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Antigen Discovery for Next-Generation Pertussis Vaccines Using Immunoproteomics and Transposon-Directed Insertion Sequencing

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Background. Despite high vaccination rates, the United States has experienced a resurgence in reported cases of pertussis after switching to the acellular pertussis vaccine, indicating a need for improved vaccines that enhance infection control.

Methods. *Bordetella pertussis* antigens recognized by convalescent-baboon serum and nasopharyngeal wash were identified by immunoproteomics and their subcellular localization predicted. Genes essential or important for persistence in the baboon airway were identified by transposon-directed insertion-site sequencing (TraDIS) analysis.

Results. In total, 314 *B. pertussis* antigens were identified by convalescent baboon serum and 748 by nasopharyngeal wash. Thirteen antigens were identified as immunogenic in baboons, essential for persistence in the airway by TraDIS, and membrane-localized: BP0840 (OmpP), Pal, OmpA2, BP1485, BamA, Pcp, MlaA, YfgL, BP2197, BP1569, MlaD, ComL, and BP0183.

Conclusions. The *B. pertussis* antigens identified as immunogenic, essential for persistence in the airway, and membrane-localized warrant further investigation for inclusion in vaccines designed to reduce or prevent carriage of bacteria in the airway of vaccinated individuals.

Keywords. antigen; baboon; *Bordetella pertussis*; immunization; immunoproteomics; TraDIS; vaccine; whooping cough.

Prior to the introduction of effective pertussis vaccines, *Bordetella pertussis* was responsible for approximately 182 000 cases and 5633 deaths per year in the United States [1]. The introduction of whole-cell pertussis (wP) vaccines led to declining numbers of reported cases with fewer than 5000 cases per year from 1968 to 1990 [2]. Although highly efficacious, reactogenicity concerns with the wP vaccines led to reduced acceptance and declining vaccination rates [3–6]. Acellular pertussis (aP) vaccines were introduced in the 1990s that were less reactogenic than the wP vaccines and demonstrated comparable efficacy over the first 18 months following vaccination [4–8]. The aP vaccines currently used in the United States include pertussis toxoid (PT), filamentous haemagglutinin (FHA), and pertactin (PRN). Some aP vaccines also include fimbriae (FIM). Despite high vaccination rates with aP vaccines, the United States has experienced a resurgence in reported pertussis cases [2]. It has been hypothesized that an important contributing factor to this resurgence is that the aP vaccines induce an immune response that is not optimal for preventing infection, carriage, and transmission [9, 10].

Infection and vaccination with the wP vaccine induce T helper type 1 (T_H1) and type 17 (T_H17) memory, and antibody responses against a wide array of bacterial antigens [11–13]. As has been demonstrated in the baboon model and in clinical data, these immune responses are well suited to prevent infection and control symptomatic disease. In contrast, the aP vaccine induces T_H2 memory with antibody responses targeting a limited set of antigens [11–13]. Neutralizing antibody responses targeting PT in the aP vaccines are likely sufficient to protect against symptomatic disease [14, 15]. However, an aP vaccine did not demonstrate a benefit in preventing infection and transmission in the baboon model. These issues have increased interest in developing more effective vaccines that combine the protection against severe disease and the safety profile of the aP vaccines with the ability to induce an immune response that prevents infection and transmission.

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For this study, a model was developed to identify candidate antigens for improved next-generation aP vaccines capable of preventing infection and transmission. The ideal antigens should meet several criteria: (1) be immunogenic in the host, (2) be exposed on the bacterial surface to induce bactericidal and/or opsonophagocytic antibodies that target the bacterial cell for clearance by the host immune response, and (3) should be required for bacterial survival or persistence in vivo to prevent emergence of variant strains due to vaccine-driven selective pressure. The importance of this last point is highlighted by the fact that one of the aP antigens, PRN, is now known to be nonessential. As a result, PRN expression has been lost from nearly all circulating strains of *B. pertussis* since the aP vaccines were introduced.

To generate data for this model we used a transposon-directed insertion-site sequencing (TraDIS) transposon library to provide the first comprehensive assessment of *B. pertussis* genes that are essential for bacterial survival in the primate airway. In addition, we used proteomics to identify antigens recognized by both serum and mucosal antibodies. By combining these datasets (Figure 1), we identified 13 putative antigens that meet all 3 criteria listed above. These antigens represent promising targets for the development of improved, next-generation aP vaccines.

METHODS

Transposon Library Production

A transposon library was constructed in *B. pertussis* D420 by multiple independent matings with *Escherichia coli* strain ST18 bearing the ep1 plasmid [16, 17] containing the gene encoding Tn*5* transposase and a mini-Tn*5* insertion cassette bearing kanamycin resistance. The mating mixture was plated on Bordet-Gengou agar plates with kanamycin (50 µg/mL) to select for D420 mini-Tn*5* chromosomal insertion mutants. This mating protocol was repeated 12 times. The resulting colonies were combined into a single transposon library and frozen in glycerol.

Bordetella pertussis Protein Lysate Preparation

B. pertussis D420 protein lysates were prepared from iron-starved bacteria to simulate growth conditions in the mammalian host [18–20]. Bacteria were cultured in iron-deficient Stainer-Scholte medium. The iron was removed from the medium with Chelex 100 resin [21, 22]. The bacterial cells were harvested at mid- to late-exponential growth phase, suspended in phosphate buffered saline (PBS), mechanically sheared at low temperature using a French pressure cell, and frozen in liquid nitrogen. The iron starvation status of the bacteria was confirmed using alcaligin siderophore detection assays [23, 24] and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins.

Immunoproteomic Identification of *Bordetella pertussis* Antigens Recognized by Baboon Antibodies Derived From Convalescent Serum and Nasopharyngeal Wash

Antibodies from pooled serum or nasopharyngeal wash (NPW) from convalescent baboons were used to pull down proteins from the *B. pertussis* D420 protein lysate. Antigen/antibody complexes were immobilized on Protein L magnetic beads (Thermo Scientific Pierce). The bound antigens were identified by reversed-phase nanoflowliquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis [25]. Identity searches were performed against the proteobacteria database. Additional details on the pull down assays and mass spectrometry are provided in the Supplemental Methods.

TraDIS Methods

Genomic DNA was isolated from *B. pertussis* cell pellets and was analyzed by Illumina sequencing according to the TraDIS method [26, 27]. The reads were mapped to the *B. pertussis* D420 genome LN849008.1 and essential genes were assigned using the script TraDIS_essential.R. TraDIS_comparison.R was used to measure the relative abundance of different mutants between the in vitro and in vivo conditions. Additional details on TraDIS methods are provided in the Supplementary Methods.

Ethics Statement

All animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with protocols approved by the Food and Drug Administration's Animal Care and Use Committee or the University of Oklahoma Health Science Center Animal Care and Use Committee, and the principles outlined in the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Resources, National Research Council.

Infection of Baboons

Olive baboons were obtained from the MD Anderson Keeling Center for Comparative Medicine, Bastrup, Texas or from the University of Oklahoma Baboon Research Resource Oklahoma City, Oklahoma. *B. pertussis* inocula were prepared from the D420 Tn*5* insertion library glycerol stocks or from wild-type D420 stocks and used to challenge baboons according to established protocols [28] with direct inoculation of 1×10^9 colony forming units (CFU)s into the trachea and nares. White blood cell counts and NPW CFUs were obtained to monitor the course of infection. Serum and NPW samples were collected at day 28 after the second challenge and pooled for immunoproteomics. Baboons infected with the *B. pertussis* D420 transposon library were euthanized on day 7 postchallenge for TraDIS and lung and trachea samples were recovered. Tissues were homogenized in 20 mL of PBS and plated on Regan-Lowe (RL) agar plates containing 10% sheep blood,

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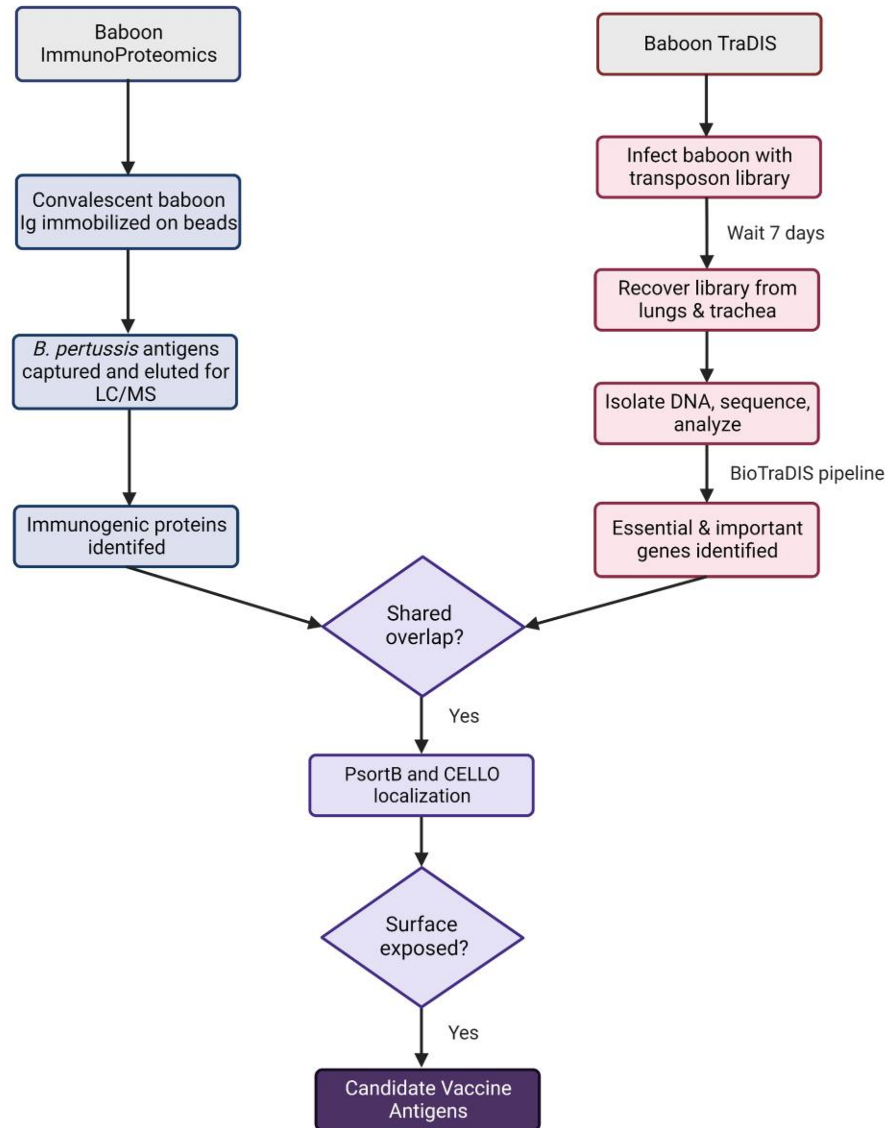

Figure 1. Experiment flow chart. Immunoproteomic and TraDIS experiments were performed in the baboon model of pertussis to select for immunogenic proteins and essential or important genes, respectively. Immunodominant proteins identified by baboon serum or nasopharyngeal wash were selected to see if their corresponding genes were essential or importanst in vitro or in vivo. Those that were also surface exposed were selected as candidate vaccine antigens. Created with BioRender.com. Abbreviations: LC/MS/MS, liquid chromatography-tandem mass spectrometry; TraDIS, transposon-directed insertion-site sequencing.

50 µg/mL kanamycin, and 40 µg/mL cephalexin for 2 days before harvesting and isolating genomic DNA from pooled plate growth. Baboons colonized by natural transmission were obtained by cohousing uninfected baboons with baboons directly inoculated with the *B. pertussis* transposon library.

Pinch Biopsies

Six baboons were directly inoculated with *B. pertussis* strain D420 as described above. Bronchoscopy was performed on each animal on day 14 and pinch biopsies of the airway mucosa were collected at 5 sites along the airway. An additional 6 uninfected baboons were cohoused with the inoculated baboons. Uninoculated baboons were followed by monitoring of CFUs in nasal washes until transmission was observed. Bronchoscopy was performed when peak infection was reached and pinch biopsies of the airway mucosa were collected at the same 5 sites along the airway. The pinch biopsies were homogenized in PBS and plated on RL agar to enumerate CFUs.

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Evaluation of Genes Essential for Fitness Encoding Immunogenic Proteins

Immunoproteomic experiments were performed in the baboon model of pertussis to identify proteins recognized by the baboon immune response following infection. TraDIS experiments were performed in the baboon model of pertussis to identify genes encoding proteins that contribute significantly to *B. pertussis* fitness in the airway during infection. Proteins that were both immunogenic and contribute to fitness were evaluated to identify those that are predicted to be exposed on the bacterial surface (Figure 1).

RESULTS

Proteomics

Immunogenic proteins from *B. pertussis* were identified using pooled serum and NPW collected from convalescent baboons 28 days after they were rechallenged with *B. pertussis* D420. Total baboon immunoglobulin from pooled convalescent baboon sera or NPW was immobilized on beads and used to pull down antigens present in a bacterial protein lysate generated from *B. pertussis* cultures grown under iron-limiting, virulent-phase conditions. The isolated proteins were identified using mass spectrometry (Supplementary Table 1). In total, 314 proteins were identified by pull-down with serum antibodies, including 34 outer membrane proteins, 7 extracellular proteins, 139 cytoplasmic proteins, and 13 proteins predicted at multiple cellular locations (Figure 2*A*). Of the proteins with multiple locations, 10 of them are predicted to be located on the outer membrane and/or to be extracellular. Similarly, 748 proteins were identified by pull-down with antibodies isolated from NPW, including 41 outer membrane proteins, 11 extracellular proteins, 392 cytoplasmic proteins, and 43 proteins at multiple locations, of which 18 may be in the outer membrane (Figure 2*B* and Supplementary Table 1). Pertussis toxin proteins (PtxA and PtxD), serotype 3 fimbriae (Fim3), pertactin (Prn), and filamentous hemagglutinin (FhaB) proteins were identified with both serum or NPW samples as well as fimbrial assembly proteins (FimB, and FimC) and FHA assembly and transport proteins (FhaB and FhaC). Other virulence-associated proteins, including BvgA, BvgS, Vag8, BrkA, TcfA, BfrD, and BrfB, were also identified.

Natural Transmission, Colonization, and Airway Distribution

A high-dose inoculum of *B. pertussis* is routinely used in the baboon model of pertussis to immediately establish infection. The degree to which the resulting distribution and bacterial load throughout the airway compares with natural infection is unknown. To determine if direct inoculation of a high-dose challenge results in comparable colonization of the airway as that observed following natural transmission, 6 baboons were inoculated with *B. pertussis* as described in the "Methods" section and 6 uninfected baboons were cohoused with the inoculated animals. The magnitude and distribution of bacteria throughout the airway was determined in all 12 animals at peak infection by quantifying bacteria from pinch biopsies collected at 5 sites along the airway of each animal. A comparable distribution of bacteria and comparable numbers of bacteria were present throughout the airway following infection by both routes (Figure 3). This supported the use of the high-dose inoculum in the baboon model of pertussis for TraDIS analysis of bacterial fitness in the baboon airway.

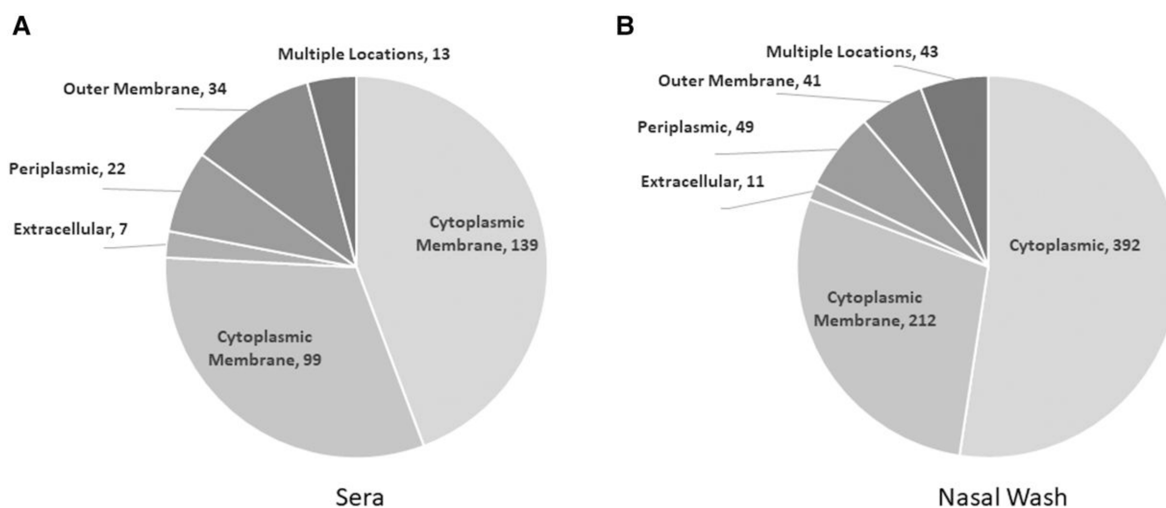

Figure 2. Subcellular locations of antigens identified by convalescent baboon serum and nasal wash. Antigenic proteins identified by convalescent baboon serum (*A*) and nasal wash (*B*) were identified by mass spectrometry. The subcellular localization of these proteins was predicted using Psortb or CELLO subcellular localization prediction methods.

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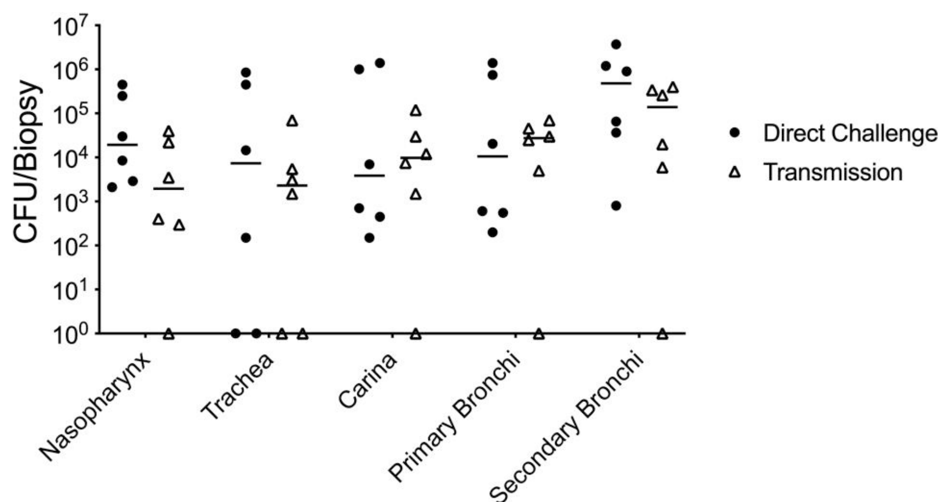

Figure 3. The magnitude and distribution of *Bordetella pertussis* cells throughout the conducting airway at peak infection is comparable following direct, high-dose challenge, and natural transmission. Six naive baboons were cohoused with 6 animals directly infected with *B. pertussis*. At peak infection, pinch biopsies were harvested from the 5 indicated sites along the conducting airway and the entire biopsy sample was diluted and plated for determination of colony-forming units (CFUs). The number of CFUs for each animal at each site are shown.

Identification of B. pertussis Genes by TraDIS

We sought to identify the *B. pertussis* genes required or important for survival in the baboon respiratory tract using TraDIS. The *B. pertussis* D420 Tn*5* transposon library had approximately 4×10^5 unique insertions distributed across the genome with an average of 50 insertions per gene (Supplementary Figure 1). Examination of the transposon library, and the inocula produced using the library, identified 249 genes lacking insertions within the first 90% of the open reading frame. The lack of insertions in these genes in the Tn*5* library and inoculum suggests the proteins encoded by these genes are essential for *B. pertussis* growth in vitro on BG medium (Supplementary Table 2).

To identify genes essential for bacterial survival in the host airway, the Tn*5* library was used to infect 4 baboons and bacteria were harvested from the lungs and trachea on day 7 postinoculation to determine gene essentiality and the abundance of different transposon insertion mutants in the airway relative to the inoculum used to infect the baboons. Genes that are required for growth in vitro may also be required for growth in vivo, but that cannot be determined in this study because they were not represented in the inocula. Transposon insertion mutants that were absent or less abundant in the baboon lung and/or trachea samples when compared to the inoculum are proposed to carry insertions in genes essential for colonization or for persistence in the airway. A total of 240 genes were determined by this analysis to be essential in the baboon airway, either in the trachea only (69 genes), the lung only (93 genes) or both sites (78 genes) (Supplementary Table 3). An additional 280 genes, although not essential, contributed significantly to persistence of *B. pertussis* in the baboon airway (Supplementary Tables 4 and 5). Of the 249 genes determined to be essential in vitro and 240 genes determined to be essential in vivo, 13 were also recognized in the baboon immunoproteomics analysis and are predicted to be localized to the outer membrane (Table 1).

DISCUSSION

Because TraDIS analysis requires high numbers of cells, it was necessary to utilize a high-dose, direct inoculation to establish infection for the TraDIS studies presented here. Although artificial, past experience with this model demonstrates that it accurately recapitulates infection in humans and our results here indicate that the magnitude and distribution of bacteria in the airway at peak infection are comparable between animals infected by direct inoculation or by natural transmission (Figure 3). It is reasonable to conclude that genes determined to be important for persistence in our model are likely to be important in natural human infections.

In the baboon model of pertussis, natural infection provides sterilizing immunity, wP vaccination significantly decreases bacterial burden and shortens the time to bacterial clearance, and aP vaccination protects against disease but does not prevent colonization or transmission [10, 29]. Adding antigens to the aP vaccine, in combination with optimizing adjuvant formulations that induce balanced $T_H1/T_H2/T_H17$ responses and optimizing the delivery route, may result in host immune responses that facilitate bacterial clearance from the airway and thereby reduce transmission. To achieve this, antigens eliciting bactericidal antibodies and/or opsonophagocytic antibodies

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Table 1. Essential Genes (In Vitro and In Vivo) Encoding Outer Membrane Proteins Identified by Convalescent Baboon Serum or NPW

Accession No.	Protein	Gene	Description	Essential Tn*5* Data Set	Proteomics Data Set
Q04064	OmpP	…	Outer membrane porin protein BP0840	In vitro	NPW and sera
Q7VU04	Pal	…	Peptidoglycan-associated protein	In vitro	NPW and sera
Q7VZG6	OmpA	*ompA2*	Outer membrane protein A	Lung and trachea	NPW and sera
Q7VY72	BP1485	…	Putative membrane protein	Lung and trachea	NPW and sera
Q7VYC2	BamA	…	Outer membrane protein assembly factor BamA	In vitro	NPW and sera
Q7VUT2	Pcp	…	Putative lipoprotein	Lung	NPW and sera
Q7VSZ7	BP3760	*mlaA*	Putative lipoprotein	Lung	NPW and sera
Q7VWL3	YfgL	*yfgL*	Outer membrane protein assembly factor BamB	In vitro	NPW and sera
Q7VWL2	BP2197	…	Ancillary SecYEG translocon subunit	In vitro	NPW and sera
Q7VXZ9	BP1569	…	Putative lipoprotein	In vitro	NPW and sera
Q7VSZ8	BP3759	*mlaD*	Mce related protein	Lung and trachea	NPW
Q7VZ03	ComL	…	Outer membrane protein assembly factor BamD	In vitro	NPW and sera
Q7W0F4	BP0183	…	Translocation and assembly module subunit TamA	In vitro	NPW

Genes known or predicted to encode outer membrane proteins identified as essential in vitro (insertions absent in inoculum) or in vivo (insertions absent in reported tissue at day 7 postchallenge) by TraDIS that were also recognized by convalescent baboon serum and/or NPW. Abbreviations: NPW, nasopharyngeal wash; TraDIS, transposon-directed insertion-site sequencing.

that bind the bacterial cell should be included. Ideally, these antigens should be sufficiently immunogenic, essential for bacterial growth in the host, and surface exposed.

We identified *B. pertussis* protein antigens using serum and NPW from convalescent baboons for immunoprecipitation and mass spectrometry. In total, 314 proteins were identified by serum antibodies and 748 proteins were identified by NPW antibodies. In another study, Gasperini et al [30] identified 389 proteins in *B. pertussis* outer membrane vesicles by proteomics and selected those with increased abundance in Bvg$^+$ (*Bordetella* virulence gene$^+$)-regulated conditions for further analysis, as proteins with increased abundance in the Bvg$^+$ phase may be related to virulence. All of their candidate Bvg$^+$-regulated proteins (BrkA, FhaB, Prn, Vag8, TcfA, BipA, SphB1, and BfrD) were also identified by our study, indicating that they are immunogenic in the baboon. In a mouse study, Raeven et al [31] described an immunoproteomic profile of a *B. pertussis* outer membrane vesicle vaccine and identified antibodies against Vag8 and BrkA, which were also identified in our pull-down studies.

In addition to immunogenicity, it is important to consider the essentiality (in vitro and in vivo) of a protein when selecting it for inclusion in an acellular vaccine. If a vaccine antigen effectively elicits antibodies that target the bacterial cell for clearance but the antigen is not required by the bacterium for survival, the bacterium could reduce or eliminate production of that antigen in the face of vaccine-mediated selective pressure. For example, pertactin is included in aP vaccines and responses to pertactin are associated with protection, but production of pertactin has been lost in most *B. pertussis* strains found circulating in countries that use pertactin-containing aP vaccines [32–36]. In our analysis, pertactin was identified as immunogenic by convalescent baboon NPW and serum as expected, but it was not identified by TraDIS as essential or even as contributing to persistence in the airway—this demonstrates the relevance of our methodology in selecting vaccine antigens. Inclusion of immunogenic but nonessential proteins in next-generation vaccines may be beneficial when the vaccines are initially introduced, but because they are not essential, it is likely that vaccine-escape variants would eventually arise, as was observed with peractin.

A caveat for using transposon libraries, such as is used in TraDIS analysis, to identify essential genes is that they cannot identify essential secreted effectors. These factors would be complemented by neighboring bacterial cells with different transposon insertions. In fact, transposon mutants lacking secreted PT were found in increased abundance in the transposon libraries both in vitro and in vivo (data not shown), highlighting that the production and secretion of PT is a metabolically intensive process and PT mutant strains were presumably able to grow at a faster rate than bacteria in the TraDIS library that do not have mutations that effect PT expression. Additionally, as noted above, TraDIS may not be able to identify genes important for initiating colonization or for infection steps that lie beyond a critical bottleneck as the method requires analysis of high numbers of bacteria.

Combining immunoproteomics with TraDIS, and predicting the gene products' cellular localization enabled us to identify candidate vaccine antigens that are immunogenic, essential for bacterial growth in the host, and surface exposed. The proteins products of BP0840 (ompP), pal, ompA, BP1485, bamA, pcp, BP3760 (mlaA), YfgL, BP2197, BP1569, BP3759 (mlaD), comL, and BP0183 met all 3 criteria (Table 1). The proteins OmpP, Pal, BamA, YfgL, BP2197, BP1569, ComL, and BP0183 are essential for growth on BG plates, which is a rich medium for *B. pertussis* growth. The requirement for these

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Table 2. **Genes Encoding Outer Membrane Proteins Identified by Convalescent Baboon Serum or NPW That Are Important But Not Essential In Vitro or In Vivo**

Locus Tag	Gene	Description	Log_2FC Lung	Log_2FC Trachea	Proteomics Data Set
BPD420_00547	*sphB1*	Autotransporter subtilisin-like protease	−6.67	−4.68	NPW and sera
BPD420_02041	*fhaB*	Filamentous hemagglutinin/adhesin	…	−3.24	NPW and sera
BPD420_01713	*omlA*	Outer membrane lipoprotein	…	−6.31	NPW
BPD420_02606	*vag8*	Autotransporter	−3.82	−3.04	NPW and sera

Genes known or predicted by PsortB or CELLO to encode outer membrane proteins, recognized by convalescent baboon serum or nasopharyngeal wash (NPW) that are in significantly decreased abundance in vivo, but were not found to be essential.

proteins could not be tested in vivo because their insertion mutants were absent from the inoculum. Because there is likely significant overlap in the genes essential for growth on BG agar and essential for growth in the airway, it is reasonable to include these 8 proteins in our list of candidate vaccine antigens. The proteins OmpA, BP1485, Pcp, MlaA, and MlaD were shown to be specifically required in vivo. Anti-Pcp antibodies have been shown to kill *Haemophilus influenzae* in serum bactericidal assays [37]. OmpA proteins in gram-negative bacteria are generally abundantly produced, antigenic, and have been implicated in cell adhesion and invasion in human endothelial cells [38, 39]. OmpA has shown promise as a carrier protein and antigen in *Klebsiella pneumoniae* and *Shigella flexneri* subunit vaccines [40, 41] and can activate antigen presenting cells and stimulate cytotoxic CD8 lymphocytes [42]. Anti-OmpA antibodies have been shown to kill *Porphyromonas gingivalis* in a complement-mediated fashion [43]. BP1485 and OmpP are novel antigens about which little is known, but when OmpP was included in a *Bordetella bronchiseptica* vaccine, it was shown to confer protection in rabbits [44]. This observation provides some proof of concept that the targets identified by our analysis may contribute to protection against *Bordetella* infection if incorporated into a vaccine. MlaD and MlaA are part of a phospholipid transport system essential for maintaining the integrity of the outer membrane [45]. While thought to primarily be localized to the inner leaflet of the outer membrane, Pal, MlaA, and YfgL may also be attractive targets for vaccine development.

Relaxation of each of our 3 criteria for antigen selection results in additional proteins to consider for inclusion in next-generation acellular vaccines. For example, 2 outer membrane-localized proteins (BPD420_01036 and BPD420_00516) were determined to be essential for bacterial survival in the trachea by TraDIS but were not identified by our baboon immunoproteomics. However, our pull-down studies are not likely to have identified all proteins recognized by the host immune response during infection. Therefore, we plan to produce these essential proteins to determine whether they are recognized by human or baboon convalescent sera. Finally, 4 outer membrane-localized proteins were determined to be immunogenic in our proteomic studies and important, but not essential, for growth by TraDIS (Table 2). Insertions in these 4 genes had significant reductions in prevalence in the baboon relative to their prevalence in the inoculum, indicating their functions are important for bacterial persistence in the airway. The contribution of these proteins to *B. pertussis* fitness in the airway may be sufficient to prevent vaccine-mediated selection against expression of these proteins in a vaccinated population. Therefore, these 4 proteins may also represent good candidates for inclusion in next-generation subunit vaccines.

We used strict criteria in our analysis and it is likely we did not identify all essential, immunogenic, outer membrane proteins, but the antigens identified represent excellent targets to be further evaluated for inclusion in next-generation pertussis vaccines designed to protect against colonization and carriage, and prevent transmission of *B. pertussis*. In future studies, utilizing the baboon model, we will evaluate the ability of these antigens to induce immune responses preventing or reducing infection, carriage, and transmission of *B. pertussis*.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that

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the editors consider relevant to the content of the manuscript have been disclosed.

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