J Antimicrob Chemother 2010; **65**: 2089–2096
doi:10.1093/jac/dkq312 Advance Access publication 27 August 2010

Journal of Antimicrobial Chemotherapy

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

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Received 23 March 2010; returned 17 May 2010; revised 21 July 2010; accepted 21 July 2010

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

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

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

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

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

Introduction

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

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

Materials and methods

Bacterial strains and culture conditions

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

DNA methods

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

β-Lactamase production testing and bro gene typing

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

RNA methods

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

Preparation of OMPs

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

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

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

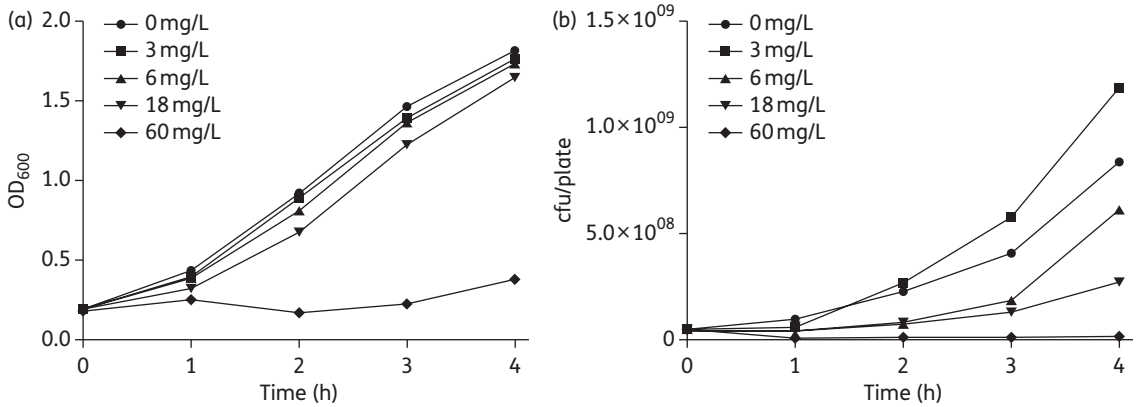

Figure 1. Time–kill curves of strain 300 for different amoxicillin concentrations (0, 3, 6, 18 and 60 mg/L). (a) OD_{600}. (b) cfu/plate.

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

SDS-PAGE and immunoblot

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

Results

PCR analysis of 52 clinical isolates

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

Down-regulation of m35 mRNA expression after amoxicillin treatment

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

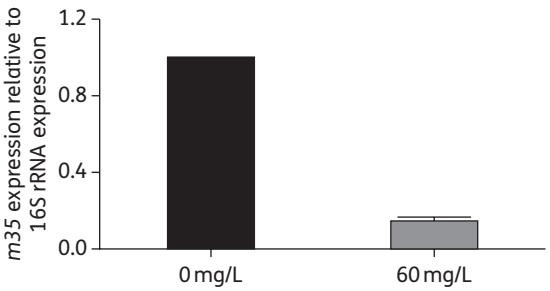

Figure 2. Down-regulation of *m35* mRNA expression during amoxicillin treatment. Quantitative real-time PCR was performed after 4 h of incubation with and without 60 mg/L amoxicillin. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate.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

M35 protein expression is also affected by amoxicillin

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

β-Lactamase production and BRO typing

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

Cold shock induces down-regulation of m35 mRNA

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

M35 protein expression is involved in the cold shock response

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

m35 mRNA expression is affected by general stress conditions

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

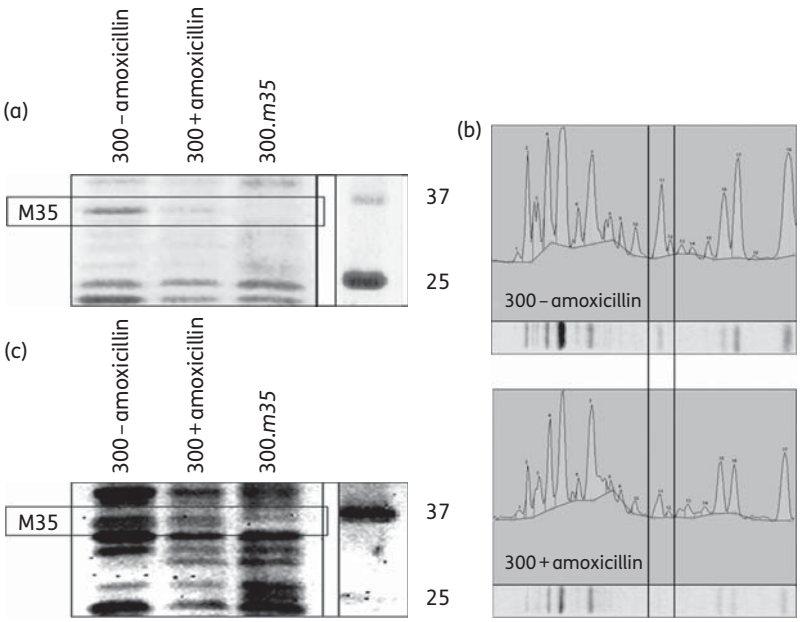

Figure 3. SDS-PAGE (a) and western blot (c) of *M. catarrhalis* OMPs (1 mg/L) from strain 300 after growth with and without 60 mg/L amoxicillin together with its respective *m35* knockout mutant as the negative control. The rectangle shows the position of the M35 protein band at 36 kDa. (b) The spectra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

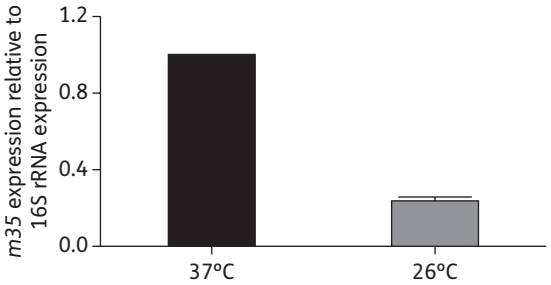

Figure 4. Down-regulation of *m35* mRNA expression after cold shock exposure. Quantitative real-time PCR was performed after 3 h of incubation at 26°C or 37°C. Up- or down-regulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented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

Discussion

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

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

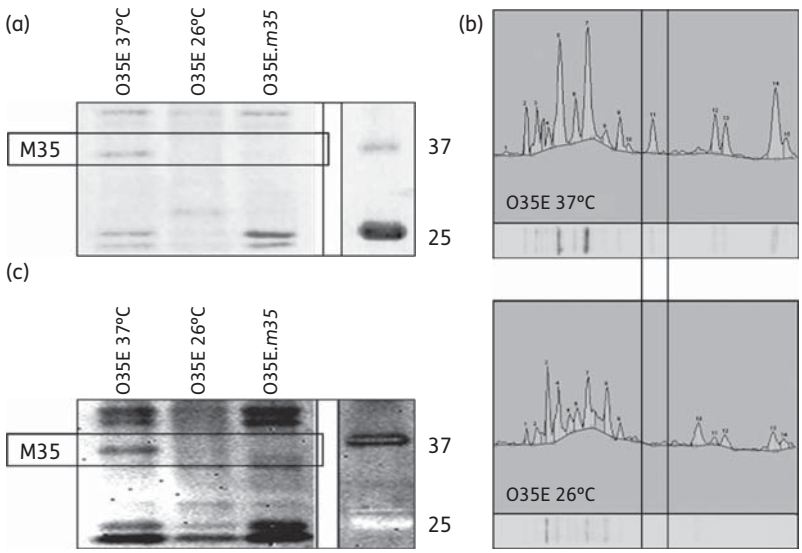

Figure 5. SDS-PAGE (a) and western blot (c) of *M. catarrhalis* OMPs (1 mg/L) from strain O35E after growth at 26°C compared with 37°C together with its respective *m35* knockout mutant as the negative control. The rectangle shows the position of the M35 protein band at 36 kDa. (b) The spectra display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

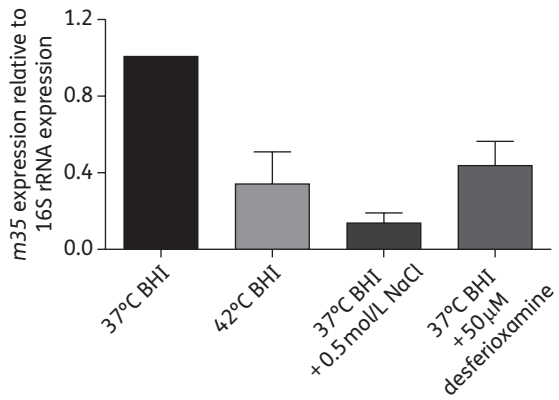

Figure 6. Down-regulation of *m35* mRNA expression after heat shock treatment, osmostress and iron depletion. Quantitative real-time PCR was performed after 3 h of incubation at 42°C, the addition of 0.5 mol/L NaCl or the addition of 50 μM desferioxamine. Up- or down-regulation was normalized to 16S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

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

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

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

Acknowledgements

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

Funding

This work was supported by the Swiss National Science Foundation (SNF) grants 3100A0-102246 and 3100A0-116053 (to C. A.).

Transparency declarations

None to declare.

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