A novel zinc binding system, ZevAB, is critical for survival of nontypeable Haemophilus influenzae in a murine lung infection model

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A Novel Zinc Binding System, ZevAB, Is Critical for Survival of Nontypeable Haemophilus influenzae in a Murine Lung Infection Model

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Nontypeable Haemophilus influenzae (NTHI) is a Gram-negative bacterial pathogen that causes upper and lower respiratory infections. Factors required for pulmonary infection by NTHI are not well understood. Previously, using high-throughput insertion tracking by deep sequencing (HITS), putative lung colonization factors were identified. Also, previous research indicates that secreted disulfide-dependent factors are important for virulence of H. influenzae. In the present study, HITS data were compared with an informatics-based list of putative substrates of the periplasmic oxidoreductase DsbA to find and characterize secreted virulence factors. This analysis resulted in identification of the “zinc binding essential for virulence” (zev) locus consisting of zevA (HI1249) and zevB (HI1248). NTHI mutants of zevA and zevB grew normally in rich medium but were defective for colonization in a mouse lung model. Mutants also exhibited severe growth defects in medium containing EDTA and were rescued by supplementation with zinc. Additionally, purified recombinant ZevA was found to bind to zinc with high affinity. Together, these data demonstrate that zevAB is a novel virulence factor important for zinc utilization of H. influenzae under conditions where zinc is limiting. Furthermore, evidence presented here suggests that zinc limitation is likely an important mechanism for host defense against pathogens during lung infection.

Nontypeable Haemophilus influenzae (NTHI) is a Gram-negative bacterial pathogen uniquely adapted to colonize the nasopharynx of healthy humans, with a carriage frequency of approximately 20 to 80% (46). An opportunistic pathogen, NTHI resides asymptomatically in the upper airways of humans but can disseminate into privileged anatomical locations, causing infections such as otitis media, sinusitis, and pneumonia (47). NTHI is also one of the most prevalent microorganisms found in the lungs of patients with exacerbations of chronic obstructive pulmonary disease (48, 49, 60) and cystic fibrosis (23, 43, 58). An effective vaccine against NTHI strains must be required for pathogenesis (30). DsbA was demonstrated to be required for pathogenesis of H. influenzae in a mouse model of bacteremia (56). Additionally, the heme-binding lipoprotein HbpA, an important factor required for growth of H. influenzae on several heme sources (26, 44), was found to be a substrate of DsbA and was required for pathogenesis. However, the defect of an hbpA mutant was not as severe as that of a dsbA mutant, suggesting that other, unidentified DsbA-dependent factors must be required for pathogenesis.

Since a DsbA-dependent protein was found to have a role in virulence of H. influenzae, we hypothesized that by examining genes required in the lung, additional DsbA-dependent factors involved in infection could be found. In this study, potential DsbA substrates were identified based on predicted extracellular localization and putative disulfide bond formation. The whole-genome fitness data generated via the HITS procedure was used to identify candidate DsbA substrates required for survival of H. influenzae in the lung. This approach led us to characterize a locus important for zinc utilization, herein referred to as “zinc binding essential for virulence"
This locus contains an operon consisting of two genes, the potential DsbA substrate zevA and a gene encoding a putative membrane protein called zevB. In contrast to the previously characterized ZnuABC zinc transport system, which is required for optimal growth in rich culture medium in vitro (39), the zevAB system is specifically required for growth under severe zinc limitation. The results of this study suggest that the lung represents a niche that exposes infecting pathogens to severe zinc limitation and that the zevAB system is required for zinc homeostasis of *H. influenzae* during pathogenesis.

**Materials and Methods**

**Strains and culture conditions.** *H. influenzae* RdAW (GenBank accession no. NZ_ACSM00000000), a capsule-deficient serotype d derivative (70), and pathogenic nontypeable *H. influenzae* strain NT127 (GenBank accession no. NZ_ACSL000000000) were grown in brain heart infusion (BHI) broth supplemented with 10 μg/ml hemin and 10 μg/ml NAD (sBHI) or on sBHI agar plates at 35°C. To generate an anearobic environment, strains were grown in anaerobic chambers with BBL GasPak Plus generators (Becton Dickinson and Company, Sparks, MD). Development of competence for transformation of *H. influenzae* was accomplished as previously described (5). For selection of Rd- and NTHI-derived strains, antibiotics were used at the following concentrations: 8 μg/ml tetracycline (Tc), 20 μg/ml kanamycin (Km), and 10 μg/ml gentamicin (Gm). For strain generation, plasmids and PCR products were constructed using standard molecular biology techniques (4). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, linearized, and used to transform *H. influenzae* strains as previously described (70).

**zevA strain construction.** The NT127 zevA strain was constructed by replacement of the coding sequence of zevA (NT127 locus tag HIAG_01363) with the Gm resistance cassette auxC1. To do this, a 707-bp PCR fragment containing the 5' flanking region of zevA, generated using primers 1249for and 1248rev (see Table S1 in the supplemental material), a 762-bp PCR product containing the coding region for the Gm resistance cassette, amplified from NT127 genomic DNA with primers 51249HA and 31249R, and a 1,661-bp PCR product containing the 3' flanking region of zevA, generated with primers 1249A and 1248rev, were joined by overlap extension PCR via complementary ends using primers 51249HA and 31248R (see Table S1 in the supplemental material), a 762-bp PCR product containing the coding region for the Gm resistance cassette, generated with primers 51249HA and 31249R, and a 1,661-bp PCR product containing the 3' flanking region of zevA, generated with primers 1249A and 1248rev, were joined by overlap extension PCR via complementary ends using primers 51249HA and 31248R (see Table S1 in the supplemental material), a 762-bp PCR product containing the coding region for the Gm resistance cassette, generated with primers 51249HA and 31249R, and a 1,661-bp PCR product containing the 3' flanking region of zevA, generated with primers 1249A and 1248rev, were joined by overlap extension PCR via complementary ends using primers 51249HA and 31249R, which bind outside the recombination junctions.

To complement the zevA deletion, zevA and upstream promoter elements were amplified by PCR with primers 51249HA and 31249R, which introduce SapI restriction sites at the termini of the fragment. The resulting 1,017-bp fragment was digested with SapI and cloned into SapI-digested vector pXT10, creating plasmid pX1249J. Plasmid pX1249J was linearized and used to transform competent R5 cells, resulting in strain R5.

**zeb strain construction.** Deletion of zevB in NT127 was also performed by replacement of the coding region of zevB (HIAG_01346) with the Km resistance gene. To increase the efficiency of mutant selection, the auxC1 promoter was replaced with the *H. influenzae* arcA promoter. First, a 1,332-bp fragment containing the 5' flanking region of zevB, amplified by PCR using NT127 genomic DNA as a template with primers 1249for and 1248BpArcA, a 762-bp fragment carrying the arcA promoter fused to the coding region of the Gm resistance gene from auxC1, amplified from a pXT10 plasmid using primers SpArcA and 3GmCt2, and a 1,326-bp fragment containing the 3' flanking region of zevB, amplified from NT127 genomic DNA using primers 1249Ahib and 1247RevB, were joined by overlap extension PCR using primers 1249for and 1247RevB. The resulting 3,638-bp fragment was used to transform competent cells of strain NTX. Gm' transformants were selected on sBHI-Gm plates, resulting in strain NTzevAG. NTzevAG was transformed with linearized "empty vector" containing Tc, resulting in strain R5.

To complement mutation of zevAB in R5, a 1,088-bp fragment containing the zevAB coding regions as well as upstream promoter elements was amplified by PCR from Rd genomic DNA with primers 51249HA and 1249for and 1249BpArcA, a 762-bp fragment carrying the arcA promoter fused to the coding region of the Gm resistance gene from auxC1, amplified from a pXT10 plasmid using primers SpArcA and 3GmCt2, and a 1,326-bp fragment containing the 3' flanking region of zevB, amplified from NT127 genomic DNA using primers 1249Ahib and 1247RevB, were joined by overlap extension PCR using primers 1249for and 1247RevB. The resulting 3,638-bp fragment was used to transform competent cells of strain NTX. Gm' transformants were selected on sBHI-Gm plates, resulting in strain NTzevAG. NTzevAG was transformed with linearized vector pXT10, creating plasmid pX1248R. Next, a 2,891-bp fragment containing the xyIF gene as well as the 5' flanking and coding regions of zevAB, amplified from plasmid pX1248R with primers pXT10xyIF and 31248Bgnt, the 762-bp Gm resistance cassette fragment described above, and a 1,794-bp fragment containing the xyB gene, amplified from pXT10 with primers 51249Bgnt and 31248Bgnt, were joined using overlap extension PCR with primers pXT10xyIF and 31248Bgnt, resulting in strain R5fnr. The resulting 5,447-bp fragment was used to transform competent cells of strain R5, resulting in strain R5fnr.
and 3FNRgent), the 762-bp Gm resistance cassette fragment described above, and a 1,794-bp fragment containing the xylB gene (amplified from pXT10 with primers 5x124RT and 3124RT, respectively). Primers RpoA5 and RpoA3 were used to amplify the RNA polymerase alpha subunit gene, as an internal reference. The real-time cycler conditions used have been described previously (71). The mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase, confirmed that the results obtained were not due to contaminating genomic DNAs (data not shown).

Reporter expression analysis. Strains were plated at a density of $2 \times 10^6$ CFU per plate on sBHI agar with or without 100 mM ZnSO$_4$ addition and incubated at 35°C overnight in either an open air incubator or an anaerobic chamber. Cells were swabbed from plates and diluted into Z buffer (42) to a concentration of $2 \times 10^7$ CFU/ml. The mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase, confirmed that the results obtained were not due to contaminating genomic DNAs (data not shown).

The mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase, confirmed that the results obtained were not due to contaminating genomic DNAs (data not shown).

Stoichiometry assays were performed as described previously (18). ZevA protein (0.8 $\mu$M) was incubated for 1 min at room temperature in the presence or absence of 50 $\mu$M ZnSO$_4$. Buffer (50 $\mu$L HEPES-NaOH, pH 7.5, 200 mM NaCl, and 10% glycerol) containing 50 $\mu$M ZnSO$_4$ was incubated in parallel as a control. After incubation, excess Zn$^{2+}$ was removed by size exclusion with Sephadex G-25 columns. Eluted protein was acid precipitated with concentric acid nitric acid overnight at room temperature. Following digestion, samples were treated with $H_2O_2$ to a final concentration of 1.5%. The concentration of Zn$^{2+}$ in each sample was measured using atomic absorption spectroscopy (AAS) (AAAnalyst 300, PerkinElmer, Waltham, MA). Average background Zn$^{2+}$ levels detected for control samples were <2% of levels determined for ZevA-Zn$^{2+}$ samples.

Determination of ZevA affinity for Zn$^{2+}$. The binding affinity of ZevA for Zn$^{2+}$ was determined using the Zn binding chromophore mag-fura-2 (Invitrogen, Carlsbad, CA) as previously described (18, 37, 67). Briefly, 10 $\mu$L ZevA protein and 20 $\mu$L mag-fura-2 were mixed and titrated with 1 mM Zn$^{2+}$. Free mag-fura-2 was determined by monitoring OD$_{366}$ of free mag-fura-2, and K$_J$ is the association constant of mag-fura-2 for ZevA$^{2+}$ (K$_J$ = $v$ (metal) / [Metal]$, I$ / $v$ (metal)) for ZevA$^{2+}$ protein association constant (K$_J$) and the apparent stoichiometry of (n) ZevA were calculated by fitting the data to the equation $v$ (metal) / [Metal] = nK$_J$ ([Metal]), where n is the ratio of moles of metal to total protein, n is the number of binding sites, and [Metal], is the free metal concentration (25).

qRT-PCR. RNA was prepared from liquid cultures of Rd using an RNeasy mini kit and an on-column DNase I kit (Qiagen, Valencia, CA). The RNA samples (5 $\mu$L) were used as templates for cDNA synthesis with Random Primer (Invitrogen, Carlsbad, CA) and RiboLock (Ambion, MA) and were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA). Quantitative reverse transcription-PCR (qRT-PCR) was performed with qSYBR green supermix (Bio-Rad Laboratories, Hercules, CA), and fluorescence was measured over time using the DNA Engine Opticon II system (MJ Research, Waltham, MA). For transcript analysis of zevA and zevR, qRT-PCR was performed with primers 51249RT and 31249RT and primers 51248RT and 31248RT, respectively. Primers RpoA5 and RpoA3 were used to amplify the RNA polymerase alpha subunit gene, rpoA, as an internal reference. The real-time cycler conditions used have been described previously (71). The mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase, confirmed that the results obtained were not due to contaminating genomic DNAs (data not shown).

Replicates were inoculated from 1 ml overnight cultures of strain H. influenzae Rd at an OD$_{600}$ of 0.01. The resulting cultures were incubated with shaking at 250 rpm and 35°C until mid-log phase before being diluted into Z buffer (42) to a concentration of $2 \times 10^7$ CFU/ml. The mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase, confirmed that the results obtained were not due to contaminating genomic DNAs (data not shown).
RESULTS

Identification of secreted factors involved in *H. influenzae* pathogenesis. An informatics-based approach was utilized to compile a database of the exported proteins in *H. influenzae* that are likely to require DsbA for oxidative folding. First, web-based prediction servers SignalP (7) and LipoP (32) were used to identify polypeptides with predicted cleavage sites for signal peptidase I (soluble exported proteins) and signal peptidase II (lipoproteins), respectively. Next, the list was refined to include only mature proteins (excluding leader peptides) with two or more cysteine residues, as these are expected to be DsbA substrates (50). The β-barrel outer membrane protein predictor (BOMP) (9) and membrane topology prediction servers TMHMM (63) and Phobius (33) were used to predict the locations of cysteines in the loops of membrane proteins, thereby excluding proteins with cysteines that are predicted to be cytoplasmic and inaccessible to DsbA. The final database includes secreted proteins involved in a variety of functional processes, which are cataloged based on Clusters of Orthologous Groups (COG) in Table S2 in the supplemental material.

Next, to identify the putative substrates of DsbA that are required for pathogenesis of *H. influenzae*, we cross-referenced the list of genes in Table S2 in the supplemental material with the set of genes previously implicated in survival of *H. influenzae* Rd in the lung, as identified by the HITS technique (22). In that study, transposon mutant libraries were acquired before and after selection for survival in the mouse lung model, and transposon/chromosomal junctions were amplified from genomic DNA, captured, sequenced, and aligned with the *H. influenzae* genome sequence. The results of the analysis were reported as a survival index (s.i.), which denotes the number of mutations detected in a given gene in the lung-selected output library divided by mutations detected in the in vitro-grown input library. Genes required during lung infection were identified on the basis of two parameters: having an s.i. of 0.3 or lower and sustaining insertions in >40% of the potential TA dinucleotide insertion sites, specific to the himar1-derived transposon used in the study (22). Comparison of our list of potential DsbA substrates to the results of HITS revealed three genes that fit these criteria: 

- **zevA** (s.i., 0.101), encoding the periplasmic component of a high-affinity zinc uptake transporter (39, 53), *nlpD* (s.i., 0.000), encoding a lipoprotein suggested to be involved in daughter cell separation (64, 65), and *H11249* (s.i., 0.106), encoding a putative protein herein referred to as ZevA. The role of *zevA* in virulence has been studied by several groups (3, 11, 14, 21, 36, 57, 75), and *nlpD* has been implicated in the virulence of *Yersinia pestis* and *Yersinia enteroocolitica* (12, 64). However, *zevA* has not been previously investigated and its role in virulence is unknown. Additionally, the putative gene 2 bp downstream of the 3′ end of the *zevA* coding sequence, *H11248*, herein referred to as *zevB*, was also found to be required for lung survival (s.i., 0.103).

The results of HITS were verified using genetic footprinting on our mutant libraries. In this technique, transposon insertions in a given region are physically mapped using PCR with a chromosome-specific primer paired with a transposon-specific primer (1, 61). The size of the amplified product corresponds to the distance between the chromosome-specific primer and the transposon insertion within that gene. In agreement with HITS, footprints of the library recovered from lung infection reveal a reduction in transposon insertions within the *zevAB* locus compared with footprints of the input library (Fig. 1). These data indicate that *zevA* and *zevB* are essential for lung colonization by *H. influenzae* Rd.

**zevAB** is required for efficient NTHI infection of the mouse lung. Mutants of *zevA* and *zevB* were generated in an NTHI clinical isolate, NT127 (28), to evaluate the roles of these genes during *in vitro* growth and lung infection. Mutants were constructed by replacement of coding regions with a drug resistance marker to generate nonpolar deletions, and complementation was conducted using an exchange vector for delivery and expression at the xylose locus (70). The complete strain set consisted of the parent strain carrying the empty vector (NTV), the *zevA* mutant carrying the empty vector (NTzEvAV), the *zevB* mutant carrying the empty vector (NTzEvBV), and complemented *zevA* and *zevB* mutants (NTzEvAX and NTzEvBX, respectively) (Table 1). When grown aerobically in sBHI medium, all strains had equivalent growth yields and generation times (NTV, 45.4 ± 1.7 min; NTzEvAV, 44.1 ± 1.3 min; NTzEvBV, 41.6 ± 0.6 min; NTzEvAC, 43.0 ± 0.5 min; and NTzEvBC, 44.2 ± 1.9 min). Ratios of CFU to optical densities at 600 nm were indistinguishable between these strains.

Next, these strains were evaluated for their ability to infect the lungs of C57BL/6 mice. This experiment was performed using mixed infections in which a competition was performed between each strain and strain NTlacZ, which expresses *E. coli lacZ* at the xyI locus. At 40 h after inoculation, average competitive indices (c.i.) were 3-fold lower for the *zevA* mutant (NTzEvAV; c.i., 0.364) and ~22-fold lower for the *zevB* mutant (NTzEvBV; c.i., 0.064) compared to that for the parent strain (NTV; c.i., 1.22) (Fig. 2). Complementation restored the ability...
TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, description, and/or relevant features</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>NT127</td>
<td>Nontypeable <em>H. influenzae</em> clinical isolate</td>
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<tr>
<td>NTV</td>
<td>NT127 xylAΔ0.8::tetAR; tetAR sequence from pXT10 replaces xylA</td>
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<tr>
<td>NTZevAV</td>
<td>NT127 Δzev:: xacC1, xylAΔ0.8::tetAR; zevA deletion mutant with tetAR Te' cassette replacing xylA</td>
<td>This study</td>
</tr>
<tr>
<td>NTZevAX</td>
<td>NT127 Δzev:: xacC1, xylAΔ0.8::zevA; zevA deletion mutant complemented with zevA expressed via the zevA promoter in place of xylA</td>
<td>This study</td>
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<tr>
<td>NTZevBV</td>
<td>NT127 Δzev:: xacC1, xylAΔ0.8::zevA; zevA mutant replacing xylA</td>
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<tr>
<td>NTZevBX</td>
<td>NT127 Δzev:: xacC1, xylAΔ0.8::zevAB; zevB deletion mutant complemented with zevAB expressed via the zevA promoter in place of xylA</td>
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<td>NTlacZ</td>
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<td>NTZnuA</td>
<td>NT127 ΔznuBC::aphI, xylAΔ0.8::tetAR; znuA deletion mutant with tetAR Te' cassette replacing xylA</td>
<td>This study</td>
</tr>
<tr>
<td>NTZevAznA</td>
<td>NTZevAV ΔznuBC::aphI; zevA and znuA double mutants</td>
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</tr>
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<td>NTZevBznA</td>
<td>NTZevBV ΔznuBC::aphI; zevB and znuA double mutants</td>
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<td>NTZnuB</td>
<td>NT127 ΔznuBC::aphI, xylAΔ0.8::tetAR; znuBC deletion mutant with tetAR Te' cassette replacing xylA</td>
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<td>NTZevAznB</td>
<td>NTZevAV ΔznuBC::aphI; zevA and znuBC double mutants</td>
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<tr>
<td>NTZevBznB</td>
<td>NTZevBV ΔznuBC::aphI; zevB and znuBC double mutants</td>
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<td>Rd AW</td>
<td>Wild type; <em>H. influenzae</em> capsule-deficient type d</td>
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<td>R5</td>
<td>Rd Δzev:: lacZ; zevAB deletion mutant with lacZ expressed via the zevA promoter (zevA reporter strain)</td>
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<td>R5X</td>
<td>R5 xylAΔ0.8::zvevAB; strain R5 complemented with zevAB expressed via the zevA promoter replacing xylA</td>
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<td>R5fmxR</td>
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<td>RdlacZ</td>
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<td>pXT10</td>
<td>Delivery vector for chromosomal expression at the xylose locus of <em>H. influenzae</em> containing xylF, xylB, xylAΔ0.8::tetAR and the tetAR tetracycline resistance cassette</td>
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<td>P1249</td>
<td>pXT10 carrying zevA from NT127 expressed from the zevA promoter</td>
<td>This study</td>
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<tr>
<td>p1248J1</td>
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<tr>
<td>pX1248R</td>
<td>pXT10 carrying zevAB from Rd expressed from the zevA promoter</td>
<td>This study</td>
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<tr>
<td>pXELacZ2</td>
<td>pXT10 carrying lacZ expressed from the xylA promoter</td>
<td>This study</td>
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FIG. 2. Effect of zevA and zevB mutations on survival of NTHI in the mouse lung infection model. Mice were coinfected with reference strain NTlacZ and the parent strain carrying the empty vector (NTV), the zevA mutant (NTZevAV), the complemented zevA mutant (NTZevAX), the zevB mutant (NTZevBV), or the complemented zevB mutant (NTZevBX). Bacteria were recovered from lung homogenates after 40 h. Competitive indices (c.i.) are calculated as the ratio of recovered CFU of the LacZ- experimental strain to recovered CFU of the LacZ+ reference strain. The symbols indicate data for individual animals, and the dashed lines indicate averages. The lower limit of detection was a c.i. of 0.001. Relevant statistical comparisons are indicated by brackets, and P values were determined using ANOVA with Tukey’s multiple-comparison test. NTV, n = 8; NTZevAV, n = 7; NTZevAX, n = 8; NTZevBV, n = 7; NTZevBX, n = 6.

of the mutants to survive in the lung, verifying that defects are specific to mutation of zevA and zevB. The complemented strains, NTZevAX and NTZevBX, exhibited competitive indices that were slightly higher than that of the parent strain, likely due to increased expression of the complementing genes due to the presence of both the zevA and xylA promoters in the constructs. To examine the possibility that mixed infection influences relative survival of the mutants, the zevA mutant was evaluated in the lung model in single-strain infections. Consistent with results obtained with mixed infections, average CFU recovery at 40 h postinfection was significantly reduced (P = 0.027) 3.5-fold for the zevA mutant compared to that for the parent strain (data not shown). In agreement with HITS analysis of Rd, these data indicate that the zevAB genes are required for colonization and survival of pathogenic NTHI in the mouse lung but are not required for normal growth in rich medium.

Bioinformatic analysis of zevA and zevB. BLAST (2) reveals that zevA and zevB of *H. influenzae* are conserved in other bacterial species within the Pasteurellaceae and Enterobacteriaceae, including several human pathogens such as *Klebsiella pneumoniae*, *Yersinia pestis*, *Proteus mirabilis*, and *Salmonella enterica* serovar Typhimurium (Table 2). In the Conserved Domains Database (CDD) (40), ZevA and ZevB are annotated by structural domain classification as the periplasmic substrate-binding protein (PBP) and the permease subunit of...
an ATP-binding cassette (ABC) transporter, respectively. ZevB is also annotated as a member of the NiCoT superfamily of potential nickel/cobalt importers and exporters (2). This superfamily is comprised of a diverse group of eight transmembrane segmented transporters with a characteristic HX4DH sequence in their second transmembrane span (17). ZevB does not show these characteristics. Moreover, modeling of ZevB using the consensus prediction of membrane protein topology server (TOPCONS) (8) indicates that it contains only the typical six transmembrane spans present in each of the two transmembrane subunits observed in ABC ATPases (13). This evidence suggests that ZevA and ZevB are partnering components of an ABC ATPase transport system probably involved in substrate influx.

**ZevA and ZevB are important for growth of *H. influenzae* during zinc limitation.** Metal-restricted growth conditions can be generated by titration of the medium with increasing concentrations of EDTA (3, 11, 54). To determine whether zevAB is involved in metal acquisition in *H. influenzae*, strains were grown in medium containing 0.25, 0.5, or 0.75 mM EDTA. Mutants of *zevA* and *zevB* were able to acquire essential nutrients for optimal growth in rich medium, as evidenced by the lack of apparent growth defects in vitro (Fig. 3A). However, medium containing 0.5 mM EDTA inhibited growth of the *zevA* and *zevB* mutants, in which growth yields at 10 h were reduced by 81% and 87%, respectively, compared to that of the parent or complemented strains, which grew normally (Fig. 3B). Supplementation with 0.25 mM EDTA had no effect on the growth of any strains, whereas 0.75 mM EDTA significantly reduced growth of the parent and complemented strains in addition to that of the mutants (data not shown).

In preliminary studies, specific metals were added to medium chelated with EDTA in an attempt to promote growth of the mutants. The addition of MgCl2, CaCl2, MnCl2, FeCl3, CoCl2, NiCl2, CuSO4, or Na2MoO4 at a concentration of either 30 or 60 μM had no significant effect on the growth of the *zevB* mutant in the presence of 0.5 mM EDTA (data not shown). In contrast, addition of 30 μM ZnSO4 partially restored the growth of the *zevB* mutant and addition of 60 μM ZnSO4 completely restored growth to the level of the parent strain. Figure 3C shows that in the presence of 0.5 mM EDTA, addition of 60 μM zinc restores both the *zevA* and *zevB* mutants to levels of the parent and complemented strains. These data suggest that zevAB is important for the growth of *H. influenzae* under conditions where free zinc is limiting.

**ZevA protein binds zinc with high affinity.** Metal binding assays were performed to determine whether ZevA binds Zn2+ and therefore can participate in metal uptake. Recombinant ZevA was cloned, expressed in *Escherichia coli*, and affinity purified, and the metal binding tag was removed. After incubation in the presence of Zn2+, the amount of metal bound to ZevA was determined by atomic absorption spectroscopy (AAS). The results indicate that ZevA binds 1.9 ± 0.3 Zn2+ ions per mol of ZevA. The affinity of ZevA for Zn2+ was determined in metal competition assays including ZevA and mag-fura-2. The fluorescent metal chelator mag-fura-2 binds

### TABLE 2. BLAST results of *H. influenzae* Rd ZevA and ZevB homologs in other pathogensa

<table>
<thead>
<tr>
<th>Organism</th>
<th>ZevA (HI1249)</th>
<th>ZevB (HI1248)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Locus ID</td>
<td>Identity (%)</td>
</tr>
<tr>
<td><em>H. influenzae</em> NT127</td>
<td>HIAG_01363</td>
<td>97</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> 342</td>
<td>Kpk_1251</td>
<td>42</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> KIM 10</td>
<td>y1329</td>
<td>43</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> HI4320</td>
<td>PMI1519</td>
<td>37</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium LT2</td>
<td>STM2552</td>
<td>37</td>
</tr>
</tbody>
</table>

a ID, identification. HI1249, 206 amino acids (aa); HI1248, 322 (aa).
$\text{Zn}^{2+}$ with a $K_a$ of $5 \times 10^7$ M$^{-1}$, and this is associated with a shift from 366 nm to 325 nm in the chelator’s spectrum maximum. Thus, the decrease in OD$_{366}$ was used to calculate the shift from 366 nm to 325 nm in the chelator’s spectrum maximum. Cooperative binding to the two $\text{Zn}^{2+}$ binding sites was not observed. Fitting the data to a Langmuir equation yielded an association constant of $\text{Zn}^{2+}$ of $1.9 \pm 0.3 \times 10^7$ M$^{-1}$. This affinity is similar to those observed for other known $\text{Zn}^{2+}$ binding proteins (15, 24, 37).

Relative contributions of $\text{zevAB}$ and $\text{znuABC}$ in zinc utilization. Aside from the ZevAB system presented here, only one other system has been implicated in zinc utilization of $H$. influenzae, the high-affinity zinc transporter $\text{ZnuABC}$. An $H$. influenzae $\text{znuA}$ mutant (originally called $pzp1$) was shown to exhibit growth defects in normal medium that could be rescued by addition of zinc, and recombinant $\text{ZnuA}$ bound up to 5 molecules of zinc per protein (39). The $\text{znuBC}$ genes have also been demonstrated to participate in zinc utilization in other organisms (11, 53), including Pasteurella multocida (21), a close relative of $H$. influenzae, and are thought to encode the ATPase ($\text{znuC}$) and membrane permease ($\text{znuB}$) components of the transporter (52, 53). To evaluate the conditions under which ZevAB and ZnuABC are needed, their relative contributions to the growth of $H$. influenzae were examined through single and double mutant analyses. First, a $\text{zevA}$ mutant was generated by replacement of its coding region with an antibiotic resistance marker, and its growth was compared to that of the $\text{zevA}$ and $\text{zevB}$ mutants. The $\text{znuA}$ mutant (NT$\text{znuA}$) exhibited an $\sim 16\%$ increase in generation time in sBHI broth relative to the parent strain (NTV), $\text{zevA}$ mutant (NT$\text{zevAV}$), and $\text{zevB}$ mutant (NT$\text{zevBV}$) (Fig. 5A). Addition of 60 μM zinc restored growth of the $\text{znuA}$ mutant in rich medium to wild-type levels (data not shown), similar to results previously reported for $\text{znuA}$ mutants of other $H$. influenzae strains (39). When grown in 0.1 mM EDTA, the $\text{znuA}$ mutant exhibited both a 21-fold reduction in growth yield at 10 h and a 2.3-fold increase in generation time compared with growth of this strain in sBHI medium, whereas the parent strain, $\text{zevA}$ mutant, and $\text{zevB}$ mutant grew normally under this condition (Fig. 5B). In the presence of 0.5 mM EDTA, the growth yield of the $\text{znuA}$ mutant was also reduced 21-fold compared to that of the parent strain (Fig. 5C). Additionally, growth yields of the $\text{zevA}$ and $\text{zevB}$ mutants were reduced by 67% compared with that of the parent strain, similar to data reported for these strains in Fig. 3B. A $\text{znuBC}$ deletion, which was also constructed by gene replacement, exhibited defects similar to those of the $\text{znuA}$ mutant during growth in rich medium or medium containing 0.5 mM EDTA (data not shown), providing evidence that $\text{ZnuBC}$ functions together with $\text{ZnuA}$ in $\text{NTHI}$ as components of the zinc transport system described previously for other organisms (11, 21, 53).

Next, to examine the role of $\text{zevAB}$ in zinc uptake in the absence of the $\text{znuABC}$ pathway, double mutants were generated by deletion of $\text{znuA}$ or $\text{znuBC}$ in either the $\text{zevA}$ or $\text{zevB}$ mutant background. The growth phenotypes of the double mutants were equal in severity to that of the $\text{znuA}$ or $\text{znuBC}$ mutant in sBHI medium, sBHI with various concentrations of EDTA (0.0025 mM, 0.005 mM, or 0.01 mM), or sBHI supplemented with various concentrations of zinc (0.001 mM, 0.003 mM, or 0.05 mM). The lack of an additive effect of $\text{zevA}$ or $\text{zevB}$ with $\text{znuA}$ or $\text{znuBC}$ suggests that $\text{ZnuAB}$ and $\text{ZnuABC}$ transport zinc through separate pathways.
zevB mutation in combination with znuA or znuBC mutation indicates that ZevAB does not substitute for ZnuABC for zinc uptake at these zinc concentrations. Taken together, these data suggest that ZevAB functions in a specialized pathway needed only when the concentration of free zinc is low in contrast to that of ZnuABC, which is essential for growth over a wide range of free zinc concentrations.

**FNR participates in regulation of the ZevAB operon.** In many species that have been examined, transcriptional regulation of bacterial genes involved in zinc uptake is mediated by the zinc-responsive metalloregulatory protein Zur (27). When zinc is abundant in the cell, dimerized Zur binds to a specific palindromic sequence in promoters to repress transcription of the zinc-responsive metalloregulatory protein Zur (27). When zinc is low in the cell, monomeric Zur binds to a specific palindromic sequence in promoters to repress transcription of zinc-responsive genes (54). However, previous studies, as well as our own BLAST searches, indicate that the Pasteurellaceae do not contain a zur homolog (21). In these organisms, zinc uptake is likely regulated by other mechanisms. For example, in *Pasteurella multocida*, znuABC is regulated by the iron response regulator Fur (21).

To investigate the potential regulation of *zevAB*, the 330-bp region directly upstream of the *zevA* translational start site from the start codon of HI1250 (negative strand relative to the *zevA* coding sequence) to the start codon of *zevA* (positive strand) was analyzed for potential transcriptional regulator binding sites. A palindromic sequence between 235 and 248 bp upstream of the HI1250 start codon that was conserved in both Rd and NT127 strains was identified (Fig. 6A). This sequence matches the known consensus binding site for the oxygen-responsive transcriptional regulator FNR (16, 41). FNR directly senses a range of low-oxygen conditions and binds to DNA to either repress genes required for aerobic growth or activate those needed for growth under low-oxygen conditions (28, 35).

To determine the expression profiles of *zevA* and *zevB*, we first examined the transcription of these genes in the Rd strain. Cultures were grown in liquid medium, and transcript levels were determined by quantitative PCR, revealing that *zevA* and *zevB* are expressed similarly to the reference gene *rpoA*, which encodes RNA polymerase. Together with their genomic organization, these data suggest that *zevA* and *zevB* may be expressed via the same promoter.

Next, to determine whether the *zevAB* genes are regulated in response to oxygen, reporter strain R5 was constructed as a *zevA* promoter-*lacZ* fusion with deletion of *zevAB* in the Rd background (Fig. 6A). A complemented reporter strain, R5X, was also generated in which the *zevAB* genes were expressed via their native promoter at the *zyl* locus in trans to the *zevA* promoter-*lacZ* fusion. When grown aerobically (sBHI agar plate), ß-Gal activities were similar between the reporter strain, R5, and the complemented reporter strain, R5X (Fig. 6B). When bacteria were grown anaerobically, ß-Gal activities were reduced for both strains by ~50% compared with aerobic levels. A strain carrying an *fnr* disruption mutation in the R5 background, R5fnr, exhibited ~80% greater ß-Gal activity than the parent reporter strain or complemented reporter strain under anaerobic conditions, effectively restoring activity to levels observed for the parent strain under aerobic conditions. The *fnr* mutant also exhibited an ~25% increase in ß-Gal activity compared with the parent strain under aerobic conditions, likely due to oxygen availability decreasing as culture density increases on the plate. Complementation restored levels of fusion expression in the *fnr* mutant to those of the wild type. Additionally, RdlacZ, a strain expressing *lacZ* via a constitutive promoter, produced equivalent ß-Gal levels in both the aerobic and anaerobic environments, suggesting that ß-Gal activity itself is not affected by the growth condition (Fig. 6B).

To examine whether free zinc concentrations affect *zevA* reporter expression, the complemented reporter strain, R5X, was grown in liquid medium in the presence or absence of 0.5 mM EDTA under aerobic conditions. In this assay, ß-Gal levels of R5X were indistinguishable between the two conditions (data not shown). When R5X was grown under either aerobic or anaerobic conditions in medium supplemented with 100 mM zinc (a concentration nontoxic to *H. influenzae*), ß-Gal levels were similar to those observed for this strain in normal sBHI medium (data not shown). Taken together, these data indicate that expression from the *zevA* promoter is unaffected by the presence or absence of *zevAB* or changes in free zinc concentrations and is repressed by FNR under oxygen-limiting...
conditions. Furthermore, a higher level of zevA promoter activity in the aerobic environment is consistent with the requirement of zevAB for lung infection, in which H. influenzae is likely to encounter high oxygen concentrations.

**DISCUSSION**

Secreted bacterial proteins mediate critical aspects of pathogenesis and often require disulfide bond formation in the periplasm to stabilize their mature structures. Due to their extracytoplasmic location and accessibility, identification of these factors may aid in the design of novel therapeutics or vaccines for combating NTHI infections. Here, we generated a list of potential substrates of the periplasmic disulfide oxidoreductase protein DsbA (see Table S2 in the supplemental material) and integrated our findings with the results of a previous study in which HITS technology was used to identify genes required for lung infection (22). Using this strategy, we identified three genes that were potentially important for lung infection, including two, _znuA_ and _nlpD_, that had previously been implicated in the virulence of several organisms (3, 11, 12, 14, 21, 36, 64, 75), and a third gene, _zevA_, whose function had not been determined. Therefore, _zevA_ and its neighboring gene, _zevB_, were investigated for their roles in the pathogenesis of a clinical NTHI isolate. NTHI mutants of _zevA_ and _zevB_ were not defective for growth _in vitro_ on rich medium compared with the parent or complemented strain but exhibited significant survival defects in a mouse lung infection model (Fig. 2). These data confirmed that _zevA_ and _zevB_ are dispensable for the virulence of _H. influenzae_ strains in the murine lung. Interestingly, several potential DsbA-dependent proteins that were not required in the lung model as determined by HITS are important for _H. influenzae_ pathogenesis in other sites of infection. For example, the hemopexin utilization protein _HxuA_ has been shown to be required for wild-type bactericidal levels in an infant rat model of _H. influenzae_ type B infection (45), and the outer membrane protein _P5_ (OmpA) is required for virulence of _NTHI_ in a chinchilla model of otitis media (62). This suggests that examination of the requirement for DsbA substrates in other _H. influenzae_ infection models is likely to reveal additional mechanisms involved in pathogenesis.

Informatics-based analysis of ZevAB suggested potential roles in metal transport, and this possibility was explored. Mutants of _zevA_ and _zevB_ were highly defective for growth in the presence of 0.5 mM EDTA (Fig. 3B), suggesting that these genes are important during metal-restricted growth. Supplementation with 60 μM zinc, but not other metals, was able to rescue the growth of the _zevA_ and _zevB_ mutants in the presence of 0.5 mM EDTA to levels of the parent and complemented strains (Fig. 3C). The concentration of zinc in sBHI medium was determined to be 40 μM using AAS. Therefore, upon chelation with EDTA, addition of 60 μM likely restores free zinc concentrations to the normal range found in sBHI media. Together, these data indicate that _zevAB_ is a novel zinc utilization pathway important for growth of _H. influenzae_ when free zinc concentrations are limiting. Consistent with this observation, we also demonstrated that ZevA protein binds to two zinc ions with high affinity (Fig. 4), similar to periplasmic binding proteins of other high-affinity zinc transporters (15, 24).

_H. influenzae_ encodes at least two zinc utilization systems, the ZnuABC system and the ZevAB system described here. Mutants of individual genes in both pathways were compared for growth levels in rich or metal-restricted medium, and growth defects of the _znuA_ mutant were consistently more severe than defects exhibited by the parent strain or _zevA_ or _zevB_ mutant in rich medium or medium containing EDTA (Fig. 5A, B, and C). These data are consistent with the role of ZnuABC as a primary zinc transport system of _H. influenzae_, required over a wide range of conditions, whereas contributions from ZevAB are important for supporting growth only when zinc concentrations are limiting. Because double mutant analysis revealed that strains lacking both Znu and Zev pathways are not completely inhibited for growth in rich medium, it is likely that an additional pathway is involved in zinc acquisition of this organism. One possibility is the PitA-dependent inorganic phosphate utilization pathway, which was proposed to be a low-affinity zinc transporter of _E. coli_ (6). Whole-genome mutant fitness analysis via HITS suggested that PitA is required for optimal growth _in vitro_ as well as for lung infection (22). Together, these observations suggest that _H. influenzae_ contains several zinc utilization pathways whose functions are specialized to maintain zinc homeostasis in diverse environments.

Consistent with our observation that these proteins participate in zinc utilization, bioinformatic analysis of ZevA and ZevB suggested that they constitute PBP and membrane permease components of an ABC-type transporter, respectively. However, the _zevAB_ locus does not encode a protein containing the Walker A and B nucleotide binding sequences known to be required by ABC transporters for activity (13). HITS analysis revealed seven genes required for lung infection that are predicted to encode ABC-type ATPases (22). Possibly, ZevAB utilizes one of these ATPases for function; however, further investigation will be needed to address this question.

In many bacterial species, genes important for zinc acquisition and homeostasis are repressed in response to elevated intracellular zinc levels by the transcriptional regulator Zur (27, 54). Because _H. influenzae_ does not contain a homolog of this regulator and expression of _zevAB_ was not influenced by exogenous zinc levels, we sought to determine whether another regulator was involved. Expression analysis using _zevA_ promoter- lacZ fusion reporter strains revealed that _zevAB_ is repressed under low-oxygen conditions by the oxygen-responsive transcriptional regulator FNR (Fig. 6B). Of note, FNR has been shown to positively regulate transcription of metal transporters, such as _nikABCDE_ nickel transport genes and _feoABC_ iron uptake genes of _E. coli_ (34, 73) and _feoABC_, _sitABC_ and _fuuA_ iron uptake genes of _Shigella flexneri_ (10, 74). To our knowledge, FNR-mediated regulation of genes involved in zinc acquisition or homeostasis has not previously been demonstrated.

A potential explanation for the repression of _zevAB_ by FNR is that maximum expression of _zevAB_ may be detrimental to the survival of _H. influenzae_ in the anaerobic environment due to excessive zinc uptake or that _zevAB_ may be dispensable during anaerobic growth and repressed to conserve resources. However, an _fnr_ mutant and a wild-type strain were equally sensitive to a range of toxic zinc concentrations during anaerobic growth, and defects in the growth of a _zevB_ mutant in
zinc-limiting medium were not significantly different under aerobic and anaerobic conditions (C. V. Rosadini, unpublished results). Ultimately, regulation of zevAB by FNR may serve as an important strategy for increasing this system’s expression exclusively in sites of infection where the bacterium encounters zinc limitation, as is likely to occur at airway mucosal surfaces, and is consistent with other reports indicating that oxygen is an important signal for modulation of virulence factor expression in *H. influenzae* (69, 71, 72).

Bacteria are thought to contain high-affinity zinc transporters as a strategy to overcome zinc limitation during infection in which the acute phase response has been shown to result in reduced plasma zinc concentrations (20, 38) with upregulation and release of the zinc binding complex calprotectin in the blood and lungs (29, 31, 55). In accord with this hypothesis, we demonstrated here that *H. influenzae* requires a specialized high-affinity zinc utilization pathway for virulence. The finding that zevAB is needed exclusively during growth under low-zinc conditions and is required for lung infection suggests that bacteria growing in the lung experience zinc limitation that may represent an immune defense for controlling pulmonary infection.

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