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Moraxella catarrhalis AcrAB-OprM Efflux Pump Contributes to Antimicrobial Resistance and Is Enhanced during Cold Shock Response

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***Moraxella catarrhalis* is a common pathogen of the human respiratory tract. Multidrug efflux pumps play a major role in antibiotic resistance and virulence in many Gram-negative organisms. In the present study, the role of the AcrAB-OprM efflux pump in antibiotic resistance was investigated by constructing mutants that lack the *acrA*, *acrB*, and *oprM* genes in *M. catarrhalis* strain O35E. We observed a moderate (1.5-fold) decrease in the MICs of amoxicillin and cefotaxime and a marked (4.7-fold) decrease in the MICs of clarithromycin for *acrA*, *acrB*, and *oprM* mutants in comparison with the wild-type O35E strain. Exposure of the *M. catarrhalis* strains O35E and 300 to amoxicillin triggered an increased transcription of all AcrAB-OprM pump genes, and exposure of strains O35E, 300, and 415 to clarithromycin enhanced the expression of *acrA* and *oprM* mRNA. Inactivation of the AcrAB-OprM efflux pump genes demonstrated a decreased ability to invade epithelial cells compared to the parental strain, suggesting that *acrA*, *acrB*, and *oprM* are required for efficient invasion of human pharyngeal epithelial cells. Cold shock increases the expression of AcrAB-OprM efflux pump genes in all three *M. catarrhalis* strains tested. Increased expression of AcrAB-OprM pump genes after cold shock leads to a lower accumulation of Hoechst 33342 (H33342), a substrate of AcrAB-OprM efflux pumps, indicating that cold shock results in increased efflux activity. In conclusion, the AcrAB-OprM efflux pump appears to play a role in the antibiotic resistance and virulence of *M. catarrhalis* and is involved in the cold shock response.**

Moraxella catarrhalis colonizes the mucosal surface of the human nasopharynx and is a major cause of acute otitis media in children and of exacerbations of chronic obstructive pulmonary disease in adults (1–4). The proportion of cases of acute otitis media caused by *M. catarrhalis* varies between 5% and 20%, with recent studies showing a relative increase in *M. catarrhalis*-caused otitis media since the introduction of routine infant immunization with the pneumococcal conjugate vaccine (2, 4–6). Furthermore, clinical studies revealed that the prevalence of pharyngeal colonization and respiratory tract infections caused by *M. catarrhalis* displays seasonal variation and increases in winter (7–10). The human nasopharyngeal flora is recurrently exposed to rapid downshifts of environmental temperature. Breathing cold air (e.g., −1°C at 10 to 20 liters/min) reduces the nasopharyngeal temperature from 34°C at room temperature to about 26°C within several minutes and for extended periods (11). Such rapid variation of temperature induces adaptive events in the residential upper respiratory tract flora that may contribute to the transition from asymptomatic colonization to infection. Our previous *in vitro* studies demonstrated that a 26°C cold shock upregulates the expression of important virulence traits, such as adherence to epithelial cells, iron acquisition, complement resistance, and immune evasion (12–14).

Adaptive resistance also involves a temporary increase in the ability of a bacterium to survive exposure to antimicrobials due to alterations in gene/protein expression as a result of an environmental trigger, e.g., temperature, stress, nutrient conditions, or subinhibitory levels of the antibiotics themselves (15). One of the main antimicrobial resistance strategies of bacteria is altered porin expression to limit intracellular access of antibiotics. Recently, we showed that *M. catarrhalis* responds to exposure to aminopenicillins by reducing the expression level of the porin M35, thereby developing adaptive resistance to these antibiotics (16). Porin M35 is also regulated by temperature, being downregulated during growth at 26°C compared to growth at 37°C.

Bacterial efflux is another important mechanism of antimicrobial resistance, and bacterial efflux pumps of the resistance-nodulation-division (RND) family confer intrinsic resistance to multiple, structurally distinct, clinically relevant classes of antimicrobials, including the β-lactams, quinolones, and aminoglycosides (17). The AcrAB-OprM tripartite efflux system is the major RND efflux system found in *M. catarrhalis* (18) and other Gram-negative bacteria (17). The pump is composed of an inner membrane RND pump (AcrB), an outer membrane channel (OprM), and a periplasmic adaptor protein (AcrA). Some studies suggest that overexpression of AcrAB is a marker of multidrug resistance (19). The multidrug-resistant phenotype of carbenicillin-resistant clinical isolates of *Pseudomonas aeruginosa* can be explained as the consequence of the overexpression of multidrug efflux systems (20). However, the role, if any, that the AcrAB-OprM efflux pump plays in *M. catarrhalis* respiratory infections has not been investigated. Our recently performed transcriptome sequencing (RNA-seq) data analysis demonstrates that the expressions of genes encoding membrane fusion

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TABLE 1 Oligonucleotides used in this study

Oligonucleotide	Procedure	Sequence (5′ to 3′)
acrA_2F	PCR	CGTGTTGCCGCCATTGAGACTT
acrA_2R	PCR	GGGAACACAGCGCGTAAGGTCA
acrB_2F	PCR	GTACTTGCCATTGGCCTTTT
acrB_2R	PCR	AGGGCTTCATGACCTACACC
oprM_2F	PCR	CTGGATTTATGGGGCAAAGT
oprM_2R	PCR	GGCATTTTGAATGGCTTTTT
16S RNA_F	PCR	AAGGTTTGATC(AC)TGG(CT)TCAG
16S RNA_R	PCR	CTTTACGCCCA(AG)T(AG)A(AT)TCCG
acrA_F	Cloning	GCTGGTCATTGGGCTGAT
acrA_R	Cloning	GTTTCGTCAGCAGCGTTCAT
acrB_F	Cloning	ATCCTATCAGCCGTGTGGAG
acrB_R	Cloning	ACACCATCGAAGACACACCA
oprM_F	Cloning	TGCACGTATCTTGACGCTCT
oprM_R	Cloning	TGGGATTTTTCGTCATCCAT
acrA_F_SYBR green	SYBR green	CTCGAAACTGTGCCAGTGAT
acrA_R_SYBR green	SYBR green	ATCAATAATGCCCGTCACCT
acrB_F_SYBR green	SYBR green	TCGCTTGAGCAACAAAAATC
acrB_R_SYBR green	SYBR green	TACAGTGCTGCCAAACACAA
oprM_F_SYBR green	SYBR green	GCCAAGTCTACAAGCAGCAA
oprM_R_SYBR green	SYBR green	ACCAATCAGCAGCTGTAACG
16S RNA _F_SYBR green	SYBR green	CAATGGGCGAAAGCCTGAT
16S RNA _R_SYBR green	SYBR green	GTGCTTTACAACCAAAAGGCCT

proteins of the RND family multidrug efflux pump (*acrA* and *acrB*) and the RND system membrane channel OprM (*oprM*) were considerably increased after exposure of *M. catarrhalis* to a 26°C cold shock (21). Therefore, consistent with these reports, it is possible that a 26°C cold shock may also influence the susceptibility of *M. catarrhalis* to several antimicrobial agents through the induction of the membrane multidrug efflux pump proteins AcrA, AcrB, and OprM.

The major aim of this study was to determine the mechanism by which the AcrAB-OprM efflux pump is involved in the susceptibility to antimicrobials. Consideration of the inducible expression of AcrAB-OprM by a cold shock led us to examine the implication of the efflux system in adaptive resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* strain O35E and clinical isolates 300 and 415 were described elsewhere (12, 14). The collection of 16 *M. catarrhalis* strains (middle ear isolates from children with acute otitis media and nasopharyngeal isolates) was provided by R. Dagan, Israel, and G. A. Syrogiannopoulos, Greece. Bacteria were cultured at 37°C and 200 rpm in brain heart infusion (BHI) broth (Difco, Detroit, MI) or on BHI agar plates in an atmosphere containing 5% CO_2. Cold shock experiments were performed as described previously (12). Bacteria were grown overnight at 37°C, resuspended in fresh medium, and grown to the mid-logarithmic phase (optical density at 600 nm [OD_{600}] of 0.3). Subsequently, bacteria were exposed to 26°C or 37°C for 3 h.

For analysis of the effects of amoxicillin and clarithromycin, bacteria were cultured in BHI broth to an OD_{600} of 0.18. Afterward, 60 μg/ml of amoxicillin (Sigma, St. Louis, MO) or 0.01 μg/ml clarithromycin was added, and bacteria were cultured for an additional 4 h (16). To quantitate viable *M. catarrhalis* at various clarithromycin concentrations, bacteria were cultured at different concentrations (0 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 0.5 μg/ml, and 1 μg/ml) for 4 h, and the OD_{600} and quantitation of CFU were determined at different time points.

DNA methods. Plasmids were isolated using the Wizard Plus SV miniprep DNA purification system (Promega Corporation, Madison, WI). *Escherichia coli* DH5α was transformed as described previously (22). Restriction enzymes were purchased from New England BioLabs (Beverly, MA). Naturally competent *M. catarrhalis* was prepared and DNA was transformed as described previously (21, 23). The *M. catarrhalis* strains (middle ear and nasopharyngeal isolates) were analyzed for the presence of *acrA*, *acrB*, and *oprM* by PCR using forward and reverse primers (acrA_2F and acrA_2R, acrB_2F and acrB_2R, and oprM_2F and oprM_2R, respectively) and visualized by 1% agarose gel electrophoresis (Table 1).

Construction of the isogenic mutants O35E.*acrA*, O35E.*acrB*, and O35E.*oprM*. The AcrAB-OprM efflux system is encoded by the *acrA acrB oprM* operon. Parts of the *acrA*, *acrB*, and *oprM* genes of strain O35E were amplified using forward and reverse primers *acrA*F1 and *acrA*R1, *acrB*F1 and *acrB*R1, and *oprM*F1and *oprM*R1, respectively (Table 1). PCR products were ligated into the EcoRI restriction site of pGEM-T Easy (Promega). The kanamycin cassette from the pUC4K vector (Pharmacia, Sweden) was ligated into the HindIII restriction site of *acrA*, into the BamHI restriction site of *acrB*, and into the HpaI restriction site of *oprM*. The resulting constructs, Δ*acrA*::*kan*, Δ*acrB*::*kan*, and Δ*oprM*::*kan*, were used for natural transformation of the competent strain O35E. Transformants were selected on BHI agar plates containing 20 μg/ml of kanamycin. Insertional inactivation of each gene was confirmed by PCR analysis and by reverse transcriptase PCR (RT-PCR). We screened a number of kanamycin-resistant mutants in which we examined the expression of other genes by isolating the RNA and performing a PCR analysis for the appropriate gene. For subsequent experiments, we selected mutant strains without downstream effects of the kanamycin cassette.

RNA preparation and quantitative reverse transcriptase PCR (qRT-PCR) assays. *M. catarrhalis* cultures were fixed with 2 volumes of RNA Protect (Qiagen, Valencia, CA) and harvested. RNA was isolated using the RNeasy kit (Qiagen) as described elsewhere (12). Residual DNA in the samples was removed using DNase I. The reverse transcription step was carried out using the Superscript II cDNA synthesis kit (Invitrogen, Basel, Switzerland) according to the manufacturer's instructions. Oligonucleotide primer pairs (Table 1) were designed for use in qRT-PCR with either PrimerExpress software (Applied Biosystems, Foster City, CA) or primer 3 (24). To assess DNA contamination, RNA samples were also run without RT. The constitutively expressed 16S rRNA gene was used as an internal control for relative quantification. Reactions for qRT-PCR were com-

TABLE 2 Susceptibilities of *M. catarrhalis* wild-type strain O35E and its mutant strains to antimicrobial agents

Measurement and antibiotic	Value for strain:			
	O35E	O35E.*acrA*	O35E.*acrB*	O35E.*oprM*
MIC (μg/ml)[a]				
Amoxicillin	1.5	1.0	1.0	1.0
Cefotaxime	0.75	0.5	0.5	0.5
Clarithromycin	0.094	0.02	0.02	0.02
Amikacin	1.5	0.75	0.75	0.75
Tobramycin	0.5	0.38	0.25	0.25
Zone of growth inhibition (mm)[b]				
Erythromycin (1.5 μg)	23.8 ± 0.5	34 ± 0.0	35 ± 0.0	33.8 ± 0.5
Rifampin (0.5 μg)	26 ± 0.0	32 ± 0.0	32.3 ± 0.5	32.8 ± 0.5
Deoxycholate (10 mg/ml)	24 ± 0.0	32.25 ± 0.5	31.75 ± 0.5	31.75 ± 1.0

[a] MICs of amoxicillin, cefotaxime, and clarithromycin were determined in two independent experiments with consistent results.
[b] Sensitivities were assessed by measuring the diameters of the zones of growth inhibition on two axes, and the means ± 1 SD were calculated. A representative of two independent experiments performed in duplicate is shown.

pleted using Power SYBR green PCR master mix (Applied Biosystems) with a two-step reaction protocol consisting of 40 cycles of 94°C for 30 s and 60°C for 1 min, followed by a dissociation phase for quality control. The 25-μl qRT-PCR mixtures contained 0.2 μM specific primers, Power SYBR green (2×), and 2 μl of cDNA (10 ng/μl). Relative quantification of gene expression was performed using the comparative threshold method. The ratios obtained after normalization were expressed as folds of change compared with untreated samples or samples isolated from bacteria exposed to 37°C.

Antimicrobial resistance testing. The MICs of amoxicillin, cefotaxime, clarithromycin, amikacin, tobramycin, gentamicin, tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole were determined by Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. We tested the MIC of rifampin and erythromycin by a gradient test (Etest). However, the antimicrobial resistance of *M. catarrhalis* to these compounds was out of range for commercially available Etest strips. Therefore, the susceptibilities of *M. catarrhalis* to rifampin, erythromycin, and deoxycholate (all purchased from Sigma) were assayed using standard disk diffusion assays (25, 26). Bacteria were cultured in BHI broth to an OD_{600} of 0.2 and spread with a cotton swab onto BHI agar plates. Sterile blank paper disks (6 mm; Becton Dickinson, Franklin Lakes, NJ) were placed on the agar surface in duplicate and saturated with a solution of the antimicrobial at various concentrations (Table 2). After 18 h of incubation at 37°C, sensitivities were assessed by measuring the diameters of the zones of growth inhibition on two axes, and the mean values were calculated. The MICs and the susceptibilities of the tested agents for the mutant strains were determined in medium without kanamycin because of the synergistic effect between two substances.

Cell lines and growth conditions. Detroit 562 pharyngeal cells (ATCC CCL-138) were maintained in Eagle's minimal essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma), 1× nonessential amino acids (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Adherence and invasion assay. The ability of *M. catarrhalis* to adhere to and invade human Detroit 562 epithelial cells *in vitro* was measured as described previously (13, 27, 28). Each strain was analyzed in triplicate in each experiment.

Accumulation of Hoechst 33342. To investigate the efflux activity of *M. catarrhalis* following a 26°C cold shock, we used an assay that measures the accumulation of the fluorescent DNA-binding dye Hoechst 33342 (H33342; Sigma), which is a substrate of the AcrAB pump in several Gram-negative pathogens, including *E. coli* and *Salmonella enterica* (29). When H33342 enters the cell, it binds to the DNA minor groove, becomes fluorescent, and can be detected by using a fluorescent plate reader. Efflux-competent cells extrude H33342 out of the cell and accumulate the dye sparingly, resulting in low levels of fluorescence. In contrast, efflux-defective cells (e.g., efflux pump mutants or heat-inactivated [hi] cells) accumulate H33342 at a higher rate, resulting in increased levels of fluorescence. Bacteria were exposed to 26°C or 37°C for 3 h, pelleted, and resuspended in NaCl (0.87%) to an OD_{600} of 0.2. H33342 was added to a final concentration of 2.5 μM. Fluorescence intensity was measured at excitation and emission wavelengths of 350 and 461 nm, respectively, using a Varioskan Flash (Thermo Scientific, Madison, WI).

Statistical analysis. Data were expressed as means ± 1 standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance with a Dunnett's correction using Prism software (GraphPad, version 5.01). A P value of <0.05 was defined as statistically significant.

RESULTS

***In vitro* growth of *M. catarrhalis acrA*, *acrB*, and *oprM* mutants and PCR and RT-PCR analyses of clinical isolates.** Standard growth curves of the O35E wild-type and mutant strains in BHI broth at 37°C revealed no significant differences in growth velocity, measured as broth density at OD_{600} (Fig. 1A). We studied 16 middle ear and nasopharyngeal isolates for the presence and transcription of *acrA*, *acrB*, and *oprM* genes of the AcrAB-OprM efflux pump. PCR and RT-PCR analyses resulted in positive products for all strains (data not shown).

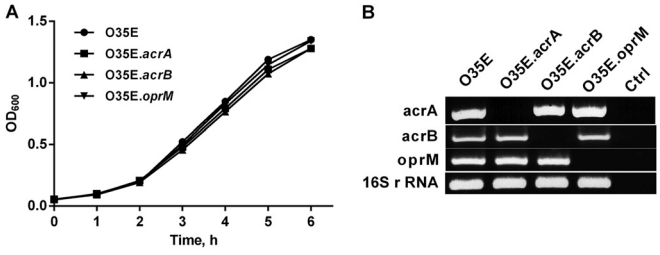

FIG 1 (A) Growth curves for wild-type O35E *M. catarrhalis* strain and its isogenic knockout strains in BHI medium at 37°C. (B) Expression of the mutated gene in each strain was determined by RT-PCR. For each strain, the product of RT-PCR using primers specific for *acrA*, *acrB*, *oprM*, or 16S rRNA, as a control gene, is shown. Ctrl indicates control reactions with no cDNA templates.

Genotypic and phenotypic characterization of the *M. catarrhalis* AcrAB-OprM operon knockout mutants. To investigate the role of the AcrAB-OprM efflux pump in *M. catarrhalis* antimicrobial resistance and virulence, we constructed isogenic knockout mutants from the wild-type strain O35E. The periplasmic adaptor protein encoded by *acrA* is the first in an operon also encoding the cognate RND pump *acrB* and the outer membrane channel *oprM*. RT-PCR was used to check the inactivation of these genes by insertion of an antibiotic resistance cassette and the expression of the downstream pump genes (Fig. 1B). The inactivation of *acrA* by insertion of the kanamycin resistance gene was nonpolar; *acrB* and *oprM* were still transcribed at normal levels. The transcriptions of *acrA* and *oprM* were not affected by disruption of the *acrB* gene. The disruption of the *oprM* gene allowed the expression of upstream genes *acrA* and *acrB*.

Role of the AcrAB-OprM efflux pump in *M. catarrhalis* antimicrobial resistance. The RND family multidrug efflux systems, which are common in Gram-negative bacteria, pump out a wide range of compounds, such as lipophilic and amphiphilic inhibitors, including antibiotics, and are responsible for intrinsic resistance to many antimicrobial agents (17, 30–32). To investigate this, Etests and susceptibility assays were performed with the mutants O35E.*acrA*, O35E.*acrB*, and O35E.*oprM* and the wild-type O35E strain. There were no differences in MICs for tetracycline, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole between wild-type and mutant strains. For amoxicillin and cefotaxime, there was a minor but consistent 1.5-fold decrease in the MICs of all mutants (1.5 μg/ml versus 1.0 μg/ml and 0.75 μg/ml versus 0.5 μg/ml, respectively) (Table 2). For clarithromycin, there was a 4.7-fold decrease in the MICs of all mutants tested in comparison with the wild-type strain (0.094 μg/ml versus 0.02 μg/ml) (Table 2). In a susceptibility test, insertional inactivation of *acrA*, *acrB*, and *oprM* also increased the antibacterial activity of many additional agents tested (erythromycin, rifampin, deoxycholic acid), implying that they are ordinarily substrates for the AcrAB-OprM efflux transporter (Table 2). Thus, *M. catarrhalis* strains lacking the efflux pump proteins display a MIC of amoxicillin, cefotaxime, and clarithromycin up to 1.5- and 4.7-fold, respectively, lower than that in the wild-type strain, indicating that the AcrAB-OprM efflux pump is involved in the susceptibility of these cells to the antimicrobials tested.

The antibiotic class of aminoglycosides is not a substrate for the *E. coli* AcrAB-TolC pump, although it is a substrate for the homologous *Yersinia pestis*, *P. aeruginosa*, and *Klebsiella pneumoniae* complexes (31, 32). The MICs of three aminoglycosides (amikacin, tobramycin, and gentamicin) were determined for *M. catarrhalis* O35E and its *acrA*, *acrB*, and *oprM* mutants. The *acrA*, *acrB*, and *oprM* mutant strains were more susceptible (2-fold) than the wild-type strain to amikacin (Table 2). The MICs of tobramycin were lower (2-fold) for the *acrB* and *oprM* knockout strains than for the wild-type strain, while there were only modest (1.3-fold) decreases in the MICs of this antibiotic in the case of O35E.*acrA* (Table 2). In contrast, gentamicin susceptibility was not affected in the *acrA*, *acrB*, and *oprM* strains. It was shown that the *S. enterica acrB* mutant strain was more susceptible to tobramycin (2-fold), whereas susceptibility to amikacin was not affected compared with that of the wild-type parental strain, reflecting substrate specificity of this pump (33).

Upregulation of *acrA*, *acrB*, and *oprM* mRNA expression after amoxicillin treatment. Reduced membrane permeability and increased active efflux—two of the main strategies used by bacteria for protection against antibiotics—are generally regulated by reduced porin expression and overexpression of efflux pumps (34, 35). Our previous findings of downregulation of *m35* porin mRNA after amoxicillin (60 μg/ml) treatment (16) prompted us to investigate the expression of the AcrAB-OprM efflux pump genes of strains O35E and 300 during amoxicillin treatment by quantitative real-time PCR. Amoxicillin treatment of strain O35E for a period of 4 h induced the upregulation of *acrA* (4-fold), *acrB* (4-fold), and *oprM* (2.5-fold) mRNA expression compared with that of bacteria grown without amoxicillin (Fig. 2). Strain 300 demonstrated similar effects, with expression of *acrA*, *acrB*, and *oprM* mRNA induced (2-fold) after amoxicillin treatment. The expression of a control gene encoding type IV pilin A (*pilA*) was not altered by amoxicillin treatment, indicating that amoxicillin induces the expression of genes that are involved in the adaptive resistance (data not shown).

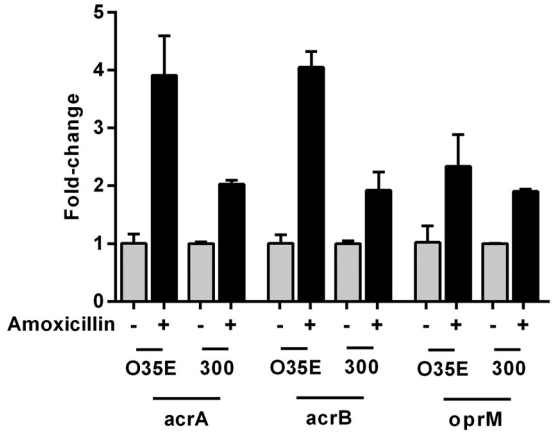

FIG 2 Upregulation of *acrA*, *acrB*, and *oprM* mRNA expression of *M. catarrhalis* strains O35E and 300 during amoxicillin treatment. Quantitative real-time PCR was performed after 4 h of incubation with and without 60 μg/ml amoxicillin. Upregulation was normalized to 16S rRNA. The graph shows one of two representative experiments done in triplicate. Data are presented as means ± 1 SD.

Expression of *acrA*, *acrB*, and *oprM* mRNA after clarithromycin treatment. Because clarithromycin was found to be a substrate for the AcrAB-OprM pump, we investigated the expression of the *acrA*, *acrB*, and *oprM* genes after clarithromycin treatment by quantitative real-time PCR. The breakpoint of bacteriostatic clarithromycin concentration was evaluated by a time-kill curve assay (Fig. 3), and the subinhibitory concentration was estimated to be 0.01 μg/ml. Clarithromycin treatment of *M. catarrhalis* strain O35E, for a period of 4 h, induced the upregulation of *acrA* (2-fold) and *oprM* (2.2-fold) mRNA expression compared with that of bacteria grown without clarithromycin (Fig. 4). Clarithromycin treatment of *M. catarrhalis* strains 300 and 415 caused the induction of *acrA* (4.5- and 2.8-fold, respectively) and *oprM* (2.4- and 3.4-fold, respectively) mRNA expression. In contrast, the expression of *acrB* mRNA in these strains remained unaffected.

The expressions of *acrA*, *acrB*, and *oprM* genes are required for efficient invasion of Detroit 562 pharyngeal epithelial cells. The abilities of a pathogen to adhere to and invade epithelial host cells are major virulence factors. Efflux pump systems have a role in mediating the adherence and uptake of bacteria into target host

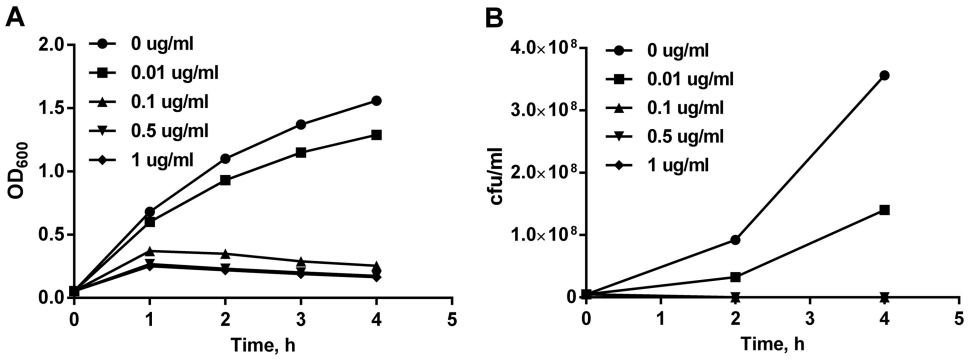

FIG 3 Time-kill curves of strain O35E for different clarithromycin concentrations (0, 0.01, 0.1, 0.5, and 1 μg/ml).

cells (29, 36, 37). To investigate if AcrAB-OprM efflux pump components mediate adherence and invasion, assays were performed on Detroit 562 pharyngeal epithelial cells. Adherence of O35E.*acrA*, O35E.*acrB*, and O35E.*oprM* mutants was as efficient as that of the O35E wild-type parent strain (data not shown). To determine if *acrA*, *acrB*, and *oprM* are required for efficient invasion, Detroit 562 cell monolayers were infected at a multiplicity of infection (MOI) of 30, and following a 3-h incubation, cells were washed, incubated with gentamicin for an additional 2 h, and then lysed, serially diluted, and plated to determine the proportion of invading bacteria. The O35E.*acrA*, O35E.*acrB*, and O35E.*oprM* strains demonstrated decreased (up to 50%) invasion compared to that of O35E (Fig. 5), indicating that *acrA*, *acrB*, and *oprM* are required for efficient invasion of human nasopharyngeal cells *in vitro*.

Serum resistance. Resistance of *M. catarrhalis* to human complement, which is associated with disease-causing isolates (38), was not impaired by the lack of AcrAB-OprM efflux pump genes (data not shown).

Growth of *M. catarrhalis acrA*, *acrB*, and *oprM* mutants after cold shock. To investigate whether inactivation of the efflux pump genes affects the growth rate following cold shock conditions, growth curves were plotted for all strains studied. When the growth at 37°C was monitored, we observed that the parent strain O35E and its knockout mutants grew similarly (Fig. 6). The growth of *M. catarrhalis acrA*, *acrB*, and *oprM* mutants, subjected to a downshift to 26°C during the mid-logarithmic phase, was slightly diminished compared to the growth of parent strain O35E (Fig. 6), indicating that the AcrAB-OprM efflux pump might play a role in the adaptation to cold shock.

Cold shock induces upregulation of *acrA*, *acrB*, and *oprM* mRNA. To confirm the RNA-seq data (21) and to investigate the contribution of the AcrAB-OprM efflux pump to the cold shock response, we assessed the *acrA*, *acrB*, and *oprM* mRNA expression levels of strain O35E exposed to either 26°C or 37°C. The expression levels of *acrA*, *acrB*, and *oprM* were significantly increased at 26°C (10.7-, 5.7-, and 6.5-fold, respectively) compared to expression at 37°C (Fig. 7). Similar expression patterns of *acrA*, *acrB*, and *oprM* were observed in *M. catarrhalis* clinical isolates 300 and 415, indicating that this effect is a general characteristic of *M. catarrhalis* (Fig. 7).

Cold shock results in the efflux upregulation of Hoechst 33342. Since cold shock increases the expression of AcrAB-OprM efflux pump genes, we examined the effect of cold shock on active efflux. The Hoechst dye is a substrate of RND efflux pumps, and its accumulation can be used to infer the level of RND efflux (29). In order to compare the levels of active efflux in bacterial growth at 26°C, accumulation of H33342 was determined and compared with bacterial growth at 37°C. The results of the H33342 accumulation assay are shown in Fig. 8. Accumulation of H33342 was

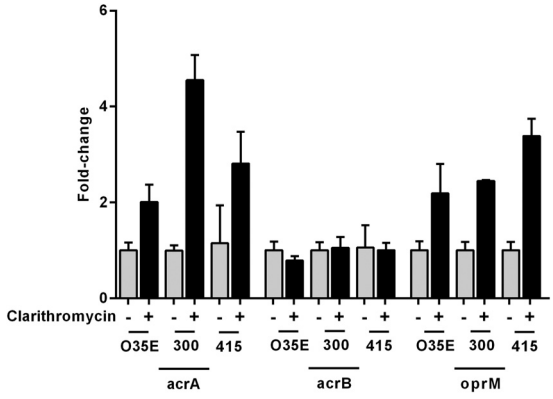

FIG 4 Expression of efflux pump AcrAB-OprM genes *acrA*, *acrB*, and *oprM* mRNA in *M. catarrhalis* strains O35E, 300, and 415 during clarithromycin treatment. Quantitative real-time PCR was performed after 4 h of incubation with and without 0.01 μg/ml clarithromycin. Upregulation was normalized to 16S rRNA. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD.

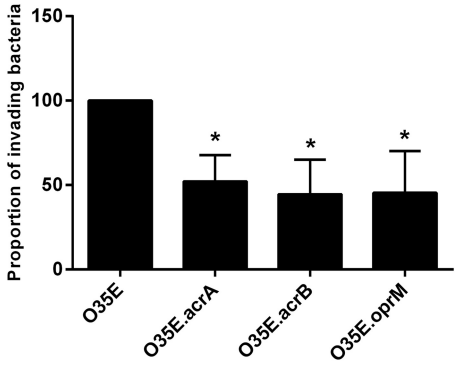

FIG 5 Ability of *M. catarrhalis* wild-type strain O35E and its knockout mutants to invade human pharyngeal epithelial cells. Data are displayed as means of four separate experiments performed in triplicate ± 1 SD. *, $P < 0.05$ for wild-type versus respective mutant.

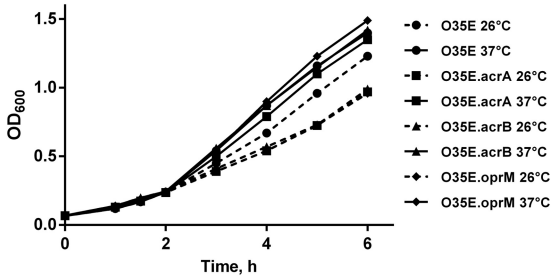

FIG 6 Growth of *M. catarrhalis* O35E and its isogenic knockout strains at 26°C compared to that at 37°C. Bacteria were grown to mid-logarithmic phase (OD_{600} = 0.25) under standard conditions at 37°C. The cultures were then split, and equal aliquots were incubated further at 26°C and 37°C, respectively.

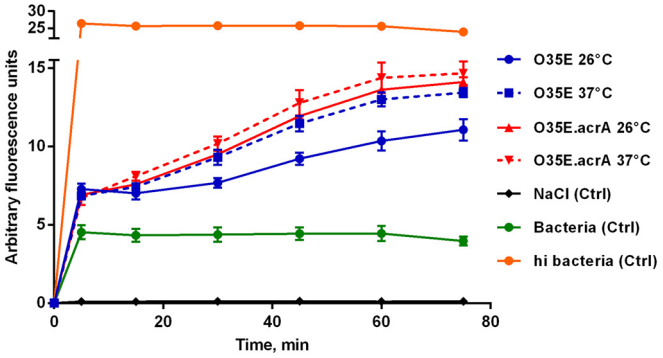

FIG 8 The accumulation of H33342 in *M. catarrhalis* strain O35E and its isogenic mutant strain O35E.*acrA* over time. Cold shock results in the efflux upregulation of H33342, a substrate of AcrAB-OprM efflux pump. Bacteria were exposed to 26°C or 37°C for 3 h, pelleted, and resuspended in NaCl (0.87%) to an OD_{600} of 0.2. H33342 was added to a final concentration of 2.5 μM. The graph shows one of three representative experiments done in triplicate. Data are presented as means ± 1 SD. Ctrl indicates control reactions with no bacteria or heat-inactivated (hi; 60°C, 1 h) bacteria.

approximately 20% lower following cold shock at 26°C than after exposure to 37°C, indicating that cold shock resulted in increased efflux activity. Inactivation of *acrA* alone increased Hoechst 33342 accumulation, indicating that the inactivation of *acrA* resulted in reduced efflux activity. Furthermore, there was no cold shock effect on the accumulation of the dye, indicating that AcrA is required for active efflux of H33342 in cold shock-induced *M. catarrhalis*.

DISCUSSION

Antimicrobial resistance is based on three major strategies: detoxify enzymes to degrade or modify antibiotics, target protection to impair target recognition and thus antimicrobial activity, and target the membrane barrier to limit intracellular access of antimicrobials (35). For *M. catarrhalis*, two major mechanisms of bacterial resistance to antimicrobials have been described: the inactivation of antimicrobials by enzymes such as β-lactamases (39) and the reduced entry of the antibiotic into bacterial cells due to reducing the number of entry channels, such as porins (16). In our previous study, we demonstrated that the M35 porin was downregulated after amoxicillin treatment, leading to increased resistance and indicating a novel resistance mechanism against aminopenicillins in *M. catarrhalis* (16). Another important mechanism of antimicrobial resistance is enhancement of the active expulsion of antibiotics out of the cells—a process termed efflux. The expression of genes encoding porins and efflux pumps is carefully regulated in order to respond to certain signals, thereby altering the resistance of a bacterium depending on the growth conditions (15).

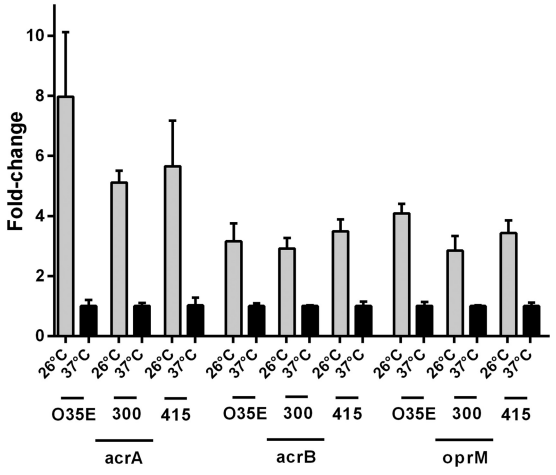

FIG 7 Upregulation of *acrA*, *acrB*, and *oprM* mRNA expression of *M. catarrhalis* strains O35E, 300, and 415 after cold shock. Quantitative real-time PCR was performed after 3 h of incubation at 26°C or 37°C. Upregulation was normalized to 16S rRNA. The graph shows one of two representative experiments done in triplicate. Data are presented as means ± 1 SD.

Sequence analysis of the genome of a variety of bacterial species has demonstrated that most of them encode several efflux pump systems. The multidrug efflux systems of *M. catarrhalis* have never before been characterized, to our knowledge, although the sequencing data for *M. catarrhalis* strain BBH18 indicate that only a few such pumps (Acr and Mtr efflux pumps) are potentially present (18). *M. catarrhalis* encodes an AcrAB-OprM efflux system homologous to the system described in *E. coli*. Most of the clinical studies have focused on the involvement of efflux pumps in antimicrobial resistance. In this study, the role of the AcrAB-OprM efflux pump in antibiotic resistance was investigated by constructing mutant strains containing *acrA*, *acrB*, and *oprM* genes in *M. catarrhalis* strain O35E. We observed a moderate (1.5-fold) decrease in the MICs of amoxicillin and cefotaxime and a marked (4.7-fold) decrease in the MICs of clarithromycin for all mutants in comparison with those for the wild-type strain. These results show that the AcrAB-OprM pump is important in clarithromycin resistance and is involved in macrolide efflux. Conversely, amoxicillin and cefotaxime resistances are only weakly mediated by the AcrAB-OprM efflux pump, suggesting that other resistance strategies, such as β-lactamases, porins (M35), or another efflux pump, may play a more important role. Furthermore, the insertional inactivation of *acrA*, *acrB*, and *oprM* increased the antibacterial activity of erythromycin, rifampin, and deoxycholic acid, indicating that this efflux pump may recognize multiple substrates, as already noted for the homologous proteins from *E. coli* (40).

Antimicrobials play a very important role in the induction of adaptive resistance by the overexpression of efflux systems (15). Several studies have shown increased bacterial efflux pump expression during antibiotic therapy, indicating that efflux pumps may play a role in survival within the host (41, 42). We found that

exposure of *M. catarrhalis* strains O35E and 300 to amoxicillin for 4 h triggered the increased expression of all AcrAB-OprM pump genes (Fig. 2), and exposure of O35E and strains 300 and 415 to clarithromycin enhanced the expression of *acrA* and *oprM* (Fig. 4). We performed the expression analysis of the AcrAB-OprM efflux pump genes after amoxicillin treatment as an extension of the previous study by our group and used the already evaluated concentration of amoxicillin (16). Growth curves for treatment with 60 μg/ml of amoxicillin indicated that this concentration was not completely bactericidal but inhibited growth for a period of about 4 h before proliferation resumed (16). The breakpoint of the bacteriostatic clarithromycin concentration was evaluated by a time-kill curve assay (Fig. 3), and the subinhibitory concentration was estimated to be 0.01 μg/ml. The different correlations of amoxicillin and clarithromycin concentrations with the MICs obtained by Etest may be attributed to different resistance strategies of bacteria upon induction by specific antibiotics. It seems that the threshold concentrations for clarithromycin required to induce the antimicrobial resistance (e.g., increased expression of efflux genes) are lower than those of amoxicillin. As we already showed, exposure of *M. catarrhalis* to amoxicillin induces downregulation of the *m35* porin to limit intracellular penetration of amoxicillin (16) and increases the expression of the AcrAB-OprM genes to enhance the active expulsion of this antibiotic.

The exposure of *M. catarrhalis* to clarithromycin increases the expression of particular components of the AcrAB-OprM efflux pump. Clarithromycin treatment of *M. catarrhalis* strains O35E, 300, and 415 caused the induction of *acrA* and *oprM* mRNA expression, while the expression of *acrB* mRNA in these strains remained unaffected. The regulatory mechanism controlling the transcription of the AcrAB-OprM genes after clarithromycin treatment is indistinct at this point and needs further investigation. However, it has been demonstrated that genes within an operon do not conform to the simple notion that they have equal levels of expression (43). Several studies revealed the existence of the internal promoters and read-through terminators in bacterial operons (43, 44). These additional promoters are often located downstream of the first gene so that only part of the operon is transcribed from the internal promoter. Similarly, some operons contain an internal read-through terminator at which partial continuation of transcription occurs (44). Additional antimicrobial strategies used by *M. catarrhalis* against clarithromycin (e.g., expression of the porin) are still unclear and need further investigation. Furthermore, it would be interesting to investigate antimicrobial resistance in *M. catarrhalis* mutant strains lacking the entire gene cluster of the AcrAB-OprM efflux pump in order to estimate the level of redundancy between potentially homologous efflux pump proteins. Our findings of the upregulation of AcrAB-OprM pump expression at the transcriptional level after treatment with amoxicillin and clarithromycin in all tested β-lactamase-producing strains combined with observations of the lower MICs for the AcrAB-OprM pump knockout mutants indicate that there is a potentially novel resistance mechanism against these antibiotics in *M. catarrhalis*.

Temperature is a critical environmental factor, and cold shock, as has been characterized for *E. coli*, affects the bacterial transcriptome in a concerted attempt to maintain essential cellular functions that favor survival under extreme and rapidly changing conditions (45). We have shown that the cold shock response is obviously an important mechanism for *M. catarrhalis* as an adaptation and survival mechanism in the nasopharyngeal habitat, as well as for the virulence and colonization abilities of *M. catarrhalis* (12–14, 16, 21). Our current results demonstrate that cold shock increases the expression of AcrAB-OprM efflux pump genes in all three *M. catarrhalis* strains tested (Fig. 7), suggesting that this effect is a general characteristic of *M. catarrhalis*. We also show that increased expression of AcrAB-OprM pump genes following incubation of *M. catarrhalis* at 26°C leads to a lower accumulation of Hoechst 33342 (Fig. 8), a substrate of the AcrAB-OprM efflux pump, indicating that cold shock results in increased efflux activity. Our findings of the upregulation of AcrAB-OprM pump expression following cold shock and pretreatment with subinhibitory concentrations of clarithromycin together with an observation of higher efflux activity upon cold shock indicate that cold shock could contribute to increased resistance of *M. catarrhalis* to this antibiotic. Since both cold shock and treatment with amoxicillin and clarithromycin induce expression of the efflux pump genes, it would be interesting to investigate whether an additive effect exists with both cold shock and antibiotic treatment. *M. catarrhalis* exposed to a 26°C cold shock might transiently increase its ability to withstand the presence of subinhibitory concentrations of an antibiotic by limiting its entry (e.g., M35 porin) into the cell and/or expelling it more efficiently, leading to increased survival of bacteria. The relative and synergistic effect of these two antimicrobial resistance mechanisms (porin loss/active efflux) in *M. catarrhalis* is still unclear and needs further investigation by constructing mutant strains lacking both components (porin/efflux pump). Since it has been shown that one stress response might help bacteria to contend with other stress conditions (46, 47), it is possible that cold shock could improve the ability of *M. catarrhalis* to survive subinhibitory concentrations of antibiotics because several pathways of stress responses are activated. Clinical studies in children demonstrated that the density of *M. catarrhalis* in the nasopharynx is positively associated with prolonged respiratory tract symptoms and a greater likelihood of purulent otitis media (48, 49).

In addition to antibiotic sensitivity, efflux pumps are used in bacteria to excrete cellular metabolites that are toxic and/or have a signaling role (50). This could potentially explain the reduced growth of the *M. catarrhalis* efflux pump mutant strains after cold shock conditions (Fig. 6). Cold shock induces the expression of AcrAB-OprM pump genes (Fig. 7), suggesting that the AcrAB-OprM efflux pump might play an important role in adaptation to cold shock. Inactivation of the AcrAB-OprM efflux pump would lead to accumulation of potentially toxic metabolites and impair growth at 26°C. On the other hand, the parent strain O35E and its knockout mutants grew similarly at 37°C, indicating that at this temperature, other efflux pumps, such as Mtr, would accomplish this function. Our RNA-seq data indicate that some components of the Mtr efflux pump (*mtrF*) are induced (1.8-fold) after exposure to 37°C (21). The contribution of other environmental triggers like heat shock or nutrient conditions to the expression of AcrAB-OprM efflux pump genes is still unclear and needs further scrutiny.

In addition to an established role in antimicrobial resistance, efflux pumps have been shown to have a role in pathogenicity in a variety of clinically relevant bacterial species, including *Salmonella*, *P. aeruginosa*, *Neisseria gonorrhoeae*, and *Campylobacter jejuni* (36). In *Salmonella*, disruption of *acrA* or *acrB* led not only to susceptibility to a range of antimicrobial compounds but also to a

reduced ability to adhere to and invade human intestinal epithelial cells (29, 37). This effect can be partially explained by the finding that many efflux pumps are also responsible for exporting host-derived substrates, such as bile salts and fatty acids. Inactivation of the AcrAB-OprM efflux pump genes of *M. catarrhalis* strain O35E demonstrated a decreased invasion ability compared with the parental strain (Fig. 5), suggesting that *acrA*, *acrB*, and *oprM* are required for efficient invasion of human nasopharyngeal cells.

In summary, we describe here the use of specific knockout mutants to investigate the effect of the AcrAB-OprM efflux system on antimicrobial resistance and virulence in *M. catarrhalis*. We demonstrate that the AcrAB-OprM efflux pump is involved in resistance to amoxicillin, cefotaxime, and clarithromycin and is required for efficient invasion of pharyngeal epithelial cells. A physiologic cold shock, as occurs when humans breathe cold air for prolonged periods, increases the expression of AcrAB-OprM pump genes *in vitro*, resulting in increased efflux activity of *M. catarrhalis*. These data support the notion that *M. catarrhalis* uses physiologic exposure to cold air to upregulate pivotal survival systems in the human pharynx that may contribute to bacterial virulence.

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REFERENCES

1. **Faden H, Duffy R, Wasielewski R, Wolf J, Krystofik D, Tung Y.** 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. J Infect Dis **175:**1440–1445. http://dx.doi.org/10.1086/516477.
2. **Palmu A, Herva E, Savolainen H, Karma P, Mäkelä PH, Kilpi T.** 2004. Association of clinical signs and symptoms with bacterial findings in acute otitis media. Clin Infect Dis **38:**234–242. http://dx.doi.org/10.1086/380642.
3. **Murphy TF, Brauer AL, Grant BJ, Sethi S.** 2005. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. Am J Respir Crit Care Med **172:**195–199. http://dx.doi.org/10.1164/rccm.200412-1747OC.
4. **Murphy TF, Parameswaran GI.** 2009. *Moraxella catarrhalis*, a human respiratory tract pathogen. Clin Infect Dis **49:**124–131. http://dx.doi.org/10.1086/599375.
5. **Eskola J, Kilpi T, Palmu A, Jokinen J, Haapakoski J, Herva E, Takala A, Kayhty H, Karma P, Kohberger R, Siber G, Makela PH.** 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med **344:**403–409. http://dx.doi.org/10.1056/NEJM200102083440602.
6. **Revai K, McCormick DP, Patel J, Grady JJ, Saeed K, Chonmaitree T.** 2006. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics **117:**1823–1829. http://dx.doi.org/10.1542/peds.2005-1983.
7. **Van Hare GF, Shurin PA.** 1987. The increasing importance of *Branhamella catarrhalis* in respiratory infections. Pediatr Infect Dis J **6:**92–94. http://dx.doi.org/10.1097/00006454-198701000-00047.
8. **Mbaki N, Rikitomi N, Nagatake T, Matsumoto K.** 1987. Correlation between *Branhamella catarrhalis* adherence to oropharyngeal cells and seasonal incidence of lower respiratory tract infections. Tohoku J Exp Med **153:**111–121. http://dx.doi.org/10.1620/tjem.153.111.
9. **Sarubbi FA, Myers JW, Williams JJ, Shell CG.** 1990. Respiratory infections caused by *Branhamella catarrhalis*. Selected epidemiologic features. Am J Med **88:**9S–14S.
10. **Hendley JO, Hayden FG, Winther B.** 2005. Weekly point prevalence of *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Moraxella catarrhalis* in the upper airways of normal young children: effect of respiratory illness and season. APMIS **113:**213–220. http://dx.doi.org/10.1111/j.1600-0463.2005.apm1130310.x.
11. **Rouadi P, Baroody FM, Abbott D, Naureckas E, Solway J, Naclerio RM.** 1999. A technique to measure the ability of the human nose to warm and humidify air. J Appl Physiol **87:**400–406.
12. **Heiniger N, Troller R, Meier PS, Aebi C.** 2005. Cold shock response of the UspA1 outer membrane adhesin of *Moraxella catarrhalis*. Infect Immun **73:**8247–8255. http://dx.doi.org/10.1128/IAI.73.12.8247-8255.2005.
13. **Spaniol V, Troller R, Aebi C.** 2009. Physiologic cold shock increases adherence of *Moraxella catarrhalis* to and secretion of interleukin 8 in human upper respiratory tract epithelial cells. J Infect Dis **200:**1593–1601. http://dx.doi.org/10.1086/644640.
14. **Spaniol V, Troller R, Schaller A, Aebi C.** 2011. Physiologic cold shock of *Moraxella catarrhalis* affects the expression of genes involved in the iron acquisition, serum resistance and immune evasion. BMC Microbiol **11:**182. http://dx.doi.org/10.1186/1471-2180-11-182.
15. **Fernandez L, Hancock RE.** 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev **25:**661–681. http://dx.doi.org/10.1128/CMR.00043-12.
16. **Jetter M, Spaniol V, Troller R, Aebi C.** 2010. Down-regulation of porin M35 in *Moraxella catarrhalis* by aminopenicillins and environmental factors and its potential contribution to the mechanism of resistance to aminopenicillins. J Antimicrob Chemother **65:**2089–2096. http://dx.doi.org/10.1093/jac/dkq312.
17. **Nikaido H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol **178:**5853–5859.
18. **de Vries SP, van Hijum SA, Schueler W, Riesbeck K, Hays JP, Hermans PW, Bootsma HJ.** 2010. Genome analysis of *Moraxella catarrhalis* strain BBH18, [corrected] a human respiratory tract pathogen. J Bacteriol **192:**3574–3583. http://dx.doi.org/10.1128/JB.00121-10.
19. **Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L.** 2011. Expression of multidrug efflux pump genes *acrAB-tolC*, *mdfA*, and *norE* in *Escherichia coli* clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob Agents Chemother **55:**921–924. http://dx.doi.org/10.1128/AAC.00996-10.
20. **Li XZ, Nikaido H, Poole K.** 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **39:**1948–1953. http://dx.doi.org/10.1128/AAC.39.9.1948.
21. **Spaniol V, Wyder S, Aebi C.** 2013. RNA-Seq-based analysis of the physiologic cold shock-induced changes in *Moraxella catarrhalis* gene expression. PLoS One **8:**e68298. http://dx.doi.org/10.1371/journal.pone.0068298.
22. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166:**557–580. http://dx.doi.org/10.1016/S0022-2836(83)80284-8.
23. **Meier PS, Troller R, Heiniger N, Hays JP, van Belkum A, Aebi C.** 2006. Unveiling electrotransformation of *Moraxella catarrhalis* as a process of natural transformation. FEMS Microbiol Lett **262:**72–76. http://dx.doi.org/10.1111/j.1574-6968.2006.00365.x.
24. **Rozen S, Skaletsky H.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol **132:**365–386.
25. **Peng D, Hong W, Choudhury BP, Carlson RW, Gu XX.** 2005. *Moraxella catarrhalis* bacterium without endotoxin, a potential vaccine candidate. Infect Immun **73:**7569–7577. http://dx.doi.org/10.1128/IAI.73.11.7569-7577.2005.
26. **Duval BD, Mathew A, Satola SW, Shafer WM.** 2010. Altered growth, pigmentation, and antimicrobial susceptibility properties of *Staphylococcus aureus* due to loss of the major cold shock gene *cspB*. Antimicrob Agents Chemother **54:**2283–2290. http://dx.doi.org/10.1128/AAC.01786-09.
27. **Spaniol V, Heiniger N, Troller R, Aebi C.** 2008. Outer membrane protein UspA1 and lipooligosaccharide are involved in invasion of human epithelial cells by *Moraxella catarrhalis*. Microbes Infect **10:**3–11. http://dx.doi.org/10.1016/j.micinf.2007.09.014.
28. **Murphy TF, Brauer AL, Kirkham C, Johnson A, Koszelak-Rosenblum M, Malkowski MG.** 2013. Role of the zinc uptake ABC transporter of *Moraxella catarrhalis* in persistence in the respiratory tract. Infect Immun **81:**3406–3413. http://dx.doi.org/10.1128/IAI.00589-13.
29. **Blair JM, La Ragione RM, Woodward MJ, Piddock LJ.** 2009. Periplasmic adaptor protein AcrA has a distinct role in the antibiotic resistance and virulence of *Salmonella enterica* serovar Typhimurium. J Antimicrob Chemother **64:**965–972. http://dx.doi.org/10.1093/jac/dkp311.
30. **Nikaido H, Pages JM.** 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev **36:**340–363. http://dx.doi.org/10.1111/j.1574-6976.2011.00290.x.
31. **Lister IM, Raftery C, Mecsas J, Levy SB.** 2012. *Yersinia pestis* AcrAB-TolC in antibiotic resistance and virulence. Antimicrob Agents Chemother **56:**1120–1123. http://dx.doi.org/10.1128/AAC.05338-11.
32. **Padilla E, Llobet E, Domenech-Sanchez A, Martinez-Martinez L, Bengoechea JA, Alberti S.** 2010. *Klebsiella pneumoniae* AcrAB efflux pump

contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother **54:**177–183. http://dx.doi.org/10.1128/AAC.00715-09.
33. **Blair JM, Smith HE, Ricci V, Lawler AJ, Thompson LJ, Piddock LJ.** 2015. Expression of homologous RND efflux pump genes is dependent upon AcrB expression: implications for efflux and virulence inhibitor design. J Antimicrob Chemother **70:**424–431.
34. **Pages JM, James CE, Winterhalter M.** 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol **6:**893–903. http://dx.doi.org/10.1038/nrmicro1994.
35. **Davin-Regli A, Bolla JM, James CE, Lavigne JP, Chevalier J, Garnotel E, Molitor A, Pages JM.** 2008. Membrane permeability and regulation of drug "influx and efflux" in enterobacterial pathogens. Curr Drug Targets **9:**750–759. http://dx.doi.org/10.2174/138945008785747824.
36. **Piddock LJ.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev **19:**382–402. http://dx.doi.org/10.1128/CMR.19.2.382-402.2006.
37. **Smith HE, Blair JM.** 2014. Redundancy in the periplasmic adaptor proteins AcrA and AcrE provides resilience and an ability to export substrates of multidrug efflux. J Antimicrob Chemother **69:**982–987. http://dx.doi.org/10.1093/jac/dkt481.
38. **Murphy S, Fitzgerald M, Mulcahy R, Keane C, Coakley D, Scott T.** 1997. Studies on haemagglutination and serum resistance status of strains of *Moraxella catarrhalis* isolated from the elderly. Gerontology **43:**277–282. http://dx.doi.org/10.1159/000213863.
39. **Khan MA, Northwood JB, Levy F, Verhaegh SJ, Farrell DJ, Van Belkum A, Hays JP.** 2010. *bro* β-lactamase and antibiotic resistances in a global cross-sectional study of *Moraxella catarrhalis* from children and adults. J Antimicrob Chemother **65:**91–97. http://dx.doi.org/10.1093/jac/dkp401.
40. **Nikaido H, Takatsuka Y.** 2009. Mechanisms of RND multidrug efflux pumps. Biochim Biophys Acta **1794:**769–781. http://dx.doi.org/10.1016/j.bbapap.2008.10.004.
41. **Hocquet D, Vogne C, El Garch F, Vejux A, Gotoh N, Lee A, Lomovskaya O, Plesiat P.** 2003. MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother **47:**1371–1375. http://dx.doi.org/10.1128/AAC.47.4.1371-1375.2003.
42. **El Garch F, Lismond A, Piddock LJ, Courvalin P, Tulkens PM, Van Bambeke F.** 2010. Fluoroquinolones induce the expression of *patA* and *patB*, which encode ABC efflux pumps in *Streptococcus pneumoniae*. J Antimicrob Chemother **65:**2076–2082. http://dx.doi.org/10.1093/jac/dkq287.
43. **Laing E, Mersinias V, Smith CP, Hubbard SJ.** 2006. Analysis of gene expression in operons of *Streptomyces coelicolor*. Genome Biol **7:**R46. http://dx.doi.org/10.1186/gb-2006-7-6-r46.
44. **de Hoon MJ, Makita Y, Nakai K, Miyano S.** 2005. Prediction of transcriptional terminators in *Bacillus subtilis* and related species. PLoS Comput Biol **1:**e25. http://dx.doi.org/10.1371/journal.pcbi.0010025.
45. **Weber MH, Marahiel MA.** 2003. Bacterial cold shock responses. Sci Prog **86:**9–75. http://dx.doi.org/10.3184/003685003783238707.
46. **Xu H, Lee HY, Ahn J.** 2008. Cross-protective effect of acid-adapted *Salmonella enterica* on resistance to lethal acid and cold stress conditions. Lett Appl Microbiol **47:**290–297. http://dx.doi.org/10.1111/j.1472-765X.2008.02429.x.
47. **Gunasekera TS, Csonka LN, Paliy O.** 2008. Genome-wide transcriptional responses of *Escherichia coli* K-12 to continuous osmotic and heat stresses. J Bacteriol **190:**3712–3720. http://dx.doi.org/10.1128/JB.01990-07.
48. **Kristo A, Uhari M, Kontiokari T, Glumoff V, Kaijalainen T, Leinonen M, Luotonen J, Koivunen P, Kujala T, Pokka T, Alho OP.** 2006. Nasal middle meatal specimen bacteriology as a predictor of the course of acute respiratory infection in children. Pediatr Infect Dis J **25:**108–112. http://dx.doi.org/10.1097/01.inf.0000201048.65828.b5.
49. **Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, Morris PS, Leach AJ.** 2006. Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord **6:**10. http://dx.doi.org/10.1186/1472-6815-6-10.
50. **Ruiz C, Levy SB.** 2014. Regulation of *acrAB* expression by cellular metabolites in *Escherichia coli*. J Antimicrob Chemother **69:**390–399. http://dx.doi.org/10.1093/jac/dkt352.