Characterization of a Ferrous Iron-Responsive Two-Component System in Nontypeable *Haemophilus influenzae*

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Nontypeable *Haemophilus influenzae* (NTHI), an opportunistic pathogen that is commonly found in the human upper respiratory tract, has only four identified two-component signal transduction systems. One of these, an ortholog to the QseBC (quorum-sensing *Escherichia coli*) system, was characterized. This system, designated *firRS*, was found to be transcribed in an operon with a gene encoding a small, predicted periplasmic protein with an unknown function, *ygiW*. The *ygiW-firRS* operon exhibited a unique feature with an attenuator present between *ygiW* and *firR* that caused the *ygiW* transcript level to be 6-fold higher than the *ygiW-firRS* transcript level. *FirRS* induced expression of *ygiW* and *firR*, demonstrating that *FirR* is an autoactivator. Unlike the QseBC system of *E. coli*, *FirRS* does not respond to epinephrine or norepinephrine. *FirRS* signal transduction was stimulated when NTHI cultures were exposed to ferrous iron or zinc but was unresponsive to ferric iron. Notably, the ferrous iron-responsive activation only occurred when a putative iron-binding site in *FirS* and the key phosphorylation aspartate in *FirR* were intact. *FirRS* was also activated when cultures were exposed to cold shock. Mutants in *ygiW*, *firR*, and *firS* were attenuated during pulmonary infection, but not otitis media. These data demonstrate that the *H. influenzae* strain 2019 *FirRS* is a two-component regulatory system that senses ferrous iron and autoregulates its own operon.

Nontypeable *Haemophilus influenzae* (NTHI) is a Gram-negative bacterium found in the upper respiratory tract of approximately 80% of people (73). As an opportunistic pathogen, NTHI can cause conjunctivitis and sinusitis in immunocompromised adults and lower respiratory tract infections in individuals with chronic obstructive pulmonary disease and cystic fibrosis (35, 45). NTHI is also responsible for one-third of otitis media cases in children under 1 year of age (45). There is currently no vaccine to protect against NTHI. While infections can be treated, NTHI is the most common cause of recurrent otitis media infections, which can lead to deafness and speech/language impediments (41).

Pathogenic bacteria require high-affinity iron acquisition systems for virulence (20, 46, 54) since the human host restricts extracellular iron levels to less than 0.1% of the body's supply (24). Indeed, iron import is important for NTHI survival in the human host. NTHI requires exogenously supplied heme or the immediate precursor to heme, protoporphyrin IX, for aerobic growth, since *H. influenzae* does not have the genes encoding the enzymes needed to make protoporphyrin IX (9). NTHI also requires iron and heme uptake to persist on the respiratory mucosa (68). For these reasons, ferric iron and heme transport have been well studied in NTHI.

Heme can be imported into NTHI by binding to the outer membrane protein HgpA, HgpB, HgpC, HxB, HxC, or Hup (3, 11, 25, 30). Ferric iron can be imported into NTHI when the FhuABCD complex binds to ferrichrome and the Tbp1/Tbp2 proteins bind to transferrin (4, 29, 56, 70, 75). To our knowledge, though, there have been no published reports of ferrous iron uptake in NTHI.

Two-component signal transduction (TCST) systems are commonly used by bacteria to sense and respond to environmental conditions. In most TCST systems, the transmembrane sensor kinase detects an environmental stimulus and activates the response regulator, which is usually a transcriptional regulator. TCST systems are known to regulate a wide variety of functions,

including the extracytoplasmic stress response (CpxRA), potassium transport (KdpDE), anoxic redox control (ArcBA), and virulence (BvgAS), to name a few (17, 58, 59, 64). While over 31 TCST systems have been identified in *Escherichia coli* and 13 in *Streptococcus pneumoniae* (69, 76), only four have been identified in *H. influenzae* genome sequences (23, 31). The identified NTHI TCST systems are orthologs to ArcAB (senses redox conditions) (19, 71, 72), NarPQ (senses nitrate-nitrite levels) (63), PhoBR (senses phosphate levels), and QseBC (senses quorum-sensing signals). To date, there are no published reports on the role of PhoBR and QseBC in *H. influenzae*.

The QseBC system responds to the host hormones epinephrine, norepinephrine, and/or bacterial autoinducer 3 (AI-3) in *Salmonella enterica* serovar Typhimurium, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Aggregatibacter actinomycetemcomitans* (7, 40, 53, 67). QseBC also has a role in biofilm production in *E. coli*, *A. hydrophila*, and *A. actinomycetemcomitans* (40, 53, 62). QseB regulates motility (6, 7, 16, 40, 61, 67) and also functions as an autoregulator, activating the expression of the *qseBC* operon in *E. coli* (15, 42, 49). Importantly, QseBC affects virulence in *A. hydrophila*, *A. actinomycetemcomitans*, *E. tarda*, and *E. coli* (40, 42, 53, 67), and swine colonization by *S. Typhimurium* (6, 7).

In our hands, *H. influenzae* QseBC does not respond to epinephrine or norepinephrine. NTHI strains are not flagellated and do not contain the virulence genes that appear to be regulated by QseBC in *E. coli*. In this report, we present evidence that the NTHI QseBC ortholog specifically senses cold temperatures, ferrous iron, and zinc, but not ferric iron or other cations. We therefore

Received 13 August 2012 Accepted 4 September 2012

Published ahead of print 7 September 2012

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doi:10.1128/JB.01465-12

propose to change the name of this TCST from QseBC to FirRS (ferrous iron responsive regulator/sensor) in *H. influenzae*, where FirR is the regulator and FirS is the sensor. We also show that, in response to ferrous iron, FirR activates its own operon, *ygiW-firRS*. Furthermore, the presence of *ygiW* and *firRS* is important to maintain a pulmonary infection in mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. influenzae* 2019 and derivatives of this strain (Table 1) were cultivated on brain heart infusion agar (Becton, Dickinson, and Company, Sparks, MD) supplemented with 10 µg/ml hemin and 10 µg/ml β-NAD (sBHI) at 37°C with 5% CO₂. RPMI 1640 medium (Sigma-Aldrich, Saint Louis, MO) was used as a chemically defined medium, and supplemented RPMI (sRPMI) was prepared with protoporphyrin IX (1 µg/ml), hypoxanthine (0.1 mg/ml), uracil (0.1 mg/ml), β-NAD (10 µg/ml), and sodium pyruvate (0.8 mM). *E. coli* strains were grown in Luria Bertani (LB) broth or on LB agar at 37°C. Ribostamycin sulfate salt (15 µg/ml) (Sigma-Aldrich), kanamycin monosulfate (62.5 µg/ml) (Fisher), chloramphenicol (34 µg/ml for *E. coli* strains; 1 µg/ml for *H. influenzae* strains) (Sigma-Aldrich), spectinomycin dihydrochloride pentahydrate (50 µg/ml for *E. coli* strains; 25 µg/ml for *H. influenzae* strains) (Sigma-Aldrich), and ampicillin sodium salt (125 µg/ml) (Fisher) were added to culture media as necessary for selection of bacterial strains carrying antibiotic resistance markers. Counterselection for transformations involving the *sacB-nptII* cassette was performed with LB agar supplemented with 5% sucrose, chlortetracycline (CTC; 1 µg/ml), hemin, and NAD (sLB).

Construction of *H. influenzae* mutants. Genetic manipulations were performed using established techniques. Restriction enzymes, Antarctic phosphatase, and T4 polymerase were obtained from New England Biolabs (Beverly, MA) and were used by following established protocols. The Expand high-fidelity PCR system (Roche Applied Science, Indianapolis, IN) was used for PCRs. Oligonucleotide primers were designed and ordered from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. Competent *H. influenzae* cells were prepared using the MIV method and transformed as described previously (32).

Construction of marked mutants. Plasmids containing *cat*-disrupted (pJJ287) and *aph3A*-disrupted (pJJ162) versions of the *ygiW* locus and an *nptII*-disrupted version of the *firRS* locus (pJJ121) (Table 1) were independently introduced into *H. influenzae* 2019 by MIV transformation, and transformants were selected on sBHI containing chloramphenicol or ribostamycin (a kanamycin analog). Putative *ygiW::aph3A* (designated strain JWJ033), *ygiW::cat* (designated strain JWJ142), and *firRS::nptII* (designated strain JWJ006) mutants were selected for further evaluation. The mutant genotypes were confirmed by PCR analysis.

Construction of unmarked deletion mutants. Unmarked deletion mutants were created using a *sacB-nptII* cassette that allows for positive and negative selection (37). Plasmids containing the *sacB-nptII*-disrupted versions of the *firR* locus (pNB113) and the *firS* locus (pKK010) (Table 1) were independently introduced into *H. influenzae* 2019 by MIV transformation, and transformants were selected on sBHI containing ribostamycin. *firR::sacB-nptII* (designated NB003) and *firS::sacB-nptII* (designated KK010) mutants were further selected based on their failure to grow on sLB supplemented with 5% sucrose and 1 µg/ml of CTC. Next, plasmids containing nonpolar deletions of *firR* (pNB110) and of *firS* (pKK009) were independently introduced into NB003 and KK010, respectively, and transformants were selected on sLB agar with 5% sucrose chlortetracycline (1 µg/ml). Putative Δ*firR* (designated NB004) and Δ*firS* (designated KK009) mutants were selected for further evaluation based on their failure to grow on sBHI with ribostamycin. The genotypes of NB004 and KK009 were confirmed by PCR analysis.

A portion of the attenuator loop and poly(U) tract between the *ygiW* and *firR* genes was mutated using splicing by overlap extension (SOEing) PCR (Fig. 1C) (34). First, two DNA fragments were amplified from NTHI 2019 genomic DNA by PCR. The first upstream DNA fragment was gen-

erated using the primer pair 1708F8/1708R10 and began with the 3' end of the *ygiW* gene and ended with the first half of the attenuator loop. The second DNA fragment was generated using the primer pair 1708F10/1708R9 and began with the nucleotides immediately after the poly(U) tract and ended with the *firR* and *firS* genes. Ten base pairs (that overlapped the last 10 bp of the upstream DNA fragment) were added to primer 1708F10. These two DNA fragments were used as the template in the SOEing reaction with the primer pair 1708F8/1708R9, so that the product from the splicing reaction included all of the nucleotides from *ygiW* to *firS* except the nucleotides that made up the last half of the attenuator loop and the poly(U) tract.

A plasmid containing the *sacB-nptII* cassette in the *firR* coding region (pJJ366) was introduced into *H. influenzae* 2019 by MIV transformation, and transformants were selected on sBHI with ribostamycin. Putative *firR::sacB-nptII* mutants were selected for further evaluation based on their failure to grow on 5% sucrose. Next, the *firR::sacB-nptII* mutants were transformed with the SOEing PCR product and selected for growth on sLB agar with 5% sucrose and CTC (1 µg/ml). A putative mutant (designated JWJ166) was selected for further evaluation based on its failure to grow on sBHI with ribostamycin. The *ygiW-firRS* region was amplified by PCR with the 1708F10/1708R10 primer set, and the mutated genotype was confirmed by sequencing this genomic DNA region.

Construction of unmarked point mutations in *firR* and *firS*. GeneTailor site-directed mutagenesis (Invitrogen) was used to generate a point mutation at the conserved aspartate residue of FirR and to construct three different mutations in a putative iron-binding motif in FirS. Mutations were created using PCR with a template that included either the *firR* gene (pJJ359) or the *firS* gene (pJJ372) (Table 1) and primers that included the respective mutated nucleotides that were engineered by following the manufacturer's guidelines (Table 2). Each of the mutated PCR products were chemically transformed into DH5α-T1^R cells, and the mutations were confirmed by sequencing either the *firR* or *firS* gene accordingly. The mutated versions of *firR* (pJJ361) and *firS* (pKS8, pKS12, and pKS13) were independently introduced into *H. influenzae* 2019 using the MIV method. Putative *firR*(D51A) (designated JWJ154), *firS*(Y149G,R150T) (designated KHS1), *firS*(D148A) (designated KHS3), and *firS*(E151G,D152S) (designated KHS4) mutants were selected for further evaluation. The genotypes of JWJ154, KHS1, KHS3, and KHS4 were confirmed by sequencing the *firR* or *firS* gene from these strains.

Construction of complemented mutants. The *ygiW::aph3*, Δ*firR*, and Δ*firS* mutants were all complemented by reintroducing the deleted gene onto the chromosome downstream of a spectinomycin resistance cassette using a previously described strategy (39). Expression of the inserted gene was driven by read-through transcription initiated at the constitutive promoter of the spectinomycin resistance gene as described previously (39). Plasmids containing the *ygiW* (pELL009), *firR* (pKS9), and *firS* (pKS14) open reading frames (ORFs) (Table 1) were independently introduced into *H. influenzae* *ygiW::aph3*(JWJ033), Δ*firR* (NB004), and Δ*firS* (KK009) mutants, respectively, by the MIV transformation method, and transformants were selected on sBHI containing spectinomycin. Putative *ygiW*⁺ (JWJ076), *firR*⁺ (KHS2), and *firS*⁺ (KHS5) colonies were selected for further evaluation. The genotypes of JWJ076, KHS2, and KHS5 were confirmed by PCR analysis of genomic DNA from these strains using primer sets that isolated part of the 601.1 vector (Table 2) and the respective gene of interest.

Construction and use of a green fluorescent protein (GFP) reporter. To measure induction of *ygiW*, a reporter vector was constructed. Primers were designed to amplify *gfpmut3* from pRSM2211 (47) and insert it into the shuttle vector pGZRS39A (70) digested with FspI and SphI. The resulting vector, pKS4, served as the recipient of target promoter fragments and an empty vector control in subsequent experiments. pKS4 was designed in a manner to allow for InFusion cloning (Clontech, Mountain View, CA) of promoter fragments into the SphI site, resulting in a translational fusion to *gfpmut3*. A 402-bp fragment containing the promoter region upstream of *ygiW*, including the *ygiW* start codon, was amplified

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ ⁻	Invitrogen catalog no. 18258-012
DH5 α -T1 ^R	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 thi-1 gyrA96 relA1 tonA</i>	Invitrogen catalog no. 12297-016
<i>Haemophilus influenzae</i>		
2019	Clinical respiratory isolate	(12)
JWJ006	2019 <i>firS</i> :: <i>nptII</i> Kn ^r	This study
JWJ033	2019 <i>ygiW</i> :: <i>aph3aA</i> Kn ^r	This study
JWJ076	2019 <i>ygiW ygiW</i> ⁺ Kn ^r Spec ^r	This study
JWJ142	2019 <i>ygiW</i> :: <i>ca</i> Cm ^r	This study
JWJ154	2019 <i>firR</i> (D51A)	This study
JWJ166	2019 (deleted attenuation loop)	This study
KHS1	2019 <i>firS</i> (Y149G,R150T)	This study
KHS2	2019 <i>firR firR</i> ⁺ Spec ^r	This study
KHS3	2019 <i>firS</i> (D148A)	This study
KHS4	2019 <i>firS</i> (E151G,D152S)	This study
KHS5	2019 <i>firS firS</i> ⁺ Spec ^r	This study
KK009	2019 Δ <i>firS</i>	This study
KK010	2019 <i>firS</i> :: <i>sacB-nptII</i>	This study
NB003	2019 <i>firR</i> :: <i>sacB-nptII</i>	This study
NB004	2019 Δ <i>firR</i>	This study
Plasmids		
p601.1-SP2	Chromosomal complementation vector; Sp ^r	(39)
pELL009	His-tag <i>ygiW</i> gene	This study
pGEM-T	ColE1-based cloning vector; Ap ^r	Promega
pGEM-T Easy	ColE1-based cloning vector; Ap ^r	Promega
pGZRS39A	pGZRS-1 based broad host range cloning vector; Kn ^r	(70)
pBSL86	Vector containing the <i>nptII</i> cassette	(2)
pACYC184	Vector containing the <i>cat</i> cassette	(13)
pJJ110	4,527-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing <i>firS-betT</i> (PCR primers 1709F1/1706R1) cloned into pGEMT	This study
pJJ121	Derivative of pJJ110 in which a 1,276-bp NruI/MfeI fragment internal to the <i>firR</i> and <i>firS</i> coding regions was replaced with the <i>nptII</i> gene from pBSL86	This study
pJJ157	573-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the downstream region of <i>ygiW</i> (PCR primers 1709F6/1709R6) cloned into pGEMT	This study
pJJ158	615-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the upstream region of <i>ygiW</i> (PCR primers 1709F7/1709R3) cloned into pGEMT	This study
pJJ160	629-bp EcoRI/KpnI fragment from pJJ158 containing the upstream region of <i>ygiW</i> cloned into pUCAT	This study
pJJ162	593-bp XbaI/HindIII fragment from pJJ157 containing the downstream region of <i>ygiW</i> cloned into XbaI/HindIII site in pJJ160	This study
pJJ260	Vector containing the <i>tetR-sacB/nptII</i> cassette	(37)
pJJ287	SmaI fragment containing the <i>cat</i> gene cloned into SmaI site in pJJ162	This study
pJJ359	1,630-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the <i>firR</i> coding region (PCR primers 1708F7/1708R7) cloned into pGEMT	This study
pJJ361	Derivative of pJJ359 in which the FirR D51A mutation has been engineered using site-directed mutagenesis and PCR primers 1708M5 and 1708M6	
pJJ366	Derivative of pJJ359 in which the <i>sacB-nptII</i> coding regions from pJJ260 was inserted into the NruI site	This study
pJJ372	3,132-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the <i>firS</i> coding region (PCR primers 1708F8/1707R3) cloned into pGEM-T Easy	This study
pJJ392	402-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the <i>ygiW</i> promoter (PCR primers qsh p-f/qsh p-r) cloned into the SphI site in pKS4	This study
pKK008	1,079-bp SacI/SmaI fragment containing the upstream region of and including the start codon of <i>firS</i> (PCR primers 1708F8/1707R2) cloned into the SacI/SmaI site in pUCAT	This study
pKK009	710-bp SmaI/SphI fragment containing the downstream region of and including the stop codon of <i>firS</i> (PCR primers 1707F3/1707R3) cloned into the SmaI/SphI site in pKK008	This study
pKK010	Derivative of pKK009 in which the <i>sacB-nptII</i> coding regions from pJJ260 was inserted into the SmaI site	This study
pKS12	Derivative of pJJ372 in which the FirS D148A mutation has been engineered using site-directed mutagenesis and PCR primers firS D-A Fwd and firS D-A Rev	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Genotype or description	Reference or source
pKS13	Derivative of pJJ372 in which the FirS E151G and D152S mutations have been engineered using site-directed mutagenesis and PCR primers firS ED-GS Fwd and firS ED-GS Rev	This study
pKS14	1,389-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the <i>firS</i> coding region (PCR primers firS ⁺ Fwd/firS ⁺ Rev) directionally cloned into the SmaI site of p601.1-SP2	This study
pKS4	747-bp <i>gfpmut3</i> gene PCR amplified from pRSM2211 (PCR primers gfp-GZRF/gfp-GZRR2) cloned into the FspI/SphI site of pGZRS39A	This study
pKS8	Derivative of pJJ372 in which the FirS Y149G and R150T mutations have been engineered using site-directed mutagenesis and PCR primers firS-SD-F and firS-SD-R	This study
pKS9	702-bp genomic DNA fragment from <i>H. influenzae</i> 2019 containing the <i>firR</i> coding region (PCR primers firR-C-F/firR-C-R) directionally cloned into the SmaI site of p601.1-SP2	This study
pNB109	414-bp SacI/SmaI fragment containing the upstream region of and including the start codon of <i>firR</i> (PCR primers 1708F8/1708R8) cloned into the SacI/SmaI site in pUCAT	This study
pNB110	Derivative of pNB109 in which the <i>sacB-nptII</i> coding regions from pJJ260 was inserted into the SmaI site	This study
pRSM2211	Vector that contains the highly stable, highly fluorescent <i>gfpmut3</i> gene; Km ^r	(47)
pUC19K3	General cloning vector	New England Biolabs
pUCAT	Derivative vector of pUC19 in which the <i>bla</i> gene has been replaced with the <i>cat</i> gene from pACYC184	This study

and inserted into pKS4, creating pJJ392, the *ygiW* reporter vector (Table 1). The vector was sequenced to confirm correct integration and orientation of the promoter fragment.

Both the reporter vector pJJ392 and the empty vector pKS4 were introduced into NTHI strains by electroporation using a previously described protocol (50). To measure the levels of GFP, samples of cultures were removed and transferred to a 96-well plate and read with a Spectra-Max M5 plate reader (Molecular Devices, Sunnyvale, CA), with an excitation wavelength of 485 nm and an emission wavelength of 518 nm. The mean fluorescence values from an empty vector control were subtracted from values obtained from strains containing the reporter to determine the relative fluorescence units (RFU) for each strain and condition.

RNA extraction. *H. influenzae* strains grown on sBHI agar overnight were inoculated into 40 ml sRPMI and incubated at 37°C with shaking at 225 rpm for 3 h (mid-exponential phase). Following incubation, the cultures were split into two flasks each with 20 ml culture. Nothing was added to one flask, and to test the response to epinephrine, norepinephrine, and serotonin, chemicals were added at various concentrations ranging from 50 to 200 μ M to the other flask. In the temperature response experiments, flasks were incubated for 30 min, with shaking at 225 rpm, with one set of flasks shaking at 37°C and the other set of flasks shaking at 9°C. In the iron response experiments, flasks were incubated for 30 min at 37°C, with shaking at 225 rpm; FeCl₂ was added to a final concentration of 100 μ M to one flask, and nothing was added to the other flask. In all cases, RNA was extracted immediately after the 30-min incubation.

RNA was extracted using the hot acid phenol method as described previously (55). DNA was removed from extracted RNA by digestion with DNase I (New England BioLabs) and cleaned up with the RNeasy minikit (Qiagen, Valencia, CA). RNA quality was assessed with an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA), and the concentration was determined using a NanoDrop ND-1000 spectrophotometer.

qRT-PCR. For quantitative real-time PCR (qRT-PCR) analysis, primer/probe sets were obtained using the custom TaqMan gene expression service (Applied Biosystems, Foster City, CA). Custom TaqMan assays were designed using the sequence of *ygiW* and *firR* from *H. influenzae* 2019, and a primer/probe set for the 16S rRNA of *H. influenzae* was designed and used as a control. The qScript one-step fast MGB qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD) was used by following the manufacturer's protocol. Reaction mixtures were prepared in triplicate using 20 ng of RNA, and transcript levels were quantified using the StepOnePlus real-time PCR system (Applied Biosystems) with StepOne analysis software. Results were calculated using the comparative threshold cycle (*C_t*) method (56) to determine the relative expression ratio between RNA sam-

ples. The primer and probe set for the 16S rRNA of *H. influenzae* was used as the endogenous reference to normalize the results. Two biological replicates were utilized in each experiment.

Northern blot analysis. The NorthernMax-gly kit (Applied Biosystems) was used to determine transcript sizes. Briefly, 15 μ g of RNA was loaded onto a 1% agarose gel with an RNA Millennium size marker (Applied Biosystems), in duplicate, and electrophoresed. The gel was destained with running buffer and the size marker visualized by UV transillumination to determine the migration distances and to generate a standard curve. The RNA was transferred to a BrightStar-Plus membrane (Applied Biosystems) using downward transfer, and the RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Agilent). The blot was cut to separate lanes, and prehybridization incubations were carried out by following the kit protocol.

Hybridization probes were prepared by PCR using the Rediprime II DNA labeling system (GE Healthcare, Piscataway, NJ). For probe synthesis, DNA fragments internal to each gene were amplified by PCR using primers 1709F8 and 1709R7 (*ygiW*) and 1708F3 and 1708R3 (*firR*) and the Expand high-fidelity PCR kit (Roche). The PCR fragments were purified with the QIAquick PCR cleanup kit (Qiagen), and 25 ng of DNA was used in the labeling reaction mixture. Radiolabeled probes were synthesized using [α -³²P]dCTP by following the protocol provided with the kit. The probe (14 μ l) was mixed with 5 ml of hybridization buffer, which was then incubated with the blots at 42°C overnight. Posthybridization washes were carried out by following the manufacturer's protocols, and the blots were exposed to film. The migration distances of hybridized bands were measured, and the size was determined using the standard curve.

Primer extension. Primer extension analysis was used to identify the transcriptional start sites for *ygiW-firRS*. Primer 1709R15 was labeled with ³²P using T4 polynucleotide kinase (New England BioLabs) and [γ -³²P]ATP (GE Healthcare). Illustra MicroSpin G-25 columns (GE Healthcare) were used to remove unincorporated ³²P. The primer extension reaction was performed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) by following the supplied protocol. After first-strand synthesis, RNA was degraded by incubation with RNase A (New England BioLabs) at 37°C for 15 min. Nucleic acids were precipitated by the addition of 300 μ l of chilled ethanol, incubation in a dry ice bath for 15 min, and centrifugation at 4°C. Dried samples were dissolved in loading buffer (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol FF, 0.025% bromophenol blue) prior to loading the sequencing gel. Sequencing reaction mixtures were prepared for each labeled primer using the SequiTherm EXCEL II DNA sequencing kit (Epicentre Technologies,

TABLE 2 Oligonucleotide primers used for PCR in this study

Designation	Sequence ^a
1706R1	5'-AGTTCGGTTTGCTCGTGTGC-3'
1707F3	5'-cccgggTTGTAGAAATGGCTTTAAATAAATG ATAT-3'
1707R2	5'-cccgggTTTCATTTTCAACTGTCTAAAGCA TATCCAACA-3'
1707R3	5'-gcatgcTGCCCCAGTGGAAACAGAGTATG-3'
1708F10	5'-gcgggcgttaATAAAGTGCAGTCAATTTTAT GAGA-3'
1708F3	5'-CTTGGTTTTCGGGTGGATTG-3'
1708F7	5'-TTTCAACAAACAGCCCTGC-3'
1708F8	5'-gagctcGCAACAATCTTCGATTAGCAACC-3'
1708M5	5'-GATGCGGTGGTATTGGCTTTAACCTT GCCT-3'
1708M6	5'-CAATACCACCGCATCATAAGGCGC-3'
1708R10	5'-TAACGCCCCGCTATCTTCAAAGA-3'
1708R3	5'-GCTTCCCCAATTTTGGCG-3'
1708R7	5'-CCCTCCGTATTGAGCAATGACAC-3'
1708R8	5'-cccgggCATAAAAAATTGACCGCACTTTAT AAAC-3'
1708R9	5'-gcatgcTCCCAATCAAGCGGTGTAG-3'
1709F1	5'-GTTTTGGTTTACTGATGGGACAGG-3'
1709F6	5'-tctagaTTATCTCTGAAGATAGCGGGCGT TAGC-3'
1709F7	5'-gaattcGGAATCAAAAGAAGGCGTAG-3'
1709F8	5'-GCAACAATCTTCGATTAGCAAC-3'
1709R15	5'-GCTAATGCGAAGATTGTGC-3'
1709R3	5'-ggtagcAACGTTTCCTTTTAAAGTTAGTTAATTC AAGC-3'
1709R6	5'-aagcttCATACGATGTGCGAGAC-3'
1709R7	5'-CGTAATACATCAAGCTCCGCTTTC-3'
gfp-GZRf	5'-GTAAAGGAGAAGAACTTTTCACTGGAG-3'
gfp-GZr2	5'-CGCCATTCAAGGCTGCTTATTTGTATAGTTCAT CCATGCCATGTG-3'
firS-SD-F	5'-TAGGGCAAGAATTAGATGGTACCGAAGATT AATT-3'
firS-SD-R	5'-TAGATTAAGAACGGGATGACGCTATTTATTA AGAG-3'
firR-C-F	5'-TGCGGTCAATTTTTTATGAG-3'
firR-C-R	5'-CGCAAAGTAAATGCTTCTATT-3'
firS ⁺ Fwd	5'-cccgggTTTAGGACAAGTTGAAAAAT-3'
firS ⁺ Rev	5'-cccgggTATTTTAAAGCCATTTCTAC-3'
firS D-A Fwd	5'-TCGAGTAGGGCAAGAATTAGCTTACCGT GAAG-3'
firS D-A Rev	5'-TAATTCCTGCCCCTACTGCGATAAATAATT CTCC-3'
firS ED-GS Fwd	5'-ACGGTAATCTAATTCTTGCCTACTGCGA TAAATA-3'
firS ED-GS Rev	5'-GCAAGAATTAGATTACCGTGGATCCTTA ATTGAAG-3'
P1709F1	5'-gagctcGCATTACAAAAACCGACCGCTG-3'
P1709R2	5'-gcatgcCATAACGACGACGAATAAGGG-3'
qsh p-f	5'-ATTACGCCAAGCTTGAATCAGGCGAAGT AGTGGCTGG-3'
qsh p-r	5'-TTCTTCTCCTTACGCATGACGTTTCCTTT TTAAGTTAGTTAATTTTC-3'

^a Lowercase letters denote a restriction enzyme site that had been added onto the primer.

Madison, WI). A PCR fragment amplified with the primers P1709F1 and P1709R2 was used as a template. Sequencing and primer extension reaction mixtures were loaded onto an 8% polyacrylamide sequencing gel. After electrophoresis, the gel was dried and exposed to film at -80°C .

Chinchilla otitis media model. The ability of NTHI to form a biofilm and persist in the middle ear was examined as described previously (5, 33). Adult chinchillas were acquired from Rauscher Chinchilla Ranch (La Rue, OH). Chinchillas anesthetized with isoflurane were infected via transbullar injection of 10^3 CFU of NTHI. Animals were euthanized at 7, 14, and 21 days postinfection, and samples were collected to determine the bacterial load in the middle ear. The bullae were aseptically opened, and the fluids and nonadherent bacteria were recovered by middle ear lavage using 1 ml of sterile phosphate-buffered saline (PBS). Both bullae were then removed and homogenized in 10 ml of sterile PBS. Samples were diluted and plated on sBHI supplemented with 3 $\mu\text{g}/\text{ml}$ vancomycin to determine the CFU in both lavage and homogenate samples.

Mouse pulmonary infection model. The procedures previously described by Johnston et al. were used to evaluate the lung colonization profiles of the *H. influenzae* strains in C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) (38). Briefly, mice were anesthetized with isoflurane then infected intranasally with 10^7 bacteria in a 50- μl volume. At each sampling point postinfection, the mice were euthanized, their lungs aseptically removed, and lung homogenates were serially diluted and plated on sBHI supplemented with 3 $\mu\text{g}/\text{ml}$ vancomycin to determine the number of viable *H. influenzae* present.

RESULTS

Identification of the two-component system FirRS in *H. influenzae* strains 86-028 NP and 2019. The genes designated NTHI2016 and NTHI2015 in the *H. influenzae* 86-028 NP genome sequence (and designated HI1708 and HI1707 in the *H. influenzae* strain Rd KW-20 genome sequence) are annotated as *qseB* and *qseC*, respectively. The products of these two genes are predicted to be homologs of the quorum-sensing TCST system QseBC of *E. coli*. In many bacteria, the QseBC TCST system serves as an important regulatory system involved in metabolism, virulence, biofilm development, and motility (6, 7, 16, 29, 40, 42, 53, 67). Here we have renamed QseBC to FirRS in NTHI since we have found no role for this TCST system in quorum-sensing or biofilm development (data not shown) and NTHI is nonmotile.

Many histidine sensor kinases, like FirS, are membrane-bound

FIG 1 (A) Schematic diagram of the *ygiW-firRS* operon. The two primary transcriptional start sites are upstream of *ygiW* and indicated by arrows. The attenuator present between *ygiW* and *firR* is represented by a loop. The attenuator is located 14 bp after the *ygiW* stop codon. (B) Northern blot analysis using internal probes for *ygiW* and *firR*. The *ygiW* probe hybridized with transcripts at 0.5 kb and 2.5 kb, with the 0.5-kb band in much greater abundance. The *firR* probe only hybridized to the 2.5-kb band. (C) The attenuator stem-loop that is present between *ygiW* and *firR*. The line shows the residues that are deleted in JWJ166.

homodimers with a periplasmic sensor domain and a cytoplasmic kinase domain (69). The sensor domain is variable due to the variety of environmental signals sensed, but the kinase domain has a conserved histidine residue in the cytoplasmic domain. Once the sensor kinase senses its signal, the histidine becomes phosphorylated by ATP (69). The NTHI 2019 FirS shares 42% amino acid identity with the *E. coli* QseC, and the His-243 residue is conserved as His-244 in the NTHI 2019 FirS ortholog.

Response regulators such as FirR are cytoplasmic proteins with a conserved receiver domain and a variable effector domain. The signal is transduced by the transfer of a phosphate from the sensor kinase to a conserved aspartate residue of the response regulator (69). The NTHI 2019 FirR shares 61% amino acid identity with the *E. coli* QseB, and the Asp-57 residue is conserved as Asp-51 in the NTHI 2019 FirR ortholog.

Directly upstream of *firRS* is a gene designated NTHI2017 in the 86-028 NP genome sequence and HI1709 in the Rd KW20 genome sequence and annotated as encoding a hypothetical protein similar to YgiW (Fig. 1A). An ortholog to *ygiW* is directly upstream of *qseBC* in *E. coli*; however, it is transcribed in the opposite orientation. YgiW is a member of the bacterial OB-fold (BOF) domain proteins (27), which are predicted periplasmic proteins with no known function. The NTHI 2019 YgiW shares 23% amino acid identity with the *S. Typhimurium* YdeI (a YgiW homolog) and 33% amino acid identity with the *S. Typhimurium* YgiW.

Northern blot analysis indicates that the *ygiW*, *firR*, and *firS* genes in NTHI 2019 are cotranscribed in an operon (Fig. 1B). This is consistent with the predicted function of the FirR and FirS products in a functional complex and with the genetic organization of the *qseBC* operons in some other bacteria (49, 62, 67). An internal probe for *ygiW* bound to RNA with approximate sizes of 0.5 kb and 2.5 kb, with the signal of the 0.5-kb band significantly stronger (Fig. 1B, lane 1). A probe for *firR* bound the same 2.5-kb RNA but not the 0.5-kb transcript (Fig. 1B, lane 2). Based on the sequence, a transcript containing *ygiW*, *firR*, and *firS* would be at least 2.5 kb, consistent with the band at 2.5 kb in the Northern blot analysis. The binding of probes for both *ygiW* and *firR* to the same 2.5-kb fragment support this. The 0.5-kb fragment bound by the *ygiW* probe indicates that a transcript comprised only of *ygiW* is also produced. There are two additional less prominent bands. We believe these represent partially degraded *ygiW*-*firRS* transcripts since both probes hybridize to each product.

Analysis of the sequence between *ygiW* and *firR* revealed the presence of a stem-loop structure with a poly(U) tract that could potentially function as an attenuator (Fig. 1C). To determine the role of the stem-loop structure, a mutant strain, JWJ166, was constructed, in which the second half of the inverted repeat and the poly(U) tract were deleted (Fig. 1C). Transcript levels of *ygiW* and *firR* were then quantified by qRT-PCR in wild-type NTHI 2019 and JWJ166. Expression of *ygiW* was higher than that of *firR* in the wild-type strain (6.2-fold), but when the attenuator was deleted, the ratio of *ygiW* expression was significantly lower than that of *firR* (only 1.5-fold). This confirms that the stem-loop structure functions as an attenuator, providing higher levels of *ygiW* transcript than those of *ygiW*-*firRS* transcript. The role for this level of control is unknown at this time.

Transcription of the *ygiW*-*firRS* operon initiates from multiple sites. The transcriptional start sites of the *ygiW*-*firRS* operon were identified using primer extension analysis. Three transcrip-

FIG 2 (A) Primer extension mapping of the *ygiW*-*firRS* transcriptional start sites. Primer extension analysis identified three transcriptional start sites in wild-type 2019, whereas only TS-2 and TS-3 are present in the NTHI *firRS* mutant. The inset shows a shorter exposure in which the doublet bands at TS-1 are more apparent. (B) Sequence upstream of the start codon of *ygiW* illustrating the transcriptional start sites (TS-1, TS-2, and TS-3) and potential -10, -35 (boxed), and +1 sites (bold letter with arrow).

tional start sites were identified, including a FirRS-dependent transcript that was not expressed in a *firRS* mutant (Fig. 2). Consensus -10 and -35 sites were easily identifiable for the constitutive transcriptional start 2 (TS-2); however, only a consensus -10 was apparent for TS-1. Of note, *E. coli* QseB binding sites were not present in the region upstream of *ygiW*.

FirR activates *ygiW*-*firRS* expression in response to cold shock in a FirRS-dependent manner. Cold temperatures affect the TCST systems CorRS and DesKR in *Pseudomonas syringae* and *Bacillus subtilis*, respectively (1, 10). Therefore, we tested whether expression of the *ygiW*-*firRS* operon was affected when exponentially grown wild-type 2019 cultures were exposed to cold temperatures. The levels of both *ygiW* and *firR* transcripts increased between 60- and 70-fold when wild-type 2019 cultures were exposed to 9°C compared to 37°C (Fig. 3). However, the levels of these transcripts did not increase in the Δ *firR* mutant NB004 or the

FIG 3 Thermoresponsive induction of *ygiW* (A) and *firR* (B). Cultures of wild-type NTHI 2019, the Δ *firR* mutant (NB004), the Δ *firS* mutant (KK009), the complemented *firR* mutant (*firR*⁺; KHS2), and the complemented *firS* mutant (*firS*⁺; KHS5) were grown in sRPMI at 37°C to early log phase and shifted to 9°C for 30 min prior to RNA extraction. Expression of each gene was measured by qRT-PCR and compared to the expression of each gene when cultures were incubated at 37°C. The data presented are means and standard deviations from two experiments, each performed in triplicate.

Δ *firS* mutant KK009, indicating that both FirR and FirS are required for activation of *ygiW-firRS* expression in response to cold temperatures. Complementation with a constitutively active chromosomal copy of *firR* (KHS2) or *firS* (KHS5) restored induction of *ygiW* and *firR* in response to the incubation at 9°C. These findings indicate that the FirRS two-component regulatory system activates expression of its own operon in response to cold shock.

FeCl₂ induces *ygiW-firRS* expression in a FirRS-dependent manner. NTHI is strictly a human pathogen without a niche outside the host (65), and internal body temperature is never 9°C. However, NTHI may be exposed to transient low temperatures, when in the nasopharynx, if the host is breathing cold air. Due to the similarity between FirRS and QseBC, we used qRT-PCR to explore the activation of FirRS by signals that have been shown to activate QseBC. Using this method, we determined that FirRS was unresponsive to epinephrine, norepinephrine, and serotonin at concentrations up to 200 μ M. We were also unable to elicit a response using spent media or fractions obtained by the AI-3 purification method described by Clarke et al. (14). Additionally, the expression of *ygiW-firRS* was unaffected by changes in oxygenation, envelope stress, or oxidative stress (data not shown).

To identify additional signals for FirRS, we constructed a green fluorescent protein (GFP) translational fusion vector containing a 402-bp DNA fragment from upstream of the *ygiW* ORF that encompassed the proximal two transcriptional start sites. We exposed exponentially grown wild-type 2019 carrying the GFP reporter to many different signals and determined if *ygiW-firRS* expression was affected, since FirR autoinduces *ygiW-firRS* ex-

pression. We then used the Phenotype MicroArray system (Biolog, Hayward, CA) to screen *ygiW-firRS* induction using the GFP reporter in response to four different concentrations of 120 different compounds, including antibiotics, sugars, salts, acids, and chelators (data not shown). Interestingly, expression of *ygiW-firRS* was most strongly induced by ZnCl₂.

Cation-responsive regulators have been shown to respond to multiple cations (8, 26), so we tested the expression of *ygiW-firRS* in response to various cations using the GFP reporter. In wild-type 2019, a 4- to 5-fold increase in GFP fluorescence is detected in response to FeCl₂ compared to an untreated control culture, and a 2-fold increase is detected in response to ZnCl₂ (Fig. 4A), which correlates to Phenotype Microarray data. The levels of GFP fluorescence in response to MnCl₂, CoCl₂, MgCl₂, and CaCl₂ were similar to a culture that had no addition. This suggests that only FeCl₂, and to a lesser degree ZnCl₂, activates *ygiW-firRS* expression in wild-type 2019.

FeCl₃, but not FeCl₂, was included in the Phenotype Microarray, and *ygiW-firRS* expression was unresponsive to FeCl₃. To test the specificity of FirRS for ferrous and ferric iron, induction of the GFP reporter was measured when exponentially grown cultures were exposed to 100 μ M and 200 μ M of FeCl₂, FeCl₃, or FeCl₃ with sodium ascorbate. Sodium ascorbate reduces FeCl₃ to FeCl₂ and keeps the iron in the reduced state (74). Significantly higher levels of *ygiW-firRS* promoter activity were detected in response to FeCl₂ and the sodium ascorbate/FeCl₃ cultures than in a control culture, but promoter activity was not induced in response to FeCl₃ (Fig. 4B). Sodium ascorbate by itself did not induce expression of the reporter, indicating that GFP expression was only increased in the presence of ferrous iron. Induction of GFP expression was induced with FeCl₂ concentrations as low as 50 μ M (data not shown). Additional studies determined that iron-mediated generation of reactive radicals was not responsible for induction, and in fact, the addition of exogenous H₂O₂ diminished Fe²⁺-mediated induction (data not shown), likely due to the rapid oxidation of ferrous iron by H₂O₂. This suggests that only ferrous iron, but not ferric iron or reactive radicals, activates FirRS signaling.

Ferrous iron could be acting as a signal for the FirRS two-component system, could be an important cofactor necessary for either FirR or FirS activity, or could be affecting a different regulator that affects *ygiW-firRS* expression. To determine if the ferrous iron-responsive induction of *ygiW-firRS* expression was FirRS dependent, we measured *ygiW-firRS* promoter activity in response to FeCl₂ in wild-type 2019, the Δ *firR* mutant NB004, the Δ *firS* mutant KK009, and complemented strains. The expression of *ygiW-firRS* significantly increases in response to FeCl₂ in wild-type 2019, but expression does not increase in either NB004 or KK009 (Fig. 4C). By restoring the *firR* gene or the *firS* gene on the chromosome in the mutant strains (KHS2 and KHS5, respectively), the Fe²⁺-responsive induction in *ygiW-firRS* expression is restored. In fact, it seems that constitutive expression of FirS, more so than for FirR, resulted in a hyperresponsive induction of *ygiW-firRS* expression, suggesting activated FirS may be the rate-limited step in the two-component process. These data suggest that Fe²⁺ induces *ygiW-firRS* expression through FirS and, subsequently, FirR.

FirS requires an iron-binding motif to sense Fe²⁺. Analysis of the FirS protein sequence revealed that FirS contains a putative iron-binding motif, DYRED, at amino acid residues 148 to 152, as

FIG 4 Induction of *ygiW-firRS* promoter activity in response to ferrous iron. Wild-type 2019 containing a GFP reporter fused to the *ygiW* promoter (pJJ392) was exposed to various conditions, and GFP fluorescence was measured to determine induction relative to background fluorescence (empty vector control). (A) Various cations were added to cultures at a 100 μ M concentration, and GFP fluorescence (RFU, relative fluorescence units) was determined. (B) FeCl₂, FeCl₃, or FeCl₃ and 5 mM sodium ascorbate (Asc) were added to cultures, and GFP fluorescence was determined. (C) GFP fluorescence in wild-type 2019 (black bars), Δ firR (gray bars; NB004), Δ firS (white bars; KK009), complemented *firR* (striped gray bars; KHS2), and complemented *firS* (striped white bars; KHS5) containing the reporter (pJJ392) in the presence or absence of FeCl₂. GFP fluorescence was measured relative to background fluorescence (empty vector control) to determine RFU. The data presented are means and standard deviations for triplicate determinations in a single experiment. The data presented here are representative of multiple (≥ 3) experiments performed, from which equivalent results and statistical trends were obtained. Statistical significance ($P \leq 0.05$), as determined by the Student two-tailed *t* test for the comparison of cultures with nothing added versus the other cultures with a compound added, is represented by an asterisk.

predicted previously for the *S. Typhimurium* homolog PreB (49). To determine if these amino acids were necessary for FirS to sense Fe²⁺ directly, we used site-directed mutagenesis to create three strains where the iron-binding motif was mutated. qRT-PCR was used to measure transcript levels of both *ygiW* and *firR* in exponentially grown cultures that were exposed to FeCl₂ or exposed to

FIG 5 Characterization of the FirS iron-binding motif. The induction of *ygiW* (A) and *firR* (B) in response to Fe²⁺ was measured by qRT-PCR. The ability of wild-type 2019, the Δ firS mutant KK009, KHS1 (*firS* Y149G, R150T), KHS3 (*firS* D148A), KHS4 (*firS* E151G, D152S), and KHS5 (*firS*⁺) to respond to Fe²⁺ was tested. Expression of each gene was measured by qRT-PCR and compared to the expression of each gene in cultures grown without the addition of exogenous FeCl₂. The data presented are means and standard deviations from two experiments, each performed in triplicate.

nothing. As seen with the GFP reporter studies, both *ygiW* and *firR* transcript levels increased at least 5-fold in response to FeCl₂ in wild-type 2019, but either no increase or a small increase in transcript levels was seen in the Δ firS mutant KK009 (Fig. 5). Both *ygiW* and *firR* transcript levels greatly increase in response to FeCl₂ in a derivative of the *firS* mutant carrying a constitutively active chromosomal copy of the *firS* gene, KHS5. Higher levels of *firR* and *ygiW* transcripts were seen in response to FeCl₂ in both *firR*(D148A) and *firR*(E151G,D152S) point mutation mutants, but not to the level of wild-type 2019, and transcript levels did not increase at all in Y149G. These mutants were still induced by cold shock, indicating the specificity of this site for Fe²⁺ signaling (data not shown). These experimental findings suggest that the tyrosine 149 and/or arginine 150 residues in the FirS protein are critical for Fe²⁺ sensing.

FirR must be phosphorylated to activate *ygiW-firRS* expression in response to FeCl₂. Two-component regulators affect expression depending on whether a key aspartate residue (at approximately amino acid 57) is phosphorylated or unphosphorylated (69). Significantly higher levels of *ygiW* transcript were detected in wild-type 2019 and the *firR* complementation strain KHS2 when exponentially grown cultures were exposed to FeCl₂ (Fig. 6). As seen with our GFP reporter studies, *ygiW* transcript levels did not increase in response to FeCl₂ in the Δ firR mutant NB004, and *ygiW* transcript levels did not increase to the level of wild-type when aspartate 51 was mutated to alanine (JWJ154). These data suggest that iron-responsive signal transduction requires the phosphorylation of FirR.

FIG 6 Characterization of *firR* mutants in Fe^{2+} -responsive induction of *ygiW*. The expression of *ygiW* in wild-type 2019, NB004 (Δ *firR*), JWJ154 (*firR* D51A), and KHS2 (*firR*⁺) was measured by qRT-PCR. Expression of each gene was measured by qRT-PCR and compared to the expression of each gene in cultures grown without the addition of exogenous FeCl_2 . The data presented are means and standard deviations from two experiments, each performed in triplicate.

The presence of YgiW and FirRS allows *H. influenzae* strains to retain their virulence in the mouse model of infection. NTHI causes otitis media and lung infections when the human host environment is compromised. To determine to what extent YgiW and FirRS protect NTHI 2019 in the host, the virulence properties of NTHI 2019 and isogenic *ygiW* and *firRS* mutants were evaluated in the chinchilla model for otitis media and the mouse model for lung infection. Isogenic *ygiW*, *firR*, *firS*, and *firRS* mutants were able to cause infection and remain as viable as wild-type 2019 and 86-028 NP in experimentally infected chinchillas (data not shown). However, the *ygiW* mutant JWJ142, the Δ *firR* mutant NB004, and the Δ *firS* mutant KK009 displayed significant attenuation compared to the parental 2019 strain in C57BL/6 mice (Fig. 7). These data provide evidence that the ability of FirRS to activate *ygiW* expression is important for the survival of *H. influenzae* 2019 in the lung environment but not in the middle ear. This may be an indication that NTHI face different challenges in the lung and middle ear and have evolved mechanisms to cope with these challenges. As we learn more about the role of FirRS signaling in the lung, these differences should become apparent.

DISCUSSION

Successful pathogens regulate gene expression in response to changes in their environment. It is also common for pathogens to use quorum sensing to coordinately regulate gene expression. There are three canonical quorum-sensing systems that have been characterized in detail: acyl-homoserine lactone signaling of Gram-negative bacteria, peptide-based signals of Gram-positive bacteria, and the AI-2 system that can be found in both Gram-negative and Gram-positive species (22). Complete homologs of these systems are not found in *H. influenzae* genome sequences (23, 31). NTHI does contain a functional LuxS that can synthesize AI-2 (5, 18), although its role in quorum sensing has not been established. An ortholog of a more recently described quorum-sensing system, the QseBC system of *E. coli*, is present in NTHI. We chose to characterize the NTHI ortholog of QseBC since that system is involved in quorum sensing and the regulation of virulence in other species.

Based on the data, our working model suggests that FirS (QseC) senses ferrous iron by directly binding Fe^{2+} in the periplasm. FirS becomes activated, leading to the phosphorylation

FIG 7 Survival of NTHI in a mouse pulmonary infection model. Mice were infected intranasally with wild-type 2019 (circles), JWJ142 (Δ *ygiW*; squares), NB004 (Δ *firR*; upward triangles), and KK009 (Δ *firS*; downward triangles), and the log₁₀ CFU in the lungs was determined at 24 and 48 h postinfection. The data presented here are representative of one experiment. Asterisks denote strains with a statistically significant difference with *P* values of 0.0317 or less compared to the wild type using the Mann-Whitney test.

and activation of FirR (QseB) in the cytoplasm. FirR activates transcription at a promoter upstream of *ygiW*, and both *ygiW* and *ygiW*-*firRS* transcripts are produced. The presence of an attenuator between *ygiW* and *firR* adds an additional level of control to the system, providing a mechanism for higher levels YgiW than the regulator. Since the *ygiW* transcript is upregulated 6-fold more than *ygiW*-*firRS*, it seems the primary function of FirRS is to activate *ygiW* expression in response to Fe^{2+} . This suggests that YgiW is the main effector of the FirRS system in NTHI. BOF proteins like YgiW are not well characterized; however, they have been implicated in stress responses (44, 57). CusF, another BOF protein, has been shown to bind to copper (24, 46), so perhaps YgiW plays a role in the resistance to high levels of Fe^{2+} or Zn^{2+} . However, we do not fully understand the role of YgiW at this time, but preliminary data suggest that YgiW may be involved in the import of ferrous iron.

Unlike the QseBC system, which responds to epinephrine, norepinephrine, and AI-3, the NTHI homolog FirRS responds to ferrous iron. FirRS is also activated when shifted to lower temperatures. While the biological relevance of this aspect of FirRS signaling is not clear at this time, it has proven useful to our studies. Thermoresponsive activation of FirRS allowed for the demonstration of autoregulation by FirR and the initial characterization of the *ygiW*-*firRS* operon. This led to the identification of ferrous iron as a signal for FirRS. Furthermore, all of these features have been confirmed in response to iron.

To date, FirRS is only the third TCST system that responds to extracellular iron to be characterized. The other two are BqsRS of *Pseudomonas aeruginosa* and PmrAB of *Salmonella* (30, 43, 73). The PmrAB system is specific for ferric iron, while the BqsRS system, like FirRS, is specific for ferrous iron. PmrAB has been shown to provide resistance to polymyxin B and high concentrations of iron (73). PmrAB is also activated by mildly acidic pH (54). BqsRS has been shown to respond specifically to ferrous iron (43) and regulates biofilm dispersal (20). Additionally, the BqsRS system activates the expression of a gene encoding a BOF superfamily protein similar to YgiW (43); however, its role has not been investigated.

Adrenergic regulation of virulence is an emerging theme in microbial pathogenesis (66). The influence of epinephrine and/or norepinephrine on bacterial gene expression has been demonstrated for enterohemorrhagic *E. coli* (EHEC) (14, 21,

61), *Salmonella enterica* (6), and *Vibrio parahaemolyticus* (52). In *E. coli*, two distinct TCST systems, QseBC and QseEF, are involved in the regulation of motility and virulence factors in response to epinephrine, norepinephrine, or AI-3 (14, 36, 60, 61). In *S. enterica*, studies by different groups have yielded conflicting results. Bearson et al. found that norepinephrine increased motility and chemotaxis genes, as well as genes required for invasion; however, QseC was not required for norepinephrine-mediated induction (6, 7). Interestingly, iron also increased motility in *S. enterica* (7). Moreira et al. also saw norepinephrine-enhanced motility and reported that only one QseC-regulated gene (*sifA*) was responsive to norepinephrine in a QseC-dependent manner (51). This is in contrast to the findings of Merighi et al., who did not see an effect by QseB/QseC (PreA/PreB) on motility but did see an influence on a number of other virulence genes, including *pmrAB* (48, 49); however, the role of epinephrine or norepinephrine was not explored. Finally, norepinephrine increased the expression of *V. parahaemolyticus* genes involved in type III secretion and virulence; however, the regulatory system has not been identified (52). Interestingly, norepinephrine has been shown to enhance iron uptake in a number of pathogens (3, 4, 25), stimulating growth and influencing gene expression. This raises the possibility that the activation of QseBC TCST systems is in response to iron rather than epinephrine. Based on the limited amount of research available on QseBC and similar TCST systems, there appears to be potential for diversity in both the members of the regulon between various organisms (even closely related genera like *Escherichia* and *Salmonella*) and the signals to which they respond. The one similarity to FirRS that we have observed is the regulation of *ygiW*. In the study by Merighi et al., *ygiW* was regulated by QseB (PreB) (49). QseBC-mediated regulation of *ygiW* has been demonstrated in *E. coli* (29); however, its role has not been investigated in detail.

It may seem surprising that FirRS would sense ferrous iron considering NTHI typically inhabits aerobic environments in which iron would most often be found in the ferric form. However, ferrous iron is present in reducing and acidic environments, so the FirRS system may function to detect shifts to these conditions. Disruption of the upper airway has been shown to result in elevated concentrations of both zinc and iron in sputum (28), and up to 110 μM Fe^{+2} has been measured in cystic fibrosis sputum samples (43). The production of phenazine by *P. aeruginosa* results in the generation of ferrous iron (68), so FirRS could potentially be involved in sensing the presence of a competitor or promote cooperation with other pathogens. Also, intraphagosomal accumulation of zinc has been demonstrated in pulmonary macrophage after the phagocytosis of *Mycobacterium tuberculosis* (9). It is possible that FirRS is involved in sensing zinc in this situation, and YgiW aids in survival. The exact role that FirRS signaling plays should become evident as we learn the function of YgiW and identify additional FirRS-regulated genes.

ACKNOWLEDGMENTS

We thank Lauren Bakaletz at Ohio State University College of Medicine and Ed Swords at Wake Forest University for assistance and training with the chinchilla otitis media model.

This work was supported by funding from NIH grant 2P20 RR020171 from the National Center for Research Resources (NCRR).

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or the NCRR.

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