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Molecular Aspects of *Moraxella catarrhalis* Pathogenesis

Stefan P. W. de Vries,¹ Hester J. Bootsma,^{1*} John P. Hays,² and Peter W. M. Hermans¹

Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands,¹ and
Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands²

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INTRODUCTION

Moraxella catarrhalis is a human-restricted, unencapsulated, gram-negative mucosal pathogen. Further, though previously thought to be a commensal of the upper respiratory tract, the bacterium is now increasingly recognized as a true pathogen of both the upper respiratory tract and the lower respiratory tract of humans. It is the third most common bacterial cause of childhood otitis media (OM) after *Haemophilus influenzae* and *Streptococcus pneumoniae*, and it is responsible for up to 20% of cases (58, 146, 149). Next to *H. influenzae*, *M. catarrhalis* is the second most common cause of exacerbations of chronic obstructive pulmonary disease (COPD), estimated to be responsible for 10 to 15% of these exacerbations, which accounts for 2 to 4 million episodes in the United States per year (99,

132). Rates of *M. catarrhalis* carriage in children and adults differ considerably. About two-thirds of all children are colonized within the first year of life, and it is expected that about half of these children will experience at least one period of OM during this year (39). In contrast, the rate of carriage in healthy adults is much lower, around 3 to 5%, though in COPD patients, *M. catarrhalis* has been detected in 5 to 32% of sputum samples (99).

Interestingly, there has been a rapid acquisition and spread of β -lactam resistance in *M. catarrhalis* in the last 20 to 30 years (21) such that approximately 95% of clinical isolates now appear to be resistant to one or more β -lactams (77). Further, it has been suggested that the production of β -lactamases by *M. catarrhalis* could protect cocolonizing pathogens from the effects of β -lactam antibiotic treatment (24, 71). In any case, the financial impact on global health care systems of the high incidence of *M. catarrhalis* colonization and disease is significant, and consequently, several research groups are currently involved in identifying and assessing the usefulness of putative *M. catarrhalis* vaccine candidates (91, 122, 144, 155).

* Corresponding author. Mailing address: Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, P.O. Box 9101 (Route 224), 6500 HB Nijmegen, The Netherlands. Phone: 31-24-3666477. Fax: 31-24-3666352. E-mail: H.Bootsma@cukz.umcn.nl.

Fortunately, our understanding of the molecular aspects of *M. catarrhalis* pathogenesis is steadily increasing. For example, it has been clearly demonstrated that *M. catarrhalis* is able to colonize the mucosal surfaces of the middle ear in OM patients (40, 55) or the lower respiratory tract in COPD patients (131, 133). To facilitate this, the bacterium possesses specific receptors allowing it to bind to host epithelia and to components of the extracellular matrix (ECM). The latter has been suggested to be especially important for adhesion to damaged epithelial cell surfaces, as may be observed, for example, in COPD patients (90, 132, 142, 143). *M. catarrhalis* also appears to be able to invade host epithelial cells (138, 141), the intracellular survival of pathogens being an important aspect of host immune evasion (31). Moreover, once attached to the host mucosal surfaces, *M. catarrhalis* has the ability to interact and/or compete with the commensal flora and has the apparatus to survive and multiply under challenging nutrient-limiting conditions. Such conditions may result in the formation of microcolonies and biofilms (54). Finally, *M. catarrhalis* has the ability to evade and survive host immune responses, a process particularly helped by its ability to withstand the effects of human serum. With these aspects in mind, this review is intended to provide a detailed overview of our current understanding of the molecular aspects of *M. catarrhalis* pathogenesis and particularly emphasizes recent publications relating to adhesion, invasion, biofilm formation, evasion of the host immune system, and nutrient acquisition.

ADHESION TO HOST EPITHELIUM AND ECM

Adhesion to mucosal surfaces is mediated by binding of several *M. catarrhalis* macromolecules to surface receptors on eukaryotic target cells or to components of the ECM. *M. catarrhalis* is an unencapsulated gram-negative bacterium, and its outer membrane comprises phospholipids, lipooligosaccharides (LOS), integral outer membrane proteins (OMPs), and lipoproteins. From various studies, it appears that *M. catarrhalis* adhesion is a multifactorial event mediated by many adhesion macromolecules. For example, pili may initiate adhesion at long range, while OMPs may be involved during more intimate contact (67, 80). In particular, several macromolecules that contribute to *M. catarrhalis* adhesion, including OMPs such as the ubiquitous surface protein A proteins (UspAs) (80), *M. catarrhalis* immunoglobulin D (IgD) binding protein/hemagglutinin (MID/Hag) (42), *M. catarrhalis* adherence protein (McaP) (145), OMP CD (72), *M. catarrhalis* filamentous Hag (FHA)-like proteins (Mha proteins) (14, 117), and surface-exposed structures, such as type IV pili (TFP) (88) and LOS structures (141), have been identified.

Adhesion and Major OMPs

Trimeric autotransporters. The *M. catarrhalis* UspAs and MID/Hag are classified as trimeric autotransporters, also known as oligomeric coiled-coil adhesins (Oca proteins) (30, 56, 70, 83). The group of trimeric autotransporters is a large protein family consisting of virulence factors of diverse pathogenic gram-negative bacteria, such as the YadA protein of *Yersinia* spp. and the Hia protein of *H. influenzae* (1, 33). Trimeric autotransporters mediate their own insertion into the

outer membrane. Their typical tripartite structure consists of (i) an N-terminal signal peptide, (ii) an N-terminal passenger domain that confers the protein's biological function, and (iii) a C-terminal translocator domain responsible for pore formation, which is required for translocation of the protein across the outer membrane (Fig. 1A). The C-terminal translocator domain consists of a β -barrel structure anchored in the outer membrane and is connected to the N-terminal head domain via a coiled-coil stalk region (30, 70).

UspAs. *M. catarrhalis* UspAs are multifunctional proteins with important adhesion properties. They can be divided into three main groups: UspA1 (88-kDa), UspA2 (62-kDa), and UspA2H (92-kDa) proteins (Fig. 1B, C, and D, respectively) (3, 80). The hybrid UspA2H protein consists of a UspA1-like domain at its N terminus and a UspA2-like domain at its C terminus (80). UspA1, UspA2, and UspA2H protrude from the bacterial cell membrane as lollipop-like structures (70). The N-terminal globular head domain (passenger domain) is most readily available for binding because it extends beyond the outer membrane. UspA1 and UspA2 share homology in the stalk region but display major differences in the head- and membrane-anchoring domains (22). In fact, the UspA proteins of different isolates are divergent but share several (apparently) interchangeable modular amino acid sequence cassettes (Fig. 1B to D), including the so-called VEEG, NINNY, LAAY, and KASS repeats. UspA variability is most evident in the N-terminal region, where UspA1 and UspA2H contain variable numbers of antiparallel β -strand-forming GGG repeats followed by a FAAG repeat, probably resulting in different sizes of the head domain. In contrast, the N-terminal part of UspA2 is highly divergent from UspA1 and UspA2H. Furthermore, UspA1 and UspA2 both have variable stalk regions, harboring the UspA1 and UspA2 variable regions (U1VR and U2VR, respectively) (22). Sequence variability of the UspA2H stalk region (tentatively designated U2HVR) has not yet been determined. The UspA1 C-terminal region is highly conserved among isolates and is composed of a NINNY-KASS-FET motif followed by C-terminal region 1. UspA2 variants are also highly conserved at the C terminus, possessing the C-terminal region 2 domain flanked by the NINNY-KASS-FET motif, except for that of *M. catarrhalis* strain TTA24, which possesses an incomplete carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1)-binding motif (see below) instead of the FET motif (22). Several groups have studied the functional role of UspAs in adhesion to human cell lines. UspA1 was found to mediate binding to Chang conjunctival epithelial cells (2, 23, 80), HEp-2 laryngeal epithelial cells (2, 92), and A549 type II alveolar epithelial cells (23, 68). UspA1 also binds to the ECM components fibronectin (143) and laminin (142). Binding to Chang cells or fibronectin was found to be mediated by the VEEG-NINNY-VEEG motif (23). Given the fact that these variable domains appear to contribute to different functional aspects of the UspA proteins, motif exchange could lead to acquisition of specific functional characteristics. In addition, this may facilitate evasion of the immune response, as potentially immunoreactive domains become exposed at the bacterial surface.

UspA1-mediated binding to host cells varies upon phase variation, which is regulated at the level of transcription by variation in a homopolymeric poly(G) tract located upstream

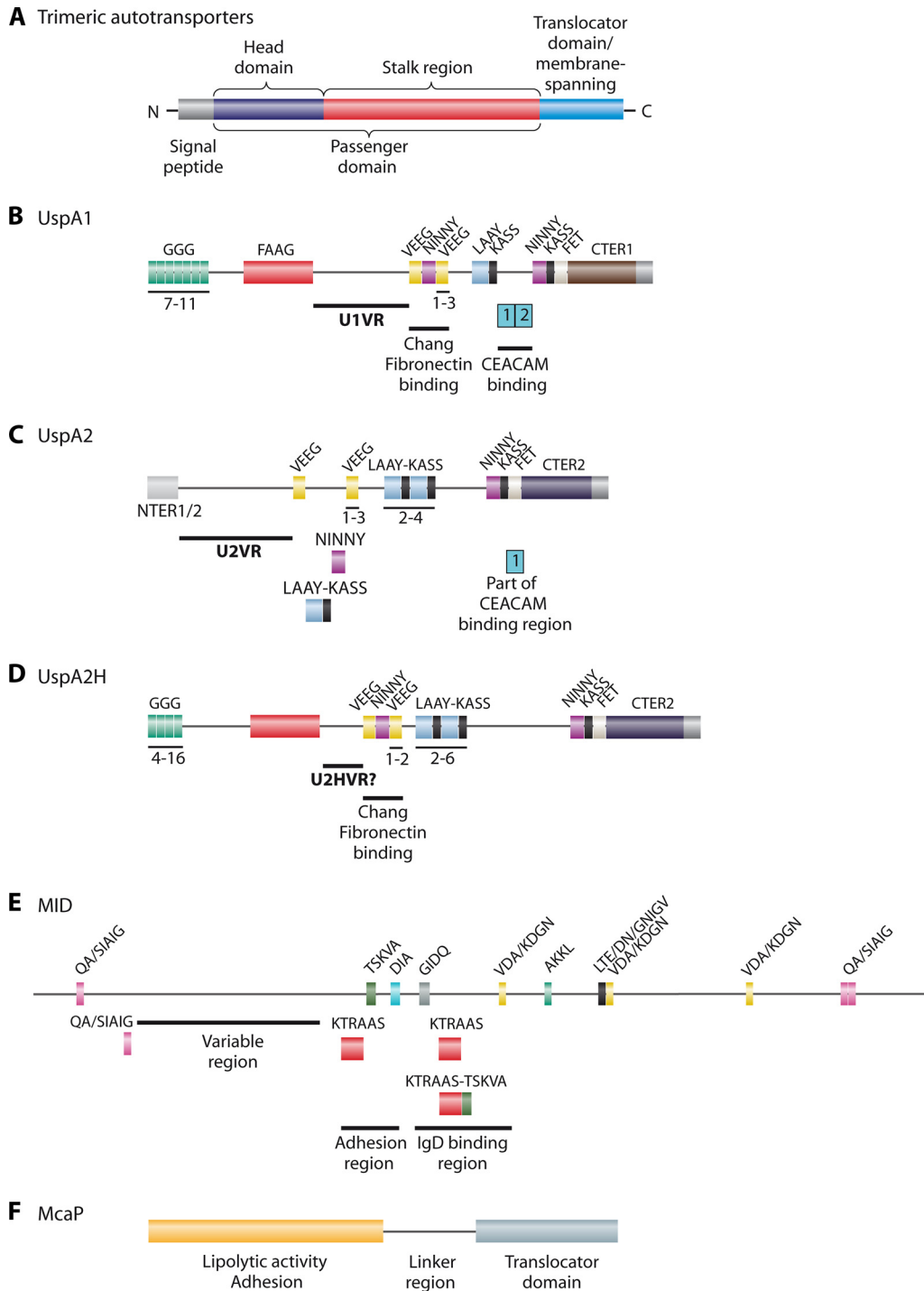


FIG. 1. Modular arrangement and functional domains of the *M. catarrhalis* trimeric autotransporter proteins. (A) Schematic representation of the basic elements of trimeric autotransporter proteins. (B, C, and D) The UspA proteins. The various sequence cassettes, as well as known functional domains, are indicated. CTER1 and CTER2, C-terminal regions 1 and 2, respectively; NTER1/2, N-terminal region 1/2. (E) MID. Indicated are the sequence motifs shared between MID variants, and the regions involved in adhesion and IgD binding. (F) Basic structural elements of McaP.

of the *uspA1* open reading frame. During DNA replication, these repeats are prone to slipped-strand mispairing, resulting in the removal of one or more G residues and thereby influencing the level of UspA1 expression (82). Further, expression

of *uspA1* correlates with the number of poly(G) residues, which in turn influences in vitro adherence capacity (82). In fact, isolates with 10 G residues express levels of *uspA1* significantly higher than those of isolates with nine G residues in

their poly(G) tract, and isolates of the same strain have been found to exist with either 9 or 10 G residues in their poly(G) tract. Interestingly, expression of *uspA1* and *uspA2* has been found to be associated with different 16S rRNA subtypes, as strains with normal *uspA1* and *uspA2* expression levels are of 16S rRNA type 1, and strains with negligible expression are predominantly of 16S rRNA types 2 and 3 (95). Additionally, the expression of *uspA1* could be induced by cold shock (26°C) in strains of all three 16S rRNA subtypes (63), although up-regulation was most prominent in strains that normally exhibited negligible *uspA1* expression, namely, those of 16S rRNA subtypes 2 and 3. Whether this cold shock-induced increase in *uspA1* expression is mediated by variation in the poly(G) tract has not yet been investigated. However, cold shock could lead to the accumulation of specific mRNAs due to transcriptional and posttranscriptional responses in a manner similar to that observed in *Streptococcus pyogenes*, in which 9% of the genes have been found to be differentially expressed upon cold shock (140). Cold shock-induced upregulation of *uspA1* could be beneficial for survival in the nasopharynx, especially the nose, which experiences regular temperature fluctuations and is generally cooler than the rest of the body (63).

One of the receptors on human epithelial cells to which UspA1 binds has been found to be the CEACAM1 (67, 68). This Ig-related glycoprotein is expressed on a variety of epithelial and endothelial cells as well as on leukocytes (52, 57). CEACAMs are also known to play a role in inter- and intracellular signaling events involved in growth and differentiation (52). Interestingly, the targeting of CEACAM receptors is also a feature of other mucosal pathogens, such as *H. influenzae* and *Neisseria meningitidis* (67). The CEACAM1 receptor itself consists of a single distal Ig variable region (IgV)-like domain and three Ig constant region 2-like domains (all four facing the extracellular environment), a transmembrane domain, and an immunoreceptor tyrosine-based inhibitory motif (ITIM) (52). UspA1 binds to the extracellular IgV-like domain of CEACAM1 on the surface of epithelial cells (68). The CEACAM1-binding motif is preceded by the highly conserved LAAY-KASS repeat and is located apart from the N-terminal head domain, namely, in the stalk region (Fig. 1B) (22, 30). CEACAM binding initiates bending of the coiled-coil stalk regions, possibly to allow closer contact to the epithelial cell surface (30). Recently, naturally occurring UspA1 variants lacking the CEACAM-binding motif were identified in several *M. catarrhalis* isolates, and these conferred different cell adhesion properties and probably elicit different host responses (23). The binding of UspA1 to CEACAM1 has been proposed to induce apoptosis in pulmonary epithelial cells, which could contribute to the pathogenesis of COPD and emphysema by inducing secondary inflammation (105). Furthermore, the interaction of UspA1 with CEACAM1 enables *M. catarrhalis* to suppress the host inflammatory response (139), a mechanism discussed in more detail in Evasion of the Host Immune System below.

The UspA2 protein does not interact with CEACAM molecules on host cells but does bind to the ECM proteins laminin (142), fibronectin (143), and vitronectin (92). Interaction of pathogens with vitronectin has been associated with both cell adhesion and complement resistance (92).

At the present, UspA2H is the least well studied of the three *M. catarrhalis* UspA proteins, although it is reported to medi-

ate binding to Chang cells, as it also possesses the Chang cell/fibronectin binding motif (Fig. 1D) (22, 80).

MID/Hag. MID (200 kDa), also referred to as Hag, has been found to be responsible for *M. catarrhalis* binding to soluble IgD and for the stimulation of high-density IgD-bearing B lymphocytes (42–44, 109). In fact, nonimmune binding of Igs by gram-positive bacteria is relatively common but rare among gram-negative bacteria (51, 75). Interestingly, *H. influenzae* also displays a strong affinity for soluble human IgD (125), although the protein involved in IgD binding has as yet been identified only in *M. catarrhalis*. MID is also a B-lymphocyte mitogenic stimulant that initiates Ig production (49), and a comparison of MIDs of five strains revealed that the overall sequence identity ranged between 65.3% and 85.0%, with some regions showing an identity up to 97% (98).

Essentially, MID/Hag is a multifunctional protein that fulfills an important role in pathogenesis. For example, MID-expressing isolates agglutinate human erythrocytes and adhere to A549 cells, and the extent of these actions has been found to correlate with MID expression levels (41). In addition, Hag mediates attachment to human middle ear epithelial cells (26, 73), NCIH292 lung epithelial cells (25), and Chang cells (25, 109). In a study by Bullard et al., isogenic *hag* mutants of *M. catarrhalis* V1171, O35E, McGHS1, and O12E were tested for their adhesion capacities (25). These strains expressed normal levels of UspA1, UspA2H, McaP, and OMP CD, but their adherence capacities were significantly lower after the deletion of *hag* (25). Additionally, a significant loss of adhesion was observed when a strain with high-level MID expression (Bc5) was incubated with an anti-UspA1 antibody (41). These results suggest that several *M. catarrhalis* adhesins act in concert in order to mediate binding to host target cells. The multimeric MID/Hag is classified as an autotransporter protein with a 10- β -barrel translocator domain and an N-terminal globular head domain with both adhesion and IgD binding activity (Fig. 1E) (56). Expression of MID/Hag is subject to translational phase variation via slipped-strand mispairing in a poly(G) tract located at the 5' end within the open reading frame (26, 98). Möllenkvist et al. showed that *mid* mRNA and MID were present in strains with 1, 2, or 3 triplets of G residues, whereas both were found to be absent in strains with 4, 7, 8, or 10 G residues. In addition to translational phase variation, it has been suggested that MID is regulated at the DNA level by *trans*-acting elements binding to the poly(G) tract or at the RNA level by regulation of the stability of the *mid* mRNA (98, 121). Interestingly, when isolates with high-level *mid* expression were subcultured multiple times, a population with low-level *mid* expression developed, and its members appeared to have lost a G residue in the poly(G) tract (98).

McaP. Adhesion studies using *uspA1* and *hag* knockout mutants indicated the presence of another adherence factor. Indeed, a gain-of-function (adhesion) approach using a nonadherent *Escherichia coli* strain led to the identification of a novel adhesin that exhibited both phospholipase B and esterase activities (145). This 62-kDa OMP, named McaP, was classified as a conventional autotransporter protein containing a 12- β -barrel translocator domain and an N-terminal passenger domain that harbors both adhesion and lipolytic activity (Fig. 1F) (83, 145). McaP was found to be highly conserved, as sequence analysis of nine isolates showed 98 to 100% identity over the

entire length of the protein (83). Recombinant *E. coli* cells expressing *mcaP* displayed increased adhesion to Chang cells, A549 cells, and 16HBE14o⁻ polarized human bronchial cells (145). Further, an *M. catarrhalis* O35E *mcaP* knockout mutant displayed reduced adherence to different epithelial cell lines and abolished esterase activity (145). However, the exact role of McaP in pathogenesis remains to be elucidated. Phospholipases are known to be involved in a diverse range of cellular functions, inducing host inflammatory responses, changing membrane composition, and altering signaling cascades (136). The importance of esterase activity for pathogenesis is currently unknown.

OMP CD. OMP CD is a heat-modifiable highly conserved porin-like protein (46 kDa) that also plays an important role in the process of adhesion (102). OMP CD functions as an adhesin for both middle ear mucin (84, 120) and A549 cells (72). It is anchored in the outer membrane via a β -barrel domain, which connects to the C-terminal domain via a linker region. Both the β -barrel domain and the linker region are important for adhesion to A549 cells, with bioinformatic analysis revealing that the linker region harbors a eukaryotic adhesion motif, the thrombospondin type III repeat (7).

Mha proteins. The FHA-like proteins MhaC, MhaB1, and MhaB2 were shown to function as a two-partner secretion locus (TPS) (14, 117). TPS systems are widespread in gram-negative bacteria and are mostly involved in the secretion of virulence proteins. A TPS typically consists of a transporter protein located in the outer membrane and the transported/secreted protein partner(s). Well-known examples include the FHA protein of *Bordetella pertussis* (69) and Hag-related protein (Hrp) of *Neisseria meningitidis* (127), both of which have also been shown to be functionally involved in adhesion. The MhaC protein is predicted to form a β -barrel porin-like structure in the outer membrane, facilitating the transport of MhaB1 and MhaB2 across the bacterial outer membrane. Disruption of the *mhaC* gene locus results in an absence of the MhaB protein in the outer membrane. All three Mha proteins are highly conserved and have been found to be expressed in several isolates (14). Bacterial mutants with mutations of the three *mha* genes (*mhaB1*, *mhaB2*, and *mhaC* mutants and an *mhaB1 mhaB2* double mutant) showed reduced binding to HEp-2 cells, whereas only the *mhaB2* mutant, the *mhaB1 mhaB2* double knockout mutant, and the *mhaC* mutant showed reduced binding to 16HBE14o⁻ cells. *M. catarrhalis* isolates O35E, O12E, and McGSHS1 with an *mhaC* knockout exhibited reduced adhesion to HEp2, Chang, and 16HBE14o⁻ cells but not to A549 or NCIH292 cells. No effect on adhesion to A549 or NCIH292 cells was seen for any of the *mha* knockout mutants (14). These results demonstrate once again that *M. catarrhalis* adhesion relies on the complex interplay between many bacterial components and target cell-specific characteristics.

Adhesion and Surface-Exposed Structures

TFP. TFP are surface-exposed filamentous polymeric structures of assembled pilin proteins. TFP mediate a wide range of cellular functions, including natural competence, adhesion to host cells, biofilm formation, and twitching motility. TFP fulfill a crucial role in pathogenesis, as a loss of TFP results in

reduced virulence for many gram-negative bacteria (34). For *M. catarrhalis*, loss of *pilA* (encoding the major pilin subunit of TFP) results in a significantly reduced adherence to Chang cells and reduced ability to colonize the chinchilla nasopharynx, suggesting that TFP promote attachment to mucosal surfaces (88). Further, in vitro assays revealed that microcolony formation and biofilm formation were abrogated in the *pilA* mutant (88).

LOS. LOS is a major component of the outer membrane and is generally considered to be important for *M. catarrhalis* virulence. It consists of an oligosaccharide core and lipid A. The LOS core oligosaccharide is connected to lipid A via Kdo (3-deoxy-D-manno-2-octulosonic acid) and is composed of glycosyl residues (Fig. 2A) (74). Several glycosyl transferases have been reported to be responsible for the formation of core or branched oligosaccharide chains (85). *M. catarrhalis* LOS is distinct from the typical lipopolysaccharides of gram-negative enteric pathogens because it lacks the O-antigen polysaccharide side chain. LOS of *M. catarrhalis* exists in three serotypes, the most prevalent being serotype A (72%), followed by serotypes B and C (21% and 2%, respectively) (150). Interestingly, a significant difference in incidence between LOS serotypes A and B is apparent among global *M. catarrhalis* isolates from children and adults, with a decrease in serotype A incidence being reported for adults (150).

LOS structures of several respiratory pathogens are known to be critical for adhesion to host target cells, including *N. meningitidis* and *H. influenzae* (50, 76). Knockout mutations of the genes involved in LOS assembly have been used to study the importance of LOS for *M. catarrhalis* adhesion (Fig. 2B). LpxA, a UDP-N-acetylglucosamine acyltransferase, is involved in the initial step of lipid A biosynthesis. An *lpxA* mutant O35E strain was found to be completely devoid of LOS, resulting in clearly diminished adhesion to Chang, A549, and HeLa cervical cells (112). This reduced adhesion to Chang cells was confirmed in another study using an O35E *lpxA* knockout mutant (141). Peng et al. examined the importance of lipid A in vivo in a mouse aerosol challenge model. Directly after aerosol challenge, numbers of *lpxA* mutants recovered from the lungs and nasopharynx were lower (20-fold and 2-fold, respectively) than the wild-type numbers. Further, the *lpxA* mutant was more easily cleared from the nasopharynx than the wild-type strain, although this difference was not observed to occur in the lungs (112). The observed changes could be due to loss of LOS interaction with host components, increased sensitivity to complement-mediated killing by lower membrane stability, or altered surface display of adhesin molecules (112). The *lpxL* and *lpxX* genes encode late acyltransferases that are responsible for the incorporation of secondary acyl chains into lipid A. An *M. catarrhalis* O35E *lpxX* knockout mutant was shown to be more efficiently cleared in a mouse pulmonary challenge model than its wild-type form (48).

The Kdo residues represent the next level of the complete LOS structure and link the lipid A moiety to the oligosaccharide core. The Kdo transferase gene *kdtA* catalyzes the addition of Kdo residues to the lipid A precursor. An O35E *kdtA* knockout mutant formed an LOS structure comprising only lipid A, without any core oligosaccharides. This *kdtA* mutant showed impaired adhesion to Chang and HeLa cells but not to A549 cells, suggesting that the contribution of Kdo to adhesion

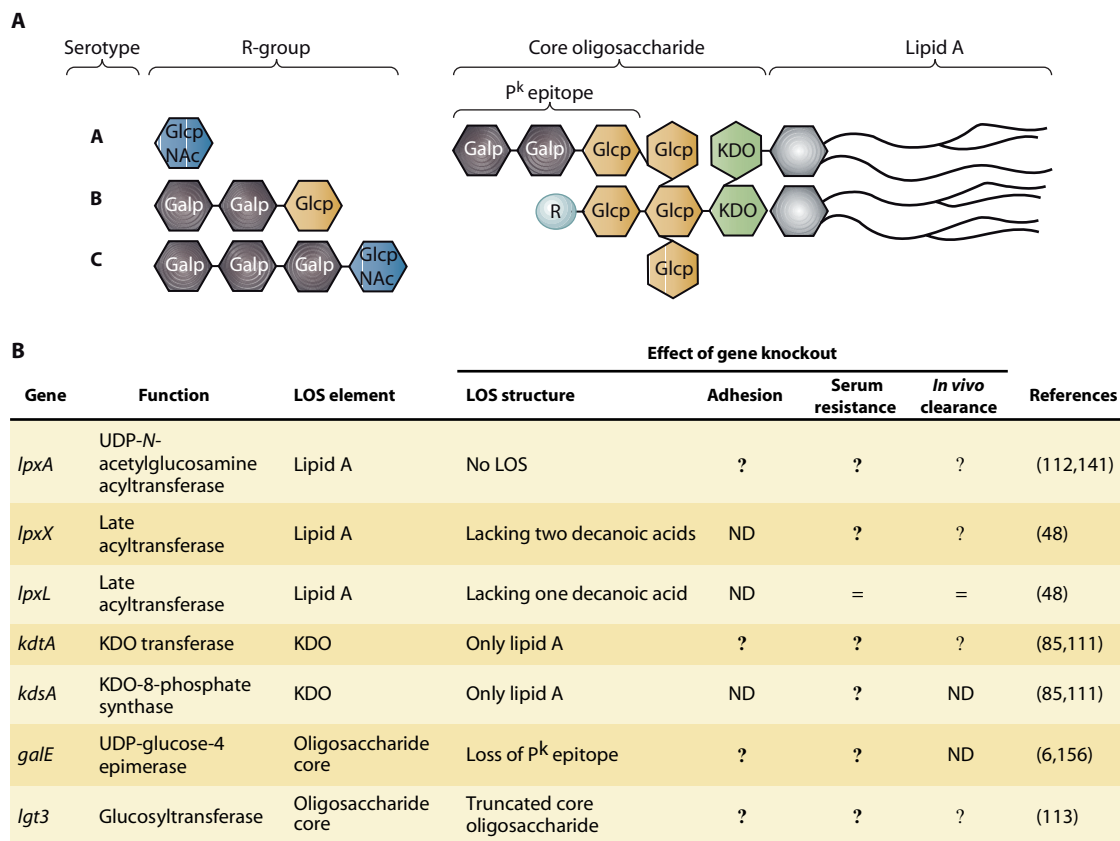


FIG. 2. *M. catarrhalis* LOS biochemical structures and phenotypes of gene knockout mutants. (A) The lipid A moiety is connected to the oligosaccharide core via a Kdo residue. The three *M. catarrhalis* LOS serotypes differ in the nature of their R group (R). (B) LOS biosynthesis genes and characteristics of knockout mutants. Abbreviations: Galp, galactose phosphate; Glcp, glucose phosphate; GlcpNAc, *N*-acetyl-glucosamine phosphate; ↑, increase; ↓, decrease; ND, not determined.

is host cell type specific. Furthermore, the *kdtA* mutant was found to be more efficiently cleared in a mouse pulmonary challenge model than its parental strain (111).

The oligosaccharide chains represent the core structure of LOS. In all three LOS serotypes, one of the oligosaccharide chains terminates with Gal α 1-4Gal β 1-4Glc, which is also known as the P^k epitope (156). Evidence for the involvement of the P^k epitope in LOS-mediated adhesion was obtained from *M. catarrhalis* 2951 (serotype A) lacking the P^k epitope as a result of a UDP-glucose-4-epimerase gene (*galE*) knockout. This *galE* mutant showed reduced attachment to a panel of pharyngeal epithelial cells isolated from healthy adults. However, treatment of the wild-type isolate with LOS or a monoclonal antibody directed against the P^k epitope did not result in reduced adhesion. The authors of the study in question suspected that the loss of the net negative surface charge caused a reduction of adhesion, suggesting that the P^k epitope itself is not an adhesin but is indirectly involved in adhesion (6). An *lgt3* (LOS glucosyltransferase gene) knockout mutant of strain O35E that possessed a truncated core oligosaccharide also exhibited a reduced adhesion capacity to Chang and HeLa cells and was more efficiently cleared in a mouse nasopharyngeal clearance model (113).

The role of LOS in *M. catarrhalis* adhesion is still open to discussion, as alterations or even the absence of LOS can lead

to a lowered membrane integrity and could affect the surface display of adhesin macromolecules in the bacterial membrane. In fact, changes in OMP composition have been demonstrated in an LOS-deficient *M. catarrhalis* O35E strain (*lpxA* mutant) (141).

INVASION OF THE HOST EPITHELIUM

Once *M. catarrhalis* has established itself at its colonization sites, the bacterium has to survive both the host immunological response and environmentally challenging conditions, such as iron limitation. Adhesion to host epithelial cells and ECM macromolecules is an essential step for facilitating pathogenesis of *M. catarrhalis*. As discussed above, OMPs and other surface-exposed macromolecules, such as TFP and LOS, may be key players in this process. However, after adhesion, a critical step of *M. catarrhalis* appears to be invasion of host cells, which would allow *M. catarrhalis* to hide from both the action of the host's immune system (cellular and humoral) and the effects of antibiotic treatment.

An elegant study by Slevogt et al. reported the potential of *M. catarrhalis* to invade different epithelial cell types (138). Scanning and transmission electron microscopy analyses of *M. catarrhalis* strain O35E adhering to BEAS-2B bronchial epithelial cells and A549 cells demonstrated phenotypic changes

in the epithelial cell surface. Both lamellipodia and filopodia were formed after bacterial adhesion, both pointing toward and eventually surrounding the *M. catarrhalis* bacterium, which indicated a macropinocytosis-like mechanism of cellular invasion. Once intracellular, the bacteria were found to reside in vacuolar structures. Furthermore, UV-killed bacteria were used to demonstrate that *M. catarrhalis* actively invades epithelial cells, as no structural changes in the epithelial membrane were observed with UV-killed bacteria and no bacteria were observed inside cells. Additionally, invasion did not appear to be strain dependent, as *M. catarrhalis* ATCC 25238 and O35E were able to invade epithelial cells to similar extents. To examine the specific host factors involved in this process, specific cellular inhibitors were used. These inhibitors revealed that cellular invasion was dependent on microfilaments, Rho-type GTPases, and phosphoinositide 3-kinase (PI3K)-dependent contractile mechanisms but not on microtubules, clathrin-coated pit formation, or tyrosine kinases (138). The exact mechanism of *M. catarrhalis* invasion into epithelial cells still needs to be unraveled, though the ability of *M. catarrhalis* to invade epithelial cells has been reported by other authors (141), who found that the level of invasion of Chang cells by *M. catarrhalis* O35E was actually fivefold higher than the level of invasion of A549 cells by the same strain. These authors also demonstrated that the addition of a purified OMP preparation resulted in reductions of both adhesion and invasion, and a lack of UspA1 (one of the major adhesins) reduced both adhesion and invasion. Further, it was shown that loss of LOS (via an *lpxA* deletion mutant) impaired both adhesion and invasion, though as already mentioned, loss of LOS appears to influence surface display of OMPs, including adhesins. Spaniol et al. (141) also utilized host-specific inhibitors to study invasion and showed that invasion was dependent on clathrin polymerization, which seemed to contradict the earlier findings of Slevogt et al. (138). Furthermore, actin polymerization was also found to contribute to the invasion process, and antibodies directed to fibronectin and integrin $\alpha 5 \beta 1$ were able to inhibit invasion (141). In another publication, Hill et al. demonstrated that blocking of the CEACAM1-binding domain with a recombinant peptide (rD-7, representing the CEACAM1-binding domain of UspA1) resulted in inhibition of cell invasion by *M. catarrhalis*, *H. influenzae*, and *N. meningitidis* (67), indicating the importance of CEACAM1 binding for both adhesion and invasion.

Recently, the invasive capacity of *M. catarrhalis* was demonstrated in vivo, with *M. catarrhalis* being detected in adenoid and tonsil tissues of patients without acute respiratory disease. This subepithelial and intracellular reservoir could potentially lead to long-term persistence of *M. catarrhalis* inside the host. Further, *M. catarrhalis* would be able to interact with B lymphocytes via MID/Hag when present within the subepithelial compartment of lymphoid tissues (62). At the present, the exact mechanism of epithelial cell invasion by *M. catarrhalis* is not fully understood, but it appears to be an active process involving several host cell and bacterial mechanisms.

BIOFILM FORMATION

Biofilm formation has already been demonstrated to be an important process involved in colonization and persistence for

(mucosal) microorganisms (32). Indeed, the capacity of *M. catarrhalis* to form biofilms has been confirmed by in vitro assays (108, 110), and *M. catarrhalis* has been found to be present in biofilms in vivo in the middle ears of OM patients (55). Pearson et al. attempted to identify the genes required for biofilm formation by use of a transposon mutagenesis approach. This led to the recognition that the presence of UspA1 positively affects biofilm formation, while the presence of Hag has a negative effect (110). Later, however, it became apparent that both UspA1 and UspA2H play a role in biofilm formation (108). Recently, Verhaegh et al. reported that *uspA2*-positive isolates were more efficient biofilm formers than isolates carrying the mutually exclusive *uspA2H* gene, suggesting that *uspA2* could also play a role in biofilm formation (150). This study analyzed a large cohort of *M. catarrhalis* isolates obtained from children and adults with respiratory disease from various geographical regions. The capacity for formation of biofilms was greater in the strains isolated from children (<5 years of age), and isolates derived from adults (>20 years of age) showed reduced incidence of *uspA2*. The role of TFP in the formation of microcolonies and biofilms in vitro was investigated by Luke et al. by using a *pilA* mutant, and they showed that microcolony formation and biofilm formation were dependent on TFP (88).

Host factor 1 (Hfq) is a global regulatory protein, highly conserved among *M. catarrhalis* strains and homologous to Hfq of *E. coli*. Hfq probably functions as an RNA chaperone that facilitates the interaction between mRNAs and their cognate small RNAs and, as such, influences the stability or translation of the mRNA. Mutation of *hfq* in *M. catarrhalis* O35E resulted in reduced growth when it was challenged with osmotic and oxidative stresses. This *hfq* knockout mutant also displayed an altered OMP composition, characterized by slightly increased expressions of CopB, OMP J, and OMP G1b. Furthermore, the *hfq* mutant strain showed a growth advantage in a biofilm system, which could be the (indirect) result of an altered outer membrane composition (13).

EVASION OF THE HOST IMMUNE SYSTEM

During colonization of mucosal surfaces, *M. catarrhalis* faces the challenge of resisting the host's innate immune system, which forms the first (nonspecific) line of defense against bacterial pathogens, before evading the host's adaptive immune response. The innate immune system comprises several key components, such as the complement system and pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), NOD-like receptors, and mannose receptors of macrophages (8).

Complement Resistance

Simply speaking, activation of the complement system via the classical, alternative, and/or mannose-binding lectin pathway leads to deposition of complement proteins on the bacterial surface, resulting in the formation of the membrane attack complex (MAC) and opsonization of bacteria for phagocytic killing. Publications have previously shown that *M. catarrhalis* may activate both the classical and alternative pathways (121) and is a weak activator of the mannose-binding lectin pathway

of the complement system (60). However, *M. catarrhalis* does possess mechanisms that have evolved to inhibit activation of complement pathways. Indeed, one important aspect of *M. catarrhalis* pathogenicity is the ability of most clinical isolates to survive complement-mediated killing by normal human serum, with almost all *M. catarrhalis* isolates recovered from OM or COPD patients being resistant to the effect of normal human serum (150). The important factors responsible for complement evasion, namely, UspA2 (2), OMP CD (72), OMP E (100), CopB (65), and LOS (6), are discussed below.

Complement Resistance and Major OMPs

UspAs. The multifunctional aspects of *M. catarrhalis* UspA proteins are illustrated by their roles in adhesion, invasion, and protection against the human complement system. The role of UspA proteins in serum resistance has been described by several authors (2, 11, 12, 16, 147). UspA2 is capable of binding several complement proteins: the C4-binding protein (C4bp) (106), C3 (107), and vitronectin (12, 92). The binding of the complement inhibitor C4bp on the surface of *M. catarrhalis* enables the bacterium to inhibit the classical complement system (Fig. 3A). A *uspA1* knockout mutant of *M. catarrhalis* was also shown to be partially impaired with respect to C4bp binding, but this effect was significantly less apparent than that in a *uspA2*-deficient strain, and this result could be explained by the C4bp affinity of UspA2 being higher than that of UspA1 (106). The UspA2 protein has also been shown to interfere with the deposition of C3b on the bacterial surface by absorbing C3 from serum and thereby preventing activation of the alternative complement pathway (Fig. 3A). Though both UspA1 and UspA2 were found to be able to bind C3, a more dominant role was again observed for UspA2. Vitronectin is a regulatory component of the complement system that inhibits the terminal stages of the complement system by blocking membrane binding of C5b-7 and inhibition of C9 polymerization and eventually inhibiting formation of the MAC (97, 134). UspA2 has been found to bind vitronectin (Fig. 3A) (12, 92, 147), with *uspA2* knockout mutants becoming more sensitive to serum-mediated killing and displaying lower vitronectin binding capacity. In addition, these *uspA2* knockout mutants contained more polymerized C9 than their isogenic counterpart (12). Interestingly, as with *uspA1*, the expression of *uspA2* is subject to phase variation, with the promoter region of the *uspA2* gene containing a variable number of AGAT tetranucleotide repeat sequences (10, 61). UspA2 expression has been found to be maximal at 18 tetranucleotide AGAT repeats, with levels of UspA2 correlating to increased vitronectin binding ability and lower deposition of polymerized C9 (10). Deletion of *uspA2* AGAT repeats results in negligible levels of UspA2 and facilitates the conversion of a phenotype of serum resistance to a phenotype of serum sensitivity (10). Clearly, the multifunctional UspA proteins are indispensable in conferring resistance to the bactericidal effect of human serum.

OMP CD. Serum resistance experiments using an O35E *ompCD* isogenic mutant, which expressed normal levels of UspA1, UspA2, and Hag, showed that this mutant was more sensitive to the effects of serum. However, expression of OMP CD in *E. coli* did not confer serum resistance to this normally

serum-sensitive bacterium (72). The mechanism facilitating OMP CD-mediated serum resistance therefore appears to be functional only in *M. catarrhalis* itself, and further studies are required to determine the exact role of OMP CD in serum resistance.

OMP E. The OMP E protein is a 50-kDa heat-modifiable protein with relatively high sequence conservation. Comparison of the OMP E amino acid sequences of 16 *M. catarrhalis* isolates to the OMP E sequence of *M. catarrhalis* strain ATCC 25240 showed levels of identity of 96.6 to 100% (101). For both *M. catarrhalis* 7169 and ATCC 25240, knockout mutants lacking OMP E appeared to be more sensitive to serum-mediated killing than their parental counterparts. Importantly, sequences of the *ompE* genes were found to remain unchanged in isolates from three patients colonized with the same strain for 3 to 9 months. Further, all three patients showed detectable IgG directed to the OMP E protein (100). However, as with OMP CD, the exact role of OMP E in mediating serum resistance remains to be elucidated.

CopB. The 81-kDa OMP CopB was the first *M. catarrhalis* protein reported to be linked to serum resistance. The effect of knocking out *copB* in *M. catarrhalis* O35E has been studied both in vitro and in vivo. The *copB* mutant showed decreased serum resistance and a lesser ability to survive in a pulmonary clearance mouse model (66). Further, CopB is a major target of the immune response against *M. catarrhalis* (64).

Complement Resistance and Surface-Exposed Structures

LOS. Along with having a role in cellular adhesion, LOS structures are important for resistance against the bactericidal activity of human serum. As with studies examining the role of LOS in adhesion, defined knockout mutants have been used to assess the contribution of LOS structural elements to serum resistance (Fig. 2B). Mutants with deletions of the enzymes responsible for synthesis of the lipid A of LOS have been tested for their ability to resist normal human serum. An *M. catarrhalis* O35E *lpxA* knockout mutant (totally devoid of LOS) and a *kdtA* knockout mutant (which contained only the lipid A moiety) were both found to be more sensitive to the bactericidal activity of human serum than their isogenic parent isolate, with the effect of normal human serum on the *lpxA* mutant being more severe than the effect on the *kdtA* mutant (111, 112). Strains with mutations of the lipid A acyltransferase enzymes, *lpxX* and *lpxL* mutants, were also tested for their sensitivity to human serum. Of these gene knockout mutants, only the *lpxX* mutant strain was clearly more sensitive to human serum than its parental O35E strain, whereas mutation of *lpxL* did not affect serum resistance (48).

The initial step in the synthesis of Kdo is catalyzed by Kdo-8-phosphatase synthase (KdsA), and the transfer of Kdo residues to lipid A is catalyzed by KdtA. Both the *kdsA* and *kdtA* knockout mutants were found to have a severely truncated form of LOS, which consisted only of lipid A without Kdo residues, and both mutants displayed increased sensitivity to serum-mediated killing (85, 111).

As mentioned previously, the *M. catarrhalis* 2951 *galE* mutant lacks the P^k epitope by loss of the terminal galactose residues. This mutant was shown to display elevated sensitivity

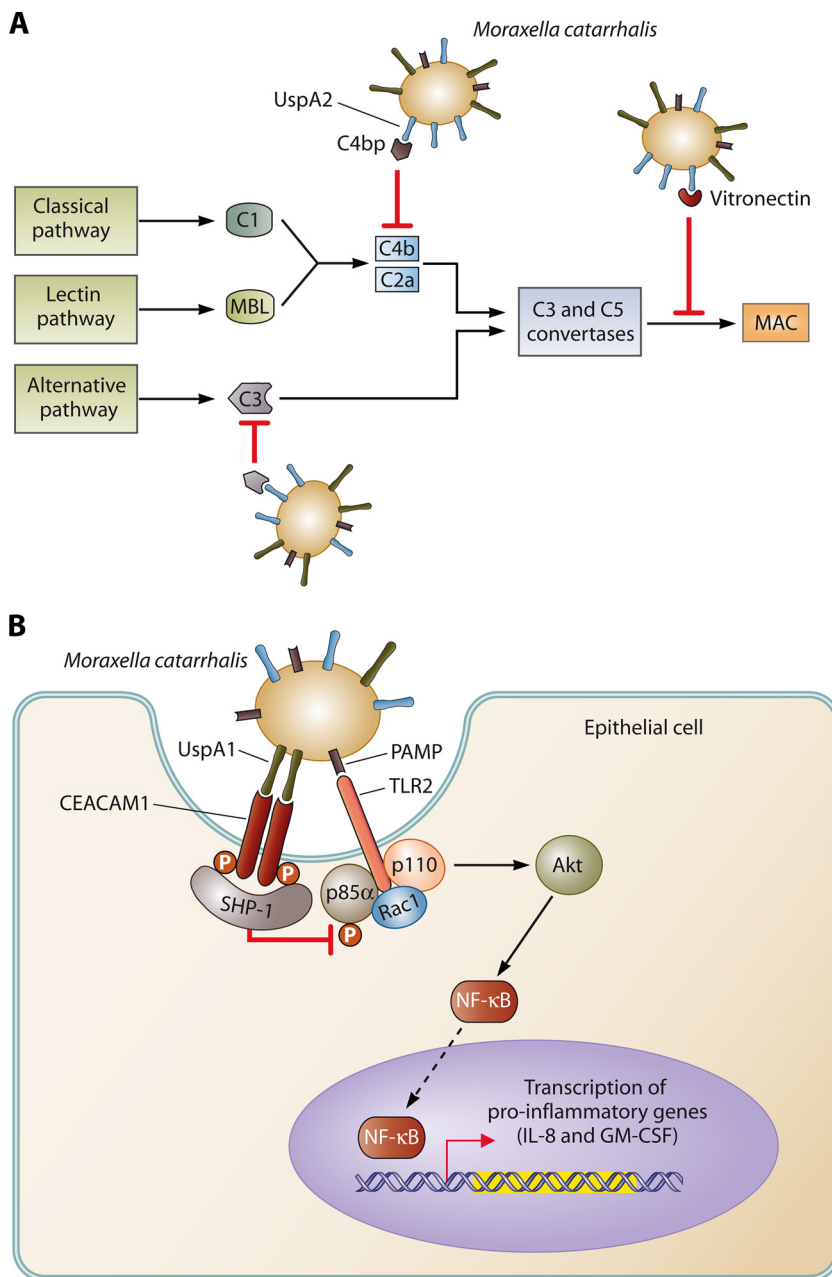


FIG. 3. *M. catarrhalis* immune evasion strategies mediated by the UspA OMPs. (A) Inhibition of the complement system. Both UspA1 and UspA2 fulfill an important role in serum resistance, with a more dominant role for UspA2. The UspA2 protein binds the complement inhibitor C4bp, enabling *M. catarrhalis* to inhibit the classical complement system. UspA2 also prevents activation of the alternative complement pathway by absorbing C3 from serum. Further, UspA2 interferes with the terminal stages of the complement system, the MAC, by binding the complement regulator protein vitronectin. (B) Inhibition of the TLR2–NF- κ B proinflammatory response. The UspA1 protein targets the CEACAM1 molecules on host epithelial cells. *M. catarrhalis* uses UspA1-mediated CEACAM1 binding to counteract the TLR2-initiated proinflammatory response. *M. catarrhalis* PAMP-mediated activation of TLR2 results in the recruitment of the p85 α regulatory subunit of PI3K and finally the transcription of proinflammatory genes, which are regulated by NF- κ B. Binding of UspA1 to CEACAM1 results in increased phosphorylation of tyrosine residues of the cytoplasmic ITIM domain of CEACAM1 and subsequently to the recruitment of SHP-1. SHP-1 inhibits the phosphorylation of p85 α , thus blocking the activation of a proinflammatory response by inhibiting the activation of the PI3K–Akt–NF- κ B pathway. Further details may be found in the text. GM-CSF, granulocyte-macrophage colony-stimulating factor.

to serum-mediated killing. Further, the P^k epitope is also present on the surface of erythrocytes and several epithelial cell types (79), and because of this immunochemical identity, no naturally occurring antibodies toward the P^k epitope of *M. catarrhalis* LOS are likely to be present in human serum. Loss

of the P^k epitope then apparently results in the exposure of a different LOS epitope and, subsequently, to the activation of the complement system (156). Further, the *lgt3* knockout mutant of strain O35E, possessing a truncated core oligosaccharide, also showed increased sensitivity to serum-mediated kill-

ing (113).

Interestingly, most knockout mutants deficient for enzymes of the LOS biosynthetic pathway also displayed slightly to more-severely impaired growth ability, as well as increased vulnerability to hydrophobic compounds. This suggests that the outer membrane structure of these mutants is more permeable and weaker than that of their parental counterpart. With these data taken together, we can conclude that the LOS structure is important for *M. catarrhalis* virulence, directly or indirectly contributing to adhesion as well as to the evasion of the immune system.

Inhibition of TLR2-Induced Proinflammatory Responses

M. catarrhalis uses adhesion and invasion mechanisms to colonize the mucosal surfaces of the human respiratory tract. At the mucosal surface, *M. catarrhalis* appears to activate an inflammatory immune response via the action of TLR2 (a PRR), which is present on the membrane of respiratory epithelial cells and is involved in the immune response (138). Like several other TLRs, TLR2 recognizes a broad range of structurally unrelated pathogen-associated molecular patterns (PAMPs) (93). Upon infection with *M. catarrhalis*, the activation of TLR2 initiates a cascade of reactions that results in the transcription of proinflammatory genes (8), such as those encoding interleukin-8 (IL-8) and the granulocyte-macrophage colony-stimulating factor (9, 137–139). PAMP-mediated activation of TLR2 results in the recruitment of the p85 α subunit of PI3K, a factor important in TLR2-mediated activation of the transcription factor NF- κ B (8, 139). However, research has shown that despite the activation of TLR2, *M. catarrhalis* is able to colonize mucosal surfaces without initiating a TLR2-mediated inflammatory response. In fact, *M. catarrhalis* inhibits this proinflammatory pathway via signaling through the CEACAM1 receptor (Fig. 3B) (139). This mechanism involves a domain present in the stalk region of UspA1, which binds to the IgV-like domain of the CEACAM1 receptor (68). UspA1 binding to the CEACAM1 receptor results in an increased phosphorylation of tyrosine residues of the cytoplasmic ITIM domain and, subsequently, to the recruitment of Src homology 2 domain-containing cytoplasmic protein tyrosine phosphatase (SHP-1). SHP-1 functions by inhibiting the phosphorylation of the p85 α regulatory subunit of PI3K, which is necessary for the activation of the PI3K–Akt–NF- κ B pathway and thus for activation of a proinflammatory response (139). Furthermore, SHP-1 is also reported to inhibit T-cell signaling (52, 104). This inhibition of the TLR2-mediated proinflammatory response (via the action of UspA1 binding to CEACAM1) may be one of the major features facilitating colonization of host epithelial surfaces by *M. catarrhalis*.

Recognition of bacterial components also occurs once intracellular pathogens have invaded host epithelial cells. For example, where TLRs are PRRs present on the outside of the cell, nucleotide-binding oligomerization domain molecules, such as NOD1, function at the intracellular level. NOD1 recognizes gram-negative peptidoglycan and subsequently triggers an NF- κ B-regulated antimicrobial response (115). With respect to *M. catarrhalis*, the importance of TLR2 and NOD1 for the induction of an NF- κ B–IL-8 inflammatory response has been investigated via gene silencing. Upon challenge with *M.*

catarrhalis, IL-8 expression was reduced in both the TLR2- and NOD1-silenced cell lines, though the impact of TLR2 silencing was more pronounced than that of NOD1 silencing. This suggests that most of the *M. catarrhalis* bacteria were found extracellularly rather than in the intracellular compartment. Moreover, even though the intracellular bacteria appeared to be present in vacuole-like structures, recognition by NOD1 still led to an IL-8 response. Apparently, either *M. catarrhalis* can escape from intracellular vacuoles or NOD1 is recruited to the vacuolar membrane (138). Altogether, inhibition of proinflammatory responses and binding of complement factors enable *M. catarrhalis* to evade the innate immune system, possibly facilitating persistent colonization of the host (Fig. 3).

NUTRIENT ACQUISITION

Efficient nutrient acquisition is one prerequisite if *M. catarrhalis* is to successfully colonize the host mucosal surfaces. Growth of *M. catarrhalis* requires several specific environmental conditions and the presence of specific nutrient components. For example, *M. catarrhalis* cannot grow under strictly anaerobic conditions (5), but enzymes involved in anaerobic metabolism are present, and they could confer the ability to grow under microaerophilic conditions (152). Typically, *M. catarrhalis* is not able to utilize exogenous carbohydrates, because it does not harbor the enzymes necessary for transport and processing of these compounds, i.e., it harbors an incomplete Embden-Meyerhof-Parnas pathway, it has an incomplete pentose cycle, and enzymes of the Entner-Doudoroff pathway are completely absent (152). *M. catarrhalis* can utilize acetate, lactate, and fatty acids for growth and is auxotrophic for arginine and possibly proline (78, 152). Other pathogens that colonize the nasopharynx, such as *N. meningitidis* and *H. influenzae*, are also able to utilize only a limited number of carbohydrates, suggesting that the nasopharynx offers a limited carbohydrate availability or a limited variety of carbohydrates (29, 152). This means that other energy-yielding biochemical pathways are likely to be especially important to *M. catarrhalis*. For instance, *M. catarrhalis* harbors an intact glyoxylate pathway, which is consistent with the ability to utilize acetate for energy generation. In addition, all enzymes of the gluconeogenic pathway are present, indicating the importance of this pathway (152). Unfortunately, the relationship between virulence and in vivo nutrient acquisition is a less-studied aspect of *M. catarrhalis* pathogenesis, although a few mechanisms have been shown to be particularly important for nutrient acquisition. These include iron acquisition (119), the nitrate respiratory chain (152), and the M35 porin, which appears to be essential for growth under nutrient-poor conditions (36).

Iron Acquisition

Iron is a key nutrient that is involved in many bacteriological processes but which is not freely available inside the host, where it is complexed with hemoglobin, heme, transferrin, or lactoferrin. Lactoferrin is the primary carrier of iron on mucosal surfaces, whereas transferrin is the most important iron carrier in serum, and the sequestration of iron is an important line of defense against pathogenic bacteria (119). The acquisition of iron by *M. catarrhalis* is mediated by several surface-

exposed iron-binding proteins: two lactoferrin-binding proteins (LbpA and LbpB) (35), two transferrin-binding proteins (TbpA and TbpB) (154), CopB (4), the heme utilization protein (HumA), (46), and the *M. catarrhalis* hemoglobin utilization protein (MhuA) (47).

M. catarrhalis strain ATCC 25240 was found to be able to utilize both human transferrin and lactoferrin as iron sources. Growth under iron-limiting conditions resulted in specific changes in the OMP composition, with at least four proteins being found only under iron-limiting conditions, one of which appeared to be CopB (28). Further, using microarrays, Wang et al. studied changes in gene expression when *M. catarrhalis* was grown under iron-limiting conditions (152). This study reported the upregulation of *lbpA*, *lbpB*, *tbpB*, and *copB* transcript levels, underscoring the importance of the proteins encoded by these genes in iron acquisition (152).

Binding of *M. catarrhalis* to both lactoferrin and transferrin sources of iron is functionally mediated by a TonB-dependent integral membrane protein (LbpA or TbpA, respectively) and a peripheral protein attached to the outer membrane via a lipid tail (LbpB or TbpB, respectively), where the LbpA or TbpA forms a channel for transport across the membrane (53). For the transferrin-binding duo, the *tbpA* gene is highly conserved between strains, whereas *tbpB* displays high-level heterogeneity (103). To study the role of Tbp in iron acquisition from transferrin, *tbpA* and *tbpB* mutants and the *tbpAB* double isogenic mutant for *M. catarrhalis* 7169 have been constructed. The *tbpA* and *tbpAB* mutants had severely reduced abilities to grow on transferrin as the sole iron source, whereas the *tbpB* mutant showed only a slight reduction in growth. This suggested that TbpA is essential for iron acquisition from transferrin and that TbpB fulfills only a facilitative role (86). This phenomenon was also confirmed for the lactoferrin-binding system in *M. catarrhalis* strain N141, in which LbpA was found to be critical for iron acquisition from lactoferrin, with LbpB facilitating this process (17).

The CopB protein appears to be linked to iron acquisition via both transferrin and lactoferrin, being one of the proteins upregulated in response to iron limitation (4, 28). An O35E *copB* mutant was severely impaired in its ability to utilize iron from transferrin or lactoferrin (4).

As well as using transferrin and lactoferrin, *M. catarrhalis* can also use heme as a sole source of iron through the action of HumA (46), although the amount of free heme on mucosal surfaces is rather low in humans (130). HumA, a 91.2-kDa OMP, originally described from *M. catarrhalis* isolate 7169, was found to bind directly to heme, and growth of a *humA*-deficient mutant was found to be impaired when heme was used as the sole iron source. Further, expression of the HumA protein was increased when the bacterium was grown in the presence of heme (46).

Another in vivo available iron source is hemoglobin; however, in most cases, it is not readily available to *M. catarrhalis*, due to the fact that hemoglobin is compartmentalized within erythrocytes. However, it is possible that hemoglobin could become available to *M. catarrhalis* during episodes of acute OM. Furano et al. demonstrated that *M. catarrhalis* was able to use hemoglobin as a source of iron during in vitro growth (47). This led to the identification of a highly conserved 107-kDa OMP, MhuA, which was detected in a collection of geograph-

ically diverse clinical isolates. The MhuA protein of *M. catarrhalis* 7169 directly binds to human hemoglobin, and an *mhuA* isogenic mutant showed reduced growth on hemoglobin as a sole iron source, though not when grown on heme or Fe(NO₃)₃ (47). Further, surface-exposed TFP also appear to be important for growth under iron-limiting conditions. Luke et al. reported an increased expression of surface-exposed pilin under iron-limiting conditions (87). Moreover, the *pilA* gene has been found to be regulated by an iron-responsive transcriptional repressor, Fur (87).

Nitrate Respiratory Chain

As a first step to identify essential metabolic pathways that are important for in vivo survival and pathogenesis, Wang et al. studied gene expression profiles of *M. catarrhalis* ATCC 43617 grown in biofilms in vitro (152). Only a few categories of genes showed increased expression in biofilms, including genes predicted to encode proteins involved in energy generation and enzymes of the nitrate respiratory chain, namely, a nitrate reductase, a nitrite reductase, and a nitric oxide reductase. Although *M. catarrhalis* cannot grow under anaerobic conditions (5), the predicted ability to reduce nitrate to nitrous oxide could provide an alternative mechanism to generate energy under lower oxygen tension (29, 152). The authors of that study also proposed that the predicted potential of nitric oxide reduction could yield some protective capacity to nitric oxide production by macrophages (152). This is supported by the fact that *M. catarrhalis* grown in the presence of nitric oxide-producing compounds spontaneously yielded colonies which were more resistant to nitric oxide (89).

M35 Porin

The M35 porin is surface exposed and constitutively expressed in several *M. catarrhalis* isolates (37). Recently, the M35 porin was found to be important for growth under nutrient-limiting conditions, as *m35*-deficient mutants show impaired growth when cultured under nutrient-poor conditions but not under nutrient-rich conditions (36). This impaired growth under nutrient-poor conditions could be reversed by the addition of glutamic acid, whereas the addition of carbohydrates had no effect. This is consistent with the fact that *M. catarrhalis* does not possess the capacity to utilize exogenous carbohydrates, with the enhanced uptake of glutamic acid possibly serving as an alternative energy source. The *m35* deletion mutant also showed an upregulation of an unknown protein of approximately 40 kDa, which the authors of that study predicted to be responsible for the enhanced uptake of glutamic acid. However, this hypothesis still needs to be confirmed (36). Furthermore, a mouse nasopharyngeal colonization model demonstrated that the M35 porin was essential for in vivo survival (36). These results suggest that M35 is a general porin important for the uptake of essential nutrients, especially under nutrient-poor conditions.

HETEROGENEITY OF THE *M. CATARRHALIS* SPECIES

Population genetic studies of *M. catarrhalis* clinical isolates rely on both molecular and nonmolecular phylogenetic tech-

niques (19, 116, 126, 148, 151, 153). Early molecular epidemiological studies indicated that *M. catarrhalis* isolates are genetically diverse and revealed that there are actually two distinct phylogenetic lineages (19, 59, 126, 148, 153). Recently, the existence of these two lineages was further confirmed by Wirth et al. via multilocus sequence typing (MLST) (153). The first of these two phylogenetic lineages, known as the serosensitive lineage, is characterized by moderate virulence, and is enriched for strains that are sensitive to complement-mediated killing (19, 148). The other, more virulent lineage, the so-called seroresistant lineage, contains predominantly complement-resistant strains (19, 148) and is enriched for strains with the ability to adhere to epithelial cells (19). The seroresistant lineage predominantly harbors isolates of 16S rRNA type 1, whereas 16S rRNA type 2/3 isolates group within the serosensitive lineage (19, 150). The *uspA1* gene was found to be associated with adherent isolates (19) and could be detected in 87 to 99% of the isolates belonging to the seroresistant lineage, whereas only 23 to 36% of isolates belonging to the serosensitive lineage harbored the *uspA1* gene (19, 153). Further, it was demonstrated that all 16S rRNA type 1 isolates express UspA1, whereas this was not an inherited property of the 16S rRNA types 2/3 (94, 150). No difference between the *uspA2* genes of the seroresistant and serosensitive lineages was observed (19), which is contradictory to the fact that *uspA2* is associated with serum resistance. However, it is conceivable that *uspA2* expression in the serosensitive isolates is below the threshold required to become functionally relevant or may even be completely abolished. In addition, Attia et al. have demonstrated that serum resistance is linked to UspA2, but it is not an inherited property of all UspA2 variants (11). The gene coding for the multifunctional Hag protein was found to be absent in almost all isolates belonging to 16S rRNA types 2/3 (17 out of 18), whereas *hag* was detected in almost all (175 out of 176) 16S rRNA type 1 isolates (150). The CopB and OmpCD proteins are associated with seroresistance, and the genes encoding them were found to be present in all isolates (19, 150). Interestingly, *copB* restriction fragment length polymorphism (RFLP) types I/III and II were almost exclusively associated with 16S rRNA type 1, whereas RFLP types 0 and IV were mostly associated with 16S rRNA types 2/3 (150). Similarly, *ompCD* RFLP type 1 isolates were grouped in 16S rRNA type 1, and all RFLP type 2 isolates were classified as being 16S rRNA types 2/3 (150). Further, LOS type B was found exclusively in isolates of 16S rRNA type 1 (150). Taken together, these results underscore the fact that putative *M. catarrhalis* virulence genes are more prevalent in isolates that are phenotypically more virulent, namely, those isolates belonging to the seroresistant 16S rRNA type 1 lineage.

The seroresistant lineage is characterized by a higher homologous recombination rate and higher mutation rate of housekeeping genes than those of the serosensitive lineage (153). Higher homologous recombination rates and horizontal gene transfer can facilitate the rapid spread and acquisition of novel virulence-associated genes in *M. catarrhalis*, as has been shown for the *bro* β -lactamase gene, whose sequence and genetic context suggested that it was acquired by interspecies gene transfer, possibly from a gram-positive bacterium (18, 20). Indeed, *M. catarrhalis* has been shown to easily acquire DNA via its natural competence for DNA transformation,

which appears to be dependent on TFP expression (87, 96). However, mobile genetic elements do not appear to play a major role in the evolution of the pathogenicity of *M. catarrhalis* compared to that for other pathogens, such as the *Bordetella* (118, 135).

The serosensitive lineage is estimated to have differentiated from its ancestral lineage about 50 million years ago, preceding the age of *Homo sapiens* (153). In contrast, based on the branch depths of seroresistant and serosensitive lineages, it has been suggested that the seroresistant lineage diverged from a common forerunner more recently, about 5 million years ago. Interestingly, this divergence coincides with hominid expansion during the Pliocene and Pleistocene (19, 153). Wirth et al. suggested that the expansion of the seroresistant lineage could have been the result of a "second host shift" accompanied by acquisition of virulence factors and/or selection of a successful clone (153). Interestingly, the closest relatives of *M. catarrhalis*, *Moraxella bovis* and *Moraxella ovis*, are infectious for cows and sheep, respectively (114). The long branch distances between the seroresistant and serosensitive lineages suggest that they have been genetically separated for a relatively long period of time, possibly due to geography or by targeting different hosts (153). It appears likely that the serosensitive lineage originally evolved in a separate mammalian species, despite the fact that modern *M. catarrhalis* isolates are specific to humans. Furthermore, it is tempting to speculate that the natural competence of and increased recombination frequency in the seroresistant lineage could have resulted in adaptation to the human host many years ago. Further, both lineages are present in the human host in the same niche, resulting in a secondary genetic contact. Wirth et al. suggested that this may have allowed "gene flow" between the lineages, with, for instance, the genetic diversity of housekeeping genes being imported into the serosensitive lineage and *uspA1* spreading in the opposite direction from the seroresistant lineage to the serosensitive lineage.

In the late 1990s, Enright and McKenzie suggested that the *M. catarrhalis* species has a population structure similar to that of *N. meningitidis*, which possesses a nonclonal (panmictic) population due to its high rate of genomic recombination, with an occasional emergence of successful clones (38). Subsequent MLST of *N. meningitidis* yielded 47 sequence types (STs) from 92 strains (128), and for *M. catarrhalis*, 173 MLST STs were observed among 268 isolates (153). In contrast, MLST of *H. influenzae* serotype b, considered to be monomorphic (clonal), yielded 29 STs from 241 strains (129). These results indicate that the *M. catarrhalis* species comprises a panmictic population of isolates, although serosensitive lineage isolates appear to be more clonal than the seroresistant lineage (153).

The two phylogenetic lineages of *M. catarrhalis* also differ with respect to their disease associations: of the strains belonging to the seroresistant lineage, 52% were isolated from diseased individuals, whereas this was the case for only 14% of the strains belonging to the serosensitive lineage (153). This phenomenon may potentially be caused by the enhanced seroresistance and adhesion properties of the seroresistant lineage (19). Verhaegh et al. performed both phenotypic and genotypic analyses on a large cohort of global clinical *M. catarrhalis* isolates cultured from children and adults with respiratory disease (150). Interestingly, the isolates cultured from the adult

TABLE 1. Major putative virulence factors of *M. catarrhalis*

Protein(s)	Adhesion and invasion	Immune evasion	Nutrient acquisition	Other function	Comments	Key reference(s)
Outer membrane proteins						
UspA1	Adhesion and invasion of epithelial cells, binding of ECM components	CEACAM1-mediated inhibition of TLR2/NF- κ B response, serum resistance, binding of complement regulators		Biofilm formation	Phase-variable expression	3, 22, 23, 30, 68, 80, 82, 105, 106, 107, 139, 141–143
UspA2	Adhesion to ECM components	Serum resistance; binding of complement regulators			Phase-variable expression	2, 3, 10, 12, 106, 107, 142, 143
UspA2H	Adhesion to epithelial cells	Serum resistance		Biofilm formation	Phase-variable expression	80, 108
MID/Hag	Adhesion to epithelial cells	Serum resistance		Negative effect on biofilm formation	Phase-variable expression	25, 26, 41, 42, 73, 98, 110
Mcap	Adhesion to epithelial cells	Serum resistance		Lipolytic activity		83, 145
OMP CD	Adhesion to epithelial cells and middle ear mucin					72, 84
Mha proteins	Adhesion to epithelial cells					
OMP E	Adhesion to epithelial cells	Serum resistance			Upregulated under iron-limiting conditions	14, 117
CopB	In vivo survival (mouse pulmonary clearance model)	Serum resistance			Upregulated under iron-limiting conditions	100, 101
TpPA and TpbB			Linked to iron acquisition via transferrin and lactoferrin		Upregulated under iron-limiting conditions	4, 66
LpPA and LpPB			Iron acquisition via transferrin		Upregulated under iron-limiting conditions	66, 86
HmuA			Iron acquisition via lactoferrin		Upregulated under iron-limiting conditions	17
MhuA			Iron acquisition via heme		Upregulated under iron-limiting conditions	46
M35			Iron acquisition via hemoglobin		In vivo survival (mouse nasopharyngeal colonization model)	47
			General porin			36, 37
Surface-exposed structures						
TFP	Adhesion to epithelial cells, binding to mucosal surface (chinchilla nasopharyngeal colonization model)			Biofilm formation, natural competence	Upregulated under iron-limiting conditions	87, 88
LOS	Adhesion to epithelial cells, involved in invasion of epithelial cells, in vivo survival (mouse pulmonary clearance model)	Serum resistance				6, 48, 85, 111–113, 141, 156

population showed a reduced incidence of *uspA2* and a drift from LOS serotype A to serotype B, possibly due to an increased incidence of anti-UspA2 and anti-LOS serotype antibodies in the adult population, although this hypothesis remains to be confirmed. Additionally, the immunodominant Hag proteins appeared to be downregulated in isolates derived from the adult population (150), also possibly indicating an immune escape mechanism. Clearly, *M. catarrhalis* is a heterogeneous and genetically flexible bacterium that possesses the potential to alter its phenotypic characteristics in order to evade the human immune response.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our understanding of the factors facilitating *M. catarrhalis* pathogenesis is far from complete. However, it has already been shown that a complex, multifactorial interaction, involving a wide range of bacterium- and host-specific macromolecules, takes place between *M. catarrhalis* and its human host. Currently known virulence factors include OMPs, LOS, and metabolic pathways, which are involved in adhesion, invasion, biofilm formation, modulation of the host immune system, and acquisition of nutrients. For reference, the major virulence factors currently known to be associated with *M. catarrhalis* pathogenesis are summarized in Table 1.

M. catarrhalis is an exclusively human pathogen, which means that the applicability of animal models to elucidate its pathogenesis is rather limited. At the present, most experiments are conducted using the mouse pulmonary clearance model and the chinchilla middle ear colonization model (66, 88, 111–113, 123). Although interpretation of the data obtained from such animal experiments is difficult, these models can still yield valuable information regarding the in vivo molecular pathogenesis of *M. catarrhalis*. However, a suitable animal model or an in vitro experimental system that accurately and reproducibly mimics *M. catarrhalis*-human interactions has yet to be developed.

In recent years, research into bacterial molecular pathogenesis has shifted toward high-throughput, genome-based technologies, generating a more comprehensive approach to broaden our knowledge about the molecular mechanisms of pathogenesis. These include the application of microarray expression profiling systems (152), as well as the use of comparative genomic hybridization for the identification of conserved genes (122). In the near future, other high-throughput methodologies, such as signature-tagged mutagenesis (124) and genomic array footprinting (GAF) (15, 27), could be successfully applied for the identification of the *M. catarrhalis* genes (conditionally) essential for pathogenesis. Recent progress in high-throughput sequencing methods and computational analysis, e.g., automated gene prediction and annotation, has led to an enormous expansion in the availability of whole-genome sequence databases. Pathogenesis-related information may be extracted from these large databases by using bioinformatics approaches such that predictions may be made regarding putative virulence mechanisms, such as adhesion, metabolic capacity, and antibiotic resistance, etc. (45). At the present, the partially annotated genome sequence of only a single *M. catarrhalis* isolate, namely, strain

ATCC 43617, is available (122, 152). As *M. catarrhalis* is a highly heterogeneous species, sequencing of many other *M. catarrhalis* isolates would greatly facilitate our understanding of *M. catarrhalis* pathogenesis.

At the moment, several research groups are focusing their attention on the identification of novel vaccine candidates for *M. catarrhalis*. However, the phase-variable expression of important virulence factors, such as UspA1 (82), UspA2 (10), UspA2H (152), and MID/Hag (98), as well as the presence of immune evasion mechanisms, e.g., differences in OMP gene incidence, drift of LOS serotypes (150), and high genotypic heterogeneity, may have major implications for the development of a successful vaccine against *M. catarrhalis*. On the other hand, several virulence factors, such as the UspA family and MID/Hag, contain conserved and immunodominant regions, indicating that antigenic variation may play a more limited role in the immune response to vaccines based on these proteins (81, 94). Nonetheless, current research indicates that a multivalent vaccine will be required to protect against *M. catarrhalis* colonization and pathogenesis.

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Stefan P. W. de Vries received his master's degree (cum laude) in Medical Microbiology from the Radboud University Nijmegen in The Netherlands in 2008. He has been a Ph.D. student in the Laboratory of Pediatric Infectious Diseases at the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, since December 2008. His specific research interests lie in the field of microbe-host interactions and the molecular aspects behind microbial virulence mechanisms. His main research focus is on the molecular aspects of *Moraxella catarrhalis* pathogenesis and the identification of novel vaccine leads.



Hester J. Bootsma is a senior scientist in the Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. Her research interest lies in microbial pathogenesis, particularly molecular epidemiology, genomics, and virulence. She completed her Ph.D. on molecular aspects of the rapid emergence of β -lactam resistance in *M. catarrhalis* at Utrecht University (1999). From 2000 to 2004, she did postdoctoral work on comparative expression analysis of *Bordetella* subspecies in the group of J. F. Miller at University of California—Los Angeles, Los Angeles. In September 2004, she returned to The Netherlands to work on an interinstitutional project focused on the development and application of a novel microarray-based tool (GAF) to identify conditionally essential genes in *S. pneumoniae*. She is currently involved in a European consortium (FP7-Pneumopath) aimed at achieving a comprehensive dissection of pneumococcus-host interactions, as well as a project in which GAF will be applied to *M. catarrhalis* to identify novel vaccine leads.



John P. Hays is currently a postdoctoral scientist working at the Department of Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands. He has received Ph.D. degrees from both Leicester University, Leicester, United Kingdom (1996), and Erasmus MC (2006) and has held postdoctoral positions at the Central Public Health Laboratory, Colindale, London, United Kingdom, and the Central Science Laboratory, York, United Kingdom. He is currently involved in several European funded projects, including those involved in vaccine development for bacterially mediated otitis media (OMVac) and antimicrobial resistance (DRESP2). Dr. Hays has been researching *M. catarrhalis* for approximately 8 years and is the instigator and head organizer of HinMax2008, a conference specifically dedicated to *H. influenzae* and *M. catarrhalis*. He is currently busy organizing the next HinMax conference, which is scheduled to take place in 2011.



Peter W. M. Hermans received his Ph.D. in 1992 at Utrecht University in The Netherlands. Subsequently, he was appointed as a senior scientist at the Armauer Hansen Research Institute in Addis Ababa, Ethiopia (1992 to 1993), as an associate professor, senior scientist, and head of the Laboratory of Pediatrics at Erasmus University Medical Centre in Rotterdam, The Netherlands (1993 to 2005), and as a principal investigator and head of the Laboratory of Pediatric Infectious Diseases at Radboud University Nijmegen Medical Center in Nijmegen, The Netherlands (2005 to the present). In 2008, he was appointed as a professor of Molecular Infectious Diseases at Radboud University Nijmegen, The Netherlands. The research of Professor Hermans aims to improve the molecular understanding of infectious diseases. In particular, the pathogenesis, immunology, and epidemiology of pediatric respiratory infectious diseases are his central research themes. His research continues to contribute to the development of novel tools to diagnose, treat, and prevent infectious diseases, especially bacterial and viral respiratory tract infections in children.



ERRATUM

Molecular Aspects of *Moraxella catarrhalis* Pathogenesis

Stefan P. W. de Vries, Hester J. Bootsma, John P. Hays, and Peter W. M. Hermans

Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre,
 Nijmegen, The Netherlands, Department of Medical Microbiology and Infectious Diseases,
 Erasmus MC, Rotterdam, The Netherlands

Volume 73, no. 3, p. 389–406, 2009. Page 394: Figure 2 should appear as shown (in panel A, note that the serotype C R group should contain two Galp residues instead of three, and in panel B, note the use of ↑ and ↓ symbols).

