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Overlapping Functionality of the Pht Proteins in Zinc Homeostasis of *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is a globally significant pathogen that causes a range of diseases, including pneumonia, sepsis, meningitis, and otitis media. Its ability to cause disease depends upon the acquisition of nutrients from its environment, including transition metal ions such as zinc. The pneumococcus employs a number of surface proteins to achieve this, among which are four highly similar polyhistidine triad (Pht) proteins. It has previously been established that these proteins collectively aid in the delivery of zinc to the ABC transporter substrate-binding protein AdcAII. Here we have investigated the contribution of each individual Pht protein to pneumococcal zinc homeostasis by analyzing mutant strains expressing only one of the four *pht* genes. Under conditions of low zinc availability, each of these mutants showed superior growth and zinc accumulation profiles relative to a mutant strain lacking all four genes, indicating that any of the four Pht proteins are able to facilitate delivery of zinc to AdcAII. However, optimal growth and zinc accumulation *in vitro* and pneumococcal survival and proliferation *in vivo* required production of all four Pht proteins, indicating that, despite their overlapping functionality, the proteins are not dispensable without incurring a fitness cost. We also show that surface-attached forms of the Pht proteins are required for zinc recruitment and that they do not contribute to defense against extracellular zinc stress.**

Streptococcus pneumoniae causes a wide range of human diseases, including pneumonia, sepsis, meningitis, and otitis media, and these diseases result in huge morbidity and mortality globally (1). Among the many surface proteins that contribute to pneumococcal pathogenicity are the polyhistidine triad (Pht) proteins, a family consisting of four members (PhtA, PhtB, PhtD, and PhtE) (2). A number of functions have been ascribed to these proteins, including binding to the complement regulator factor H (3) and adherence to host epithelial cells (4, 5). However, to date, the majority of attention has focused on their contribution to metal ion homeostasis.

The defining feature of the Pht proteins is the presence of multiple copies of a histidine triad (HXXHXH) motif. Each motif within each of the Pht proteins is potentially capable of binding one Zn^{2+} ion, depending on protein conformational considerations (6). Zinc acquisition is critical for the survival of *S. pneumoniae* during infection and occurs via the ATP-binding cassette (ABC) transporter AdcBC after delivery of the metal via the substrate-binding proteins (SBPs) AdcA and/or AdcAII (7–11). There is now substantial evidence supporting the hypothesis that an important role of the Pht proteins is to facilitate Zn^{2+} acquisition via AdcAII (6, 9, 12). The *phtD* and *adcAII* genes are cotranscribed, and their expression is coregulated by cellular Zn^{2+} abundance. The two proteins have been shown to interact with one another by *in vitro* cross-linking and immunoprecipitation experiments (6, 12). Recently we showed that the growth of a Δ*phtABDE* Δ*adcA* mutant was highly attenuated in media containing a low concentration of available Zn^{2+} compared to the growth of the Δ*adcA* strain, and the *in vivo* fitness of a Δ*phtABDE* Δ*adcA* mutant was significantly less than that of the Δ*phtABDE* Δ*adcAII* strain (9). Collectively, these data are consistent with AdcA being able to acquire Zn^{2+} independently, whereas AdcAII requires the Pht proteins to be present for optimal functionality (9).

However, an important, yet unanswered, question is whether each of the four Pht proteins is able to aid in the delivery of Zn^{2+} to AdcAII or whether this function is confined to only certain members of the protein family. This issue has significance for our understanding of pneumococcal Zn^{2+} homeostasis, and it also has implications for vaccine development. The Pht proteins, and PhtD in particular, are targets of several novel protein-based pneumococcal vaccines currently in development. The efficacy of these formulations may depend upon stimulation of an opsonophagocytic antibody response against these surface antigens as well as blockade of their function by antibody binding (13–16). As a consequence, if the Pht proteins are redundant, cross-reactive antibodies that bind to all four proteins would be required to elicit protection based on blockade of the function(s) of the proteins, whereas if they are not redundant, such a protective effect may be derived from single Pht protein-specific antibodies. While the Pht proteins share high sequence similarity, suggesting that they may all be able to perform the same function, only *phtD* is cotranscribed with *adcAII*. Thus, it is plausible that PhtD alone may contribute to Zn^{2+} acquisition. A limited number of previous studies have shed some light on the issue of redundancy among the protein family. Ogunniyi et al. found that deletion of all four *pht* genes led to increased complement deposition on the pneumococcal surface and increased survival time after intraperitoneal challenge in a mouse model of disease relative to the wild type (3). However, strains with one or two *pht* genes deleted did not show significant differences from the wild type, leading to the conclusion that the Pht proteins are functionally redundant (3). However, triple *pht* mutants (i.e., strains with only one *pht* gene) were not tested in

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TABLE 1 *S. pneumoniae* strains used in this study

S. pneumoniae strain	Description or characteristic(s)	Source or reference
D39	Serotype 2	NCTC7466
Δ*adcA* mutant		9
Δ*phtABDE* Δ*adcAII* mutant		9
Δ*phtABDE* Δ*adcA* mutant		9
Δ*phtBDE* Δ*adcA* mutant	Expresses only PhtA of the four Pht proteins	This study
Δ*phtADE* Δ*adcA* mutant	Expresses only PhtB of the four Pht proteins	This study
Δ*phtABE* Δ*adcA* mutant	Expresses only PhtD of the four Pht proteins	This study
Δ*phtABD* Δ*adcA* mutant	Expresses only PhtE of the four Pht proteins	This study
PhtD 3 Ala Δ*adcA* mutant	D39 Δ*phtABDE* mutant complemented with *phtD* with amino acid substitutions R26A, H27A, and Q28A; *adcA* replaced by chloramphenicol acetyltransferase gene	19
PhtE 3 Ala Δ*adcA* mutant	D39 Δ*phtABDE* mutant complemented with *phtE* with amino acid substitutions Q27A, H28A, and R29A; *adcA* replaced by chloramphenicol acetyltransferase gene	19
Δ*adcAII* mutant		9

this study, limiting the strength of the conclusions that could be drawn. In another study, Rioux et al. reported that deletion of all four *pht* genes was necessary to observe a phenotype of impaired growth in chemically defined medium deficient in cations, whereas single, double, or triple (Δ*phtBDE*) mutants behaved similarly to the wild type (17). However, deletion of individual *pht* genes has been shown to cause a significant reduction in pneumococcal adherence to nasopharyngeal and lung cell lines, suggesting that the Pht proteins are not fully redundant in this role (4, 5).

The aim of this study was to further investigate potential functional redundancy in the Pht protein family, with specific emphasis on their role in Zn^{2+} homeostasis. In order to resolve earlier conflicting evidence regarding Pht protein redundancy, we assessed their functionality in a more comprehensive manner than has been performed previously by generating and examining the phenotypes of all four possible *pht* triple mutants (Δ*phtBDE*, Δ*phtADE*, Δ*phtABE*, and Δ*phtABD*), allowing examination of the ability of each Pht protein to contribute to Zn^{2+} acquisition in the absence of the three other Pht proteins.

MATERIALS AND METHODS

Strains and growth media. Defined, nonpolar deletion replacement or unmarked pneumococcal mutants of *Streptococcus pneumoniae* strain D39 lacking *phtA*, *phtB*, *phtD*, *phtE* or *adcA* had been generated previously (3, 9). Combination mutants used in this study were generated by amplifying the relevant loci from the preexisting mutants by PCR and using these loci to transform the appropriate background strain using established methods (18, 19). PCR, DNA sequencing, and immunoblotting were used to confirm that the desired mutations had been introduced. A complete list of strains used in this study is shown in Table 1. Opaque phase variants were used in all experiments. Bacteria were routinely grown at 37°C in a CO_2-enriched atmosphere on Columbia agar supplemented with 5% (vol/vol) horse blood. If the bacteria would be used to challenge mice, they were grown in serum broth (10% heat-inactivated horse serum in nutrient broth). Alternatively, cation-defined medium (CDM) (prepared as described previously [9, 20]) was used. To assess the effect of Zn^{2+} starvation, the chelating agent *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) was supplemented at an equimolar concentration to Zn^{2+} determined to be in CDM by inductively coupled plasma mass spectrometry (ICP-MS) to provide CDM-TPEN. A single batch of CDM was used for all experiments in this study, and it was supplemented with 13 μM TPEN to yield CDM-TPEN when required.

Growth analysis. For growth curve measurements, wild-type *S. pneumoniae* D39 and mutant strains were grown in CDM with 1 μM $MnSO_4$ until they reached an optical density at 600 nm (OD_{600}) of 0.4. They were then subcultured into 200 μl CDM, CDM-TPEN, or CDM supplemented with $ZnSO_4$. The bacteria were incubated at 37°C, and growth was monitored by measurement of the OD_{600} at 30-min intervals.

Whole-cell metal ion accumulation. *S. pneumoniae* strains were grown in CDM-TPEN to an OD_{600} of 0.4. The bacteria were washed three times with phosphate-buffered saline (PBS) containing 5 mM EDTA and then washed twice with PBS, with centrifugation at 12,000 × *g* for 10 min at 4°C between each wash. Bacterial pellets were desiccated by heating to 95°C overnight. The dry mass of cellular material was recorded and then resuspended in 35% HNO_3. The metal ion content of the dissolved cellular material was measured on an Agilent 7500cx inductively coupled plasma mass spectrometer (Adelaide Microscopy).

Quantitative immunoblot and qRT-PCR analyses. Wild-type and mutant *S. pneumoniae* bacteria were grown under the same conditions as for the whole-cell metal ion accumulation experiments. After the cultures reached an OD_{600} of 0.4, they were split into samples to be processed for protein and RNA extraction. For protein extraction, cells were incubated with 0.1% sodium deoxycholate (Sigma-Aldrich) at 37°C for 60 min to induce lysis. Protein concentrations were determined (DC Bio-Rad protein assay; Bio-Rad), and 20 μg of total protein was loaded into each lane. After electrophoretic separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane using the iBlot (Life Technologies) system. The blots were incubated with previously generated murine antisera against the relevant Pht protein (16). The blots were then incubated with anti-mouse IRDye 800 and scanned using an Odyssey infrared imaging system (LI-COR Biosciences). Band intensities were measured using the manufacturer's application software. For measurement of absolute molar quantities of protein, a standard curve with a range of quantities of the relevant purified Pht protein was included in the Western blot and used to calibrate the band intensities measured for cell lysates against absolute amounts, and the predicted molecular weights of the proteins were used to convert to molar quantities (16).

For RNA extraction and reverse transcription-PCR (RT-PCR) analysis, 500 μl of culture was mixed with 1 ml of RNA protect (Qiagen). RNA was extracted and purified using an RNeasy Protect Bacteria minikit (Qiagen) after enzymatic lysis using lysozyme and mutanolysin, all according to the manufacturer's instructions. The total RNA samples were treated with DNase I (Roche), and quantitative RT-PCR (qRT-PCR) was carried out using a SuperScript III one-step RT-PCR kit (Invitrogen) on an LC480 real-time cycler (Roche). Transcription levels of genes analyzed were normalized to those obtained for 16S rRNA. Primer sequences are shown in Table 2.

Assessment of bacterial fitness by *in vivo* competition experiments. Outbred 5- to 6-week-old female CD1 (Swiss) mice were used in all animal experiments. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal; Rhone-Merieux) at a dose of 66 μg per

TABLE 2 Oligonucleotide primers used for qRT-PCR in this study

Primer[a]	Sequence (5′→3′)
16s_F	CATGCAAGTAGAACGCTGAA
16s_R	TGTCATGCAACATCCACTCT
phtA_F	AGTCTGAAAGCCAATGCAACAG
phtA_R	CCTTACTTACAGATGAAGGATTAC
phtB_F	CCAACAGAGGAACCAGAAGAAG
phtB_R	TAATTGGATTCTGGATTTTTCC
phtD_F	GTATTAGACAAAATGCTGTGGAG
phtD_R	CTGTATAGGAGTCGGTTGACTTTC
phtE_F	GGAACAGTTGAGAACCAACCA
phtE_R	TAGGGTCACTCCCCACATTC

[a] The direction of the primer is indicated by the letter at the end of the primer designation as follows: F, forward; R, reverse.

g of body weight, followed by intranasal administration of 50 μl of bacterial suspension containing an equal mixture of the two mutant strains, with a total of approximately 1×10^7 CFU. The challenge dose and input ratio were confirmed retrospectively by serial dilution and plating on blood agar followed by patching of colonies onto blood agar plates with and without antibiotic to allow discrimination of the two strains.

At 24 and 48 h postchallenge, mice were euthanized by CO_2 asphyxiation. Blood was collected from the posterior vena cava by using a syringe. The pleural cavity was lavaged with 1 ml sterile PBS containing 2 mM EDTA introduced through the diaphragm. Pulmonary vasculature was perfused by infusion of sterile PBS through the heart. The lungs were subsequently excised. The trachea was then exposed, and 1 ml of PBS containing 0.5% (wt/vol) trypsin (Life Technologies) was inserted into the tracheal end of the upper respiratory tract and collected from the nose (nasal wash). Last, the nasopharynx/upper palate was excised (nasal tissue). Tissues were homogenized, and all samples were serially diluted and plated on blood agar. Colonies were subsequently patched onto blood plates with and without the relevant antibiotic to allow discrimination of the two mutant strains and determination of output ratios. Competitive indices (the ratio of one mutant strain to the other mutant strain relative to the input ratio) were then calculated and compared to a theoretical value of 1 (which would indicate no difference in fitness between strains) by one-sample *t* tests.

All procedures performed in this study were conducted with a view to minimizing the discomfort of the animals and used the minimum numbers of animals to generate reproducible and statistically significant data. All experiments were approved by the University of Adelaide Animal Ethics Committee (Animal Welfare Assurance number A5491-01; project approval number S-2013-053) and were performed in strict adherence to guidelines dictated by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

RESULTS

Growth of *pht*-deficient strains under conditions of low Zn^{2+} availability. In order to examine the contributions of the individual Pht proteins to Zn^{2+} homeostasis, mutant strains were constructed lacking all possible combinations of three out of the four *pht* genes (Δ*phtBDE*, Δ*phtADE*, Δ*phtABE*, and Δ*phtABD* mutants). Our prior study showed that the contribution of the Pht proteins to Zn^{2+} acquisition by AdcAII was most pronounced when the other Zn^{2+}-recruiting substrate-binding protein (SBP), AdcA, was absent (9). Consequently, we also deleted the *adcA* gene from all strains in order to obviate its effect on Zn^{2+} uptake. Therefore, the resultant mutant strains were entirely reliant upon AdcAII and either PhtA, PhtB, PhtD, or PhtE alone for Zn^{2+} import. A complete list of strains used in this study is shown in Table 1.

For growth experiments, cation-defined medium (CDM) was prepared, and its concentration of Zn^{2+} was measured by ICP-MS. As Zn^{2+} cannot be completely removed from any culture medium, we then restricted the bioavailability of Zn^{2+} by use of the chelating agent TPEN at an equimolar concentration to Zn^{2+}, as determined by ICP-MS of the CDM, to yield the culture medium CDM-TPEN, as described previously (9, 20). CDM-TPEN contains negligible bioavailable Zn^{2+}, as TPEN has a K_D (equilibrium dissociation constant) for Zn^{2+} of $\sim 2.6 \times 10^{-16}$ M. As a consequence, bacteria grown in this medium are critically dependent on bacterial high-affinity Zn^{2+}-recruiting proteins, which have experimentally reported K_Ds for Zn^{2+} in the nanomolar range. Initially, growth of the wild-type, Δ*adcA*, Δ*phtABDE* Δ*adcAII*, and Δ*phtABDE* Δ*adcA* strains was compared (Fig. 1A). Of the latter two strains, only the Δ*phtABDE* Δ*adcAII* mutant was able to grow (albeit to a much lesser extent than the wild type), consistent with our previous observation that AdcAII is more reliant on the Pht proteins to obtain Zn^{2+} than AdcA is (9). We then examined strains with only one of the four *pht* genes in the absence of AdcA (Fig. 1B). These mutant strains displayed a much lower rate of growth and lower final cell density than the Δ*adcA* mutant, which grew in a manner indistinguishable from the wild type. However, the mutant strains containing a single *pht* gene all showed superior growth compared to the Δ*phtABDE* Δ*adcA* strain, which was not able to grow in CDM-TPEN. There was no defect in the growth of any of the strains in the absence of TPEN (Fig. 1C), indicating that the differences observed were caused by the lack of available Zn^{2+} in the medium. Therefore, these results show that all four of the Pht proteins are capable of contributing to Zn^{2+} recruitment by AdcAII. However, these results also show that the Pht proteins are not fully redundant, in the sense of being dispensable, as strains producing only one Pht protein did not grow as effectively as when all four proteins were present.

Surface attachment of Pht proteins is required for Zn^{2+} recruitment. In a previous study, we identified amino acids in PhtD and PhtE that are critical for the proteins' surface attachment. We further showed that a significant proportion (up to 50% in strain D39) of the total amount of each Pht protein produced was released into the culture medium during planktonic growth, even in wild-type strains (19). It is not clear what biological role, if any, this material performs. As a consequence, we sought to investigate whether surface attachment of the Pht proteins was required for Zn^{2+} acquisition. To assess this, we used strains containing only a single Pht protein, into which the specific amino acid substitutions R26A, H27A, and Q28A in PhtD or Q27A, H28A, and R29A in PhtE had been introduced, resulting in complete loss of surface attachment of the proteins (19). These mutant strains were also generated without *adcA* to elucidate the impact of surface attachment of PhtD or PhtE on AdcAII Zn^{2+} uptake in this background. The growth of the resultant strains (PhtD 3 Ala Δ*adcA* and PhtE 3 Ala Δ*adcA*) in CDM-TPEN was compared to the Δ*phtABE* Δ*adcA* and Δ*phtABD* Δ*adcA* strains. The results, shown in Fig. 2, demonstrate that the PhtD 3 Ala Δ*adcA* and PhtE 3 Ala Δ*adcA* strains were unable to grow in CDM-TPEN, indicating that surface attachment of PhtD and PhtE is essential for their contribution to Zn^{2+} acquisition.

The Pht proteins do not aid in defense against extracellular Zn^{2+} stress. The results of this work and prior studies support the involvement of the Pht proteins in facilitating Zn^{2+} acquisition from the environment under conditions where its availability is tightly restricted (9). However, it is also plausible that

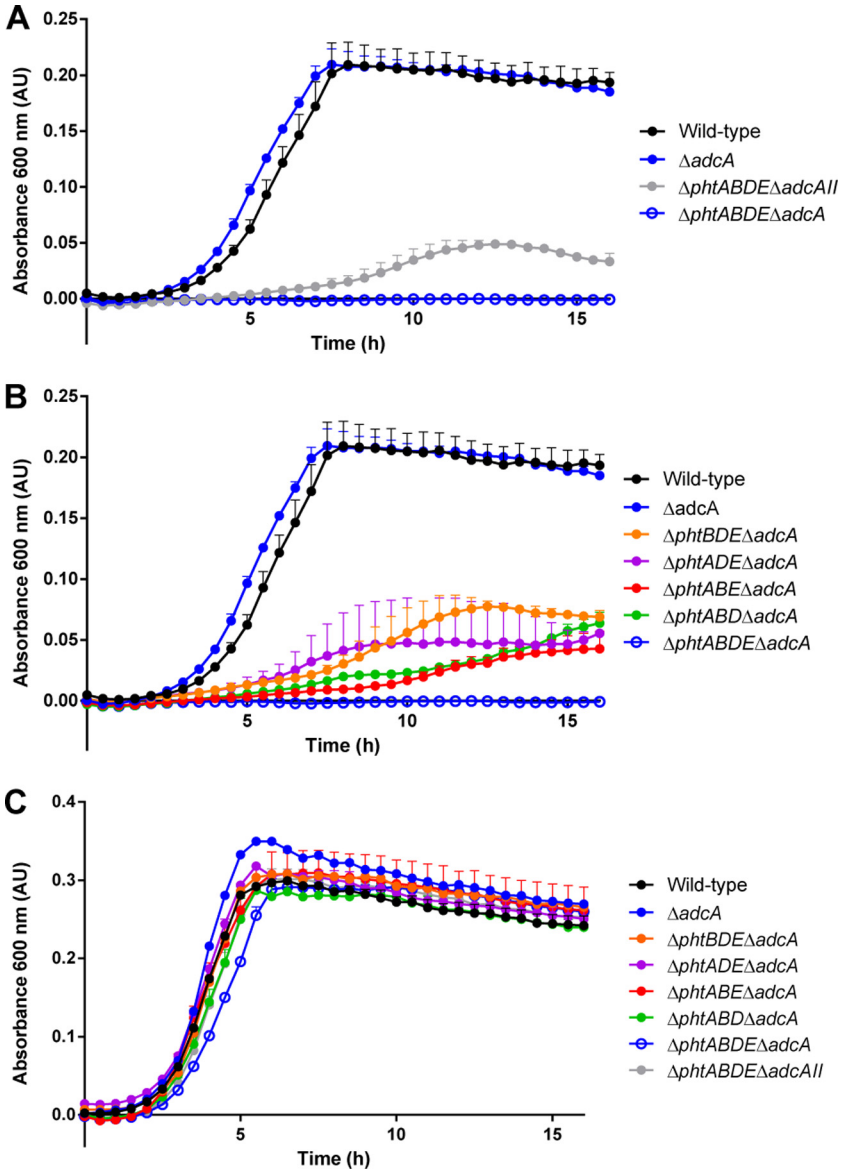

FIG 1 *In vitro* growth measurements of pneumococcal strains. (A and B) The indicated strains were grown in CDM-TPEN (i.e., under Zn^{2+}-restricted conditions) at 37°C, and growth was monitored by OD_{600} measurements every 30 min. The absorbance at 600 nm or OD_{600} is shown in arbitrary units (AU). (C) Control growth in unsupplemented CDM (i.e., Zn^{2+}-replete conditions). Data are representative mean (plus standard error of the mean [SEM] [error bar]) OD_{600} measurements from three independent biological experiments.

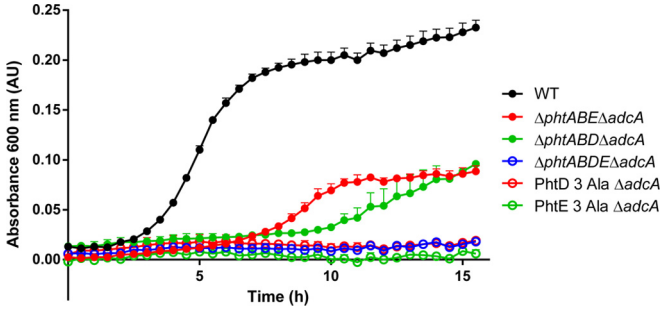

FIG 2 *In vitro* growth comparison of strains with or without surface-attached Pht proteins. Bacteria were grown in CDM-TPEN (i.e., under Zn^{2+}-restricted conditions) at 37°C, and growth was monitored by OD_{600} measurements every 30 min. Data are representative mean (plus SEM) OD_{600} measurements from three independent biological experiments. WT, wild type.

they could help defend the pneumococcus when an excess of the metal ion is present in the extracellular environment, which exerts a toxic effect on the bacteria by interfering with the physiological function of the ABC permease, PsaBCA, in importing the essential transition metal ion manganese (20, 21). It has been speculated that the Pht proteins may aid in protecting the PsaBCA permease from extracellular Zn^{2+} by binding excess ions and acting as a "sink" (12, 22). To test this hypothesis, wild-type and Δ*phtABDE* pneumococci were first grown in CDM-TPEN to induce maximal expression of *pht* genes (6, 9) and then subcultured into CDM supplemented with increasing concentrations of $ZnSO_4$ (Fig. 3). As expected, high extracellular concentrations of Zn^{2+} impaired bacterial growth and resulted in reduced final cell densities. However, no phe-

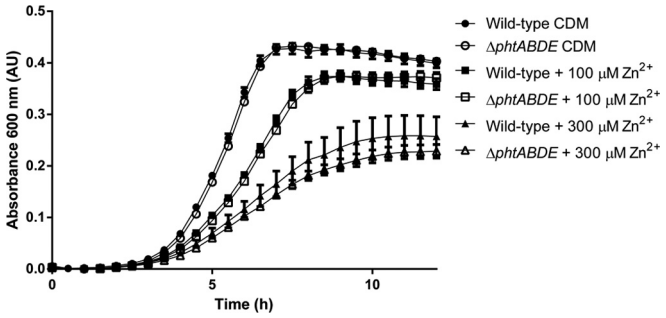

FIG 3 *In vitro* growth comparison of wild-type and Δ*phtABDE* strains grown under conditions of high extracellular Zn^{2+} stress. Bacteria were grown in CDM supplemented with $ZnSO_4$ at 37°C, and growth was monitored by OD_{600} measurements every 30 min. Data are representative mean (plus SEM) OD_{600} measurements from three independent biological experiments.

notypic differences were apparent between the wild-type and Δ*phtABDE* strains in any of the culturing conditions, indicating that the Pht proteins did not protect the pneumococcus against extracellular Zn^{2+} stress under the conditions examined.

Metal ion accumulation of *pht*-deficient strains under conditions of low Zn^{2+} availability. To further examine the impact of possessing only one *pht* gene, whole-cell metal accumulation was measured for all relevant mutant strains after growth in CDM-TPEN to an OD_{600} of 0.4 (Fig. 4). It should be noted that all mutants required approximately 5 h to reach this density, except for the Δ*phtABDE* Δ*adcA* control strain, which took approximately 8 h. This strain also had the smallest amount of intracellular Zn^{2+}, and since Zn^{2+} acquisition was the major limiting factor for its growth, we infer that the 30 μg of Zn^{2+} per g cells (dry weight) it was found to contain represents the minimum required for pneumococcal viability. In contrast, Zn^{2+} acquisition in the Δ*phtABDE* Δ*adcAII* strain, where AdcA was the only protein present responsible for uptake of the metal, was impaired to a lesser extent, consistent with our prior results (9). In the strains carrying only one of the four *pht* genes, Zn^{2+} accumulation was more than for the Δ*phtABDE* Δ*adcA* strain. This showed that all of the Pht proteins were capable of aiding in Zn^{2+} acquisition via AdcAII, consistent with the phenotypic growth experiments (Fig. 1B). Intriguingly, the Δ*phtABD* Δ*adcA* strain (i.e., AdcAII and PhtE present) showed more Zn^{2+} accumulation than the other strains with a single *pht* gene. Statistical analysis by one-way analysis of variance (ANOVA) with Dunnett's posttest to compare all strains to the Δ*phtABDE* Δ*adcA* mutant showed significant differences in all cases except for the Δ*phtBDE* Δ*adcA* and Δ*phtADE* Δ*adcA* strains. The same test was also applied to compare all strains to the Δ*adcA* mutant; of the strains with only one *pht* gene, only the Δ*phtABD* Δ*adcA* mutant did not show a significant difference. Taken together, these results show that production of only one Pht protein allows more Zn^{2+} accumulation than when all four Pht proteins are absent but less than when the four Pht proteins are present.

Expression levels of individual *pht* genes in *pht*-deficient strains. To complement the above analyses, changes in expression levels of each of the Pht proteins in Zn^{2+}-replete and Zn^{2+}-starved conditions were measured. We hypothesized that strains lacking *adcA* and three out of the four *pht* genes would show greater expression levels of their one remaining *pht* gene under Zn^{2+}-starved conditions than the *adcA* mutant, since as shown by the previous experiments, they cannot accumulate as much Zn^{2+} and face a greater challenge to grow in CDM-TPEN. Conversely, it

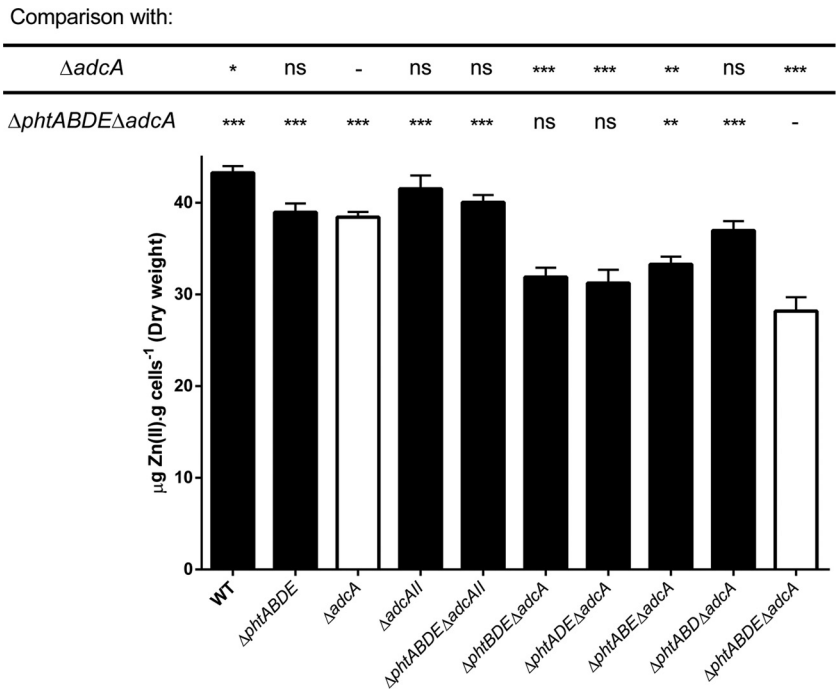

FIG 4 Whole-cell Zn^{2+} accumulation of wild-type and *pht* or *adc* mutant strains determined by ICP-MS. Error bars indicate SEMs. All strains were compared to the Δ*adcA* and Δ*phtABDE* Δ*adcA* mutants (white bars) by one-way ANOVA using Dunnett's posttest, and statistical significance is indicated as follows: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are means (plus SEMs) from a minimum of three biological replicates.

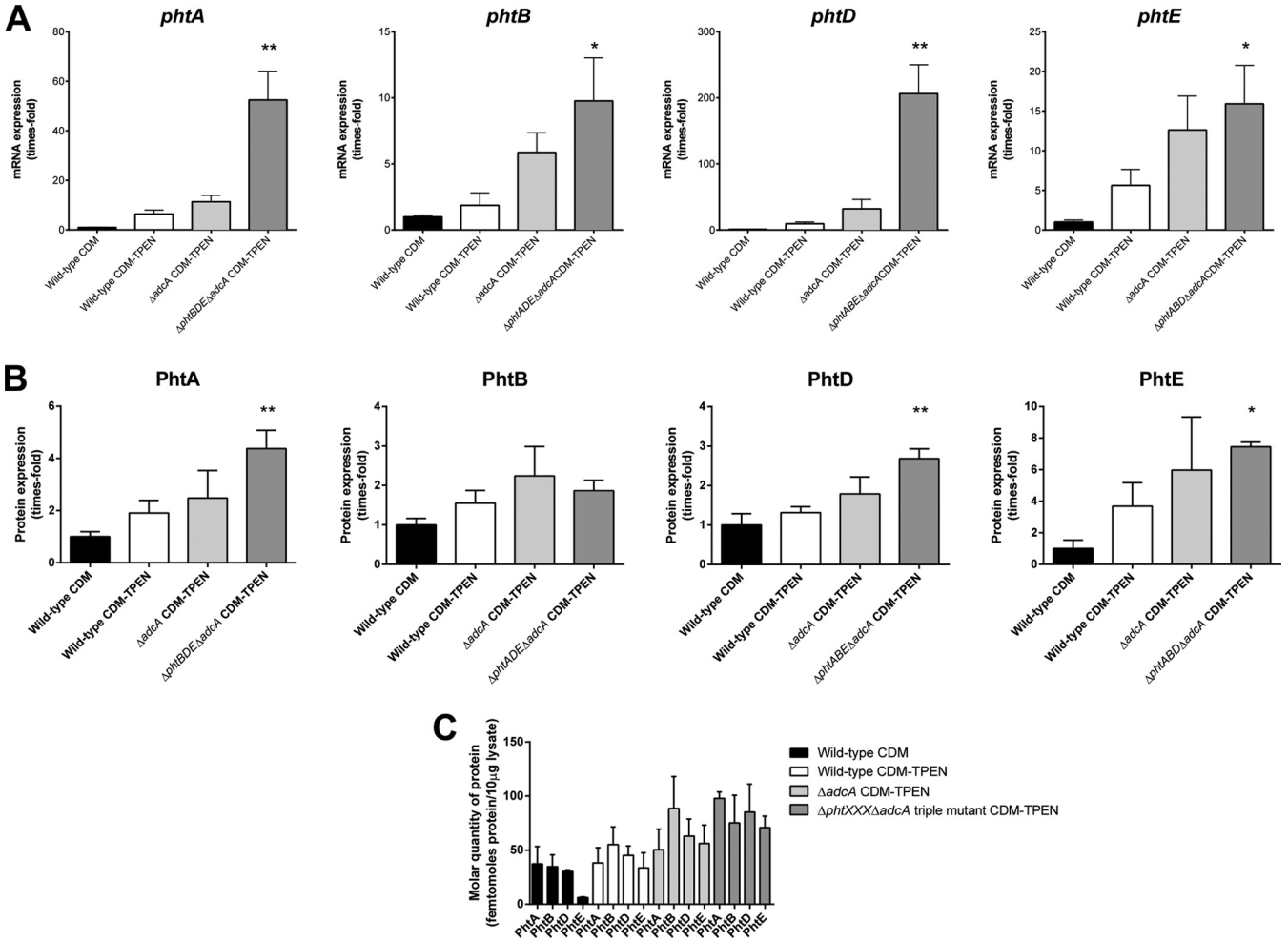

FIG 5 Analysis of Pht expression levels in wild-type (white bars), Δ*adcA* (light gray bars), or Δ*phtXXX* (*XXX* represents three *pht* loci) Δ*adcA* (dark gray bars) strains grown in CDM-TPEN, compared to the wild-type strain grown in CDM (black bars). (A and B) mRNA (A) and protein (B) expression levels are shown, normalized to levels in the wild type grown in CDM. For mRNA, measurements were made relative to 16S rRNA. Error bars depict SEMs. Strains were compared to the wild type grown in CDM by one-way ANOVA using Dunnett's posttest, and statistically significant differences where present are indicated as follows: *, $P < 0.05$; **, $P < 0.01$. (C) Absolute protein expression levels of PhtA, PhtB, PhtD, and PhtE, expressed as femtomoles per 10 μg of bacterial lysate. Results are representative of three independent experiments.

was also feasible that there would be no difference in *pht* expression between these strains if *pht* expression were already maximally derepressed in the Δ*adcA* mutant. To investigate this, strains were grown in CDM-TPEN, and mRNA and protein expression levels of each of the *pht* genes and proteins was measured by qRT-PCR and Western blotting, respectively, and compared to that of the wild type grown in CDM (Fig. 5). The results reveal that, in general, *pht* expression was greatest in strains lacking *adcA* and three out of four *pht* genes, consistent with the difficulty these strains experience in obtaining sufficient quantities of Zn^{2+} to grow. Furthermore, *phtA* and *phtD* transcripts were more abundant in their respective triple *pht* mutant strains than in the strain lacking *adcA* alone. In contrast, expression levels of *phtB* and *phtE* were similar in the Δ*adcA* mutant and the relevant Δ*adcA* triple *pht* mutant, although a minor but statistically significant difference was observed for *phtE*. For a control, the expression of the remaining intact Pht proteins in the triple mutants was measured after growth in CDM, and no differences were found from that of the wild type, indicating that the induction of expression was specifically caused by the addition of TPEN to the medium (data not shown).

To complement this analysis, we also quantified the absolute level of protein expression of each of the Pht proteins, since if one of the proteins were much more abundant in molar terms than any of the others, it could be speculated that it would play a more important role in Zn^{2+} import. However, as shown in Fig. 5C, the amounts of the Pht protein were largely similar in the given mutant strains, with the exception of PhtE in the wild type grown in CDM. The expression of this protein appears to be more strongly repressed in this medium, allowing for a greater fold increase when strains are grown in CDM-TPEN.

Collectively, these data show that expression of PhtB and PhtE was already maximally derepressed in the Δ*adcA* mutant and could not be further increased even when the degree of Zn^{2+} starvation was increased by deletion of the other three *pht* genes. In contrast, PhtA and PhtD could be further derepressed under these circumstances.

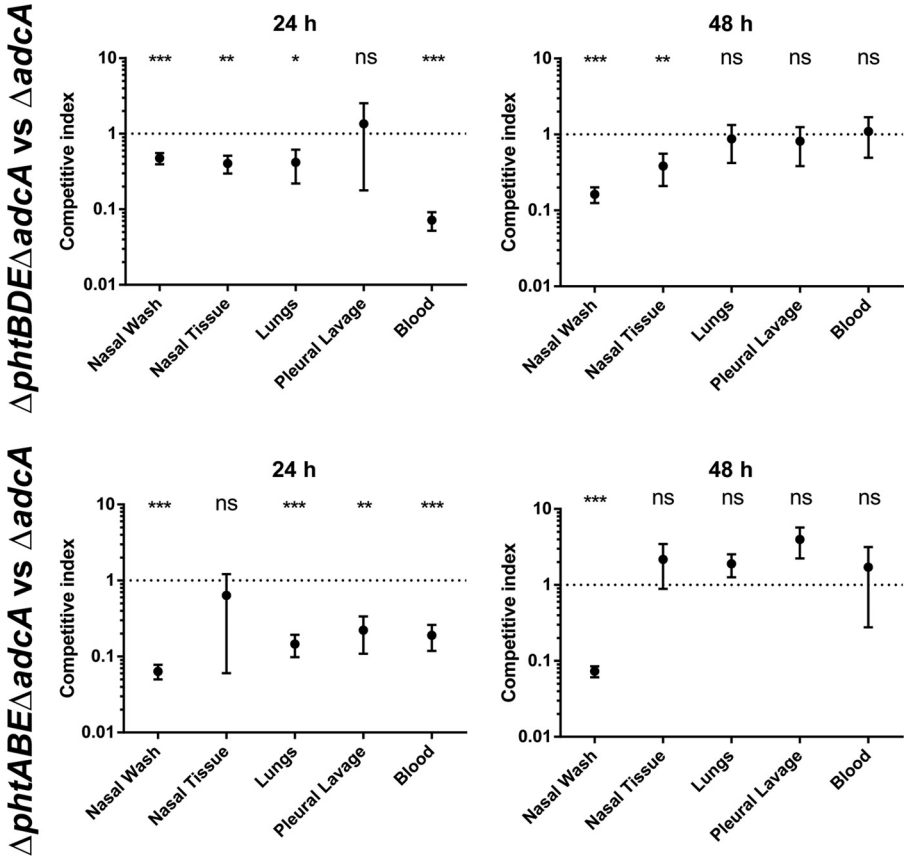

FIG 6 *In vivo* competition between the Δ*adcA* mutant and strains lacking three out of four *pht* genes (Δ*phtBDE* Δ*adcA* [top graphs] and Δ*phtABE* Δ*adcA* [bottom graphs] mutants). Eight mice were used per time point per group. Results are shown as mean (±SEM) competitive indices (the ratio of Δ*phtXXX* Δ*adcA* bacteria to Δ*adcA* bacteria corrected for the input ratio). The mean competitive index was compared to a theoretical mean of 1 (dotted line) by one-sample *t* tests, and statistical significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Redundancy of Pht proteins *in vivo*: four Phts are better than one. To compare the fitness of the *pht*-deficient strains *in vivo*, a series of competition experiments were performed in which mice were infected intranasally with a mixed culture of two mutant strains. After 24 and 48 h, mice were euthanized, and bacteria were enumerated from the indicated niches. Results are displayed as competitive indices, calculated as the ratio of the number of antibiotic-resistant bacteria to the control strain relative to the input ratio (Fig. 6).

We first sought to examine the impact of loss of three out of four *pht* genes *in vivo*, so the Δ*adcA* mutant was used in competition with the Δ*phtBDE* Δ*adcA* mutants and with the Δ*phtABE* Δ*adcA* mutants. The experiments were restricted to these mutant strains due to ethical considerations regarding the number of mice required to assess each strain. The "PhtA-only" and "PhtD-only" strains were selected as PhtD is the leading vaccine candidate and thus of the greatest interest, while PhtA is encoded at a separate gene locus from the gene encoding PhtD and may provide a different insight into the functional roles of these proteins. After 24 h, the strain expressing only PhtA (Δ*phtBDE* Δ*adcA* mutant) was significantly less competitive than the control Δ*adcA* mutant in all niches except for pleural lavage fluid, while the strain expressing only PhtD (Δ*phtABE* Δ*adcA* mutant) was significantly less competitive in all niches except for nasal tissue at this time point. At 48 h, the strain expressing only PhtA was significantly attenuated in the nasal wash fluid and nasal tissue, and the strain expressing only PhtD was significantly attenuated in the nasal wash fluid alone. These results suggest that the Pht proteins are not fully redundant *in vivo*, since the strains containing only one out of the four *pht* genes were significantly less competitive in a number of instances compared to the Δ*adcA* mutant, which had all four *pht* genes intact. It is also apparent that production of all four Pht proteins may be of greatest importance at the early stage of infection, as in most niches, the differences present at 24 h were no longer apparent after 48 h.

The Pht proteins have roles outside facilitating Zn^{2+} acquisition through AdcAII. It was also of interest to assess whether the Pht proteins had role(s) outside facilitating Zn^{2+} acquisition through AdcAII. To achieve this, a competition experiment was performed using the Δ*adcAII* and Δ*phtABDE* Δ*adcAII* strains. The results of the competition experiment are shown in Fig. 7. The Δ*adcAII* mutant was significantly more competitive after 24 h in the lungs, pleural lavage fluid, and blood, indicating that Pht proteins do contribute to pathogenesis independently of AdcAII at this time point. However, this was not the case after 48 h. Interestingly, the strain lacking *pht* genes was significantly more competitive in the nasal wash fluid at this time point. For reference, the absolute numbers of CFU recovered from all animal model experiments (as opposed to competitive indices) are shown in Fig. 8.

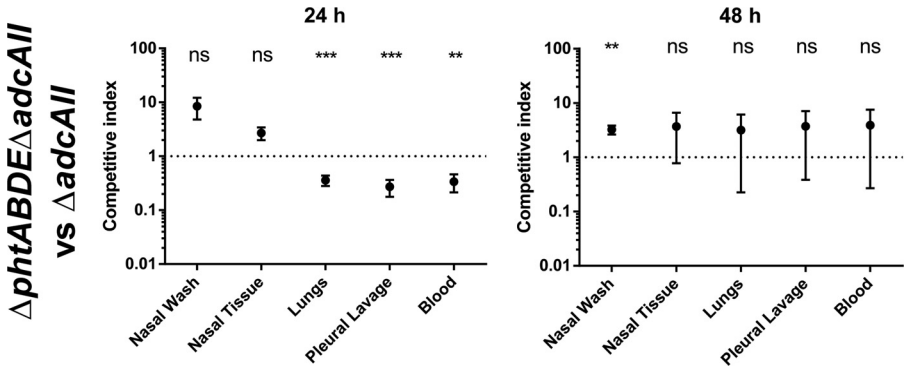

FIG 7 *In vivo* competition between the Δ*phtABDE* Δ*adcAII* and Δ*adcAII* mutants. Eight mice were used per time point. Results are shown as mean (±SEM) competitive indices (the ratio of Δ*phtABDE* Δ*adcAII* bacteria to Δ*adcAII* bacteria corrected for the input ratio). The mean competitive index was compared to a theoretical mean of 1 (dotted line) by one-sample *t* tests, and statistical significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

DISCUSSION

The Pht proteins are an important family of surface-associated virulence factors with potential to be used in novel pneumococcal protein-based vaccines (13–16). In this work we have investigated whether the four Pht proteins act redundantly in contributing to Zn^{2+} homeostasis. Under conditions of low Zn^{2+} availability, the presence of any one of the four proteins permitted superior growth and increased intracellular Zn^{2+} accumulation relative to when all four were absent. These findings demonstrate that all four of the Pht proteins aid in facilitating pneumococcal Zn^{2+} acquisition by AdcAII. Notably, they also clarify the debate over the contribution of PhtE, the most divergent member of the family in terms of primary structure (32% identity with the other family members, compared to over 60% identity shared between PhtA, PhtB, and PhtD [2]), to Zn^{2+} homeostasis. Indeed, the Δ*phtABD* Δ*adcA* strain showed the greatest whole-cell accumulation of Zn^{2+} among the strains producing only one Pht protein. However, despite this observation, the growth experiments did not reveal any consistent differences between the four mutant strains with a single *pht* gene. Thus, these findings indicate that each Pht

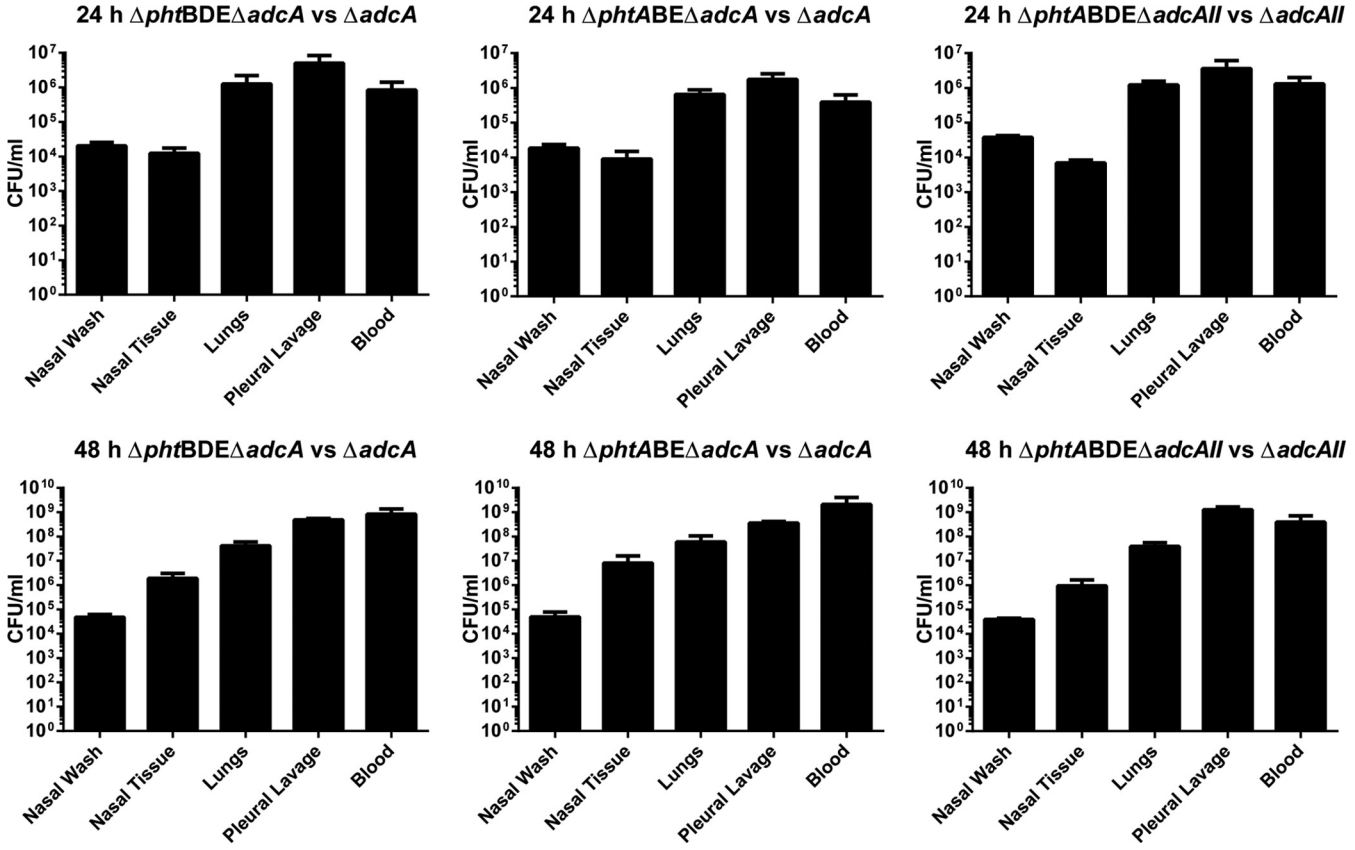

FIG 8 Numbers of CFU recovered from animal model experiments. Values are shown as means plus SEMs.

protein has a similar capacity to facilitate Zn^{2+} import via AdcAII. We also observed that surface attachment is required for the proteins to play this role, thereby ruling out the possibility of a siderophore-like mechanism for Zn^{2+} recruitment. Furthermore, our data also indicate that the Pht proteins do not contribute to defense against extracellular Zn^{2+} stress.

A recent study concluded that Zn^{2+} transfer from PhtD to AdcAII involves a direct interaction (12). Since the metal-binding site of AdcAII is buried ~10 to 15 Å beneath the surface of the protein (23), free Zn^{2+} ions may not be easily acquired by this site. A direct interaction with a Zn^{2+}-loaded Pht protein may enable binding of the metal ion by inducing a conformational change in AdcAII to present the Zn^{2+} to the metal-binding site. This interaction may serve an analogous role to the proposed capture of Zn^{2+} in AdcA by its ZinT-like domain and histidine-rich loop region (9). While the possibility of a direct interaction of AdcAII with PhtA, PhtB, and PhtE has not been examined, we may speculate that they would also operate to deliver Zn^{2+} to AdcAII in this direct manner. This would presumably predominantly involve the highly conserved N termini of the Pht proteins, which are also closer to the cell membrane (and thus to AdcAII) than the more surface-exposed C-terminal regions. However, the immunoprecipitation experiments described by Bersch et al. (12) used purified cell membranes, which may not accurately represent the conditions found in intact cells; furthermore, a stable complex between PhtD and AdcAII could not be detected by nuclear magnetic resonance (NMR) spectroscopy in their study (12). Previous cross-linking evidence purportedly showing that the two purified proteins interact with one another *in vitro* was also somewhat weak, since only a small proportion of the purified PhtD became associated with AdcAII, in spite of the latter being present at 8-fold molar excess (6). Therefore, the possibility that transfer of Zn^{2+} to AdcAII by the Pht proteins may occur in the absence of a physical interaction cannot be discounted. The transfer of Zn^{2+} to AdcAII may instead simply rely upon the Pht proteins increasing the metal's local concentration near the cell membrane, thereby increasing the frequency of the metal encountering AdcAII. Further experimental investigation will be required to discriminate between the direct and indirect models of Zn^{2+} delivery to AdcAII by the Pht proteins.

Previous studies have either pointed to complete redundancy among the Pht proteins (3, 17) or observed a phenotypic impact when only one *pht* gene was deleted, consistent with the proteins being nonredundant (4). In this study, we observed partial redundancy, whereby strains producing only one Pht protein showed growth phenotypes and Zn^{2+} accumulation levels intermediate between those of strains producing either all four Pht proteins or no Pht protein. Simply put, in terms of Zn^{2+} acquisition, production of one Pht protein was better than none, but not as effective as having all four. Thus, the Pht proteins have overlapping functionality, and while the data reflect that they all aid in Zn^{2+} acquisition via AdcAII, they are not redundant in the sense of being dispensable, as deletion of three out of four of the genes led to a fitness cost both *in vitro* and *in vivo*.

This was also reflected in the observed levels of expression of each of the Pht proteins, which in general terms increased in proportion to the severity of the challenge of obtaining a sufficient quantity of Zn^{2+} experienced by the particular strain under examination. Thus, strains that lacked three of the four *pht* genes (as well as *adcA*) showed the greatest levels of *pht* expression when grown in CDM-TPEN. However, differences were observed between the Pht proteins with respect to when their expression became maximally derepressed. PhtB and PhtE protein expression appeared to be fully derepressed under Zn^{2+} restriction irrespective of whether the other *pht* genes were present. In contrast, expression of PhtA and PhtD showed further derepression in the absence of the other Pht proteins. Thus, there are differences in the degree of regulation of the *pht* genes in response to Zn^{2+} restriction and the ability of the pneumococcus to acquire it. This may indicate that carrying four *pht* genes could be of benefit to pneumococcal Zn^{2+} acquisition by allowing several "layered" increases in the expression of proteins required for this process, depending on the precise extent of the challenge the bacteria face in acquiring a sufficient quantity of the metal. This observation also mirrors our previous findings that the fold change in expression of AdcAII is greater than that of AdcA when Zn^{2+}-restricted and Zn^{2+}-replete conditions are compared. That finding led to the conclusion that the role of AdcA is to provide a baseline level of Zn^{2+} recruitment, with AdcAII providing a greater enhancement in Zn^{2+} acquisition when the ion is particularly scarce (9). It is interesting to note that PhtE showed a greater fold change in protein levels than the other Pht proteins, and this may be linked to the slightly elevated Zn^{2+} accumulation in the "PhtE-only" Δ*phtABD* Δ*adcA* mutant compared to the other mutants lacking three out of four *pht* genes. The presence of six histidine triad motifs in PhtE, which is one more than is found in the other three pneumococcal Pht proteins, may also contribute to the increased Zn^{2+} accumulation in this mutant. Furthermore, these results further support the notion of overlapping functionality of the Pht proteins, in that they suggest that the impairment in growth of the strains lacking three *pht* genes is due to a lower total amount of Pht protein expressed in each strain. Intriguingly, a degree of cross-reactivity was observed in the Western blots between different Pht family members for each antiserum (the antisera were generated against individual Pht proteins [16]), as has been observed previously (13). Immunization with only one Pht protein may therefore be sufficient to induce protective immunity targeting multiple members of the protein family.

The results of the animal experiments were in agreement with the conclusion from the *in vitro* work that the Pht proteins are all capable of performing the same role in Zn^{2+} uptake, but they are not dispensable without incurring a fitness cost. A clear attenuation in fitness *in vivo* was observed for strains producing either PhtA or PhtD alone compared to the Δ*adcA* control strain (Fig. 6), consistent with production of more than one Pht protein being beneficial in pneumococcal pathogenesis. We also observed that, in the absence of AdcAII, the Pht proteins do still contribute to pathogenesis at 24 h postinfection. Our experiments thus far have clearly demonstrated a role for the Pht proteins in aiding Zn^{2+} acquisition by AdcAII but have not definitively determined whether they also contribute to the functionality of AdcA; such a role could explain the results observed here. Alternatively, the proposed adherence and factor H binding properties of the Pht proteins, or a combination of all of these roles, may account for this AdcAII-independent contribution to pathogenesis (3–5). The finding that the Δ*phtABDE* Δ*adcAII* mutant was more competitive in the nasal wash after 48 h may also be explained by the Pht proteins playing a role in adherence to host surfaces, since this strain may be expected to adhere more weakly than the Δ*adcAII* mutant and thus be overrepresented in the nasal wash sample.

Across both sets of animal experiments, it can be seen that in most cases mutants lacking *pht* genes were significantly attenuated in various niches at 24 h postinfection, but they had recovered by 48 h. This implies that the challenge of acquiring a sufficient

amount of Zn^{2+} is more profound for the pneumococcus at the earlier stages of infection. Indeed, this is supported by our previous observation that single mutants lacking *adcA* or *adcAII* are attenuated compared to the wild type in the nasopharynx at 24 but not 48 h postinfection (9). We may speculate that the array of Zn^{2+}-binding proteins employed by *S. pneumoniae* reflects the severity of this challenge. The pneumococcus is unusual among pathogenic bacteria that infect the nasopharynx in its possession of two Zn^{2+}-specific SBPs. These, in conjunction with the Pht proteins, may provide an advantage over other microorganisms with which the pneumococcus has to compete to colonize the nasopharynx, since they may allow more-effective acquisition of the limited quantities of available Zn^{2+}. We have previously shown that, at 48 h postinfection, host Zn^{2+} levels are elevated and that this may be an immune mechanism used to expose the pneumococcus to a toxic excess of Zn^{2+} (20). The release of Zn^{2+} is consistent with the recovery (in terms of competitive indices) of strains that are deficient in Zn^{2+} uptake at this time point observed in this study, since the increase in abundance of the metal means its acquisition would no longer pose a significant challenge. Furthermore, such a recovery for strains lacking *pht* genes is also consistent with the Pht proteins not contributing to defense against Zn^{2+} toxicity, as we found to be the case *in vitro*.

In other streptococci, *adcAII* homologues are often found with only one *pht* gene in the genome (24). Given that we have shown that all four Pht proteins of *S. pneumoniae* have overlapping functionality with respect to aiding in delivery of Zn^{2+} to AdcAII, it is not apparent what the advantage of producing four such proteins is, as opposed to perhaps simply producing a greater quantity of one Pht protein. Further studies will be required to examine whether each Pht protein does make a distinct contribution to pneumococcal fitness, either in terms of Zn^{2+} uptake or in an alternative virulence role. Such understanding will be crucially important to guide future vaccine development targeting this intriguing family of surface proteins.

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