Outer membrane protein M35 is an essential general porin for *Moraxella catarrhalis*

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Abstract

_Moraxella catarrhalis_ is a Gram negative respiratory pathogen that is an important causative agent for otitis media and exacerbations of chronic obstructive pulmonary disease. We have previously predicted the outer membrane protein M35 to be a general porin and in the current study we have investigated the function of M35 and its importance for survival of _M. catarrhalis in vivo_. Lipid bilayer experiments reveal that refolded M35 functions as a channel that is typical of gram-negative bacterial porins. M35 forms wide and water-filled channels with a single-channel of about 1.25 nS in 1 M KCl solution and has only a small selectivity for cations over anions. When the _in vitro_ growth characteristics of two M35 deletion mutant strains of _M. catarrhalis_ were compared to the wildtype parent isolates, the growth of the mutant strains was only inhibited under nutrient-poor conditions. This growth defect could be eliminated by additional glutamic acid, but not additional aspartic acid, glycine, sucrose or glucose. The mutant strains compensated for the lack of M35 by enhancing their uptake of glutamic acid and this enhanced rate of glutamic acid uptake was attributed to the compensatory up-regulation of a novel 40 kDa protein. M35 was also found to be essential for nasal colonisation of mice, demonstrating that its presence is essential for survival of _M. catarrhalis in vivo_. These results suggest that M35 is a general porin that is necessary for the uptake of important energy sources by _M. catarrhalis_ and that it is likely that M35 is an essential functional protein for _in vivo_ colonisation.
**Introduction**

*Moraxella catarrhalis* is a Gram-negative bacterium that is mainly responsible for respiratory tract infections such as otitis media, sinusitis and exacerbations of chronic obstructive pulmonary disease (COPD) (25, 31, 43). On rare occasions, it can also cause more serious diseases such as meningitis and septicaemia (16, 28).

All Gram-negative species investigated have been found to produce porins (34). Outer membrane porins are passive pores that allow the influx and efflux of hydrophilic nutrients and waste products across the outer membranes of Gram negative bacteria (34). In most species general porins are expressed in very high levels, accounting for the majority of the OMPs and this leads to a high degree of permeability of the outer membrane to small hydrophilic molecules (1, 26). General porins, such as OmpF and OmpC from *Escherichia coli*, are permeable to small (< 600 Da) hydrophilic molecules whereas substrate-specific porins, like the sucrose-specific porin ScrY from *Salmonella typhimurium* (26), have binding sites for particular molecules that enhance the efficiency of their diffusion.

Substrate-specific porins increase the efficiency of acquisition of nutrients that are available at low extracellular concentration and would therefore not diffuse through general porins at a high enough rate to sustain an adequate supply to the bacterial cell (34). These specific channels are not truly specific for one particular substrate but rather facilitate increased diffusion of a particular class of molecule, for example ScrY mentioned above increases permeability of the membrane to sugars other than sucrose, including glucose, fructose, arabinose, maltose, lactose, raffinose and maltodextrins (34). Slow diffusion of other small molecules is also usually possible through the pores. These
specific channels are generally regulated in response to environmental stimuli, such as osmolarity or specific nutrient availability, and complement the general porins, which mediate the high degree of permeability of the outer membrane. An interesting exception is the outer membrane of *Pseudomonas aeruginosa* that has very low permeability in comparison to other species. It overcomes this by producing many different substrate-specific porins that allow it to acquire the necessary substrates from its environment (3, 39).

M35 was the first *M. catarrhalis* protein identified that had homology with other known porins (20). To date, no other *M. catarrhalis* OMPs have been positively identified as porins. Murphy et al. (33) suggested that OMP CD may be a porin, based on homology with OprF from *P. aeruginosa* that is itself a homolog of the *E. coli* OmpA protein (34), and *M. catarrhalis* OMP E shares homology with the FadL family of fatty acid transporters (32). M35 is a 36.1 kDa surface exposed protein that demonstrates homology with the *Klebsiella pneumoniae* porin K36 and the *Escherichia coli* porin OmpC (20). M35 is unusually highly conserved for a porin, which are often variable in the external loop regions due to factors such as immunological selective pressure (4, 19, 38). The high level of conservation of M35 across strains and the likelihood that it is a porin together suggest that M35 may be a particularly important functional protein for *M. catarrhalis*. Additionally, immunisation with M35 enhances bacterial clearance in the murine pulmonary challenge model (unpublished data) and M35 may therefore be of interest as a potential vaccine antigen since it is a highly conserved, surface exposed protein that is likely to be important for survival of *M. catarrhalis in vivo*. This study aimed to confirm that M35 is a porin and to determine whether any uptake of sugars,
amino acids or antibiotics required M35 in vitro. We checked the ability of refolded M35 to form channels in lipid bilayer membranes. The results suggest that this protein forms general diffusion channels with a size similar to OmpC of \textit{E. coli} K12 but with a much smaller selectivity (12) and that M35 is essential for the survival of \textit{M. catarrhalis} \textit{in vivo}. In the absence of M35 a novel 40 kDa protein was up-regulated and this protein may have been responsible for enhanced uptake of glutamic acid by \textit{M. catarrhalis}.

**Methods**

**Black Lipid Bilayer Experiments**

The methods used for painted black lipid bilayer experiments were previously described in detail (10). The experimental set up consisted of a Teflon cell with two water-filled compartments divided by a thin wall and connected by a small circular hole. The hole had an area of about 0.3 to 0.5 mm². Membranes were formed across the hole from a 1\% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane. The aqueous salt (analytical grade) solutions were used unbuffered and had a pH of 6. Refolded M35 was added to one or both sides of the membrane from concentrated protein solutions. Ag/AgCl electrodes (with salt bridges) were connected in series to a voltage source and a current amplifier (Keithley 427). The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart (Rikadenki, Freiburg, Germany) or a tape recorder. Zero current membrane potentials were measured with a Keithley 617 electrometer 5-10 minutes after a 5-fold salt gradient was established across the membranes containing about 100 to 1000 channels (12).
Insertional inactivation of the *M35* gene

A kanamycin resistance cassette was inserted in place of nucleotides 349 to 444 of the *M35* gene, taking advantage of the *Eco*RI restriction site contained in the M35-L3 insert described previously for the construction of the M35loop3− recombinant protein (20). This construct was then PCR amplified and used to naturally transform *M. catarrhalis* isolates 4223 and K114 using the method described by Furano and Campagnari (22).

The *M. catarrhalis* isolates were grown to early log phase in BHI broth (Oxoid) and a 100 µL aliquot was spread on to CBA plates containing 5 % partially lysed horse blood. After PCR amplification the M35KAN amplicon was purified using the Qiagen MinElute PCR Purification kit and quantitated by agarose gel electrophoresis and comparison with the Invitrogen E-gel Low Range Quantitative DNA Ladder. An aliquot containing approximately 20 ng of DNA was then spotted on to each agar plate and incubated for approximately 5 hours at 37 ºC with 5 % CO₂ to allow the *M. catarrhalis* cells to take up the DNA. Bacterial cells within the area the DNA was spotted on to were then swabbed on to CBA plates containing 25 µg/mL kanamycin and incubated overnight at 37 ºC with 5 % CO₂. *M. catarrhalis* cells that were able to grow in the presence of kanamycin were likely to have recombined the M35KAN construct into the genome and were grown overnight again in the presence of kanamycin before storing at -80 ºC in BHI media containing 15 % glycerol.

SDS-PAGE analysis and western blotting with anti-M35 mouse serum confirmed deletion of the M35 protein and replacement of the *M35* gene by the M35KAN construct was confirmed by DNA sequencing. The deletion mutant strains were named 4223ΔM35
and K114ΔM35, respectively, and 25 µ/mL kanamycin was included in the media used for their growth unless specified otherwise.

Antibiotic sensitivity

Antibiotic susceptibility of *M. catarrhalis* was tested by the disc diffusion method. Several different isolates of *M. catarrhalis* were grown on Mueller-Hinton agar under standard growth conditions and a single colony was used to inoculate 10 mL of Mueller-Hinton broth. The broth culture was grown for 5 hours at 37 ºC with shaking at 200 rpm and the resulting culture was diluted with fresh Mueller-Hinton broth to give a turbidity equivalent to a 0.5 McFarland standard. A sterile swab was used to inoculate a Mueller-Hinton agar plate with this culture and the antibiotic sensitivity discs (Oxoid) were placed on the agar. The antibiotics used were cefotaxime (5 µg) and imipenem (10 µg). The plates were incubated overnight (18 hours) at 37 ºC with 5 % CO₂ and the zone of inhibition was measured. All isolates were tested in triplicate.

Growth comparison of deletion mutants with wildtype isolates

Growth of 4223ΔM35 and K114ΔM35 on CBA plates did not appear to differ in colony morphology from the wildtype isolates. To establish whether the mutant strains were adversely affected by the deletion of M35 their growth rates in BHI broth were compared to the respective wildtype isolates. Overnight cultures of each isolate in BHI broth were used to inoculate fresh media to an OD at 600 nm (OD₆₀₀) of 0.1 and these cultures were incubated at 37 ºC with shaking at 220 rpm. The OD₆₀₀ was recorded every 30 minutes for 4 hours and eight serial 10-fold dilutions were spotted on to CBA plates to
confirm that the cell density was accurately represented by the OD$_{600}$ readings. This was repeated three times to confirm the repeatability of the results.

Growth analysis in the presence of additional sugars or amino acids

10 % BHI broth was prepared by diluting BHI broth made according to the manufacturer’s instructions with sterile water 1/10. For growth rate analysis, cultures contained 1.5 mL neat BHI, the appropriate volume of additional substrate (2 M stock, 0.22 µm filtered) to give the desired final concentration, the appropriate volume of the overnight starter culture to give an OD$_{600}$ of 0.1 and the necessary volume of sterile water to give a total volume of 15 mL. The cultures were incubated at 37 ºC with shaking at 220 rpm for 3-4 hours and samples were taken every 30 minutes to record the OD$_{600}$. The substrates tested were glucose, sucrose, glycine, aspartic acid and glutamic acid and the concentrations tested were 0, 10, 50, 100 and 200 mM.

The OD$_{600}$ was graphed against time for each culture and the rate coefficient (k) was determined from the first order rate equation. The rate coefficient was determined by linear regression of ln(OD$_t$/OD$_0$) versus time (t), as described by the integrated rate equation ln(OD$_t$/OD$_0$) = kt. Triplicate k values were determined for each condition from independent cultures and the average k values were graphed against the concentration of additional substrate added to the cultures.

Uptake assay

An uptake assay following the method outlined by Monaco et al. (30), with some modifications, was used to assess the affect deletion of M35 has on glutamic acid uptake by M. catarrhalis. Isolates 4223 and 4223ΔM35 were grown overnight on CBA,
harvested, washed twice in buffer A (50 mM K$_2$HPO$_4$, 0.5 mM MgCl$_2$, pH 6.9) and the
concentration was adjusted to 1 x 10$^9$ CFU/mL using the OD at 400 nm in buffer B
(buffer A with 300 µg/mL chloramphenicol). The cell suspensions (in buffer A) were
titrated by serial 10-fold dilution and spotting on to CBA plates to ensure that the
concentration of viable cells in the two cell suspensions was equivalent. The cell
suspensions were kept on ice until needed.

For the assay 20 µL of bacterial suspension was added to 20 µL of buffer C (buffer A
with 3 % glycerol and 40 mM NaCl) warmed at 37 °C for 10-15 minutes to energise the
bacteria. Since it is not known whether the glycerol included by Monaco et al. (30) for
their study of *N. meningitidis* would have the desired effect on *M. catarrhalis*, acetate and
lactate were also added for some experiments. Lactate and acetate were chosen because
*M. catarrhalis* has been shown to utilise both of these compounds as carbon sources (5,
37). For such assays buffer C also contained 0.02 % (w/v) acetate and 0.02 % (w/v)
lactate.

A 20 µL solution of L-[G-3H]glutamic acid (2.04 TBq/mmol) at three times the
desired final concentration was added to the energised cell suspensions to start the assay.
To stop the assay at the indicated time points 50 µL of the cell suspension was filtered
through 13 mm Millipore Express PLUS 0.45 µm membrane filters held in disc filter
holders attached to a vacuum manifold. The filters were immediately washed with 5 mL
of buffer A and allowed to air dry before being placed in scintillation vials containing
250 µL of DMSO to dissolve them. Once the filters had dissolved, 4 mL of BCS
Scintillation Cocktail (Amersham Biosciences) was added and the radioactivity was
counted using a liquid scintillation analyser (Tri-Carb 1600CA, Packard). The counts per
minute were converted to disintegrations per minute (DPM) using a previously developed quench curve.

Nasal colonisation

The ability of the wildtype and M35 deletion mutant strains to survive in the nasopharynx of mice was assessed using a previously developed colonisation assay (27). *M. catarrhalis* 4223 and 4223ΔM35 were grown overnight on CBA, harvested and washed twice in sterile PBS. The concentrations of the bacterial suspensions were adjusted to $2 \times 10^{10}$ CFU/mL and this was confirmed by titration. Ten male BALB/c mice were anaesthetised by intraperitoneal injection with 200 µL of a 1:2 mixture of Zoletil (stock 100 mg/ml) and Xylazine (stock 20 mg/ml) in PBS and nasally inoculated with 5 µL of each bacterial suspension.

After three days five mice from each group were euthanised and the bacteria recovered by nasal lavage with 50 µL sterile PBS per nare. The recovered bacteria were evaluated by spotting 20 µL of four 10-fold serial dilutions in PBS on to CBA plates. The colony morphologies, Gram stain characteristics and approximate number of colonies in each spot were assessed after overnight incubation. This was repeated with the remaining five mice in each group seven days after the inoculation and bacterial recovery from five uninoculated control mice was also assessed at both time points. This procedure was undertaken as a qualitative assessment because the volume and quality of nasal lavage fluid collected was not adequately uniform between individual mice to allow quantitative evaluation. The nasal colonisation assay was conducted with the approval of the University of Canberra Committee for Ethics in Animal Experimentation.
Statistical analysis

Growth coefficients (k) for each concentration were compared between the wildtype and deletion mutants using a two-way analysis of variance followed by Bonferroni’s multiple comparison test using GraphPad Prism 4.0b. The values were also compared between different concentrations for the same strains using one-way analysis of variance followed by Bonferroni’s multiple comparison test using GraphPad Prism 4.0b.

Results

Interaction of the M35 protein with lipid bilayer membranes

Conductance measurements were performed with lipid bilayer membranes to study the interaction of refolded M35 protein with artificial membranes. Refolded M35mat (20) was diluted 1:100 with a 1% Genapol solution and 1 µL (approx. 2.5 µg) was added to one or both sides of black membranes. The addition of the refolded M35 protein in such a small concentration (about 500 ng/ml) to one or both sides of the lipid membranes resulted in a strong increase of the conductance. The conductance increase was not sudden but it was a function of time after the addition of the M35 protein to membranes in the black state. During about 20 to 30 min the membrane conductance increased by several orders of magnitude above that of membranes without the protein (from about 0.02 µS/cm² to 150 µS/cm²). Only a small further increase (as compared with the initial one) occurred after that time. Control experiments with Genapol alone at the same concentration as in the experiments with protein demonstrated that the membrane activity was caused by the presence of the refolded M35 and not by the detergent.
Single Channel Analysis

The addition of smaller concentrations of refolded M35 (about 50 ng/ml) to lipid bilayer membranes allowed the resolution of stepwise conductance increases. Figure 1 shows a single channel recording in the presence of the M35 protein. It was added 5 min after the membrane was in the black state to both sides of the membrane. Shortly after the addition of the protein the current increased in step-like fashion that is typical for reconstitution of Gram-negative bacterial porins into lipid bilayers (7, 10). The current steps had a long lifetime (mean lifetime more than 5 min.). Interestingly, the steps showed some flickering indicating that the channels were not in a stable configuration that would be typical for channel formation by gram-negative bacterial porins.

Figure 2 shows a histogram of the conductance fluctuations observed under the conditions of Figure 1 (20 mV membrane potential; 1 M KCl, pH 6). Besides a major conductance step of about 1.25 nS (about 20% of all conductance fluctuations) we also observed channels with a higher and smaller single-channel conductance, indicating that the channel conductance showed a broad distribution (Fig. 2). It has to be noted that this is also not typical for Gram-negative bacterial porins that normally exhibit defined single-channel conductance (9, 10, 13). Studies with different detergents used for refolding did not change these unusual characteristics, which may indicate that it reflected intrinsic properties of the M35 channels.

Conductance experiments were also performed with salts containing ions other than K⁺ and Cl⁻. These experiments were done to gain some insight into the biophysical properties of the channel formed by M35 of M. catarrhalis. The results summarised in Table 1 show that the channel is probably only moderately selective. This conclusion can
be derived from experiments in which KCl was replaced by LiCl or KCH₃COO. The exchange of the mobile ions K⁺ and Cl⁻ by the less mobile ions Li⁺ and acetate⁻ indicates that cations and anions are permeable through M35 of *M. catarrhalis*. The replacement of K⁺ by the less mobile Li⁺ has a somewhat greater influence on the channel conductance than the replacement of Cl⁻ by acetate⁻. This means that the porin forms a wide slightly cation-selective channel, which has only small field strength inside and no small selectivity filter (i.e. no binding site). Table 1 also shows the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase. The values for G always corresponded to that of the main maximum of the histograms, i.e. to the 1.25 nS peak in the case of 1 M KCl. Measurements were performed down to 0.1 M KCl. We observed an almost linear relationship between single-channel conductance and KCl-concentration, which would be expected for wide water-filled channels similar to those formed by many gram-negative bacterial porins (7, 8, 45). This means that the selectivity of the M35 channel is not influenced by the presence of a large excess of positively or negatively charged groups in or near the channel opening. Under the low voltage conditions of Figure 1, all the steps were directed upwards, which indicated that the channels were always in the open state.

**Selectivity of the M35 channel of *M. catarrhalis***

Zero-current membrane potential measurements were performed to obtain further information on the molecular structure of the M35 channel. The experiments were performed as described in Material and Methods. After incorporation of a sufficient number of channels into the membranes, the salt concentration on one side of the membranes was raised fivefold from 100 mM to 500 mM and the zero-current potential
was measured 5 min after every increase of the salt gradient across the membrane. For KCl the more dilute side of the membrane (100 mM) became slightly positive and had on average a potential of about 9 mV. Analysis of the membrane potential using the Goldman-Hodgkin-Katz equation (11) confirmed the assumption that anions and cations are permeable through the channel. The ratio of the permeability $P_{\text{cation}}$ and $P_{\text{anion}}$ was 1.73 for KCl suggesting small cation selectivity for M35 of *M. catarrhalis*.

**Creation of M35 deletion mutants.**

M35 deletion mutants of *M. catarrhalis* isolates 4223 and K114 were created by insertion of a kanamycin resistance cassette into the M35 gene. Analysis of the deletion mutant strains by SDS-PAGE indicated that the M35 band was not present (fig. 3 A) and western blotting showed that no proteins recognised by anti-M35 mouse serum were expressed (fig. 3 B), confirming that the band had not simply shifted and become obscured by other protein bands. DNA sequencing of the deletion mutant strains also confirmed that the M35KAN construct had replaced M35 as expected. The 4223ΔM35 #1 strain also appears to be missing a very high molecular weight protein or protein aggregate so strain #2 was chosen for use in the further experiments (fig. 3).

**Analysis of the *M. catarrhalis* protein profile.**

The protein profiles of 4223, K114, 4223ΔM35 and K114ΔM35 isolates were analysed using the Quantity One software package (Bio-Rad) after separation by 12 % SDS-PAGE (fig. 3 A). No differences were found between the mutants and their wildtype parent strains other than the high molecular weight protein missing from 4223ΔM35 #1 when the whole profile was analysed.
When the section of the gel most likely to contain porins (approximately 30-50 kDa) was cropped (fig. 4A) and analysed more closely it was found that a doublet had been identified as being only a single band in the analysis of the whole protein profile. When this doublet was analysed as two individual bands a difference was found. The relative density of the bands within this cropped section was compared between lanes as shown in figure 4. The lack of M35 in the mutant strains can be seen in both figures and an approximately 40 kDa protein band was found to have a higher relative density in 4223ΔM35 compared to 4223.

**Effect of M35 deletion on antibiotic sensitivity**

*M. catarrhalis* isolates 4223, 4223ΔM35, K114, K114ΔM35, ID78LN266, K65 and ATCC 25240 were tested for their sensitivity to cefotaxime and imipenem to assess the potential change in permeability caused by deletion of M35. Table 2 shows the annular radii of the zones of inhibition observed around the sensitivity discs, measured to the nearest 0.5 mm. There are no published breakpoints for *M. catarrhalis* sensitivity to these antibiotics, however the accepted breakpoint for the majority of other strains is 6 mm (6). Using this breakpoint, all the *M. catarrhalis* strains tested were found to be sensitive to both antibiotics and deletion of M35 did not result in a decrease in susceptibility to either antibiotic. K65 was found to be less sensitive to cefotaxime than the other strains, with a significantly smaller annular radius (P < 0.0001).

**Growth rate comparisons**

The M35 deletion mutants consistently grew at the same rate as their wildtype parent strains in undiluted BHI broth media (fig. 5A). Colony counts from these cultures
also confirmed that the OD$_{600}$ readings reflected the viable cell count. When the growth rate was compared between 4223ΔM35 and its wildtype parent strain in 10 % BHI media, it was found that the deletion mutant was consistently impaired in its growth (fig. 5 B). K114 and K114ΔM35 did not grow consistently in the diluted media (data not shown), thus isolate 4223 and its M35 mutant, 4223ΔM35, were selected for further study.

**Affect of sugars and amino acids on growth rate in dilute media**

The rate coefficient (k) from the first order rate equation describing the log-phase growth of the bacteria in each culture condition was used to compare the growth rates of the deletion mutant and wildtype isolate in the different growth conditions. Without the addition of sugars or amino acids to the diluted media, the wildtype strain was found to have a k value of approximately 0.1, approximately twice that of the deletion mutant. This difference was significant (P < 0.001).

Figure 6 shows the effect that each sugar (glucose, sucrose) and amino acid (glycine, aspartic acid, glutamic acid) tested had on the growth rates of the M35 deletion mutant and wildtype parent strain at differing concentrations. The growth of the M35 deletion mutant and the wildtype were both unaffected by the presence of additional sucrose or aspartic acid (fig. 6 B and D). The addition of glucose or glycine did not affect the growth of the wildtype but had an inhibitory affect on the growth of the M35 deletion mutant (fig. 6 A and C). In all of these cases the difference in growth rate between the deletion mutant and wildtype strain was significantly different, as indicated in the figures. Additional glutamic acid did not affect the growth of the wildtype but enhanced the growth of the mutant up to a similar growth rate as the wildtype (fig. 6 E).
At the lowest concentrations the difference in growth rate between the deletion mutant and wildtype strain was significantly different, however the difference was no longer significant when the concentration of glutamic acid was increased to 50 mM or more. The increase in growth rate of the deletion mutant strain between the 0 mM glutamic acid control culture and the 50 mM, 100 mM and 200 mM glutamic acid cultures were all statistically different (P < 0.01, P < 0.01 and P < 0.001, respectively).

**Effect of M35 deletion on rate of glutamic acid uptake**

Both the deletion mutant and wildtype strains of *M. catarrhalis* were found to take up the labeled glutamic acid (fig. 7) and that they did so approximately ten times faster if acetate and lactate were included in the energising solution (fig. 7 B). It can also be seen that the deletion mutant takes up the glutamic acid much faster than the wildtype isolate. The results shown in figure 7 are representative results from multiple assays and titration of the bacteria confirmed that both cell suspensions contained equivalent concentrations of live *M. catarrhalis*.

**Nasal colonisation**

The effect that deletion of M35 had on the longevity of nasal colonisation of mice by *M. catarrhalis* was tested using a previously described mouse model (27). Three days after inoculating mice with the wildtype isolate, *M. catarrhalis* was recovered from two of the five mice. Seven days after inoculation *M. catarrhalis* was recovered from four of the five mice. *M. catarrhalis* was not recovered from any of the mice inoculated with the mutant strain (4223ΔM35) after either three or seven days. No colonies with *M. catarrhalis*-like morphology were recovered from the sham-inoculated groups.
Discussion

Previously it was predicted from DNA alignments and structural predictions that M35 is an outer membrane porin (20), though the previous study did not show whether M35 functioned as a porin. Lipid bilayer analysis undertaken on M35 demonstrated that this protein can form channels in a black lipid bilayer typical of Gram-negative porins. M35 forms wide, water-filled channels with a single-channel of approximately 1.25 nS in 1 M KCl solution and has only a small selectivity for cations over anions. This analysis suggests that M35 forms general diffusion channels with a size similar to OmpC of E. coli K12 but with a much smaller selectivity (12). Further analysis of M35 may be warranted to investigate the atypical behaviour of this protein in comparison to other Gram-negative porins, which usually display a more defined single-channel conductance.

To further analyse the functional role of M35 two isogenic deletion mutants lacking the M35 protein were created by insertional inactivation of the M35. Growth of the mutant strains in both nutrient-rich and nutrient-poor media was then compared to the growth of the wildtype parent strains to gain a preliminary insight into how important M35 is for the growth of M. catarrhalis. Growth of the M. catarrhalis mutant strains lacking M35 did not appear to be impaired in the nutrient-rich BHI broth media, however their growth was significantly impaired when the media was diluted to 10 %.

In the middle of last century Fitting and Scherp (21) demonstrated that M. catarrhalis (then known as Neisseria catarrhalis) required amino acids for growth and did not utilise glucose from the media. They also observed that glutamic acid and aniline were metabolised to form ammonia and that growth could not occur without a mixture of amino acids. These results are consistent with the recent report from Wang et al. (44) that
*M. catarrhalis* does not possess the necessary genes for utilisation of exogenous carbohydrates. Their analysis of the *M. catarrhalis* genome found that the necessary genes for the Embden-Meyerhof-Parnas, Entner-Doudoroff or pentose cycle glycolytic pathways are not present, and that *M. catarrhalis* presumably relies upon the glyoxylate pathway for which it does possess the necessary genes. Wang et al. (44) also found that *M. catarrhalis* does not have the genes necessary for ammonia assimilation, consistent with the observation by Fitting and Scherp (21) that ammonia is exported into the medium by *M. catarrhalis* during growth. The lack of ability to utilise exogenous sugars is consistent with the standard laboratory tests used to distinguish *M. catarrhalis* from *Neisseria* species, in which it is seen that *M. catarrhalis* does not produce acid from any of the simple sugars included. It has also been reported that *M. catarrhalis* can utilise acetate (35, 37), lactate (5) and fatty acids (36) for growth and several studies have found absolute requirements for different amino acids including arginine and proline (5, 14, 24).

The selection of sugars and amino acids for the growth rate analysis of the deletion mutant strains was based on this knowledge of the nutritional requirements of *M. catarrhalis*. It was predicted that the addition of amino acids to the media could enhance growth but the addition of sugars would not affect the growth of *M. catarrhalis*. It was observed that the impairment of growth of the deletion mutant (4223ΔM35) in nutrient-poor media could be ameliorated by the addition of sufficient glutamic acid. This was similar to the pattern reported for deletion of substrate-specific porins from *P. aeruginosa*, which results in an impairment of growth by the mutant strains in nutrient-deficient media but not in nutrient-rich media. In nutrient-rich media the mutant strains are able to acquire sufficient nutrients non-specifically through other porins, but in the
nutrient-poor media the mutant strains cannot overcome the low concentration of
substrate without the higher efficiency specific-porins (39).

Thus, it seemed possible that M35 is necessary for efficient uptake of glutamic acid. However, in contrast to this hypothesis, the M35 deletion mutant actually took up glutamic acid faster than the wildtype parent strain. Additionally, conductance experiments showed that M35 does not contain a binding site. This would suggest that the deletion mutant can compensate for the lack of M35 by up-regulating another protein that enhances its ability to take up glutamic acid. It seems likely that the 40 kDa protein that was up-regulated by the M35 deletion mutant (fig. 4) was responsible for this enhanced rate of glutamic acid uptake.

When the similarly structured amino acid, aspartic acid, was tested it did not enhance the growth of the mutant. While these two amino acids are likely to cross the outer membrane through the same porin due to their similar structures (42), aspartic acid is not as central to metabolic pathways as glutamic acid and it appears that it cannot be utilised as a secondary energy source in the same way as glutamic acid.

Glycine is known to have an antibacterial affect on many different species of bacteria, including Helicobacter pylori, Bacillus subtilis and Staphylococcus aureus, by interfering with the synthesis of peptidoglycan (23, 29). This may account for the observation that additional glycine inhibited the growth of the mutant strain. The wildtype strain was not significantly affected by the additional glycine, however Hammes et al. (23) found that the range of concentrations of glycine needed to inhibit growth of the different species they tested was up to 1.33 M. The inhibitory affect of glycine has not been demonstrated with M. catarrhalis previously so the necessary concentration is unknown, thus it is
possible that 200 mM was simply not a high enough concentration to inhibit the growth of the wildtype *M. catarrhalis*. The mutant strain was already stressed by nutritional deficiency and this appears to have made it more susceptible to the potential antibacterial effects of glycine.

Glucose and sucrose were included in this study as control nutrients that were not expected to have any affect on the growth of either the wildtype or mutant strains, since *M. catarrhalis* cannot utilise exogenous carbohydrate (44). As expected, neither additional glucose nor sucrose was able to enhance the growth of either strain, however glucose was found to inhibit the growth of the M35 deletion mutant. It is possible that glucose causes this inhibitory affect by reducing the acquisition of other nutrients from the media through competition for outer membrane porins. That is, there may be an outer membrane porin that has a channel large enough to allow the diffusion of glucose and at high concentrations this unnecessary sugar prevents the diffusion of nutrients that would normally be acquired through this porin. With the lack of M35 already reducing permeability of the *M. catarrhalis* outer membrane, such competition may be enough to entirely prevent growth of the mutant when the glucose concentration was 200 mM (fig. 5 A). The same was not seen with sucrose, however as this disaccharide is a much larger molecule than the monosaccharide glucose it may not be small enough to pass through any of the porins present in the *M. catarrhalis* outer membrane. Vasserot et al. (42) observed similar competitive inhibition with *Oenococcus aeni*, when a high concentration of aspartic acid added to the media competitively inhibited the uptake of glutamic acid thus reduced growth of the bacteria.
The analysis of growth of the M35 deletion mutant in dilute media was not able to
determine a specific role for M35 in nutrient uptake, although it seems highly likely that
M35 is a general porin. This study did however lead to the identification of the 40 kDa
protein potentially involved in glutamic acid uptake. Until this protein has been
characterised it is unknown whether it is another porin or if it is an inner membrane
transport molecule or associated protein.

The observation that M35 is a general porin that is constitutively expressed and that is
necessary for efficient growth of *M. catarrhalis* in nutrient-poor conditions suggested that
it may also be essential for survival *in vivo*. Krishnamurthy et al. (27) found that when
mice are inoculated with a sufficiently high concentration of *M. catarrhalis* nasal
colonisation can be established that persists for at least 14 days. This model was used to
assess whether the M35 deletion mutant strain (4223ΔM35) can survive *in vivo*.

*M. catarrhalis* was recovered from some of the mice inoculated with the wildtype strain
after three and seven days of colonisation, however it was not recovered from any of the
mice inoculated with the mutant strain. It would appear that the mutant strain cannot
survive *in vivo* for the same reasons that its growth is inhibited in the nutrient-poor
conditions *in vitro*; the lack of M35 reduces the permeability of the outer membrane to
essential nutrients sufficiently to restrict growth. The necessity of M35 for survival *in
vivo* also indirectly confirms that it is expressed *in vivo*. This enhances the apparent
suitability of M35 as a potential vaccine antigen because a protein that is essential for
survival is unlikely to be significantly altered in expression level or antigenicity.

Additionally, if it is possible to block M35 *in vivo* this could be used as a therapeutic
strategy to specifically target *M. catarrhalis*. 
It has been shown in several different species that changes in the expression of outer membrane porins can affect antibiotic susceptibility. For example the *P. aeruginosa* porin OprD is necessary for the uptake of both basic amino acids and the antibiotic imipenem (41). Removal of OprD leads to a lower susceptibility to imipenem since the antibiotic cannot enter the cell. Other species, such as *Klebsiella pneumoniae* (18) and *Enterobacter aerogenes* (15), have also been found to develop resistance to antibiotics by reducing the expression of outer membrane porins.

Additionally, the amino acid substitution previously reported in the *M. catarrhalis* isolate ID78LN266 (20) is identical to that seen in the Omp36 osmoporin from *E. aerogenes* that lead to greater resistance to β-lactam antibiotics (17, 40). This porin shows a high level of sequence similarity to the OmpK36 porin from *K. pneumoniae*, a porin with which M35 also shares significant sequence similarity (20). This mutation leads to increased susceptibility to a range of β-lactam antibiotics, most significantly ceftazidime and cefotaxime but sensitivity to cefepime, cefpirome and imipenem was also effected (40).

Thus, a preliminary investigation was undertaken to assess the role M35 may play in the permeability of antibiotics across the outer membrane and the possibility that alterations to M35 could affect antibiotic sensitivity. Cefotaxime was selected as a representative cephalosporin and imipenem was selected as a representative carbapenem. When sensitivity of the *M. catarrhalis* M35 deletion mutants to these antibiotics was tested and compared with the sensitivity of their parent strains there was no difference in sensitivity, demonstrating that M35 does not play a significant role in permeability of the outer membrane to these particular antibiotics. The K65 isolate is known to produce β-
lacatamase and as expected this isolate was found to be less sensitive to cefotaxime than the other isolates. This was consistent with observations that the *M. catarrhalis* isolates that produce β-lactamases have higher MIC against this antibiotic than those that do not produce β-lactamases (2).

As previously mentioned, *M. catarrhalis* isolate ID78LN266 has an identical mutation to one in an *E. aerogenes* porin that significantly effects antibiotic sensitivity. However, *M. catarrhalis* ID78LN266 was not found to be any more sensitive than the other isolates to either cefotaxime or imipenem. It appears that if these two antibiotics are able to cross the outer membrane of *M. catarrhalis* through M35 they are also able to enter the cell through other porins. While it is still possible that M35 is the sole entry point for other antibiotics, these results demonstrate that if antibacterial approaches involving the blocking of M35 as a means of starving the bacteria were to be developed, such interventions would not have the undesired side-effect of enhancing resistance to these antibiotics. That is, if it is possible to block M35 *in vivo* it would reduce the ability of *M. catarrhalis* to take up essential nutrients, but antibiotics would still be able to enter the cell.

This study has shown that M35 is indeed an outer membrane porin, the first functionally analysed for *M. catarrhalis*. M35 is permeable to both cations and anions, with a small selectivity for cations. Deletion of M35 did not affect sensitivity of *M. catarrhalis* to the antibiotics cefotaxime or imipenem. A novel approximately 40 kDa protein was found to be up-regulated in the M35 deletion mutant (4223∆M35) and we hypothesise that this protein is responsible for the significantly enhanced uptake of glutamic acid by the M35 deletion mutant in comparison to the wildtype *M. catarrhalis*. 

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This protein presumably compensates for the lack of M35 by increasing the permeability of the outer membrane to secondary energy sources, such as glutamic acid. The deletion of M35 prevented the mutant strains from being able to survive within the nasal cavities of mice, demonstrating that it is an essential protein for in vivo survival of M. catarrhalis.

Acknowledgments

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References


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41. Trias, J., and H. Nikaido. 1990. Protein D2 channel of the Pseudomonas aeruginosa outer membrane has a binding site for basic amino acids and peptides. Journal of Biological Chemistry 265:15680-4.


Table 1. Average single-channel conductance, G, of the M35 porin of M. catarrhalis in different salt solutionsa.
<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration [M]</th>
<th>G [nS]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiCl</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>KCH$_3$COO (pH 7)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*The membranes were formed of 1% diphytanoyl phosphatidylcholine dissolved in n-decane. The aqueous solutions were used unbuffered and had a pH of 6 unless otherwise indicated. The applied voltage was 20 mV and the temperature was 20°C. The average single-channel conductance, G, was calculated from at least 80 single events derived from measurements of at least four individual membranes.

**Table 2.** Annular radius (mm) of the zone of inhibition surrounding cefotaxime and imipenem sensitivity discs.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Cefotaxime$^a$ (5 mg)</th>
<th>Imipenem$^a$ (10 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4223</td>
<td>11.5 ± 0.6</td>
<td>17.3 ± 0.3</td>
</tr>
<tr>
<td>4223ΔM35</td>
<td>11.8 ± 0.8</td>
<td>17.7 ± 0.6</td>
</tr>
<tr>
<td>K114</td>
<td>12.3 ± 0.5</td>
<td>14.2 ± 0.6</td>
</tr>
<tr>
<td>K114ΔM35</td>
<td>10.7 ± 1.6</td>
<td>12.8 ± 0.3</td>
</tr>
<tr>
<td>ID78LN266</td>
<td>10.8 ± 0.8</td>
<td>17.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>K65</td>
<td>ATCC 25240</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Value</td>
<td>7.8 ± 0.8</td>
<td>13.2 ± 0.3</td>
</tr>
<tr>
<td>Value</td>
<td>18.7 ± 0.3</td>
<td>15.8 ± 0.0</td>
</tr>
</tbody>
</table>

*Values are the average annular radius (mm) ± standard deviation.*

**Figure 1.** Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane in the presence of M35 porin of *M. catarrhalis*. The aqueous phase contained 1 M KCl pH 6 and 50 ng/mL M35 protein. The applied membrane potential was 20 mV; T = 20°C.
Figure 2. Histogram of the probability of the occurrence of a given conductivity unit observed with membranes formed of diphytanoyl phosphatidylcholine/n-decane in the presence of M35 porin of *M. catarrhalis*, taken from experiments similar to that one shown in Figure 1. The aqueous phase contained 1 M KCl, pH 6 and 50 ng/mL M35 porin. The applied membrane potential was 20 mV; T = 20°C. The average single-channel conductance was 1.25 nS for 133 single-channel events.
Figure 3. A: SDS-PAGE analysis of 4223ΔM35 and K114ΔM35. Lane 1: Low molecular weight markers, lane 2: wildtype 4223, lane 3: 4223ΔM35 #1, lane 4: 4223ΔM35 #2, lane 5: wildtype K114, lane 6: K114ΔM35 #1, lane 7: K114ΔM35 #2.

B: Western blot analysis of 4223ΔM35 and K114ΔM35. Lane 1: See-Blue plus 2 prestained markers, lane 2: wildtype 4223, lane 3: 4223ΔM35 #1, lane 4: 4223ΔM35 #2, lane 5: wildtype K114, lane 6: K114ΔM35 #1, lane 7: K114ΔM35 #2

Figure 4. The cropped part of the SDS-PAGE gel showing that protein profiles of 4223 and 4223ΔM35 reanalysed using Quantity One software (Bio-Rad).

A: The part of the gel that was reanalysed. The doublet that was up-regulated in the 4223 deletion mutants is indicated by the arrows. The 45 kDa marker is shown.

B: Density plot for 4223 and 4223ΔM35. Rf value = relative front within cropped image. M35 and the up-regulated protein are indicated.
Figure 5. Representative data for the comparison of the growth of 4223 with 4223ΔM35. Growth in (A) standard BHI broth and (B) 10% BHI broth.
**Figure 6.** The effect of additional (A) glucose, (B) sucrose, (C) glycine, (D) aspartic acid or (E) glutamic acid on the growth of *M. catarrhalis* 4223 and 4223ΔM35. Graphs show the average rate coefficient (k) for three replicates. Error bars indicate standard deviation and asterisks indicate statistical significance.
Figure 7. Uptake of L-[G-³H]-glutamic acid by *M. catarrhalis*. (A) energised with 3 % glycerol; (B) energised with 3 % glycerol, 0.02 % acetate and 0.02 % lactate. DPM, disintegrations per minute.