Hemoglobin Induces Early and Robust Biofilm Development in *Streptococcus pneumoniae* by a Pathway That Involves *comC* but Not the Cognate *comDE* Two-Component System

Fahmina Akhter,^a Edroyal Womack,^a Jorge E. Vidal,^b Yoann Le Breton,^{c*} Kevin S. McIver,^c Shrikant Pawar,^{a*} Zehava Eichenbaum^a

^aDepartment of Biology, Georgia State University, Atlanta, Georgia, USA

^bDepartment of Microbiology and Immunology, University of Mississippi Medical Center, Jackson, Mississippi, USA

^cDepartment of Cell Biology and Molecular Genetics, Maryland Pathogen Research Institute, University of Maryland—College Park, College Park, Maryland, USA

ABSTRACT *Streptococcus pneumoniae* grows in biofilms during both asymptomatic colonization and infection. Pneumococcal biofilms on abiotic surfaces exhibit delayed growth and lower biomass and lack the structures seen on epithelial cells or during nasopharyngeal carriage. We show here that adding hemoglobin to the medium activated unusually early and vigorous biofilm growth in multiple *S. pneumoniae* serotypes grown in batch cultures on abiotic surfaces. Human blood (but not serum, heme, or iron) also stimulated biofilms, and the pore-forming pneumolysin, *ply*, was required for this induction. *S. pneumoniae* transitioning from planktonic into sessile growth in the presence of hemoglobin displayed an extensive transcriptome remodeling within 1 and 2 h. Differentially expressed genes included those involved in the metabolism of carbohydrates, nucleotides, amino acid, and lipids. The switch into adherent states also influenced the expression of several regulatory systems, including the *comCDE* genes. Inactivation of *comC* resulted in 67% reduction in biofilm formation, while the deletion of *comD* or *comE* had limited or no effect, respectively. These observations suggest a novel route for CSP-1 signaling independent of the cognate ComDE two-component system. Biofilm induction and the associated transcriptome remodeling suggest hemoglobin serves as a signal for host colonization in pneumococcus.

KEYWORDS RNA-Seq, *Streptococcus pneumoniae*, biofilms, hemoglobin

Streptococcus pneumoniae causes ~15 million cases of severe infections each year, leading to approximately half a million deaths in children (1, 2). Antibiotic resistance was reported in more than 30% of invasive pneumococcal disease in 2017 (3, 4), leading the World Health Organization to classify *S. pneumoniae* in 2017 as one of the top 12 priority pathogens (3–5).

S. pneumoniae establishment in the human upper respiratory tract involves the formation of bacterial aggregates developed into biofilms. In addition to playing a crucial stage in nasopharyngeal establishment, pneumococcal biofilms also take part in infections, such as rhinosinusitis (6), otitis media (7), and pneumonia (8–10). Local spread and bronchoaspiration may allow *S. pneumoniae* to breach the epithelial and endothelial barriers and penetrate tissues, providing access to the bloodstream. Circulating *S. pneumoniae* can then reach and invade the heart tissue, creating microscopic lesions within the myocardium that contain intracellular biofilms (11, 12). Biofilm growth serves a central role in the pneumococcal carriage and disease states by helping *S. pneumoniae* compete with the native flora, serving as reservoirs for spread, conferring antibiotic resistance, and facilitating the escape of the host immune responses (13, 14).

Several *ex vivo* systems for pneumococcal biofilms that facilitate dense bacterial

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Address correspondence to Zehava Eichenbaum, zeichen@gsu.edu.

* Present address: Yoann Le Breton, Wound Infections Department, Bacterial Diseases Branch, The Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; Shrikant Pawar, Yale Center for Genome Analysis, Yale University, New Haven, Connecticut, USA.

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communities with pronounced structures were developed. These include static or continuous flow bioreactors with live cultures of respiratory or mucosal epithelial cells (8–10, 15–18). Curiously, biofilm growth *in vitro* on abiotic surfaces is much more limited than in the *ex vivo* models mentioned above and requires various treatments such as replenished growth medium, an inoculum with logarithmic cells, or supplementation with a synthetic competence-stimulating peptide (CSP) (19, 20). Even under these conditions, *S. pneumoniae* grown in batch cultures develops biofilms on abiotic surfaces after a relatively long incubation period.

The aptitude for biofilm formation varies between pneumococcal isolates, and various bacterial elements impact this process. For example, capsule production is considered adversarial; unencapsulated strains produce more biofilms, mutants in genes influencing capsule production show increased biofilm formation (21), and biofilm pneumococci downregulate capsule production (19, 21–23). Free sialic acid supports *S. pneumoniae* biofilms (24), and inactivation of the bacterial neuraminidase, *nanA*, which cleaves terminal sialic acid from the host glycans, attenuates biofilm development (18). The choline residues of *S. pneumoniae* teichoic and lipoteichoic acids also play a role in biofilms, and the choline uptake operon, *lic*, contributes to the formation of the biofilm matrix (10, 13). *S. pneumoniae*'s choline-binding proteins (CBPs), which are anchored to the cell wall by binding the surface choline residues, include various enzymes and adhesins and interact with DNA *in vitro* (25). Incubation of *S. pneumoniae* with a high concentration of choline chloride inhibits the enzymatic activity, leads to the release of some CBPs from the surface, and attenuates biofilm formation (19). Still, the contribution of individual CBPs to biofilms varies. The loss of some CBPs (e.g., the murine enzymes *lytA*, *lytC*, and *lytB* or the adhesins *cbpA*, *pcpA*, and *pspA*) results in decreased biofilms, but inactivation of other CBPs (e.g., *pce* and *cbpD*) has no impact (25).

The two quorum-sensing systems, *luxS/AI-2* and *com*, also impact biofilm development (15, 26). A mutant in *luxS*, an important enzyme-producing AI-2, produces less biofilm than the wild-type strain during middle ear infection in rats (27) and model systems involving either abiotic surfaces or human respiratory epithelial cells (15, 28). Similarly, inactivation of the *comC* gene (encoding the competence-stimulating peptide [CSP]) impairs biofilms on abiotic surfaces (29) and human respiratory epithelial cells (15). Interestingly, the AI-2 autoinducer is needed during the early stages of biofilm formation and the CSP-1 peptide promotes biofilm stability and maturation on abiotic surfaces (29). In summary, biofilm development by *S. pneumoniae* is multifactorial and multiple regulatory circuits influence it.

We recently discovered that addition of hemoglobin to the culture medium triggers unusually robust growth in *S. pneumoniae* and an extensive transcriptome shift that includes many metabolic genes and virulence factors necessary for nasopharyngeal colonization and lung infections (30). Here, we followed up on these observations and tested the hypothesis that hemoglobin also influences *S. pneumoniae* biofilm development.

RESULTS

Hemoglobin induces an early formation of *S. pneumoniae* biofilms *in vitro*. The addition of human hemoglobin to iron-depleted Todd-Hewitt broth containing yeast extract (THYB), THYB with the iron chelator 2,2'-di-pyridyl [THYB-DP], or regular THYB (iron complete) facilitates vigorous pneumococcal growth that exceeded the one observed when the media were supplemented with free iron or heme (30). To better understand these observations, we tested here whether additional heme sources found in the host impact *S. pneumoniae* growth. THYB-DP was inoculated with *S. pneumoniae* D39 (serotype 2), and the cultures were grown in microtiter plates for 18 h in the presence of equine myoglobin or human serum. THYB-DP, as well as THYB-DP supplemented with free heme, iron, or hemoglobin, served as the controls (Fig. 1). The addition of 20 μ M myoglobin or serum (5 to 40% [vol/vol]) reinstated growth in the iron-deprived medium that surpassed the one observed in medium complemented

FIG 1 Myoglobin and serum promotes 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 DP, THYB with DP and 2 mM FeNO₃, 10 μ M heme, 10% human serum, 20 μ M equine myoglobin, or 20 μ M hemoglobin. (B) THYB, THYB supplemented 80 μ M FeNO₃, 10 μ M heme, 10% human serum, 20 μ M equine myoglobin, or 20 μ M hemoglobin. The data are representative of three independent experiments performed in triplicates; error bars indicate the standard deviations (SD).

with iron or heme (Fig. 1A and data not shown). As with hemoglobin, the addition of myoglobin and serum improved growth also in standard THYB (Fig. 1B). 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.

We noticed that *S. pneumoniae* cultures grown for 18 h 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. 2A) and iron-depleted THYB (Fig. 2B). Biofilm induction by hemoglobin occurred in a dose-dependent manner, starting with 0.5 μ M hemoglobin in THYB (Fig. 2A) or 5 μ M hemoglobin in THYB-DP (Fig. 2B). We did not observe biofilm formation with heme or free iron. Importantly, myoglobin or serum did not trigger biofilm formation, although they supported more pneumococcal growth. The addition of a control protein (bovine serum albumin [BSA], which has a molecular weight [MW] similar to that of hemoglobin) or denatured hemoglobin also did not stimulate biofilms. To control for the possible involvement of contaminant in the hemoglobin preparation, we filtered the hemoglobin solution using 10,000-MW cutoff and tested both fractions. Although filtered hemoglobin induced biofilms, the flowthrough did not (Fig. 2C). Finally, hemoglobin induced biofilms also in different *S. pneumoniae* strains (Fig.

FIG 2 Hemoglobin induces biofilm formation in *S. pneumoniae*. THYB was inoculated with *S. pneumoniae* cells grown on BAPs ($OD_{600} = 0.05$). Cultures were grown in microtiter plates; biofilms (18 h) produced by D39 (serotype 2) are shown. (A) THYB, THYB with $80 \mu\text{M}$ FeNO_3 , $10 \mu\text{M}$ heme, $20 \mu\text{M}$ equine myoglobin, 10% human serum, $20 \mu\text{M}$ BSA, or 0.5 to $20 \mu\text{M}$ hemoglobin. (B) THYB with 3 mM DP, THYB with DP and 2 mM FeNO_3 , $10 \mu\text{M}$ heme, $20 \mu\text{M}$ equine myoglobin, 10% human serum, $20 \mu\text{M}$ BSA, or 0.5 to $20 \mu\text{M}$ hemoglobin. (C) THYB with $20 \mu\text{M}$ 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 $20 \mu\text{M}$ 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 $\Delta\text{spbhp-37}$ (stripes) strains grown in THYB or in THYB with 5 to $20 \mu\text{M}$ hemoglobin. The data show means $\pm$ 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 $\Delta\text{spbhp-37}$ in panel E [Student *t* test]).

2D), 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, Spbhp-37, contributes to the iron acquisition from hemoglobin (31) and a D39 $\Delta\text{spbhp-37}$ mutant exhibits an attenuated growth compared to the parental strain also in regular THYB supplemented with hemoglobin (30). Examining the $\Delta\text{spbhp-37}$ mutant for biofilm formation revealed that it produced similar or slightly higher biofilm biomass compared to the wild-type strain (Fig. 2E), suggesting biofilm formation is independent of this heme uptake protein.

Pneumococci grown in THYB supplemented with hemoglobin formed noticeable biofilm biomass as early as 6 h postinoculation, peaking within 18 h of incubation. A lower level of biomass was observed at the 24- and 48-h time points (Fig. 3A). In contrast, cells grown in THYB produced little to no biofilms up to 48 h of incubation. In the presence of hemoglobin, we recovered 3.6-fold more live cells from the microplate surface compared to cultures that grew in THYB alone ($[2.2 \pm 0.57] \times 10^8$ versus $[6.1 \pm 1.94] \times 10^7$, respectively, $P = 0.05$). We also used a more sensitive, fluorescence-based method and confocal microscopy to investigate the timing of biofilm formation. Biofilms produced by strain D39 were visualized by staining the pneumococcal capsule and DNA. Confocal images (xy optical sections) revealed a rapid formation of biofilms

FIG 3 Hemoglobin induces unusually early and robust biofilms. THYB with 20 μ M hemoglobin was inoculated with D39 grown on BAPs (starting OD₆₀₀ = 0.05). (A) Cultures were grown in microtiter plates, and biofilm was quantified at different time points. The data are expressed as means $\pm$ the SD of at least two independent experiments each performed in triplicates. The asterisks denote statistically significant difference: $P \leq 0.05$ (THYB-Hb versus THYB [Student *t* test]). (B and C) Confocal micrographs of biofilms produced by GFP-expressing D39 (28) grown in 8-well glass wells: THYB (B) or THYB with 20 μ M hemoglobin (C). The DNA was treated with TO-PRO-3 (green), and the capsule was treated with Alexa Fluor 555-labeled anti-serotype specific antibodies (red).

that started at 2 h postinoculation and plateau at 8 h when D39 was incubated with hemoglobin (Fig. 3C).

Ferric iron ($\sim 50 \mu$ M) promotes biofilms in *S. pneumoniae* grown in semisynthetic medium (C+Y) (26, 32). We examined biofilm formation in THYB or THYB-DP supplemented with ferric iron (50 μ M to 2 mM). The iron did not impact biofilm formation under our experimental conditions, perhaps because of the different assay and growth conditions (THYB may contain more iron than C+Y). These observations are consistent with the finding that inactivation of *spbhp-37* did not hamper biofilm formation (Fig. 2E). Still, since the iron stimulation of biofilm is dependent on *luxS*, we tested the impact of hemoglobin on growth and biofilm formation by a *luxS* mutant (28). As Fig. 4 shows, hemoglobin stimulated growth and biofilms in the $\Delta luxS$ and isogenic D39 parental strains to a similar extent. Together, these observations suggest that *S. pneumoniae* biofilm reaction to hemoglobin is mediated by a mechanism that is different from the reported response to ferric iron and is independent of *luxS*.

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

FIG 4 *S. pneumoniae* growth and biofilm induction by hemoglobin are independent of *luxS*. (A and B) Growth (A) and biofilms (B) by isogenic D39 wild-type (red) and $\Delta luxS$ (purple) strains grown in THYB (empty symbols and bars) or in THYB with 20 μ M hemoglobin (full symbols and bars). Biofilms were visualized and quantified after 18 h. NS, not statistically significant (+Hb, WT versus $\Delta luxS$ [Student *t* test]). The data are representative of at least two experiments performed in triplicates.

FIG 5 Blood cells activate biofilm formation in a *ply*-dependent manner. Fresh medium was inoculated (starting OD₆₀₀ = 0.05) with wild-type (WT) or Δ *ply* 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 THYB (empty) or THYB with 0.3% washed human blood cells (filled) (B) and in THYB (empty) or THYB with 20 μ M hemoglobin (stripes) (C). The data are expressed as means $\pm$ 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)]).

blood cells (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 (Fig. 5A). Since Ply releases hemoglobin from blood cells *in vitro* (33–35), we sought to determine whether the pneumolysin is needed for the induction of biofilms by blood. Previously described *ply* knockouts in both D39 and TIGR4 backgrounds (36) did not produce biofilms when grown in THYB supplemented with blood (Fig. 5B), suggesting that the absence of hemolytic activity prevents biofilm induction by erythrocytes in the *ply* mutants. *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 (Fig. 5C). Therefore, similar to our previous findings (36), the data suggest that Ply contributes to biofilm development independently of its hemolytic activity.

Compared to pneumococci that remain planktonic, *S. pneumoniae* transitioning into an adherent state modulates its transcriptome within 1 and 2 h after hemoglobin addition. We recently described the significant growth enhancement and transcriptome remodeling hemoglobin is inducing in planktonic *S. pneumoniae* (30). Since we found that hemoglobin also triggers exceptionally early biofilm development, we aimed to probe the molecular events contributing to this prompt change in *S. pneumoniae* lifestyle. We rationalized that shortly after hemoglobin addition, genes needed for this transition will exhibit differential gene expression compared to pneumococci that remained planktonic. Hence, we investigated the *S. pneumoniae* transcriptome among planktonic and adherent cells 1 and 2 h after hemoglobin treatment by RNA-Seq (transcriptome sequencing). Since only a small number of pneumococci grown in THYB are found in an adherent state even after 18 h of incubation (Fig. 3A), we could not recover sufficient sessile cells from the cultures grown without hemoglobin at these early time points. In the absence of this group, we could not compare the transcriptome in early adherent state between cells grown with and without hemoglobin. Still, comparing between early adherent and planktonic pneumococci (both grown with hemoglobin) allows us to learn what takes place in cells that are just transitioning from free into sessile growth under the influence of hemoglobin.

Analysis of the global transcriptome revealed that newly attached pneumococci underwent an extensive transcriptome shift compared to cells that remained free living (see Table S1 in the supplemental material). Using quantitative reverse transcription-PCR (qRT-PCR) on a subset of regulated genes, we validated the RNA-Seq findings (see Fig. S3). Within the first hour after the addition of hemoglobin, 322 and 136 genes were up- and downregulated in adherent cells, respectively (Fig. 6A and B). Most of the transcriptome changes took place within the first hour after hemoglobin addition, and in the second hour, only 81 genes were induced, and 35 were repressed compared to cells that remained planktonic. Figure 6C illustrates the functional gene categories

FIG 6 *S. pneumoniae* transition from planktonic to biofilm growth in the presence of hemoglobin involves significant transcriptome remodeling. A Venn diagram (using R) of differentially expressed genes in adherent compared to planktonic cells (fold change ≥ 2) 1 h (pink and yellow [A and B, respectively]) and 2 h (green and purple [A and B, respectively]) after hemoglobin addition is shown. (A) Upregulated genes. (B) Downregulated genes. (C) Genes exhibiting altered expression (fold change ≥ 2) within 1 and/or 2 h of growth with hemoglobin are included.

most influenced by the switch into an adherent state within 1 and/or 2 h posttreatment. These include genes involved in carbohydrate uptake and metabolism, as well as bacteriocins production and quorum sensing. The data also showed that 13 genes involved in translation were repressed in newly adherent cells. In addition, 17 genes that contribute to translation and three for transcription were activated in adherent cells compared to planktonic cells grown with hemoglobin. The choline moiety of *S. pneumoniae* teichoic acids is important for biofilm formation. We noted that five genes involved in choline uptake (*proWX* and *proV*), metabolism (*pck*), or binding (*cbpF* and *cbpD*) were activated and that the CBP, *pcpA*, was repressed (see Table S1). The expression of *lytB*, a peptidoglycan hydrolase that binds choline, was induced by hemoglobin during planktonic growth (30; accession number [PRJNA626052](#)) and remained expressed to a similar level in adherent pneumococci (data not shown; accession number [PRJNA642413](#)).

Many genes that facilitate carbohydrate transport and metabolism were differentially expressed in sessile pneumococci compared to planktonic cells at the 1- and/or 2-h time point (Fig. 6C). Of the 63 induced genes, the glycerol uptake and metabolism operon, *glpKOF* (encoding a glycerol kinase, glycerol-3-phosphate oxidase, and a glycerol facilitator, respectively) exhibited the highest activation (Fig. 7A). Carbohydrate

FIG 7 Genes activated in *S. pneumoniae* transitioning into biofilms. The relative expression of selected genes that are upregulated in adherent versus planktonic cells (both grown with hemoglobin) at 1 h after hemoglobin addition (y axis) is plotted for D39 genes (x axis). (A) The log₂-fold change (≥ 2 -fold) of genes involved in the transport or metabolism of sugars derived from the host glycans. Shown are gene cluster encoding PTS (blue), enzymes (gray), transporter (orange), facilitator (green), and transcriptional regulator (white). Genes (within the cluster) encoding hypothetical proteins are not shown. (B) Genes or gene clusters involved in various functions. Region of diversity 12 (RD12) encodes a lantibiotic-synthesis gene cluster (38, 39).

transporters and enzymes that were activated during the shift into adherent state included several systems for the uptake and catabolism of the carbohydrate moieties of the host glycans such as sialic acid, β -glucosides, galactose, and mannose (Fig. 7A). The capsule and saccharide synthesis genes (*cps2P*, *cps2L*, *rfbC*, and *rfbB*) were induced 2-fold in the first hour. We also noticed an activation (4- to 6-fold) of the glycogen synthesis operon, *glgBCDA*, which converts glucose 1-phosphate to glycogen. By reducing the cellular availability of glucose, *glgBCDA* induction can end up limiting the amount of UDP-GlcNAc required for capsule synthesis (37).

In the first hour after hemoglobin treatment, the transition from planktonic to sessile growth was also associated with the induction of quorum-sensing and several two-component systems (TCS) (Fig. 7B). We previously found that the signaling peptide gene *tprA/phrA* (SPD_1746) is activated 2-fold in planktonic cells 1 h after hemoglobin addition (30; accession [PRJNA626052](https://pub.ncbi.nlm.nih.gov/record/PRJNA626052)). In sessile cells, we observed an earlier and more robust induction of *tprA/phrA* along with the associated lantibiotic-synthesis gene cluster (encoded by region of diversity 12 [38, 39]). Notably, the TrpA/PhrA-controlled

gene cluster is not expressed in regular laboratory medium, but its transcription is activated during heart infection (11). The lantibiotic immunity genes—*blpYZ*, the *comCDE* operon encoding the CSP-1 peptide, and the *comDE* TCS—were also induced, together with several genes from the *com* regulon (i.e., *amiEDCA*, *cbpD*, and *cinA*). Altogether, five TCSs were activated in the early stage of the switch into the adherent state reflecting the complexity of the transition in lifestyle.

In addition to the changes mentioned above, we noted the induction of enzymes acting on molecules found in the host tissue and extracellular matrix. These enzymes include putative hyaluronate lyase (SPD_0287) and heparinase (SPD_0300), as well as the neuraminidase, *nanA* (Fig. 7B), which is essential for biofilm formation in the nasopharynx (40). Genes involved in heme uptake (i.e., *piuBC* and *spbhp-22* operon) (41–43) and heme binding (i.e., *glnA* and *malX* [44]) were also induced by *S. pneumoniae* cells recovered from the plate surface compared to free-living pneumococci (Fig. 7). A number of characterized and putative proteins involved in zinc acquisition and enzymes that require zinc were activated, as well in the early stages of sessile growth. On the other hand, the ferrichrome-binding protein, *piaA* (45), and the virulence factors, *pfbA* (46), *pavA* (47, 48), and *pspA* (49), were all repressed in biofilm cells compared to planktonic *S. pneumoniae* growing with hemoglobin. In summary, the *S. pneumoniae* switch into sessile growth is associated with a large shift in gene expression and involves a multifaceted regulatory scheme.

Biofilm formation in the presence of hemoglobin involves the *comC* gene but not the related TCS *comDE*. 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* (15, 29, 50), the TCS *ciaRH*, and *yesMN* and in the histidine kinase *vicK* (*vicR* is an essential gene) were tested for growth (see Fig. S1) and biofilm formation (see Fig. S2 and S8) with or without hemoglobin. Other than the Δ *ciaRH* mutant, the mutants grew similarly to the wild type in THYB (see Fig. S1A) and created minimal but similar biomass on the plate surface after 18 h of incubation in THYB (see Fig. S2). The *ciaRH* mutant exhibited severely attenuated growth and a reduced biofilm biomass in THYB. Hemoglobin enhanced the growth of all strains, including that of the *ciaRH* mutant (see Fig. S1B). Compared to the wild-type strain, we did not observe a significant difference in biofilm generated by the Δ *yesMN* and Δ *vicK* mutants after 18 h of incubation (Fig. 8A). However, inactivation of *ciaRH* or *comC* resulted in a 37% ($P = 0.01$) or a 67% ($P = 0.001$) reduction, respectively.

As described above, *comC* inactivation was the most detrimental for biofilm development, and this phenotype was specific to THYB with hemoglobin. To explore this further, we examined individual deletion mutants in the histidine kinase CSP-receptor (*comD*) and the response regulator (*comE*) (29, 51, 52), which consists of the canonical pathway for CSP-1 signaling. Surprisingly, *comD* deletion resulted only in a minor reduction in biofilm production ($\sim 20\%$, $P = 0.04$), and the deletion of *comE* had no impact (Fig. 8B). Supplementing the medium with synthetic CSP-1 complemented the Δ *comC* and Δ *comD* phenotypes but had no significant influence on biofilm production by the Δ *comE* or the wild-type strains grown with or without hemoglobin (Fig. 8B). Together, the data demonstrate that hemoglobin triggers biofilms in a pathway that involves CSP-1 signaling. Importantly, in the presence of hemoglobin, *comC* contributes to biofilm development mostly independently of its cognate TCS *comDE*.

DISCUSSION

Biofilms are important for asymptomatic pneumococcal carriage as well as disease, and the pathogen alternates between planktonic and biofilm growth as it spreads from the upper respiratory tract and establishes infections at different sites (20, 22, 47, 53). The factors that promote one or the other lifestyle choices in *S. pneumoniae* are not fully understood. Iron availability affects biofilm formation in multiple bacterial pathogens, including *S. pneumoniae*. The heme in hemoglobin and other host proteins

FIG 8 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 OD₆₀₀ = 0.05). (A and B) Biofilm biomass produced by *S. pneumoniae* grown after 18 h in THYB or THYB with 20 μ M hemoglobin (Hb) (A and B), 200 ng/ml CSP1 (B), or Hb and CSP1 (B). The data are expressed as the means $\pm$ 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 +Hb versus -Hb and of Hb versus Hb+CSP-1 [two-way ANOVA]).

is the largest pool of iron within the human body (54–56). Here, we investigated the influence of the host hemoglobin and additional heme sources on *S. pneumoniae* growth and lifestyle.

Experiments with myoglobin and serum revealed that, like hemoglobin (30), they both stimulated pneumococcal planktonic growth in iron-depleted and iron-complete THYB beyond the level seen with free heme, iron, or in regular medium (Fig. 1). Therefore, growth induction is shared by several hemoproteins, although human hemoglobin has the most pronounced effect. Earlier analysis revealed an equal amount of intracellular iron in *S. pneumoniae* grown in THYB either with or without hemoglobin (30). This observation does not support the notion that the growth benefits of hemoproteins stem from increased iron bioavailability. Free iron and heme can be harmful due to the formation of reactive oxygen species and the lipophilic nature of heme (57, 58); it is possible that hemoproteins offer a less-damaging form of iron. Interestingly, the human pathogens *Staphylococcus aureus* and *Streptococcus pyogenes* exhibit a preference for heme iron over ferric iron (from transferrin or in a free form) (57, 58). It took longer for *S. pneumoniae* to initiate growth in THYB-DP with myoglobin compared to hemoglobin and serum. We used human hemoglobin and serum in this study; it is plausible that the strictly human pathogen, *S. pneumoniae*, is better adjusted to use human heme sources than the equine myoglobin.

S. pneumoniae, an avid colonizer of the human nasopharynx, exhibits surprisingly limited biofilm development on abiotic surfaces (8, 18, 53). One of the central

discoveries we made in this study is that hemoglobin stimulates not only planktonic growth but also triggers robust biofilms in static models with multiple pneumococcal strains (Fig. 2). Control experiments established that biofilm activation is specific to hemoglobin and requires the protein in its native form. Notably, myoglobin and serum did not impact biofilm formation. Therefore, the hemoglobin-only activation of biofilms is probably mechanistically separated from growth induction that is shared by several hemoproteins (Fig. 1). In the presence of hemoglobin, pneumococcal biofilm growth on either polystyrene or glass surfaces was remarkably early (Fig. 3). The finding that the simple addition of hemoglobin triggered early and vigorous biofilm growth under growth conditions that otherwise do not facilitate significant biofilm formation, even after 48 h, implicates hemoglobin as one of the host factors that promote pneumococcal biofilm development during infection.

Growth in iron-restricted conditions stimulate biofilm in some pathogenic bacteria (e.g., *Acinetobacter baumannii* [59], *S. mutans*, and staphylococci [60]). Still, in other pathogens, such as *Pseudomonas aeruginosa* (61, 62), biofilm formation is activated by the presence of iron. A previous study showed that *S. pneumoniae* belongs to the second group and that iron promotes biofilms on abiotic surfaces in pneumococci grown in C+Y medium (26). While hemoglobin can provide *S. pneumoniae* with heme iron, the data suggest that, in our model system, hemoglobin induces biofilm in another way. Free iron or heme had no impact on biofilms in THYB (Fig. 2A). The Δ *spbhp-37* mutant was not impacted in hemoglobin induction of biofilms (Fig. 2E), although its growth on heme and hemoglobin iron is attenuated (30). Lastly, stimulation of pneumococcal biofilm by iron requires *luxS*, which promotes iron import, but the experiments with a Δ *luxS* mutant revealed no difference in hemoglobin-dependent biofilm formation (Fig. 4). We thus suggest that hemoglobin directly activates biofilms in *S. pneumoniae* independently of its ability to donate iron.

Hemoglobin is found in nasal sections as a result of local bleed caused by trauma, dry conditions, or the use of medications (63–65). Infections can also promote the release of hemoglobin into the cellular environment due to hemolytic activity and the host immune response. Pneumolysin (Ply) can release hemoglobin from erythrocytes (33–35). The addition of blood cells to the growth medium also promoted pneumococcal biofilms, and this activity required the *ply* gene (Fig. 5). Therefore, the presence of labile hemoglobin and blood in the upper respiratory tract secretions may stimulate biofilm formation and *S. pneumoniae* colonization. The same could take place also in other sites in which *S. pneumoniae* develops biofilms (e.g., the ear and the lungs).

Only two studies implicate hemoglobin as a host colonization factor. The addition of hemoglobin to a defined medium restored surface binding and adherence by the pathogenic yeast *Candida albicans*, which are otherwise observed only during growth in a complex medium or *in vivo* (66). This hemoglobin activity is independent of iron acquisition and is mediated by a receptor on the yeast surface. Similarly, hemoglobin in nasal secretions enhances nasal colonization by *S. aureus* and promotes surface adherence and biofilm formation *in vitro* (64). Staphylococcal biofilms were observed with either apo or holo hemoglobin, but not with free heme or myoglobin, and was independent of the staphylococcal hemoglobin receptors IsdB, and IsdH. Therefore, in both *C. albicans* and *S. aureus*, the colonization response is specific to hemoglobin and independent of the hemoglobin ability to provide the bacteria with heme iron. To the best of our knowledge, these are the only incidences in which the host hemoglobin was reported to impact microbial colonization. Still, the similarity between the response to hemoglobin in *S. pneumoniae*, *S. aureus*, and even by the yeast *C. albicans*, is significant and raises the possibility that other pathogens might have evolved to respond to the host hemoglobin in a similar manner.

Transcriptome analysis showed that newly adherent *S. pneumoniae* exhibits major changes in gene expression compared to cells that remained planktonic after hemoglobin treatment (Fig. 6). Newly sessile *S. pneumoniae* activated genes involved in the metabolism of carbohydrates, amino acids, lipids, and nucleotides, as well as enzymes

involved in host interactions (e.g., *nan A*), redox, and cell wall functions. These pneumococci also reduced the expression of many genes involved in protein synthesis, the energy-generating ATP synthase, and some virulence factors (Fig. 6 and 7). The transcriptome changes in new adherent cells contain some overlap and trends similar to changes in gene expression recorded for early pneumococcal biofilms at later time points (20, 53, 67). Hence, these shifts in gene expression are likely needed early to facilitate the *S. pneumoniae* switch into sessile growth.

Hemoglobin induces in planktonic cells genes involved in the use of the host glycan as a carbon source (30) (accession [PRJNA626052](#)). In this study, the comparison between planktonic and sessile cells grown with hemoglobin revealed in adherent cells the activation of additional operons and gene clusters that facilitate the uptake and metabolism of glycan-derived sugars (Fig. 7A). Some of the phosphotransferase system (PTS) that were activated by hemoglobin in planktonic growth remained expressed to the same level in newly sessile cells (e.g., SPD_0293-95, which recognizes several sugars, including fructose and *N*-acetylglucosamine [data not shown]). Other systems (e.g., the galactose PTS encoded by SPD_0066-69) were further induced when cells grown with hemoglobin transitioned into an adherent state (data not shown). In summary, *S. pneumoniae* transitioning into sessile growth express many genes that allow it to take advantage of the sugars found in mucin and/or the epithelial surfaces, which are largely depleted of free glucose.

We previously noted that hemoglobin induces the expression of the zinc uptake gene, *phtD*, in planktonic *S. pneumoniae* (30). Here, we observed an additional activation of *phtD* in cells that switched into sessile growth, along with the induction of the other zinc uptake genes in *S. pneumoniae* and a number of zinc-dependent enzymes (Fig. 7B). These changes in gene expression, which suggest an increase in zinc uptake and use, are consistent with the positive correlation between zinc levels and pneumococcal aggregation and biofilm formation previously reported (68).

The induction of several TCS accompanied the pneumococcal transition into biofilms (Fig. 7B). Mutant analysis of selected genes demonstrated that only *comC* and *ciaRH* play a major role in the biofilm development under our experimental condition (Fig. 8A). The observation that the *ciaRH* mutant is also impacted in growth (see Fig. S1A) and biofilm development in regular THYB (see Fig. S2) 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 (69–72). Curiously, the addition of hemoglobin improved the mutant's planktonic growth in THYB significantly (see Fig. S1B), raising the possibility that hemoglobin activates signaling downstream of the *ciaRH* signaling pathway.

The *comC* mutant exhibited a significant defect in biofilm growth in the presence of hemoglobin (Fig. 8A). Moreover, while exogenically added CSP-1 complemented the *comC* phenotype in THYB containing hemoglobin (Fig. 8B), 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 the 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 (32). 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.

In summary, the addition of hemoglobin to *S. pneumoniae* culture grown on abiotic

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
Strains		
<i>S. pneumoniae</i>		
D39	Avery strain, clinical isolate (capsular serotype 2), CSP1	79, 80
D39 (SPJV01)	GFP-expressing strain, D39/pMV158GFP (Tet ^r)	28
TIGR4	Invasive clinical isolate (capsular serotype 4), CSP2	81
8655	Invasive isolate (capsular serotype 6B), CSP2	CDC
3875	Invasive isolate (capsular serotype 6B), CSP1	CDC
Δ spbhp-37 (SPZE1)	D39 Δ spbhp-37::ermB (Ery ^r)	30
Δ ply (SPJV14)	D39 Δ ply::ermB (Ery ^r)	36
Δ ply (SPJV18)	TIGR4 Δ ply::ermB (Ery ^r)	36
Δ luxS (SPJV05)	D39 Δ luxS::ermB (Ery ^r)	28
Δ comC (SPJV10)	D39 Δ comC::ermB (Ery ^r)	15
Δ comD (SPJV31)	D39 Δ comD::catP (Cm ^r)	This study
Δ comE (SPJV32)	D39 Δ comE::catP (Cm ^r)	This study
Δ ciaRH (SPZE2)	D39 Δ ciaRH::ermC (Ery ^r)	This study
Δ yesMN (SPZE3)	D39 Δ yesMN::ermC (Ery ^r)	This study
Δ vicK (SPZE4)	D39 Δ vicK::cat194 (Cm ^r)	This study
<i>E. coli</i>		
OneShot Top10	Cloning host	Invitrogen
Plasmids		
pMV158GFP	<i>S. pneumoniae</i> mobilizable plasmid containing the green fluorescent protein gene	82
pJRS233	Temperature-sensitive shuttle vector encoding the ermC gene (Ery ^r)	83
pDC123	Gram-positive vector encoding the cat-194 gene (Cm ^r)	84
pAF105	pUC19-derived plasmid with the Δ ciaRH::ermC allele and the bla gene (Amp ^r)	This study
pAF106	pUC19-derived plasmid with the Δ vicK::cat194 allele and the bla gene	This study
pAF108	pUC19-derived plasmid with the Δ yesMN::ermC allele and the bla gene	This study

surfaces (without any other change in medium composition and growth condition) allows for rapid and robust biofilm development. Our findings suggest that the host hemoglobin serves as a colonization/attachment signal during infection, influencing the pneumococcal interchange between planktonic and sessile states. Hemoglobin promotes biofilms and colonization in two other microbial pathogens, suggesting a possible new theme in host-microbe interactions (64, 66).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. pneumoniae* strains used in this study are listed in Table 1. Frozen *S. pneumoniae* stocks were prepared in the medium skim milk-tryptone-glucose-glycerin (STGG), as described previously (73), and kept at -80°C . *S. pneumoniae* STGG stocks were plated on tryptic soy blood agar plates (BAPs) and incubated overnight at 37°C under microaerophilic conditions. *S. pneumoniae* was grown in Todd-Hewitt broth containing 0.5% (wt/vol) yeast extract (THYB). One or more of the following supplements were added to the growth medium as indicated: the iron chelator 2,2'-di-pyridyl (ACROS Organics); bovine hemin, BSA, human hemoglobin, and equine myoglobin (Mb; all from Sigma-Aldrich); ferric nitrate nonahydrate (FeNO_3 ; Fisher Scientific); whole human blood (BioIVT); human serum (Innovative Research); and competence-stimulating peptide (CSP1, 200 ng/ml; Annaspec). The antibiotics erythromycin (0.5 $\mu\text{g/ml}$), chloramphenicol (4 $\mu\text{g/ml}$), and tetracycline (1 $\mu\text{g/ml}$) were added to the BAPs when needed. In some experiments, heat-inactivated hemoglobin (99 $^{\circ}\text{C}$ for 5 min) or filtered hemoglobin (Amicon Ultra centrifugal filters, MWCO 10,000) or the flowthrough fraction was added to the growth medium.

Growth assays. Fresh THYB medium (with or without supplements) was inoculated with *S. pneumoniae* cells (serotypes 2, 4, and 6; Table 1) collected from BAPs after 18 h of incubation (starting culture optical density at 600 nm [OD_{600}] of 0.05). Cell cultures (200 $\mu\text{l/well}$) were grown in 96-well microtiter plates (Costar 3595; Corning) incubated at 37°C . The culture OD_{600} was recorded at 1-h intervals for the indicated times using (SpectraMax M2 spectrophotometer; Molecular Devices). For each growth condition, we used wells containing only the medium (and supplements when appropriate) as the blank. Bacterial growth was tested in triplicates.

Biofilms in microtiter plates. Fresh THYB medium (with or without supplements) was inoculated with *S. pneumoniae* cells (serotypes 2, 4, and 6) as described above, and the cultures were grown in 96-well microtiter plates for up to 48 h. In some experiments, whole human blood suspensions (0.1, 0.3, and 0.5%) were prepared in sterile phosphate-buffered saline (PBS), washed three times by centrifugation (500 $\times g$, 5 min, 4°C), and the blood cells were suspended in THYB prior to inoculation with *S. pneumoniae*. In other experiments, normal pooled human serum was added to the THYB (2.5 to 40%),

and the medium was filter sterilized and used in the assay. Crystal violet staining was used to quantitate the biofilm biomass at different time points, as indicated in the text. The growth medium was removed by aspiration, and plates were washed five times with ddH₂O and allowed to dry for 15 min. Crystal violet (0.1%) was added, and the plates were incubated for 15 min with slow shaking (Lab-Line Maxi-Rotator). The wells were washed and allowed to dry for 15 min before the addition of 95% ethanol. The suspensions were mixed vigorously by pipetting after 15 min of incubation at room temperature. The absorbance at 590 nm was determined by a SpectraMax M2 spectrophotometer (Molecular Devices). To determine viability, biofilms were washed twice with sterile PBS at designated time points, and the plates were sonicated for 15 s in a Cole-Parmer ultrasonic cleaner, followed by vigorous pipetting; the cells were then collected. Cell counts (CFU/ml) were determined by plating into BAPs.

Confocal microscopy. Overnight BAP cultures of D39 were used to inoculate 8-well glass plates as with the microtiter assay described above. At the indicated time points, the wells were washed three times with PBS, fixed with 2% paraformaldehyde, and stained at room temperature, as previously described (15). The capsule was stained with a rabbit polyclonal antibody against serotype 2 capsule (i.e., D39) conjugated with Alexa Fluor 555 (Molecular Probes, Invitrogen) for 30 min. Nucleic acids were stained with TO-PRO-3 (Molecular Probes) for 15 min. After three washes with PBS, the plates were mounted with Vectashield mounting medium (Vector Laboratories) and analyzed with a Zeiss LSM 510 confocal microscope. Confocal images were analyzed with an LSM image browser, v4.0.2.121.

RNA preparation from early adherent pneumococci 1 and 2 h after hemoglobin treatment. Cell growth and RNA preparation were performed, as we recently described in Akhter et al. (30). Briefly, fresh THYB was inoculated with *S. pneumoniae* D39 (serotype 2) cells from frozen logarithmic stocks (starting culture OD₆₀₀ of 0.02), and the cultures were grown in 12-well microtiter plates (2 ml/well) at 37°C. Next, 20 μM hemoglobin (in 0.9% saline) or 0.9% saline (negative control) was added to the growing cells at the early logarithmic phase (after ~3 h, OD₆₀₀ = 0.2 to 0.3). The cultures were allowed to continue to grow and the medium was removed 1 or 2 h after hemoglobin treatment, and the plates were washed with sterile PBS (1 ml). Adherent cells were released by sonication in PBS (1 ml) for 15 s. Samples were collected and processed with RNA protect reagent (Qiagen) according to the manufacturer's recommendations, and the pellet was stored at -80°C. Cell samples were suspended in 700 μl of TRIzol with 300 mg of acid-washed glass beads (Sigma Life Science), followed by disruption by vortexing. Total RNA (two biological replicates) was prepared by using a Direct-Zol RNA MiniPrep kit (Zymo Research). DNA was removed by using a Turbo DNase-free kit (Life Technologies). rRNA was eliminated with a Ribo-Zero Magnetic kit for Gram-positive bacteria (Epicenter). RNA Quality and quantity were assessed by using a 2100 Bioanalyzer (Agilent) and a NanoDrop 8000 spectrophotometer (Thermo Scientific), respectively.

RNA-Seq analysis. RNAseq 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 sequencing facility at the University of Maryland—College Park using the Illumina HiSeq 1500 platform. Data were generated in the standard Sanger FastQ format, and raw reads were deposited with the Sequence Read Archive at the National Center for Biotechnology Institute (accession number PRJNA642413). Read quality was evaluated using FastQC software, and mapping against the *S. pneumoniae* D39 genome was completed using Bowtie package alignment software (74). The read count or raw count data for all genes were acquired using the Featurecount package (75). These raw count data files were then used in the DESeq2 package (76) to calculate differential expression analysis 1 and 2 h after hemoglobin addition between newly adherent (samples collected as described above) and planktonic *S. pneumoniae* (data derived from four biological replicates collected and analyzed as described in Akhter et al. [30]). The DESeq2 package was used for differential expression analysis with raw count data for all samples for pairwise comparison.

qRT-PCR analysis. Quantitative reverse transcription-PCR analysis was carried out using a Power SYBR Green RNA-to-Ct 1-Step kit (Applied Biosystems) and a 7500 Fast Real-Time PCR machine (Applied Biosystems) according to the manufacturers' specifications. A total of 25 ng of RNA (from two independent replicates) was used per qRT-PCRs, and each reaction was performed in duplicate. The 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. qRT-PCR validation of RNA-Seq done with RNA samples from planktonic *S. pneumoniae* is described in Akhter et al. (30).

Construction of mutant strains. The plasmids used in this study are listed in Table 1, and the primers are shown in Table 2. To generate a deletion mutation in *ciaRH*, *yesMN*, and *vicK* genes, mutant alleles containing an antibiotic resistance gene flanked by the 5' and 3' genomic regions of the targeted genes were prepared for each mutant using the GeneArt seamless cloning kit (Thermo Fisher Scientific). Briefly, the appropriate genomic segments were amplified from the D39 chromosome. The *ermC* gene, *cat-194*, and the pUC19 vector were amplified from plasmids pJRS233, pDC123, and pUC19-L, respectively. All PCR fragments were purified using a Minelute PCR purification kit (Qiagen) and cloned into One-Shot Top10 *E. coli*, generating plasmids pAF105 (Δ*ciaRH*), pAF106 (Δ*vicK*), and pAF108 (Δ*yesMN*). The Δ*comD* and Δ*comE* mutants were prepared by replacing the wild-type gene with a truncated fragment containing the *catP* gene. This fragment was prepared by PCR splicing, as previously described (77). The DNA fragments containing the mutant alleles were amplified from and introduced into competent D39 cells according to standard protocols (78). The mutants were selected on BAPs containing the appropriate antibiotics, and the mutation was confirmed by PCR.

TABLE 2 Primers used in this study

Target	Primer, sequence (5'–3')	Comment
pUC19-L	ZE 774-L, TGATTCTCGGCATGCAAGCTTGGCGTAATCAT	<i>ciaRH</i> deletion
	ZE 775-R, TAAGACTCGTACCGAGCTCGAATTCAGTGGCC	
5' region of <i>ciaRH</i>	ZE 776-L, CTCGGTACGAGTCTTATCTGGTGGTTTCAGCT	<i>ciaRH</i> deletion
	ZE 777-R, CACACGGTCATGAGAACTCCTCCTTATTA	
<i>ermC</i>	ZE 778-L, TTCTCATGACCGTGTGCTCTACGACCAAACT	<i>ciaRH</i> deletion
	ZE 779-R, GCATTATCCCGTGAATTCCTTAACTTA	
3' region of <i>ciaRH</i>	ZE 780-L, TTCCACGGGATAATGCCGTCAAGTATACTGAG	<i>ciaRH</i> deletion
	ZE 781-R, TGCATGCCGAGAATCATGCCCGTAAGAAAATT	
pUC19-L	ZE 798-L, ATGTTTTGGGCATGCAAGCTTGGCGTAATCAT	<i>yesMN</i> deletion
	ZE 799-R, CTTGAGACGTACCGAGCTCGAATTCAGTGGCC	
5' region of <i>yesMN</i>	ZE 800-L, CTCGGTACGTCTCAAGCAACCTGATTTTCTAT	<i>yesMN</i> deletion
	ZE 801-R, CACACGGTATCATTTTCAACATAGAGGTCATC	
<i>ermC</i>	ZE 802-L, GAAATGATACCGTGTGCTCTACGACCAAACT	<i>yesMN</i> deletion
	ZE 803-R, TATTGACCCCGTGAATTCCTTAACTTA	
3' region of <i>yesMN</i>	ZE 804-L, TTCCACGGGGTCAATACCGTATGAATGAAAAT	<i>yesMN</i> deletion
	ZE 805-R, TGCATGCCCAAAACATAGCCAAACGTAAGTATA	
pUC19-L	ZE 782-L, AGAAAGAAGGCATGCAAGCTTGGCGTAATCAT	<i>VicK</i> deletion
	ZE 783-R, CACAATAGGTACCGAGCTCGAATTCAGTGGCC	
5' region of <i>vick</i>	ZE 784-L, CTCGGTACCTATTGTGCTTCTGACTATTTTT	<i>vick</i> deletion
	ZE 785-R, CATCGGTCTCAAGCATTATTTCTCATGTAATA	
<i>cat-194</i>	ZE 786-L, ATGCTTGAGACCGATGATGAAGAAAAGAATT	<i>vick</i> deletion
	ZE 787-R, TCACTCTTTTATAAAAGCCAGTCATTAGGCCT	
3' region of <i>vick</i>	ZE 788-L, TTTTATAAAAGAGTGAATACGGCAAGGGTTCA	<i>vick</i> deletion
	ZE 789-R, TGCATGCCTTCTTCTATATCTCTGTCAATGG	
5' region of <i>comD</i>	SL91, CTTACCAATAATGCGC	<i>comD</i> deletion
	SL98, CGGTTTTCTAATGTCACTAACTAGACAAATCATTAGATTTAAGAGG	
5' region of <i>comE</i>	SL99, GCTTATCGATACCGTCGAAAAGGATAAAGGTAGTCC	<i>comD</i> deletion
	SL96, GGTTGTCAAATCTTCC	
<i>catP</i>	SL97, CCTCTTAAATCTAATGATTTGTCTAGTTAGTGACATTAGAAAACCG	<i>comD</i> deletion
	SL100, GGACTACCTTTATCCTTTTCGACGGTATCGATAAGC	
5' region of <i>comE</i>	MS93, TAGTCAAAGCAAATCATAAATTGCG	<i>comE</i> deletion
	MS99, GCTTATCGATACCGTCGAATATTCTCTAGTCTCACTTGATGTTT	
3' region of <i>comE</i>	MS100, CGGTTTTCTAATGTCACTAACTCTCAAAAGTGATTGACAATTAGC	<i>comE</i> deletion
	MS96, CATGCTCATCAGAAAAGAGACGC	
<i>catP</i>	MS101, GAACATCAAGTGAGACTAGAGAGAATATTGACGGTATCGATAAGC	<i>comE</i> deletion
	MS102, GCTAATTGTCAATCACTTTTGAGAGTTAGTGACATTAGAAAACCG	
<i>comD</i>	ZE 856-L, GATTTGGTTCGTATCATGAGC	qRT-PCR
	ZE 857-R, GGAGTCATCGTCATTTTACATG	
<i>yesM</i>	ZE 858-L, GATTCCCAAGTTTACCTTGCAAC	qRT-PCR
	ZE 859-R, CGACATTCCTCTACCATTATCG	
<i>piuB</i>	ZE 864-L, TGATTTGACCAGCAGACCTG	qRT-PCR
	ZE 865-R, CTGTAATCGGTGCAGCAAACTG	
<i>phrA</i>	ZE 874-L, GTTACATTTGCAATTGCTAGGTG	qRT-PCR
	ZE 875-R, TTGCACTCGAATCTCCAATTG	
SPD_1587	ZE 665-L, ATGCAGGCAGAAGGAATAGAAG	qRT-PCR
	ZE 666-R, TTGGCAAACAACCTTCTCATC	

Data availability. We have deposited all RNA-Seq raw sequencing reads with the Sequence Read Archive at the National Center for Biotechnology Institute (accession number [PRJNA642413](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA642413)) for public availability.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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