Outer Membrane Vesicles (OMV)-based and Proteomics-driven Antigen Selection Identifies Novel Factors Contributing to *Bordetella pertussis* Adhesion to Epithelial Cells*\(^*\)\(^{\#}\)

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Despite high vaccination coverage world-wide, whooping cough, a highly contagious disease caused by *Bordetella pertussis*, is recently increasing in occurrence suggesting that novel vaccine formulations targeted at the prevention of colonization and transmission should be investigated. To identify new candidates for inclusion in the acellular formulation, we used spontaneously released outer membrane vesicles (OMV)\(^1\) as a potential source of key adhesins. The enrichment of Bvg+ OMV with adhesins and the ability of anti-OMV serum to inhibit the adhesion of *B. pertussis* to lung epithelial cells *in vitro* were demonstrated. We employed a proteomic approach to identify the differentially expressed proteins in OMV purified from bacteria in the Bvg+ and Bvg− virulence phases, thus comparing the outer membrane protein pattern of this pathogen in its virulent or avirulent state. Six of the most abundant outer membrane proteins were selected as candidates to be evaluated for their adhesive properties and vaccine potential. We generated *E. coli* strains singularly expressing the selected proteins and assessed their ability to adhere to lung epithelial cells *in vitro*. Four out of the selected proteins conferred adhesive ability to *E. coli*. Three of the candidates were specifically detected by anti-OMV mouse serum suggesting that these proteins are immunogenic antigens able to elicit an antibody response when displayed on the OMV. Anti-OMV serum was able to inhibit only BrkA-expressing *E. coli* adhesion to lung epithelial cells. Finally, stand-alone immunization of mice with recombinant BrkA resulted in significant protection against infection of the lower respiratory tract after challenge with *B. pertussis*. Taken together, these data support the inclusion of BrkA and possibly further adhesins to the current acellular pertussis vaccines to improve the impact of vaccination on the bacterial clearance. *Molecular & Cellular Proteomics* 17: 10.1074/mcp.RA117.000045, 205–215, 2018.

*Bordetella pertussis* is a Gram-negative bacterium, obligate human pathogen and causative agent of whooping cough, a highly contagious disease which is recently increasing in occurrence despite high vaccination coverage world-wide (1–3). The resurgence of pertussis over the last two decades has been suggested to be because of many factors including improved diagnostics and pathogen evolution but also to waning immunity following vaccination with the acellular formulation (aP) which replaced the more reactogenic whole-cell vaccine (wP) (4–7).

Acellular pertussis vaccines are currently available from different manufacturers and include up to five different components (*Pertussis Toxin* (PT)), Filamentous Hemagglutinin (FHA), 69kDa outer-membrane protein (also known as Pertactin), fimbral-2 and fimbral-3 antigens) in different concentrations and with different adjuvants. All the aP vaccine components are highly regulated by the BvgAS two component system which enables *B. pertussis* to respond to extracellular stimuli and modulate the concerted activation of all the virulence genes acting like a master switch among clearly distinct phenotypic phases (8). Therefore, Bvg-activated proteins are mainly associated with colonization, toxicity and host immune evasion and represent potential vaccine candidates (9).

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\(^{1}\) The abbreviations used are: OMV, Outer Membrane Vesicles; aP, acellular pertussis vaccine; wP, whole-cell pertussis vaccine; 69K, Pertactin; FHA, Filamentous Hemagglutinin; IPTG, isopropyl  β- D-thiogalactopyranoside; CDS, Coding DNA Sequence; LB, Luria Bertani broth; HLB, Hydrophilic-Lipophilic Balance; UPLC; Ultra Performance Liquid Chromatography; HCD, Higher-energy C-trap dis-association; AGC, Automatic Gain Control; FDR, False Discovery Rate; CFU, Colony Forming Unit; NTA, Nanoparticle Tracking Analysis; DDA, Data-Dependent Acquisition.

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Importantly, several studies including the recent employment of the baboon infection model have shown that the acellular vaccine is able to prevent the clinical symptoms of the disease but not the colonization of the airways, leading to an increased risk of transmission and consequent bacterial spread throughout the population (10). Moreover, strains belonging to the *pbp3* lineage have emerged in recent years, showing a higher level of PT and loss of Pertactin (11); therefore, aP vaccines may be less efficient in eliciting toxin-neutralizing and anti-adhesive antibodies against these new circulating strains. Taken together, all these data suggest that a new generation vaccine against pertussis able to shorten bacterial colonization by inclusion of new protective antigens is needed (12).

To identify new adhesins to be included in a novel vaccine formulation we used outer membrane vesicles (OMV) as a potential source for the identification of protective antigens. OMV are blebs of the outer membrane which are spontaneously released by all Gram-negative bacteria during growth and they contain periplasmic proteins in their lumen and outer membrane proteins and lipidproteins in their natural conformation and architectural context (13, 14).

In this study, we isolated OMV from the pathogen in its virulent (Bvg+) or avirulent (Bvg−) phase and employing a proteomic approach we selected six Bvg-regulated candidates to be subsequently evaluated for their adhesive properties and vaccine potential. Indeed, OMV are far more suitable than Outer Membrane Protein (OMP) preparations for proteomic analysis because of the lack of contaminants deriving from other cellular compartments such as the cytoplasm. Finally, we evaluated whether a stand-alone immunization with BrkA could confer protection in a mouse aerosol challenge model of infection.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The following *B. pertussis* strains were used in this study: Tohama I-derivative BPS36 (15) and BPS37 (16) and W28 PT 9k/129G (17). Bacteria were stored at −80 °C and recovered by plating on Bordet-Gengou (BG) agar plates, supplemented with 15% (v/v) sheep blood, for 3 days at 37 °C. Bacteria were then inoculated at initial 600 nm optical density (OD600) of 0.05–0.1 in Stainer-Scholte medium supplemented with 0.4% (w/v) l-cysteine monohydrochloride, 0.1% (w/v) FeSO4, 0.2% (w/v) ascorbic acid, 0.04% (w/v) nicotinic acid, 1% (w/v) reduced glutathione. Cultures were grown in rotary shakers at 37 °C. Recombinant DH5α *E. coli* strains were stored at −80 °C, recovered by plating on LB agar plates supplemented with 20 μg/ml chloramphenicol for 16 h at 37 °C. For liquid cultures, bacteria were inoculated in LB medium supplemented with 20 μg/ml chloramphenicol and were grown in rotary shakers at 37 °C for 16 h.

**Generation of E. coli Strains Expressing Heterologous B. pertussis Candidate Adhesins**—*E. coli* strain DH5α was transformed with a range of 6 plasmids based on the broad host range vector pMMB208 (18) which was modified to express the candidate adhesins. The Apal-Xb fragment containing the *lacI* gene and the IPTG-inducible pTac promoter was substituted with an expressing cassette consisting of a constitutive *B. pertussis* promoter and the full-length coding sequences for *brkA*, *sphB1*, *vag8*, *tcfA*, *bipA* and *bfrD*. The promoter and the coding sequences were amplified from *B. pertussis* W28 9k/129G genomic DNA using the following primers: promoter F (ccc GGGCCTTCCTTAGTAGAAGCTTTG) and promoter R (ccc AGGGTTAATCTGGTTATTGAGA); *brkA* F (ccc AGGTCATTATGATCTCAGTATAGCTCCGA) and *brkA* R (ccc TTGATCGTACGGAATGTTGAGCTG); *sphB1* F (ccc AAAGCTTTAGTTAGCCGGGCGCCGCGCCT) and *sphB1* R (ccc TTGATACGTAGTGTTAGTTGAGCTG); *tcfA* F (ccc AAAGCTTATGCATCTGCCGCGCCT) and *tcfA* R (ccc TTGATACGTAGTGTTAGTTGAGCTG); *bipA* F (ccc AAAGCTTTAGTAAAGAAGATTTACGTTT) and *bipA* R (ccc TTGATACGTAGTGTTAGTTGAGCTG); *bfrD* F (ccc AAAGCTTATGAAATGTTTCTAGGTTTCCCATCC) and *bfrD* R (ccc TTGATACGTAGTGTTAGTTGAGCTG).

**OMV Purification**—Cell-free supernatants from liquid cultures of BPS36 and BPS37 were recovered after a 3-day growth in 250 ml baffled flasks. The liquid-air volume ratio resulted critical for OMV production yield and was kept at 1:5 ratio. Bacteria were then pelleted through centrifugation at 5000 × g for 30 min. Supernatants were recovered and filtered through 0.22 μm Stericup filters (Millipore, Billerica, MA). After ultracentrifugation at 175,000 × g for 2 h at 4 °C, the resulting OMV pellet was washed with Dulbecco’s Phosphate-Buffered Saline (D-PBS), further ultracentrifuged at 175,000 × g for 2 h and finally resuspended in 100 μl D-PBS. OMV were quantified through the Lowry assay (DC Protein Assay, BioRad, Hercules, CA) for total protein content following the manufacturer’s instructions.

**SDS-PAGE Analysis**—For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, 10 μg (protein content) of OMV samples were resuspended in 20 μl Tris-HCl (pH 8.0) buffer containing 8% (v/v) SDS and 10 μl DTT, boiled for 5 min, separated on NuPAGE™ Novex™ 4–12% polyacrylamide Bis-Tris Protein Gels (Invitrogen) and stained with Coomassie Blue R-250.

**Nanoparticle Tracking Analysis**—A NanoSight NS300 instrument (Malvern Ltd.) was used to determine OMV particle size and concentration as previously described (19). Briefly, OMV can be observed by light scattering using a light microscope. Sequential videos are recorded and the NTA software can track the Brownian movement of individual vesicles and calculate the size and concentration of OMV. Samples at the protein concentration of 1 mg/ml were diluted 1:500 or 1:1000 in D-PBS and loaded in the sample chamber. Five videos per sample were recorded for 60 s and size of individual OMVs and total amount of OMV particles were analyzed by Nanoparticle Tracking Analysis 3.2 software (NanoSight Ltd., Malvern, United Kingdom). All measurements were performed at room temperature.

**Generation of Mouse Immune Sera**—BALB/c mice (10 female/group, 6-week old) (Charles River Laboratories International Inc., Wilmington, MA) received three intraperitoneal immunizations, with a 4-week interval, with aluminum hydroxide adjuvanted OMV from *B. pertussis* strain W28 9k/129G (2.5 μg per dose) or pW vaccine (NIBSC) at one fifteenth of a human dose. Sera were collected before immunization and 2 weeks after the third immunization. Control mice immunized with adjuvant only were included in the experiments. All animal experiments were performed in compliance with the Italian law with the approval of the local Animal Welfare Body (AWB 2014/06) followed by authorization of Italian Ministry of Health.

**Adhesion Assay**—The AS49 cell line (Human epithelial alveolar basal adenocarcinoma, ATCC CCL-185) was maintained in Ham’s F-12K medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA) and anti-
bacteria were grown for 16 h in liquid culture and then pelleted. Liters of bacteria/serum mixtures were transferred in triplicate onto B. pertussis used for OMV adhesion) and one-way ANOVA with Dunnett's multiple comparison test (for E. coli adhesion).

**Adhesion Inhibition Assay**—For B. pertussis adhesion inhibition assay, bacteria were grown for 16 h in liquid culture and then pelleted at 8,000 g for 5 min and resuspended at OD_{600} 0.1 in F12-K medium. One hundred microliters of the bacterial suspension were transferred in triplicate onto plated A549 cells. Infected cells were incubated for 3 h at 37 °C. After extensive washing to remove unbound bacteria, cells were fixed with 3.7% (v/v) formaldehyde for 20 min, blocked with 3% (w/v) BSA and incubated with Alexa Fluor 488 rabbit anti-mouse IgG (1:500) (Molecular Probes) for 30 min. After three washes with D-PBS, fluorescence was measured at excitation/emission 485/535 nm by Tecan Infinite F200PRO microplate reader.

For E. coli adhesion assay, bacteria were grown for 16 h in liquid culture and then washed with D-PBS, centrifuged at 8,000 × g for 5 min and resuspended at OD_{600} 0.5. For the fluorescent labeling of B. pertussis cells, a volume of 445 μl of bacterial suspension was transferred in triplicate onto plated A549 cells. Infected cells were incubated for 3 h at 37 °C. After extensive washing to remove unbound bacteria, cells were fixed with 3.7% (v/v) formaldehyde for 20 min, blocked with D-PBS containing 3% (w/v) BSA for 15 min and incubated with rabbit anti-E. coli polyclonal antibodies diluted in D-PBS with 1% (w/v) BSA (1:500) for 1 h. After washes, samples were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:500) (Molecular Probes, Eugene, OR) for 1 h. After three washes with D-PBS, fluorescence was measured at excitation/emission 485/535 nm by Tecan Infinite F200PRO microplate reader. Adhesion assays were performed 3 times on different days; statistical analyses were performed using unpaired t test (for B. pertussis OMV adhesion) and one-way ANOVA with Dunnett’s multiple comparison test (for E. coli adhesion).

**Proteomic Study of B. pertussis OMV Detects Novel Antigens**—For each of three biological replicates of OMV purified from Bvg+ and Bvg− strains, three LC-MS/MS acquisitions were performed (technical replicates). Because the Bvg regulon is intrinsically very sensitive to little environmental changes (such as culture media composition and temperature) and given that we are not using controlled growth settings like fermentors, we decided to analyze each biological replicate independently.

The percentage of each protein in the total sample was calculated for each biological replicate according to the corresponding peak area (averaged among the three technical replicates) and the theoretical molecular weight (MW) using the following formula:

\[
\% \text{Protein}_X = \frac{\text{AvgAreaProtein}_X \times \text{MWProtein}_X}{\sum (\text{AvgAreaProtein}_X \times \text{MWProtein}_X)}
\]

Then, the fold change of each protein amount in Bvg+ versus Bvg− was calculated. Finally, we set 2 parameters to define Bvg+ phase specificity: a protein is considered specific to Bvg+ OMV if its amount is at least 4-fold higher in Bvg+ versus Bvg− and if it represents less than 0.5% of the total amount of proteins in Bvg− OMV.

**Proteomic Data Analysis**—The mass spectrometric raw data were processed with the PEAKS software ver. 8 (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) for de novo sequencing, database matching identification and label free quantification. Raw mass spectrometry data were deposited in the publically accessible repository MassIVE (Project number: MSV000081702; Proteome Exchange PXD008179). Peptide scoring for identification was based on a database search with an initial allowed mass deviation of the precursor ion of up to 15 ppm. The allowed fragment mass deviation was 0.05 Da. Protein identification from MS/MS spectra was performed against B. pertussis Tohama I NCBI protein database (3,425 protein entries, 25,203 sequences), database matching identification and label free quantification. Raw mass spectrometry data were deposited in the publicly accessible repository MassIVE (Project number: MSV000081702; Proteome Exchange PXD008179). Peptide scoring for identification was based on a database search with an initial allowed mass deviation of the precursor ion of up to 15 ppm. The allowed fragment mass deviation was 0.05 Da. Protein identification from MS/MS spectra was performed against B. pertussis Tohama I NCBI protein database (3,425 protein entries, release date: November 6, 2001) combined with common contaminants (human keratins and autoproteolytic fragments of trypsin) with a False discovery rate (FDR) set at 0.1%. FDR is defined as the ratio between the false peptide-spectrum match (PSMs) and the total number of PSMs above the score threshold. PEAKS software employs the decoy fusion method and concatenate the decoy and target sequences of the same protein together as a “fused” sequence (21). Enzyme specificity was set as C-terminal to Arg and Lys, without allowing cleavage at proline bonds and a maximum of four missed cleavages. N-terminal pyroGlu, Met oxidation and Gln/Asn deamidation were set as variable modifications. No fixed modifications were set for the protein search. Tryptic digestion from rabbit phosphoarylase B (Waters) was used as internal standard for label free quantification (2 pmol/μl) using a mass tolerance of 20 ppm, a retention time shift tolerance of 2 min, minimum 3 different peptides with a FDR set at 0.1%.
Bioinformatics—PSORTb version 3.0.2 was used for the prediction of protein cellular compartment (http://www.psort.org/psortb/) (22). Further refinement was performed for lipoprotein annotation that were sorted from unknown identifications using the precompiled genome annotation by DOLOP (http://www.mrc-lmb.cam.ac.uk/genomes/dolop/) (23).

Flow Cytometry—E. coli strains were grown for 16 h in liquid culture. Bacteria were then pelleted and washed with d-PBS at 8000 × g for 5 min. Bacteria were then blocked with d-PBS containing 3% (w/v) BSA for 15 min and incubated with mouse anti-OMV serum diluted in d-PBS with 1% (w/v) BSA (1:500) for 1 h. After washes, samples were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500) (Molecular Probes) for 30 min. Finally, bacteria were fixed with 3.7% (w/v) formaldehyde for 20 min and flow cytometry analysis was performed using FACS Canto II flow cytometer (BD Biosciences, San Jose, CA).

Recombinant Antigen Production—The gene fragment encoding BrkA, corresponding to protein residues 43–726, was amplified by PCR from B. pertussis W28 9K/129G genomic DNA. The PCR fragment was cloned into the pET15-TEV vector, a modified version of the pET15 vector (Novagen, Merck, Darmstadt, Germany), constructed to express N-terminal His-tagged (TEV cleavable) proteins by replacing the multiple cloning site of pET15 with a His-TEV-ccdB-chloramphenicol cassette amplified from the SpeedET vector (24). Protein expression was performed in E. coli BL21 (DE3) cells, by using EnPresso B growth systems (Biosilta, Oulu, Finland) supplemented with 100 μg/ml ampicillin. Bacteria were grown at 30 °C for 12 h, and recombinant protein expression was then induced by the addition of 1 mm isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for additional 24 h. Proteins were extracted from the insoluble fraction with 6 M of guanidinium chloride and then purified by immobilized metal ion affinity chromatography (IMAC) using HITRAP in 8 M urea, 100 mM NaH2PO4 (pH 8) 10 mM Tris HCl (pH 8), 500 mM imidazole (GE Healthcare Life Sciences) and refolded by multistep dialysis in 50 mM NaH2PO4 (pH 7.5), 300 mM NaCl, 1% (w/v) glycerol.

Mouse Aerosol Challenge Model—BALB/c mice (10 female/group, 6-week old) (Charles River Laboratories International Inc.) received three intraperitoneal immunizations, with a 4 weeks interval, with aluminum hydroxide adjuvanted recombinant BrkA (10 μg per dose) or with adjuvant only. A bacterial suspension of BP536 from a 24-hour culture was generated at 2 × 108 CFU/ml in d-PBS with 1% (w/v) casein salts (Sigma). BrkA-vaccinated and control mice were placed in a closed chamber in which the bacterial suspension was administered as an aerosol for 10 min using a nebulizer device with an administration rate of 0.5 ml/min. B. pertussis infection was monitored by performing colony forming units (CFU) counts on lungs from groups of 10 mice 7 days after aerosol challenge. Lungs were aseptically removed and homogenized in 2 ml of sterile physiological saline buffer with 1% (w/v) casein salts on ice. Ten microfilters of undiluted homogenate or of serially diluted homogenates were spotted in triplicate onto Bordet-Gengou agar plates, and the number of CFU was estimated after 3 days of incubation at 37 °C. Statistical analysis was performed using unpaired t test (Mann-Whitney test).

RESULTS

OMV Purification, Visualization, Enumeration and Sizing—To identify possible Bvg-regulated outer membrane adhesins, we compared the protein composition of OMV from two B. pertussis strains, the Tohama I-derivatives BP536 and BP537 representative of the Bvg+ and Bvg− phases, respectively. OMV were purified using a detergent-free method from cell-free culture supernatants through ultracentrifugation. Equal amounts with respect to total protein quantities from both preparations were loaded onto SDS-PAGE gel (Fig. 1A) showing significant differences between the two samples representing Bvg+ and Bvg− phases. Several high molecular weight protein bands appear to be exclusive to Bvg+ OMV, as well as two major bands with apparent molecular weight of 42 and 32 kDa, whereas one intense protein band of apparent molecular weight around 40 kDa seems to be majorly expressed in Bvg− OMV. The 40 kDa protein band was excised, destained and digested with trypsin for peptide mass fingerprinting (PMF) identification: outer membrane porin protein BP0840 was identified with a sequence coverage of 27% (supplemental Fig. S1). To evaluate the actual number and the size of vesicles we used nanoparticle tracking analysis (NTA), a method for direct, real-time visualization of nanoparticles in liquids through the tracking of the Brownian movement of individual particles, as previously reported (25). OMV samples from BP536 and BP537, normalized to the same protein con-
performed in triplicates. Results represent mean ± S.D. of one representative of three independent experiments each performed in triplicates. *** = p < 0.001. B, Sera from 10 mice immunized with either OMV, whole bacteria or aluminum hydroxide as control were pooled, serially diluted in infection medium and incubated with labeled wild-type *B. pertussis* BP536 for 1 h. A549 cells were then infected with the bacteria/sera mixtures for 1 h and, after extensive washes to remove unbound bacteria, cell-associated bacteria were quantified by fluorescence reading at Ex/Em 485/535 nm. Results represent mean ± S.D. of one representative of three independent experiments each performed in triplicates.

Fig. 2. **Adhesiveness of *B. pertussis* OMV and impact of anti-OMV serum on *B. pertussis* adhesion to A549 cells:** A, Cells were incubated with OMV (10 ng/μl) for 6 h and, after extensive washes to remove unbound vesicles, cells were fixed and stained with mouse anti-OMV primary antibody and with AlexaFluor488-conjugated anti-mouse secondary antibody. Cell-associated OMV were quantified by fluorescence reading at Ex/Em 485/535 nm. Results represent mean ± S.D. of one representative of three independent experiments each performed in triplicates, **” = p < 0.001.** B, Sera from 10 mice immunized with either OMV, whole bacteria or aluminum hydroxide as control were pooled, serially diluted in infection medium and incubated with labeled wild-type *B. pertussis* BP536 for 1 h. A549 cells were then infected with the bacteria/sera mixtures for 1 h and, after extensive washes to remove unbound bacteria, cell-associated bacteria were quantified by fluorescence reading at Ex/Em 485/535 nm. Results represent mean ± S.D. of one representative of three independent experiments each performed in triplicates.

**Adhesion of *B. pertussis* OMV to Human Respiratory Cells and Inhibition of *B. pertussis* Adhesion with Anti-OMV Serum—** To validate the employment of Bvg+ OMV as a source of adhesins to be selected as potential vaccine candidates, we checked whether Bvg+ OMV were enriched with adhesive molecules in comparison to Bvg– OMV. The two different OMV samples were added to seeded A549 respiratory epithelial cells and their ability to adhere was measured by immuno-fluorescence using mouse anti-OMV serum: we found that only Bvg+ OMV were able to bind to A549 cells (Fig. 2A). This result prompted us to determine whether antibodies raised against antigens present in Bvg+ OMV could have anti-adhesive properties on *B. pertussis*. We tested the ability of anti-OMV sera to inhibit adhesion of BP536 to A549 respiratory epithelial cells. Fluorescent bacteria were pre-incubated with mouse pooled sera in a range of four dilutions and A549 cells were infected as described previously (26). Mouse anti-sera were raised to Bvg+ OMV and whole bacteria, adsorbed to aluminum hydroxide and collected after the third immunization together with control sera from mice immunized with adjuvant only. We found that anti-OMV serum conferred a substantial reduction of adhering bacteria even at low serum concentrations. Interestingly sera raised against whole bacteria, therefore targeting the entire membrane antigen repertoire, showed a comparable kinetic of inhibition. Anti-alum serum was included in the experiments as negative control and, as expected, did not induce any inhibition of *B. pertussis* adhesion (Fig. 2B).

**Comparative Proteomic Analysis—**OMV samples from BP536 and BP537 were digested by trypsin after TCA precipitation. Proteomic analysis was performed by LC-MS/MS using a DDA approach. A total of 247, 155, and 372 proteins were quantified in the first, second and third biological replicate, respectively (supplemental Table S1). This variability was expected considering the high sensitivity of the Bvg regulon to slight environmental changes, as also proven in previous reports (27, 28). Therefore, each biological replicate was analyzed independently to identify the differentially expressed proteins in the Bvg+ versus Bvg– phase. Even if some proteins were identified exclusively in the Bvg+ phase, we set a threshold to determine the Bvg+ specificity: proteins showing a 4-fold increase in Bvg+ versus Bvg– phase and representing less than 0.5% of the total amount of proteins in Bvg– OMV were considered Bvg+ specific. Despite the mentioned differences in the absolute quantity of each protein in each biological replicate, ~64% of the total protein amount in Bvg+ OMV appeared to be Bvg+ specific (Fig. 3A) and represented less than 0.6% of the total protein amount of Bvg– OMV. The localization of the quantified proteins was predicted according to PSORTb software and refined for lipoprotein annotation using DOLOP software. The analysis predicted ~90 and ~80% of the total protein amount to be either outer membrane proteins, lipoproteins, extracellular or periplasmic...
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![Diagram A](image1.png)

**Fig. 3.** Comparative proteomic analysis of *B. pertussis* OMV: protein quantification through LC-MS/MS and prediction of their subcellular localization. A, Bvg+ specific proteins amount: Bvg+ specific proteins (green) were identified as proteins showing a 4-fold amount increase in Bvg+ versus Bvg− OMV and representing less than 0.5% of the total amount of Bvg− OMV. B, Distribution of quantified proteins in the distinct subcellular localizations based on prediction with PSORTb and DOLOP software: Outer Membrane (OM), Periplasm (P), Lipoprotein (L), Inner Membrane (IM), Cytoplasm (C), Extracellular (Ex), Unknown (U). R1 = biological replicate 1; R2 = biological replicate 2; R3 = biological replicate 3.

proteins, in Bvg+ and Bvg− OMV respectively and in all three biological replicates (Fig. 3B). Around 10% of the total protein amount had an unknown prediction of localization in both OMV samples but only a minority of proteins was predicted to be inner membrane or cytoplasmic, thus confirming that vesicles are generated by outer membrane blebbing and not by bacterial lysis.

**Vaccine Antigen Selection**—We applied three main criteria for antigen selection: putative antigens should be Bvg+ phase specific (i.e. showing at least a 4-fold increase as compared with Bvg−), they should have a predicted outer membrane localization according to PSORTb and they should represent at least 1% of the total amount of proteins present in Bvg+ OMV. These criteria must be true in at least 2 out of the three biological replicates. The list of the resulting selected antigens is reported in Table I. Excluding the proteins already part of the currently available aP formulation, we focused on six promising candidates to be assessed for their adhesive properties and vaccine potential: BrkA, Vag8, TcfA, SphB1, BipA, and BfrD. Interestingly, five of the selected antigens had a predicted autotransporter structure and they were identified with high amino acid sequence coverage in both the passenger domain and the β-barrel translocator domain, indicating that both domains are associated to OMV (supplemental Fig. S2).

**Recombinant *E. coli* Strains Adhesion and Adhesion Inhibition Assays**—In order to evaluate the adhesive properties of each single protein, we exploited *E. coli* as an heterologous background system for surface exposure of the selected antigens. We generated *E. coli* strains constitutively expressing the selected full-length proteins and checked for their ability to bind to A549 respiratory epithelial cells as compared with wild type *E. coli*. A549 cells were infected with *E. coli* strains for three hours, then washed to remove unbound bacteria, fixed and stained with an anti-*E. coli* antibody. We found that BrkA, SphB1, Vag8 and TcfA conferred adhesive ability to *E. coli* when expressed on the membrane. On the contrary, heterologous expression of BipA and BfrD had no effect on strain adhesiveness (Fig. 4A). We continued the analysis by testing if the *E. coli* strains singularly expressing the selected antigens were detected with the anti-OMV serum we had generated. By flow cytometry, anti-OMV serum was able to recognize the specific heterologously expressed BrkA, Vag8 and BipA (Fig. 4B). This result showed that not only BrkA, Vag8 and BipA antibodies had been elicited after mice immunization with *B. pertussis* Bvg+ OMV, but also that these proteins are properly expressed and surface-exposed on *E. coli*. Finally, to determine whether the inhibition of *B. pertussis* adhesion promoted by anti-OMV serum correlated with the presence of specific antibodies against the selected antigens we tested its inhibitory effect on the adhesion of recombinant *E. coli* strains. We found that anti-OMV serum caused reduction of adhering *E. coli* at high serum concentration only when BrkA was expressed on the bacterial surface. Unexpectedly, Vag8-expressing *E. coli* was not inhibited despite the presence of specific anti-Vag8 antibodies in the serum (Fig. 4C).

**Recombinant BrkA Protein Production and Vaccination**—To analyze the protective potential of BrkA, recombinant Histagged protein was expressed, purified and used to immunize mice three times, 4 weeks apart. Fifteen days after the final vaccination, mice were challenged with aerosolized *B. pertussis* and 7 days later the bacterial load in the lungs was evaluated by CFU counting. This experiment showed that immunization with recombinant BrkA resulted in about 100
specific proteins were predicted to be outer membrane proteins of the total protein amount in Bvg identification of hundreds of proteins and showed that analysis between OMV from Bvg/H11001 antibodies after immunization. The comparative proteomic proteins present in Bvg/H11001 adhesion is likely determined by the additive effects of different epithelial cells. Clearly, the whole inhibitory effect on bacterial adhesion to the same cell line. Interestingly, Bvg + OMV, but not Bvg – OMV, were able to adhere to human respiratory cells, thus confirming their enrichment with Bvg-activated adhesins. Moreover, anti-OMV sera proved to be particularly powerful in preventing B. pertussis adhesion to respiratory epithelial cells. Clearly, the whole inhibitory effect on bacterial adhesion is likely determined by the additive effects of different antibodies targeting various antigens displayed by OMV and present on B. pertussis surface. We therefore decided to deepen the analysis at the single protein level, looking for proteins present in Bvg + OMV that could elicit anti-adhesive antibodies after immunization. The comparative proteomic analysis between OMV from Bvg + and Bvg – allowed the identification of hundreds of proteins and showed that ~64% of the total protein amount in Bvg + OMV are specific for that phase and their amount drops to ~0.6% in Bvg – OMV. This finding agrees with the well-established regulation of the virulence genes by the BvgAS two-component system in B. pertussis. Interestingly, almost the totality of the Bvg + specific proteins were predicted to be outer membrane proteins and among them we found the best known and characterized B. pertussis adhesins such as FHA, 69K and Fimbriae. Also, Pertussis Toxin was identified as Bvg+ specific whereas Adenylyl Cyclase Toxin and Dermoeneotic Toxin were not identified at all. On the other hand, there was a general increase in the amount of all the non Bvg+ specific proteins in Bvg – OMV and it is tempting to hypothesize that most of the proteins in Bvg – OMV are overrepresented to compensate for the lack of the Bvg+ outer membrane proteins. The outer membrane porin protein BP0840 perfectly supported this hypothesis, resulting to be the most abundant protein in Bvg – OMV in all the biological replicates. The NTA analysis further supported the hypothesis of a compensative mechanism, showing no differences in size distribution and quantity of proteins per vesicle in both OMV samples. The protein composition of OMV described in the present study differs substantially from what has been shown in a previous report (28). Although the high quantity of BrkA and Vag8 is confirmed in both studies, the overall composition in terms of presence and relative abundance is drastically different. This is likely because of the distinct OMV purification methods and B. pertussis strains employed in the two studies. The proteomic analysis presented in this study allowed the identification of an initial list of putative antigens, which were narrowed down to 8 interesting candidates. This antigen selection resulted in seven known B. pertussis protective antigens including two of the current aP antigens, thus validating our strategy to identify virulence factors. It is intriguing to underline that proteomic analysis of two currently circulating B. pertussis strains under in vitro Bvg+ and Bvg- conditions (29), combined to in silico prediction for surface expression, resulted in a panel of top 15 candidates which perfectly mirrors the OMV-based antigen selection described in our study. Then, albeit with variations in the strains and methods employed, the membrane composition of OMV seems to closely reflect the composition of the bacterial outer membrane. We decided to focus our attention on the most abundant Bvg+ outer membrane proteins and interestingly five autotransporter proteins were at the top of

| Accession | Protein name [Bordetella pertussis Tohama I] | Replicate 1 | Replicate 2 | Replicate 3 |
|-----------|--------------------------------------------|-------------|-------------|-------------|
| NP_882013.1 | BrkA autotransporterab | 12.96% | 10.69% | 19.21% |
| NP_880571.1 | FHA filamentous hemagglutinin/adhesina | 17.95% | 15.33% | 1.43% |
| NP_879839.1 | 69K pertactin autotransporterca | 5.44% | 11.48% | 10.68% |
| NP_879797.1 | TcaA tracheal colonization factorab | 4.26% | 3.28% | 6.95% |
| NP_879893.1 | BipA outer membrane ligand binding proteinab | 1.56% | 5.27% | 9.06% |
| NP_879974.1 | TcfA tracheal colonization factorab | 4.26% | 3.28% | 6.95% |
| NP_880571.1 | FHA filamentous hemagglutinin/adhesina | 17.95% | 15.33% | 1.43% |
| NP_882013.1 | BrkA autotransporterab | 12.96% | 10.69% | 19.21% |
| NP_880571.1 | FHA filamentous hemagglutinin/adhesina | 17.95% | 15.33% | 1.43% |
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| NP_879974.1 | TcfA tracheal colonization factorab | 4.26% | 3.28% | 6.95% |

a = known B. pertussis protective antigens.
b = antigen selected for further analysis.
c = antigen currently included in a commercial vaccine.

TABLE I
OMV-based antigen selection. Antigens were selected from the total proteins quantified in OMV based on the following criteria: Bvg regulation, localization prediction, and abundance. Percentages refer to the total protein composition of OMV from either Bvg+ or Bvg– phase in the three biological replicates.

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our list. Autotransporters are possibly the simplest bacterial secretion systems (type V): they consist only of a single poly-peptide chain organized in a passenger and translocator domain and they can autonomously translocate across the outer membrane (30). Therefore, the heterologous expression of the autotransporters on the surface of *E. coli* was selected to characterize their adhesive properties. This approach allowed the demonstration that BrkA, TcfA, SphB1 and Vag8 conferred to *E. coli* a significant increased ability to adhere to lung epithelial cells. To the best of our knowledge, this is the first time that *B. pertussis* autotransporters were rationally characterized for their adhesive properties exploiting a heterologous *E. coli* background. The involvement in adhesion of BrkA was previously demonstrated in *in vitro* analysis and in mouse infection studies with *B. pertussis* mutant strains showing 2-fold and 2-log reduction in the ability to adhere to cell lines and to colonize the murine lungs, respectively (31, 32). Also, the loss of TcfA was previously shown to cause 10-fold reduction in the number of bacteria isolated from tracheas after *B. pertussis* aerosol challenge (33). Nevertheless, deletion of individual virulence factor genes could have limited effects on the ability of *B. pertussis* to efficiently infect the respiratory tract of mice, suggesting they may perform redundant functions. This was the case of a Vag8 deletion mutant, which was
as efficient as the parental *B. pertussis* strain in colonizing murine lungs (34). On the contrary, our study showed that Vag8 is also involved in adhesion and not only in serum resistance as previously described (35). In other cases, the generation of single knock-outs could be misleading. In fact, SphB1 loss has an indirect effect on Filamentous Hemagglutinin which is not cleaved anymore from *B. pertussis* surface therefore rendering the mutant strain even more adhesive than the wild-type parental strain (36) but in this study, we showed that SphB1 itself can contribute to the overall adhesiveness of *B. pertussis*. Two of the heterologously expressed antigens, BipA and BfrD, did not confer an adhesive phenotype to *E. coli*. Although this result could be expected for BfrD, which is reported to be involved in iron acquisition (37), it was quite controversial for BipA, which is partially characterized in the literature as the major adhesin in the intermediate phase between Bvg+ and Bvg− (38). This virulence phase (Bvg(i)) was described to be involved in the transmission of *B. pertussis* from host to host and a BipA deletion mutant of a Bvg(i)-locked *B. pertussis* strain displayed a reduced ability to colonize the nasal cavity of mice compared with the parental strain (39). BipA was also identified as the most abundant surface-associated biofilm protein (40) therefore it is likely involved in bacterium-bacterium interactions as well as host-pathogen interactions. Our results were in contrast with the previous reports but, in the absence of specific antibodies, we could not rule out that BipA is not properly expressed and surface exposed on *E. coli*. We therefore took advantage of sera raised against OMV to explore whether the heterologously expressed antigens were recognized on the surface of *E. coli*. Only three proteins, BrkA, Vag8 and BipA, were specifically recognized on *E. coli* surface by anti-OMV serum, thus excluding the possibility that BipA was not properly surface-exposed on the outer membrane. Finally, we checked the ability of anti-OMV serum to inhibit recombinant *E. coli* adhesion to lung A549 cells. The inhibitory effect of anti-OMV serum was only observed on the adhesion of BrkA-expressing *E. coli*, demonstrating the functionality of anti-BrkA antibodies. Consistently with the fact that SphB1 and TcfA-expressing *E. coli* strains were not recognized by anti-OMV serum, no inhibition was observed on the adhesiveness of these strains. Unexpectedly, anti-OMV serum was not able to inhibit Vag8-expressing *E. coli* strain; whether this is because of low antibody level or to weak antibody functionality needs further clarification. As far as BfrD is concerned, the protein did not result to have a contribution in adhesion nor to be recognized by anti-OMV serum on *E. coli* surface, therefore was excluded from the adhesion inhibition assay. Still, given its predicted structure of integral outer membrane protein, we cannot exclude that it is not properly expressed on the surface of *E. coli*. The FACS analysis using anti-OMV serum on *E. coli* strains also gave us an indirect result on the ability of the selected antigens to be immunogenic in the context of OMV. Immunization of mice with OMV results in a wide antibody repertoire somehow reflecting what happens during a natural infection with *B. pertussis* or during a vaccination with wP. Clearly, immunogenicity does not necessarily correlate with protein abundance and, in the complexity of the OMV lipid bilayer, some abundant proteins might not induce that high antibody response, whereas some less abundant proteins might be immunodominant and induce higher responses. Although the presence of anti-BrkA, anti-Vag8 and anti-BipA antibodies correlated with high abundance of these proteins on OMV, the absence of anti-SphB1 and anti-TcfA antibodies was unexpected given the high quantity of these proteins on vesicles. Nevertheless, immunization of mice with recombinant proteins could have a completely different outcome and elicit specific anti-adhesive antibodies, as previously demonstrated. Indeed, the protective role of autotransporters is a peculiar trait of *B. pertussis* and thus far, five autotransporters have been shown to confer protection in the mouse model (either aerosol or intranasal challenge) when expressed as recombinant proteins, including Prn (41), TcfA (42), SphB1 (29), Vag8 (29), and BipA (40) suggesting that *B. pertussis* evolved this class of proteins as virulence strategy to colonize the host. Here, we demonstrated that immunization with recombinant BrkA resulted in significant protection against lower respiratory tract infection of *B. pertussis* in the mouse aerosol challenge model. This was different with respect to what previously reported using the mouse intranasal challenge model, where BrkA resulted as an added value only when formulated with aP and not as stand-alone vaccine (43); however, the two studies differ both in the immunization route and in the bacterial infection administration. Finally, we contributed to unravel the mechanism of protection induced by anti-BrkA antibodies showing the inhibitory properties against the bacterial adhesion. In conclusion,
BrkA proved to be a promising candidate antigen to improve existing aP vaccines for use in humans. Whether such multidomain aP vaccines that include BrkA and possibly further virulence factors of *B. pertussis* other than pertussis toxin, filamentous hemagglutinin, pertactin and fimbriae have superior efficacy over existing aP vaccines remains to be determined.

Taken together, these results suggest that spontaneously released OMV from *B. pertussis* provide a potential source of protective antigens able to specifically target the colonization step. The inclusion of BrkA and possibly further adhesins may provide a higher protection to current aP vaccines answering the need of improving the impact of vaccination on the bacterial clearance.

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DATA AVAILABILITY

Raw mass spectrometry data were deposited in the publicly accessible repository MassIVE (Project number: MSV000081702; Proteome Exchange PXD008179).

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