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# Hemoglobin stimulates vigorous growth of *Streptococcus pneumoniae* and shapes the pathogen's global transcriptome

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***Streptococcus pneumoniae*** **(Spn) must acquire iron from the host to establish infection. We examined the impact of hemoglobin, the largest iron reservoir in the body, on pneumococcal physiology. Supplementation with hemoglobin allowed Spn to resume growth in an iron-deplete medium. Pneumococcal growth with hemoglobin was unusually robust, exhibiting a prolonged logarithmic growth, higher biomass, and extended viability in both iron-deplete and standard medium. We observed the hemoglobin-dependent response in multiple serotypes, but not with other host proteins, free iron, or heme. Remarkably, hemoglobin induced a sizable transcriptome remodeling, effecting virulence and metabolism in particular genes facilitating host glycoconjugates use. Accordingly, Spn was more adapted to grow on the human α – 1 acid glycoprotein as a sugar source with hemoglobin. A mutant in the hemoglobin/heme-binding protein Spbhp-37 was impaired for growth on heme and hemoglobin iron. The mutant exhibited reduced growth and iron content when grown in THYB and hemoglobin. In summary, the data show that hemoglobin is highly beneficial for Spn cultivation in vitro and suggest that hemoglobin might drive the pathogen adaptation in vivo. The hemoglobin receptor, Spbhp-37, plays a role in mediating the positive influence of hemoglobin. These novel findings provide intriguing insights into pneumococcal interactions with its obligate human host.**

*Streptococcus pneumoniae* (Spn) is a significant human pathogen that causes illnesses ranging in severity from common otitis media infections to invasive diseases such as pneumonia, bacteremia, and meningitis. Pneumococcal pneumonia is also a significant risk factor for the development of cardiac diseases and heart failure[1,2]. Altogether, the toll of Spn on human health is substantial, and the pathogen is responsible for ~ 15 million infections each year and about half a million deaths in children worldwide[3,4]. Pneumococci commonly colonize the human nasopharynx, and Spn can persist asymptomatically in healthy individuals for several weeks and up to a few months[5]. From the nasopharynx, Spn can be transmitted among hosts[6] and spread to other organs. Young children (< 5 years of age), the elderly, and immunocompromised persons are the most susceptible individuals to pneumococcal infections[7–9]. While colonization of the upper respiratory tract is a pre-requisite for pneumococcal pathogenesis and infectivity, the factors that govern the establishment of Spn in the human host and the pathogen's transition into a virulent state, are not fully appreciated.

Healthy individuals typically avoid infection by *S. pneumoniae*. Still, the opportunistic pathogen can thrive in susceptible hosts and cause serious ailments. *S. pneumoniae,* though, is a fastidious bacterium that in vitro exhibits relatively weak growth. Several studies were undertaken to optimize streptococcal cultivation in a complex

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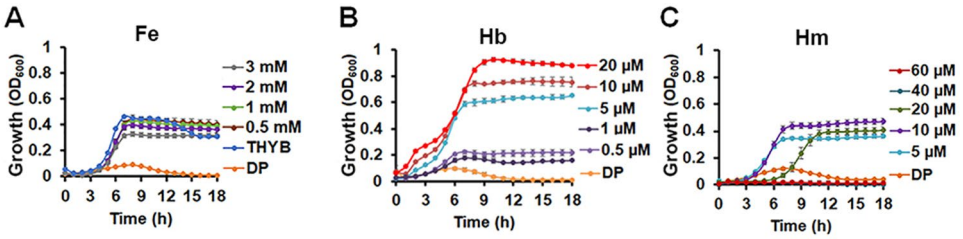


**Figure 1.** Hemoglobin-dependent growth of Spn D39 in iron-deplete medium. THYB was inoculated with D39 cells grown on BAPs (18 h, starting $O.D._{600} = 0.05$). Shown is growth in (**A**) THYB, THYB with 3 mM 2, 2′-Dipyridyl (DP), THYB with DP and 0.5–3 mM of $FeNO_3$ (Fe); (**B**) THYB with DP supplemented with 0.5 µM -20 µM hemoglobin (Hb); and (**C**) THYB with DP supplemented with 5–60 µM heme (Hm). The data are representative of three independent experiments performed in triplicates; error bars indicate SD.

(e.g., Todd-Hewitt broth containing yeast extract (THYB) and Tryptic Soy Broth) or defined media[10–12]. Pre-cultivation (i.e., inoculating with cells collected from a logarithmic-phase culture), medium replenishment, and growth in bioreactors with controlled growth parameters (such as pH and oxygen levels) were found to improve pneumococcal growth, although often in a strain-dependent manner[10]. Inoculating THYB with cells grown overnight in either broth or solid medium typically results in a long lag period that is followed by a relatively short exponential growth. The culture enters the stationary phase with low optical density ($O.D._{600}$ of 0.3–0.5, see references[13,14] for examples). In addition to the limited biomass, Spn cultures remain viable for a much shorter time when compared to other bacteria. An autolysis mechanism that is activated upon entry into the stationary phase or in response to inhibition of the cell-wall synthesis is often responsible for the decreased viability observed during in vitro cultivation. A threshold concentration of the extracellular amidase, LytA, determines the onset of autolysis. During infection, however, the cell-wall degrading LytA is needed for fratricidal lysis and virulence[15,16]. The pneumococcal pyruvate oxidase, SpxB, and its metabolic by-product $H_2O_2$ also contribute to pneumococcal killing during the stationary phase of growth[17].

Within the human body, high-affinity proteins sequester iron and reduce the metal bioavailability for invading microbes in a process called nutritional immunity. During colonization and the courses of infections, pneumococci must gain access and retrieve the iron it needs for growth from host proteins that carry heme or metal iron[18–20]. Most of the metal in the human body is in heme within the erythrocyte hemoglobin (67% of the total body iron). Myoglobin and cytochrome represent most of the remaining heme pool (3.5 and 3%). Iron is also highly sequestered in the extracellular compartment. The serum proteins hemopexin and transferrin respectively remove the small amounts of free heme and iron in the serum. The host lactoferrin sequesters ferric ions from secretions and near the infection site. Spn readily grows on heme or hemoglobin iron[21] but is unable to obtain the metal from transferrin and lactoferrin[21]. The pneumococcal mechanisms that facilitate the capture and uptake of heme are only partially understood[22,23]. The ABC transporter PiuBCDA (also known as Pit1BCDA) is the only recognized heme importer in Spn. PiuA, the ligand-binding component, binds heme and hemoglobin in vitro, and inactivation of the *piuBCDA* genes partially impairs Spn growth on heme iron[24]. Spbhp-37, is the second substrate-binding protein in Spn that interacts with hemoglobin and heme[25]. Spbhp-37 antiserum inhibits pneumococcal growth on hemoglobin iron. However, a mutant in *spbhp-37* was not described, and it's not known which transporter works with this substrate-binding protein for heme import. A recent report implicated an additional Spn protein, Spbhp-22, in iron uptake. In vitro assays showed that Spbhp-22 binds heme, but the mechanism by which this cytoplasmic protein promotes iron or heme intake remains unclear[26]. Since hemoglobin is the primary source of iron during infection, we hypothesized that hemoglobin is vital for pneumococcal pathophysiology.

## Results

**Hemoglobin stimulates unusually robust growth of Spn in batch cultures.** To test the use of hemoglobin iron by Spn, we adopted the growth assay from our studies with the related Group A Streptococcus[27]. We cultivated Spn in fresh THYB, with or without the iron chelator 2, 2′-Dipyridyl (DP), and different iron supplements (hemoglobin, heme, and $FeNO_3$) (Fig. 1). Spn did not grow in THYB-DP, but growth was restored when we supplemented the medium with ferric iron (≥ 0.5 mM, Fig. 1A), demonstrating that DP prevents pneumococcal cultivation by limiting iron bioavailability. Supplementation with hemoglobin (Fig. 1B) or heme (Fig. 1C) also supported Spn growth in THYB-DP in a dose-dependent manner. Higher amounts of $FeNO_3$ compared to a heme source (mM vs. µM, respectively) were needed to support growth, because DP chelates metal iron but not heme[27,28]. Surprisingly, hemoglobin facilitated a much better growth in THYB-DP compared with free iron (Fig. 1B). In the presence of hemoglobin, the culture displayed only a brief lag phase, which was followed by a prolonged (and somewhat bi-phasic) exponential period, reaching a higher maximal optical density. Free heme also supports Spn growth in THYB-DP, but growth was not as robust as with hemoglobin, and heme became a growth-inhibitory above ten µM (Fig. 1C).

Spn growth in THYB-DP with hemoglobin (≥ 5 µM) exceeded the one observed in standard medium (THYB, Fig. 1A,B). We tested the impact of adding 0.5–20 µM hemoglobin to iron complete THYB. Remarkably, hemoglobin stirred vigorous growth in a dose-dependent manner (Fig. 2A). Incubation with 20 µM serum albumin, which has a similar molecular weight (~ 60 kDa), resulted only in a minimal growth improvement (Fig. 2B).

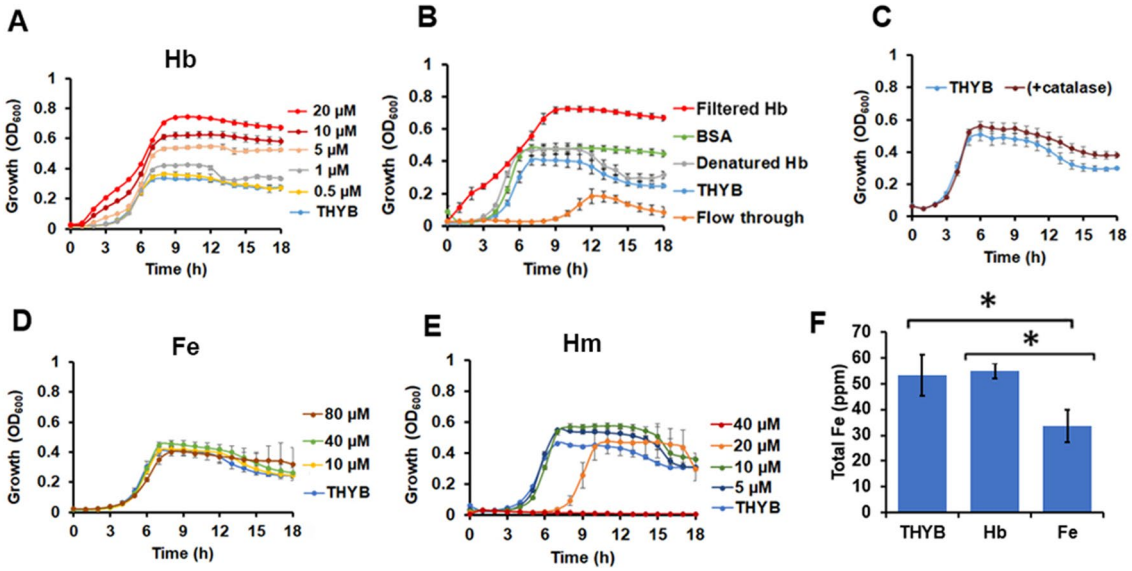


**Figure 2.** Hemoglobin stimulates a robust growth of Spn D39 in standard (iron-complete) medium. THYB was inoculated with D39 cells grown on BAPs (18 h, starting $O.D._{600} = 0.05$. Shown is growth in (**A**) THYB with 0–20 μM hemoglobin (Hb); (**B**) THYB with 20 μM BSA, denatured hemoglobin, filtered 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 $FeNO_3$ (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 Hb vs. Fe, Student's t-test).

| Incubation time (h) | Conditions[a] | Total (CFU/ml) |
|---|---|---|
| 8 | NS | $2.6 \pm 1.3 \times 10^8$ |
| | Hb | $5.5 \pm 1.1 \times 10^8$ |
| 18 | NS | $4.7 \pm 3.1 \times 10^2$ |
| | Hb | $8.5 \pm 6.4 \times 10^3$ |

**Table 1.** Effect of hemoglobin supplementation on Spn growth. [a]NS denotes THYB without supplements, Hb denotes THYB supplemented with 20 μM hemoglobin. Statistical analysis: 8 h, *P = 0.02*; 18 h, *P = 0.04* (Student's t-test).

Supplementation with hemoglobin that was heat-inactivated did not promote growth. To rule out the possibility that a contaminant in our hemoglobin preparation was responsible for the observed enhanced growth, we filtered the hemoglobin solution using 10,000 MW cutoff and tested both fractions for growth impact. The filtered hemoglobin retained activity while the flow-through was somewhat growth-inhibitory (Fig. 2B). It seemed possible that the positive growth induced by hemoglobin results from the intrinsic peroxidase activity of hemoglobin[29], leading to protection from hydrogen peroxide[30]. To test this hypothesis, we grew Spn in THYB and added catalase to scavenge hydrogen peroxide. Results in Fig. 2C demonstrates that supplementation with catalase had a minimal impact on Spn cultivation.

Supplementation with an equimolar amount of iron (10–80 μM $FeNO_3$, each hemoglobin molecule has four heme groups) did not have a significant effect (Fig. 2D). The addition of free heme to THYB did not improve growth at the low μM range and was inhibitory above ten μM (Fig. 2E). To evaluate whether the enhanced growth was due to an improved uptake of iron when pneumococci are incubated with hemoglobin, we measured intracellular iron levels by ICP-MS (Fig. 2F). Equal amounts of iron were found in cells grown in THYB or THYB with 20 μM hemoglobin. Surprisingly, supplementation with 80 μM $FeNO_3$ resulted in an actual reduction in the total iron cellular level.

We next tested the viability of cells at different time points during growth in THYB with and without 20 μM hemoglobin. Consistent with the higher optical density, in the presence of hemoglobin, viable count at 8-h post-inoculation was about twice that of cultures grown without hemoglobin (Table 1). Cells exhibited a sharp decline in viability after overnight incubation (18 h) in both THYB and THYB with hemoglobin. However, 18-fold more viable cells were obtained in cultures grown with hemoglobin compared to cultures cultivated in THYB alone

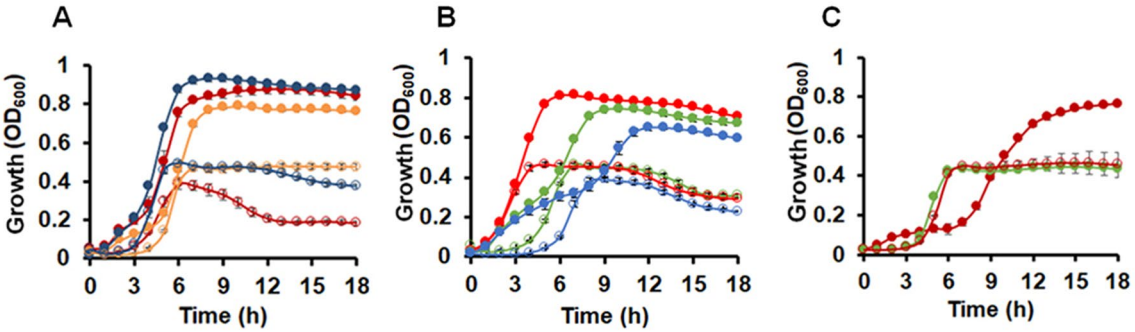


**Figure 3.** Hemoglobin stimulates Spn growth independently of the strain or the growth assay. Shown is Spn growth in fresh THYB (empty symbols) or THYB with 20 μM hemoglobin (full symbols). The culture starting $O.D._{600}$ is indicated. (**A**) TIGR4 (red), and the clinical isolates 3,875 (blue), and 8,655 (yellow) grown on BAPs (18 h) was used as the inoculum ($O.D._{600} = 0.05$). (**B**) THYB was inoculated with D39 cells from frozen logarithmic cultures ($O.D._{600} = 0.02$, red), THYB cultures (18 h, $O.D._{600} = 0.05$, blue), or cell from BAPs (18 h, $O.D._{600} = 0.05$, green). (**C**) D39 growth in CAT medium (empty symbols), CAT medium with 20 μM hemoglobin (red, full symbols) or 20 μM human serum albumin (green, full symbols). The data are representative of three independent experiments performed in triplicates; error bars indicate SD.

(Table 1, 18 h). In summary, hemoglobin-induced growth and viability are a hemoglobin-dependent phenomenon observed in both iron-deplete and iron-complete medium.

**Hemoglobin modulates Spn growth independently of the strain or the growth assay.** Next, we asked if the response to hemoglobin is widespread among pneumococcal strains, and thereby tested the impact of hemoglobin on TIGR4 and two clinical isolates. These experiments revealed that hemoglobin stimulated growth in all three strains (Fig. 3A). The positive impact of hemoglobin might only be seen in our experimental system whereby Spn are grown on blood agar plates prior to inoculating THYB. To examine if this is the case, we inoculated fresh THYB with an overnight culture grown in THYB (Fig. 3B). Compared to cells collected from BAPs, bacteria that came from liquid culture (in THYB) exhibited a more prolonged lag phase and yielded lower final biomass. Nonetheless, the addition of hemoglobin still significantly improved the second round of growth in THYB in these cells. We next used frozen logarithmic cells grown in THYB to start new THYB cultures with and without hemoglobin (Fig. 3B). Inoculating the medium with logarithmic cells abolished the lag phase and improved overall growth. Still, supplementation with hemoglobin enhanced growth even further compared to THYB alone. We also tested the effect of hemoglobin on pneumococcal growth in Casein-Tryptone (CAT) medium. When we inoculated CAT medium with Spn cells collected from BAPs, the bacterial growth was comparable to the one seen in THYB inoculated with cells from BAPs. The addition of hemoglobin to CAT, however, altered the bacterial growth pattern and yield. In the presence of hemoglobin, the pneumococci exhibited only limited growth for the first six hours in CAT. This initial phase was followed by extended exponential growth that resulted in higher biomass compared to pneumococci growing in CAT only (Fig. 3C). Supplementing with human serum albumin in CAT as a control exhibited similar growth as seen in THYB (Fig. 3C). In summary, hemoglobin dramatically impacts growth in batch cultures of multiple pneumococcal strains regardless of the assay, or media used.

**Hemoglobin induced a sizable transcriptome remodeling.** Since hemoglobin has a considerable influence on growth, we asked how it may affect pneumococcal gene expression. Hemoglobin or saline (as a control) was added to Spn cultures grown in THYB at the early log phase and culture samples were collected one- and two-hours post-treatment. RNA was isolated from four biological replicates for each growth condition and analyzed by RNA-Seq. This global transcriptome study revealed that the addition of hemoglobin to the growth medium resulted in a significant shift in gene expression (Supplementary Table S1). 59 Spn genes were up-regulated and 18 genes were down-regulated (at least two-fold) in response to hemoglobin within one hour (Fig. 4A,B), and a total of 100 and 45 genes were induced or repressed respectively two hours post-treatment. qRT-PCR on a subset of regulated genes validated the RNA seq findings (Fig. S1).

Many of the genes that were differentially expressed in response to hemoglobin are related to the import and biosynthesis of nucleotides, amino acids, fatty acids, and lipids (Fig. 5). Notably, hemoglobin activated the transcription of several metabolic genes and genes encoding virulence factors needed for Spn nasopharyngeal colonization and infections. These include the genes of the oligopeptide-binding proteins, *aliA* and *aliB*[31], the PGN hydrolase, *lytB*[32], the gene encoding the pneumococcal histidine triad protein D, *phtD*[33] and the surface adhesin, *pavB* (Fig. 5A)[34]. Hemoglobin also repressed many metabolic genes of which, it is noteworthy that the whole operon coding for proteins of a heme importer, *piuBCDA,* was down-regulated by incubation with hemoglobin (SPD_1649-52, Fig. 5B)[35]. The expression of the heme/hemoglobin binding protein, *spbhp-37,* which also promotes Spn use of hemoglobin iron[25,36], was high but not differentially expressed (Fig. 5C).

Many of the hemoglobin-responding genes are related to carbohydrate intake and conversion (Fig. 6). The *bgu* operon[37] encoding a lactose type phosphotransferase system (PTS) (SPD_1830-33) exhibited the most robust

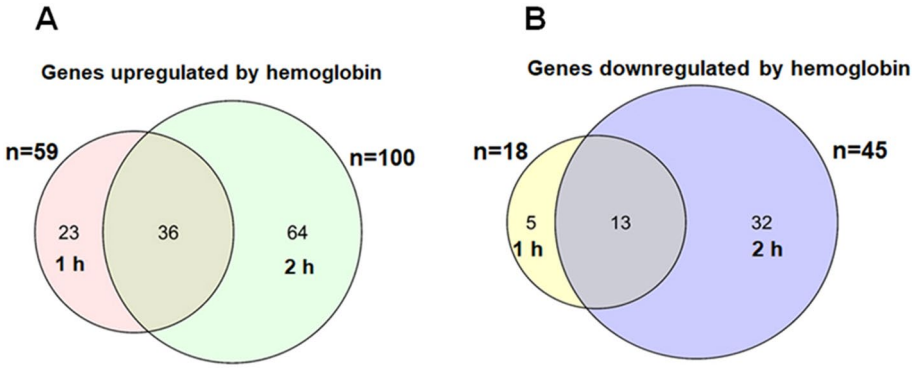


**Figure 4.** The addition of hemoglobin to the culture medium triggers a significant transcriptome remodeling in Spn. Venn diagram (using R) of differentially expressed genes in D39 culture (fold change ≥ 2). (**A**) Genes up-regulated by hemoglobin. (**B**) Genes down-regulated by hemoglobin.

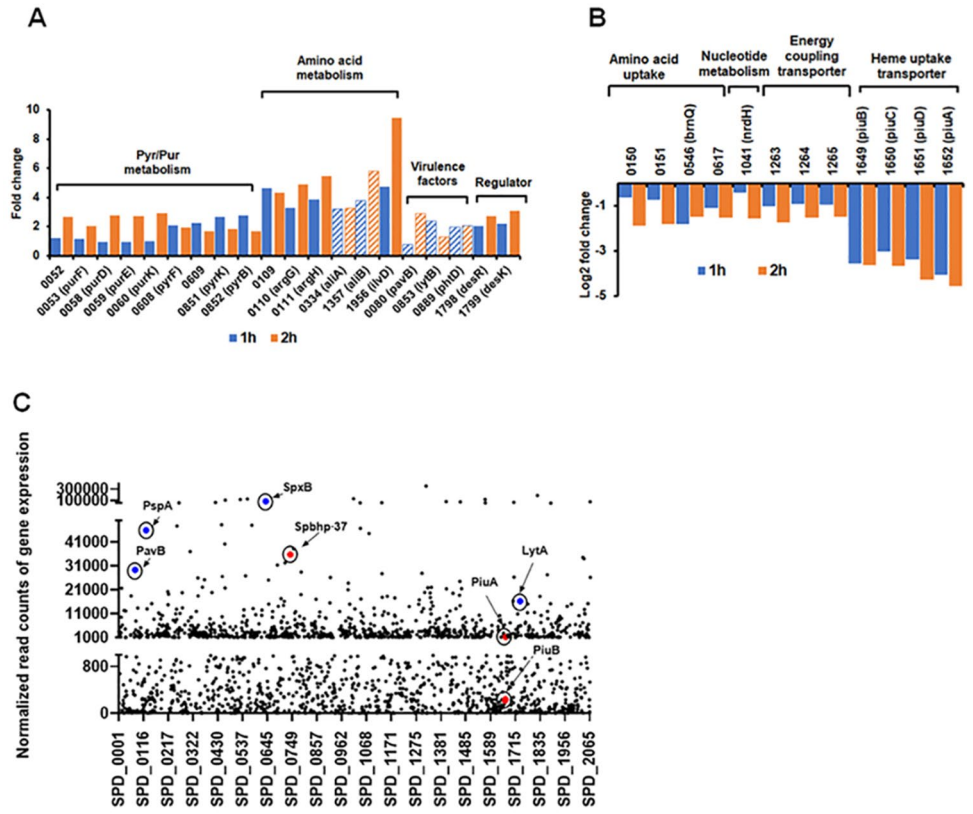


**Figure 5.** Hemoglobin activates Spn genes vital for host colonization. The relative expression of genes involved in metabolism, nutrient uptake, virulence, or regulation, at 1 h and 2 h post hemoglobin addition (Y-axis) is plotted for D39 genes (X-axis). (**A**) Up-regulated genes. Stripes indicate involvement in nasopharyngeal colonization. (**B**) Down-regulated genes. (**C**) Dot plot representation of gene expression levels depicting average normalized RNA-seq read counts (Y-axis) for cells with 1 h post-hemoglobin treatment is plotted for D39 genes (X-axis). Genes encoding heme/hemoglobin binding (red) or virulence factors (blue) are highlighted.

induction post hemoglobin addition (sixfold and 16–25-fold in the first and second hours respectively, Fig. 6A). In vitro, this PTS system mediates the use of beta-glucosides such as amygdalin or cellobiose[38]. During infection, however, β-linked disaccharides found in glycosaminoglycans of the host extracellular matrix are the proposed substrates of the *bgu* PTS. Other hemoglobin responding loci involved in carbohydrate metabolism include the *lacABCD* genes (SPD_1050-54, the tagatose pathway enzymes), *bgaA* and *bgaC* enzymes, and the associated galactose and mannose type PTS systems (SPD_0559-62 and 0,067–71 respectively). A mannose-type PTS system (SPD_0293-5) that is involved in the use of sulfated-glycosaminoglycan (e.g., hyaluronic acid)[38] was up-regulated

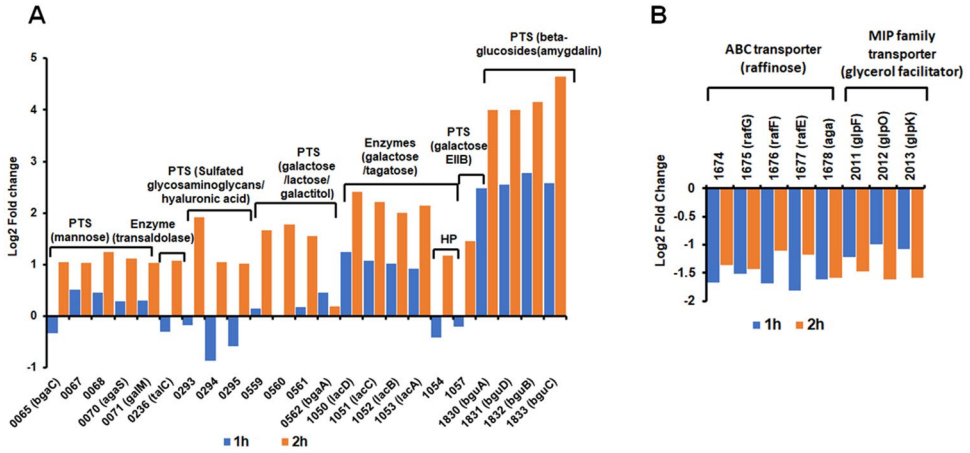


**Figure 6.** Hemoglobin up-regulates Spn genes involved in host glycoconjugate use. $Log_2$-fold changes in gene expression levels (Y-axis) is plotted for D39 genes (X-axis). (**A**) PTSs, enzymes, and hypothetical protein (HP). (**B**) down-regulated sugar transporters.

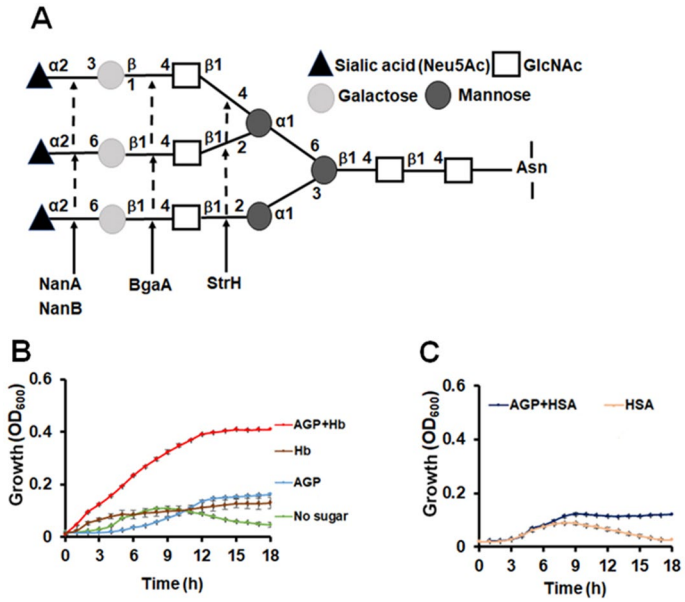


**Figure 7.** Hemoglobin facilitates Spn growth on a human glycoprotein. (**A**) Schematic representation of the human α − 1-acid glycoprotein (AGP), as described in[58]. Arrows indicate the cleavage sites of the Spn enzymes, neuraminidase (NanA), galactosidase (BgaA), and N-acetylglucosaminidase (StrH). Fresh medium was inoculated with D39 grown on BAPs (18 h, starting $O.D._{600} = 0.05$). (**B**) Shown is Spn growth in sugar-free CAT medium (no glucose added), or sugar-free CAT with 5 mg/ml AGP, and/or 20 μM hemoglobin (+Hb). (**C**) The same as in (A), only that 20 μM human serum albumin (+HSA) was added instead of hemoglobin. The data are representative of three independent experiments performed in triplicates; error bars indicate SD.

in the second hour of hemoglobin treatment (Fig. 6A). In contrast, hemoglobin down-regulated the expression of the ABC transporter (*rafGFE*) that imports the trisaccharide raffinose and of a glycerol facilitator (Fig. 6B).

**Hemoglobin promotes pneumococcal growth on host glycoconjugates in vitro.** Since hemoglobin induced the expression of PTSs and enzymes that are known or predicted to be involved in the use of host glycans, we hypothesized that hemoglobin enhances the ability of Spn to use these host molecules as nutrients. To test this hypothesis, we examined the impact of hemoglobin on Spn growth on the human α − 1-acid glycoprotein, AGP (N-linked glycan, Fig. 7A). Spn growth in CAT medium (which contains glucose) was similar to that seen in THYB (Fig. 3C). The removal of the glucose from the CAT broth, however, impeded growth (Fig. 7B). Spn grew rapidly when the sugar-free CAT was supplemented with both AGP and hemoglobin, but not in sugar-free CAT medium supplemented with either AGP or hemoglobin individually (Fig. 7B). The addition of

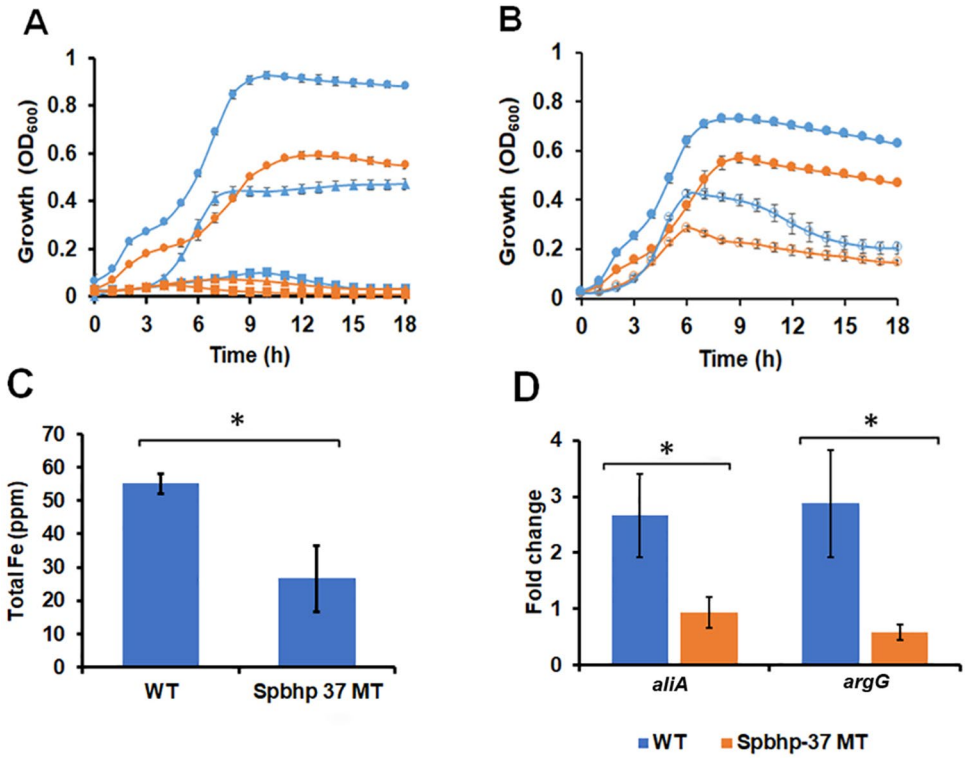


**Figure 8.** The heme/hemoglobin binding protein, Spbhp-37, plays a role in mediating the positive impact of hemoglobin in Spn. Fresh medium was inoculated with D39 (blue) and the isogenic Δ*spbhp-37* strain (orange) grown on BAPs (18 h, starting O.D.600 = 0.05). Shown is growth in (**A**) THYB with 3 mM 2, 2′-Dipyridyl (DP), THYB with 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 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 Δ*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).

human serum albumin as a control did not promote growth in sugar-free CAT with or without AGP (Fig. 7C). These data indicate that hemoglobin promotes pneumococcal use of AGP in vitro and suggests it may enhance the use of host glycoproteins as a carbohydrate source in vivo.

**A *spbhp-37* deletion mutant exhibits a lessened growth response to hemoglobin.** Although hemoglobin stimulates Spn growth in both iron-complete and iron-deplete THYB, it still seemed possible that the positive influence of hemoglobin is related to its ability to donate heme. THYB supplementation with hemoglobin repressed transcription of the heme importer genes *piuBCDA,* allowing for only very low expression (Fig. 5C). The expression of *spbhp-37,* the second heme/hemoglobin receptor with a role in heme uptake, did not change in response to hemoglobin. Still, *spbhp-37* transcription was relatively high under our experimental condition (Fig. 5C). In silico analysis predicted that Spbhp-37 is expressed as a monocistronic mRNA[39]. To see if Spbhp-37 plays a role in the response, we generated a deletion mutant (replacing *spbhp-37* ORF with that of *ermB)* and tested its impact on hemoglobin growth induction (Fig. 8). The Δ*spbhp-37* mutant was not able to grow in THYB-DP supplemented with free heme and exhibited growth attenuation when grown in THYB-DP supplemented with hemoglobin (Fig. 8A). The mutant also had a growth phenotype in THYB alone. Although Δ*spbhp-37* growth was improved in the presence of hemoglobin, it was still reduced compared with the wild type strain (Fig. 8B). ICP-MS analysis demonstrated a significant reduction in total iron levels in the Δ*spbhp-37* compare with the parental strain in cells grown in THYB with 20 μM hemoglobin (Fig. 8C). qRT-PCR analysis demonstrated that the expression of the *aliA* and *argG* genes was not induced in response to hemoglobin in the Δ*spbhp-37* mutant as it did in the wildtype strain (Fig. 8D). These observations confirm a role for Spbhp-37 in iron uptake and suggest that this hemoglobin/heme-binding protein plays a role in the Spn response to hemoglobin.

## Discussion

In this study, we demonstrated that hemoglobin, a host hemoprotein, has a substantial impact on Spn physiology and planktonic growth. The data reveal that the presence of hemoglobin greatly benefits pneumococcal growth and viability in complex laboratory media, redirecting the pneumococcal transcriptome and metabolic capacity. This appears to enhance the ability of Spn to use host glycoproteins as a source of carbohydrates, providing a potential fitness advantage during colonization and infection. The data also suggest that the heme uptake protein, Spbhp-37, plays a role in the positive influence of hemoglobin on Spn physiology. Below we discuss how these intriguing findings advance our current understanding of Spn interactions with its obligate human host.

**Hemoglobin facilitates vigorous growth in iron-depleted medium.** The reinstatement of Spn growth in the iron-depleted medium, THYB-DP, by hemoglobin or heme (Fig. 1B,C) shows that Spn can metabolize heme iron, as it was previously reported[21,25,35]. Hemoglobin iron, however, appears particularly beneficial, triggering strong Spn growth surpassing that seen with free iron or heme alone (Fig. 1). This is the first report to describe the unusually robust growth of Spn upon hemoglobin supplementation in an otherwise iron-deplete medium. Why had other investigators not seen this exquisite hemoglobin-specific enhanced growth? Technical differences, such as the use of the chelating resin, Chelex-100, might have masked the positive impact of hemoglobin in prior investigations[21,25,35]. Chelex-100 has broad specificity, and treatment with this resin might have limited the availability of additional cations that are needed for full Spn growth. Furthermore, the long precultivation in iron-deprived medium prior to the addition of hemoglobin used in some studies[21,25,35], might also have limited maximal growth with hemoglobin.

THYB-DP supplemented with heme above 5–10 µM (equivalent to 20–40 µM hemoglobin) was growth inhibitory. In excess, heme is harmful to many bacteria, including the related *Streptococcus pyogenes*, due to its lipophilic and oxidative nature, which damages the bacterial membranes, proteins, and nucleic acids[40,41]. The negative impact that is inherent to the presence of free heme in the medium may prevent the benefit of heme that is provided by hemoglobin. Hemoglobin likely delivers the heme directly to surface receptors and membrane transporters for import[24,36,42]. Compared to free heme, heme that is delivered from one protein to another likely has fewer opportunities to damage the cell envelope.

**Spn growth stimulation is hemoglobin-dependent and utilizes a unique mechanism.** Supplementing THYB with hemoglobin enhances pneumococcal growth, shortening the lag period, and increasing maximal biomass and viability (Fig. 2A,B and Table 1). Other host proteins, inactivated hemoglobin, or the flow-through collected after hemoglobin filtration, did not have this effect. Hence, it is hemoglobin in its native form, rather than a nonspecific increase in peptides and amino acids availability, or the presence of low molecular weight contaminants, that is advantageous for Spn growth. Spn is often grown with a source of catalase (e.g., blood) to neutralize a large amount of hydrogen peroxide this bacterium can produce. If inhibition of $H_2O_2$ by hemoglobin allowed enhanced growth, then the addition of catalase to THYB would have improved growth, but this did not occur (Fig. 2C), suggesting that hemoglobin does not extend growth by scavenging the $H_2O_2$ from the medium.

Supplementation with ferric iron did not enhance Spn growth in THYB, suggesting that the iron levels in this medium are not growth-limiting for Spn (Fig. 2D). Moreover, the cellular levels of iron in cells grown in THYB with hemoglobin are similar to those found in cells grown in regular THYB (Fig. 2F). Therefore, hemoglobin growth benefits are not derived by facilitating an increase in the total iron level in Spn. Hemoglobin, however, might have increased the cellular heme-to-iron ratio compared with cells that grew in THYB. Hence, we speculate that hemoglobin promotes growth, at least in part, by donating heme and that heme is more growth beneficial then metal iron. The finding that Spn imports less of the metal when THYB is enriched with ferric iron (Fig. 2F) supports the idea that heme might be more useful for the pathogen's physiology than free iron. Other pathogenic bacteria, such as *Staphylococcus aureus* and *S. pyogenes,* demonstrate a preference for heme when supplied with both free iron and heme[43,44]. Canonical heme degradation results in the production of biliverdin and its reduced product bilirubin[45], both molecules scavenge various ROS and are considered potent antioxidants[46,47]. Perhaps, similar to the human host, heme catabolism by Spn leads to the production of protective end products and thus is valuable beyond iron release. Studies in our laboratories are underway to address this hypothesis.

The positive response to hemoglobin was rigorously assessed in the current study and found to be shared by multiple pneumococcal strains and independent of the growth assay used (Fig. 3). The addition of hemoglobin shortens the lag period in THYB inoculated with overnight cultures that grew either on BAPs or in THYB (Fig. 3B). Nevertheless, cells that were collected from BAPs exhibited improved growth. It's possible that the presence of hemoglobin is one of the growth-promoting factors in BAPs, which are commonly used for Spn cultivation. Notably, the addition of hemoglobin to THYB inoculated with logarithmic cells still enhanced growth (Fig. 3B). Hence, hemoglobin acts by both stimulating growth in resting cells and by extending the growth period before an exponentially growing culture enter the stationary phase. Spn growth with hemoglobin appeared biphasic in all conditions, other than in cultures inoculated with logarithmic cells. The formation of a two-step growth curve was more apparent in the experiments using Spn grown on THYB as the inoculum (Fig. 3B), in CAT medium (Fig. 3C), and with the Δ*spbhp-37* mutant (Fig. 8A,B). We have not explored in detail this biphasic growth pattern. Still, the absence of the first growth phase in cultures using logarithmic cultures implies that the initial hemoglobin impact on resting cells can be separated from its influence on exponentially growing cells.

It is not fully understood why Spn exhibits limited growth in batch cultures and enters early into the stationary phase. Acidification and other growth conditions were implicated as growth limiting factors. Nevertheless, at least some Spn strains (such as D39), still demonstrated limited exponential growth even under conditions where the pH and other factors such as temperature and atmosphere were controlled[10,48]. The data here show

that hemoglobin extends Spn growth in medium that was not otherwise replenished, without pH control, with or without pre-cultivation. Therefore, it's possible that hemoglobin (directly and/or via the heme it donates) redirects a regulatory mechanism(s) that otherwise leads to premature growth arrest during in vitro cultivation. Interestingly, hemoglobin did not influence the expression of *lytA* and *spxB,* which cause Spn death, but the expression of these two genes was high under our experimental conditions (Fig. 5C). It is worth noting that for our transcriptome studies, hemoglobin was added at the early exponential growth, and gene expression was analyzed one and two hours after treatment; thus, we can't rule out a later change in *lytA* and *spxB* expression.

**Hemoglobin has a broad impact on Spn transcriptome suggesting host adaptation.** Hemoglobin triggered significant transcriptome remodeling (Fig. 4). Various genes with an established role in colonization were activated (Fig. 5A), including the *aliA/aliB* genes. The lipoproteins, AliA, and AliB, are paralogs of AmiA, the oligopeptide-binding component of the Ami system, which is used by the auxotroph Spn to import oligopeptides[49]. Inactivation of *ami-aliA/aliB* attenuates adherence to pulmonary epithelial cells[50], and these proteins are important for the initial colonization of the nasopharynx[31]. Hemoglobin also induced the PGN hydrolase gene, *lytB,* whose activity is necessary for the adherence and invasion of human lung epithelial cells[32]. The colonizing factor PavB also positively responded to hemoglobin. PavB contributes to nasopharyngeal colonization and is highly expressed during heart infection[34,51]. Similarly, hemoglobin induced the arginine biosynthesis genes, *argGH*, which are needed for growth in the lung, blood, and cerebrospinal fluids[52]. Hence, Spn transcriptome response to hemoglobin suggests the pathogen perceives the presence of hemoglobin as a cue for the host environment.

**Hemoglobin activates pneumococcal utilization of glycoproteins found in the host mucin and the epithelial mucosa.** Hemoglobin had a strong influence on the expression of many genes involved in the uptake and use of host-derived sugars found in the mucosa and extracellular matrix (Fig. 6). Spn can ferment about 32 different carbohydrates in vitro[38], and over 30% of the transporters in the Spn genome are dedicated to carbohydrate uptake[53], many of which contribute to colonization and pathogenesis. Free sugars, however, are not typically available in the upper respiratory tract[54,55]; rather, they are linked to the glycoproteins (i.e., O- and N-linked glycan and glycosaminoglycan) found on the epithelial cell lining and in mucin[56,57]. Sequential deglycosylation by various pneumococcal exo-glycosidases (e.g., BgaA, BgaC, NanA, NanB, and StrH) allow Spn to cleave the sugars from the host glycan for uptake ([57–59] and Fig. 7A). Our transcriptome analysis suggests that hemoglobin activates the expression of genes needed for the release, import, or catabolism of the host glycan derived monosaccharides, galactose and mannose, glycosaminoglycan disaccharides (e.g., hyaluronic acid), and the beta-glucosides amygdalin and cellobiose (Fig. 6A). Therefore, like mucin[60], hemoglobin induces the expression of Spn genes required for growth on sugars derived from the host glycans. In vitro growth assays confirmed that Spn could use the human α − 1-acid glycoprotein AGP (N-linked glycan) as a carbohydrate source when grown in the presence of hemoglobin (Fig. 7B), but not in the presence of human serum albumin, HSA, as a control (Fig. 7C). Hemoglobin down-regulated the operons encoding the glycerol MIP family transporter, *glp,* and the raffinose transporter (Fig. 6B). Interestingly, Spn ear isolates demonstrate a reduced capacity to use the sugar raffinose comparing with blood isolates[61].

**The hemoglobin/heme receptor Spbhp-37 has a role in mediating the hemoglobin response and growth benefits.** PiuA and Spbhp-*37* are the only two hemoglobin (and heme) receptors with an established role in heme uptake. Hemoglobin, however, represses expression of the *piuBCDA* transporter, while the expression of *spbhp-37* remained high whether hemoglobin was added or not (more than 30-fold above *piuA*, Fig. 5C). Using a *Δspbhp-37* mutant, we showed that Spbhp-37 plays an important role in the use of heme and hemoglobin iron (Fig. 8A). This is consistent with the observation that inhibition of Spbhp-37 by antibody interfered with Spn growth on hemoglobin iron[25]. Spn, however, was still able to utilize hemoglobin, likely due to redundancy in heme uptake systems. The Δ*spbhp-37* mutant did not grow well in THYB, the addition of hemoglobin improved growth only partially, and lower iron levels were found in mutant cells grown in THYB with hemoglobin (Fig. 8C). These observations suggest that Spbhp-37 has a redundant role in mediating the growth benefit of hemoglobin, possibly by capturing heme from hemoglobin. Notably, hemoglobin failed to induce the expression of the *aliA* and *argG* genes in the Δ*spbhp-37* mutant as it did in the wildtype strain (Fig. 8D). Hence, *spbhp-37* contributes to the shift in gene expression caused by hemoglobin. Further investigations are needed to determine if Spbhp-37 influence on gene expression depends on its role in heme import.

Spn is listed as a severe threat in the 2019 CDC antimicrobial-resistant (AMR) report[62]. Therefore, it is critical to gain insights into Spn pathophysiology to better design new treatment modalities. Herein, we have demonstrated that hemoglobin greatly benefits pneumococcal cultivation in vitro, promoting growth and viability, and causes a transcriptome shift that is likely to advance Spn preparation for colonization and infection. Hence, this study makes significant contributions to the understanding of pneumococcal growth requirements and adaptation to its obligate human host. Additional work is needed to describe the mechanism by which Spn perceives and responds to hemoglobin and the effect of hemoglobin on Spn during infection.

## Experimental procedures

**Bacterial strains and growth media.** The Spn strains used in this study are listed in Table 2. Frozen Spn stocks were prepared in the medium Skim milk-Tryptone-Glucose-Glycerin (STGG) as described[63] and kept at -80 °C. Spn STGG stocks were plated on Tryptic Soy blood agar plates (BAPs) and incubated overnight at 37 °C under microaerophilic conditions. Spn was also grown in Todd-Hewitt broth containing 0.5% (w/vol) yeast extract (THYB) or in Casein-Tryptone (CAT) medium[64] containing Bacto Casitone 10 g/l, Bacto Tryptone

| *S. pneumoniae* | Description | Source or references |
|---|---|---|
| D39 | Avery strain, clinical isolate, WT (capsular serotype 2), CSP1 | [72,73] |
| TIGR4 | Invasive clinical isolate, WT (capsular serotype 4), CSP2 | [53] |
| 8,655 | Invasive isolate (serotype 6B), CSP2 | CDC |
| 3,875 | Invasive isolate (serotype 6B), CSP1 | CDC |
| Δ*spbhp-37* (SPZE1) | D39-derivative *spbhp-37* null mutant, Ery$^r$ | This study |
| ***E. coli*** | | |
| One shot Top-10 | Cloning strain | Invitrogen |
| **Plasmids** | | |
| pAF104 | Seamless cloning vector pUC19, Amp$^r$ | This study |
| pCR2.1 TOPO | Cloning vector, Ery$^r$ | [67] |

**Table 2.** Strains used in this study.

10 g/l, Yeast Extract 1 g/l, NaCl 5 g/l, 0.5 M $K_2HPO_4$ (30 ml/l) and 200 U/µl of catalase. One or more of the following supplements were added to the growth medium as indicated: The iron chelator 2, 2′-Dipyridyl (ACROS Organics), bovine hemin (Sigma Aldrich), human hemoglobin (Sigma Aldrich), Ferric nitrate nonahydrate ($FeNO_3$, Thermo Fisher Scientific), bovine serum albumin (BSA, Sigma Aldrich), 200 U/µl of catalase (Sigma Aldrich), glucose (Sigma Aldrich), human α − 1 acid glycoprotein (AGP, Sigma Aldrich), or fatty-acid free human serum albumin (Sigma Aldrich). Some experiments used frozen logarithmic Spn stocks as the starting inoculum. To prepare such stocks, Spn cells growing in THYB were collected at the early logarithmic phase of growth ($O.D._{600}$ = 0.2–0.3), and glycerol was added to the culture to a final 10% (vol/vol) and stored at − 80 °C.

**Growth assays.** Fresh medium (with or without supplements) was inoculated with Spn cells collected from BAPs following overnight incubation (starting $O.D._{600}$ = 0.05) or from frozen logarithmic culture (starting culture $O.D._{600}$ = 0.02) as indicated. Cell cultures (200 µl per well) were allowed to grow in 96-well microtiter plates (Corning) incubated at 37 °C. The culture $O.D._{600}$ was recorded at 1 h intervals for 18 h using a SpectraMax M2 spectrophotometer (Molecular Device). For each growth condition, we used wells containing only the medium (and supplements when appropriate) as the blank. Bacterial growth was tested in triplicates. To determine cell viability, culture samples were collected at designated time points, serially diluted in 0.9% saline, and plated in triplicates into BAP.

**Total iron determination by ICP-MS.** Fresh THYB medium containing 80 µM $FeNO_3$ or 20 µM hemoglobin was inoculated with Spn grown on BAPs (starting $O.D._{600}$ = 0.05) and incubated at 37 °C for 6 h in 12 well microtiter plates. Culture samples (6 ml, $O.D._{600}$ = 1) were washed three times with phosphate-buffered saline (PBS) prior to collection. The cell pellet was digested and analyzed (Center for Applied Isotope Studies, University of Georgia, Athens, GA) as described[65,66].

**Construction of Δ*spbhp-37* mutant.** The plasmids used in this study are listed in Table 2 and the primers in Table 3. We cloned a Δ*spbhp-37* mutant in Spn strain D39 by replacing the *spbhp-37* coding sequence with that of *ermB* (erythromycin resistance[67]), such that the *ermB* ORF is under the transcriptional control of *spbhp-37* promoter and terminator. The mutant allele containing *ermB* ORF flanked by the 5′ and 3′ genomic regions of the *spbhp-37* 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 740-L/ZE 741-R and ZE 744-L/ZE 745-R. The *ermB* gene and the pUC19 vector were amplified from pCR2.1 topo and pUC19-L plasmids using the primer sets ZE 742-L/ZE 743-R and ZE 738-L/ZE 739-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 pAF104. The resulting allele was then amplified (from pAF104) and transformed into competent D39 cells using standard protocols[68]. The mutants were selected on BAPs containing erythromycin (0.5 µg/ml). The mutation was confirmed by PCR in the resulting erythromycin-resistant clones using the primer set ZE 740-L/ZE 745-R. qRT-PCR analysis confirmed that the Δ*spbhp-37* mutation did not change the expression of the downstream gene, SPD_0740 (Fig. S2).

**Spn growth with human α − 1 acid glycoprotein and serum albumin.** To remove contaminating free sugars, human α − 1 acid glycoprotein (AGP, 10 mg/ml) was dialyzed in water using Slide-A-Lyzer G2 Dialysis Cassette (10 K MWCO, Thermo Fisher Scientific) as described[58]. The samples were then concentrated with Amicon Ultra-0.5 mL Centrifugal Filter Unit (10 K MWCO, Millipore Sigma), reconstituted in CAT medium (5 mg/ml) and filter sterilized. Spn D39 grown overnight on BAP were collected, washed and suspended in CAT. These cell suspensions were used to inoculate fresh CAT medium with or without glucose (0.5% (w/v), AGP (5 mg/ml) or HSA (20 µM). Bacterial growth in the presence or absence of 20 µM hemoglobin in 96-well microtiter plates was monitored as described above.

| Target | Primers | Sequence (5′–3′) | Comments |
|---|---|---|---|
| *pUC19-L* | ZE 738-L | TATCAAAGGGCATGCAAGCTTGGCGTAATCAT | Cloning |
| | ZE 739-R | ACTGTGCAGTACCGAGCTCGAATTCACTGGCC | |
| *5′-region of spbhp 37* | ZE 740-L | CTCGGTACTGCACAGTAGTAGGTTTCCCTTTG | Cloning |
| | ZE 741-R | TTGTTCATTACTGAACCTCCTAAATAAGATGT | |
| *ermB* | ZE 742-L | GTTCAGTAATGAACAAAAATATAAAATATTCT | Cloning |
| | ZE 743-R | CATCAAGGCGACTCATAGAATTATTTCCTCCC | |
| *3′-region of spbhp 37* | ZE 744-L | ATGAGTCGCCTTGATGGAAGCGTAAAAGTTCC | Cloning |
| | ZE 745-R | TGCATGCCCTTTGATAGACAAAACCACTTCTT | |
| *aliB* | ZE 878-L | GGACTGTTTCTCAGGACGGTTTG | qPCR |
| | ZE 879-R | CAGCTGCATATTGCAAACCTGTC | |
| *ilvD* | ZE 880-L | CCTGGTATGCGTTTCTCTCTAAC | qPCR |
| | ZE 881-R | AGCAATCATAGATCCAGGCATG | |
| *aliA* | ZE 882-L | GGTCACTTATGGGGATGAATGG | qPCR |
| | ZE 883-R | GGAATGTCACACCTTCTGCTTG | |
| *argG* | ZE 884-L | CCTTGGTATCTGCCTTGAGC | qPCR |
| | ZE 885-R | GATCCAAGGCTGCAATCGATAC | |
| *SPD_1803* | ZE 886-L | GGATTGGATGAGGATTTCTACC | qPCR |
| | ZE 887-R | CTTCTCTAACAAGCCAAGACATG | |
| *piuB* | ZE 864-L | TGATTTCGACCAGCAGACCTG | qPCR |
| | ZE 865-R | CTGTACTCGGTGCAGCAAACTG | |
| *rafE* | ZE 964-L | GCTTGCTCCAACTATGGTAAATC | qPCR |
| | ZE 965-R | CATTGACGACTTTGACCTTGATC | |
| *brnQ* | ZE 966-L | CTCTATCTGGAGAACATTTTCTTCC | qPCR |
| | ZE 967-R | GCTATCTTCGTTGAAATCTCGTAG | |
| *SPD_0740* | ZE 962-L | GTCATTGAGATGCGTGATATTACC | qPCR |
| | ZE 963-R | CATGTTCATTAGCGTGGACTTAC | |
| *gyrB* | ZE 661-L | GGCACTGTATGGTATCACACAAG | qPCR |
| | ZE 662-R | TCTCTAAATTGGGAGCGAATGTC | |

**Table 3.** Primers used in this study.

**RNA-Seq analysis.** Fresh THYB was inoculated with Spn cells from frozen logarithmic stocks (starting culture $O.D._{600} = 0.02$) and the cultures were allowed to grow in 12 well microtiter plates (2 ml per well) at 37 °C. 20 µM hemoglobin (in 0.9% saline) or 0.9% saline (negative control) was added to the growing cells at the early logarithmic phase ($O.D._{600} = 0.2–0.3$). Cultures samples (four biological replicates for each condition) were collected and mixed with RNA protect reagent (Qiagen) following the manufacturer's recommendations. Cells were then collected by centrifugation and stored at -80 °C. For RNA preparation, cell samples were suspended in 700 µl of Trizol with 300 mg of acid-washed glass beads (Sigma Life Science) and disrupted by vortexing. Total RNA was isolated using the Direct-zol RNA MiniPrep kit (Zymo Research). DNA was removed using the Turbo DNase-free kit (Life Technologies). rRNA was eliminated with the Ribo-Zero Magnetic kit for Gram-positive bacteria (Epicenter). RNA Quality and quantity were assessed using a 2,100 Bioanalyzer (Agilent) and NanoDrop 8,000 spectrophotometer (Thermo Fisher Scientific), respectively. Directional RNA-Seq libraries were created using the ScriptSeq v2 RNA-Seq Library Preparation kit (Illumina) according to the manufacturer's instructions. A rapid-run 100 bp single-read DNA sequencing was performed at the Institute for Bioscience and Biotechnology Research (IBBR) Sequencing Facility at the University of Maryland, College Park, using the Illumina HiSeq 1,500 platform. Data were generated in the standard Sanger FastQ format and raw reads were deposited with the Sequence Read Archive (SRA) at the National Center for Biotechnology Institute (accession PRJNA626052). Read quality was evaluated using FastQC software, and mapping against the Spn D39 genome using Bowtie package alignment software[69]. The read count or raw count data for all genes were acquired using Feature count package[70]. These raw count data files were then used in DESeq2 package[71] to calculate differential expression analysis of all samples for pairwise comparison. Graph Pad Prism (version 8.3.1) was used to prepare dot plot representation of gene expression levels using normalized RNA-seq read counts.

**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 7,500 Fast Real-Time 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 3. The relative expression was normalized to the endogenous control *gyrB* gene and fold changes were calculated using the comparative $2^{-\Delta\Delta CT}$ method.

## Data availability

The authors have deposited all RNA-seq raw sequencing reads with the Sequence Read Archive (SRA) at the National Center for Biotechnology Institute (accession PRJNA626052) for public availability.

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## Author contributions

F.A., E.W., J.E.V., K.S.M. and Z.E. conceived and contributed to the design and implementation of the research, and to the analysis of the results. F.A., E.W., and Y.L.B. carried out the experiments. S.P. and F.A. conducted bioinformatics analyses. F.A., and Z.E. wrote the manuscript in consultation with E.W., J.E.V., Y.L.B. and K.S.M.

## Competing interests
The authors declare no competing interests.

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