Comprehensive Antigen Screening Identifies \textit{Moraxella catarrhalis} Proteins That Induce Protection in a Mouse Pulmonary Clearance Model

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Abstract

\textit{Moraxella catarrhalis} is one of the three most common causative bacterial pathogens of otitis media, however no effective vaccine against \textit{M. catarrhalis} has been developed so far. To identify \textit{M. catarrhalis} vaccine candidate antigens, we used carefully selected sera from children with otitis media and healthy individuals to screen small-fragment genomic libraries that are expressed to display frame-selected peptides on a bacterial cell surface. This ANTIGENome technology led to the identification of 214 antigens, 23 of which were selected by \textit{in vitro} or \textit{in vivo} studies for additional characterization. Eight of the 23 candidates were tested in a \textit{Moraxella} mouse pulmonary clearance model, and 3 of these antigens induced significantly faster bacterial clearance compared to adjuvant or to the previously characterized antigen OmpCD. The most significant protection data were obtained with the antigen MCR\_1416 (Msp22), which was further investigated for its biological function by \textit{in vitro} studies suggesting that Msp22 is a heme binding protein. This study comprises one of the most exhaustive studies to identify potential vaccine candidate antigens against the bacterial pathogen \textit{M. catarrhalis}.

Introduction

\textit{Moraxella catarrhalis} is a gram-negative aerobic diplococcus and an exclusive human respiratory pathogen that for a long time used to be considered a purely human commensal [1]. However, \textit{M. catarrhalis} is the third most frequent bacterial pathogen causing otitis media disease in children (after \textit{Streptococcus pneumoniae} and non-typeable \textit{Haemophilus influenzae} (NTHI)), and is a major cause of exacerbations in adults with chronic obstructive pulmonary disease (COPD) [2]. Further, between 50–85\% of all children experience at least one acute otitis media (AOM) episode before 3 years of age [3,4], and the disease is associated with high costs. In addition, chronic and frequent recurrent AOM can lead to delayed speech development and language learning, due to hearing impairment. Moreover, complications including mastoiditis, and in rare cases even meningitis, may develop as a result of such middle ear infections [5,6].

Since otitis media (OM) is a polymicrobial disease, an effective vaccine will have to protect against the 3 main bacterial causative agents of OM, including \textit{M. catarrhalis}, and several vaccine related studies have already been performed to identify potential single vaccine candidates. These include various outer membrane proteins (OMPs) and lipooligosaccharide [7,8]. Others have used a genome-wide data mining approach to identify novel antigens [9]. Of the putative antigens so far identified, the ubiquitous surface proteins A (UspA1, UspA2, and UspA2B) [10,11], involved in adherence [12] and serum resistance [13], have been shown to provide some protection in animal models using active vaccination or passive immunization strategies. Other potential candidates include the IgD-binding protein Hag/MID, a human epithelial cell adhesin and B cell mitogen, [14], and it has been reported that a monoclonal antibody specific for the outer membrane protein CopB, an iron-regulated protein involved in iron uptake from transferrin and lactoferrin, enhanced pulmonary clearance of \textit{M. catarrhalis} in a mouse model [15]. Finally, the porin OmpCD, an adhesin, was reported to enhance pulmonary clearance upon immunization [16], and at the time that this research project began, appeared to be the most appropriate
potential vaccine candidate to act as a positive control in in vivo immunization experiments.

The ANTIGENome technology offers another approach in the search for vaccine candidates and has been successfully applied to identify novel protective antigens from several other bacterial pathogens [17,18,19]. The technology generates many thousands of potential peptide antigen candidates that are then screened using magnetic-activated cell sorting (MACS) methods against well characterized human sera to identify novel protein vaccine candidates.

We have applied this technology and selected 214 protein candidates, among them the previously described protective proteins, UspA, Hag, CopB and OmpCD. Further validation by in vitro assays and finally in a murine model of Moraxella pulmonary clearance identified three proteins from M. catarrhalis as novel protective vaccine candidates. The functional characterization of one of these candidates, the surface protein Msp22, in Moraxella showed that it possesses heme-dependent peroxidase activity.

Materials and Methods

Ethical statement

All human serum samples used for these studies were collected according to the general national ethical guidelines and upon consent from individual subjects. Sera from healthy individuals were collected for this and similar studies by Intercell with written consent given by each individual specifically for this study. Collection of sera at the Erasmus University Medical Center (Rotterdam) was approved specifically for this study by the medical ethical Committee of the Erasmus MC (MEC-2-12-176) with patient consent given (or informed consent by parents or guardians in case of children). Human sera were also collected at the Semmelweis University as approved specifically for this study by the ethical committee of Semmelweis University. All animal experiments were approved by Stockholm's Norra djurforsokstämman and were conducted in agreement with the European Communities Council Directive 86/609/EEC and the Swedish animal protection legislation. Mice were scored and sacrificed according to the obtained ethical permission.

Bacterial strains and growth conditions

M. catarrhalis strain RH4 was originally isolated from the blood of an infected patient [20] and strain BBH18 was from the sputum of a COPD patient during an exacerbation [21]. Both strains were obtained from Arne Forsgren and Kristian Riesbeck (Malmo University Hospital, Sweden). Bacteria were grown in brain heart infusion (BHI) broth at 37°C with shaking (180 rpm) or on Columbia agar supplemented with 5% sheep blood (Biome´rieux, Austria) or horse blood at 37°C.

Additional M. catarrhalis strains and clinical isolates (strains from various clinical samples (ear, sinus, nasal cavity and middle ear punctuate samples) were obtained from the Pediatric department at Semmelweis University (Budapest, Hungary), Erasmus University Medical Center (Rotterdam, The Netherlands) or were commercially acquired from GR Micro (London, UK). The following strains (Origin and IDs listed) from GR Micro isolated from patients with acute otitis media were used for the gene conservation studies: Australia (1090122, 1090127, 1091216), Belgium (1510233, 1041218, 3041116, 3041117), Canada (1022133, 1022357, 2022135), France (1502130, 3502122, 3530129), Germany (3517132, 3518116, 3519121, 3522120), Hungary (3650122, 3650134), Italy (1530120, 2530126, 3530121), Japan (2081130, 2085119, 3079119), Portugal (2560117), South Africa (3681122), South Korea (1070122, 1071124, 2070120), Spain (2554135, 3552130, 3553117), Sweden (3590123, 3590127, 3590135), Taiwan (3696117, 3696119, 3696126), Turkey (2660116, 2660119, 2660122), United States (1001118, 1001207, 1009124, 1009125).

E. coli cells were grown in LB broth at 37°C with shaking or on LB plates containing appropriate antibiotics (kanamycin and/or ampicillin).

For human sera adsorption, E. coli (DH5α transformed with pHIE11/pMAL9.1 [22]) cells were grown to exponential phase and induced with 0.1 mM IPTG. The bacteria were harvested after one hour and washed three times with ice cold 2x PBS. Prior to addition to serum samples, the pellet was re-suspended in PBS (100 μL per 100 mL culture).

Selection of human sera for library screening

A comprehensive collection of serum samples was obtained from the Department of Pediatrics, Semmelweis University (Budapest), the Erasmus University Medical Center (Rotterdam), and from Intercell AG (Vienna, Austria). In addition to the sera from otitis media patients, sera from healthy individuals or from patients in other disease groups (asthmatic patients, allergy) were also included in the studies, serving as relevant controls. All sera were aliquoted and stored at ~80°C prior to use.

For the preparation of serum pools, all human sera were analyzed by ELISA and Western blot with M. catarrhalis cell lysates. The sera containing high titer of antibodies and showing a diversity of bands in the Western blot were selected to create serum pools of 5 individual sera per pool.

ELISA on bacterial cell lysates and recombinant proteins

M. catarrhalis cells were grown in liquid medium at 37°C, 5% CO2 until a late log phase was reached. Cells were harvested by centrifugation (1,000 g, 10 min, 4°C) and washed twice with PBS. Bacteria were re-suspended in PBS containing protease inhibitors and then lysed on ice by sonication (2 min, pulse 5, 100% power) and the supernatant was collected by centrifugation. ELISA plates (96F Cert, Maxisorb Nunc-Immuno plate, 439454, Denmark) were coated with either bacterial lysates or recombinant proteins and human serum samples were tested at 3-fold dilutions from 1:50 to 1:36,450. Highly specific Horse Radish Peroxidase (HRP)-conjugated goat anti-human IgG (Southern Biotech, 2040-05, USA) was used for signal detection.

Peptide ELISA

N-terminally biotin-labeled peptides were coated onto streptavidin ELISA plates (Nunc, Denmark) at 5 μg/mL (in a 100 μL volume) and incubated overnight at 4°C. Sera were tested in duplicate at a 1:1000 dilution. Horse Radish Peroxidase (HRP)-conjugated anti-human IgG antibodies (Southern Biotech) were used according to the manufacturer’s recommendation (1:1000 dilution). ABTS was used as a substrate for HRP and the absorbance read at 405 nm.

Preparation of IgGs from human serum pools

Prior to library screening, human serum pools were adsorbed against E. coli (DH5α transformed with pHIE11/pMAL9.1) cells in order to reduce background. The cell suspension was added to the serum pools (150 μL cell suspension per 800 μL serum) and rotated overnight at 4°C. The next day, the mixture was centrifuged and the supernatants were transferred into a clean tube. The whole procedure was carried out three times for each single serum pool.
The E. coli adsorbed human sera were heat-inactivated at 56°C for 45 min and centrifuged to remove precipitated proteins. The supernatant was filtered using a 0.22 μm syringe filter (Costar, USA) and IgGs were purified and biotinylated with the reagents provided by Pierce Biotechnology (USA), as previously described [17], and subsequently used for library screening.

Construction of bacterial surface display libraries

Bacterial surface display libraries were generated as previously described [17,22]. Briefly, genomic DNA from M. catarrhalis BBH18 was fragmented by DNase I digest (LambD library, DNase Shotgun Cleavage Kit (Novagen, USA)) or sonication (FnuA library, Sonopuls Ultrasonic Homogenizer HD2200 (Bandelin, Germany)). Blunted DNA fragments of 50–200 bp or 150–600 bp were ligated with the >5ul digested frame-selection vector pMAL431. pMAL431 containing 50–150 bp or 150–600 bp DNA fragments from M. catarrhalis was transformed into DH10B electrocompetent E. coli cells (Invitrogen, USA). Plasmid DNA was isolated from the pool of transformed clones, and the DNA inserts cloned into the platform vectors pMAL4.1 (FnuA, 150–600 bp) and pHIE14 (LambD, 50–150 bp) for surface display.

MACS screening

MACS (magnetic-activated cell sorting) screening using bacterial surface display libraries was performed as described previously [17,22].

Cloning, expression and purification of recombinant M. catarrhalis proteins in E. coli

For recombinant expression of M. catarrhalis antigens, the PCR amplified gene or gene fragments to be expressed were cloned into pET28b+, a vector containing a kanamycin resistance cassette as well as a T7-RNA polymerase promoter. All proteins were expressed with N- or C-terminal His-tags without possible signal peptides. Protein expression was analyzed in small scale (2 mL) centrifugation of lysed bacterial cultures and analysis of soluble (supernatant) and insoluble (pellet) fractions. Western blot with anti-His-tag antibodies was performed to confirm the expression of the recombinant protein. Proteins were purified from 2 L DH10B cultures carrying the pET28b+ (+) vector containing a kanamycin resistance cassette as well as a T7-RNA polymerase promoter. All proteins were expressed with N- or C-terminal His-tags without possible signal peptides. Protein expression was analyzed in small scale (2 mL) centrifugation of lysed bacterial cultures and analysis of soluble (supernatant) and insoluble (pellet) fractions. Western blot with anti-His-tag antibodies was performed to confirm the expression of the recombinant protein. Proteins were purified from 2 L DH10B cultures carrying the pET28b+ (+) vector containing a kanamycin resistance cassette as well as a T7-RNA polymerase promoter. All proteins were expressed with N- or C-terminal His-tags without possible signal peptides. Protein expression was analyzed in small scale (2 mL)

Preparation of outer membrane vesicles from M. catarrhalis

Cells from a 1.5 L culture (without or with 2μM Desferal) were harvested (4,500 rpm, 4°C, 60 min) and washed with PBS. They were resuspended in 50 mL EDTA buffer (0.05 M Na2HPO4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4) and incubated at 56°C for 30 min at 75 rpm agitation with glass beads (1.7–2 mm). The culture was centrifuged (3,500 rpm, room temperature, 15 min) twice, and the supernatant containing the membrane vesicles was ultracentrifuged (40,000 rpm, 4°C, 90 min). The pellet was washed with PBS (40,000 rpm, 4°C, 90 min).

Finally, the pellet was re-suspended in 500 μL PBS.

Generation of mouse immune serum against M. catarrhalis recombinant protein Msp22

Msp22 with a His-tag at the C-terminus and expressed without lipidation in E. coli was purified using IMAC columns and utilized for the generation of Msp22-specific immune serum in mice. Female NMRI mice 6–8 weeks of age were bled by tail vein puncture to generate pre-immune sera, and were immunized three times intraperitoneally with 50 μg recombinant antigen per immunization, using Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) as adjuvant. Terminal bleeds were collected via the orbital sinus. Sera were heat-inactivated at 56°C for 30 minutes.

Immunization and challenge of mice

Five to seven-week-old female C57BL/6J mice were kept under specific pathogen-free conditions in a standardized 12 hours light/dark cycle and received commercial food and water *ad libitum*. Before immunization on Day 0, 10 μL of blood was withdrawn from each mouse to prepare pre-immune serum samples. On days 0, 21 and 42, intranasal immunization of groups of 10 mice as controls with PBS or Intercell's proprietary adjuvant IC31® [23] and with the respective adjuvanted proteins was performed as follows: 17.5 μL protein solution was mixed with 2.5 μL IC31® (2000 nmol/mL KLK; 80 nmol/mL ODN1a), incubated for 30 minutes at room temperature and used to immunize mice within one hour of preparation. Adjuvant control mice received 17.5 μL 50 mM Tris/HCl pH 8.0 mixed with 2.5 μL IC31®. Immune sera were obtained on Day 63 (3 weeks after the last boost) and frozen at −20°C for storage.

Twenty-one days after the last boost, mice were infected intranasally with 40 μL (20 μL per nostril) live *M. catarrhalis* strain RH4, equaling approximately 5 × 10⁶ CFU. For mouse inoculation, *M. catarrhalis* strain RH4 was grown in BHI broth to an OD₅₆₀ of 0.4. Bacteria were pelleted and re-suspended in PBS. Mice were held in a head-up vertical position during the inoculation and kept in that position for at least 10 seconds after the inoculation.

Euthanasia, tissue collection and bacterial culture

Mice were euthanized at 6 hours post-infection. Both lungs were removed, placed in 1 mL PBS plus protease inhibitor (Roche, Germany), homogenized using cell strainers (100 μm, Becton Dickinson and Company, USA) and used for serial plating to quantify viable bacteria.

For the evaluation of bacterial clearance due to immunization with recombinant proteins, several independent experiments were performed and the CFU in the lungs of the mice were normalized to an infectious dose of approximately 5 × 10⁶ CFU bacteria (actual
Preparation of M. catarrhalis lysates

M. catarrhalis RH1 or BBH18 lysates were prepared from cultures grown in BHI broth. The cells were harvested, washed and re-suspended in PBS, then sonicated on ice using 2 × 30 second bursts. The protein concentration was measured using BCA protein assay reagent (Pierce Biotechnology).

Generation of the msp22 gene deletion mutant

The M. catarrhalis gene deletion mutant msp22Δ was generated by amplifying a ~500 bp region up- and downstream of the msp22 gene from genomic DNA using the following oligonucleotide primers: 8666−5’TGTATTTGCGGTAGATGTG-3’; 8667−5’CCACTAAGTTAGACCGGAGTGGTCTATTGGCATAAG-3’; 8668−5’GGGCTAAATCTGGCGAGGTATGTGAAACATGAGCAGCAGCTAAG-3’; 8669−5’GATGCCATGACATCACTACCAATCTT-3’. The flanking regions of the genes were ligated by overlap-extension PCR with a spectinomycin resistance cassette that was derived from the vector pR412T7 [24]. M. catarrhalis cells were rendered competent by washing with PBS containing 0.15% bovine gelatin. Transformation was achieved by adding the DNA fragments to the competent cell cultures, and subsequent plating on spectinomycin-containing blood agar plates (100 µg/mL). The numbers of CFUs were counted after overnight incubation at 37°C. Msp22 gene-specific PCRs, sequencing and Southern blot analysis were performed to confirm the presence of the gene deletion.

Msp22 cloning for complementation, expression and purification in M. catarrhalis BHI18 using complementation plasmid pEMCHJ04-KAN

The complete msp22 gene and a region of approximately 200 bp upstream of the gene was amplified using genomic DNA as template and primers 8825 and 8826, and cloned into pEMCHJ04-KAN [25] resulting in pEMCHJ04-KAN-Msp22. Mini prep DNA of pEMCHJ04-KAN-Msp22 and primers 8825 and 8860 (primer containing 6xHis-tag) were used for PCR amplification (see listing below). The resulting fragment was BamH1/Pst1 digested and ligated with BamH1/Pst1 digested pEMCHJ04-KAN (pEMCHJ04-KAN-Msp22-HIS). Transformation of the ligation into competent M. catarrhalis wild type and gene deletion mutant msp22Δ cells was performed as described above. Transformed cells were plated on blood agar containing 100 µg/mL Kan. Clones were analyzed by colony PCR using the following primers: 8825−5’ATATATGGATCCCATACAATAATTGCGGTGTTTGGG-3’; 8826−5’ATATATCTGCGACTATTITTTTCTTATAGCCTATGGG-3’; 8835−5’ACTTCTTTTGCTAGGTTAAGGA-3’; 8836−5’ACAAATTGTGTAGCGGTCTCTG-3’; 8860−5’AAACACAGCGATGTGTTGGTTCTTCTTATAGG-3’. The cells were harvested by centrifugation and frozen at −20°C until use.

The pellet was thawed and re-suspended in lysis buffer (50 mM Tris/HCl pH 8.0, 500 mM NaCl, 0.1% Triton X-100) containing protease inhibitors. Sonication on ice was performed 7 × 2 min (5 × 10% cycle, 100% power), and soluble and insoluble fractions were separated by centrifugation. Small scale Western blot analysis of crude lysate, soluble and insoluble fractions was performed to determine the solubility of the protein. The protein in the soluble fraction was purified using an IMAC affinity column. The protein bound to the column was washed with Tris/NaCl buffer (50 mM Tris/HCl pH 8.0, 500 mM NaCl, 0.5 mM DTT) containing 0.1% Triton X-100 (wash 0), buffer only (wash 1), 20 mM imidazole (wash 2) and 40 mM imidazole (wash 3) and then eluted in 50 mM Tris/HCl pH 8.0, 150 mM NaCl, 250 mM imidazole.

Luminol based heme staining

For heme staining, the protocol by Feissner et al. [26] was used. Briefly, SDS-PAGE was performed using non-reducing loading buffer and samples were not heat treated prior to loading. Proteins were subsequently blotted onto a nitrocellulose membrane, washed with PBS and incubated with the substrate luminol (SuperSignal West Femto Maximum Sensitivity Substrate Trial Kit, Pierce Biotechnology). Luminol/Enhancer solution and Stable Peroxidase Buffer were mixed at a 1:1 ratio and added to the membrane, followed by exposure of the membrane to light-sensitive film, allowing the detection of proteins with heme-dependent peroxidase activity.

Results

Human sera for antigen identification recognize M. catarrhalis proteins

Antigen identification using human sera relies on the assumption that candidate antigens have induced seroconversion or an immune response in patients recovering from infection or in healthy individuals upon encounter with the pathogen without developing disease. For identification of M. catarrhalis vaccine candidate antigens, 414 sera from patients (children 1–10 years of age) with otitis media were collected over a three year period. This serum collection included 147 serum pairs taken from the same individual during acute and convalescent disease phase (294 samples) and 120 single serum samples taken either from the acute or convalescent phase from different patients. Human sera were further collected from children suffering from respiratory allergies or asthma (2–18 years) and healthy adults (18–40 years) having no recent history of middle ear disease or M. catarrhalis infection. The sera containing high titer of antibodies as measured by ELISA and showing a diversity of bands in the Western blot using whole M. catarrhalis cell lysate were selected to create four different serum pools for antigen selection by bacterial surface display (Table 1). In general, good antibody levels against Moraxella lysates were detected in the majority of sera, but we could not observe significant differences in IgG levels between samples obtained from patients in acute and convalescent phase. The sera for pooling were therefore selected mainly based on ELISA titer and Western blotting. Sera from both, healthy individuals and patients, had higher ELISA titers than the sera from patients with recurrent AOM, while the latter showed a more homogeneous banding pattern in Western blot as compared to the individual sera included in the other pools. Serum pool PMC36 contained sera from young patients (2–18 years) with respiratory allergies, PMC37 serum pool was derived from children with asthma (5–17 years), PMC39 serum pool included sera from the patients with recurrent
represented by a total number of 1.6 insert sizes of 39 bp (LamB/1), 87 bp (LamB/2) and 199 bp of each library were sequenced in order to determine the quality of platforms on the bacterial cell surface. Approximately 600 clones of M. catarrhalis libraries were generated consisting of less well characterized proteins, such as a TonB dependent hits [28,29] and CopB (35 hits [30,31]). However, a number of antigens were preferably selected when screening the FhuA library. These antigens and MhuA (MCR_0739, 15 hits) were frequently detected in the human IgG pools that were initially used for library screening. The most frequently selected antigens in all screens included the immunodominant regions of these proteins. The selection of 6 additional antigens were selected against 3 UspA2H and 4 (IC20 IgG pool) antigens. Therefore, serum pools IC20 and PMc39 were additionally adsorbed against 3 UspA2H and 4 (IC20 IgG pool) Hag/MID clones for adsorption resulted in a strong reduction of Hag/MID clones in the screen using P39 serum pool, therefore a second LamB library was generated with a larger average insert size.

Screening of the three genomic libraries was performed using IgGs purified from the four serum pools, resulting in 13 individual bacterial surface display screens (3 LamB screen with the LamB/1 library, 4 LamB screens with the LamB/2 library, and 6 FhuA screens) to identify novel vaccine antigens. Approximately 800 clones per screen were sequenced and the results matched to annotated ORFs using BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A problem that occurred in the initial screens was the frequent selection of the Hag/MID, UspA1/UspA2H antigens. Therefore, serum pools IC20 and PMc39 were additionally adsorbed against 3 UspA2H and 4 (IC20 IgG pool) Hag/MID clones that covered the immunodominant regions of these proteins. The selection of 6 Hag/MID library clones for adsorption resulted in a strong reduction of Hag/MID clones in the screen using P39 serum pool, and a relative increase in the selection of the remaining antigens.

In total, 214 candidates were selected by the ANTIGENome approach and positively confirmed by Western blot analysis using the human IgG pools that were initially used for library screening. The most frequently selected antigens in all screens included the previously published antigens Hag/MID (493 hits [27]), the UspA1 and UspA2H proteins (131 hits [10]) as well as LbpB (39 hits [28,29]) and CopB (35 hits [30,31]). However, a number of less well characterized proteins, such as a TonB dependent receptor (MCR_0076, 13 hits), an outer membrane protein (MCR_1742, 24 hits), a carboxypeptidase (MCR_1010, 48 hits), and MhuA (MCR_0739, 13 hits) were frequently detected in addition to these well characterized antigens. Certain antigens were preferably selected when screening the FhuA library. These candidates included among others: Hag/MID (FluA: 349 hits vs. LamB: 144 hits); UspA2H (FluA: 81 hits vs. LamB: 4 hits); UspA1 (FluA: 39 hits vs. LamB: 7 hits); and the aconitate hydratase (FluA: 42 hits vs. LamB: 1 hit). In contrast, McmA was found 29 times in LamB screens, but was only selected once using the FhuA library. Many other antigens were identified equally frequent in both screens. These results confirm that the ANTIGENome technology is a valuable and comprehensive approach for the identification of novel antigens as potential vaccine candidates. Moreover, the utilization of two different surface display libraries, expressing smaller (LamB) and larger (FhuA) peptides, may also – besides mainly linear epitopes – allow for the selection of conformational epitopes.

Following initial antigen identification, several in vitro and in vivo analyses were performed to further reduce the number of selected vaccine candidates. Initially, all 214 candidates were tested for their gene distribution among 47 M. catarrhalis isolates. Based on this PCR analysis, 196 antigens were present in at least 43 of 47 Moraxella strains, whereas only 18 candidates were present in less than 90% of all isolates tested.

In order to evaluate the immunogenicity of individual antigens in humans, an ELISA using synthetic peptides corresponding to the epitope bearing regions of the antigenic proteins identified by the genomic screens was performed using the individual sera from the four human serum pools. The peptides were designed based on bioinformatic analysis of the selected clones encoding immunogenic epitopes and synthesized with an N-terminal biotin-tag. In case of longer antigenic fragments (more than 26 amino acid residues), overlapping peptides were generated. The 402 peptides were selected from 110 antigens according to their frequency of being selected by the antigenic screens as well as their annotation (e.g. predicted to be surface located, antigenic or secreted peptides/proteins). The 50 most reactive peptides are listed in Tables 2 and 3. Several of the most reactive peptides corresponded to antigens frequently found in the screens, such as PcnB/McrA, MsrAB) were found in all membrane preparations, whole

### Table 1. Human sera selected for antigen identification by peptide library screening.

| Serum pool | Individual sera | Source | Purpose |
|------------|-----------------|--------|---------|
| P39        | P4060.2, P4070.2, P4072.2, P4010.2, P4115.2 | Patients with otitis media; age: 1-10 years | Antigen selection |
| IC20       | IC58B, IC648, IC658, IC668, IC69B | Healthy individuals; age: 18–40 years | Antigen selection |
| P36        | P3792, P3801, P3819, P3832, P3861 | Patients with respiratory allergies; age: 2–18 years | Control; other condition |
| P37        | P3918, P3923, P3941, P3943, P3965 | Patients with asthma; age: 5–17 years | Control; other condition |

Pool P39 consisted of individual sera collected from OM patients during the convalescent disease phase. Pool IC20 contained sera from healthy individuals. The additional 2 pools were used as controls for otitis media-unrelated antigen reactivity: Pool P37 (patients with asthma, age: 5–17 years). Pool P36 (patients with respiratory allergies, 8 months–18 years of age).

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A final selection of 23 promising antigens for recombinant protein production and further in vivo evaluation was made based on the number of screen hits, data obtained from the serological studies, and the bioinformatic and gene distribution analyses (see Table 4). All 25 candidates were present in at least 44 of the 47 tested Moraxella isolates and the majority of the antigens were predicted to be localized in the outer membrane. In addition, proteomic studies with M. catarrhalis membrane fractions were performed to support antigen selection (data not shown). As shown in Table 4, four candidates (OppA, M16-like peptidase, MhuA and MsrAB) were found in all membrane preparations, whole
Table 2. ELISA data for the 50 most reactive *M. catarrhalis* peptides – Average ELISA titers for groups of sera.

| Peptide     | Annotation                                               | Average (OM) | Average (Asthma) | Average (Healthy) | Average (All) |
|-------------|----------------------------------------------------------|--------------|------------------|-------------------|---------------|
| MCR_1292-02 | phosphatidylethanolamine Kdo2-lipid A phosphoethanolamine transferase | 553          | 486              | 507               | 518           |
| MCR_0412-03 | hypothetical protein                                      | 413          | 396              | 412               | 407           |
| MCR_1728-03 | Ppx/Gpa phosphatase                                       | 445          | 410              | 294               | 387           |
| MCR_1387-01 | ribonuclease PH                                           | 428          | 474              | 219               | 377           |
| MCR_1836-07 | poly(A) polymerase                                        | 479          | 358              | 260               | 373           |
| MCR_0169-04 | excinuclease ABC subunit A                                | 511          | 394              | 154               | 363           |
| MCR_1494-02 | chaperonin protein Cpn10                                  | 431          | 390              | 239               | 359           |
| MCR_0081-02 | prol y endopeptidase                                      | 348          | 377              | 317               | 347           |
| MCR_1728-05 | Ppx/Gpa phosphatase                                       | 347          | 351              | 276               | 326           |
| MCR_1596-01 | phospholipid/glycerol acyltransferase                     | 333          | 265              | 332               | 311           |
| MCR_1690-04 | extracellular solute-binding protein family 3             | 280          | 401              | 241               | 306           |
| MCR_0036-01 | glutamate-cysteine ligase                                 | 334          | 353              | 211               | 302           |
| MCR_0604-04 | Fe-S protein assembly chaperone HscA                      | 316          | 291              | 244               | 286           |
| MCR_1619-10 | ribonuclease E                                            | 303          | 347              | 178               | 277           |
| MCR_1200-01 | 2-isopropylmalate synthase                                | 370          | 244              | 173               | 269           |
| MCR_0036-03 | glutamate-cysteine ligase                                 | 233          | 240              | 338               | 268           |
| MCR_1283-01 | glycine dehydrogenase                                     | 340          | 287              | 153               | 265           |
| MCR_0092-01 | 3-ketoacyl-CoA thiolase FadA                              | 301          | 260              | 211               | 260           |
| MCR_1683-02 | DNA polymerase I                                          | 304          | 298              | 140               | 251           |
| MCR_1681-01 | peptide chain release factor 3                            | 280          | 231              | 216               | 245           |
| MCR_1596-02 | phospholipid/glycerol acyltransferase                     | 230          | 271              | 230               | 243           |
| MCR_1487-01 | ubiquinone biosynthesis hydroxylase                       | 268          | 283              | 158               | 238           |
| MCR_0131-02 | nitric oxide reductase NorB                               | 250          | 251              | 144               | 217           |
| MCR_1320-02 | cbb3-type cytochrome c oxidase subunit CcoP               | 243          | 273              | 111               | 211           |
| MCR_0321-03 | lysophospholipase-like protein                            | 217          | 201              | 181               | 201           |
| MCR_0604-02 | Fe-S protein assembly chaperone HscA                      | 271          | 165              | 152               | 201           |
| MCR_0131-04 | nitric oxide reductase NorB                               | 263          | 247              | 69                | 197           |
| MCR_0996-04 | hypothetical protein                                      | 248          | 225              | 107               | 197           |
| MCR_0934-05 | polyphosphate kinase 2                                    | 257          | 204              | 108               | 194           |
| MCR_1003-02 | LysM domain protein                                       | 169          | 300              | 114               | 193           |
| MCR_1735-02 | M48 family zinc metallopeptidase                          | 213          | 231              | 129               | 192           |
| MCR_1295-02 | leucyl-tRNA synthetase                                    | 222          | 208              | 119               | 186           |
| MCR_0439-03 | penicillin-binding protein 1A                             | 194          | 200              | 154               | 184           |
| MCR_0078-01 | hypothetical protein                                      | 193          | 211              | 136               | 180           |
| MCR_0169-03 | excinuclease ABC subunit A                                | 240          | 226              | 58                | 179           |
| MCR_1672-02 | pepSY-associated membrane protein                         | 149          | 229              | 163               | 178           |
| MCR_0692-03 | hypothetical protein                                      | 216          | 213              | 96                | 177           |
| MCR_0092-02 | 3-ketoacyl-CoA thiolase FadA                              | 276          | 153              | 78                | 176           |
| MCR_0258-01 | DNA-directed RNA polymerase subunit beta'                 | 170          | 156              | 194               | 173           |
| MCR_0321-04 | lysophospholipase-like protein                            | 191          | 180              | 132               | 169           |
| MCR_0791-02 | nicotinate-nucleotide diphosphorylase                     | 184          | 205              | 113               | 168           |
| MCR_0625-01 | penicillin-binding protein 1B                             | 210          | 177              | 106               | 167           |
| MCR_0394-04 | aconitase                                                | 200          | 93               | 201               | 167           |
| MCR_0136-02 | conserved hypothetical protein                            | 178          | 191              | 129               | 166           |
| MCR_1690-01 | extracellular solute-binding protein family 3             | 153          | 207              | 136               | 164           |
| MCR_1652-02 | peptidase M16 inactive domain protein McmA                | 183          | 206              | 92                | 162           |
| MCR_1350-06 | tRNA uridine S-carboxymethylaminomethyl modification enzyme GidA | 221          | 178              | 63                | 158           |
| MCR_0405-01 | tetratricopeptide repeat family protein                   | 145          | 202              | 129               | 158           |
| MCR_1295-01 | leucyl-tRNA synthetase                                    | 184          | 134              | 129               | 151           |
membrane, outer membrane vesicles and outer membrane vesicles isolated from cultures grown in iron-depleted medium (as a variety of virulence factors are induced by low iron levels). Three candidates (hypothetical proteins MCR_0063, MCR_0691, MCR_0692) were found in the whole membrane and in outer membrane vesicles, and seven further candidates were detected in one of the three membrane preparations.

Three candidate vaccine antigens demonstrated protection in vivo

Of the 23 candidates selected by the ANTIGENome technology, we evaluated 8 well conserved (see Table 5) and readily recombinant expressed antigens that had shown some promise in a preliminary mouse study in more detail for their potential to elicit protective immune response in vivo (Figure 1). The rate of *M. catarrhalis* clearance from mouse lungs in response to immunization with recombinant antigens was assessed using a mouse pulmonary clearance model (Figure 2). Mice were immunized intranasally 3 times at 3 week intervals and challenged intranasally with 40 μL of approximately 5 x 10⁶ live *M. catarrhalis* RH4 (actual CFU varied between 3.8 x 10⁶ to 5.9 x 10⁶) 3 weeks after the last boost. Bacterial CFU were determined in lungs 6 hours post infection and systemic antibody titers after vaccination of mice were determined by ELISA (Figure 2).

Groups of mice immunized with recombinant proteins MCR_1416, MCR_1303, MCR_0076-1, MCR_1010, MCR_0196, MCR_1003-1, MCR_0996 and MCR_0686 expressed in *E. coli* showed a greater or comparable clearance of bacteria from lungs compared to the positive control protein OmpCD (Figure 2A, B). The effect was statistically significant for MCR_1416 with one log reduction in bacterial recovery compared to mice immunized with adjuvant alone (IC31⁰) (p<0.01) (Figure 2A). Further, there was also a significant reduction in bacterial load for MCR_1303 (p<0.05) and MCR_0076-1 (p<0.05) compared to IC31⁰ alone, when sterile lung cultures were removed from the analysis (Figure 2B). The exclusion of sterile cultures was considered reasonable, based on the observation that negative (sterile) lung cultures appeared randomly between 0 to 3 in the PBS groups, the number of sterile lung cultures in the immunized mice occurred with the same frequency as in the PBS groups (between 0 and 4). Therefore, the sterile lung cultures were more likely to represent a technical artifact (infection failure), rather than elimination of bacteria. While significant protection was observed for MCR_1416, MCR_1303 and MCR_0076-1, protection was lower for the other candidates despite strong antibody responses as measured by IgG ELISA (Figure 2C). In contrast, the IgG response was very low for MCR_0076-1 and MCR_1010, while the level of protection was higher than for the positive control protein OmpCD. This observation indicated that factors other than antibody responses may contribute to protection against *M. catarrhalis*.

Systemic human antibody responses against the selected antigens are not induced upon infection

In order to evaluate the human immune response for the 8 selected recombinant antigens upon natural infection, additional serological studies were performed with ELISA and Luminex xMAP® technology, using a collection of 164 individual sera from children with otitis media collected during the acute and convalescent disease phase. Sera from healthy individuals were tested in parallel in order to compare antigen specific responses between healthy adults and children with otitis media. We detected antibodies against all eight antigens in the 20 paired acute/convalescent serum samples from children with otitis media, however IgG end titers were relatively low (<2000) and no significant antigen specific seroconversion (defined as ≥2 fold increase in the convalescent IgG titer) was detected in any of the donors (data not shown). We also examined the median antibody titers between healthy donors and otitis media patients, however no statistically significant difference was seen (data not shown). Moreover, we detected a decrease in median systemic IgG titers against the antigen MCR_1303 in convalescent sera compared to acute sera (data not shown). These results are in agreement with the peptide ELISA data, as no increase in antibody titer was detected for these antigens in sera from otitis media patients during an OM episode when the paired serum samples were collected.

**MCR_1416 exhibits heme-dependent peroxidase activity**

The recombinant antigen showing the highest protection in the pulmonary clearance model was further studied for its biological function. MCR_1416 has previously been identified as *Moraxella* surface protein 22 (Msp22) [9] and shows homology to cytochrome c, it containing one CXXCH motif (residues 142 to 146). C-type cytochromes are characterized by covalent attachment of heme to the protein via two thioether bonds formed between the heme vinyl groups and the cysteine sulfurs in a CXXCH peptide motif [32]. Since Msp22 also contains this motif, we set out to determine whether it binds heme and exhibits heme-dependent peroxidase activity. Heme staining was performed according to the method of Feissner et al. [26] using luminol as substrate for the heme-dependent peroxidase activity.

In order to try to ensure that native lipidated Msp22 protein was recovered possessing its correct native conformational folding, Msp22 with its native signal sequence and a C-terminal His-tag was expressed in *M. catarrhalis* (i.e. its native host), using the complementation vector pEMCJH04-KAN. We complemented the wild type strain with the plasmid expressed Msp22 in order to increase the yield of purification from *M. catarrhalis*. Subsequently,
Table 3. ELISA data for the 50 most reactive *M. catarrhalis* peptides – ELISA titers for individual sera.

| Peptide         | OM Average | Asthma Average | Healthy individuals Average |
|-----------------|------------|----------------|-----------------------------|
| MCR_1292-02     | 677        | 388            | 789                         |
| MCR_0412-03     | 602        | 68             | 226                         |
| MCR_1728-03     | 612        | 352            | 197                         |
| MCR_1387-01     | 506        | 397            | 172                         |
| MCR_1836-07     | 569        | 624            | 433                         |
| MCR_0169-04     | 472        | 1008           | 159                         |
| MCR_1494-02     | 462        | 408            | 196                         |
| MCR_0081-02     | 522        | 83             | 199                         |
| MCR_1728-05     | 520        | 58             | 180                         |
| MCR_1596-01     | 236        | 306            | 117                         |
| MCR_1690-04     | 139        | 248            | 120                         |
| MCR_0036-01     | 353        | 215            | 117                         |
| MCR_0604-04     | 334        | 398            | 303                         |
| MCR_1619-10     | 396        | 0              | 83                          |
| MCR_1200-01     | 398        | 784            | 103                         |
| MCR_0036-03     | 277        | 106            | 275                         |
| MCR_1283-01     | 346        | 338            | 115                         |
| MCR_0092-01     | 187        | 822            | 76                          |
| MCR_1683-02     | 458        | 9              | 121                         |
| MCR_1681-01     | 284        | 725            | 109                         |
| MCR_1596-02     | 180        | 271            | 98                          |
| MCR_1487-01     | 158        | 132            | 84                          |
| MCR_0131-02     | 173        | 468            | 141                         |
| MCR_1320-01     | 158        | 112            | 60                          |
| MCR_0321-03     | 214        | 126            | 0                           |
| MCR_0604-02     | 186        | 346            | 161                         |
| MCR_0131-04     | 31         | 945            | 79                          |
| MCR_0996-04     | 352        | 260            | 11                           |
Msp22 was obtained from the soluble fraction and purified on an IMAC column. Western blot analyses of the column eluate (using extracts from *M. catarrhalis* with or without pEMCJH04-KAN-Msp22-HIS) and immune sera against recombinant MCR_1416 and anti-penta-His antibody revealed successful purification of His-tagged Msp22 (Figure 3). These experiments also showed that the Msp22 protein as produced by wild type *M. catarrhalis* is recognized by antibodies induced in mice by the recombinant *E. coli* protein.

For heme detection experiments, samples were prepared using a non-reducing sample buffer and were not heated prior to SDS-PAGE, which was performed under denaturing conditions. Bacterial lysates of the wild type, the *msp22* gene deletion mutant and the complemented strains all served as additional heme controls. Positive signals were obtained for hemoglobin, purified Msp22 and the cell lysates expressing Msp22, indicating the presence of heme-dependent peroxidase activity. No signal was detected for the negative control protein BSA (Figure 4).

### Table 3. Cont.

| Peptide     | OM Asthma | Healthy individuals |
|-------------|-----------|---------------------|
|             | P406 | P407 | P407 | P410 | P411 | P412 | P39 | P39 | P39 | P39 | P39 | ICS | ICS | ICS | ICS | ICS | Aver |
| MCR_0934-05 | 232 | 376 | 48 | 449 | 182 | 256 | 254 | 301 | 258 | 193 | 15 | 42 | 68 | 0 | 29 | 400 | 194 |
| MCR_1003-02 | 124 | 233 | 0 | 432 | 40 | 182 | 727 | 216 | 199 | 242 | 118 | 73 | 76 | 0 | 143 | 280 | 193 |
| MCR_1735-02 | 122 | 202 | 35 | 474 | 215 | 229 | 238 | 298 | 207 | 167 | 244 | 74 | 88 | 0 | 84 | 399 | 192 |
| MCR_1295-02 | 223 | 65 | 35 | 593 | 228 | 189 | 282 | 295 | 210 | 254 | 0 | 69 | 67 | 0 | 58 | 400 | 186 |
| MCR_0078-01 | 177 | 0 | 132 | 462 | 177 | 207 | 319 | 284 | 252 | 198 | 0 | 73 | 97 | 0 | 0 | 508 | 180 |
| MCR_0169-03 | 176 | 576 | 59 | 359 | 74 | 193 | 404 | 259 | 100 | 322 | 45 | 30 | 10 | 0 | 17 | 233 | 179 |
| MCR_1672-02 | 247 | 290 | 78 | 168 | 100 | 10 | 240 | 101 | 410 | 112 | 282 | 123 | 99 | 143 | 60 | 390 | 178 |
| MCR_0692-03 | 348 | 229 | 56 | 341 | 163 | 158 | 205 | 266 | 256 | 148 | 189 | 95 | 70 | 0 | 48 | 265 | 177 |
| MCR_0092-02 | 325 | 313 | 226 | 97 | 427 | 265 | 103 | 67 | 201 | 89 | 307 | 117 | 114 | 0 | 69 | 0 | 131 | 176 |
| MCR_0258-01 | 157 | 143 | 34 | 468 | 102 | 114 | 193 | 240 | 132 | 216 | 0 | 52 | 0 | 0 | 666 | 253 | 173 |
| MCR_0321-04 | 199 | 119 | 0 | 341 | 191 | 296 | 252 | 192 | 19 | 202 | 235 | 168 | 66 | 0 | 94 | 332 | 169 |
| MCR_0791-02 | 285 | 61 | 80 | 308 | 216 | 152 | 218 | 214 | 153 | 233 | 206 | 39 | 159 | 0 | 78 | 287 | 168 |
| MCR_0625-01 | 202 | 139 | 53 | 476 | 204 | 187 | 226 | 272 | 200 | 188 | 0 | 35 | 14 | 0 | 46 | 0 | 405 | 167 |
| MCR_0394-04 | 297 | 513 | 76 | 44 | 202 | 65 | 78 | 51 | 98 | 81 | 159 | 490 | 2 | 0 | 305 | 208 | 167 |
| MCR_0136-02 | 83 | 154 | 58 | 368 | 200 | 202 | 237 | 278 | 170 | 166 | 103 | 150 | 65 | 0 | 70 | 359 | 166 |
| MCR_1690-01 | 162 | 136 | 91 | 306 | 112 | 108 | 205 | 165 | 350 | 152 | 164 | 172 | 157 | 0 | 56 | 294 | 164 |
| MCR_1652-02 | 109 | 119 | 4 | 474 | 159 | 234 | 297 | 278 | 241 | 158 | 57 | 57 | 11 | 0 | 13 | 380 | 162 |
| MCR_1350-06 | 245 | 317 | 143 | 307 | 165 | 147 | 193 | 242 | 145 | 126 | 185 | 1 | 10 | 0 | 0 | 306 | 158 |
| MCR_0405-01 | 128 | 202 | 45 | 237 | 150 | 106 | 179 | 229 | 239 | 116 | 249 | 43 | 42 | 292 | 35 | 232 | 158 |
| MCR_1295-01 | 158 | 167 | 71 | 224 | 216 | 265 | 164 | 122 | 109 | 169 | 104 | 131 | 117 | 0 | 70 | 329 | 151 |
| MCR_0405-03 | 534 | 106 | 46 | 232 | 154 | 29 | 214 | 129 | 118 | 101 | 132 | 168 | 154 | 0 | 70 | 225 | 151 |

For legend see Table 2.
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absence of the respective protein band at 17 kDa in the msp22 gene deletion mutant, the presence of a strong signal in the complemented strain, and a weak signal in the wild type strain suggested that the 17 kDa hemoprotein was indeed Msp22.

**Discussion**

Over the last three decades, *M. catarrhalis* has become recognized as an important pathogen of the human respiratory tract [33,34,35,36,37,38,39]. However, even though *M. catarrhalis* is the third most frequent bacterial pathogen to be associated with otitis media and is a major cause of exacerbations of COPD in adults, none of the currently available bacterial vaccines developed to prevent these diseases include *M. catarrhalis* antigens [8]. Therefore, the aim of this study was to comprehensively identify potential vaccine targets of *M. catarrhalis* by applying the ANTIGENome technology that had previously been developed by Intercell AG (Vienna, Austria), and which had been previously successfully used for vaccine discovery for several other bacterial pathogens [17,18,40]. Genomic libraries displaying multiple antigens previously identified in other studies. Hag/MID has been described as an adhesin, a hemagglutinin, and a stimulator of B cells [41,42], whilst UspA1 functions as an adhesin and a transporter [43,44]. Further, immunization with UspA1 has been shown to induce bactericidal antibodies in mice and humans [11]. In fact, the detection of these well-known candidate antigens shows the value of the ANTIGENome technology in identifying potential vaccine candidates, also including potentially novel vaccine candidates. Indeed, using this technology, allowed the identification of 214 antigenic candidates from the respiratory tract was measured at 6 to 9 hrs post-infection. It is precisely for this reason that the clearance of *M. catarrhalis* pulmonary clearance model. The fact that *M. catarrhalis* is a strictly human pathogen, which does not induce active infection in animals, means that there is currently no clinically relevant model for *M. catarrhalis* vaccination studies available, especially for studies that adequately mimic otitis media infection in humans. For this reason, the mouse pulmonary clearance model is the most frequently used animal model to test the ability of antigens to generate a protective immune response against *M. catarrhalis* [45]. However, it is known that mice do not develop pneumonia and are able to clear the *M. catarrhalis* bacteria relatively quickly in this model (within 6-24 hours), and in this study, *M. catarrhalis* clearance occurred within 24 hrs post-infection. It is precisely for this reason that the clearance of *M. catarrhalis* from the respiratory tract was measured at 6 to 9 hrs post-infection when using this animal model, rather than
measuring total bacterial clearance at 24 hrs (by which time non-vaccination related factors could have influenced the clearance of the *M. catarrhalis* bacteria) [46]. Based on our preliminary studies with heat killed bacteria and the OmpCD antigen, the optimal end point for *M. catarrhalis* strain RH4 in our model was 6 hrs post-infection. This model was found to be reproducible, as we detected similar clearance rates for the tested antigens in up to 6 independent experiments. Nevertheless, it should be noted that the clearance rate of bacteria from the lungs of vaccinated mice (a measure of the efficacy of vaccination) was based on an actual increase in clearance rate compared to the normal clearance rate observed in unvaccinated control mice. In our study, this meant that the maximum clearance rate we observed using this model lay in the range of 0.5 to 1.0 log10 when compared to negative controls. However, our results are in agreement with similar studies that have previously been performed using putative *M. catarrhalis* vaccine antigen candidates [47,48,49].

Using our comprehensive screening technology, we eventually selected 8 out of the 23 proteins that possessed the potential to become vaccine candidates for testing in a mouse pulmonary clearance model. Three of these protein antigens showed beneficial effects on bacterial clearance from mouse lungs after mucosal immunization: 1) MCR_1416 (Msp22), a candidate also previously identified by Ruckdeschel and colleagues [9,49]; 2) MCR_1303 (OppA), an oligopeptide permease A [50] and 3) MCR_0076, the “plug” domain of a TonB-dependent receptor. The fact that similar results and clearance rates were obtained independently by other investigators for Msp22 [49] and OppA [50] using different experimental set-ups, indicates that these proteins are indeed promising vaccine candidates. MCR_0076,

![Figure 1. Structural features of 8 potential *M. catarrhalis* vaccine candidates.](image-url)

**Figure 1. Structural features of 8 potential *M. catarrhalis* vaccine candidates.** MCR_0076, TonB-dependent receptor; MCR_0196, MltB; lytic murein transglycosylase; MCR_0686, peptide methionine sulfoxide reductase MsrA/MsrB; MCR_0996, hypothetical protein; MCR_1003, LysM domain protein; MCR_1010, D-alanyl-D-alanine carboxypeptidase; MCR_1303, oligopeptide ABC transport system substrate binding protein; MCR_1416, cytochrome c class II, Msp22. SP, signal peptide; LP, signal peptide for lipidation; Plug, an independent folding subunit blocking the pore until the channel is bound by a ligand; PGBD1, peptidoglycan binding-like; MsrA, methionine sulfoxide reductase A; SelR, seleno protein R; LysM, lysine motif; SBP bac 5, bacterial extracellular solute-binding protein family 5. Light grey bars represent the recombinant protein (fragments). Thin black bars delineate epitope containing regions covered by clones selected by the ANTIGENome technology with human IgGs. doi:10.1371/journal.pone.0064422.g001
the plug domain of TonB-dependent receptor, is situated within the beta-barrel structure and appears to be more conserved than the barrel. This plug domain is an independent folding subunit blocking the pore until the channel is bound by a ligand and causes the structural and functional differences between these transporters and porins [51,52,53]. TonB-dependent receptors have previously been reported to be potential vaccine antigens and important virulence factors [54,55,56] and should thus be taken into consideration and analyzed in more detail for *M. catarrhalis*. The *oppA* gene (MCR_1303) encodes an oligopeptide permease that belongs to the ABC transport system. These types of transporters have been shown to play a role in virulence, to be immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57]. The Msp22 antigen (MCR_1416) induced the most significant immunogenic and to be potential vaccine candidates [57].

The heme group of type c cytochromes accepts electrons from the bc1 complex and transfers them to the cytochrome oxidase complex. Among other functions, cytochrome c has heme-dependent peroxidase activity and plays a role in initiation of apoptosis in more complex organisms [58,59,60,61]. Based on its homology to cytochrome c and its heme binding, Msp22 may also function in the electron transfer via its heme-dependent peroxidase activity. Besides its important role for cytochrome function, heme is also the most abundant source of iron in the human body [62]. Not surprisingly, due to very limited free iron availability in the human host, many pathogens have evolved mechanisms to utilize heme containing proteins as iron sources. Recently, two *M. catarrhalis* proteins have been shown to acquire iron from hemin and heme complexes [63,64]. Therefore, Msp22 could also be involved in iron acquisition from heme and heme-containing compounds. Interestingly, it was recently suggested that Msp22 has a potential role in divalent ion transport [50]. An investigation into the mechanism of heme binding and the contribution of the CXXCH motif was recently performed for two putative cytochrome c peroxidases of *Campylobacter jejuni* [26,65]. While these proteins exhibited heme binding, site-directed mutations within the CXXCH motif resulted in unstable proteins excluding...
them from further analysis [65]. Whether this holds true also for *M. catarrhalis* Msp22 remains to be elucidated.

As targets for protective immune responses need to be accessible on the bacterial surface and knowing that Msp22 has been annotated as a putative surface protein, we attempted to confirm the cell surface location of Msp22. However, using flow cytometry of both wild type and Msp22 overexpressing strains and polyclonal anti-Msp22 mouse sera, we could not detect this protein on the bacterial surface. This suggests that the protein is not surface exposed under the *in vitro* growth conditions tested in these studies. In order to elicit a protective immune response, one may speculate that Msp22 may become transiently exposed to the host’s immune system during infection. Unlike Msp22, OppA is accessible on the bacterial surface *in vitro* [50] as confirmed by our studies (data not shown), and antibody mediated neutralization of bacteria is therefore likely to be an important protective immune mechanism complementing native immune defenses against this antigen.

Interestingly, in agreement with the data obtained by other researchers in this field [50], we could not detect significant differences in the antibody titers against the 8 tested antigens in: 1) sera from children with otitis media in the acute compared to the convalescent disease phase, or 2) in sera from children compared to sera from healthy individuals. The natural systemic IgG response observed in humans has therefore not provided any further validation of our selected eight antigens, but the selection as a vaccine candidate was rather based on the pulmonary clearance model. Furthermore, although UspA1, UspA2 and Hag/MID antigen specific antibodies were frequently found in both children and healthy individuals [66,67], there is no clear evidence that natural immune responses raised against other putative vaccine candidates contribute to protection. The question whether naturally induced antibodies against any *M. catarrhalis* antigens play a role in protection against otitis media has been previously raised [50], and our observations confirm that further investigations into the immune mechanisms operating during *M. catarrhalis* infection induced by this pathogen will be required. In addition, naturally occurring antibodies may exhibit different epitope specificity and avidity, compared to vaccine induced antibodies. But more importantly, systemic IgG levels do not adequately reflect mucosal immune responses. Thus, if mucosal immunity is more critical for protection against *M. catarrhalis*, serological studies based on serum samples collected from otitis media patients may be of limited value. Such a discrepancy between mucosal and systemic serological immune responses was

**Figure 3.** Detection of recombinant MCR_1416 (Msp22) expressed and purified from *M. catarrhalis*. Equal volumes of eluates obtained from IMAC columns from extracts of *M. catarrhalis* complemented with His-tagged MCR_1416 (eluate A) or wild type strain (not complemented; negative control) were separated by SDS-PAGE and immunoblotted using immune serum against recombinant Msp22 (left panel) and antibody against the His-tag (right panel). doi:10.1371/journal.pone.0064422.g003

**Figure 4.** Msp22 shows heme-dependent peroxidase activity. The specificity of the heme stain for Msp22 is demonstrated by staining of lysates from the wild type, and gene deletion mutant strains as well as the BBH18 strain transformed with pEMCJH04-KAN-Msp22. Hemoglobin (positive control), BSA (negative control). wt, wild type *M. catarrhalis* BBH18; wt c*, wild type *M. catarrhalis* BBH18 transformed with pEMCJH04-KAN-Msp22; msp22 Δ, msp22 gene deletion mutant; msp22Δ c* msp22 gene deletion mutant transformed with pEMCJH04-KAN-Msp22. The position of Msp22 is marked with an arrow. doi:10.1371/journal.pone.0064422.g004
previously detected in otitis media patients against *M. catarrhalis* outer membrane proteins [68]. In addition, the role of T cells for protection and B cell activity stimulation remains to be elucidated. Most recent studies suggested that *M. catarrhalis* is able to modulate mucosal epithelial responses and B cell adaptive immunity in such a way as to hinder the generation of antibodies with a correct function and epitope specificity [69,70]. If this indeed turns out to be the case, vaccination with *M. catarrhalis* would be an extremely valuable approach in preventing infection by this pathogen. In terms of antigen validation, the detection of a natural immune response against the selected antigens indicated that they were expressed in vivo upon infection of the human host.

In conclusion, comprehensive screening using the ANTIGENE-Mome technology has led to the identification of 214 antigenic proteins, with 3 of these being shown to provide protection against *M. catarrhalis* colonization in a mouse pulmonary model. The results confirm that further evaluation of these proteins as vaccine candidates in additional functional studies and in clinically relevant *Moraxella* otitis media models is warranted.

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**Author Contributions**

Conceived and designed the experiments: MS PB SV AN MH SS UL JH AM BH-N. Performed the experiments: MS PB SV AN WE EM CH. Analyzed the data: MS PB SV AN MH SS WE EM CH EN UL JH AM BH-N. Contributed reagents/materials/analysis tools: AN MH SS UL JH AM BH-N. Wrote the paper: MS PB SV SS WE EM CH JH AM BH-N.

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