

Pneumococci growing on met-hemoglobin in $THYB_{NTA}$ experience stronger iron starvation signals compared with those growing in $THYB_{Chx}$.

I noticed that all three strains tested for growth on met-hemoglobin iron grew faster and reached higher biomass in $THYB_{Chx}$ repleted with met-hemoglobin compared to repleted $THYB_{NTA}$ (Fig. 14C and 15D compared with 14E and 14F). To gain more insights into

pneumococcal physiology during cultivation using met-hemoglobin as a single source of iron we compared the expression of the pneumococcal genes implicated in iron or heme uptake between cells grown in $THYB_{Chx}$ or $THYB_{NTA}$ supplemented with limiting amounts (5 mM) met-hemoglobin. RNA was prepared from samples collected at the mid logarithmic phase of growth, and the expression of selected iron homeostasis genes was analyzed by RT-PCR (Fig. 18). Although both media were supplemented with equal amounts of met-hemoglobin, we observed differential expression of several transporters. The most pronounced change was the 10-fold induction of *pitA2* (*spd_1609*) during growth in $THYB_{NTA}$ compared with $THYB_{Chx}$. Smaller but significant induction of the *piuB* (1.9 fold) and *pitB* (spd-0225, 1.7 fold) was also observed. The elevated expression of *pitB* during growth in $THYB_{NTA}$ compared with $THYB_{Chx}$, is consistent with the observation that the Δ*piuBCDA* strain imported less heme than the WT during growth in this medium, while the *piu* loss did not impact heme content during growth in $THYB_{Chx}$. Interestingly, cells grown on hemoglobin iron in the presence of NTA downregulate the expression of *spd_0090* (0.54 fold), which is part of a recently described heme importer, and of *piaA* (0.43 fold), a ferrochrome binding protein [37]. The repression of *spd_0090* and *piaA* genes suggest different regulation modalities for these transporters.



Genes	Fold Change
piuB	1.9±0.37
spd_0090	0.54±.090
spd_0310	0.81±0.20
pitA2 (spd_1609)	10.0±3.5
piaA	0.43±0.015
pitB (spd_0225)	1.7±0.70

Figure 18: S. pneumoniae-grown on met-Hemoglobin iron in an NTA-containing medium experienced iron more stress compared to cells grown in iron deplete medium w/o chelator.

Cells grown in $THYB_{Chx}$ or $THYB_{NTA}$ supplemented with 5 µM hemoglobin were harvested at the mid-log phase (OD_{600} ~0.4), RNA was prepared, and gene expression was determined by qRT-PCR. Data derived from two bioreplicates processed each in duplicates is shown in bar graph and a table format. Values smaller than 1 indicate down regulation.

3.6 Discussion

The human pathogen, *S. pneumoniae* requires iron and can readily obtain the metal from heme and host hemoproteins, with hemoglobin the most growth beneficial source compared with other iron sources [29, 30]. Still, the molecular mechanisms by which pneumococci obtains and import heme are only partially described and the literature contains inconsistent reports regarding the function of transporters implicated in iron acquisition [33, 36, 37, 41-43]. Additionally, it is unknown how this important pathogen degrades heme to liberate the iron. One confounding factor that was largely overlooked in previous studies of iron metabolism in *S. pneumoniae* is that in the presence of oxygen, the bacterium produces and releases to the extracellular environment copious amounts of H_2O_2, which can rapidly interact with free, and hemoglobin-

bound iron. While H_2O_2 degrades both ferrous- and ferric-heme that is free in solution [81, 82], the fate of the heme that is bound to hemoglobin depends on the iron redox state [69, 81-83]. In this study we begun describing the role of H_2O_2 production in iron acquisition by *S. pneumoniae*, uncovering intimate relationships between iron metabolism and H_2O_2 production.

Examining the impact of met-hemoglobin on pneumococcal cultures showed that the millimolar concentration of H_2O_2 produced by the pneumococcal SpxB and LctO enzymes are removed from the medium in the presence of micromolar amount of met-hemoglobin (Fig. 11B). This catalytic removal of H_2O_2 is consistent with the catalase-like activity of met-Hemoglobin, whereby H_2O_2 acts as both an electron donor and acceptor in an oxidation reduction cycle of $HbFe^{+3}$ and $HbFe^{+4}$ [81]. H_2O_2 removal likely reduces the consequential oxidative stress pneumococcus experiences during growth and can help explain the dramatic growth benefits we observed when *S. pneumoniae* is cultivated in the present of met-hemoglobin [30]. Interestingly, *spxB* expression augments the release of Ply by *S. pneumoniae* [73], which in turn promotes erythrocyte lysis and the release hemoglobin to the medium [72]. Hence, it is plausible that *in vivo*, H_2O_2 producing pneumococci (such as those colonizing the respiratory track) also benefit from the removal of H_2O_2 in their vicinity by met-hemoglobin.

Met-hemoglobin is stable in cell free medium, but it falls out of solution during incubation with growing *S. pneumoniae*. I notice a much faster decrease in soluble met-hemoglobin level in the WT cultures compared with the Δ*spx*BΔ*lctO* mutant (Fig. 10C). Oxidation of met-hemoglobin by H_2O_2 produces reactive amino acid radicals, destabilizes the globin chain, and promotes met-hemoglobin conversion to hemichrome (met-hemoglobin complexes in which a histidine residue, distal or external, binds to the iron 6^{th} position). Since hemichrome readily precipitate out of solution forming Heinz bodies *in vivo* [84], it is likely that

hemichrome formation in the presence of H_2O_2 enhances the loss of met-hemoglobin from solution. We observed met-Hemoglobin precipitation also in Δ*spxB*Δ*lctO* cultures, although less than with the WT. Hence additional streptococcal-dependent but H_2O_2 independent mechanisms (possibly the reduction on cell surface [85]), also lead to met-hemoglobin denaturation. The hemoglobin that falls out of solution likely supports the robust biofilm growth we observe when *S. pneumoniae* is grown in the presence of met-hemoglobin [84].

It is well documented that H_2O_2 degrades free heme and heme that is bound to oxy-hemoglobin [68, 69, 82, 86], while its reactions with met-hemoglobin heme do not lead to iron release [86]. Hence, we were surprised to find that incubation of met-hemoglobin with H_2O_2 producing streptococci led to accumulation of free iron in the medium, albeit in low amounts (Fig. 12A). Extracellular iron accumulation was not observed with the Δ*spxB*Δ*lctO* mutant, and externally added catalase blocked this buildup in the WT cultures (Fig. 13A). These observations suggest that iron is released extracellularly from met-hemoglobin heme by a H_2O_2-dependent process. Incubation of met-hemoglobin in spent medium that contain H_2O_2 did not lead to iron release from met-hemoglobin (Fig. 13B), indicating that extracellular degradation of the heme in met-hemoglobin also requires the presence of pneumococcal cells.

Pneumococcus has two surface-exposed thioredoxin proteins, Etrx1 and Etrx2 that help it cope with oxidated stress by maintaining a reductive outward environment [32]. It seems possible that these enzymes reduce the ferric iron in met-hemoglobin molecules, making it suspectable to degradation by H_2O_2. Alternatively, binding of met-hemoglobin to pneumococcal receptors may prompt heme release, which is then degraded by H_2O_2. The contribution of H_2O_2-mediated degradation of free heme that was pumped out by the cell cannot be overruled as well. Still, the low amounts of iron found in the medium (Fig. 13A) compared with the relatively high

concentration of met-hemoglobin (Fig. 16) support a model by which there is a localized iron release from met-hemoglobin, which is in turn taken up by the cells.

To examine how extracellular heme degradation impact pneumococcal iron metabolism we compared growth, and cellular heme and iron content among pneumococci grown on met-hemoglobin iron. All tested strains were able to grow (Fig. 14) and accumulated heme (Fig. 15A, and 15B) in either $THYB_{Chx}$ or $THYB_{NTA}$ supplemented with met-hemoglobin. The *piuBCDA* mutant accrued significantly lower heme amounts compared with the parent WT strain, when grown in $THYB_{NTA}$ (orange and blue symbols, Fig. 15B). This phenotype indicates that active uptake promotes cellular heme accumulation and establishes the Piu proteins as an important iron acquisition mechanism when *S. pneumoniae* is dependent on external heme supply. Despite these differences in heme content, the growth of the Δ*piuBCDA* mutant was like that of the WT parent (Fig. 14), suggesting other transporters compensate for the loss of the Piu proteins.

Cellular heme levels peaked during the mid-logarithmic phase of growth in most cultures and then decreased. It is possible that intracellular heme catabolism and heme export facilitated this decrease in heme levels during long incubation. Since the free iron in the medium is chelated, pneumococci cultivated in $THYB_{NTA}$ with met-hemoglobin must degrade the heme in the intracellular compartment to obtain the metal. Hence, the growth of the Δ*spxB*Δ*lctO* on met-hemoglobin in $THYB_{NTA}$ indicates the presence of penumococal mechanisms for intracellular heme-degradation that are independent of H_2O_2. Interestingly, the only sample in which heme level did not decline after prolong incubation, was that of Δ*spxB*Δ*lctO* cells grown on met-hemoglobin in $THYB_{Chx}$. This observation suggests that H_2O_2 may also contributes to intracellular heme degradation. It is also possible that H_2O_2-independent heme degradation come

into play only in the absence of H_2O_2 production and without free iron source (such as in $THYB_{NTA}$). The pneumococcal mediators of catalytic heme degradation are unknown at this time, as heme degrading enzymes were not described, and the pathogen does not encode homologs of recognized heme-degrading proteins. Nevertheless, catalytic heme degradation is likely critical during infection in sites with low oxygen tention (where pneumoocci do not produce H_2O_2).

I found less heme in the WT compared with the Δ*spxB*Δ*lctO* mutant, when the bacteria were grown on met-hemoglobin iron (blue and gray symbols, Fig. 15A and 15B). These differences in heme content were 5 versus 2.5-fold larger when the cells were cultivated in $THYB_{Chx}$ than in $THYB_{NTA}$ in which pneumococci growth was dependent on heme import. Despite the difference in cellular heme, both strains contained equal amount of iron (Fig. 15C). These findings imply that the WT strain incorporates more free iron than heme. It is also possible that intracellular heme degradation or export is enhanced in the H_2O_2-producing cells. Still, the quarter reduction in heme content within the WT strain when grown in $THYB_{Chx}$ compared with $THYB_{NTA}$ (blue symbols, Fig. 15A and 15B) supports the notion that the WT strain take up more iron than heme during growth on met-hemoglobin iron.

I found that inactivation of the genes for the H_2O_2 producing enzymes, *spxB* and *lctO* resulted in wide activation of transporters for iron (*pit 1* and *pit 2*, 8.7-9-fold), heme (*spd_0090*, 5.2-fold) and an unknown iron complex (*pia*, 1.6-fold, Fig. 16). I hypothesize that the likely increase in iron import contributes to the resistance to iron stress exhibited by the Δ*spxB*Δ*lctO* mutant (Fig. 15A and 15B). The strong repression of the *piu* expression (0.067) is intriguing and may reflect the complexity of *piu* regulation. The transcription of the *piu* genes is regulated by multiple proteins including CodY and the orphan response regulator, RitR [35, 87] and

environmental conditions such as oxygen tension, oxidation stress, and nutrition status. Nevertheless, there was significant activation of other iron related transporters in the Δ*spxB*Δ*lctO* strain.

All pneumococcal strains grew to higher cell density on met-hemoglobin iron in $THYB_{Chx}$ compared with $THYB_{NTA}$ (Fig. 14C to 14F). In addition, WT pneumococci grown on 5 µM met-hemoglobin as an iron source exhibited 10-fold activation of the metal iron transporter, *pit2* and a small increase in the expression of *pit1* and *piu* genes (1.7- and 1.9- fold respectively) when grown in in $THYB_{NTA}$ compared with $THYB_{Chx}$ (Fig. 18). This observation implies that the transcription of the *pit1*, *pit 2* and *piu* genes is responsive to iron (who's availability in the medium is limited in $THYB_{NTA}$) and not heme. The molecular mechanism for such iron regulation remain remains elusive, however, since *S. pneumoniae* doesn't code for obvious homologs of the iron-dependent repressors from the Fur families. A potential DtxR family member named SmrB has been identified. SmrB resembles the regulator of Mn uptake in *Treponema pallidum*, TroR, but its role in iron metabolism is unknown [35]. Both media were supplemented with limiting but equal amounts of met-hemoglobin. Hence, it seems that pneumococci that are not allowed to benefit from external heme degradation by H_2O_2 perceive a more severe iron stress compared with those that grow without an external chelator. Together, these observations provide additional support to the idea that that extracellular heme degradation by H_2O_2 provides pneumococci with nutritional iron from met-hemoglobin.

S. pneumoniae is a versatile pathogen that can spread from the upper respiratory track and survive in multiple niches in the host. In this study, we establish H_2O_2 is a key mediator in pneumococcal use of the host heme and hemoproteins as iron sources. I expect that *in vivo*, the H_2O_2-dependent pathway is important particularly during nasopharyngeal and lungs

colonization. Invasive progressions, such as with bacteremia likely requires pneumococci to rely on additional mechanisms for iron acquisition that are independent of H_2O_2. My findings suggest that H_2O_2 interactions with hemoglobin promotes pneumococcal pathophysiology in patients with sickle cell anemia. The hemoglobin is such patients (HbS) is more sensitive to H_2O_2 and ferrous HbS (HbS (Fe2+)) auto-oxidizes to the ferric (Fe3+) (met-Hb) form at a nearly double rate as normal hemoglobin (HbA) [88].

3.7 Future Directions

Pathogenic microbes who rely on the import of heme as an iron source must transcribe additional mechanisms to release the metal iron from the protoporphyrin ring of heme [9]. Oxidative degradation by an endogenous heme oxygenase is the most common approach used by bacteria to breakdown heme. Canonical heme oxygenase pathways are used in aerobic environments, however anaerobic pathways for heme degradation have also been reported. Endogenous H_2O_2 may non-enzymatically degrade heme to verdoheme so it is reasonable to speculate that endogenous H_2O_2 production by *S. pneumoniae* can assist with intracellular degradation of heme *in vivo* [89]. Interestingly, the strains with knockouts of H_2O_2 producing genes *spxB* and *lctO* are more reliant on heme import to meet iron demands and can still degrade heme to release iron indicating that *S. pneumoniae* encode additional mechanisms to degrade heme that are independent on H_2O_2 production. The first prokaryotic heme oxygenase was discovered by screening a library of mutants in *Corynebacterium diphtheriae* that were unable to grow in medium containing heme and hemoglobin as an iron source [90]. Investigations into a genetic screen in the background of Δ*spxB*Δ*lctO* for mutants that are unable to grow on heme sources can be adapted for *S. pneumoniae.*

4 CHAPTER 3: THE HIGHLY ABUNDANT LIPOPROTEIN, SPD_0739, INDIRECTLY IMPACTS IRON METABOLISM IN PNEUMOCOCCUS

4.1 Abstract

Streptococcus pneumoniae (S. pneumoniae) is a common commensal of the nasal-pharynx and the leading cause of invasive diseases such as bacterial pneumonia especially in young children and the elderly. The protein Spd_0739 is the substrate-binding component of a conserved pneumococcal transporter characterized as a nucleoside importer. In addition to nucleoside binding, Spd_0739 was also reported to interact with heme and hemoglobin and was linked to heme acquisition. Using binding assays to a purified Spd_739 and whole cells we confirmed Spd_0739 interactions with heme and show that it contributes to hemoglobin binding to pneumococcal cell surface. Nevertheless, analysis of single and double mutants in *spd_0739* and the heme transporter *piuBCDA* indicated that Spd_0739 inhibits iron and heme uptake in *S. pneumoniae* and that its absence renders the expression of PiuBCDA growth inhibitory. Externally added nucleoside enhanced pneumococcal growth under iron limitation and the pathogen's use of hemoglobin iron. In summary, this study reveals a new connection between iron and nucleotide metabolism in *S. pneumoniae* and implicates Spd_0739 as a mediator of this association.

4.2 Introduction

Streptococcus pneumoniae is a leading human pathogen, which can colonize the nasal pharynx early in childhood and continue to persist asymptomatically. Colonizing pneumococci can shift into a virulent state and cause a disease spectrum that ranges from mild to life-threatening conditions, including otitis media infections, bacterial pneumonia, bacteremia, and

meningitis [91]. Pneumococcal burden is the highest in adults over 50 and children younger than 5 years of age, with more than 800,000 deaths of children reported annually worldwide. The World Health Organization predicts that with the rise in aging population, hospitalization for pneumococcal diseases will increase by 96% and will cost about $5 billion per year by 2040 [20, 24, 25, 63, 92-94]. Current immunization programs are based on conjugated vaccines that cover prevalent pneumococcal capsule types. These vaccines protect from only a fraction of the pneumococcal serotypes. Hence, immunization led to an increase in human carriage of non-vaccine serotypes and infections by vaccine-escape isolates have been reported [22-24, 63, 95-97].

Lipoproteins are peripherally anchored membrane proteins involved in a range of vital process including cell wall metabolism, cell division, and signal transduction [98]. These proteins decorate the surface of Gram-positive organisms, where their functions may include nutrient acquisition as the substrate binding components of ABC (ATP) transporters, cell adhesions, and interactions with the host immune system. Because of their surface exposure and importance to bacterial growth and host interactions, lipoproteins are often targeted for vaccines and therapeutics against Gram-positive pathogen [58, 85, 91, 97-100].

Iron is an essential nutrient for most bacteria including *S. pneumoniae* [8-13, 17, 26, 27, 43, 101, 102]. In mammals, free iron is found in extremely low (and far below the concentration required for bacterial growth) due to abundance of iron sequestering mechanisms. Non-heme iron (20-30% of the total iron in the body) serves as a cofactor for iron enzymes (often in Fe-S clusters) and the spare metal is sequestered by the intracellular storage protein, ferritin, or found in hemosiderin complexes. About 75% of the iron in the human body is heme bound to hemoglobin and hemoproteins such as myoglobin and heme-containing cytochromes [2]. Hence,

S. pneumoniae ability to use hemoglobin as an iron source, allow it to take advantage of the largest iron reservoir in the host [30]. Furthermore, we recently discovered that this pathogen uses hemoglobin not only as a key source of iron but also as a host molecule that impacts planktonic growth, biofilm development, and adaptation to the mucosal surface environment [29, 30]. Despite their great influence on pneumococcal physiology and pathogenesis, the molecular mechanisms that facilitate the use of hemoglobin as an iron source or a signaling molecule are only partially recognized or described.

Bacterial pathogens rely on elegant protein machineries to compete with the host for its heme and to transport it into the bacterial cell. To shuttle heme across the cell envelope Gram-positive bacteria often use heme relay proteins that include surfaces exposed proteins that remove heme from the host hemoproteins and deliver it across the peptidoglycan layers to the substate-binding proteins of membrane-embedded ABC transporters [14, 16, 103]. To date, such heme relay mechanism has not been described in pneumococcus, but a few ABC transporters have been linked to the import of heme across the cytoplasmic membrane [34, 36, 37, 42, 43, 104]

The gene tagged as *spd_0739* in D39 serotype 2 and its orthologue *sp_0845* in TIGR4 serotype 3, encodes a lipoprotein that has been connected to the uptake of heme (under the name Spbhp-37 [104]) and nucleosides (as PnrA [39, 58]). Spbhp-37 was isolated from pneumococcal extract over heme agarose column [28]. Evidence supporting a role in heme metabolism included the observations that *in vitro* Spbhp-37 binds hemoglobin in high affinity and the findings that the addition of Spbhp-37-antiserum to the culture medium interferes with pneumococcal growth on hemoglobin as an iron source [104]. Previous bioinformatic analysis, however, identified *sp_0845* as the substrate binding component of a nucleoside transporter and named it PnrA [39,

95]. The *pnrBCD* genes, encoding the rest of the transporter components, are found downstream to *pnrA*. The PnrA protein is highly conserved among pneumococcal serotypes, a plentiful component of the pneumococcal cell envelope, and is immunogenic [95]. The purified protein was crystalized with adenosine, guanosine, cytidine, thymidine and uridine, but additional analysis revealed preference for purine ribonucleosides [39]. Knockout of *pnrA* in either TIGR4 and D39 results in reduced uptake of guanosine and resistance to the toxic nucleoside analog, 5FU [95]. Finally, the genes preceding *pnrA* (*spd 0739* in D39), *spd_0736*, *0737*, and *0738*, in D39) code for a putative pyrimidine phosphorylase (*pdp)*, a deoxyribose aldolase (*deoC*) and a cytidine deaminase (*cdd-1*). Hence, in addition to the reports described above, the chromosomal location of *spd_0739* also associates this binding protein to nucleotide metabolism.

We recently demonstrated that a knockout of *spd_0739* (aka *spbhp-37* in [40]) exhibits impaired growth when cultivated in THYB or iron-depleted THYB supplemented with hemoglobin [30]. Moreover, unlike the wildtype parent, the Δ*spd_0739* mutant cannot grow in iron-deplete THYB supplemented with free heme [30]. Hence, our previous studies also implicate *spd_0739* to pneumococcal use of hemoglobin as an iron source. A review of the literature concerning *pnrA* or *spbhp-37* and the identities of corresponding genes in the published genome of *S. pneumoniae* allowed me to determine that *spbhp-37* and *pnrA* represent the same pneumococcal gene (which belong to the core genome of *S. pneumoniae*). Therefore, in this study we refer to it as *spd_0739* and address the conundrum of *spd_0739* function in pneumococcal biology.

4.3 Materials and Methods

Bacterial growth and media

Frozen stocks of *S. pneumoniae* were preserved in Skim milk-Tryptone-Glucose-Glycerin (STGG) as described [74] and stored at -80°C. *S. pneumoniae* cells from STGG stock, were plated on Trypic Soy blood agar plates (BAPs) and incubated at 37°C. Cells collected from BAPs following overnight incubation were used to inoculate fresh medium in a starting OD_{600} of 0.05. Pneumococci were grown in Todd-Hewitt both containing 0.5% (w/vol) yeast extract (THYB), iron-depleted THYB ($THYB_{NTA}$) or iron-depleted THYB supplemented with hemoglobin. $THYB_{NTA}$ was prepared by adding 3mM Nitrilotriacetic acid (NTA), 0.55mM $ZnSO_4$ and 0.55 mM $MnCl_2$ to fresh THYB followed by filter sterilization (0.45-micron filters). Hemoglobin stock solutions were prepared fresh by resuspending lyophilized power of human hemoglobin (Sigma Aldrich) in 1 x PBS (1 mM). Hemoglobin was then added at the indicated concentration and the medium was then filter sterilized (0.45-micron filters). Supplementations with nucleosides received a 0.5 mM cocktail of adenosine, guanosine, cytidine, uridine and thymidine (Sigma-Aldrich). Pneumococci were grown in 96-well flat bottom tissue culture plates at 37°C in a Multiscan Skyhigh spectrophotometer (Thermo Scientific). Optical density was measured hourly after brief shaking.

Determination of Heme Content

Fresh $THYB_{NTA}$ was supplemented with 20 µM hemoglobin, inoculated with *S. pneumoniae* OD_{600} 0.05 in 12 well flat bottom microtiter plates and incubated at 37°C. 6 mL culture samples (at OD_{600} 1.0) were collected at mid-log as determined by growth kinetics (Figure 2A). The cells were harvested by centrifugation, washed three times within PBS, resuspended in 2 ml DMSO

and sonicated (20% amplitude for 30s). Heme was extracted by acidified chloroform and its concentration determined using a standard curve made with hemin solutions in DMSO as described [76]. Briefly, 2 mL of 50 mM glycine buffer, pH 2.0, 0.1 mL of 4 N HCl (pH 2.0), 0.2 mL of 5 M NaCl (pH 2.0) and 2 mL of chloroform: isopropanol was added to the cell lysates. The reactions were mixed vigorously and were allowed to incubate at room temperature for 1 min. Absorbance of the organic phase at 388, 450 and 330 nm were recorded and fed into the correction equation $Ac = 2 \times A388 - (A450 + A330)$. Hemoglobin (if was present in the culture medium) was removed by filtration prior to heme extractions.

Whole Cell ELISA

Overnight grown cultures grown with 200 U/µL catalase (Sigma Aldrich) (5 mL) of *S. pneumoniae* were harvested, washed with PBST (5 mL) 3 times, and used to coat microplates (OD_{600} 1.0 50 µL) overnight at 4°C at OD600 1.0. After blocking, cells were allowed to interact with concentrations of hemoglobin in PBS (0-7.5 µM) for 1 hour at 37ºC. 1º Anti-Human hemoglobin antibody (1:8000) (Sigma) the 2º AP-Conjugated Anti-Rabbit antibodies (1:10000) (Sigma) allowed to interact with the coated wells for 1 hour at RT and binding was detected with PNPP substrate solution (ThermoFisher).

Quantification of Hydrogen Peroxide Concentration in Pneumococcal Growth Media

Pneumococcal cells collected from BAP following overnight growth at 37°C were used to inoculate fresh of THYB or THYB supplemented with 20µM met-hemoglobin at OD_{600} 0.05 (6 mL in 15mL falcon tubes). Supernatant was prepared from culture samples by centrifugation and filtration (0.45-micron) and the hemoglobin was removed by filtration with Ultra-15 centricon

filters (molecular wight cutoff of 30,000, Amicon). The sample H_2O_2 content was measured using the Quantitative Peroxide Assay Kit (ThermoFisher) per manufacturer's instructions. A serial dilution of 30% H_2O_2 in THYB was used to generate a standard curve, from which we a derived the H_2O_2 concentration in media samples.

Purification of SPD_0739

The pEW111 (expressing His6-Spd_0739) plasmid was transformed into Invitrogen Chemically Competent BL21(DE3) cells before each expression. All others were grown overnight from a glycerol stock and then diluted in fresh LB media. Cultures were grown at 37°C with 225 rpm until they reached an OD_{600} of 0.8, induced with 0.5 mM isopropyl β-D-1-tiogalactopyranosie (IPTG), and incubated overnight at 20°C with 180 rpm. The following day, cells were harvested by centrifugation. His6-SPD_0739 expressing cells were resuspended in 20 mM Tris (pH 8.0), 100 mM NaCl, and 0.1% Triton X-100. One CompleteEDTA-free Protease inhibitor tablet (Roche #1183670001) per 500 mL of grown culture was added before sonification. The cellular debris was pelleted by centrifugation at 20,000 × g for 30 min at 4°C, and the lysate was filtered using a 0.45 µM filter unit. Protein was purified on an AKTA FPLC using Cytiva HisTrap HP columns. Imidazole was removed via buffer exchange in Buffer A without imidazole (50 mM potassium phosphate, 250 mM NaCl) using Ultra-15 centricon filters (molecular wight cutoff of 30,000, Amicon).

Protein purification and size were confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Figure 19). Protein concentration was determined by the ThermoScientific Lowry Protein Assay Kit (23240). The buffer used for reconstitutions and

degradations consisted of 20 mM sodium phosphate and 500 mM NaCl (pH 7.4). Arginine (30 mM) and 10% glycerol were added to the buffer for protein storage.

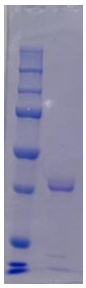


Figure 19 SDS-PAGE of purified His6-SPD_0739

Heme Titration

A stock solution of hemin chloride in DMSO was prepared. The absorbance of a 1:1000 dilution of the stock solution at 404 nm was recorded and the concentration of hemin chloride in the stock solution was calculated using Beer's law (A= εbc where hemin in DMSO ε_{404}=188,000 $m^{-1}cm^{-1}$. Protein samples were diluted in His-tag running buffer A to 10 µM. Absorbance from 250-700 nm was recorded before addition of hemin chloride. Hemin chloride was added to 100 µl aliquots of 10 µM protein to a final hemin chloride concentration of 2 µM, incubated with stirring at RT for 5 minutes and the absorbance from 250-700 nm was scanned and recorded. This was repeated for hemin chloride concentrations of 2 µM, 4 µM, 6 µM and 8 µM, 10 µM, 20 µM, 30 µM, and 40 µM. *His*-tag buffer A alone was similarly incubated with 2 µM, 4 µM, 6 µM and 8 µM, 10 µM, 20 µM, 30 µM, and 40 µM of hemin chloride. These heme-containing buffer solutions were scanned as blanks for the UV-visible spectra of the protein solution containing corresponding concentrations of hemin chloride.

4.4 Strains and Primers

Table 3: Chapter 3 **Strains and p**lasmids

S. pneumoniae	**Description**	**Source or references**
D39	Avery strain, clinical isolate, WT (capsular serotype 2), CSP1	[77, 78]
D39 Δ*spd_0739 (ermB)*	D39 derivative with knockout of *spd_0739*	[30]
D39 Δ*piuBCDA*	D39 derivative with Δ*piuBCDA*::*ermC* mutation (Erm^R)	This study
E. coli		
One shot Top10	Cloning strain	Invitrogen
BL21 (DE3)	Expression strain	Invitrogen
Plasmids		
pAF103	Plasmid for allelic replacement of Δ*piuBCDE::ermC* with pUC19 backbone, Amp^r	This study
pEW109	Plasmid for allelic replacement of Δspd_0739::spec with pUC19 backbone, Amp^r	This study
pEW111	Plasmid for expression of His6x-Spd_0739 without signal peptide with pET151 backbone, Amp^r	This study

Table 4: Chapter 3 Primers

Target	**Primer name**	**Sequence (5'-3')**	**Comments**
Puc19L	ZE 730-FW	GTCTGGAAGGCATGCAAGCTTGGCGTAATCAT	Sense primer for cloning of the pUC19 segment of pAF103
pUC19L	ZE 731-RV	GAACATGAGTACCGAGCTCGAATTCACTGGCC	Antisense primer for cloning of the pUC19

			segment of pAF103
5'piuB & upstream region (D39)	ZE 732 FW	CTCGGTACTCATGTTCTCTTCGACTGCTTCTC	Sense primer for cloning of the left arm of *piu* segment of pAF103
5'piuB & upstream region (D39)	ZE 733 RV	CACACGGTGGTAATAGTCTGCATGAGAAGGC C	Antisense primer for cloning of the left arm *piu* segment of pAF103
ermC (pJRS233)	ZE 734-FW	CTATTACCACCGTGTGCTCTACGACCAAAAC T	Sense primer for cloning of the *ermC* segment of pAF103
ermC (pJSR233)	ZE 735-RV	TTGCTTGCCAAAGCTGCCGACAACACGGGAG C	Antisense primer for cloning of the *ermC* segment of pAF103
3' *piuA* & downstream region (D39)	ZE 736-FW	CAGCTTTGGCAAGCAAGGATGACTACTGGAC T	Sense primer for cloning of the right arm of *piu* segment of pAF103
3' *piuA* & downstream region (D39)	ZE 737-RV	TGCATGCCTTCCAGACCACTTTTCCCTTAAAC	Antisense primer for cloning of the right arm *piu* segment of pAF103
Puc19L	ZE 964-FW	ATCAAAGAGGCATGCAAGCTTGGCGTAATCA T	Sense primer for cloning of the pUC19 segment of pEW109
Puc19R	ZE 965-RV	GTTCAACCGTACCGAGCTCGAATTCACTGGC C	Antisense primer for cloning of the pUC19 segment of pEW109

5’ SPD_0739 upstream region (D39)	ZE 966-FW	CTCGGTACGGTTGAACATGCTAAAAAAGGACT	Sense primer for cloning of the left arm of *spd_0739* segment of pEW109
5’ SPD_0739 upstream region (D39	ZE 967-RV	ATCCTCCTCATTACTGAACCTCCTAAATAAGA	Antisense primer for cloning of the left arm of *spd_0739* segment of pEW109
Spec cassette (pJRS525)	ZE 968-FW	CAGTAATGAGGAGGATATATTTGAATACATAC	Sense primer for cloning of the *spec* segment of pEW109
Spec cassette (pJRS525)	ZE 969-RV	TCCATCCATTATAATTTTTTTAATCTGTTATT	Antisense primer for cloning of the *spec* segment of pEW109
3’ SPD_0739 downstream region (D39)	ZE 970-FW	AATTATAATGGATGGAAGTCATTTCTTAGGAA	Sense primer for cloning of the right arm of *spd_0739* segment of pEW109
3’ SPD_0739 downstream region (D39)	ZE 971-RV	TGCATGCCTCTTTGATAGACAAAACCACTTCT	Antisense primer for cloning of the right arm of *spd_0739* segment of pEW109
5’ SPD_0739 upstream region (D39) without signal peptide	ZE 966-FW	CACCGGTAACCGCTCTTCT	Sense primer for cloning of *spd_0739* in pET151 backbone (pEW111)
3’ SPD_0739 upstream region (D39)	ZE 963-RV	TTATTTTTCAGGAACTTTTACGC	Antisense primer for cloning of *spd_0739* in

			pET151 backbone (pEW111)
gyrB (D39)	ZE 661-FW	GGCACTGTATGGTATCACACAAG	qRT-PCR
gyrB RV	ZE 662	TCTCTAAATTGGGAGCGAATGTC	qRT-PCR
piuB FW	ZE 864	TGATTTCGACCAGCAGACCTG	qRT-PCR
piuB RV	ZE 865	CTGTACTCGGTGCAGCAAACTG	qRT-PCR
Pit 2 (Spd_1609) FW	ZE 149-FW	ATTGCCCGTCCTGTACCACC	qRT-PCR
Pit 2 (Spd_1609) RV	ZE 150-RV	TGCTGCTTGCTCTGGAGGTT	qRT-PCR
piaA FW	ZE 151-FW	AAAAATGGTGCCGTTGCTGT	qRT-PCR
piaA RV	ZE 152-RV	AAAGTGGTGTTGGAGTGCATGA	qRT-PCR
SPD_0225 (pit1) FW	ZE 153-FW	CCAAGCGAAGTTTGGCCTCC	qRT-PCR
SPD_0225 (pit1)	ZE 154-RV	CACCTTGGCATGCGCCTTAG	qRT-PCR
SPD_0310 FW	ZE 155-FW	TGATCAAGGCAGCGGCTGTA	qRT-PCR
SPD_0310 RV	ZE 156-RV	GAAACTGGTGGACCAGCCCT	qRT-PCR
SPD_0090 FW	ZE 171-FW	ACGGTCCAGAAGGCAAGAACT	qRT-PCR
SPD_0090 RV	ZE 172-RV	ACCAGTGTTCCATCCACCCA	qRT-PCR
CodY FW	ZE 167-FW	CCGCCATGGTGACAGCAGTA	qRT-PCR
CodY RV	ZE 168-RV	ATCGGGGATTCGCCTTGGTT	qRT-PCR

4.4.1 Plasmid Construction

Construction of Δspd_0739 with spectinomycin resistance

Δspd_0739 (spec) mutant in *S. pneumoniae* strain D39 by replacing the *spd_0739* coding sequence with that of *spec* (spectinomycin) in a Δ*piuBCDA::ermC* knockout, such that the *spec*

ORF is under the transcriptional control of *spd_0739* promoter and terminator. The mutant allele containing spec ORF flanked by the 5′ and 3′ genomic regions of the *spd_0739* gene was prepared using the Gene art seamless cloning kit (Thermo fisher scientific). Briefly, the appropriate genomic segments were amplified from D39 chromosome using the primer sets ZE 966-F /ZE 967-R and ZE 970-F/ZE 971-R. The spec gene and the pUC19 vector were amplified from pJRS525 and pUC19-L plasmids using the primer sets ZE 968-F/ZE 969-R and ZE 964-F/ZE 965-R respectively. All PCR fragments were purified (using the MinElute PCR Purification Kit, Qiagen) and cloned into One Shot TOP10 *E. coli* strain, generating plasmid pEW109 (Figure 20). The resulting allele was then amplified (from pEW109) and transformed into competent D39 cells using standard protocols [80]. The mutants were selected on BAPs containing spectinomycin (100 µg/ml). The mutation was confirmed by PCR in the resulting erythromycin-resistant clones using the primer set ZE 966-F/ZE 971-R.

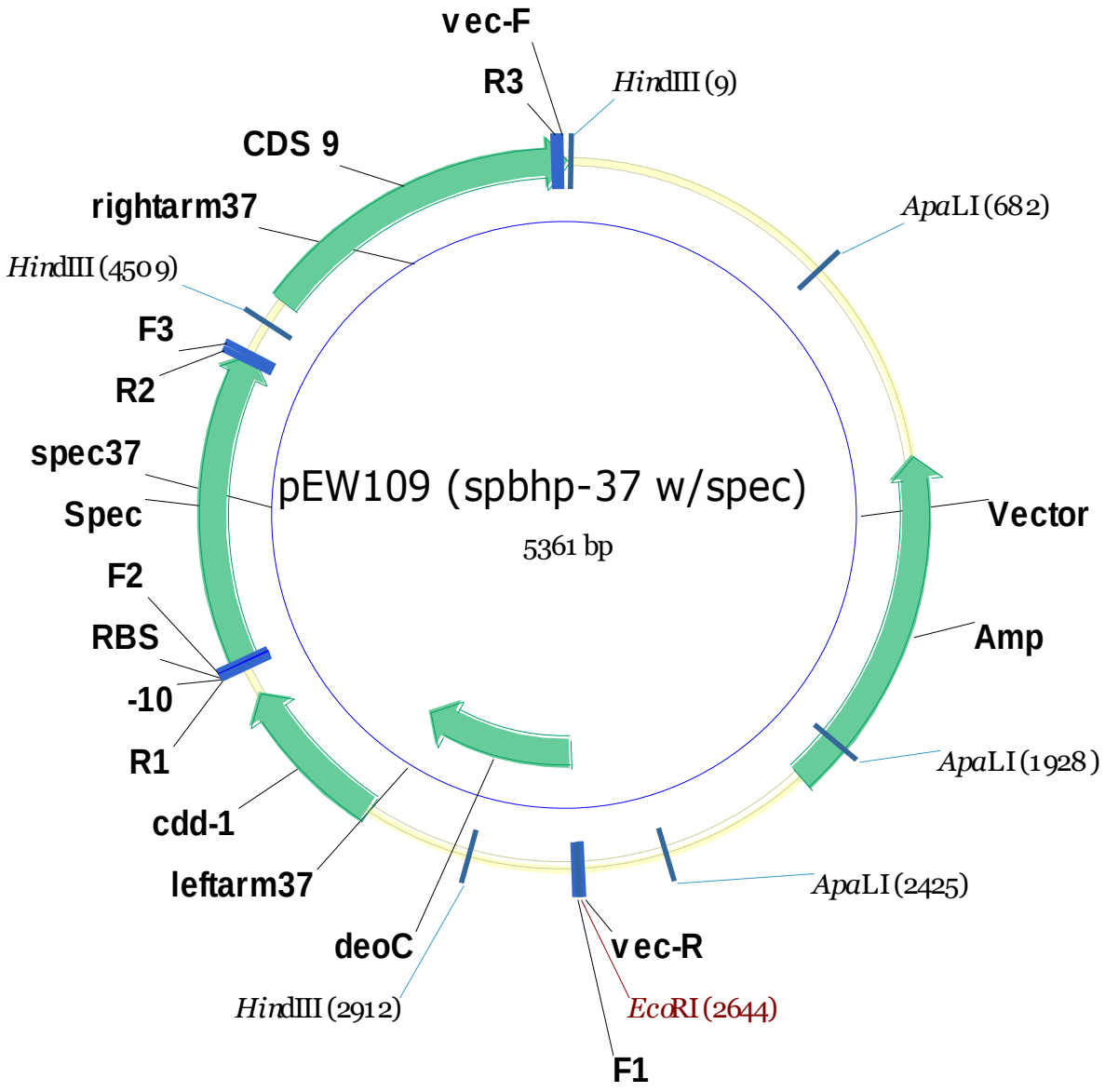


Figure 20: Illustration of pEW109 plasmid

Construction of His6-Spd_0739

Spd_0739 was amplified without a signal peptide from *S. pneumoniae* D39 strain using primer pair ZE966-F/ZE697-R. Resulting amplification was cloned into *E. coli* Top10 cloning stain with pET151 backbone to generate pEW111 (Figure 22). pEW111 was transformed into *E. coli* BL21 (DE3) expression strain.

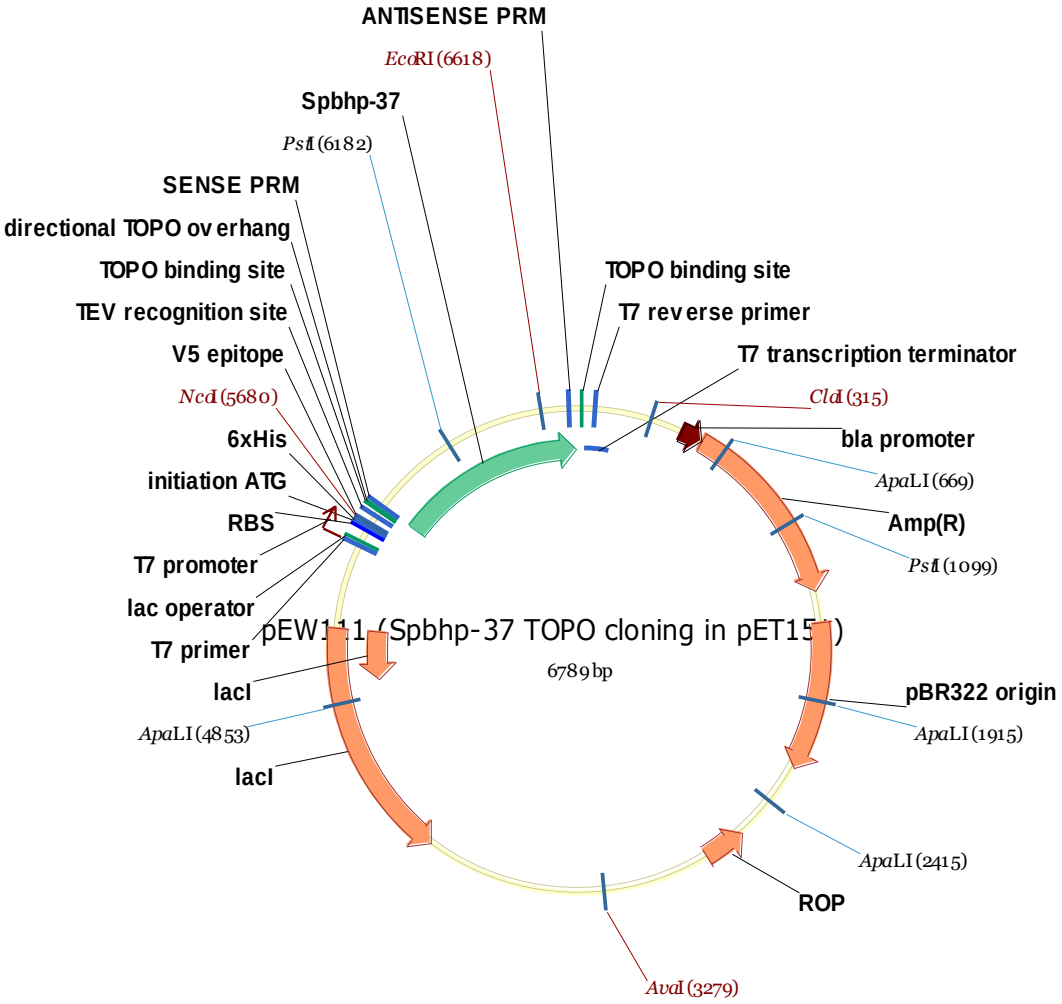


Figure 21: Illustration of pEW111 plasmid

4.5 Results

The pneumococcal Spd_0739 protein does not facilitate heme import and has an inhibitory effect on function of the heme importer PiuBCDA

Spd_0739 binding to heme and hemoglobin [40] and Δ*spd_0739* growth phenotype [30] point to a role in heme uptake. To further examine this possibility, I deleted the *spd_0739* gene in the Δ*piuBCDA* background and compared the phenotype of the Δ*spd_0739*Δ*piuBCDA* double mutant with those of the isogenic wildtype, and the two single mutants. The *piuBCDA* genes encode an ABC transporter suggested to import heme and norepinephrine-iron complex [34-36, 42, 105] and a Δ*piuBCDA* mutant accumulates less heme compared with the isogenic wild-type parent, when cultivated on hemoglobin iron (Chapter 2). We therefore hypothesize that if Spd_0739 and PiuBCDA pathways independently contribute to heme import, then the loss of both systems will aggravate the phenotype of individual mutations in *spd-0739* or *piuBCDA.* I monitored the growth of the isogenic series during cultivation in THYB (Figure 22A) and in the iron-deplete, $THYB_{NTA}$ (THYB containing 3mM NTA), supplemented with 5 or 20 µM hemoglobin (Figure 22B and 22C respectively). NTA chelates free iron and therefore in its presence, heme from hemoglobin is the only form of the metal available for bacterial use. As expected, bacterial growth was completely inhibited in $THYB_{NTA}$ unless hemoglobin was added to the medium. The deletion of *spd_0739* resulted in growth impairment in regular THYB. This phenotype worsened when the mutant was cultured in $THYB_{NTA}$ containing 5 µM hemoglobin, with the mutant exhibiting 12 hour long lag phase followed by a short logarithmic growth, eventually yielding a very low optical density (Figure 22B). In $THYB_{NTA}$ containing 20 µM hemoglobin, the Δ*spd_0739* mutant grew better but still exhibited repressed growth compared with the wildtype strain. Inactivation of *piuBCDA* by itself did not impact bacterial growth in

THYB or $THYB_{NTA}$ with 5 µM hemoglobin (Figure 22A and 22B), but this strain grew a bit slower than the wildtype in $THYB_{NTA}$ with 20 µM hemoglobin (Figure 22C). These observations were consistent with findings our and others' previous findings using alternative iron chelators [36, 41]. Surprisingly, the Δ*spd_0739*Δ*piuBCDA* double knockout grew much better than the single *spd_0739* mutant. The double mutant exhibited only a small growth attenuation when cultivated in $THYB_{NTA}$ with 5 µM hemoglobin and grew nearly as the Δ*piuBCDA* strain in $THYB_{NTA}$ supplemented with 20 µM hemoglobin. Hence, inactivating *piuBCDA* revives the growth phenotype resulted from the loss of *spd-0739.* Still, the double mutant did not grow as well as the wildtype or the *piuBCDA* mutant in iron deficient medium. These observations do not support the notion that *spd_0739* promotes heme import and imply that the loss of *spd_0739* render *piuBCDA* expression toxic for pneumococci.

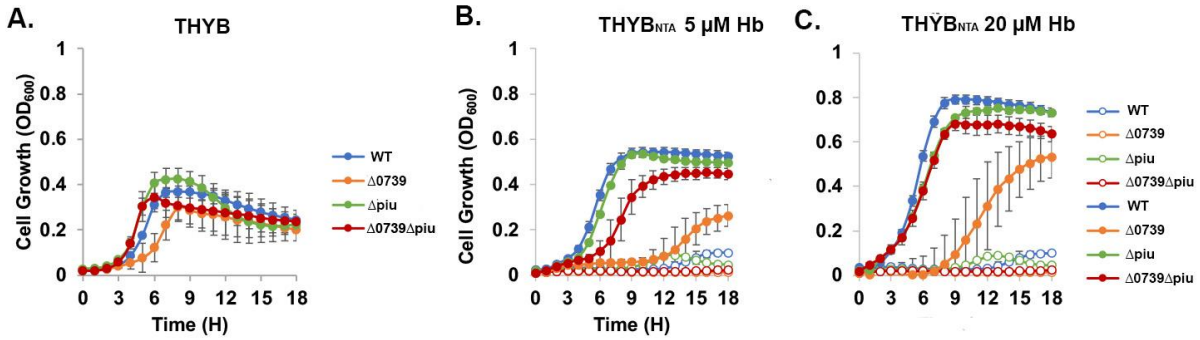
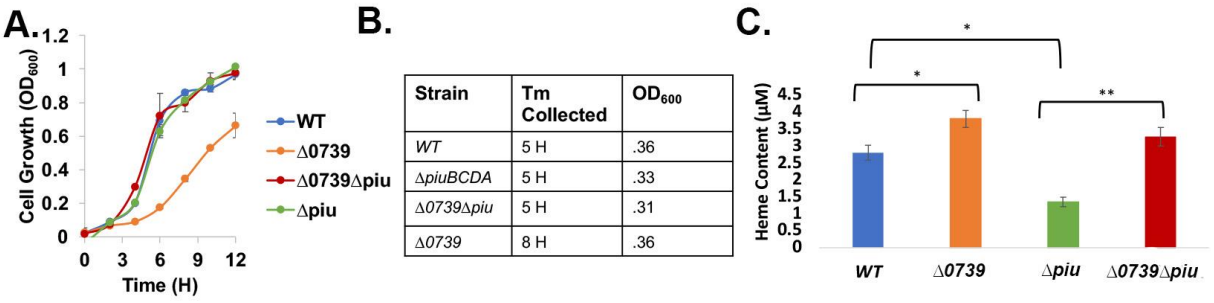


Figure 22: The impaired growth of the Δspd_0739 strain is alleviated by the inactivation of the piuBCDA heme transporter.

Shown is growth of *S. pneumoniae* in regular THYB (A) or in THYB containing 3mM of the iron chelator NTA ($THYB_{NTA}$) supplemented with 5 µM hemoglobin (B) or 20 µM hemoglobin (C). Cells of D39 WT (blue), Δ *spd_0739* (orange), Δ*piuBCDA* (green) and Δ*spd_0739*Δ*piuBCDA* (red) isogenic strains collected from blood agar plates were used to inoculate fresh media in microtiter plates at OD_{600} 0.05. The cultures were incubated at 37ºC and optical density was recorded. Open circles represent cultures grown in $THYB_{NTA}$ and closed circles represent those cultivated in THYB or $THYB_{NTA}$ with hemoglobin. All experiments were

done in triplicates and repeated at least two more times. Each curve shown is derived from an average of three bioreplicates from representative experiments.

Next, we sought to characterize the impact of the Δ*spd_0739* and Δ*spd_0739*Δ*piuBCDA* mutations on heme accumulation. Since we found that heme cellular content peaks in the mid exponential growth and then begin to decline (Chapter 2), I compared the heme accumulation in cells collected at the mid logarithmic phase of growth (~ 0.3 OD_{600}, Figure 23A and 23B). Samples of the isogenic D39 WT, Δ*spd_0739*, Δ*piuBCDA* and Δ*spd_0739*Δ*piuBCDA* strains cultivated in $THYB_{NTA}$ with 20 µM hemoglobin were collected and heme content in the cell lysate was determined as described (Figure 2C). Consistent with our previous findings, I recover about half (48%) of the amount of heme from the Δ*piuBCDA* compared with the wildtype strain. To our surprise, the Δ*spd_0739 mutant* accumulated 26% more heme then the wildtype strain. Moreover, inactivating the *piuBDCA* genes in the background of the Δ*spd_0739* returned heme uptake to the wildtype levels. These data indicate that Spd_0739 inhibits heme uptake by PiuBCDA independent mechanisms.



Strain	Tm Collected	OD_{600}
WT	5 H	.36
ΔpiuBCDA	5 H	.33
Δ0739Δpiu	5 H	.31
Δ0739	8 H	.36

Figure 23: Inactivation of spd_0739 results in higher heme accumulation in the mutant strain compared with the parental WT strain.

A. Cells of D39 WT (blue), Δ*spd_0739* (orange), Δ*piuBCDA* (green), and Δ*spd_0739*Δ*piuBCDA* isogenic strains were allowed to grow in $THYB_{NTA}$ supplemented with 20 µM hemoglobin in 12-

well microtiter plates at 37ºC (**A**). Cells from culture samples at mid-log phase were collected (**B)** washed and the heme content in the cell lysates was determined by the acidified chloroform method (**Ref**). Shown is heme content in samples standerized by culture OD_{600} (**C**). Samples were processed from two biological replicates. A one-way ANOVA with Tukey post-hoc was used to determine statistical significance, where * indicates a $P \leq 0.05$ and ** ≤ 0.01.

The Spd_0739 protein was recovered from pneumococcal lysate by hemin affinity column and was reported to interact with hemoglobin *in vitro* by Blot overlay (Far Western blot) and surface plasmon resonance studies [104]. Given our findings that the PiuBDCA system imposes a growth inhibitory effect in the absence of Spd_0739 (Figure 23), and the negative impact on cellular heme level (Figure 24), I reexamined Spd_0739 binding to heme and evaluated its contribution to hemoglobin binding to pneumococcal surface. I tested heme binding to a recombinant a recombinant Spd_0739 by absorbance spectroscopy. Incubating a purified Spd_0739 with increasing concentration of heme resulted in the formation of the typical absorbance maximum at the Soret region (416 nm), confirming that it binds heme *in vitro*. I next, tested hemoglobin binding to pneumococcal surface by ELISA. Overnight culture of the isogenic D39 WT, Δ*spd_0739*, Δ*piuBCDA* and Δ*spd_0739*Δ*piuBCDA* were used to coat a 96-well microtiter plate. The penumococi were then allowed to interact with increasing concentrations of hemoglobin and binding was detcted using by antibody (Figure 24B). I included catalase in the medium to prevent hemoglobin denaturing and falling out of solution due to interactions with the endogenously producted H_2O_2 (Chapter 2). Deleting either the *spd_0739* or *piuBDCA* genes resulted in 30-35% reduction in pneumococcal binding to hemoglobin. These observations are consistent with previous findings that both PiuA and Spd_0739 bind hemoglobin *in vitro* and suggest that both pathways contribute to the interactions of hemoglobin with the pneumococcal cells *in vivo*. Surprisingly, deleting both *spd_0739* and *piuBCDA* did not have an accumulative effect, and the double mutant bound hemoglobin to the same extend as the single mutants. This

observation implies that hemoglobin binding by PiuA and Spd_0739 was interconnected somehow.

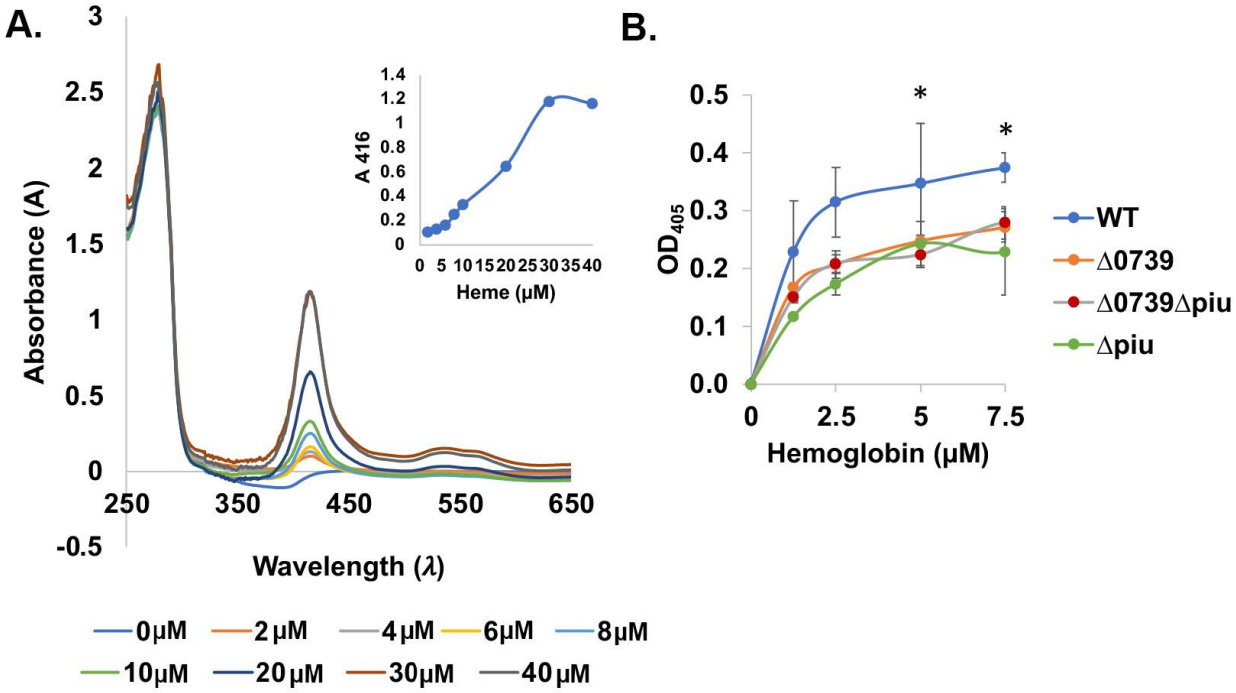


Figure 24: A recombinant Spd-0739 protein binds heme in vitro and contributes to hemoglobin binding to intact pneumococci.

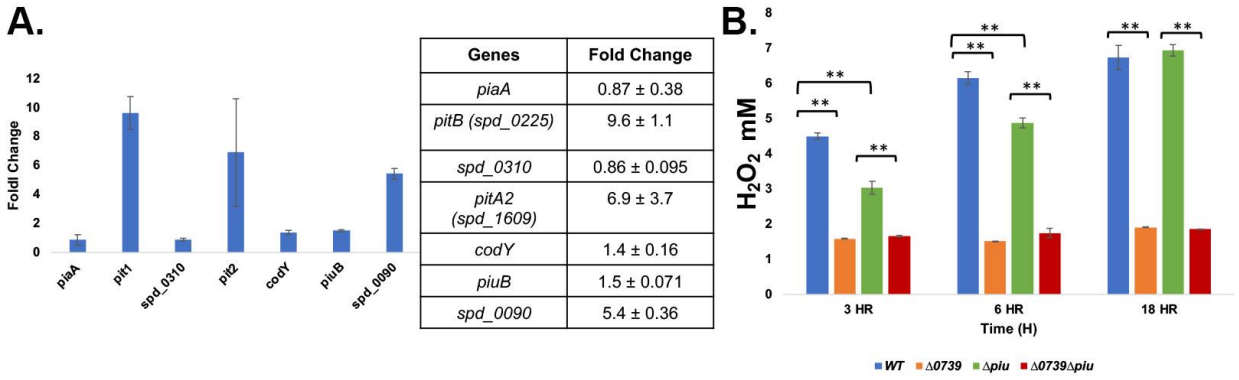
A. Heme binding by Spd_0739 protein. Purified His_6-tagged Spd_0739 (10 μM) was allowed to incubate Buffer A w/o imidazole with increasing increments of heme and the absorbance (250-650 nm) was monitored using a UV/Vis Spectrophotometer after 5 min incubation at RT. Buffer A with equimolar concentrations of heme was used as blank. The growing Soret peak (416 nm) indicates heme binding to Spd-0739. The insert shows heme binding plotted as a function of the heme concentration in the sample. **B.** Hemoglobin binding by whole pneumococcal cells. Overnight cultures (OD_{600} 1.0) were used to coat 96-well plates overnight. Unbound cells were removed, and the coated wells were allowed to interact with increasing concentrations of hemoglobin for one hour at 37ºC. Wells coated with only cells without hemoglobin were used as a black and subtracted from the values. Hemoglobin binding was detected using rabbit hemoglobin antiserum and anti-rabbit antibody conjugated to alkaline phosphates. The data derived from experiments done in duplicated and repeat at least two times. Statistical analysis of each concentration was process via one way ANOVA with Tukey post-hoc was used to determine statistical significance, where * indicates a $P \leq 0.05$.

Inactivation of *spd_0739* is pleotropic and results in the upregulation of iron and heme transporters and reduces production of endogenous hydrogen peroxide

The observation that the Δ*spd_0739* mutant accumulated more heme compared with the wildtype strain suggest Spd_0739 inhibits heme import. Following up on these observations we tested how the removal of *spd_0739* impacts the expression of iron and heme related genes. Exponentially growing pneumococci cultivated in THYB were harvested and the expression of selected genes was analyzed by RT-PCR (Figure 25A). This study revealed 5-fold upregulation of *spd_0090,* the substrate binding protein of a putative aldouronate transport system that was recently implicated in heme import [37]. In addition Δ*spd_0739* exhibited induced expression for the ferric uptake genes *pitB (spd_0225,* ~ 10 fold) [41, 42] and *pitA2 (spd_1609,* ~ 7 fold)[41]. I did not see any significant changes in *piuB* expression or *codY*, a nutritional regulator of *piuB*. Similarly the deletion of *spd_0739* did not impact the ferrochrome binding protein, *piaA* [33, 66] or *spd_0310,* which was recently suggested to function as heme chaperon [106].

Several reports indicate that endogenous hydrogen peroxide production and iron transport are linked *S. pneumoniae* [31, 72]. Hence, I compared hydrogen peroxide production among the isogenic D39 WT, Δ*spd_0739*, Δ*piuBCDA* and Δ*spd_0739*Δ*piuBCDA* strains (Figure 25B). Testing the amount of hydrogen peroxide accumulated in pneumococcal cultures during cultivation in THYB revealed that the removal of *spd_0739* resulted in about 70% decrease in hydrogen peroxide, still about 1.5 mM of hydrogen peroxide were detected in the culture medium of this strain. Removal of the *piuBCDA* genes delayed the production, with Δ*piuBCDA* exhibiting reduced amounts in sample collected during the exponential growth, but eventually

accumulating equal concentration at the wildtype strain. The phenotype of the double mutant was the same as the single *spd_0739* inactivation.



Genes	Fold Change
piaA	0.87 ± 0.38
pitB (spd_0225)	9.6 ± 1.1
spd_0310	0.86 ± 0.095
pitA2 (spd_1609)	6.9 ± 3.7
codY	1.4 ± 0.16
piuB	1.5 ± 0.071
spd_0090	5.4 ± 0.36

Figure 25: Inactivation of the spd_0739 gene results in the upregulation of iron and heme transporters and a reduced production of hydrogen peroxide.

A. Fold change in the expression in of iron related genes in the Δ*spd_0739* mutant compared to the isogenic WT strain as determined by qRT-PCR. RNA was prepared from cells grown in THYB up to the mid log phase of growth**.** Data derived from two bioreplicates and processed in duplicates is shown in a bar graph and table format. **B.** The sample H_2O_2 content was measured using the Quantitative Peroxide Assay Kit (ThermoFisher) per manufacturer's instructions. Shown are hydrogen peroxide concentration in the culture supernatant of D39 WT, Δ*spd_0739*, Δ*piuBCDA* and Δ*spd_0739*Δ*piuBCDA* strains grown in THYB. Samples were processed from two biological replicates done in duplicates. A one-way ANOVA with Tukey post-hoc was used to determine statistical significance, where * indicates a $P \leq 0.05$ and ** ≤ 0.01.

The addition of nucleosides improves pneumococcal use of hemoglobin as an iron source.

Like it's orthologs from other bacteria, the pneumococcal Spd_0739 plays a role in ribonucleosides salvaging [38, 39, 58, 95, 107]. Since we found that Spd_0739 also influences iron and heme uptake in *S. pneumoniae*, we next tested how nucleosides impact the Δ*spd_0739* growth and use of hemoglobin as an iron source (Figure 26). Inspection of the wild type and Δ*spd_0739* growth in THYB revealed that the addition of a nucleoside's mixture to the medium inhibited growth (Figure 26A). When inoculated into THYB supplemented nucleosides, both the wildtype and the mutant strains exhibited a much longer lag phase (9 and 3 hours with and

without nucleosides respectively). The wildtype also reached a lower maximal biomass in the supplemented medium compared to regular THYB. These observations are consistent with the previous reports describing growth inhibition by nucleosides addition in pneumococci grown in RPMI medium [39].

Unlike the negative impact nucleosides had in THYB, testing growth in $THYB_{NTA}$ revealed that the addition of nucleosides is beneficial in this iron deplete medium (Figure 26B). While the growth of both the wildtype and Δ*spd_0739* strains was significantly inhibited in $THYB_{NTA}$, the addition of nucleosides allowed for some residual growth that was absent in $THYB_{NTA}$ alone. Nucleosides addition improved growth also in THYB and $THYB_{NTA}$ supplemented with 20 µM hemoglobin, facilitating higher biomass by both the wildtype and the Δ*spd_0739* strains. Still, the Δ*spd_0739* exhibited a longer leg and grew to a lower final OD_{600} compared with the wildtype (Figure 26C-D).

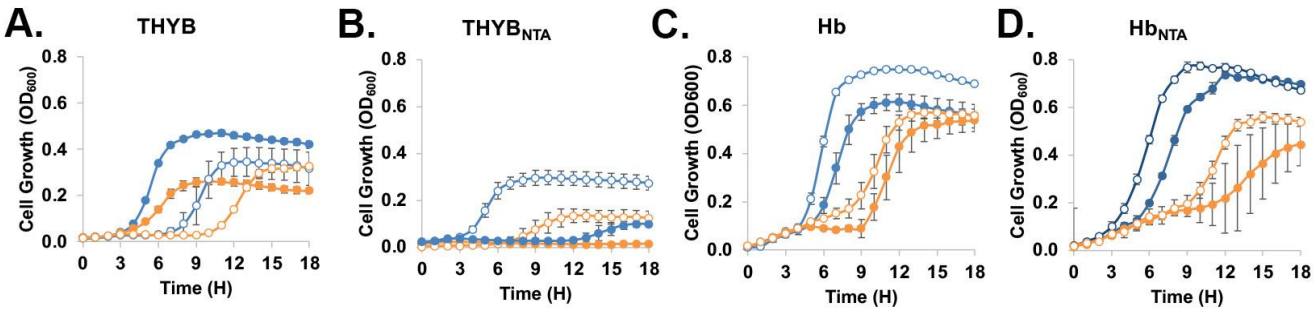


Figure 26: Externally added nucleosides benefit growth in iron depletion and during growth on Heme-iron.

Cells of the wild-type D39 (blue) and isogenic mutant *spd_0739* (orange) were grown in (**A**) THYB (**B**) $THYB_{NTA}$ and (**C**) THYB supplemented with 20 µM hemoglobin with supplementation with a mixture of nucleosides (**D**) $THYB_{NTA}$ supplemented with 20 µM hemoglobin with supplementation with a mixture of nucleosides (0.5 mM adenosine, guanosine, cytidine, uridine and thymidine (Sigma-Aldrich)), empty symbols) as described in Figure 1. Growth is expressed in optical density (OD_{600}) as measured every hour for 18 hours. All

experiments were done in triplicates and repeated twice. Each curve shown is derived from an average of three bioreplicates from representative experiments.

4.6 Discussion

In this study we investigated the role of Spd_0739 in pneumococcal physiology, discovering a new link between iron and nucleotide metabolism in this important human pathogen. *Spd_0739* encodes the binding component of an ABC transporter (*spd_0739- 0742*). This highly expressed lipoprotein is a protective antigen conserved among pneumococcal serotypes [91]. A deletion mutant in *spd_0739* is delayed in developing pneumonia post-intranasal inoculation in a murine model and exhibits reduced fitness in competition experiments [39]. While characterized as a promising vaccine target that serves an important function during infection, the exact role of Spd_0739 in pneumococcal physiology is not clear. First identified as a carbohydrate transporter[108], the Spd_0739-0742 homologs in the TIGR4 strain (*sp*_0845 to *sp_0848*) were later recognized as a nucleoside importer, named PnrABCD. Independent search for heme binding proteins in pneumococcal lysate, however, recovered Sp_0845 (g.i. 15900732, aka Spbhp37 in [40]) and additional investigations demonstrated hemoglobin binding and linked this protein to the use of hemoglobin as an iron source [30].

The phenotype displayed by the Δ*spd_0739* during growth on hemoglobin iron, led us to believe that like PiuA, Spd_0739 is a heme-binding protein that promotes heme import. The loss of two heme importers is expected to aggravate the phenotype resulted from the loss of an individual transporters. Hence, the finding that inactivation of the heme importer PiuBCDA, rescued much of the growth phenotype of the Δ*spd_0739* in $THYB_{NTA}$ supplemented with hemoglobin (Figure 22) argues strongly against our original assumption. I therefore tested the

alternative hypothesis that Spd_0739 acts to limit heme import and in its absences the activity of PiuBCDA becomes toxic.

Spd_0739 inhibits iron and heme uptake in S. pneumoniae.

Monitoring cellular heme levels, we found that the Δ*spd_0739* accumulated more heme than the wildtype, supporting the new hypothesis (Figure 23). Interestingly, inactivation of *piuBCDA* together with *spd_0739* genes restored heme to the wildtype level, and above that observed with the ΔpiuBCDA strain. This suggest Spd_0739 deters heme import also by mechanisms that are independent of the PiuBCDA proteins. Gene expression studies revealed that the loss of Spd_0739 resulted in the upregulation of multiple iron uptake proteins including the recently described heme transporter Spd_0090 (Figure 25). Together these data demonstrate that Spd_0739 is not a heme import protein and instead identify it as a protein with a major inhibitory role on iron and heme import.

I previously reported that endogenously produced hydrogen peroxide promotes extracellular heme degradation, enabling the acquisition of iron from hemoglobin and enable iron acquisition form hemoglobin. Moreover, pneumococcal Δ*spxB*Δ*lctO* mutant that cannot produce hydrogen peroxide upregulated several iron and heme uptake. Here, I found that inactivation of *spd_0739* resulted in a similar induction of iron and heme transporter. In addition, we discovered that this mutant produced significantly less hydrogen peroxide compared with the parent strain. These observations establish that inactivation of *spd_0739* is pleotropic and raise the possibility Spd_0739 influence on the expression of iron and heme uptake genes is related to its impact on hydrogen peroxide production.

Spd_0739 contributes to hemoglobin binding on pneumococcal surface, possibly modulating heme acquisition by the PiuBCDA proteins.

Growth phenotypes suggest the absences of Spd_0739 renders the PiuBCDA toxic (Figure 1). The Piu transports mediates the update of heme and ferric-catechol complexes (e.g., norepinephrine and enterobactin) [34-36, 42, 105]. The expression of this system is repressed by RitR, in the presence of oxidative stress, and the pleotropic repressor, CodY, in a GTP and branched chain amino acids dependent manner [35, 42, 87, 105, 109]. Interestingly, *codY* knockout mutant accumulates suppressor mutations in the *piu* genes [87], indicating that unregulated expression of the Piu system has a toxic effect. Nevertheless, expression analysis in this study revealed that inactivation of *spd_0739* had only a minor impact on the expression of *codY* (1.4 fold) and *piuB* (1.5 fold, Figure 25A), which is not likely sufficient to account for the sever growth phenotype this Δspd_739 mutant exhibits.

Both PiuA and Spd_0739 were reported to bind heme and hemoglobin *in vitro*. I confirmed that a purified Spd_0739 binds heme using absorbance spectroscopy (Figure 24A). ELISA with whole pneumococci revealed that inactivation of *spd_0739*, *piuBCDA*, or both systems each resulted in 30-35% loss of hemoglobin binding o pneumococcal surface. Since I found that it binds heme and hemoglobin but do not promote heme uptake, I suggest that Spd_0739 interactions with hemoglobin reduce the ability of PiuA to obtain heme from hemoglobin. Since the double mutant exhibit the same binding capacity of a single mutation in Spd_0379 or PiuBCDA, it seems possible that both Spd_0739 and PiuA bind to hemoglobin together.

Nucleotide and iron metabolism are interconnected in S. pneumoniae.

In addition to their role in DNA and RNA metabolism, nucleosides are important for the activation of precursors and the function of coenzymes [38]. Pneumococci encode for the enzymes required for nucleoside synthesis, but the pathogen benefits from externally supplied nucleosides [39, 95]. Spd_0739 binds and promote nucleosides scavenging [39, 95]. Here, we found that while the addition of nucleotides had an inhibitory effect on pneumococcal growth in THYB, their addition improved growth in iron-depleted medium and in medium supplemented with hemoglobin. It seems possible that nucleotides enhance somehow the ability of pneumococci to use heme as an iron source or manage its toxicity. In addition, Spd_0739 binding to heme and hemoglobin and its impact on the function of PiuBCDA and on the uptake of iron and heme overall might be influenced by its binding to nucleosides. In summary, this study establishes Spd_0739 as a modulator of iron and heme uptake in *S. pneumoniae* and reveals a close link between nucleoside and iron metabolism in this impotent human pathogen.

4.7 Future Work

Spd_0739 is an important virulence factor and is a strong candidate pneumococcal vaccination studies [95, 110]. However, the function of this lipoprotein *in vivo* requires further study as this protein seems to impact both heme and nucleoside metabolism [30, 39, 40, 95]. Inactivation of *spd_0739* may induce a global change in the pneumococcal transcriptome. Future genome wide analysis comparing WT and isogenic *spd_0739* knockout in the presence of nucleosides or heme may help provide better clarity to the importance of this lipoprotein and its transport system.

5 DRUG-RESISTANT GRAM-NEGATIVE BACTERIA THROUGH THE DEVELOPMENT OF POTENT SMALL-MOLECULE ADJUVANTS

This section discusses work of the same title published in 2022 in the American Chemical Society (ACS) Infectious Diseases [111].

5.1 Background

Alexander Fleming who famously discovered penicillin, predicted the inevitable rise of antibiotic resistance in his 1945 Nobel acceptance speech [112]. Since then, bacteria showing resistance to at least three different classes of antimicrobials, defined as multidrug resistant (MDR), have become common, especially in hospitals. Pathogen deemed superbugs are emerging that are resistant to all current antibiotics, leaving few options for therapeutics causing chronic illness and even death in the most extreme cases. To compound this problem, the rate of new drug discovery is decreasing and causes a hefty economic burden.

Strategies utilized by bacteria to avoid destruction by antimicrobials include reducing the permeability for antibiotics and reduced expression of porins, over-expression of efflux pumps and metabolizing enzymes for drug degradation, and reduced affinity towards drug targets. Multidrug resistance is more prevalent in Gram-negative bacteria because of the existence of an outer membrane (OM) which add an addition barrier that impedes drug delivery. Compounds that can permeabilize the OM of Gram-negatives and increase uptake of antibiotic would rejuvenate the current arsenal of drugs without the need to develop novel antibiotics.

In this study, a novel bacterial sensitizer scaffold named MD-124, was described to sensitize the OM of Gram-negative bacteria by binding to LPS, damaging the OM integrity and

increasing permeability for antibiotics. MD-124 can also significantly sensitize drug-resistant Gram-negative bacteria, including carbapenem-resistant, colistin-resistant, and other MDR strains, toward existing antibiotics. MD-124 can sensitize Gram-negative bacteria, including MDR strains, to the point that it allows the use of some narrow-spectrum antibiotics to be effective treatment options. Besides its potent sensitization effect, MD-124 also exhibited a very low propensity to induce bacterial resistance with low toxicity to host tissues. In a skin burn infection model, MD-124 enabled the use of novobiocin to treat Gram-negative bacterial skin infection. MD-124 also increased the efficacy of clindamycin in treating carbapenem-resistant Escherichia. Further, MD-124 was shown to sensitize mcr-1-expressing Gram-negative bacteria toward polymyxin B in a bacterial skin infection model. Here, I determined the efficacy of MD-124 in a murine model of systemic infection using both wild-type (WT) and drug resistant *E. coli* strains.

5.2 Major findings

5.2.1 MD-124-Antibiotic Combinations Show Potent Antimicrobial Effects in a Systemic Infection Model in Mice.

Briefly, each mouse was infected with 10^7 CFUs of *E. coli* (WT or NDM-1-expressing) intraperitoneally (i.p). One hour post infection, the mice were subjected to different treatments through i.p. injection. Survival of the mice in each group was monitored for 72 h (Figure 27D). As shown in Figure 27E, all mice treated with saline died from infection within 18 h post infection. Novobiocin was chosen to pair with MD-124 for the same reason as in the ex vivo model. Single dose of combination of MD-124 (10 mg/kg) and novobiocin (80 mg/kg) treatment increased the survival rate from 0% (novobiocin or MD-124 only) to 93% in the case of WT *E. coli* (ATCC 25922) infection. Encouraged by those results, we then tested if MD-124 was

effective against drug-resistant strains. We tested MD-124 in the mice infected with NDM-1-expressing E. coli and observed a similar effect (Figure 27F). MD-124 (10 mg/kg) and novobiocin (80 mg/kg) combination group achieved a survival rate of 87%, while the MD-124 only and novobiocin only group had a survival rate of 0 and 6.7%, respectively. Mice that survived after 72 h in the MD-124 and novobiocin combination group showed normal body temperature, recovered body weight, and normal vibrant behavior, indicating a cure from the otherwise lethal infection. Taken together, these results showed that MD-124 extended the spectrum of novobiocin and potentiated novobiocin for treating drug-resistant Gram-negative bacterial infection *in vivo* and greatly increased the efficacy of the tested antibiotic.

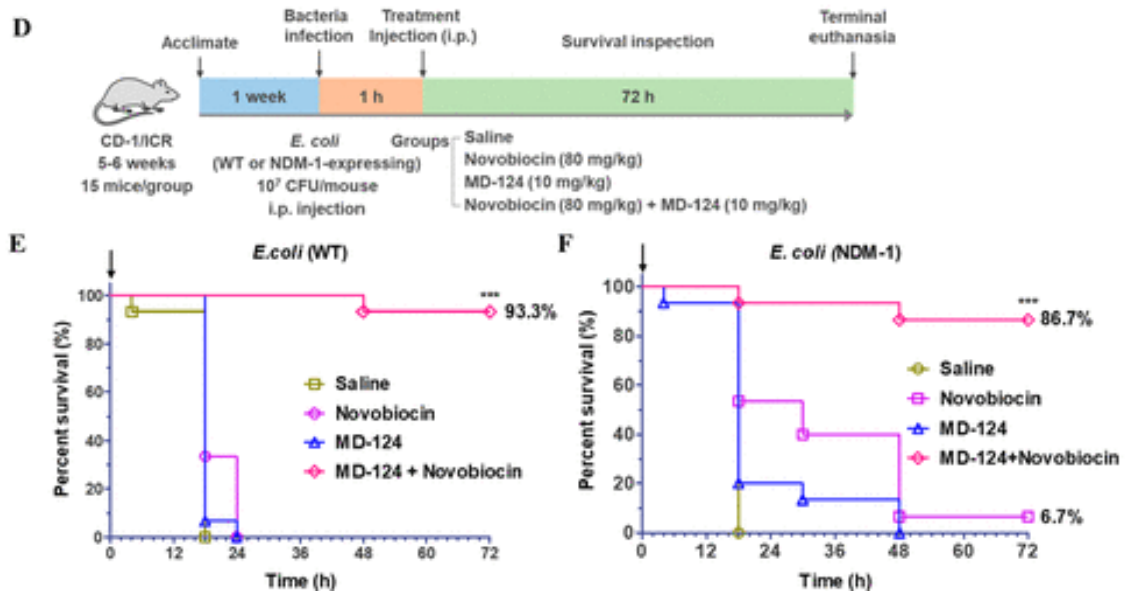


Figure 27: Validation of MD-124 efficacy in vivo systemic infection model in mice (D–F).

(D) Schematic illustration of the experimental procedures of a systemic infection model in mice. (E, F) MD-124 and novobiocin combinations significantly increased the survival rates of mice after infection by WT *E. coli* (E) and NDM-1-expressing *E. coli* (F). Arrows in (E, F) indicate the treatment time. The concentration for MD-124 and novobiocin is 10 and 80 mg/kg,

respectively. The same concentration was used for novobiocin and MD-124 in the combination treatment groups. For (E,F), n = 15 biologically independent animals per group. ***P < 0.001 vs antibiotics or MD-124 alone group. Statistical analysis using Log-rank (Mantel–Cox) test.

5.4 CONCLUSION

The rise of multidrug resistance in bacteria is increasingly becoming a major threat to public health. The decreasing rate of new drug discover foreshadows a future where patients are forced to live with chronic illness or even death due to once treatable pathogens that are now resistant to all available therapeutics. Therefore, there is an urgent need for new approaches to tackling multidrug resistant bacteria. The OM of Gram-negative bacteria is a barrier for overcome in order to tackle drug resistance. Reduce permeability for antibiotics and reduction of porin protein expressions of the OM are major mechanisms utilized by Gram-negatives to resist drug susceptibility. MD-124 sensitized Gram-negative bacteria to narrow scale antibiotics by binding to Lipid A of LPS, therefore increasing permeability for drug delivery through the outer membrane. In this study, I showed that total lethally can be prevented in a murine model when drug delivery of antibiotics is partnered with MD-124. This protection extended to infections with *E. coli* strains expressing *mcr-1* which can modify phosphate groups on lipid A, resulting in decreased negative charged density on the Gram-negative OM and subsequent resistance to several antibiotics. While promising, Gram-negative can employ different mechanism to avoid eradication by antibiotics other than reducing permeability. For example, some bacteria can upregulate efflux channels or enzymatically modifying antibiotics once delivered across the OM. Future works are encouraged to investigate to potency of MD-124 in strains that resist antibiotics via efflux or enzymatic degradation.

6 CONCLUDING REMARKS

The aim of this study was to explain the growth benefits of hemoglobin and to describe the molecular mechanisms that facilitate iron acquisition in *S. pneumoniae.* In this dissertation, I demonstrated that pneumococcus readily metabolizes hemoglobin as an iron source increasing overall biomass, and that hemoglobin signals the transition into host colonization, regulates the use of alternative metabolites found in the host, and promotes the induction of biofilm [29, 30]. *In vivo*, the iron sequestered in hemoglobin represents most of the iron in the host. Pneumococci can access hemoglobin by promoting RBC hemolysis by the pneumococcal pneumolysin, Ply [29]. Other Gram-positive microbes such as *Streptococcus pyogenes [18, 113]* and *Staphylococcus aureus [17, 114, 115]* removes the iron-bound heme from hemoglobin and shuttle it using a relay of cell-wall anchored receptors and deliver it into the cell via membrane bound transporters. This dissertation demonstrates a unique mechanism of iron acquisition from hemoglobin that relies on the innate ability of *S. pneumoniae* to produce robust amounts of hydrogen peroxide when in aerobic environments. Hemoglobin has long been known to react with hydrogen peroxide by different reaction pathways depending on the redox state of heme iron [68, 69, 83, 86]. The hemoglobin-H_2O_2 reactions either consume hydrogen peroxide or the degrade of the protoporphyrin ring and release iron [68, 69, 81]. However, to my knowledge this study is the first to demonstrate that endogenously produced hydrogen peroxide can contribute to the acquisition of iron from hemoglobin in pathogenic bacteria.

In this dissertation, I demonstrate that hydrogen peroxide producing pneumococci gradually releases iron to the extracellular milieu during growth in hemoglobin media. I then validated that as cells that did not produce hydrogen peroxide, due to a double knockout of *spxB* and *lctO,* took up heme and upregulated several iron and iron-complex uptake systems. While *S.*

pneumoniae colonizes the areas of the host where oxygen is present, e.g., the nasal-pharyx and lower respiratory system, this pathogen can become invasive and reach niches that are considered anoxic, such as the blood [25, 44, 64]. Hydrogen peroxide contributions to iron metabolism in *S. pneumoniae*, must be considered when one aims to explain the mechanism used by pneumococcus to metabolize the host hemoglobin in either in the aerobic mucosal environments or in invasive anoxic settings. Figure (28) illustrates how *S. pneumoniae* may process hemoglobin in aerobic or anoxic host niches. Hemoglobin is present in the host nasal-pharynx due to cellular damage from viral infections, hemolysis, or trauma [67, 116]. With the production of pneumococcal hydrogen peroxide, free iron and heme can be liberated from hemoglobin and imported by the iron transporters previously mentioned. It is also important to recognize that *S. pneumoniae* may persists in polymicrobial environments with other host commensals. Reactions with host hemoglobin and pneumococcal hydrogen peroxide liberates iron into the sounding which may benefit other microbes. In addition, these interactions reduce the toxic burden of the hydrogen peroxide on the neighboring organisms. Pneumococci express a few iron importers; previous attempts to attenuate pneumococcal growth on hemoglobin iron revealed that such phenotypes require knockouts in two or more of these metal iron transporters [43]. Extracellular degradation of hemoglobin by hydrogen peroxide, likely provide the ligand for these uptake systems.

As pneumococci transition into an invasive state and reach the blood, they likely rely on canonical heme import mechanisms without the assistance of hydrogen peroxide. While there are a few lipoproteins than been implicated in heme import, further studies are required to better understand the hierarchy in these redundant mechanisms. The protein Spd_0739 in the D39 strain (tagged as Sp_0845 in TIGR4 was suggested to import heme transport [30, 40], however

further analysis reveals that rather than contributing to heme import, this lipoprotein is buffering the toxic effects of *piuBDCA* activity and has a pleotropic consequence on the expression of iron and heme importers and hydrogen peroxide production.

In summary, this dissertation exposed the strong association between hydrogen peroxide, nucleotide metabolisms, and iron hemostasis in *S. pneumoniae*, redefining in *S. pneumoniae* and likely in other hydrogen peroxide producing pathogens. Previous studies failed to consider the contribution of the endogenously produced hydrogen peroxide and the associated release of free iron into the medium when investigating mechanisms that facilitate the use of heme and hemoglobin as a source of iron. Uncovering these contributions will help deconvolute previous observations, which may wrongly link ferric transporters to the import of heme.

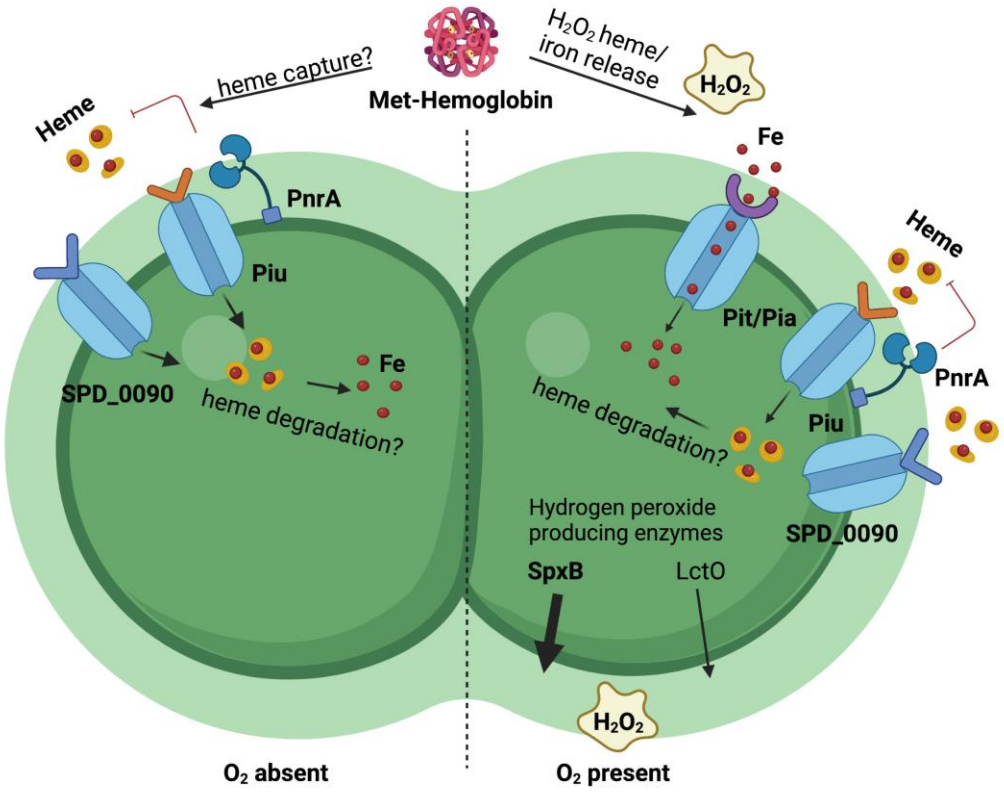


Figure 28: A schematic illustration of pneumococcal iron scavenging from hemoglobin with consideration to the production of endogenous H_2O_2.

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7 APPENDICES

7.1 Appendix A

Following is a chronological list of all the publications I have obtained during my graduate program at Georgia State University.

Akhter F, **Womack E**, Vidal JE, Le Breton Y, McIver KS, Pawar S, Eichenbaum Z. *Hemoglobin stimulates vigorous growth of Streptococcus pneumoniae and shapes the pathogen's global transcriptome.* Sci Rep. 2020 Sep 16;10(1):15202. doi: 10.1038/s41598-020-71910-1. PMID: 32938947; PMCID: PMC7494912.

Akhter F, **Womack E**, Vidal JE, Le Breton Y, McIver KS, Pawar S, Eichenbaum Z. *Hemoglobin Induces Early and Robust Biofilm Development in Streptococcus pneumoniae by a Pathway That Involves comC but Not the Cognate comDE Two-Component System*. Infect Immun. 2021 Mar 17;89(4):e00779-20. doi: 10.1128/IAI.00779-20. PMID: 33397818; PMCID: PMC8090938.

Yu B, Roy Choudhury M, Yang X, Benoit SL, **Womack E**, Van Mouwerik Lyles K, Acharya A, Kumar A, Yang C, Pavlova A, Zhu M, Yuan Z, Gumbart JC, Boykin DW, Maier RJ, Eichenbaum Z, Wang B. *Restoring and Enhancing the Potency of Existing Antibiotics against Drug-Resistant Gram-Negative Bacteria through the Development of Potent Small-Molecule Adjuvants.* ACS Infect Dis. 2022 Aug 12;8(8):1491-1508. doi: 10.1021/acsinfecdis.2c00121. Epub 2022 Jul 8. PMID: 35801980.

Womack E, Alibayov B, Vidal JE, Z Eichenbaum. *Endogenously produced H_2O_2 is intimately involved in iron metabolism in Streptococcus pneumoniae*. 2023. (TBA).

Womack E, Z Eichenbaum. *The highly abundant ligand binding protein, spd_0739, indirectly impacts iron metabolism in Streptococcus pneumoniae*. 2023.(TBA).

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