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Moraxella catarrhalis M35 Is a General Porin That Is Important for Growth under Nutrient-Limiting Conditions and in the Nasopharynges of Mice▽

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***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 conductance 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 wild-type parent isolates, the growth of the mutant strains was inhibited only 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 upregulation of a protein of approximately 40 kDa. M35 was also found to be essential for nasal colonization 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 colonization.**

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 (26, 32, 43). On rare occasions, it can also cause more serious diseases, such as meningitis and septicemia (16, 29).

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 at very high levels, accounting for the majority of the outer membrane proteins (OMPs), and this leads to a high degree of permeability of the outer membrane to small hydrophilic molecules (1, 27). 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 enterica* serovar Typhimurium (27), have binding sites for particular molecules that enhance the efficiency of 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; porins 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

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porin together suggest that M35 may be a particularly important functional protein for *M. catarrhalis*. Additionally, immunization 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 *E. coli* K-12 but with a much smaller selectivity (12) and that M35 is essential for the survival of *M. catarrhalis* in vivo. In the absence of M35, a 40-kDa protein was upregulated, and this protein may have been responsible for enhanced uptake of glutamic acid by *M. catarrhalis*.

MATERIALS AND METHODS

Black lipid bilayer experiments. The methods used for planar black lipid bilayer experiments were previously described in detail (10). The experimental setup 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 mm^2. 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 and 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 to 10 min after a fivefold salt gradient was established across the membranes containing about 100 to 1,000 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 EcoRI restriction site contained in the M35loop3$^-$ 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 nucleotide sequence of the M35 gene from isolate 4223 has previously been deposited in GenBank with accession number AY905611.

The *M. catarrhalis* isolates were grown to early log phase in brain heart infusion (BHI) broth (Oxoid), and a 100-μl aliquot was spread onto chocolate blood agar (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 onto each agar plate and incubated for approximately 5 h at 37°C with 5% CO_2 to allow the *M. catarrhalis* cells to take up the DNA. Bacterial cells within the area of the DNA spot were then swabbed onto CBA plates containing 25 μg/ml kanamycin and incubated overnight at 37°C with 5% CO_2. *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°C in BHI medium containing 15% glycerol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blotting with anti-M35 mouse serum confirmed deletion of the M35 protein, and replacement of the middle 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 μg/ml kanamycin was included in the medium 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 h at 37°C 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 h) at 37°C with 5% CO_2, and the zone of inhibition was measured. All isolates were tested in triplicate.

Growth comparison of deletion mutants with wild-type isolates. The morphology of colonies of *M. catarrhalis* 4223ΔM35 and K114ΔM35 grown on CBA plates did not appear to differ from that of the wild-type 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 wild-type isolates. Overnight cultures of each isolate in BHI broth were used to inoculate fresh medium to an OD at 600 nm (OD_{600}) of 0.1, and these cultures were incubated at 37°C with shaking at 220 rpm. The OD_{600} was recorded every 30 min for 4 h, and eight serial 10-fold dilutions were spotted onto 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. BHI broth (10%) 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°C with shaking at 220 rpm for 3 to 4 h, and samples were taken every 30 min 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. k was determined by linear regression of $\ln(OD_t/OD_0)$ versus time (t), as described by the integrated rate equation $\ln(OD_t/OD_0) = kt$, where OD_0 is the optical density at time zero. 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. (31) with some modifications was used to assess the effect that deleting M35 has on glutamic acid uptake by *M. catarrhalis*. Isolates 4223 and 4223ΔM35 were grown overnight on CBA, harvested, and washed twice in buffer A (50 mM K_2HPO_4, 0.5 mM $MgCl_2$ [pH 6.9]), and by looking at the OD_{400}, the concentration was adjusted to 1×10^9 CFU/ml 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 onto CBA plates to ensure that the concentration of viable cells in the two cell suspensions were equivalent. The cell suspensions were kept on ice until needed.

For the uptake assay, 20 μl of bacterial suspension was added to 20 μl of buffer C (buffer A with 3% glycerol and 40 mM NaCl) and warmed at 37°C for 10 to 15 min to energize the bacteria. Since it was not known whether the glycerol included by Monaco et al. (31) for their study of *Neisseria 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 utilize both of these compounds as carbon sources (5, 37). For such assays, buffer C also contained 0.02% (wt/vol) acetate and 0.02% (wt/vol) lactate.

A 20-μl solution of L-[G-^{3}H]glutamic acid (2.04 TBq/mmol) at three times the desired final concentration was added to the energized 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 dimethyl sulfoxide 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 analyzer (Tri-Carb 1600CA; Packard). The counts per minute were converted to disintegrations per minute (dpm) using a previously developed quench curve.

Nasal colonization. The ability of the wild-type and M35 deletion mutant strains to survive in the nasopharynges of mice was assessed using a recently developed colonization assay (A. Krishnamurthy, J. McGrath, A. W. Cripps, and J. M. Kyd, submitted for publication). *M. catarrhalis* 4223 and 4223ΔM35 were grown overnight on CBA, harvested, and washed twice in sterile phosphate-buffered saline (PBS). The concentrations of the bacterial suspensions were adjusted to 2×10^{10} CFU/ml, and this concentration was confirmed by titration. Ten male BALB/c mice were anesthetized by intraperitoneal injection with 200

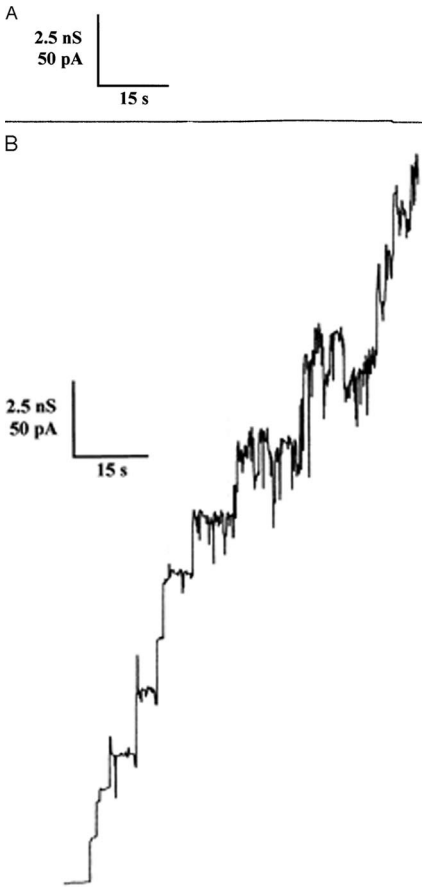

FIG. 1. Single-channel recording of a diphytanoyl phosphatidylcholine–*n*-decane membrane without protein added (A) and in the presence of M35 porin of *M. catarrhalis* (B). The aqueous phase contained 1 M KCl (pH 6) and 50 ng/ml M35 protein. The applied membrane potential was 20 mV, and the temperature was 20°C.

μl of a 1:2 mixture of Zoletil (100-mg/ml stock) and Xylazine (20-mg/ml stock) in PBS and nasally inoculated with 5 μl of each bacterial suspension.

After 3 days, five mice from each group were euthanized, and the bacteria were 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 onto 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 7 days after 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 were not adequately uniform between individual mice to allow quantitative evaluation. The nasal colonization assay was conducted with the approval of the University of Canberra Committee for Ethics in Animal Experimentation.

Statistical analysis. Growth rate coefficients (k) for each concentration were compared between the wild-type 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 (approximately 2.5 μg) was added to one or both sides of planar bilayer 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 planar bilayer state. For 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/cm^2 to 150 μS/cm^2). Only a small further increase (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.

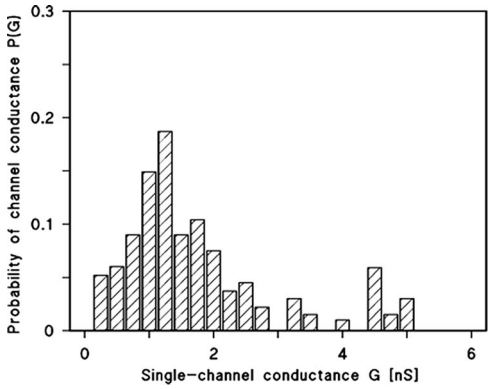

FIG. 2. Histogram of the probability of the occurrence of a given conductivity unit observed with membranes formed of diphytanoyl phosphatidylcholine–*n*-decane in the presence of M35 porin from *M. catarrhalis*, taken from experiments similar to the one shown in Fig. 1. The aqueous phase contained 1 M KCl (pH 6) and 50 ng/ml M35 porin. The applied membrane potential was 20 mV, and the temperature was 20°C. The average single-channel conductance was 1.25 nS for 133 single-channel events.

Single-channel analysis. The addition of lower 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. M35 was added 5 min after the membrane was in the black (planar) state to both sides of the membrane. Shortly after the addition of the protein, the current increased in a 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 of 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 Fig. 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). The distribution was broader than is typical for gram-negative bacterial porins (9, 10, 13), which may be related to the refolding of the porin.

TABLE 1. Average single-channel conductance of the M35 porin of *M. catarrhalis* in different salt solutions[a]

Salt	Concn (M)	*G* (nS)
LiCl	1.0	0.75
KCl	0.1	0.15
	0.3	0.50
	1.0	1.25
	3.0	3.5
KCH_3COO (pH 7)	1.0	1.0

[a] 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.

However, 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^- to gain further insight into the biophysical properties of the channel formed by M35 of *M. catarrhalis*. Experiments in which KCl was replaced by LiCl or KCH_3COO, summarized in Table 1, showed that the channel was probably only moderately selective. The exchange of the mobile ions K^+ and Cl^- by the less mobile ions Li^+ and acetate (negative charge) indicated that M35 was permeable to both cations and anions. The replacement of K^+ by the less mobile Li^+ had a somewhat greater influence on the channel conductance than the replacement of Cl^- by acetate (negative charge). This indicated that the porin formed a wide slightly cation-selective channel, which had 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 was 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 Fig. 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. 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 for the cation/permeability for the 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 in place of nucleotides 349 to 444. Analysis of the deletion mutant strains by SDS-PAGE indicated that the M35 band was not present (Fig. 3A), and Western blotting showed that no proteins recognized by anti-M35 mouse serum were expressed (Fig. 3B), 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 the middle (nucleotides 349 to 444) of M35 as expected. The 4223ΔM35 strain 1 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 *M. catarrhalis* 4223, K114, 4223ΔM35, and K114ΔM35 isolates were analyzed using the Quantity One software package (Bio-Rad) after separation by 12% SDS-PAGE (Fig. 3A). No differences were found between the mutants and their wild-type parent strains other than the high-molecular-weight protein missing from 4223ΔM35 strain 1 when the whole profile was analyzed.

When the section of the gel most likely to contain porins (approximately 30 to 50 kDa) was cropped (Fig. 4A) and analyzed more closely, it was found that a doublet had been identified as being a single band in the analysis of the whole protein profile. When this doublet was analyzed 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 Fig. 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 strain 4223ΔM35 than in strain 4223.

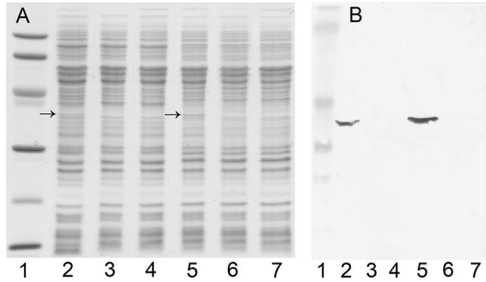

FIG. 3. (A) SDS-PAGE analysis of *M. catarrhalis* 4223ΔM35 and K114ΔM35. Lane 1, low-molecular-weight markers; lane 2, wild-type 4223; lane 3, 4223ΔM35 strain 1; lane 4, 4223ΔM35 strain 2; lane 5, wild-type K114; lane 6, K114ΔM35 strain 1; lane 7, K114ΔM35 strain 2. The small black arrows indicate the location of M35 in the wild-type strains. (B) Western blot analysis of 4223ΔM35 and K114ΔM35. Lane 1, See-Blue plus two prestained markers; lane 2, wild-type 4223; lane 3, 4223ΔM35 strain 1; lane 4, 4223ΔM35 strain 2; lane 5, wild-type K114; lane 6, K114ΔM35 strain 1; lane 7, K114ΔM35 strain 2.

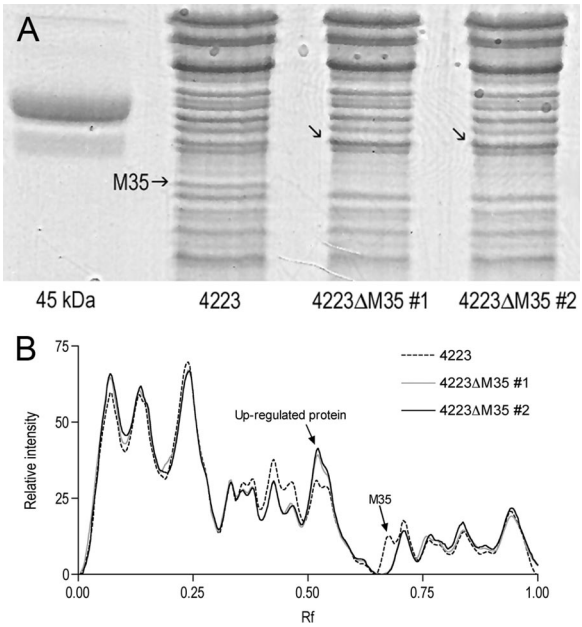

FIG. 4. Analysis of *M. catarrhalis* 4223 and 4223ΔM35 for protein band presence and density. (A) Enlargement of the segment of the SDS-polyacrylamide gel corresponding to the protein profiles analyzed using Quantity One software (Bio-Rad). The doublet that was upregulated in the 4223 deletion mutants is indicated by the small black arrows. The 45-kDa marker is shown in the leftmost lane. 4223ΔM35 strain 1 (#1) and strain 2 are shown in the right two lanes. (B) Density plot for 4223 and 4223ΔM35. The Rf value is the relative front within the displayed image. Density profiles corresponding to M35 and the upregulated protein are indicated by the arrows.

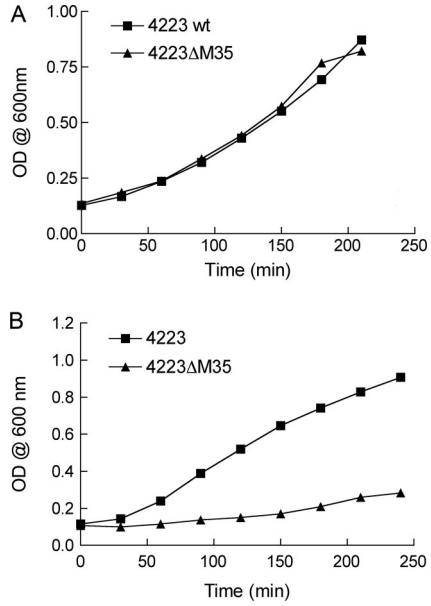

FIG. 5. Representative data for the comparison of the growth of *M. catarrhalis* 4223 (wild type [wt]) with 4223ΔM35. Growth in standard BHI broth (A) and 10% BHI broth (B) is shown. Optical density at 600 nm is shown on the *y* axes.

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, but 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. Strain K65 was found to be less sensitive to cefotaxime than the other strains, with a significantly smaller annular radius ($P < 0.001$).

TABLE 2. Annular radius of the zone of inhibition surrounding cefotaxime and imipenem sensitivity discs

Isolate	Annular radius (mm)[a]	
	Cefotaxime (5 mg)	Imipenem (10 mg)
4223	11.5 ± 0.6	17.3 ± 0.3
4223ΔM35	11.8 ± 0.8	17.7 ± 0.6
K114	12.3 ± 0.5	14.2 ± 0.6
K114ΔM35	10.7 ± 1.6	12.8 ± 0.3
ID78LN266	10.8 ± 0.8	17.0 ± 0.6
K65	7.8 ± 0.8	18.7 ± 0.3
ATCC 25240	13.2 ± 0.3	15.8 ± 0.0

[a] Values are averages ± standard deviations.

Growth rate comparisons. The M35 deletion mutants consistently grew at the same rate as their wild-type parent strains in undiluted BHI broth medium (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 mutant strain 4223ΔM35 and its wild-type parent strain in 10% BHI medium, it was found that the deletion mutant was consistently impaired in its growth (Fig. 5B). Strains K114 and K114ΔM35 did not grow consistently in the diluted media (data not shown). K114 is a highly piliated strain compared with the 4223 strain (28), but whether this characteristic has the capacity to impede porin function in a nutrient-reduced environment is not known. Therefore, isolate 4223 and its M35 mutant, 4223ΔM35, were selected for further uptake studies.

Effect of sugars and amino acids on growth rate in dilute media. The rate coefficient from the first-order rate equation describing the log growth phase of the bacteria in each culture condition was used to compare the growth rates of the deletion mutant and wild-type isolate in the different growth conditions. Without the addition of sugars or amino acids to the diluted media, the wild-type 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 or sucrose) and amino acid (glycine, aspartic acid, or glutamic acid) tested had on the growth rates of the M35 deletion mutant and wild-type parent strain at differing concentrations. The growth of the M35 deletion mutant and the wild-type strain were both unaffected by the presence of additional sucrose or aspartic acid (Fig. 6B and D). The addition of glucose or glycine did not

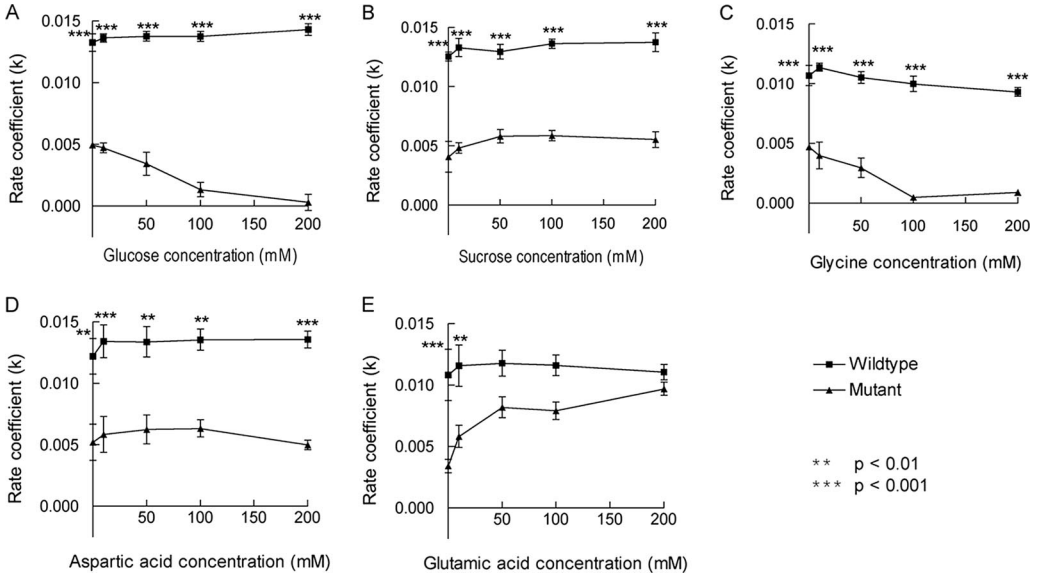

FIG. 6. Effect of additional glucose (A), sucrose (B), glycine (C), aspartic acid (D), or glutamic acid (E) on the growth of *M. catarrhalis* 4223 and 4223ΔM35 in 10% BHI medium. Graphs show the average rate coefficient (*k*) for three replicates. Error bars indicate standard deviations.

affect the growth of the wild type but had an inhibitory effect on the growth of the M35 deletion mutant (Fig. 6A and C). In all of these cases, the difference in growth rate between the deletion mutant and wild-type strain was significantly different, as indicated in the figures. Additional glutamic acid did not affect the growth of the wild type but enhanced the growth of the mutant up to a growth rate similar to that of the wild type (Fig. 6E).

At the lowest concentrations, the difference in growth rate between the deletion mutant and wild-type 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 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 wild-type strains of *M. catarrhalis* were found to take up the labeled glutamic acid (Fig. 7) and did so approximately 10 times faster if acetate and lactate were included in the energizing solution (Fig. 7B). It was also found that the deletion mutant took up the glutamic acid much faster than the wild-type isolate did. The results shown in Fig. 7 are representative of multiple assays, and the equivalence of the concentration and viability of the two strains of bacteria in the cell suspensions was confirmed.

Nasal colonization. The effect that deletion of M35 had on the longevity of nasal colonization of mice by *M. catarrhalis* was tested using a recently developed mouse model (Krishnamurthy et al., submitted). Three days after the mice were inoculated with the wild-type 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 3 or 7 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 in the

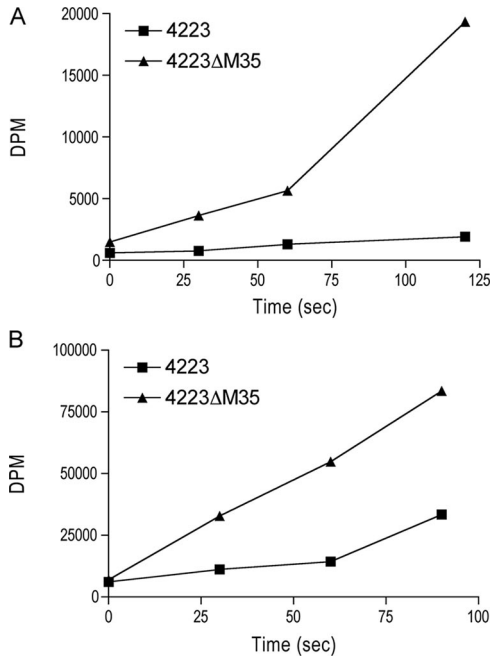

FIG. 7. Uptake of L-[G-^{3}H]glutamic acid by *M. catarrhalis*. *M. catarrhalis* cells were energized with 3% glycerol (A) or with 3% glycerol, 0.02% acetate and 0.02% lactate (B). DPM, disintegrations per minute.

current study of M35 demonstrated that this protein could form channels in a black lipid bilayer typical of gram-negative porins. M35 formed wide, water-filled channels with a single channel of approximately 1.25 nS in 1 M KCl solution and had only a small selectivity for cations over anions. This analysis suggests that M35 forms general diffusion channels with a size and selectivity smaller than those for OmpC of *E. coli* K-12, if M35 is indeed trimeric as suggested by our previous study (12, 20).

To further analyze the functional role of M35, two isogenic deletion mutants lacking the M35 protein were created by insertional inactivation of the M35 gene. Growth rate comparisons of the mutant and wild-type strains in both nutrient-rich and nutrient-poor media showed that while the mutant strains were not impaired by their lack of M35 in the nutrient-rich conditions, their growth was significantly impaired when the medium was diluted to 10%.

In the middle of the last century, Fitting and Scherp (21) demonstrated that *M. catarrhalis* (then known as *Neisseria catarrhalis*) required amino acids for growth and did not utilize glucose from the media. A more recent report from Wang et al. (44) confirmed that *M. catarrhalis* did not possess the necessary genes for utilization of exogenous carbohydrates, as *M. catarrhalis* lacks the necessary genes for the Embden-Meyerhof-Parnas, Entner-Doudoroff, and pentose cycle glycolytic pathways, so *M. catarrhalis* presumably relies upon the glyoxylate pathway for which it does possess the necessary genes. It has also been reported that *M. catarrhalis* can utilize 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, 25).

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 that 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 nonspecifically 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 was 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 wild-type parent strain did. Additionally, conductance experiments showed that M35 does not contain a binding site. This would suggest that the deletion mutant compensated for the lack of M35 by upregulating another protein that enhanced its ability to take up glutamic acid. It seems likely that the 40-kDa protein that was upregulated by the M35 deletion mutant (Fig. 4) was responsible for this enhanced rate of glutamic acid uptake, though this has yet to be confirmed.

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 utilized by *M. catarrhalis* as a secondary energy source in the same way as glutamic acid can.

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 (24, 30). This may account for the observation that additional glycine inhibited the growth of the mutant strain. The wild-type strain was not significantly affected by the additional glycine; however, Hammes et al. (24) 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 effect of glycine has not previously been demonstrated with *M. catarrhalis*, so the necessary concentration is unknown; thus, it is possible that 200 mM was simply not high enough to inhibit the growth of the wild-type *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 effect on the growth of either the wild-type or mutant strains, since *M. catarrhalis* cannot utilize 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. Glucose is often responsible for carbon catabolite repression in other species (23); however, as *M. catarrhalis* does not utilize glucose, such a system seems unlikely to be responsible for the reduced growth seen in the presence of excess glucose. It is also possible that glucose causes the inhibitory effect 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. 5A). Vasserot et al. (42) observed similar competitive inhibition with *Oenococcus oeni*, when a high concentration of aspartic acid added to the medium competitively inhibited the uptake of glutamic acid, thus reducing 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 has observed the upregulation of a 40-kDa protein that may potentially be involved in glutamic acid uptake. Until this protein has been characterized, it is unknown whether it is another porin or if it is an inner membrane transport molecule or -associated protein.

The observation that M35 was a general porin that was constitutively expressed and was 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. (submitted) found that when mice were inoculated with a sufficiently high concentration of *M. catarrhalis*, nasal colonization could be established that persisted for at least 14 days. This colonization model was used to assess whether the M35 deletion mutant strain (4223ΔM35) could survive in vivo. *M. catarrhalis* was recovered from some of the mice inoculated with the wild-type strain after 3 and 7 days of colonization; however, no bacteria were recovered from any of the mice inoculated with the mutant strain. It would appear that the mutant strain could not survive in vivo for the same reasons that its growth was inhibited in the nutrient-poor conditions in vitro, that is, the lack of M35 reduced the permeability of the outer membrane to essential nutrients enough to restrict growth. The necessity of M35 for survival in vivo also indirectly suggested that it was 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, further studies might investigate whether it is possible to block M35 in vivo 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 of aspartic acid replacing glycine in loop 3 previously reported in the *M. catarrhalis* isolate ID78LN266 (20) was identical to that seen in the Omp36 osmoporin from *E. aerogenes* that led 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 affected (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 the 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 the permeability of the outer membrane to these particular antibiotics. The K65 isolate is known to produce β-lactamase, and as expected, this isolate was found to be less sensitive to cefotaxime than the other isolates were. This was consistent with observations that the *M. catarrhalis* isolates that produce β-lactamases have a higher MIC against this antibiotic than those that do not produce β-lactamases (2).

As previously mentioned, the *M. catarrhalis* isolate ID78LN266 has the same mutation of glycine to aspartic acid found in an *E. aerogenes* porin that was reported to be responsible for significantly affecting its 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 were possible to block M35 in vivo, it would reduce the ability of *M. catarrhalis* to take up essential nutrients, but other antibiotics would still be able to enter the cell.

This study has shown that M35 is indeed an outer membrane porin, the first functionally analyzed for *M. catarrhalis*. M35 was permeable to both cations and anions, with a small selectivity for cations. Deletion of M35 did not affect the sensitivity of *M. catarrhalis* to the antibiotic cefotaxime or imipenem. A protein of approximately 40 kDa was found to be upregulated in the M35 deletion mutant (4223ΔM35), and it is hypothesized that this protein may be responsible for the enhanced uptake of 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*.

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