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Phenotypical characterisation of *Moraxella catarrhalis* porin M35 and the 16S rRNA type 2/3 subpopulation

Graduate School for Cellular and Biomedical Sciences
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PhD Thesis

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ABBREVIATIONS

AOM	Acute otitis media
BHI	Brain heart infusion
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule-1
Cfu	Colony forming units
CopB	Conserved outer membrane protein B
COPD	Chronic obstructive pulmonary disease
DAPI	4', 6'-diamidino-2-phenylindole
2-D Gel	2-dimensional gel electrophoresis
EDTA	Ethylene-diamino-tetra-acetate
FGF-2	Fibroblast growth factor-2
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
Hag	Hemagglutinin
HEPES	Hydroxyethyle-1-piperazinyl-ethansulfonic acid
HumA	Hemin utilization protein of *Moraxella catarrhalis*
ICAM-1	Inter cellular adhesion molecule
Ig	Immune globulin
IL	Interleukin
IFNγ	Interferon-γ
LB	Luria-Bertani medium
LbpA	Lactoferrin-binding proteins A
LbpB	Lactoferrin-binding protein B
LOS	Lipooligosaccharide
M35	Outer membrane porin M35

MALDI-TOF	Matrix assisted laser desorption/ionization - time of flight
MCP-1	Monocyte chemotactic protein-1
MIC	Minimum inhibitory concentration
MID	*M. catarrhalis* IgD-binding protein
McaP	*M. catarrhalis* adherence protein
MhuA	*M. catarrhalis* haemoglobin-utilization protein
OD	Optical density
OlpA	*M. catarrhalis* Opa-like protein
OM	Outer membrane
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PCV7	Heptavalent pneumococcal conjugate vaccine
PBS	Phosphate-buffered saline
RFLP	Restriction fragment length polymorphism
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TEM	Transmission electron microscopy
TbpA	Transferrin-binding protein A
TbpB	Transferrin-binding protein B
TNFα	Tumor necrose factor α
UspA1	Ubiquitous surface protein A1
UspA2	Ubiquitous surface protein A2
UspA2H	Ubiquitous surface protien A2 Hybrid
VEGF	Vascular endothelial growth factor

OVERVIEW AND SUMMARY OF THE THESIS

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Moraxella catarrhalis is a gram-negative diplococcus which exclusively colonizes the human nasopharynx exerting a dual function as a commensal inhabitant as well as a pathogen of the upper respiratory tract. Next to *Streptococcus pneumoniae* and *Haemophilus influenzae, M. catarrhalis* is the third major cause of acute otitis media (AOM) in young children. Furthermore it causes 10% of all bacterial exacerbations in patients with chronic obstructive pulmonary disease (COPD). Since the introduction of the pneumococcal conjugate vaccine PCV7, a shift in the pharyngeal colonization and the species causing AOM, from *S. pneumoniae* to *M. catarrhalis* and *H. Influenzae* could be observed. The recognition of *M. catarrhalis* as a pathogen and the increasing infection rates focused research endeavors towards the development of a vaccine and the investigation oft he still poorly understood host-pathogen interactions.

AIM 1

The outer membrane protein M35 is a highly conserved porin of type 1 strains of *M. catarrhalis*. It was previously shown that M35 is involved in the uptake of essential nutrients required for bacterial growth and for nasal colonization in mice.

The first aim of this thesis focused on the:

(i) Characterization of the M35 phenotype regarding functionality and putative virulence factors
(ii) Determination of the potential of M35 as a vaccine candidate
(iii) Analysis of the degree of M35 conservation in type 2/3 strains

The experiments were performed with three different wild-type strains and their respective isogenic *m35* mutants of the *M. catarrhalis* 16S rRNA type 1 subpopulation, namely O35E, 300 and 415. All examined strains were indistinguishable with respect to the phenotypes of autoagglutination, serum resistance, iron acquisition from human lactoferrin, adherence to and invasion of respiratory tract epithelial cells as well as pro-inflammatory stimulation of human monocytes **(chapter 3.1)**. To determine whether M35 is detected as an antigen by the human host immune system and thus of potential value as a vaccine candidate, immunoblot analyses were performed. Results revealed the presence of anti-M35 IgA in the human saliva from various healthy donors **(chapter 3.1)**. A high degree of conservation of M35 had previously been described for *M. catarrhalis* type 1 strains, only. DNA sequencing of *m35* from the phylogenetic subpopulation type 2/3 strain 287 revealed 94.2% and 92.8% identity on the DNA and amino acid levels, respectively, in comparison with type 1 strains **(chapter 3.1)**. For investigating M35 being involved in the uptake of antimicrobial agents, the wild type strains O35E, 300 and 415, respectively, and their *m35* knockout mutants were tested by examining their antimicrobial susceptibilities to 15 different agents by E-tests®. Differences in the Minimum Inhibitory Concentration (MIC) between wild-type and mutant strains were found for eight antibiotics, whereas the biggest differences could be observed for ampicillin and amoxicillin. A statistically significant 2.5 to 2.9-fold MIC increase ($p<0.03$) in the *m35* mutants compared to their respective wild type strains **(chapter 3.1)** was found. Screening of 52 middle ear isolates concerning the presence or absence of *m35* resulted in positive PCR products for all tested strains. The analysis of *m35* mRNA expression after amoxicillin treatment by real time PCR, showed an 24-85% down regulation compared to the respective amoxicillin-free controls in all three strains tested **(chapter 3.2).** Also, the immunoblot analysis of protein concentrations revealed lower M35 expression after growth in presence of amoxicillin than in its absence **(chapter 3.2)**. The investigation of M35 involved

in further stress responses resulted in porin down regulation induced by the growth at 26°C and 42°C, at hyperosmolar stress as well as at conditions of iron restriction **(chapter 3.2)**.

In conclusion, a potential new resistance mechanism against aminopenicillins in *M. catarrhalis* was established, which could affect amoxicillin therapy in a clinically relevant manner. Furthermore, the results indicate a major role of M35 in general stress responses of *M. catarrhalis*. The presence of IgA antibodies in healthy human donors indicates that M35 is expressed *in vivo* and recognized as a mucosal antigen by the human host. However, further analyses have to be performed to identify immunoreactive epitopes and in elucidating differences in the *m35* sequence of the type 2/3 strains, before M35 can be considered as a potential vaccine candidate.

AIM 2

M. catarrhalis consists of two different phylogenetic subpopulations discernible by the sequence of their 16S rRNA subunit: The phylogenetically more recent - 5 million years old - type 1 lineage and the older – 50 million years old - type 2/3 lineage. Type 1 strains are associated with virulence factors such as the ability to adhere to and invade human epithelial cells, complement resistance, natural competence, as well as adaptation abilities to changing environmental conditions in the host. In contrast, the type 2/3 subpopulation is generally serum sensitive, typically lacks the expression of proteins important for type 1 virulence traits, and presents the smaller proportion of clinical isolates compared to type 1 strains. Therefore, type 2/3 strains are regarded as the 'less virulent' lineage and research thus far mainly focused on genetic analyses.

The second project investigated in detail:

(i) Putative type 2/3 virulence traits with regard to their ability to invade human epithelial cells as well as their pro-inflammatory activity
(ii) The influence of cold shock treatment on type 2/3 strains with respect to adhesion and invasion respectively their protein expression

The investigation of different type 2/3 strains resulted in significantly greater invasion ratios into Chang conjunctival cells, A549 lung cells, Hep-2 laryngeal and Detroit 562 pharyngeal cells compared to type 1 strains **(chapter 3.3)**. Intracellular type 2/3 bacteria could be identified by electron microscopy **(chapter 3.3)**. To determine the host cell structures involved in the process of invasion of type 2/3 strains compared to type 1 strains, 'invasion-inhibition-experiments' into Detroit 562 cells were performed. Results indicated that the same host cell structures were involved – actin but no clathrin polymerization – in invasion for both lineages **(chapter 3.3)**. Long-term experiments to investigate the fate of the invaded bacteria showed no long-time persistence, but indicated that bacteria evaded the intracellular space and proliferated extracellularly **(chapter 3.3)**. Analysis of the pro-inflammatory stimuli exerted by the type 2/3 lineage resulted in an IL-8, IL-6, MCP-1 and VEGF response, respectively, but not in IL-1β or TNFα release of Detroit 562 cells. These findings were consistent with type 1 bacteria **(chapter 3.3)**.

In summary, the significantly greater invasion ability of type 2/3 strains compared with type 1 strains as well as the comparable pro-inflammatory stimulation demonstrate putative novel virulence factors of the previously considered ‘non virulent’ lineage. These findings, together with their still largely unknown outer membrane surface structures and functions, warrant further investigations of this phylogenetically old *M. catarrhalis* subpopulation.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 *Moraxella catarrhalis*

Moraxella catarrhalis, a gram-negative aerobic diplococcus, is an important and common human respiratory tract pathogen whose clinical significance was unnoticed for the most of the past century [1-3]. It was long regarded as a harmless upper respiratory tract commensal organism until new diagnostic methods helped to identify and recognize it as being involved in upper respiratory tract infections [3-5]. *M. catarrhalis*, previously known as *Branhamella catarrhalis* or *Neisseria catarrhalis*, consists of two different phylogentic subpopulations differing in their 16S rRNA subunit. Type 1 is the phylogenetically more recent subpopulation exisiting around 5 million years. In contrast, to the 16S type 2/3 subpopulation, which is the older lineage (approximately 50 million years), type 1 isolates are generally associated with virulence factors such as complement resistance, the ability to adhere to and invade human epithelial cells, biofilm formation, natural competence for DNA transformation and cold shock response. Type 2/3 isolates are mainly complement sensitive, lack natural competence, poorly adhere to human epithelial cells in vitro and appear less virulent because of reduced or absent expression of virulence-associated outer membrane proteins (OMP) [6-11]. The mentioned long branch distances between the seroresistant and serosensitive lineages suggest that they have been genetically separated for a relatively long time period, possibly because of geography or different hosts [11]. It is been discussed that the serosensitve subpopulation originally evolved in a separate mammalian species whereas the seroresistant *M. catarrhalis* evolved as an exclusively human pathogen. Nevertheless, both lineages colonize the human host and the same niche, possibly resulting in a “gene flow” between the subpopulations and the exchange of housekeeping genes and therefore specific adaptation of 16S type 2/3 genes to humans. The two phylogenetic lineages also differ in their

frequency among clinical isolates from diseased people. More than 50% of the isolates are from the seroresistant 16S rRNA type 1 and just 14% from the serosensitive 16S rRNA type 2/3 subpopulation [11].

1.1.1 Clinical significance of infections caused by *Moraxella catarrhalis*

The recognition of *M. catarrhalis* as a respiratory tract pathogen led to a comprehensive analysis of the microbiology, epidemiology, pathogenesis, genetics and host response within the last two decades. The two most common infectious diseases caused by *M. catarrhalis* are acute otitis media (AOM) and exacerbations of chronic obstructive pulmonary disease (COPD). Clinical studies show that *M. catarrhalis* causes 15%-20% of AOM in young children up to three years of age (figure 1) [12-18] as well as 10% of bacterial exacerbations of COPD [19]. In addition, ~20% of bacterial sinusitis, and rare cases of bacteraemia, pneumonia and other invasive infections are caused by *M. catarrhalis* [3].

1.1.2 *Moraxella catarrhalis* and otitis media

Acute otitis media is the most common bacterial infectious disease in childhood and the most common reason why children receive antibiotics. Up to 80% of children have at least one episode of acute middle ear infection, 30% suffer three episodes or even more [20]. *M. catarrhalis* is the third major pathogen causing bacterial AOM next to *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae (*figure 1) [3].

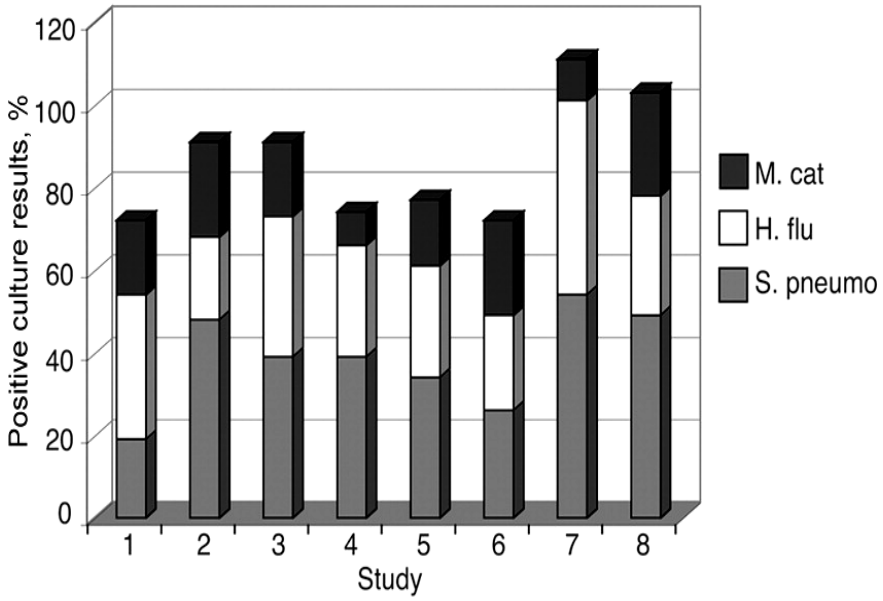

FIGURE 1

Bacterial causes of otitis media. *Results presented of 8 studies published from 1992 to 2006.[12-18]. Bars represent the percentage of middle ear fluid samples that were positive by culture. The black portions of the bars represent Moraxella catarrhalis, the white portions represent nontypeable Haemophilus influenzae and the gray portions represent Streptococcus pneumoniae. Figure adapted from Murphy et al 2009 [3].*

The pathogenesis of otitis media involves migration of bacterial pathogens from the nasopharynx to the middle ear. Therefore nasopharyngeal colonization patterns are important determinants. New nasopharyngeal colonization studies recently show that since the introduction of the pneumococcal conjugate vaccine PCV7 colonization by *M. catarrhalis* and *M. catarrhalis*-caused infections increased by about 40% and 18%, respectively (figure 2) [21].

Children who come down with more than four episodes of acute otitis media or at least eight months of middle ear effusion in one year are defined as otitis prone [22]. These children run the risk of suffering permanent hearing loss, with the result of delays in speech and language development as well as cognitive skills [23]. PCR (polymerase chain reaction) analyses of samples isolated from children with otitis media with effusion identified bacterial DNA from *H. influenzae*, *M. catarrhalis* or *S. pneumoniae* in up to 80% of cases [24-27]. Furthermore, *M. catarrhalis* DNA was detected in larger proportions in cases of otitis media with effusion than of acute otitis media. This could be explained by the ability of *M. catarrhalis* to form biofilms, which are increasingly recognized as playing an important role in recurrent otitis media and otitis media with effusion [24-28].

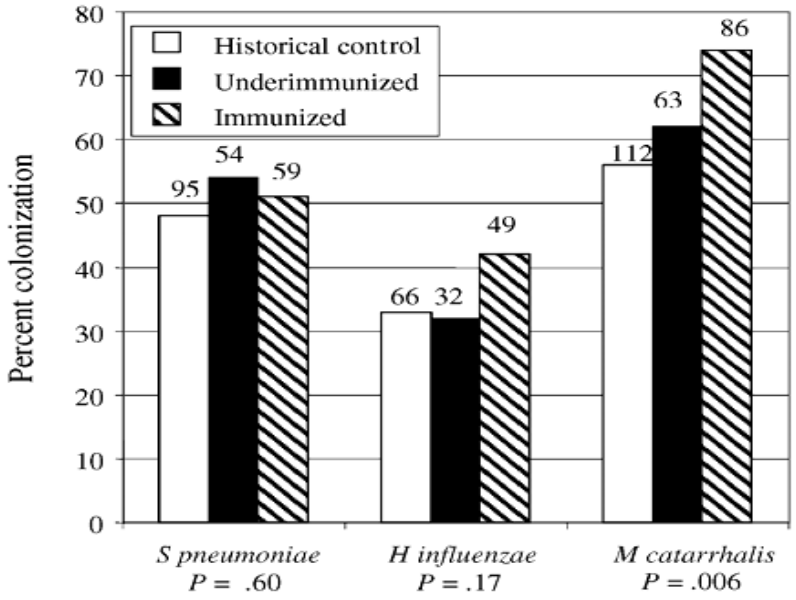

FIGURE 2

Nasopharyngeal colonization with the three main bacterial causes (S. pneumoniae, H. influenzae and M. catarrhalis) of otitis media at the time of AOM proportional to the vaccine status. Children may be colonized with >1 type of bacteria. The numbers above each bar indicate the total number of cases in each group. Figure adapted from Revai et al. 2006 [21].

1.1.3 *Moraxella catarrhalis* and COPD

COPD – chronic obstructive pulmonary disease is often accompanied by exacerbations – intermittent worsening of symptoms – such as increased sputum production, sputum purulence and dyspnoea. Estimations indicate that one-half of exacerbations are caused by bacterial infections [29], predominantly by *H. influenzae*, but also by *M. catarrhalis* and *S. pneumoniae*. Studies suggest that *M. catarrhalis* is an important cause of exacerbations of COPD [30-32] evidenced by: (a) the presence of *M. catarrhalis* in lower airways detected by bronchoscopy [31, 33-35]; (b) the acquisition of new strains during exacerbation [36, 37]; (c) the development of strain specific protection [37]; (d) new systemic and/or mucosal antibody responses [37-41], and (e) an increased airway inflammation in *M. catarrhalis* culture positive exacerbation [42, 43].

1.1.4 Antimicrobial chemotherapy and resistance development

Antimicrobial chemotherapy of otitis media, is the most frequent reason for antibiotic prescriptions in the United States [23, 44-47], based on a therapeutic benefit of about 10-25% [48], the prevention of prolonged periods of decreased hearing secondary to middle ear effusion, and the prevention of suppurative complications resulting from contiguous spread of infection, e.g., mastoiditis [49].

Standard or high dose amoxicillin is still the recommended therapeutic standard in Europe as well as in the USA [47, 50, 51]. The routine use of antibiotics in the otitis media therapy implies an enormous selective pressure on the upper respiratory flora, resulting in an increase of treatment failures – related on β-lactamase producing or otherwise drug resistant isolates [47]. Especially the spread of antimicrobial resistant isolates of *M. catarrhalis*

increased dramatically over the last decades. Over 95% of all clinical isolates of *M. catarrhalis* are penicillin resistant - producing either BRO-1 or BRO-2 β-lactamase [3, 52-55]. Additionally, resistance to cefaclor and cefuroxime has recently been reported. Eighty % of the tested *M. catarrhalis* isolates from the UK and Ireland showed resistance to cefaclor and 5% to cefuroxime [56].

1.2 *Moraxella catarrhalis* outer membrane porin M35

The outer membrane protein M35 of *M. catarrhalis* was first described in 2005 by Easton et al. [57]. M35 is a highly conserved protein in *M. catarrhalis* 16S rRNA type 1 strains. Protein structure analysis demonstrated high homology to classic gram-negative porins, such as OMP C from *Escherichia coli* and OMP K36 from *Klebsiella pneumoniae* [57]. Porins are water-filled open channels that span the outer membrane of gram-negative bacteria, chloroplasts and mitochondria, which allow the passive penetration of hydrophilic molecules [58-60]. For gram-negative bacteria different porin types have been characterized and could be classified according to their specific activity, their functional structure and their regulation and expression [58-62]. The predicted structure of M35 consists of 8 surface loops and 16 antiparallel β-sheets (figure 3) [57]. It forms wide water-filled channels with a single-channel conductance of about 1.25 nS and display some selectivity for cations compared to anions [63]**.**

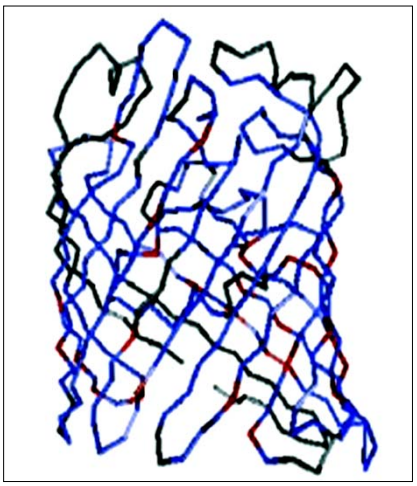

FIGURE 3

Three-dimensional alignment of the M35 amino acid sequence with OMP K36 from Klebsiella pneumoniae*. Red indicates residues that are identical between the two proteins, blue represents similar amino acids, and grey represents non identity. Figure adapted from Eastona et al. 2005 [57].*

Further analysis show that M35 is involved in the uptake of the essential nutrient glutamic acid, but not aspartic acid, glycine, sucrose, or glucose, and indicates an essential function in the survival and colonization of the nasopharynx in mice [63].

1.3 Porins in antimicrobial resistance mechanisms

Antimicrobial resistance of bacteria is an alarming global development with a continuous increase of multidrug resistant strains [64]. The routine but often unnecessary or non-specific use of antibiotics leads to a tremendous selective pressure on the bacteria resulting in the development of complex survival strategies. There are three main strategies bacteria developed for antimicrobial resistance: (a) the membrane barrier which limits the intracellular access of a drug, (b) the production of detoxifying enzymes which degrade or modify an antibiotic, and (c) the target protection barrier which affects the target recognition and therefore the antimicrobial activity [65].

Limiting the intracellular access of an antimicrobial agent by the outer membrane or cell wall of the bacteria can be achieved either by active efflux pumps, which transport the substance out of the periplasm or by inhibiting the influx of the drug. The influx into the bacterial cell is mainly controlled by porins. Therefore, it is not astonishing that one of the main resistance mechanisms is based on the regulation of of porin expression. There are four known alterations in porin expression (figure 4): (i) Decreased expression of the wild type porin, (ii) normal expression of a restricted porin, (iii) normal expression of a mutated porin, mostly based on a point mutation (iv) and normal expression of a wild type porin with a channel blocker [62, 66]. Those altered expressions can be induced after contact with the antibiotic and may be reversible (e.g., decreased expression or porin exchange), or irreversible (e.g., mutated porins). These findings highlight the efficiency of the bacterial adaptive response. Limiting the internal accumulation of antibiotics provides time for the development of additional resistance mechanisms such as the target modification or drug inactivation.

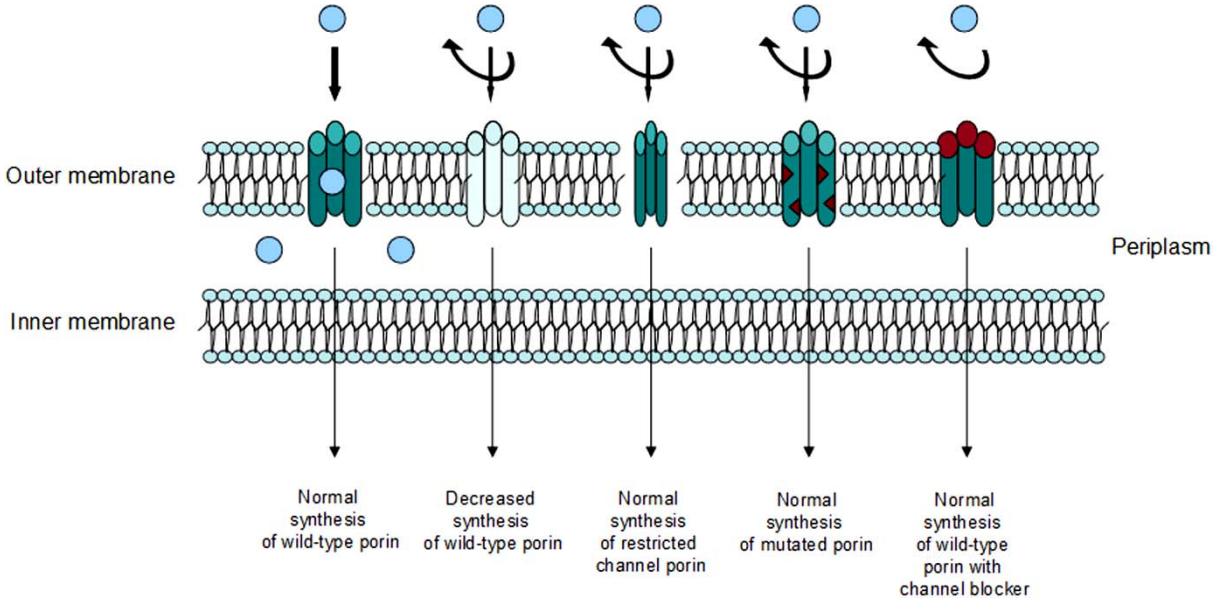

FIGURE 4

Antimicrobial resistance mechanisms associated with alterations in porin expression. *The blue circles represent the antibiotic molecule. The turquoise cylinders represent the porin subunits. The thickness of the straight arrows reflects the level of penetration through the channel. The curved arrows reflect the uptake failure of the substance by an decreased expression of the porin (light turquoise), an exchange in the porin type (restricted porin; smaller cylinders), the expression of a mutated porin (structural changes indicated by red triangles) which affects the function of the porin and the normal synthesis of the porin but with pore blocking molecules (indicated by red circles).*

1.4 Pathogenesis

The characteristics of a pathogen which distinguishes it from harmless or commensal bacteria is the ability to escape the host defence and lead to an infection of the host. Therefore five main steps must be achieved by the bacteria: (a) adherence to and colonization of the host surface (b) the invasion into the host tissue (c) successful proliferation inside the host (d) escape or resistance against the host immune defense (e) and the injury of the host.

Since the recognition of *M. catarrhalis* as a pathogen and the growing interest in developing a vaccine, extensive research has been performed to investigate this species regarding (i) its virulence mechanisms, (ii) its outer membrane compounds to identify potential vaccine candidates and (iii) its infection cycle.

1.4.1 Colonization of the respiratory tract

The colonization of the respiratory tract by *M. catarrhalis* is highly dependent on the host's age but also on health, genetic background of the population, socio-economic conditions and seasonal variations. The rate of colonization in adults is very low (1%-5%), whereas colonization rates in children vary between 80% and 100% [67-69]. Colonization starts early in the first months of life and seems to be highest in the first two years. Thereafter, it decreases to less than 10% in older children and adults [67-70]. This obvious relationship between decreased colonization rate and increased age correlates with the development of the systemic humoral immunity against *M. catarrhalis* [71-74].

1.4.2 Virulence factors

Table 1 shows all investigated components involved in the pathogenesis of *M. catarrhalis* as well as their phenotypic characterisation known to date. Five main virulence mechanisms could be identified: Bacterial attachment, invasion, nutrient acquisition, interactions with the host immune system and biofilm production.

1.4.2.1 Bacterial attachment

Bacterial colonization is initiated by the adhesion to the host's mucosal surface by binding to surface receptors or extracellular components. Adhesion of *M. catarrhalis* is by now well investigated and appears as a multifactorial event mediated by several adhesin molecules. The outer membrane proteins (OMP) UspA1, UspA2, UspA2H, Hag/MID, OMP CD, McaP and the lipooligosaccharide LOS could be identified as contributing to bacterial attachment to different cell types [75]. The highly conserved UspA proteins – the most extensively studied OMPs of *M. catarrhalis* - are able to bind to the extracellular matrix proteins fibronectin [76], laminin [77], vitronectin [78] as well as to the carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) [79]. Another well investigated and also highly conserved OMP is the adhesin Hag/MID (also called immunoglobulin D-binding protein). Hag is a member of an autotransporter family called oligomeric coiled coil adhesins (Oca), and thus related to the OMP YadA from *Yersinia enterocolitica* and the adhesin Hia of *H. influenza* [80, 81]. It could be shown that Hag is able to bind to type II alveolar epithelial cells and middle ear epithelial cells [80, 82, 83].

TABLE 1 Characteristics of outer membrane components of Moraxella catarrhalis

Component	MW(kDa)	16SrRNA type	Function	Reference
UspA1	88	**Type 1**	**Adhesion** Binding to Chang, Hep-2, A549 and specific interaction with CEACAM1; Binding to fibronectin/α5β1-integrin, laminin;	[76-79, 84-86]
			Invasion Binding to fibronectin/α5β1-integrin	[87]
			Biofilm formation	[88]
			Induction of IL-8 release in human epithelial cells	[89, 90]
			Binding to CEACAM-1	[91]
		Type 2	**23-36% carry the gene but no or less expression**	[6, 11, 92, 93]
UspA2	62	**Type 1**	**Adhesion** Binding to fibronectin/α5β1-integrin and laminin	[77]
			Serum resistance Vitronectin- and C3-binding	[78, 94]
			IL-8 production activation of protein kinase C	[90]
		Type 2	**All strains carry the gene but there is no serum resistance associated with UspA2**	[6]
UspA2H	92	**Type 1**	**Adhesion** Binding to Chang; 'hybrid' of UspA1 and UspA2	[85]
			Biofilm formation	[95]
		Type 2	**Unknown**	
Hag/MID	200	**Type 1**	**Adhesion** Binding to A549 and human middle aer epithelial cells	[80, 82, 83, 95]
			Autoagglutination and Biofilm formation Involved in auto- and haemagglutination;	[88, 96]
			IgD-binding High affinity to human IgD; B cell mitogenic;	[97-99]
		Type 2	**Gene is absent in almost all isolates (17 of 18)**	[93]

OMP CD	46	**Type 1**	**Adhesion** Binding to A549 and middle ear mucin	[100, 101] [100]
			Serum resistance	
			Carry gene for RFLP type I	
		Type 2	**Carry gene for RFLP type II**	[93]
McaP	62	**Type 1**	**Adhesion** Binding to Chang and A549	[102]
		Type 2	**Unknown**	
LOS	10	**Type 1**	**Adhesion** Binding to Chang and HeLa	[103]
			Serum resistance	[104]
			Invasion	[105]
			Induction of pro-inflammatory cytokine release and ICAM-1 expression	[87] [106]
		Type 2	**Carry no LOS B gene**	[93]
CopB	81	**Type 1**	**Serum resistance**	[107]
			Nutrient acquisition Iron acquisition from human lactoferrin and transferrin;	[108]
			Carry genes for CopB RFLP type I/III and II.	[93]
		Type 2	**Carry genes for CopB RFLP type 0 and IV**	[93]
OMP E	50	**Type 1**	**Serum resistance**	[109]
		Type 2	**Unknown**	
LbpA, LbpB	110, 100	**Type 1**	**Nutrient acquisition** Iron extraction from human lactoferrin	[110, 111]
		Type 2	**Unknown**	
TbpA, TbpB	110, 75-80	**Type 1**	**Nutrient acquisition** Iron extraction from human transferrin	[112, 113]
		Type 2	**Unknown**	
M35	76	**Type 1**	**Nutrient acquisition** Porin involved in the uptake of glutamic acid	[57, 63]
			Nasopharyngeal colonization in mice	[63]
		Type 2	**Unknown**	

MhuA	107	**Type 1**	**Nutrient acquisition** Haemoglobin-utilization protein	[114]
		Type 2	**Unknown**	
HumA		**Type 1**	**Nutrient acquisition** Hemin-uptake	[115]
		Type 2	**Unknown**	
OMP J1/2	19 and 16	**Type 1**	**Unknown**	[116]
		Type 2	**Unknown**	
OMP G1a/b	29 and 28	**Type 1**	G1a: putative copper-binding lipoprotein	[117]
		Type 2	**Unknown**	
OlpA	24	**Type 1**	Homologies to *Neisseria* Opa protein adhesins	[118]
		Type 2	**Unknown**	

Chang, human conjunctival epithelial cells; HEp-2, human pharyngeal epithelial cells; A549, lung epithelial cells; HeLa; human epithelia carcinoma cell line; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; C3, third component of complement; full names of OMPs will be found in the list of abbreviations.

1.4.2.2 Invasion

After the successful colonization of the host surface by adhesion, the second step leading to invasive infection is to invade the host cell and break through the physical barrier of the host defence. It could be previously shown that the preciously considered ‘obligate’ extracellular pathogen *M. catarrhalis* is also able to invade human epithelial cells *in vitro* (figure 5) [87, 119] and *in vivo* (resected tonsils and adenoids) [120]. Studies indicate that invasion is an active process of both the bacteria and the host cell [90], and that the bacterial outer membrane components UspA1 and LOS are involved in this process [87]. The invasion mechanism itself is not yet fully understood. Slevogt et al. [119] demonstrated that

microfilaments, Rhotype GTPase and phosphoinositide 3-kinase (PI3K) -dependent contractile mechanisms are involved in the invasion of A549 and BEAS-2B cells, whereas Spaniol et al. found clathrin polymerization as well as actin polymerization involved in the invasion of Chang conjunctival cells [87].

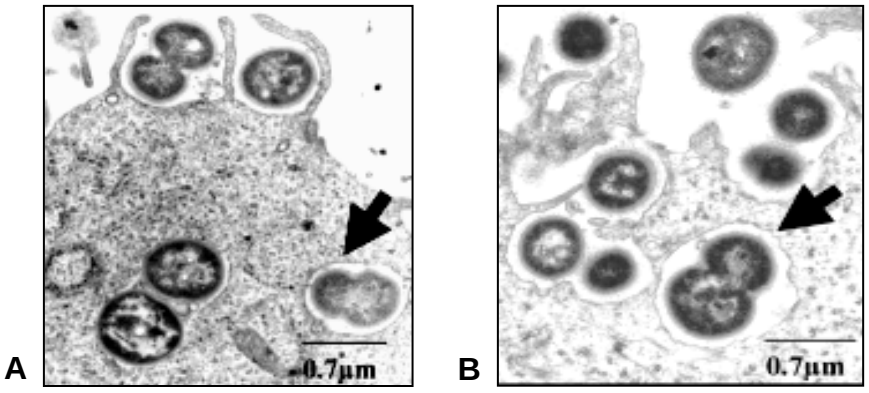

FIGURE 5

Invasion of M. catarrhalis in pulmonary epithelial BEAS-2B and A549 cells. *Transmission electron micrographs (TEMs) demonstrating cross-sections of the epithelial cell monolayer showing lamellipodia enclosing M. catarrhalis in a macropinocytotic ingestion. (A) The black arrow show intracellular bacteria surrounded by a vacuole in BEAS-2B cells. (B) Encapsulated intracellular M. catarrhalis (black arrows) in A549 cells. Adapted from Slevogt et al. Cell Microbiol 2007 [119].*

1.4.2.3 Nutrient acquisition

Nutrient acquisition is a further key element in the infection of the host. Devoid of it, successful proliferation would not be possible. *M. catarrhalis* needs special environmental conditions and the presence of specific nutrients. A essential nutrient for many bacteriological processes is iron. Elementary iron is not freely available inside the host but

complexed with haemoglobin, heme, transferrin or lactoferrin. Therefore, special receptors and mechanisms are required and indeed seven OMPs could be identified, which are involved in the iron acquisition by *M. catarrhalis* so far. These include CopB, which is involved in the iron acquisition from lactoferrin and transferrin [108], the lactoferrin-binding proteins A and B (LbpA and LpbB) [110, 111], the transferrin-binding protein A and B (TbpA and TbpB) [112], the haemoglobin utilization protein MhuAb [114] and the heme utilization protein HumA [115].

1.4.2.4 Interactions with the host immune response

The innate and adaptive immune response system protects the host against invading pathogens with a complex network of tissue, cells and molecules working together to eradicate the intruder.

Evasion of the complement

Complement resistance is one of the major virulence factors of *M. catarrhalis*. The majority of type 1 strains are serum resistant and several OMPs have been reported to be involved in this survival strategy: UspA2, UspA2H OMP CD, LOS, CopB and OMP E [78, 94] [100, 104, 105, 109]. The main components are UspA2 and UspA2H which interfere with the classical and the alternative complement pathways by binding, respectively, to the complement inhibitor C4 binding protein (C4bp) and neutralizing C3 [78, 94]. It could also been shown that *M. catarrhalis* is able to inhibit the direct formation of the membrane attack complex by vitronectin binding [78].

Immune response to M. catarrhalis

Activation of the innate immune response via specific and nonspecific immune cells generally leads to the production and release of pro-inflammatory cytokines resulting in a further activation and attraction of secondary immune cells. Several studies could identify different outer membrane components of *M. catarrhalis* and also mechanisms involved in the induction of pro-inflammatory cytokine release by human respiratory epithelium and monocytes. Slevogt et al. demonstrated the induction of interleukin-8 (IL-8) release by the UspA2-dependent activation of protein kinase C through the nuclear factor (NF-κB) signalling pathway [90] in pulmonary epithelial cells. Also, the UspA1 adhesin is involved in the induction of pro-inflammatory IL-8 production in respiratory epthelium [89, 90]. This effect is mainly dependent on the interaction of UspA1 with the toll-like receptor TLR2 [90, 119]. Interestingly, it was found that this IL-8 induction is inhibited by an - also UspA1-dependent - interaction with CEACAM-1 [90] representing a new strategy of pathogen evasion. Additionally, also the *M. catarrhalis* lipooligossaccharide LOS could be identified as an inductor of the pro-inflammatory cytokine production and ICAM-1 expression on human monocytes. This activation processes via toll like receptor 4 (TLR4) and the CD14-dependent pathway [106].

IgD-binding and B-cell activation

It could be shown that *M. catarrhalis* is able to bind IgD via the IgD-binding molecule MID/Hag [97-99] resulting in the activation of B-cells in a thymus-independent manner – independently without physical T-cell help [99]. B-cell superantigens induce a strong proliferative response of B-cells and enable the bacterium to circumvent the adaptive immune system and enhance its virulence. Basically, superantigens are known from several gram-positive bacteria – such as protein A of *Staphylococcus aureus* (SpA) [121] – but are quite rare among gram-negative bacteria. In addition to *M. catarrhalis*, superantigens are

currently only known in *H. influenzae* [122] *and Yersinia pseudotuberculosis* [123]. MID, which is present in a large number of isolates, is able to bind both soluble and membrane bound IgD. The B-cell activation is initiated by a nonimmune cross-linking of the IgD constant heavy chain region 1 and leads to an upregulation of different B-cell surface molecules and, importantly, induces no apoptosis in the B-cells [124]. However, IgD binding alone is obviously not enough to induce a strong proliferative response, but Jendholm et al. [125] found a particular TLR9 activation parallel to the IgD signaling.

1.4.2.5 Biofilm production

Biofilm production is a very effective strategy of bacteria to colonize and also persist in the host. It enables the bacteria to protect themselves from the host's immune system but also from antimicrobial substances. Additionally, the existence of biofilms supports the exchange and uptake of DNA. Simultaneously, biofilm production displays a big problem not only in surgery, but also in chronic diseases such as chronic middle ear infections [28]. Just a few studies have been performed *in vitro* to demonstrate that *M. catarrhalis* is able to produce biofilms in whose formation UspA1, UspA2H and MID are involved [88, 95].

1.4.2.6 Cold shock response and its effect on the virulence

During colonization and infection of the host, bacteria are continuously confronted with changing environmental conditions, such as altered pH values, osmolarity, nutrition depletion or varying temperatures. Therefore, the bacteria developed special strategies to adapt to these variable conditions - the so-called stress responses. The best investigated stress

response of *M. catarrhalis* is the cold shock response – induced after a temperature shift of about 10°C – occurring *in vivo* after breathing cold air [126]. This cold shock response results mainly in the transcriptional regulation of the expression of different proteins, which are important for the survival under the new conditions, but also in the upregulation of OMP involved in virulence mechanisms e.g. in adhesion. It could be shown that the adhesin UspA1 is upregulated after growth at 26°C compared to 37°C [127] resulting in a significantly higher adhesion ratio of the cold shock-treated bacteria to the human host epithelium as well as a higher induction of IL-8 release by respiratory cells [89].

1.4.3 Virulence factors of the 'non-virulent' lineage

As it becomes also apparent from table 1, the information about the 16S rRNA type 2/3 is exceptional scant. Apart from some information about genes - known from type 1 – and their expression status, no further information is available. It could be shown that the UspA1 adhesin - which is present in 87% to 99% of the serum resistant lineage – is carried only by 23% to 36% of type 2/3 strains, and not all of them express it [6, 11, 92, 93]. These findings would explain the poor capacity to adhere to epithelial cells of serum sensitive isolates [6]. Interestingly, there were no differences found in the respective proportions carrying a UspA2 gene [6], although the UspA2 protein is one of the main proteins in type 1 strains and associated to serum resistance. A possible explanation would be that type 2/3 strains express low levels of UspA2, i.e., below the functionally relevant threshold. Also, the gene for the multifunctional Hag/MID protein was found to be absent in almost all isolates of the 16S rRNA tpye 2/3 subpopulation [93]. The outer membrane proteins CopB and OmpCD are also associated to serum resistance and found in all type 1 isolates [6]. Studies utilizing restriction fragment length polymorphism (RFLP) analysis are able to classify CopB as well as OmpCD

into different subgroups. CopB can be distinguished into the types 0, I/III,II and IV, and OmpCD in type I and II. Investigations regarding differences between type 1 and type 2/3 now demonstrated that CopB types I/III and II were almost exclusively related to the complement resistant lineage whereas types 0 and IV were mostly associated with the complement sensitive population [93]. Similarly, results for OmpCD demonstrated that RFLP type I was associated with 16S rRNA type 1 and RFLP type II with 16S rRNA type 2/3 [93]. Finally LOS B – one of three LOS serotypes (A, B and C) - was also found exclusively in 16S rRNA type 1 isolates [93].

AIMS OF THE THESIS

2. AIMS OF THE THESIS

The increasing incidence of acute otitis media (AOM) infections caused by *Moraxella catarrhalis* intensified the research focused on the identification of possible vaccine candidates. Furthermore, objectives of previous studies were mainly the still poorly understood pathogenesis and virulence of the bacterium. Recent data elucidated that *M. catarrhalis* consists of two different phylogenetic subpopulations – the 16S rRNA type 1 and type 2/3 lineages. Previous investigations of *M. catarrhalis* mainly focused on the 16S rRNA type 1 lineage.

2.1 Aim 1

Outer membrane components serving as immunogens (i.e., potential vaccine candidates) should be recognized by the host immune system and should be highly conserved within all strains of a given species. M35, a highly conserved porin of 16S type 1 is virtually completely conserved among type 1 strains and may thus qualify as a potential vaccine candidate, even though immunodominant epitopes are yet to be identified. In detail the following questions should be addressed within the first part:

(i) Characterization of the M35 phenotype regarding functionality and putative virulence factors

(ii) Determination of the potential of M35 as a possible vaccine candidate

(iii) Analysis of the degree of M35 conservation in type 2/3 strains

2.2 Aim 2

The two phylogenetic subpopulations of *M. catarrhalis* differ in their 16S rRNA subunit and in the time range of their phylogenetic development. Serum sensitivity was recognized as mainly attributed to type 2/3 isolates, which, furthermore, lack the carriage or expression of – for the virulence genes or proteins important to type 1 strains. As previous focus of research on the serum sensitive lineage is limited mainly on genotypic investigations, the second part of the thesis consists of:

(i) The determination of putative type 2/3 virulence traits with regard to their ability to invade human epithelial cells as well as their pro-inflammatory activity

(ii) The investigation of the influence of cold shock treatment of type 2/3 strains with respect to adhesion and invasion respectively their protein expression

THESIS RESULTS

3. THESIS RESULTS

3.1 Outer membrane porin M35 of *Moraxella catarrhalis* mediates susceptibility to aminopenicillins

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Outer membrane porin M35 of *Moraxella catarrhalis* mediates susceptibility to aminopenicillins

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Abstract

Background: The outer membrane protein M35 is a conserved porin of type 1 strains of the respiratory pathogen *Moraxella catarrhalis*. It was previously shown that M35 is involved in the uptake of essential nutrients required for bacterial growth and for nasal colonization in mice. The aim of this study was (i) to characterize the potential roles of M35 in the host-pathogen interactions considering the known multifunctionality of porins and (ii) to characterize the degree of conservation in the phylogenetic older subpopulation (type 2) of *M. catarrhalis*.

Results: Isogenic *m35* mutants of the type 1 strains O35E, 300 and 415 were tested for their antimicrobial susceptibility against 15 different agents. Differences in the MIC (Minimum Inhibitory Concentration) between wild-type and mutant strains were found for eight antibiotics. For ampicillin and amoxicillin, we observed a statistically significant 2.5 to 2.9-fold MIC increase ($p < 0.03$) in the *m35* mutants. Immunoblot analysis demonstrated that human saliva contains anti-M35 IgA. Wild-type strains and their respective *m35* mutants were indistinguishable with respect to the phenotypes of autoagglutination, serum resistance, iron acquisition from human lactoferrin, adherence to and invasion of respiratory tract epithelial cells, and proinflammatory stimulation of human monocytes. DNA sequencing of *m35* from the phylogenetic subpopulation type 2 strain 287 revealed 94.2% and 92.8% identity on the DNA and amino acid levels, respectively, in comparison with type 1 strains.

Conclusion: The increase in MIC for ampicillin and amoxicillin, respectively, in the M35-deficient mutants indicates that this porin affects the outer membrane permeability for aminopenicillins in a clinically relevant manner. The presence of IgA antibodies in healthy human donors indicates that M35 is expressed *in vivo* and recognized as a mucosal antigen by the human host. However, immunoblot analysis of human saliva suggests the possibility of antigenic variation of immunoreactive epitopes, which warrants further analysis before M35 can be considered a potential vaccine candidate.

Background

Moraxella catarrhalis is an exclusively human, mucosal respiratory tract commensal and pathogen causing between 5% [1] and 20% of cases of acute otitis media in children [2] across all regions of the world. The recent introduction of routine infant immunization with pneumococcal conjugate vaccines has - in some studies [3] - led to a substantial increase in otitis media caused by *M. catarrhalis* [3]. It is thus a major cause of the most common bacterial infection in children requiring medical attention. *M. catarrhalis* also triggers approximately 10% acute exacerbations of chronic obstructive pulmonary disease (COPD) in adults [4]

In our attempts to identify cold shock regulated outer membrane proteins (OMP) of *M. catarrhalis* [5] we investigated a recently described OMP called M35. We found no evidence of cold shock regulation, but the construction of an isogenic mutant lacking the expression of a currently incompletely described OMP of *M. catarrhalis* provided us with the opportunity to conduct a phenotypic analysis of the function of M35. Meanwhile, in an elegant series of experiments, Easton and co-workers [6] demonstrated that M35 is a typical Gram-negative OM porin, which also is essential for short-term nasal colonization of mice. Importantly, porins of Gram-negative bacteria not only assure bacterial homeostasis by acting as transport channels, but are also known to afford virulence mechanisms such as adhesion, invasion [7-11], and pro-inflammatory stimulation. [11-17]. In addition, porins are often involved in antimicrobial resistance [18-26]. Porins of *M. catarrhalis* have received little attention in the scientific literature. Gotho et al. described the permeability for beta-lactam antibiotics across the OM of *M. catarrhalis* suggesting that porins may be involved [27]. Lafontaine et al investigated the porin-like OMP CD, which acts as an adhesin on lung cells [7]. Thus, M35 is currently the only well characterized porin of *M. catarrhalis* [6,28].

The aims of the present study were (i) to provide an overview of phenotypic differences between the strains O35E, 300 and 415 and their respective isogenic *m35* mutants, (ii) to investigate whether M35 is a human mucosal antigen and thus a potential vaccine candidate, (iii) to evaluate the role of M35 in the susceptibility of *M. catarrhalis* to various classes of antimicrobial agents, and (iv) to provide the DNA sequence *m35* of strain 287, which is a representative of the phylogenetically older major lineage (type 2) of *M. catarrhalis* [29].

Methods

Bacterial strains and culture conditions

The *M. catarrhalis* strains and their isogenic *m35* mutants used in this study are listed in Table 1. All strains were cultured at 37°C and 150-200 rpm in brain heart infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plates in an atmosphere containing 5% CO_2. Media were supplemented with kanamycin (20 μg/ml) for culturing of the mutants. To investigate growth under different osmotic conditions, strains were cultured in BHI broth overnight at 37°C and 150 rpm. One ml of overnight culture was diluted 1:100 in fresh BHI supplemented with 0.25 M, 0.5 M or 1.0 M NaCl, respectively, and incubated at 37°C and 150 rpm. During cultivation to the stationary phase cell density was measured at OD_{600}. The effect of exposure to different acidic environments was measured by growing bacteria in BHI broth overnight, harvesting and resuspending them in 20 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 25 mM L-arginine adjusted to pH 4.0, pH 5.0, pH 6.0, or pH 7.0, respectively. Suspensions were incubated for 2 h and 4 h, respectively, at 37°C, and the number of viable bacteria was quantified by plating of serial dilutions. Iron utilization experiments were performed by a disk feeding assay applying 5 μl of iron-saturated human lactoferrin (10 mg/ml) to sterile filter disks [30]. BHI agar plates were previously iron depleted by adding deferoxamine mesylate (Desferal, Novartis, Basel, Switzerland) to a final concentration of 30 μM and incubated at 4°C overnight before use. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) agar plates or in LB broth.

DNA methods

Plasmids were isolated using the Wizard Plus SV Miniprep DNA purification system (Promega Corp., Madison, Wis.) DH5α was transformed as described previously [31]. Restriction enzymes were purchased from New England

Table 1: Bacterial strains used in this study

Strain	Description	Source or reference
M. catarrhalis O35E	middle ear isolate	[65]
M. catarrhalis O35E.*m35*	isogenic mutant strain, kan^R	this study
M. catarrhalis 300	nasopharyngeal isolate from child	[35]
M. catarrhalis 300.*m35E*	isogenic mutant strain, kan^R	this study
M. catarrhalis 415	nasopharyngeal isolate from child	[35]
M. catarrhalis 415.*m35*	isogenic mutant strain, kan^R	this study
M. catarrhalis 287	nasopharyngeal isolate from child	[35]
Escherichial coli DH5α	Host strain for plasmid constructs	[66]

Biolabs, Inc., Beverly, MA. Electrocompetent *M. catarrhalis* was prepared and DNA was electroporated as described [32]. DNA sequencing was performed by using an ABI PRISM 310 genetic analyzer (PE Biosystems, Rotkreuz, Switzerland) with the Big Dye Terminator cycle sequencing ready reaction kit (PE Biosystems, Rotkreuz, Switzerland). Sequences were analyzed with the Lasergene software (DNASTAR Inc., Madison, WI). For sequencing of *m35* of strain 287, DNA was amplified using the primers *m35*B5 (5'-TCGATACCAGAACACTACCTAAGC-3'), *m35*F2 (5' -GTCTGAGGGCAAGGTAGGCG-3'), *m35*RMJ1 (5' -CGTAGCAGTTTTCATCTCACCAC 3'), *m35*F3 (5'-CTTGCTCTAGCAACCGCAG-3'), *m35*R3 (5'-GCAAGACCTAGGTAAGTATC-3') and *m35*FMJ4 (5'-TGCGTGCATGGGTCGTGA-3').

*Construction of the isogenic mutants O35E.*m35, *300.*m35 *and 415.*m35

Part of the *m35* gene of the strains O35E, 300 and 415, respectively, was amplified using forward primer *m35*F3 (5'-CTTGCTCTAGCAACCGCAG-3') and reverse primer *m35*B5 (5'-TCGATACCAGAACACTACCTAAGC-3'). PCR products were ligated into the *BamH*I restriction site of pGEM-T-Easy pUC4K (Promega, Madison, USA). The kanamycin cassette was ligated into the *Avr*II restriction site of the *m35* insert. The resulting construct, *Δm35:kan*, was used for electroporation of the competent strains O35E, 300 and 415, respectively. Transformants were selected on BHI agar plates containing 20 μg/ml of kanamycin. Insertional inactivation of *m35* was confirmed by PCR analysis, sequencing, Southern blot analysis (data not shown) and immunoblotting (figure 1).

Preparation of OMP

OMP were prepared by the EDTA buffer method as described [33]. Bacteria were harvested from a stationary phase culture, resuspended in EDTA buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, ph 7.4), homogenized and incubated at 55°C at 300 rpm for 1 h. Cells and cell debris were eliminated by centrifugation at 10,000 × g for 15 min at 4°C. Finally, OMP were collected by ultracentrifugation at 100.00 × g for 2 h at 4°C.

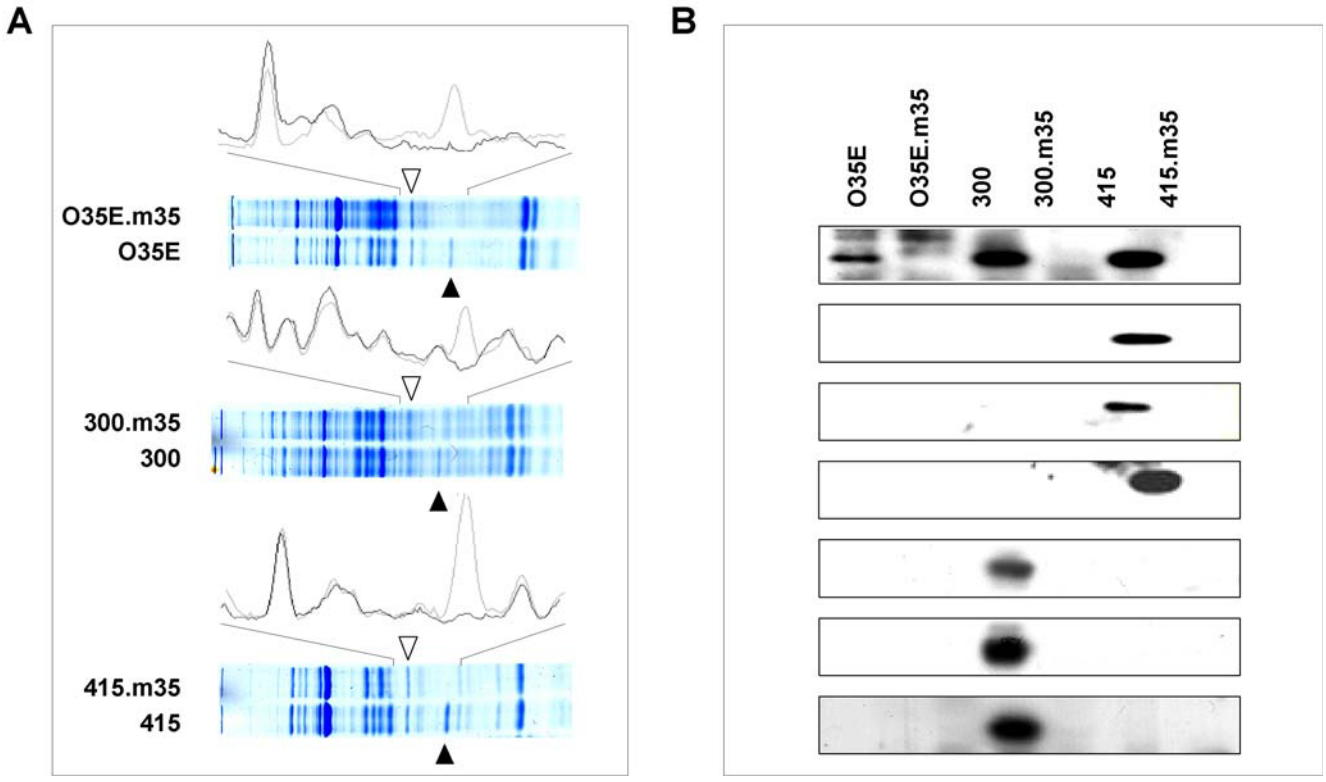

Figure 1
(A) SDS PAGE of *M. catarrhalis* OMP of the strains O35E, 300 and 415 together with their respective *m35* knock-out mutants. Black triangles show the position of the M35 protein band at 36 kDa. White triangles show the position of the 40 kDa protein. The spectra display the intensity of each protein band determined by the AlphaEaseFC® software. Light lines show the wild-type strain, dark lines show the respective M35 mutant strains. (B) Western Blot analysis for human salivary IgA against M35 of the strains O35E, 300 and 415 from seven healthy donors. The isogenic *m35* mutants were included as negative controls.

2D-Gel electrophoresis and MALDI-TOF

Analysis of M35 and other OMP spots of strain O35E was performed a described previously [34], except for the precipitation of the OMP, which was omitted.

SDS-PAGE gel electrophoresis and immunoblot for detection of human anti-M35 IgA

Samples were resolved by SDS-PAGE using a 7.5% polyacrylamide gel. Band intensity was quantified using the AlphaEaseFC® program from Inotech, Inc. Antibody detection was performed by Western blot analysis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, MA). IgA binding was detected using human saliva samples as primary antibody source and goat anti-human IgA, respectively, labeled with horseradish peroxidase (SIGMA) as secondary antibody. Super Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) was used for detection of antibody binding. Unstimulated human saliva was collected from healthy volunteers using Salivette sponges® (Sarstedt, Nümbrecht, Germany), centrifuged for 5 minutes at 2000 rpm and stored at -20°C. All volunteers were laboratory researchers and provided oral informed consent. Sampling of saliva from healthy volunteers was approved by the local ethics committee.

Antimicrobial resistance testing

The minimum inhibitory concentrations (MIC) of penicillin, ampicillin, amoxicillin, amoxicillin-clavulanate, cefuroxime, ceftriaxone, imipenem, meropenem, erythromycin, doxycycline, gentamicin, vancomycin, ciprofloxacin, levofloxacin, and moxifloxacin were determined by E-test® (AB Biodisk, Sweden) according to the manufacturer's instructions.

Autoagglutination and serum bactericidal assay

Overnight cultures were resuspended in PBS and adjusted to an OD_{600} of 2.0 in glass tubes. OD_{600} of the supernatants were determined after 15 and 60 minutes, respectively. Serum bactericidal assay were performed as previously reported [35].

Human cell lines and growth conditions

Chang conjunctival cells and A549 lung cells were maintained in Eagle's minimal essential medium (Invitrogen, Basel, Switzerland) supplemented with 10% of heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in 5% CO_2. The THP-1 human monocytic cell line was maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 0.05 mM β-mercaptoethanol, 10 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Adherence and Invasion assay

The ability of *M. catarrhalis* to adhere to and invade human epithelial cells *in vitro* was measured as described previously [36]. Adherence and invasion was assessed on both Chang conjunctival cells and A549 lung cells as described [36,37]. Each strain was analyzed in triplicate in each experiment.

Proinflammatory activity of M35 on human monocytes

The pro-inflammatory potential of *M. catarrhalis* OMP was described previously [38]. To investigate if M35 is an important mediator of proinflammatory cytokine release on the bacterial cell surface, THP-1 cells (1×10^6/ml) were stimulated with different concentrations (1×10^5/ml, 1×10^6/ml, or 1×10^7/ml) of heat inactivated strain O35E or the O35E.*m35* mutant and incubated for 18 h at 37°C and 5% CO_2. After incubation, cells were centrifuged for 2 min at 11,800 × g and supernatants were stored at -80°C. Cytokines were measured using the R&D Systems DY208 for human CXCL8/IL-8 and R&D Systems DY210 for human TNFα/TNFSF1A (R&D, Minneapolis, USA), respectively.

Statistical analysis

Comparison of several test series was evaluated by analysis of variance (ANOVA). The significance of differences between treatment and control groups was determined using the two-tailed *t*-test. $P < 0.05$ was considered as statistically significant. Each value represents the mean ± one standard deviation of at least three independent experiments performed in triplicate.

Results

In vitro *growth of m35 mutants*

Standard growth curves of the three wild-type/mutant pairs in BHI broth revealed no difference in growth velocity measured as broth density at OD_{600} (data not shown). Because porins are frequently involved in stress responses of bacteria against changes in osmolarity or pH [39-41], we investigated the wild-type/mutant pairs with respect to growth at various osmotic (supplementation of BHI with 0.25, 0.5 and 1 M NaCl) and acidic (pH 4-7) conditions (figure 2). Again, wild-type strains and their respective mutants behaved identically.

M35 *knockout is not associated with upregulation of a 40 kDa OMP*

Easton et al. [6] described the upregulation of a 40 kDa protein in one of their isogenic *m35* mutants. In order to confirm this observation, we compared Coomassie-blue stained OMP profiles of our three strains with their respective *m35* mutants, but failed to detect any discernible upregulation of other OMP as determined by measuring protein band intensities (figure 1A). Thus, removal of M35 does not appear to affect the OMP composition

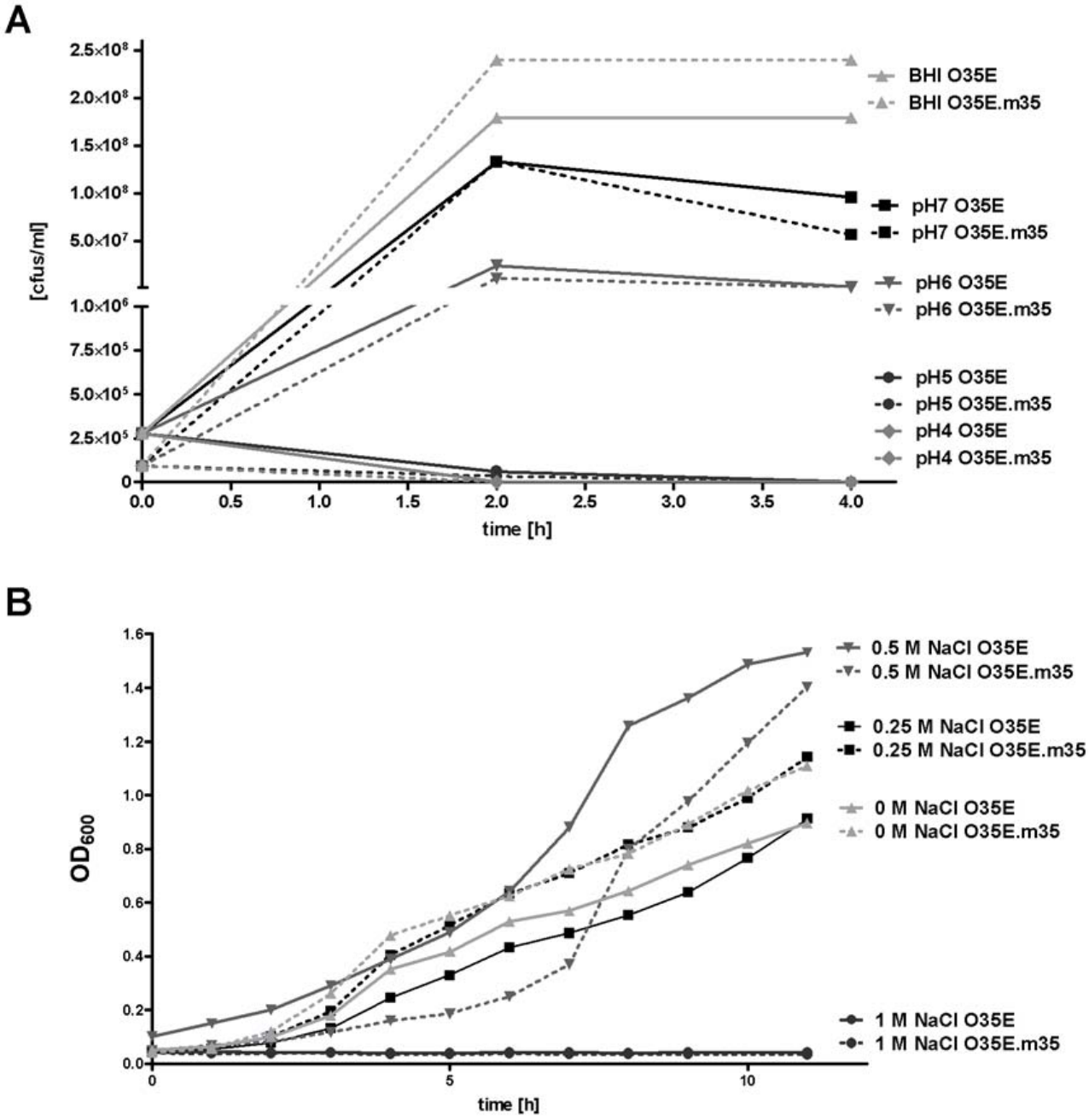

Figure 2

Growth of *M. catarrhalis* O35E and its isogenic *m35* mutant under acidic (A) and osmotic (B) stress. (A) The effect of exposure to different acidic conditions was measured by growing bacteria in BHI adjusted to pH 4.0, pH 5.0, pH 6.0, or pH 7.0, respectively. Suspensions were incubated for 2 h and 4 h, respectively, and the number of viable bacteria was quantified by plating on BHI agar plates. (B) Different osmotic conditions were investigated by culturing bacteria in BHI supplemented with 0.25 M, 0.5 M or 1.0 M NaCl. During cultivation to the stationary phase bacterial density was measured at OD_{600}.

when bacteria are grown in BHI. Taken together, the data presented thus far indicate that M35 is not essential for growth *in vitro* and that its removal from the OM does not otherwise affect the OMP composition.

M35 is expressed in vivo

The human mucosal antibody response to OM components of *M. catarrhalis* has been described in detail [42-45], but M35 has never been paid specific attention. In order to search for human antibodies against M35, OMP of the strains O35E, 300, 415 and their isogenic mutants were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with human saliva for detection of IgA. Western Blot analysis for anti-M35 IgA with 7 different donors resulted in antibody signals to all three strains with the strains 300 and 415 displaying broader immunogenicity than O35E (figure 1B). Four of seven donor saliva contained anti-M35 IgA against strain 300 and 415, respectively. One donor displayed anti-M35 IgA for all three strains. OMP of the *m35* mutants were used as negative controls (figure 1B). These data suggest that *M. catarrhalis* expresses M35 *in vivo* and that the mucosa-associated lymphoid tissue recognizes M35 as an antigen.

Aminopenicillin susceptibility is mediated by M35

One of the major bacterial strategies for drug resistance is barrier protection, which limits the intracellular access of antimicrobial agents [18]. The influx of large, charged molecules is controlled by porins, which allow passive penetration of hydrophilic molecules of several classes of antibiotics [19-21]. Thus, porin-mediated OM permeability is expected to affect susceptibility to antimicrobial agents [18,22]. To investigate this, E-tests were performed with the mutants O35E.*m35*, 300.*m35*, 415.*m35* and their respective wild-type parent strains. There were no differences in MIC for penicillin G, ceftriaxone, meropenem, erythromycin, doxycycline, gentamicin, and vancomycin between wild-type and mutant strains, respectively. For quinolones (ciprofloxacin, levofloxacin and moxifloxacin), cefuroxime and imipenem there was a minor, but consistent ~1.4-fold increase in the MIC of the mutants (data not shown). For ampicillin and amoxicillin, however, there was a statistically significant increase in the MIC of the mutants (2.5 to 2.9-fold) in comparison with their respective wild-types (figure 3A/B) ($p = 0.003$-0.023). Interestingly, at an approximately 10-fold lower level, this was also found for amoxicillin-clavulanate (figure 3C).

Expression of M35 and putative virulence traits of M. catarrhalis

The capacity to autoagglutinate is mediated by hemagglutinin (also called *Moraxella* IgD-binding protein) [46], but some hemagglutinin knock-out mutants still autoagglutinate (unpublished data). Thus, we investigated whether the absence of M35 affected autoagglutination, but failed to identify any difference between strain O35E and O35E.*m35* (data not shown). Similarly, resistance of *M. catarrhalis* to human complement, which is associated with disease-causing isolates [47-49] and which requires expression of several OMP [37,50-52], was not impaired by the lack of M35 (data not shown). Growth of *M. catarrhalis in vivo* is dependent on the ability to acquire iron from the human host by retrieving Fe^{3+} from iron-containing host proteins by a number of specific binding and uptake systems [30,53-59]. Because of its abundance on mucosal surfaces, we chose to investigate the ability of the *m35* mutant to use iron bound to human lactoferrin using a standard disk feeding assay on iron depleted BHI agar plates [30]. The experiment resulted in no differences in growth between the three *m35* mutants and their respective wild-type parents (data not shown).

Adherence and invasion of the m35 *mutant*

The abilities of a pathogen to adhere to and invade epithelial host cells, respectively, are major virulence factors. Adhesins and invasins usually are OMP [5,36,60,61], some of which also act as porins [7-11]. To investigate if M35 mediates adherence and invasion assays were performed on Chang conjunctival cells as well as on A549 lung cells. Adherence of the O35E.*m35* mutant was as efficient as that of its wild-type parent strain (figures 4A/B). Similarly, no differences were found for the capacity to invade these cell types (figures 4C/D) in gentamicin protection assays.

Proinflammatory activity of M35 on human monocytes

Proinflammatory activity is typically induced by OMP, lipopolysaccharide or lipoteichoic acids. Porins have also been described to induce proinflammatory cascades by activating innate immune receptors mediating the expression of several chemokines and cytokines [11-17]. We investigated M35 with regard to its proinflammatory effect on human monocytes. THP-1 cells were stimulated with strain O35E or its O35E.*m35* mutant overnight and cytokine release in the supernatant was measured by determining the concentrations of IL-8 and TNFα by ELISA. There was no difference in the release of IL-8 and TNFα, respectively, between wild-type and mutant (data not shown).

M35 *sequence analysis of type 2 strain 287*

M35 is nearly 100% conserved among type 1 strains of *M. catarrhalis* [28]. To determine if this is also true for the other major phylogenetic subpopulations of *M. catarrhalis*, *m35* of type 2 strain 287 was sequenced and analyzed by bioinformatics. In comparison with type 1 strain O35E, there was a divergence of 5.8% at the DNA level consisting mainly of point mutations (figure 5). Unexpectedly, these DNA mutations lead to an even greater

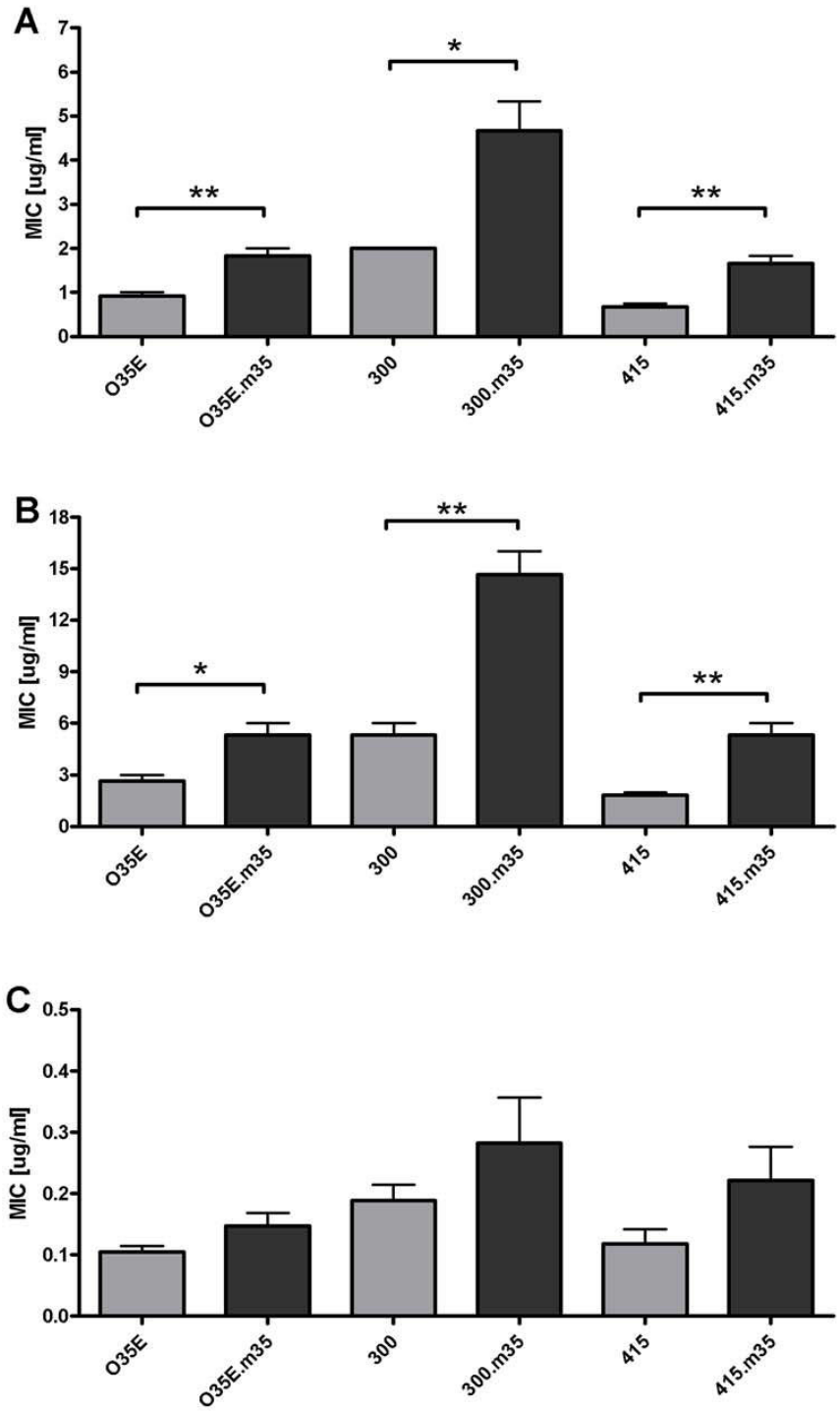

Figure 3

E-tests of the strains O35E, 300 and 415 and their respective isogenic *m35* mutants with ampicillin (A), amoxicillin (B) and amocixillin-clavulanate (C). Bacteria were cultured on agar plates together with E-test strips overnight. Bars show the minimum inhibitory concentration (MIC) for each antibiotic. Data are presented as means ± 1 SD (n = 3). * $p < 0.05$ for wild-type vs. respective mutant.

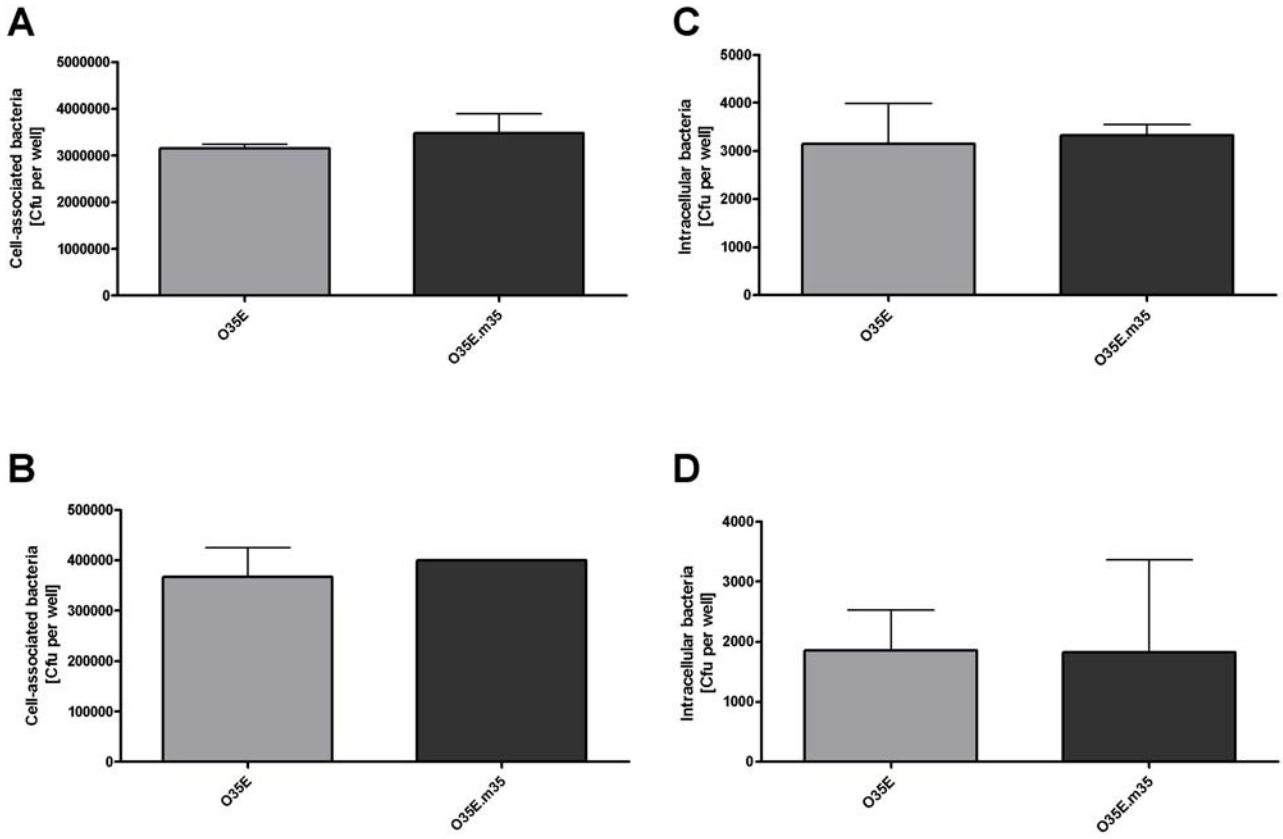

Figure 4

Adherence to Chang conjunctival cells (A) and A549 lung cells (B) and invasion into Chang cells (C) and A549 cells (D) by *M. catarrhalis* O35E and its isogenic knock-out mutant O35E. *m35*. The cells were infected and, after 30 min for adherence and 3 h for invasion, total cell-associated bacteria or intracellular bacteria, respectively, were quantitated by dilution plating. Data are represented as means ± 1 SD (n = 3) of at least three separate experiments.

divergence of 6.1% at the amino acid sequence level (figure 6). This corresponds to a substantial number of missense mutations.

Discussion

Porins are essential components of the gram-negative outer membrane and contribute to nutrient transport, antimicrobial resistance, response to osmostress and other processes, which are essential for bacterial homeostasis. M35 is the first functionally characterized OM porin of *M. catarrhalis* [28] and as of today all isolates examined carry a highly conserved *m35* gene on their chromosome. This may indicate that strains not expressing M35 are not viable *in vivo*, but the only evidence in support of this hypothesis is the observation that a *m35* mutant was unable to colonize the nasal mucosa of mice [6], which are not a natural host species for *M. catarrhalis*.

Our observation that healthy humans have mucosal IgA directed against M35 indicates that this protein is expressed *in vivo*. However, the fact that some salivary samples did not recognize all three M35 proteins tested suggests that either (i) antigenic variation occurs at immunoreactive, surface exposed epitopes, or (ii) it is a weak antigen and some individuals lose or never acquire anti-M35 IgA, or (iii) some isolates lack expression of M35 *in vivo*. If the latter were the case, screening of large collections of clinical *M. catarrhalis* isolates should identify strains either lacking a *m35* gene or isolates carrying silent genes. The answer to this question is of clinical relevance, because our data indicate that the absence of M35 is a previously unknown mechanism of aminopenicillin resistance in *M. catarrhalis*. This effect could occur in *vivo* by alterations in porin expression to prevent antibiotic influx, which is a well known mechanism of resistance in other pathogens [18,22], and which often is associated with the expression of degradative enzymes to confer high level resistance [23,24]. The specificity of M35 for aminopenicillins could be explained by an effect Bezrukov et al described for OmpF of *E. coli*. They found that the

Figure 5
***M35* gene DNA sequence of type 1 strain O35E compared with type 2 strain 287**. The red squares indicate diverse nucleotides between the two strains.

Figure 6
Deduced M35 amino acid sequence of type 1 strain O35E compared with type 2 strain 287. The red squares indicate diverse amino acids between the two strains.

nature and position of specific charges on both the antibiotic molecule and the porin play a major role in these interactions [25,26]. Strong binding to the pore correlates with high diffusion rates whereas weak binding is associated with reduced diffusion. As of today, the only known mechanism of aminopenicillin resistance in *M. catarrhalis* is the expression of one of three chromosomally encoded BRO betalactamases, which are detectable in greater than 90% of clinical isolates [62] and explain the fact that the MIC for amoxicillin-clavulanate in our experiments was at least 10-fold lower than the MIC for amoxicillin. This finding indicates that clavulanate penetrates the OM by ways other than M35. It also demonstrates that, in the presence of clavulanate, the reduced amount of aminopenicillins still penetrating the OM in *m35* mutants suffices to inhibit growth. Thus, it appears that clavulanate inhibits essentially all betalactamase activity available and that naturally occurring isolates lacking M35 would not

currently pose a substantial therapeutic problem in patients treated with betalactamase-resistant betalactams. However, standard dose or high dose amoxicillin still is the therapeutic standard for antimicrobial therapy of acute otitis media. Based on our data (figure 3), currently accepted pharmakinetic/pharmcodynamic (PK/PD) breakpoints for resistance against standard dose (≥ 1.0 μg/ml) or high dose amoxicillin (≥ 8 μg/ml), respectively, [63] thus predict that isolates lacking functional M35 may display clinically relevant aminopenicillin resistance. This is particularly relevant for the treatment of acute otitis media. Drug concentrations reached in the middle ear cavity are low in comparison with serum concentrations [64] and treatment failure is typically caused by insufficient drug concentrations in the middle ear fluid [64]. The list of betalactam antibiotics tested in this study is not exhaustive and it is conceivable that other drugs may also be affected by *m35* mutations. Thus, further studies are needed to explore the potential impact of *m35* mutants on antimicrobial treatment failures.

Multiple phenotypic tests that we carried out with three wild-type/mutant pairs failed to uncover an additional functions attributable to M35. None of these results is particularly surprising. The strength of these "negative" data lies precisely in the fact that we did not study one, but three different isolates and their respective mutants, which, taken together, provide firm evidence that M35 is not involved these various phenotypes in *vitro*. The analysis of three different wild-type/mutant pairs also lead to the conclusion that knocking-out *m35* does not necessarily upregulate expression of a 40 kDa OMP as stated by Easton et al (figure 1A). Because these authors used a different strain, it is conceivable that they observed a strain-specific phenomenon, which does not represent the entire species. Finally, we sequenced *m35* of a strain belonging to the phylogenetically old, second subpopulation of *M. catarrhalis* [29], which differs from the younger subpopulation by a considerably larger genetic diversity [29]. Indeed, we found a substantial number of sequence deviations, which, interestingly, were even greater at the amino acid level than at the DNA level. It is thus conceivable that type 2 strains exhibit functional and/or antigenic differences with respect to M35, which warrant further investigation.

Conclusion

The significant increases in MIC for ampicillin and amoxicillin of the *m35* mutants indicate that the OM porin M35 is involved in the uptake of aminopenicillins. This is a previously unknown mechanism of resistance in *M. catarrhalis*. It remains to be elucidated whether naturally occurring, disease causing strains of *M. catarrhalis* devoid of functional M35 exist, and whether they may contribute to clinical treatment failure. The fact that normal human saliva contains anti-IgA indicates that M35 is expressed *in vivo*, but that antigenic variation may be greater than previously appreciated. Thus, further studies are needed before M35 can be considered a potential vaccine candidate against *M. catarrhalis*.

Abbreviations

ANOVA: analysis of variance; BHI: brain-heart infusion; COPD: chronic obstructive pulmonary disease; EDTA: ethylene-diamino-tetra-acetate; HEPES: Hydroxyethyl-1-piperazinyl-ethansulfonic acid; IL-8: interleukin-8; LB: Luria-Bertani medium; MALDI-TOF: matrix-assisted laser desorption/ionisation-time of flight; OD: optical density; OM: outer membrane; OMP: outer membrane protein; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PVDF: polyvinylidene difluoride; RPMI: Roswell Park Memorial Institute medium; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TbpB: transferring-binding protein B; TNFα: tumor-necrosis factor α.

Authors' contributions

MJ participated in conceiving the study, conducted the majority of the experimental work and drafted the manuscript. NH constructed the *m35* mutants of the strains O35E, 300 and 415. VS participated in conceiving the study. RT performed and interpreted the comparative SDS-PAGE analyses of wild-type and mutant strains. AS performed and analysed the MALDI-TOF experiments. CA was the principal investigator, conceived the study and finalized the manuscript. All authors read and approved the final manuscript.

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3.2 Down-regulation of porin M35 in *Moraxella catarrhalis* by aminopenicillins and environmental factors and its potential contribution to the mechanism of resistance to aminopenicillins

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Down-regulation of porin M35 in *Moraxella catarrhalis* by aminopenicillins and environmental factors and its potential contribution to the mechanism of resistance to aminopenicillins

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Objectives: The outer membrane protein M35 of *Moraxella catarrhalis* is an antigenically conserved porin. Knocking out M35 significantly increases the MICs of aminopenicillins. The aim of this study was to determine the biological mechanism of this potentially new antimicrobial resistance mechanism of *M. catarrhalis* and the behaviour of M35 in general stress situations.

Methods: PCR using *m35*-specific primers was used to detect the *m35* gene in clinical isolates. The *m35* mRNA expression of strains 300, O35E and 415 after exposure to amoxicillin and different stress conditions was measured by real-time PCR and normalized in relation to their 16S rRNA expression. The expression of M35 protein was analysed by SDS-PAGE and western blotting.

Results: Screening of 52 middle ear isolates resulted in positive PCR products for all tested strains. The analysis of m35 mRNA expression after amoxicillin treatment showed 24%–85% down-regulation compared with the respective amoxicillin-free controls in all three strains tested. Also, analysis of protein concentrations revealed lower M35 expression after growth with amoxicillin. Investigation of M35 during general stress responses showed down-regulation of the porin with growth at 26°C and 42°C, under hyperosmolar stress and under iron restriction.

Conclusions: The reduced expression of M35 after aminopenicillin exposure indicates a novel resistance mechanism against aminopenicillins in *M. catarrhalis*, which may be relevant *in vivo*. The differences in expression after different stress treatments demonstrate that M35 is involved in general stress responses.

Keywords: acute otitis media, amoxicillin, antimicrobial susceptibility, stress response

Introduction

Moraxella catarrhalis is a Gram-negative diplococcus and an exclusively human pathogen, mainly involved in exacerbations of chronic obstructive pulmonary diseases (COPD) in adults and acute otitis media in young children.[1–5] The proportion of cases of acute otitis media caused by *M. catarrhalis* varies between 5% and 20%, with recent studies showing an increase of *M. catarrhalis*-caused otitis media since the introduction of routine infant immunization with pneumococcal conjugate vaccine.[2–4,6,7] Acute otitis media treated with standard or high-dose amoxicillin is still the recommended therapeutic standard in Europe as well as in the USA.[8–11] Treatment failures after the use of amoxicillin are documented in different studies and are usually related to infections with β-lactamase-producing strains of *Haemophilus influenzae* or *M. catarrhalis* or a drug-resistant strain of *Streptococcus pneumoniae*.[2,11–15] More than 90% of *M. catarrhalis* isolated worldwide are resistant to penicillin and until now the only known resistance mechanism has been the production of one of two bro β-lactamases (BRO-1 and BRO-2).[2,16–19] We previously demonstrated that *M. catarrhalis* strains lacking the outer membrane protein (OMP) M35 display an MIC of aminopenicillins up to 3-fold higher in comparison with their respective wild-types, indicating that M35 is involved in the susceptibility of the organism to these antimicrobials.[20] M35 is a highly conserved porin in type 1 strains of *M. catarrhalis*; it is involved in nutrient uptake, appears essential for nasal colonization in mice and results in a mucosal immune response manifested as specific IgA in human saliva.[20–22] Porins are water-filled open channels in the outer membrane of bacteria and allow the passive penetration of hydrophilic molecules. They can be differentiated by their activity

(non-specific or specific), their functional structure and their regulation and expression pattern.[23–27] By their function as passive transport channels they are not only involved in the uptake of nutrients but also of drugs such as antimicrobials.[27,28] Therefore it is not surprising that one of the main antimicrobial resistance strategies of bacteria is altered porin expression to limit intracellular access of antibiotics. In particular, antimicrobial resistance to β-lactam antibiotics is often described as a combination of the production of β-lactamases and altered porin expression, which appears to be highly efficient.[27–37] Not only antimicrobials, however, induce alterations in porin expression, but also general stressors, such as nutrient deprivation, temperature changes, osmolarity, pH changes and iron depletion.[38–42] The major aim of this study was to determine the mechanism by which M35 is involved in the susceptibility to aminopenicillins. Secondarily, we observed M35 expression in response to general bacterial stress factors.

Materials and methods

Bacterial strains and culture conditions

The *M. catarrhalis* strains and their isogenic *m35* mutants used in this study are listed in Table 1. All strains were cultured at 37°C and 150–200 rpm in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) or on BHI agar plates in an atmosphere containing 5% CO_2. Media were supplemented with kanamycin (20 mg/L) for culturing of the mutants. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) agar plates or in LB broth. For analysis of the effects of amoxicillin, bacteria were cultured in BHI broth to an optical density (600 nm) (OD_{600}) of 0.18. Afterwards, 60 mg/L amoxicillin (Sigma-Aldrich, Steinheim, Germany) was added and bacteria were cultured for an additional 4 h. To quantify viable *M. catarrhalis* at various amoxicillin concentrations, bacteria were cultured at different concentrations (0, 6, 18, 60 mg/L) of the antibiotic for 4 h, and both the OD_{600} and cfu were determined at different timepoints (Figure 1). For temperature experiments, bacteria were cultured to an OD_{600} of 0.3 before exposing them to 26°C, 42°C or 37°C, for 3 h. M35 expression under hyperosmolar stress and iron depletion conditions was analysed by adding 0.5 mol/L NaCl or 50 μM desferioxamine to the BHI medium at a culture density (OD_{600}) of 0.3.

DNA methods

Plasmids were isolated using the Wizard Plus SV Miniprep DNA purification system (Promega, Madison, WI, USA). *E. coli* DH5α was transformed as described previously.[43] Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). Electrocompetent *M. catarrhalis* was prepared and DNA was electroporated as described.[44] DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PE Biosystems, Rotkreuz, Switzerland) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). *M. catarrhalis* strains 1–52 (middle ear isolates provided by Prof. Ron Dagan, Beer-Sheva, Israel) were analysed for the presence of *m35* by PCR using forward primer M35F3 (5′-CTTGCTCTAGCAACCGCAG-3′) and reverse primer M35_R_MJ1 (5′-CGTAGCAGTTTTCATCTCACCAC-3′) and visualized by 1% agarose gel electrophoresis.

β-Lactamase production testing and bro gene typing

Isolates were investigated for β-lactamase production by the nitrocefin disc test (BD, Basel, Switzerland). β-Lactamase BRO typing was performed by sequencing the putative promoter region of the *bro* gene, described elsewhere, which shows clear sequence differences between BRO-1 and BRO-2.[19] DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer (PE Biosystems) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequences were analysed with Lasergene software (DNASTAR, Madison, WI, USA). To sequence the relevant *bro* gene region, DNA was amplified using the primers *bro_F_MJ* (5′-TATCGCACCCCGTAGGACAA-3′) and *bro_R_MJ* (5′-GTAAGGAATTGGTTTTGCGGTATC-3′).

RNA methods

RNA for *m35* mRNA expression analysis was isolated and used for cDNA synthesis as described elsewhere.[45] Quantitative real-time PCR was performed in triplicate for both target (*m35*) and normalizer [16S ribosomal RNA (rRNA)] genes. No-template controls and reverse transcriptase-negative controls were included in each run. Primers and probes for *m35* were purchased from Applied Biosystems (Rotkreuz, Switzerland). The forward primer was M35ANYF (5′-GCCTTTGCTTATGTATCACCTGAGT-3′), the reverse primer was M35ANYR (5′-GCATTGATAGGGCCTGTGCTA-3′) and the TaqMan probe was 5′-(FAM)CACCCACACCAAACTG(TAMRA)-3′. Primers and probes for 16S rRNA were used as described elsewhere.[45,46] Relative quantification of gene expression was performed using the comparative threshold method. The ratios obtained after normalization were expressed as fold changes compared with untreated samples.

Preparation of OMPs

OMPs were prepared by the EDTA buffer method as described.[47] Bacteria were harvested after amoxicillin or cold shock treatment, respectively, as described, resuspended in EDTA buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4), homogenized and incubated at 55°C at 300 rpm

Table 1. *M. catarrhalis* strains and their isogenic *m35* mutants used in this study

Strain	Description	MIC (mg/L)[20]	β-Lactamase production	BRO type	Reference or source
Isolates 1-52	middle ear isolates from children with acute otitis media	not done	not done	not done	Beer-Sheva, Israel
O35E	parent strain; middle ear isolate	3	+	BRO-1	71
O35E.*m35*	isogenic *m35* mutant; kanamycin resistant	6	+	BRO-1	this study
300	parent strain; nasopharyngeal isolate	6	+	BRO-1	72,73
300.*m35*	isogenic *m35* mutant; kanamycin resistant	16	+	BRO-1	this study
415	parent strain; nasopharyngeal isolate	2	+	BRO-1	72,73
415.*m35*	isogenic *m35* mutant; kanamycin resistant	6	+	BRO-1	this study
DH5α (*E. coli*)	host strain for plasmid constructs	not done	not done	not done	74

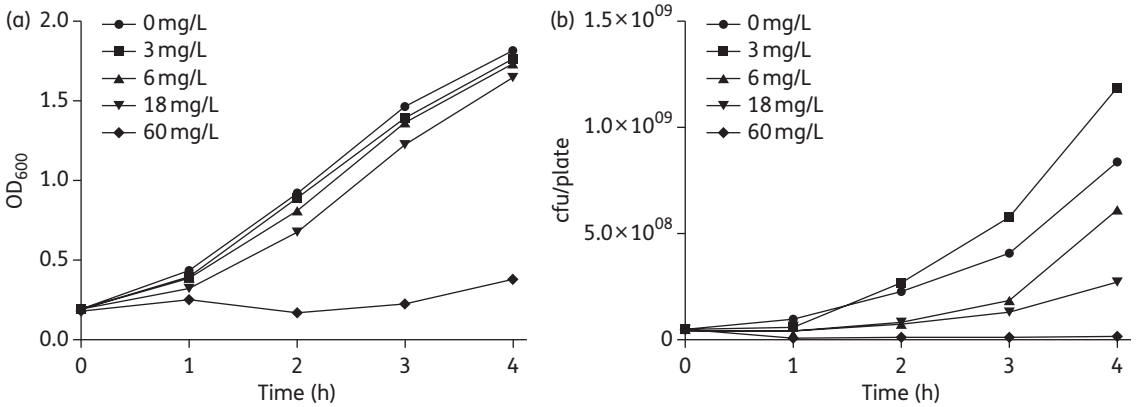

Figure 1. Time–kill curves of strain 300 for different amoxicillin concentrations (0, 3, 6, 18 and 60 mg/L). (a) OD_{600}. (b) cfu/plate.

for 1 h. Cells and cell debris were eliminated by centrifugation at 10000 **g** for 15 min at 4°C. Finally, OMPs were collected by ultracentrifugation at 100000 **g** for 2 h at 4°C.

SDS-PAGE and immunoblot

Samples were resolved by SDS-PAGE using a 10% polyacrylamide gel. To compare protein expression between treated and untreated samples, the protein concentration loaded on the SDS-PAGE was always 1 mg/L. Band intensity was quantified using the AlphaEaseFC program (Inotech, San Leandro, CA, USA). Antibody detection was performed by western blot analysis as described elsewhere.[20] Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). IgA binding was detected using human saliva samples as the primary antibody source and goat anti-human IgA labelled with horseradish peroxidase (Sigma) as the secondary antibody. SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL, USA) was used for detection of antibody binding. Unstimulated human saliva was collected from healthy volunteers using Salivette sponges (Sarstedt, Nümbrecht, Germany), centrifuged for 5 min at 2000 rpm and stored at −20°C. All volunteers were laboratory researchers and provided oral informed consent. Sampling of saliva from healthy volunteers was approved by the local ethics committee. OMPs of *m35* knockout mutants were used as negative controls for their respective wild-type strains. Construction of the isogenic mutants O35E.*m35*, 300.*m35* and 415.*m35* has been described elsewhere.[20]

Results

PCR analysis of 52 clinical isolates

Our previous findings of increased amoxicillin MIC for *m35* knockout mutants compared with their respective wild-type parent strains prompted us to investigate the possibility of naturally occurring clinical middle ear isolates lacking an *m35* gene.[20] We studied 52 middle ear isolates obtained from Prof. Ron Dagan (Beer-Sheva, Israel). PCR resulted in positive products for all 52 strains.

Down-regulation of m35 mRNA expression after amoxicillin treatment

Reduced membrane permeability—one of the main strategies used by bacteria for protection against antibiotics—is generally regulated by altered porin expression.[27,28] Consequently, we investigated the *m35* mRNA expression of strains 300, O35E and 415 during amoxicillin treatment by quantitative real-time PCR. The breakpoint of bactericidal amoxicillin concentration was evaluated by a time–kill curve assay and was found to be at 60 mg/L (Figure 1), which correlates with the MIC for the wild-type strain 300 depending on the inoculum, which is more than 10 times higher in this experimental setting than in antimicrobial resistance testing using Etests.[20] Growth curves with 60 mg/L amoxicillin demonstrated that this concentration seems not to be completely bactericidal but inhibits growth for a period of ∼4 h before proliferation resumes (data not shown). Compared with bacteria grown without amoxicillin, strain 300 grown with 60 mg/L amoxicillin showed a decrease of 85±2% in its *m35* mRNA expression (Figure 2). Strain O35E demonstrated a similar effect, i.e. 73.7±24% down-regulation of *m35* mRNA after amoxicillin treatment (data not shown). In strain 415, down-regulation was only 24±9%. We also observed down-regulation of *m35* mRNA transcription in strain 300 after treatment with a subinhibitory concentration of amoxicillin (15 mg/L). This effect, however, was less pronounced (33±5.6%) than with an amoxicillin concentration at the MIC (data not shown).

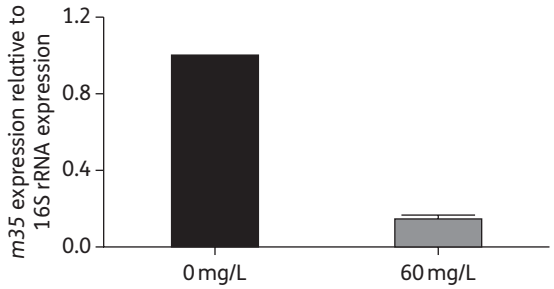

Figure 2. Down-regulation of *m35* mRNA expression during amoxicillin treatment. Quantitative real-time PCR was performed after 4 h of incubation with and without 60 mg/L amoxicillin. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means±1 SD (n=3).

M35 protein expression is also affected by amoxicillin

To determine that a reduction of mRNA copy number translates into less M35 protein in the outer membrane, OMPs from bacteria grown with and without amoxicillin were isolated and protein expression was analysed by SDS-PAGE and western blotting. Strain 300 (Figure 3) as well as strains O35E and 415 (data not shown) expressed less M35 protein both on the SDS gel (Figure 3) and in western blot analysis (Figure 3). These data demonstrate that *M. catarrhalis* senses the presence of amoxicillin in the medium and subsequently down-regulates M35 porin expression.

β-Lactamase production and BRO typing

The investigation of the β-lactamase production of all three strains and its *m35* knockout mutants showed that all strains produced β-lactamases. The sequences of the putative promoter region of the *bro* gene were identical to those published by Bootsma *et al.*[19] and identified all our strains as BRO-1 β-lactamase producers.

Cold shock induces down-regulation of m35 mRNA

Porins involved in antimicrobial resistance have often been described to take part in general stress responses.[38–42] One of the best known stress responses of *M. catarrhalis* is the cold shock response, which alters the expression of specific OMPs after exposure to 26°C.[45,48] In the course of our further studies, and emphasizing that the expression of M35 appeared to be particularly stress-sensitive, we thus investigated the response of *m35* induced by general stress stimuli such as cold shock treatment. Indeed, all three strains—O35E, 300 and 415—showed an effect reminiscent of amoxicillin exposure in that expression was down-regulated after growth at 26°C in comparison with 37°C. Strains O35E (Figure 4), 300 and 415 demonstrated down-regulation of 76 ± 4%, 57.6 ± 3% (data not shown) and 52.2 ± 19%, respectively.

M35 protein expression is involved in the cold shock response

To prove that down-regulation induced by cold shock is not only a transcriptional event, OMPs from cold shock- and non-cold shock-treated bacteria were isolated and analysed by SDS-PAGE and western blotting. Indeed, M35 protein expression was clearly down-regulated in strain O35E (Figure 5) as well as in strains 300 and 415 (data not shown).

m35 mRNA expression is affected by general stress conditions

Cold shock is not the only stress condition that affects *M. catarrhalis*. Amongst others, heat shock, altered osmolarity and iron depletion occur in their mucosal habitat. Thus, we analysed *m35* mRNA expression under these conditions. The analysis revealed *m35* down-regulation after growth at 42°C (66 ± 29%), as well as in response to hyperosmolar stress (86 ± 14%) and iron depletion (56.3 ± 22%) (Figure 6). Taken together, these data indicate that the level of *m35* is influenced by various environmental variables, which may consequently affect susceptibility to aminopenicillins in the mucosal habitat of the respiratory tract.

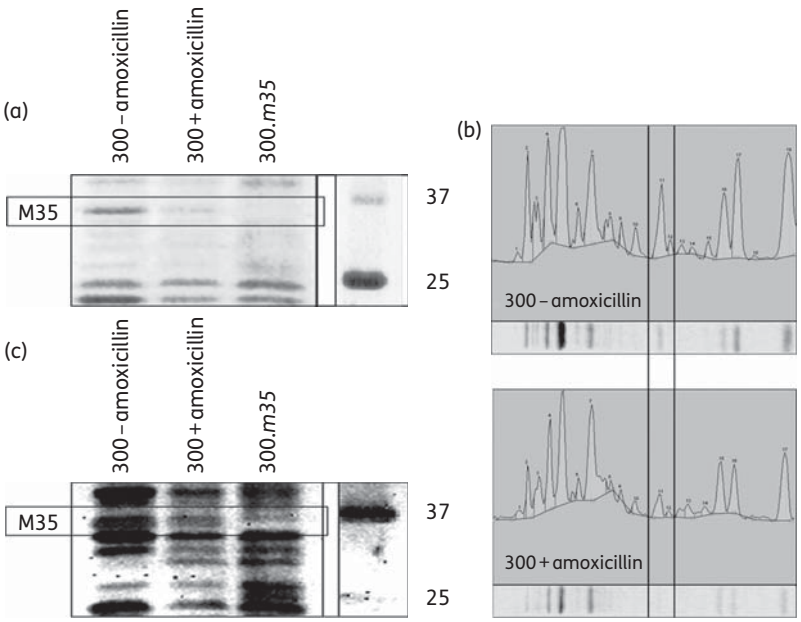

Figure 3. SDS-PAGE (a) and western blot (c) of *M. catarrhalis* OMPs (1 mg/L) from strain 300 after growth with and without 60 mg/L amoxicillin together with its respective *m35* knockout mutant as the negative control. The rectangle shows the position of the M35 protein band at 36 kDa. (b) The spectra display the intensity of each SDS-PAGE protein band from strain 300 grown without amoxicillin and with 60 mg/L amoxicillin proportional to the total protein intensity.

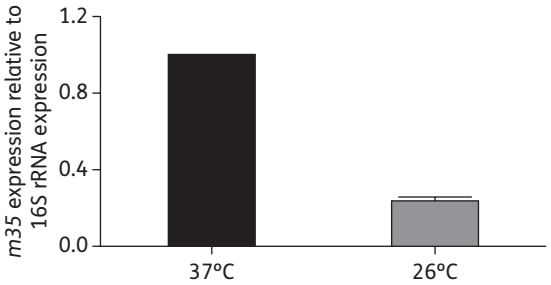

Figure 4. Down-regulation of *m35* mRNA expression after cold shock exposure. Quantitative real-time PCR was performed after 3 h of incubation at 26°C or 37°C. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD ($n = 3$).

Discussion

Antimicrobial resistance is based on three major strategies: detoxifying enzymes to degrade or modify antibiotics; target protection to impair target recognition and thus antimicrobial activity; and the membrane barrier to limit intracellular access of antimicrobials.[49] For *M. catarrhalis*, until now just one of these three strategies has been described—the production of two different chromosomal β-lactamases.[16] More than 95% of clinical isolates are resistant to penicillin and it was shown recently that 80% of all strains tested in the UK and Ireland were resistant to cefaclor and 5% to cefuroxime.[16,50–54] Drug resistance to β-lactam antibiotics is often associated with a second major resistance strategy—reduced outer membrane permeability.[23,26,27,49,55] Reduced membrane permeability can be the result of altered porin expression or the presence of a mutated porin.[23,26–28,49,55]

Our analysis of 52 clinical middle ear isolates using PCR amplification with conserved primers failed to identify strains lacking an *m35* gene on their chromosome. This finding corroborates previous data indicating that *m35* is a highly conserved porin gene and indicates that *in vitro* aminopenicillin susceptibility must be mediated by means other than lack of porin expression.[21] Easton *et al.*[21] demonstrated that M35 is structurally homologous to classical Gram-negative porins, such as OmpC from *E. coli* and OmpK36 (OmpC homologue) from *Klebsiella pneumoniae*. Both porin types are involved in antimicrobial resistance mechanisms and are described in detail elsewhere.[27,28] Several investigators have shown altered porin expression during antibiotic therapy, resulting in specific antimicrobial resistances, dependent on the ability of the bacteria to produce β-lactamase, their general porin composition and the antimicrobial substance they are confronted with.[27–29,33,34,36,37,56,57] Our findings of the down-regulation of M35 expression at the transcriptional level as well as at the protein level in all three β-lactamase-producing strains tested, together with our previous observations of the significantly higher aminopenicillin MICs for the M35 knockout mutants, indicate that we have found a potentially novel resistance mechanism against aminopenicillins in *M. catarrhalis*.[20] The MICs of ampicillin and amoxicillin for the M35 knockout strains were up to 3-fold greater than for their respective wild-type strains, and even amoxicillin/clavulanate displayed the same effect.[20] The mechanism seems to be similar to those described for *K. pneumoniae* OmpK36 and Omp36 porin (OmpC homologue) from *Enterobacter aerogenes*.[27,28,33,34,37,56,56–62] Down-regulation of OmpK36 leads, depending on which β-lactamase the isolate is producing, to resistance to oxyimino and zwitterionic cephalosporins or carbapenems.[59–62] In *E. aerogenes*, loss of Omp36 leads mainly to imipenem resistance.[56] This demonstrates an additional analogy to our findings. The specificity for aminopenicillins—which are also zwitterionic molecules—is reminiscent of the physical characteristics of OmpK36 and Omp36.[63,64] Nevertheless, the differences between the down-regulation of M35

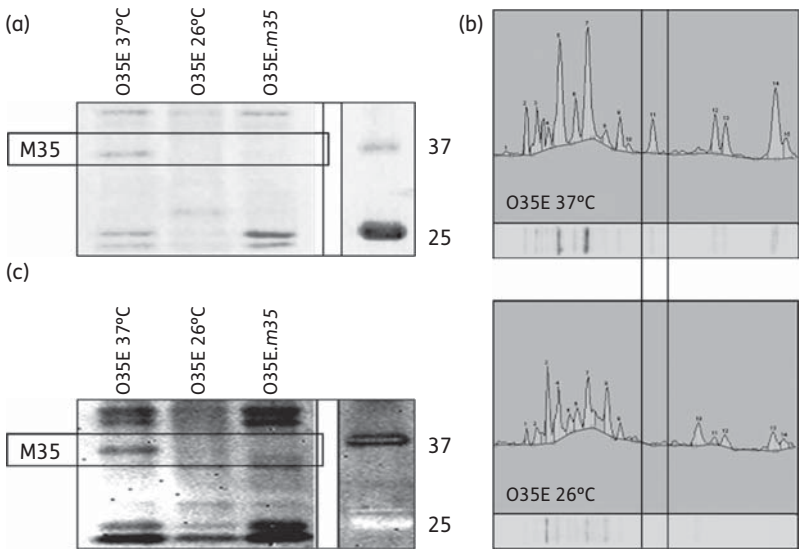

Figure 5. SDS-PAGE (a) and western blot (c) of *M. catarrhalis* OMPs (1 mg/L) from strain O35E after growth at 26°C compared with 37°C together with its respective *m35* knockout mutant as the negative control. The rectangle shows the position of the M35 protein band at 36 kDa. (b) The spectra display the intensity of each SDS-PAGE protein band from strain 300 grown at 26°C and 37°C proportional to the whole protein intensity.

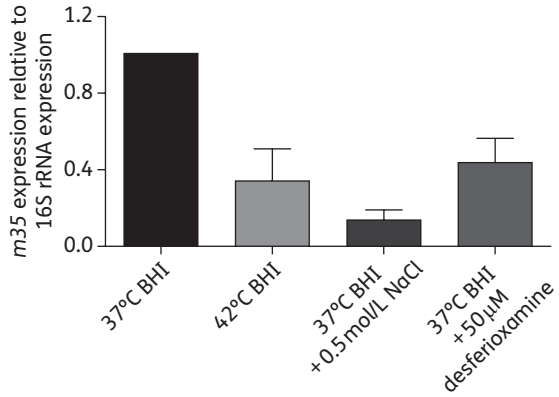

Figure 6. Down-regulation of *m35* mRNA expression after heat shock treatment, osmostress and iron depletion. Quantitative real-time PCR was performed after 3 h of incubation at 42°C, the addition of 0.5 mol/L NaCl or the addition of 50 μM desferioxamine. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD ($n = 3$).

after amoxicillin treatment between strain O35E and 415 with the same MICs for wild-type and mutants, respectively, indicate that there must be a regulation mechanism whose complexity exceeds β-lactamase production and protein down-regulation.[20]

The bacterial stress response is based on a complex network of regulatory systems—a cascade of alterations in gene expression and protein activity that favour survival under extreme and rapidly changing conditions.[39,65] Porins, especially those involved in antimicrobial resistance, such as those of the OmpF and OmpC-like porin families, are often reported to be associated with general stress responses.[40–42] Cold shock as well as heat shock responses are well known mechanisms and have been researched intensively in *E. coli* and *Bacillus subtilis*.[40,42,66,67] We have shown previously that the cold shock response is obviously an important mechanism for *M. catarrhalis* as an adaptation and survival mechanism in the nasopharyngeal habitat, but also regarding its virulence and colonization abilities.[45,48,68] Begic and Worobec[42] showed that temperature is also a predominant effector of expression regulation of OmpF and OmpC in *Serratia marcescens*. This organism is a β-lactam-resistant Gram-negative bacterium, whose porins—OmpF and OmpC—show high similarities to those of *E. coli* and are also involved in β-lactam resistance by decreasing outer membrane permeability.[38,42,69,70] By analysing the regulation of these porins, these authors showed that OmpF is up-regulated and OmpC is down-regulated after growth at 28°C, but the opposite way round after growth at 42°C.[42] The same effect of OmpC regulation, comparable to our findings in M35 after cold shock treatment (Figures 4 and 5), has been described in *E. coli* several times.[40–42] M35 down-regulation after exposure to 42°C has not been described until now. The general effect described in all Gram-negative bacteria is up-regulation of the OmpC-like protein after heat shock treatment, whereas OmpF is down-regulated. However, antagonistic regulation of OmpF and OmpC is apparently not the general mechanism in all *E. coli* strains.[40,42] Allen *et al.*[41] observed down-regulation of both OmpF and OmpC after cold shock treatment in *E. coli* O157:H7. Certainly, the physiological basis for porin regulation under temperature stress conditions is still unclear and needs further investigation. Likewise, our findings concerning the down-regulation of M35 under high osmolarity conditions need further scrutiny. Generally, the OmpC-like porin is down-regulated at low osmolarities and up-regulated at high osmolarity conditions—similar to our findings under temperature stress conditions.[40,42] However, it is currently not known whether *M. catarrhalis* possesses an OmpF-like porin acting as an M35 antagonist. It is conceivable that *M. catarrhalis* expresses only the OmpC-like porin M35 comparable to *K. pneumoniae* strains expressing extended-spectrum β-lactamase (ESBL). These ESBL-expressing strains produce only the OmpC-like porin OmpK36.[34,58] This could explain—together with the high specificity of molecule transport—why the role of M35 in stress response regulation is clearly different from that of OmpC-like porins associated with an antagonist. The expression of an OmpC-like porin in response to iron depletion has, to our knowledge, also never been described before, but substantiates the notion that the regulation of M35 is an important mechanism allowing the survival of *M. catarrhalis* under changing environmental conditions.

In summary, we describe here a new antimicrobial resistance mechanism in *M. catarrhalis* against aminopenicillins, which obviously could lead to significantly higher MICs that would affect the use of amoxicillin in the therapy of acute otitis media caused by *M. catarrhalis* in a critical manner.[20] To verify these findings *in vivo* and clarify its clinical relevance regarding amoxicillin therapy of acute otitis media caused by *M. catarrhalis*, further analyses will be needed. In particular, it will be necessary to elucidate whether down-regulation of M35 during treatment may contribute to treatment failure caused by isolates that have been shown to be susceptible *in vitro*.

Acknowledgements

Professor George Syrogiannopoulos (Larissa, Greece) provided the nasopharyngeal *M. catarrhalis* isolates 300 and 415.

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Transparency declarations

None to declare.

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3.3 Virulence factors of *Moraxella catarrhalis* 16S rRNA type 2/3 strains

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ABSTRACT

Objectives: The gram-negative human pathogen *Moraxella catarrhalis* consists of two different phylogenetic subpopulations discernible by their 16S rRNA sequence. Type 1 – the more recent population - is associated with serum resistance and typical virulence factors such as the ability to adhere and invade human epithelial cells. The type 2/3 subpopulation is the phylogenetically older, generally serum sensitive and is regarded as the less virulent lineage of *M. catarrhalis.* Until now, research mainly focused on the genotypic analysis of the type 2/3 subpopulation. The aim of this study was to investigate this lineage regarding its ability to invade human epithelial cells as well as its pro-inflammatory potential.

Results: Comparative investigation of the ability to invade into four different human epithelial cell lines demonstrated a significantly greater invasion ratio of type 2/3 strains compared to type 1 strains. Type 2/3 bacteria were identified intracellularly by electron microscopy. The host cell structures involved in invasion appear to be the same for both lineages. Long-term experiments failed to demonstrate long-time persistence but indicated externalization of primarily intracellular bacteria over time and extracellular proliferation. Testing the pro-inflammatory activity of type 2/3 on Detroit 652 nasopharygeal cells resulted in an IL-8, IL-6, MCP-1 and VEGF response, but no IL-1β or TNFα release.

Conclusion: The significantly greater invasion ability of type 2/3 strains compared with type 1 strains as well as the comparable pro-inflammatory activity suggest the presence of unknown virulence factors in the ‘non virulent’ lineage and warrant further investigation into its pathogenic potential.

INTRODUCTION

Moraxella catarrhalis - a gram-negative diplococcus – is an exclusively human pathogen and the third major cause for bacterial acute otitis media (AOM) in young children up to three years of age, as well as a major cause of bacterial exacerbations in adult patients with chronic obstructive pulmonary disease (COPD) (19).

Phylogenetically, *M. catarrhalis* consists of two different subpopulations, which differ in their 16S rRNA subunit (2, 12, 24, 32, 34). Type 1 is generally considered the more virulent population (6, 14, 20, 34). Type 1 strains display typical virulence factors such as complement resistance, the ability to adhere to and invade human epithelial cells, biofilm formation, cold shock response, and the interaction with both the innate and the adaptive immune system (6, 14, 20, 25-28, 30, 34). Also, the number of type 1 isolates associated with clinical symptoms and infectious diseases appears to be higher than that of the type 2/3 strains (6). The latter subpopulation, in contrast, has not until now been phenotypically characterized in detail. Type 2/3 has been described as the phylogenetically older population, being estimated to have evolved about 50 million years ago, while the type 1 subpopulation is considered substantially younger (5 million years) (34). Type 2/3 bacteria generally are complement sensitive, lack natural competence, and poorly adhere to human epithelial cells in vitro (2). Genomic and proteomic analysis show that they lack putative type 1 virulence factors such as the multifunctional hemagglutinin (hag), also known as Moraxella IgD binding protein (MID) or failed to express outer membrane proteins (OMP) such as UspA1, which is an adhesin and invasin, or UspA2, which is essential for complement resistance (2, 6, 33, 34). Also, CopB and OmpCD, show differences in RFLP analysis between 16S rRNA type 1 and type 2/3 strains(33). Further, LOS type B - also an important invasin and proinflammatory agent - was found exclusively in type 1 isolates (6, 33). Moreover, the fact that type 2/3 strains are far less commonly isolated from diseased patients

than type 1 strains, supports the notion that the former constitute the less virulent subpopulation (6, 34).

The aim of this study was to investigate representative isolates from the type 2/3 subpopulation regarding the expression of established virulence factors such as the ability to invade human host cells, their pro inflammatory activity on human epithelial cells as well as their cold shock response.

METHODS

Bacterial strains and culture conditions. The *M. catarrhalis* strains used in this study are listed in Table 1. All strains were cultured at 37°C and 150-200 rpm in brain heart infusion (BHI) broth (Difco, Becton Dickinson, Franklin Lakes, USA) or on BHI agar plates in an atmosphere containing 5% CO_2. Media were supplemented with kanamycin (20 µg/ml) for culturing the green fluorescent protein (GFP) mutants. To investigate growth under cold shock conditions, strains were cultured in BHI broth at 37°C and 150 rpm to an OD_{600} = 0.3 and exposed subsequently to growth for 3h at 37°C or 26°C, respectively. During cultivation to the stationary phase cell density was measured at OD_{600}. *Escherichia coli* DH5α was grown on Luria-Bertani (LB) agar plates or in LB broth.

DNA methods. Plasmids were isolated using the Wizard Plus SV Miniprep DNA purification system (Promega Corp., Madison, USA) DH5α was transformed as described previously (11). Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, MA. Electrocompetent *M. catarrhalis* was prepared and DNA was electroporated as described (16).

***M. catarrhalis* GFP construction.** In the first step the plasmid pGFP mut 3.1 (Clontech Labs., Mountain View, USA) was digested with *Pst*I and *Hind*III to isolate the GFP gene fragment and integrate it into the plasmid pBluescript II KS+ (pBs II KS+). Secondly, pBluescript II KS+ as well as the *M. catarrhalis* plasmid pEMCJH04 (kindly provided by J. P.

Hays, Rotterdam, Netherlands) were digested with *BamH*I and *Sal*I overnight at 37°C. Digested pEMCJH04 and the GFP gene fragment were ligated with T4 ligase and transformed into *E. coli* DH5α. Therefore, 10µl of the ligation reaction was incubated with 100 µl of competent *E. coli* DH5α for 30min on ice, 2min at 42°C and 1h at 37°C in 1ml LB medium. Afterwards bacteria were plated out and incubated at 37°C and 5% CO_2. Positive GFP bacteria were selected by LB agar plates containing 20 µg/ml kanamycin, and immune fluorescence microscopy. After overexpression by *E. coli*, plasmids were isolated and transformed into *M. catarrhalis* by electroporation with 1.25kV/cm and 1h incubation at 37°C in 1ml BHI medium, before plating out on agar plates and incubation at 37°C and 5% CO_2. Positive GFP *M. catarrhalis* colonies were selected on 20 µg/ml kanamycin BHI agar plates, by immune fluorescence microscopy, and plasmid digest with *BamH*I and *Sal*I. All enzymes were purchased from New England BioLabs (Bioconcept, Allschwil, Switzerland).

Human cell lines and growth conditions. Chang conjunctival cells, A549 lung cells and Hep-2 laryngeal cells were maintained in Eagle's minimal essential medium (Invitrogen, San Diego, USA) supplemented with 10% of heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C in 5% CO_2. Detroit 562 pharyngeal cells were maintained in Eagle minimal essential medium (Invitrogen), supplemented with 10% of heat-inactivated fetal calf serum (FCS), 2 mmol/L L‑glutamine, 1 mmol/L sodium pyruvate (Sigma, St. Louis, USA), 1% nonessential amino acids (Sigma), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO_2.

Adherence and Invasion assay. The ability of *M. catarrhalis* type 2/3 strains to adhere to and invade human epithelial cells *in vitro* was measured as described previously (29). Adherence and invasion was assessed on Chang conjunctival cells, A549 lung cells, Hep-2 laryngeal cells and Detroit 562 pharyngeal cells as described (1, 29). Invasion blocking experiments were performed as described previously (29). Briefly, Detroit 562 pharyngeal cells were treated with cytochalasin D (Sigma) – to determinate the role of the

actin cytoskeleton – and sucrose (Sigma) to study the involvement of clathrin pits in the internalization process of the bacteria (29). Each strain was analyzed in triplicate in each experiment.

Persistence and evasion testing. To investigate whether internalized bacteria persist within the cells and/or evade over time, long time experiments were performed. For determination of intracellular persistence, Detroit 562 pharyngeal cells were infected as described above, incubated for 3h, treated with 200 µg/ml of gentamicin for 2h and cells were cultivated for an additional 19h and 43h, respectively, with 20 µg/ml gentamicin in the medium to ascertain that we detected intracellular bacteria only, i.e., those initially inoculated for 3h. Samples were plated out after trypsinisation and lysis as described in the invasion experiment to count the number of bacterial colonies per well. To investigate how long intracellular bacteria remained viable, cells were infected and treated as described for the previous experiment. After 2h of high dose gentamicin (200 µg/ml) cells were cultivated with 20 µg/ml gentamicin for additional 10 hours. Every 2 h samples were plated out to quantify the intracellular bacteria. Evasion experiments were performed as decribed for the persistence experiments but without low-dose gentamicin (20 mg/l) for the long-term cultivation of 19 h and 43 h, respectively. Before plating out the bacteria, the samples were treated again with 200 µg/ml gentamicin to kill all *extracellular* bacteria. To quantify *extracellular* bacteria in the supernatant, the supernatant was plated out at every time point before and after gentamicin treatment.

Long time experiments with GFP bacteria and immune fluorescence microscopy. Experiments were performed as described above for the evasion experiments but with the type 2/3 GFP mutant strain 287.GFP. Detroit 562 pharyngeal cells were seeded out in lab-tek chamber slides (8 wells, TPX, Nunc, Thermo Fisher Scientific, Waltham, USA). After 3h, 24h and 48h of infection, the cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature and washed again 3 times with PBS. Actin

was labeled with rhodamine phalloidine (Invitrogen), nuclei were stained with DAPI (Sigma). Antibodies were diluted in Tris-buffered saline that contained 0.25% of bovine serum albumin (Sigma). After additional washing steps, samples were embedded into mowiol (Calbiochem, Darmstadt, Germany) and analysed by immune fluorescence microscopy. Pictures for quantification of bacteria were chosen by systematic random sampling and counted out by the Photo-Capt Software (Vilber Lourmart., Marne-la-Vallée, France).

Preparation of OMP. OMP were prepared by the EDTA buffer method as described (18). Bacteria were harvested from a stationary phase culture, resuspended in EDTA buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, ph 7.4), homogenized and incubated at 55°C at 300 rpm for 1 h. Cells and cell debris were eliminated by centrifugation at 10,000 x g for 15 min at 4°C. Finally, OMP were collected by ultracentrifugation at 100.00 x g for 2 h at 4°C.

SDS-PAGE gel electrophoresis and Western Blot. Samples were resolved by SDS-PAGE using a 7.5% polyacrylamide gel. Band intensity was quantified using the AlphaEaseFC® program from AlphaInotech, Inc (Cell Biosciences, Santa Clara, USA). Antibody detection was performed by Western blot analysis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Billerica, USA). IgA binding was detected using human saliva samples as primary antibody source and goat anti-human IgA, respectively, labeled with horseradish peroxidase (Sigma) as secondary antibody. Super Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co, Thermo Fisher Scientific) was used for detection of antibody binding.

2D-Gel electrophoresis. Analysis of the OMP expression of the type 2 strain 287 at 26°C compared to 37°C was performed as described previously (24), except for the precipitation of the OMP, which was omitted.

Electron microscopy. To visualize invasion and intracellular location of bacteria, infected cells were investigated by transmission electron microscopy (TEM). Cells grown to confluency on microporous filter inserts (0.4µm pore size, BD Falcon, Allschwil, Switzerland)

in 12-multiwell plates were chemically fixed with buffered 2.5% glutaraldehyde (Agar Scientific Ltd.; Essex, UK), 1.0% osmium tetroxide (Oxkem Ltd.; Reading, UK) and 0.5% uranyl acetate (Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland), dehydrated in a graded series of ethanol, and embedded in Epon resin (Fluka) according to standard protocols (9). From the embedded cell inserts, ultrathin sections of 60 nm thickness were cut, transferred onto Formvar-coated 200-mesh copper grids (0.4 % Formvar, Ted Pella Inc. USA) and post-stained with uranyl acetate and lead citrate (Ultrostain; Laurylab, Saint-Fons, France) (9).

Proinflammatory activity of type 2 bacteria compared to type 1 bacteria on human epithelial cells. The proinflammatory potential of *M. catarrhalis* type 1 strains was described previously (25-28, 30). To investigate the proinflammatory cytokine induction by type 2/3 strains, Detroit 562 pharyngeal cells ($3x10^5$/ml) were stimulated by different concentrations ($3x10^5$/ml, $3x10^6$/ml, or $3x10^7$/ml) of heat inactivated type 2/3 strain 287 or heat-inactivated type 1 strain O35E and incubated for 18h at 37°C and 5% CO_2. After incubation, cells were centrifuged for 2 min at 11,800 x g and supernatants were stored at -80°C. Cytokines were measured by the Bio-Plex 200 System (BioRad, Reinach, Switzerland) using the MILLIPLEX MAP Kit, Human Cytokine/Chemokine 96-Well Plate Assay (Millipore).

Statistical analysis. The significance of differences between treatment and control groups was determined using the two-tailed *t*-test. $P < 0.05$ was considered as statistically significant. Each value represents the mean ± one standard deviation of at least three independent experiments performed in triplicate.

RESULTS

Type 2/3 strains invade epithelial cells significantly better than type 1 strains. The ability of *M. catarrhalis* type 1 strains to invade human epithelial cells has been described both in vitro and in vivo (13, 27, 29). Our aim was to investigate the ability of type

2/3 strains to invade various epithelial host cell types in comparison to type 1 strains. Type 2/3 strain 287 demonstrates significantly greater invasion into Chang, A549, Hep2, and Detroit cells, compared to type 1 strain O35E (figure 1). Type 2/3 strain 458 invades similarly, albeit somewhat less efficient than strain 287. We found similar results for other type 2/3 strains (22 and 482) compared with the type 1 strains O35E and 610 tested with the cell types Chang and Detroit 562 (data not shown).

Type 2/3 strain 287 invades epithelia as shown by electron microscopy. To demonstrate that type 2/3 strain 287 truly invades Detroit 562 cells, electron microscope pictures were made by TEM. Both type 2/3 strain 287 and type 1 strain O35E were identified intracellularly, as well as the process of invasion of the bacteria into Detroit cells (figure 2). Intracellular bacteria were directly surrounded by cytoplasm without any vacuole. Furthermore internalized bacteria could be observed as single cells rather than as diplococci. Also it appeared that more type 2/3 penetrated the epithelial cells than type 1 strain O35E.

Cell structures involved in type 2/3 and type 1 invasion, respectively are similar. Structures and mechanisms involved in invasion of *M. catarrhalis* are described for various cell types (27, 29). Microfilaments, Rhotype GTPases and phosphoinositide 3-kinase (PI3K) as well as clathrin and actin could be identified as contributing in the active process of internalization of *M. catarrhalis* into host cells. (25, 29). Figure 3 shows the inhibition of invasion of type 2/3 strain 287 (B) compared to type 1 strain O35E (A) into Detroit 562 cells by sucrose and cytochalasin D. Both strains were significantly inhibited in their invasion by cytochalasin D at 1µg/ml, 5 µg/ml and 10 µg/ml whereas sucrose did not affect invasion in any tested concentration. Strain 287 was more efficiently inhibited by cytochalasin D (92.6% using 1 µg/ml) than strain O35E (40.3% inhibition).

Lack of intracellular long time persistence. To determine the fate of intracellular bacteria after invasion and how they persist in the cells over time, persistence tests were performed and bacteria were plated out after 24h and 48h of cultivation after infection in the

presence of a constant concentration of 20 μg/ml gentamicin in the medium. Neither after 24 h nor after 48 h could any living bacteria be detected (data not shown).

Evasion of intracellular bacteria over time and extracellular proliferation.

The results of the persistence testing clearly indicate that intracellular bacteria do not remain viable for 24 h or longer. Long-term experiments without antibiotics in the supernatant should indicate, whether bacteria are able to escape epithelial cells and proliferate extracellularly. In fact, there is a continuous increase in extracellular bacteria after both 24h as well as 48h although all extracellular bacteria were killed by 200 μg/ml of gentamicin after 3h hours of infection (figure 4). Investigations of invasion still revealed intracellular bacteria after 24h and 48h, the numbers were much smaller than after 3h of infection (figure 4). Counting of intracellular bacteria every two hours after 2h of 200μg/ml of gentamicin treatment demonstrated both a time-dependent decrease of intracellular bacteria and a time-dependant increase of extracellular bacteria (data not shown).

Confirmation of bacterial evasion over time using GFP labelling.

To visualize extracellular proliferation after 2h of high-dose gentamicin exposure aimed at the killing of all extracellular bacteria, long-term experiments with strain 287.GFP and Detroit 562 cells were performed. Analysis by immune fluorescence microscopy revealed a duplication of the approximate bacterial count after 24h and 48h, respectively, compared with the time point at the end of the 3h of infection period (figure 5 and 6).

Type 2/3 strain 287 induces proinflammatory responses similar to type 1 strain O35E. The induction of proinflammatory cytokine release and the subsequent initiation of inflammation has been described repeatedly for type 1 strains (7, 25, 26, 30). Type 2/3 strains, however, have not previously been investigated. Thus, we evaluated the proinflammatory potential of type 2/3 strain 287 on Detroit 562 cells in comparison with the type 1 strain O35E. The human cytokines interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrose factor α (TNFα), interleukin-10 (IL-10), interferon-γ (IFNγ), interleukin 6 (IL-6),

monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and granulocyte macrophage colony-stimulating factor (GM-CSF) were measured by a microsphere based multiplex assay. There was no cytokine release for FGF-2, GM-CSF, IFNγ, IL-1β, IL-10 and TNFα at all (data not shown). IL-6, IL-8, MCP-1 and VEGF release was induced by both bacterial strains in a MOI-dependent manner (figure 7). Both types were similar in the extent of their induction and pattern of proinflammatory activation.

Adherence and invasion of type 2/3 strain 287 is not affected by cold shock treatment. As previously described, cold shock induces increased adherence and interleukin-8 release to and of human epithelial cells, respectively, induced by type 1 strains (14, 30). To investigate whether type 2/3 strains display a similar cold shock response after growth at 26°C compared to 37°C, adhesion and invasion assays were performed. However, neither adhesion nor invasion ratios were affected by cold shock treatment in a significant manner for the tested type 2/3 strain 287.

Protein expression is not significantly altered after growth at 26°C compared to 37°C. Bacterial stress responses are generally reflected by up- or down-regulation of specific proteins such as OMP (10, 14, 30). Such regulatory changes were also observed in type 1 strains after cold shock exposure, both on the RNA and the protein level (14, 30). The investigation of protein expression by type 2/3 strain 287 at 26°C compared to 37°C, however, revealed no significant differences in 2D-Gel analysis of OMP (data not shown).

DISCUSSION

The ability to invade host cells enables a pathogen to escape the hosts' immune system and break through its physical barrier – two of the hosts' main lines of defence. These aspects turn the process of invasion into an important survival strategy for the pathogen and a potentially detrimental virulence factor for its host.

Moraxella catarrhalis is a primary extracellular bacterium and type 1 strains show a nearly 100% ability to adhere and therefore colonize human host cells. Recently, however, it could be demonstrated that *M. catarrhalis* has – both in vitro and in vivo (resected tonsils and adenoids) – the additional capacity to invade human epithelial cells (13, 27, 29). Also, a number of potential mechanisms involved in the internalization of *M. catarrhalis* have been identified. Slevogt et al. demonstrated that microfilaments, Rho type GTPases, and phosphoinositide 3-kinase (PI3K) - dependent contractile mechanisms are involved for the invasion into BEAS-2B bronchial epithelial cells and A549 cells. Spaniol et al. observed that clathrin as well as actin polymerization is involved in the invasion into Chang conjunctival cells (27, 29). In addition, specific bacterial outer membrane structures could be identified. Loss of the major adhesin UspA1, as well as the lack of LOS affected not only adherence but also invasion (29).

Our findings that type 2/3 strains appear to have a greater capacity to invade host cells than type 1 strain sheds a new light on this seemingly less virulent subpopulation. This is an unexpected finding if we consider that type 2/3 strains do not express known invasins that mediate both adherence and invasion of type 1 strains. Thus, mechanisms other than those identified in the latter may mediate adhesion and invasion by type 2/3 strains. Our data indicate that adhesion ratios - after 30 min of infection - in the strains 287 and 458, respectively, are approximately 30%, but nevertheless their association after 3h of infection seems to be the same or even greater than that of type 1 strains. On the other hand, invasion inhibition experiments show that the involved host cell structures may be the same for type 1 and type 2/3 strains, respectively. For both types, invasion into Detroit cells was inhibited in a dose dependant manner by cytochalasin D, which inhibits actin polymerization, whereas sucrose, which would indicate the presence of clathrin-coated pits did not prevent invasion. As previously shown for type 1 strains, these data also suggest that the involved internalization mechanisms are host cell specific, as has been shown by Slevogt et al. and

Spaniol et al., who differed in their findings concerning the involvement of clathrin polymerization (27, 29). Slevogt et al. failed to identify any involvement of clathrin polymerization for A549 and BEAS-2B cells – exactly as we did with Detroit 562 cells – whereas Spaniol et al. identified both clathrin as well as actin polymerization is being involved in bacterial internalization into Chang cells (27, 29). These findings were corroborated by electron microscopy, which showed more intracellular type 2/3 bacteria than type 1 bacteria and - on the other hand – visualized an internalization process which clearly differed from what has been demonstrated by Slevogt et al.. Interestingly, in this respect our electron microscopy data were indistinguishable for the two different bacterial subpopulations (27). For Detroit cells there was no clear macropinocytosis-like mechanism visible neither for type 1 strain O35E nor for type 2/3 strain 287.

The results of our long-term experiments regarding the fate of intracellular bacteria after invasion led us to the conclusion that there is no intracellular persistence of *M. catarrhalis* – neither for type 1 strains (data not published) nor for type 2/3 strains. The experiments indicate that bacteria obviously evade the host cells over time and proliferate extracellularly. LDH cytotoxicity tests show an increase of LDH release in infected cells over time (data not published), which indicates the decay of cells, presumably caused by infection. If they are able to establish a cycle of re-infection, this will need to be further investigated, but it would indeed represent a common mechanism for serum sensitive bacteria to escape the complement system and survive intracellularly (5). This would also challenge the concept that less clinical isolates of type 2/3 circulate among humans compared to type 1 strains (6, 34). Typically, clinical isolates are obtained by swab sampling, which detects pathogens on the epithelial surface rather than intra- or subepithelial bacteria.

As of today no data regarding the proinflammatory potential of type 2/3 strains have been published. One could postulate that because of their lack in UspA1 expression, type 2/3 strains induce less epithelial inflammation than type 1 strains. In contrast, the results of our

cytokine profile demonstrate that type 2/3 strain 287 induces a very similar cytokine release in Detroit 562 cells when compared with type 1 strain O35E. Not only the same amounts of IL-8, IL-6, MCP-1 and VEGF, but also the same types of cytokines were induced. Both types show no IL-1β production, which was also observed by Fink et al. for A549 cells, and also other respiratory bacterial pathogens display this type of cytokine profile (3, 4, 7, 8). These findings indicate that *M. catarrhalis* type 2/3 has different stimulating structures than those known of type 1, and show the same high virulence state regarding their proinflammatory activity.

The *M. catarrhalis* type 1 subpopulation is able to react on a temperature downshift of approximately 10°C – which could occur in the nasopharynx in vivo after breathing cold air – with a so-called cold shock response (14, 21, 22, 30). Thereby, the expression of various proteins is up- or downregulated, such as the OMP UspA1 - an important adhesin and invasin as well as a proinflammatory stimulus for human epithelial cells (30). UspA1 is strongly upregulated after exposure to 26°C and this leads to a significantly greater adhesion and proinflammatory activity – which indicates that *M. catarrhalis* could increase its virulence after exposure to cold air (30). Our findings for *M. catarrhalis* type 2/3 strains, however, indicate that they show no cold shock response. We saw no significant differences for the phenotypes of adhesion or invasion, as well as no significant differences in protein expression using 2D gel electrophoresis. These results could be possibly explained by the fact that type 2/3 strains overall are not well adapted to the extracellular life. They are less able to adhere, typically are complement sensitive but show excellent invasion abilities. It is conceivable that type 2/3 strains display a different survival and colonisation strategy compared to type 1 strains and, as one possible consequence, do not need cold shock adaptation.

In summary, here we demonstrate previously undescribed, putative *M. catarrhalis* type 2/3 virulence mechanisms. The ability to invade human epithelial cells in a significantly

greater extent than type 1 strains as well as their comparable proinflammatory activity on human epithelial cells characterize a new picture of this so called non virulent subpopulation. Also, their ability to associate to the epithelium, together with their still largely unknown outer membrane structures and functions warrant further investigations of type 2/3 strains.

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FIGURE LEGENDS

Figure 1

Invasion ratio (proportion of the inoculum) of type 1 strain O35E, type 2/3 strain 287 and type 2/3 strain 458 into Chang conjunctival cells (A), A549 lung cells (B), Hep-2 laryngeal (C) and Detroit 562 pharyngeal cells (D). Data are represented as means ± 1 SD (n=3) of at least three separate experiments. * $p < 0.05$ for strain O35E vs. strain 287 respectively strain 458.

Figure 2

Transmission electron micrographs of *M. catarrhalis* type 2/3 strain 287 (A-E) and type 1 strain O35E (F-H), respectively, in human Detroit 562 pharyngeal cells. Black arrows indicate extracellular respectively to the cell surface attached bacteria. White arrows indicate intracellular bacteria. Pictures B, D and E show the internalization process of the extracellular bacteria to the intracellular.

Figure 3

Inhibition of the invasion into Detroit 562 pharyngeal cells of type 1 strain O35E (A) and type 2/3 strain 287 (B) by Sucrose (0.05M, 0.1M, 0.2M) and cytochalasin D (0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml). Data are represented as means ± 1 SD (n=3) of at least three separate experiments. * $p < 0.05$ for inhibitor free controls (co) vs. the respective inhibitor concentrations.

Figure 4

(A) Intracellular bacteria (cfu/well) at 3 h, 24 h and 48 h after infection of Detroit 562 pharyngeal cells. All samples were treated with 200 µg/ml gentamicin after the first 3 h and additionally after 24 h respectively 48 h of infection to kill all *extracellular* bacteria. (B)

Extracellular bacteria in the supernatant (cfu /well) after 3 h, 24 h and 48 h of Detroit 562 pharyngeal cell infection before and after 2 h of 200µg/ml gentamicin treatment. Data are represented as means ± 1 SD (n=9) of at least three separate experiments.

Figure 5

Over time evasion and proliferation of *M. catarrhalis* strain 287.GFP (green) after 3 h (A), after 24 h (B) and 48 h (C) of infection. Actin is labelled with rhodamine phalloidine (red) and the nucleus with DAPI (blue). (D) Intracellular bacteria (yellow) after 48h from above and the side view in- and onto the cell.

Figure 6

GFP bacteria strain 287 (bacteria / 0.61mm^2) after 3 h, 24 h and 48 h of infection. All samples were treated with 200 µg/ml gentamicin after the first 3 h of infection to kill all *extracellular* bacteria. Data are represented as means ± 1 SD (n=18) of at least three separate experiments. * $p < 0.05$ for 3h vs. 24 h respectively 48 h.

Figure 7

Cytokine profile of Detroit 562 pharyngeal cells induced by type 1 strain O35E and type 2/3 strain 287. Cytokine release (pg/ml) of human IL-6 (A), IL-8 (B), MCP-1 (C) and VEGF (D) after 18 h of incubation. Data are represented as means ± 1 SD (n=9) of at least three separate experiments.

TABLES

Table 1

Strains used in this study.

Strain	Description	Source or reference
M. catarrhalis O35E	Type 1 strain; middle ear isolate	(15)
M. catarrhalis 610	Type 1 strain; nasopharyngeal isolate	(17, 31)
M. catarrhalis 287	Type 2 strain; nasopharyngeal isolate	(17, 31)
M. catarrhalis 22	Type 2 strain; nasopharyngeal isolate	(17, 31)
M. catarrhalis 458	Type 3 strain; nasopharyngeal isolate	(17, 31)
M. catarrhalis 482	Type 3 strain; nasopharyngeal isolate	(17, 31)
M. catarrhalis 287.GFP	Type 2 strain; nasopharyngeal isolate	this study
Escherichial coli DH5α	Host strain for plasmid constructs	(23)

FIGURES

Figure 1

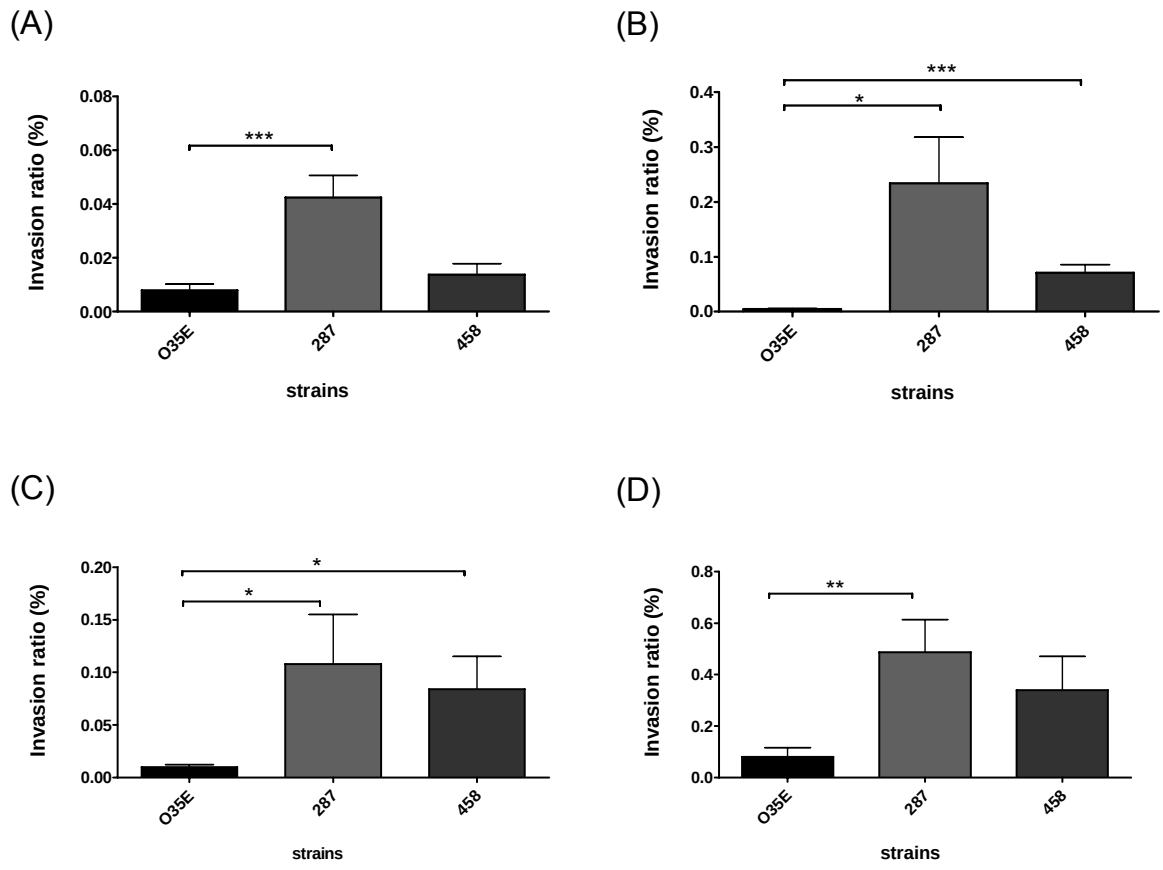


Figure 2

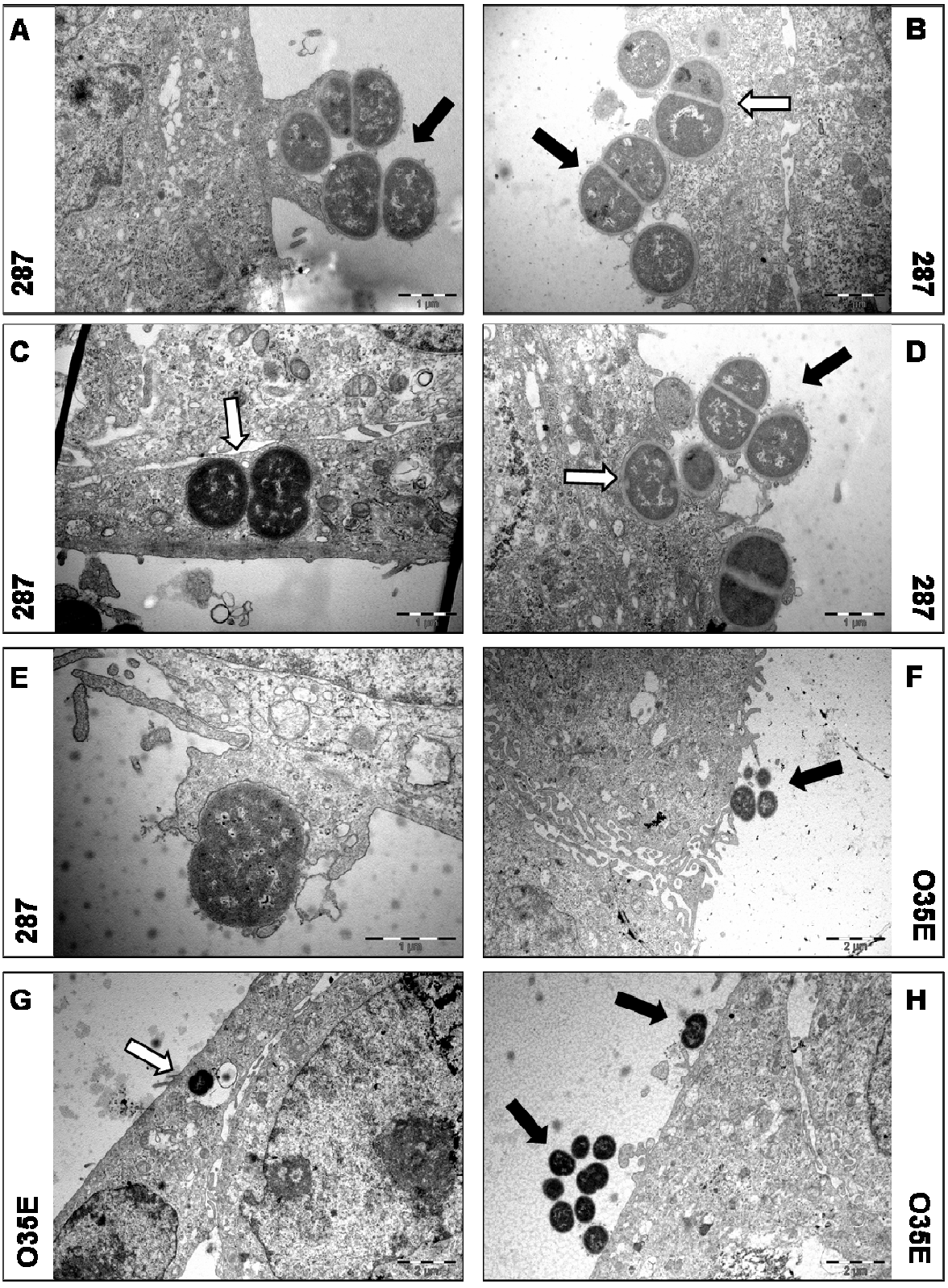


Figure 3

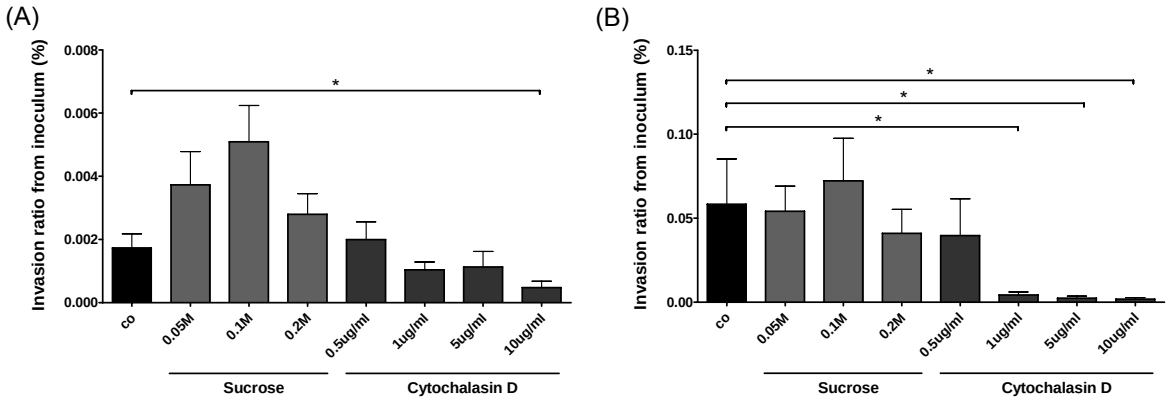

(A)
Invasion ratio from inoculum (%)
0.008
0.006
0.004
0.002
0.000
*
co
0.05M
0.1M
0.2M
0.5ug/ml
1ug/ml
5ug/ml
10ug/ml
Sucrose
Cytochalasin D
(B)
Invasion ratio from inoculum (%)
0.15
0.10
0.05
0.00
*
*
*
co
0.05M
0.1M
0.2M
0.5ug/ml
1ug/ml
5ug/ml
10ug/ml
Sucrose
Cytochalasin D

Figure 4

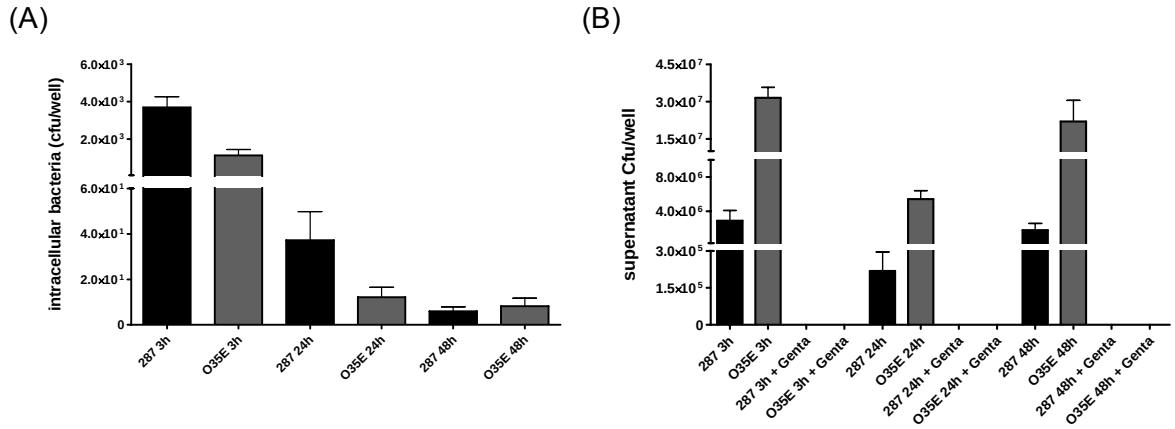

(A)
intracellular bacteria (cfu/well)
6.0x10 3
4.0x10 3
2.0x10 3
6.0x10 1
4.0x10 1
2.0x10 1
0
287 3h
O35E 3h
287 24h
O35E 24h
287 48h
O35E 48h
(B)
supernatant Cfu/well
4.5x10 7
3.0x10 7
1.5x10 7
8.0x10 6
4.0x10 6
3.0x10 5
1.5x10 5
0
287 3h
O35E 3h
287 3h + Genta
O35E 3h + Genta
287 24h
O35E 24h
287 24h + Genta
O35E 24h + Genta
287 48h
O35E 48h
287 48h + Genta
O35E 48h + Genta

Figure 5

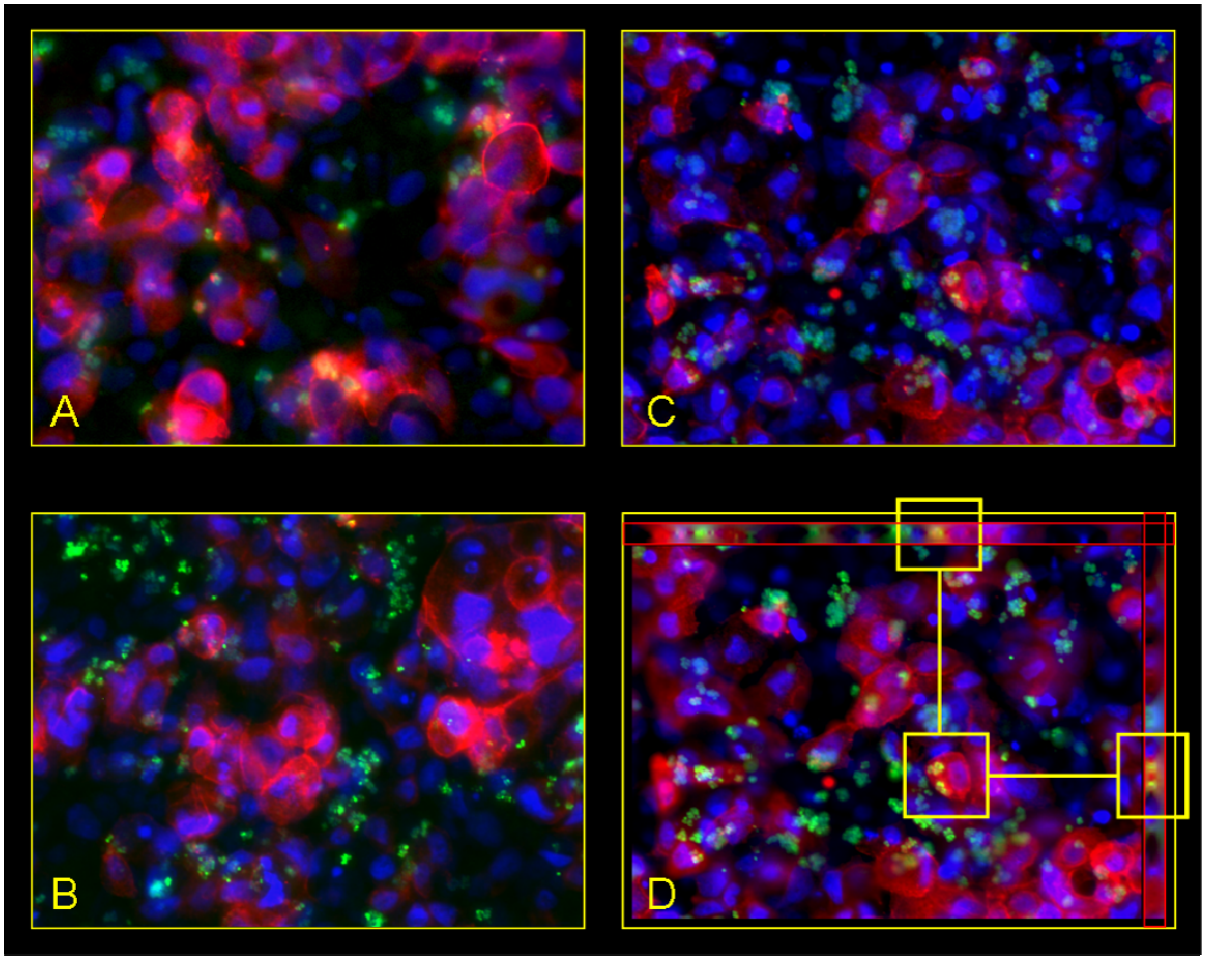


Figure 6

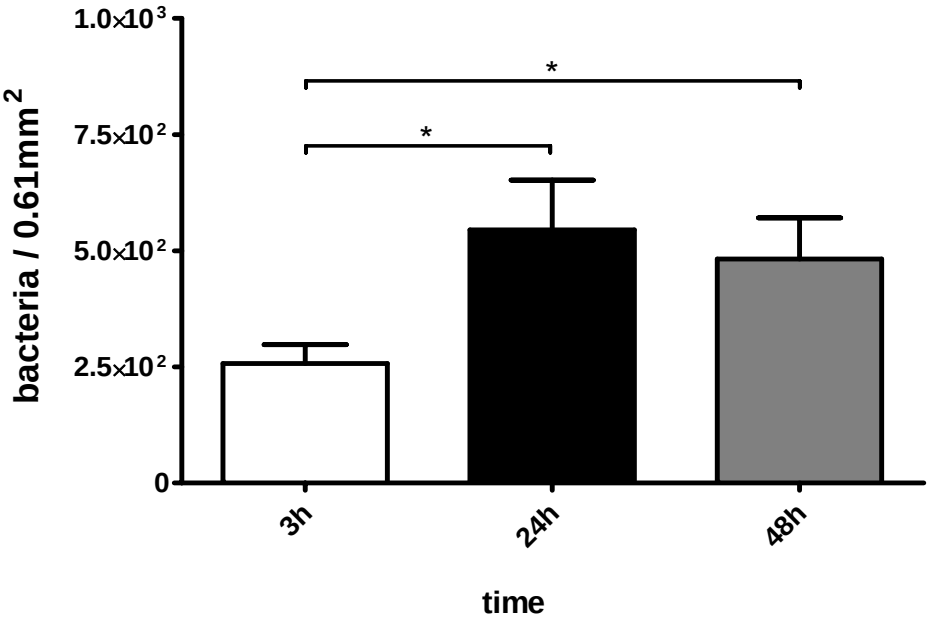
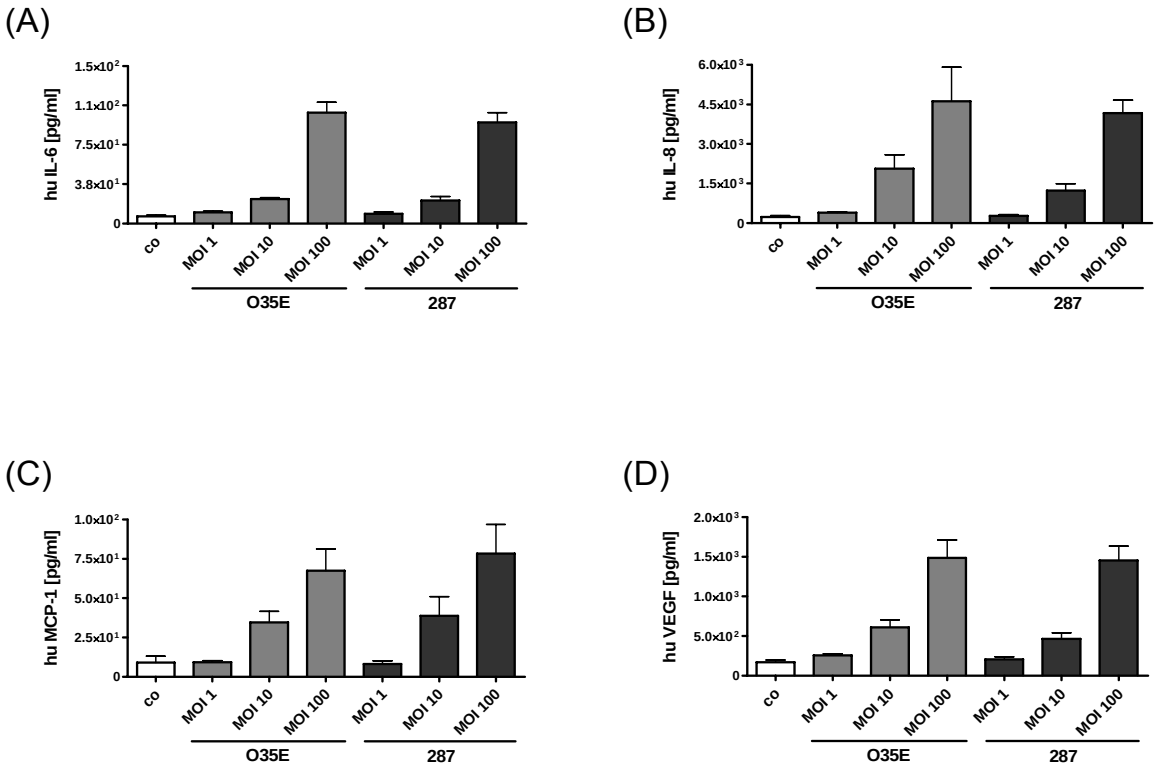


Figure 7


(A)
hu IL-6 [pg/ml]
1.5x10²
1.1x10²
7.5x10¹
3.8x10¹
0
co
MOI 1
MOI 10
MOI 100
MOI 1
MOI 10
MOI 100
O35E
287
(B)
hu IL-8 [pg/ml]
6.0x10³
4.5x10³
3.0x10³
1.5x10³
0
co
MOI 1
MOI 10
MOI 100
MOI 1
MOI 10
MOI 100
O35E
287
(C)
hu MCP-1 [pg/ml]
1.0x10²
7.5x10¹
5.0x10¹
2.5x10¹
0
co
MOI 1
MOI 10
MOI 100
MOI 1
MOI 10
MOI 100
O35E
287
(D)
hu VEGF [pg/ml]
2.0x10³
1.5x10³
1.0x10³
5.0x10²
0
co
MOI 1
MOI 10
MOI 100
MOI 1
MOI 10
MOI 100
O35E
287

DISCUSSION AND OUTLOOK

4. DISCUSSION AND OUTLOOK

Approximately 80% of all children below three years of age undergo at least one acute otitis media, which is one of the main reasons for antimicrobial prescriptions in children. With a fraction of 15-20% and a continuously increasing prevalence *M. catarrhalis* is the third major bacterial pathogen causing AOM. This thesis provides new insights into antimicrobial defence strategies of *M. catarrhalis* against aminopenicillins and characterizes new virulence factors of the *M. catarrhalis* 16S rRNA type 2/3 lineage.

AIM1

The high degree of DNA sequence conservation of M35 in *M. catarrhalis* 16S rRNA type 1 strains [57] renders this outer membrane porin a potential vaccine candidate. It is also an attractive OMP for additional phenotypic analyses including potential functions in virulence.

In their capacity as passive transport channels, porins of gram-negative bacteria are - in addition to nutrient uptake - also in the absorption of selected antimicrobial substances [62, 66]. However, porins are not only involved in the uptake but also in limiting antibiotic access to the bacterial cell. By altering their porin expression or functionality, bacteria create a physiological barrier for drugs. Therefore, bacterial porins hold an important position in antimicrobial resistance mechanisms [62, 66]. For example, resistance against β–lactam antibiotics often consists of a highly efficient combination of the production of detoxifying enzymes – β-lactamases – and an altered porin expression. OmpC from *Escherichia coli,* OmpK36 of *Klebsiella pneumoniae* and Omp36 (an OmpC homologue) from *Enterobacter aerogenes*, porins which show a high structural homology to M35 [57], exhibit a β-lactam-antimicrobial treatment-induced down-regulation in their porin expression [62, 66, 128-137]. Our findings of significantly higher minimum inhibitory concentrations (MIC) in all tested *m35*

knock out mutants indicate the involvement of M35 in the uptake and susceptibility to aminopenicillins. The down-regulation of the porin on both the transcriptional as well as the translational level after amoxicillin treatment would display a common regulation mechanism of porin-associated antimicrobial resistance. To our knowledge this is the first study investigating a porin involved in antimicrobial resistance of *M. catarrhalis*. The only currently known resistance mechanism for *M. catarrhalis* has been the production of two different β-lactamases, BRO1 and BRO2 [3, 52]. If our *in vitro* findings affect the still recommended amoxicillin therapy of AOM patients *in vivo***,** further investigations are needed. In particular, experimental settings would have to be created to study the possibility that *M. catarrhalis* become resistant to amoxicillin during therapy because of M35 down-regulation induced by the latter.

Porins such as OmpC and OmpK36 could be shown as responding with a stress response not only to antimicrobial treatment, but also to environmental variables such as altered osmolarities, temperature or pH [138-141]. The down-regulation of M35 after cold and heat shock treatment, respectively , growth at enhanced osmolarity and iron depleted conditions indicates a major role of M35 in general stress responses of *M. catarrhalis.* Whether *M. catarrhalis* has an antagonist to M35, which is upregulated in case of M35 down-regulation comparable to the situation in *E. coli* or *K. pneumoniae* [140-142] still requires further analysis.

The high degree of genetic conservation of M35 in type 1 strains [57] predestinates this porin as a possible vaccine candidate. The detection of human IgA antibodies by immunoblot analysis indicates that M35 is expressed *in vivo* and recognized as a mucosal antigen. However, the results suggest the possibility of antigenic variation of the immunoreactive epitopes and challenge the usefulness as a vaccine candidate. The differences between the

m35 sequence of the type 2/3 strains versus type 1 strains are in line with this issue and demand additional studies. Analyzing the type 2/3 M35 protein regarding structure and function as well as the anti-M35-antibody response appears essential. Overall the porin should be analysed by searching for highly conserved epitopes, which induce bactericidal IgG antibodies.

AIM 2

Since *M. catarrhalis* consists of the two phylogenetic lineages which differ not only in their 16S rRNA subunit but apparently also in their degree of virulence [6, 8, 9, 11, 93, 143] research mainly focused on the more virulent 16S rRNA type 1 subpopulation. Information about the type 2/3 lineage was limited largely to genetic information on individual genes or their expression, and was compared to those, who play a role in type 1 virulence [144]. Phenotypically, the dominant difference is serum sensitivity, which can be found for almost all type 2/3 strains tested [6, 9, 93]. Their inability to adhere to epithelial cells is a somewhat less distinctive feature [6]. Nevertheless clinical isolates of *M. catarrhalis* type 2/3 strains are clearly represented, albeit in a lower proportion than type 1 strains. [11].

Our findings of significantly higher invasion ratios of type 2/3 compared to type 1 strains, together with the observation that intracellular bacteria escape into the extracellular space for replication, open new perspectives regarding the potential degree of virulence of this serum sensitive lineage. Greater invasion ability could protect bacteria against host clearance and may explain the low proportion of clinical type 2/3 isolates obtained by swab sampling on the mucosal surface. Previous studies identified internalized *M. catarrhalis* in the lamina propria of human lymphoid tissue *in vivo* [120]. It remains to be analysed whether this epithelial/subepithelial niche is a preferred habitat of type 2/3 strains, and whether the

complement sensitive strains show a greater ability to invade adenoids and tonsils than the complement resistant lineage.

Internalization of typically extracellular bacteria could be shown in several studies of gram-positive as well as gram-negative bacteria [145-150]. Different internalization pathways could be identified [145, 151-153]. For *M. catarrhalis*, our findings of the inhibition experiments and our analysis of the TEM images indicate a internalization mechanism, which differs from macropinocytosis as described by Slevogt et al. [119]. Thus, cellular invasion by *M. catarrhalis* requires further investigation. In particular additional studies will have to address whether the mechanism of internalization is cell type-specific and what molecular pathways underly each mechanism identified. Furthermore, adhesins specific for type 2/3 should be identified. Preliminary data indicate that type 2/3 strains are indeed able to associate with epithelial cells, but ligands and receptors are currently unknown.

The degree of virulence pertaining to interactions with the host immune system could be defined by an organism's ability to escape the immune system, but also by its pro-inflammatory activity. The serum sensitive lineage of *M. catarrhalis* induces the same cytokine profile as well as the same magnitude of cytokine release in comparison with the serum resistant lineage. These findings suggest that both pathogens are recognized with the same intensity by the host cells and therefore are similarly virulent. On the other hand, results indicate that the type 2/3 lineage has different stimulating structures than those known from type.

Epidemiologically it would be interesting to perform 16S rRNA type colonization studies in healthy but also diseased children and adults. Such an endeavour could supplement studies by Verhaegh et al. who compared isolates cultured from adults with those from children and

identified a drift from LOS serotype A to serotype B and a reduced incidence of *uspA2* as well as a downregulation of Hag, all of which were regarded as adaptations in order to evade the human immune response [93]. These findings are similar to the characteristics found in type 2/3.

M. catarrhalis 16S rRNS type 2/3 strains are poorly investigated and are - due to their differences to type 1 - regarded as the less virulent. The results of this work give first insights into the pathogenesis of this serum sensitive lineage and question the assumption of reduced virulence. However, our data also unveiled a number of further questions, which await detailed analysis. Above all, the still largely unknown outer membrane surface structures and their functions require further investigations.

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7. APPENDIX

7.1 Declaration of Originality

Last name, first name: Jetter Marion

Matriculation number: 07-106-511

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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