Use of the Chinchilla Model for Nasopharyngeal Colonization To Study Gene Expression by *Moraxella catarrhalis*

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Young adult chinchillas were atraumatically inoculated with *Moraxella catarrhalis* via the nasal route. Detailed histopathologic examination of nasopharyngeal tissues isolated from these *M. catarrhalis*-infected animals revealed the presence of significant inflammation within the epithelium. Absence of similar histopathologic findings in sham-inoculated animals confirmed that *M. catarrhalis* was exposed to significant host-derived factors in this environment. Twenty-four hours after inoculation, viable *M. catarrhalis* organisms were recovered from the nasal cavity and nasopharynx of the animals in numbers sufficient for DNA microarray analysis. More than 100 *M. catarrhalis* genes were upregulated *in vivo*, including open reading frames (ORFs) encoding proteins that are involved in a truncated denitrification pathway or in the oxidative stress response, as well as several putative transcriptional regulators. Additionally, 200 *M. catarrhalis* genes were found to be downregulated when this bacterium was introduced into the nasopharynx. These downregulated genes included ORFs encoding several well-characterized *M. catarrhalis* surface proteins including Hag, McaP, and MchA1. Real-time reverse transcriptase PCR (RT-PCR) was utilized as a stringent control to validate the results of *in vivo* gene expression patterns as measured by DNA microarray analysis. Inactivation of one of the genes (MC ORF 1550) that was upregulated *in vivo* resulted in a decrease in the ability of *M. catarrhalis* to survive in the chinchilla nasopharynx over a 3-day period. This is the first evaluation of global transcriptome expression by *M. catarrhalis* cells *in vivo*.

Moraxella catarrhalis is a Gram-negative mucosal pathogen that has attracted increased interest within the scientific and medical communities for its role in several clinically significant human infections. The bacterium is a cause of upper respiratory tract infections including sinusitis and otitis media in healthy children (10, 17, 62). More recently, *M. catarrhalis* has been shown to be involved in conjunctivitis in children (9) and in acute exacerbations of chronic sinusitis in adults (11). Additionally, in adults, it is an important etiologic agent of exacerbations of chronic obstructive pulmonary disease (COPD) (54, 55, 62). It has been estimated that *M. catarrhalis* is responsible for up to 10% of exacerbations of COPD in the United States, a finding which translates into as many as 4 million infections per year (43).

For *M. catarrhalis* to cause clinical disease, it typically must spread from its initial site of colonization in the nasopharynx into either the middle ear or the lower respiratory tract. It is believed that biofilm formation is an important event involved in colonization of the nasopharynx, and a recent study demonstrated that *M. catarrhalis* was present in a biofilm in the middle ear of children with chronic otitis media (25). It is likely that *M. catarrhalis* exists in a biofilm together with other normal flora in the nasopharynx. Until relatively recently, no studies had been performed in an *in vivo* environment to identify and better characterize the bacterial factors involved with colonization of the nasopharynx by *M. catarrhalis*. However, utilizing a chinchilla model, Luke et al. (36) demonstrated that type IV pili are important for colonization by *M. catarrhalis* in this animal model.

Previous studies have examined the human antibody response to known surface proteins of *M. catarrhalis* as a surrogate for identification of bacterial genes expressed *in vivo* (for a representative example, see reference 42), and one study was able to detect

mRNA from a small number of selected *M. catarrhalis* genes in nasopharyngeal secretions from young children with acute respiratory tract illness (39). The demonstration that the chinchilla nasopharynx can be colonized by *M. catarrhalis* (5, 36), together with the development of *M. catarrhalis* DNA microarrays (19, 65), presented the opportunity for utilizing this animal model for identification of bacterial genes expressed *in vivo*. There is ample evidence that bacterial gene expression profiles can be altered by growth in the *in vivo* environment, including studies of *Streptococcus pyogenes* in soft tissue (22), *Helicobacter pylori* in the stomachs of gerbils (53), nontypeable *Haemophilus influenzae* in the middle ear of chinchillas (38), *Yersinia pestis* in murine lungs (34), and uropathogenic *Escherichia coli* in the murine urinary tract (24).

In this study, we utilized DNA microarray technology and the chinchilla model to study the bacterial gene expression patterns of *M. catarrhalis* introduced into an *in vivo* environment. Detailed histopathologic analysis demonstrated that the chinchilla is capable of producing a vigorous mucosal inflammatory response to the presence of this bacterium. *M. catarrhalis* genes that were mark-

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TABLE 1 Bacterial strains used in this study

<i>M. catarrhalis</i> strain	Genotype or description	Reference or source
O35E	Wild-type disease isolate	61
O35E.118	O35E variant expressing a high level of UspA1 protein	34
O35E.118.rpsL	Spontaneous streptomycin-resistant mutant of O35E.118	This study
O35EΔ <i>mapA</i>	O35E with a spectinomycin resistance cartridge inserted into the <i>mapA</i> gene	30
O35E::spec	O35E with a spectinomycin resistance cartridge inserted into the intergenic region between <i>galE</i> and <i>glmS</i>	This study
O35EΔ1550	O35E with a kanamycin resistance cartridge inserted into a deletion in MC ORF 1550	This study
ATCC 43617	Wild-type disease isolate	American Type Culture Collection

edly upregulated (i.e., at least 4-fold) *in vivo* included open reading frames (ORFs) encoding proteins involved in a truncated denitrification pathway (66), in resistance to oxidative stress (28), and several putative transcriptional regulators. Inactivation of one of these upregulated genes caused a decrease in the ability of *M. catarrhalis* to persist in the chinchilla nasopharynx. Among those genes downregulated *in vivo* were several encoding previously studied major surface proteins of *M. catarrhalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type *M. catarrhalis* strain O35E and its derivatives that were used in this study are listed in Table 1. The wild-type *M. catarrhalis* strain ATCC 43617 (65) has been described. Brain heart infusion (BHI) (Difco/Becton Dickinson, Sparks, MD) was utilized as the base medium in this study, and broth cultures were incubated at 37°C with aeration. BHI medium was supplemented with vancomycin (V) (10 μg/ml), trimethoprim lactate (T) (5 μg/ml), dihydrostreptomycin sulfate (S) (100 μg/ml or 750 μg/ml), spectinomycin (15 μg/ml), kanamycin (15 μg/ml), or carbenicillin (5 μg/ml) when appropriate. All BHI agar plates were incubated at 37°C in an atmosphere containing 95% air and 5% CO₂.

Generation of a spontaneous streptomycin-resistant O35E mutant. *M. catarrhalis* O35E.118 expresses a maximal level of the UspA1 adhesin, typical of wild-type strains, due to the presence of 10 G residues in the poly-G tract located in the 5' untranslated region (5'-UTR) of the *uspA1* gene (33). *M. catarrhalis* strain O35E.118 was grown in 10 ml BHI broth to a density of 1×10^9 to 3×10^9 CFU/ml, which corresponds to an optical density at 600 nm (OD₆₀₀) of ~0.9. The cells were harvested by centrifugation and suspended in 1 ml of BHI broth, and 150-μl aliquots were spread on BHI agar plates containing streptomycin (750 μg/ml). After a 24-h incubation, one of the streptomycin-resistant colonies was picked and passaged twice on BHI agar containing streptomycin (100 μg/ml). Chromosomal DNA from this spontaneous streptomycin-resistant mutant was isolated with the Easy-DNA kit (Invitrogen, Carlsbad, CA). Nucleotide sequence analysis of this mutant's *rpsL* gene detected a single nucleotide change that caused a single amino acid change (K79R). This spontaneous streptomycin-resistant mutant was designated O35E.118.rpsL.

Preparation of outer membrane vesicles and SDS-PAGE. Outer membrane vesicles of *M. catarrhalis* strains O35E.118 and O35E.118.rpsL were prepared from agar plate-grown bacteria essentially as described previously (44), with the exception that the final, high-speed centrifugation step was performed for 2 h. SDS-PAGE and Coomassie blue staining were performed using standard methods.

Inoculation of the chinchilla nasopharynx with *M. catarrhalis*. This method is adapted from that described by Bakaletz et al. (5). In the present study, the term "nasopharynx" will be used to include both the nasal cavity and anterior portion of the chinchilla nasopharynx as defined by Jurcisek et al. (32). Healthy male adult (18- to 24-month-old) chinchillas (*Chinchilla lanigera*) (Ryerson Chinchilla Ranch, Plymouth, OH) (average mass of 550 g to 750 g) were anesthetized by intramuscular injection of ketamine (10 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA) and

xylazine (2 mg/kg) (Lloyd Laboratories, Shenandoah, IA). Once anesthetized, the animal was placed in the sternal recumbent position. *M. catarrhalis* O35E.118.rpsL was grown in BHI broth to late logarithmic phase (OD₆₀₀, ~0.9) and harvested by centrifugation at $3,800 \times g$ at 4°C for 5 min. The pellet was subsequently resuspended in phosphate-buffered saline (PBS) containing 0.15% gelatin (PBS-G) (which helps sustain bacterial viability *in vitro*). This bacterial suspension was serially diluted and plated on BHI agar containing vancomycin, trimethoprim, and streptomycin (VTS) to determine the number of CFU introduced into the nasopharynx. To complete the inoculation procedure, a 1-cc syringe with a 25-gauge needle attached was used to administer 150 μl of the bacterial suspension in a dropwise manner into the anesthetized chinchilla's nares. The animal subsequently inhaled these droplets into its nasal passageways. Approximately 75 μl of the bacterial suspension was administered to each naris. Utilizing this method, approximately 5×10^8 CFU of *M. catarrhalis* were inoculated into the nares of each animal.

Recovery of viable *M. catarrhalis* from the chinchilla nasopharynx.

Depending on the experimental objective, viable *M. catarrhalis* organisms were collected by either nasopharyngeal lavage or homogenization of the nasal and ethmoid tissues. With the nasopharyngeal lavage technique, sterile PBS-G was instilled into one naris of the anesthetized chinchilla in 10- to 15-μl portions. A blunt-ended 1-cc syringe (with the needle removed) was then inserted into the contralateral naris, and aspiration of the nasal passageway was performed. This procedure was performed on both nares in an alternating fashion until a total of 500 μl of sterile PBS-G had been instilled into the animal's nose. The collected fluid was then serially diluted in BHI broth and plated on BHI-VTS agar.

The turbinate homogenization method required euthanasia of the anesthetized animal. Once adequate anesthesia was achieved, the animal was placed in the supine position, and a subxyphoid approach was utilized to puncture the heart with a 18-gauge needle attached to a 12-cc syringe. Intracardiac aspiration was performed to remove approximately 10 cc of blood from the animal. The syringe was exchanged for a 1-cc syringe preloaded with 1.0 ml of Euthasol (Virbac Animal Health, Fort Worth, TX). Subsequently, intracardiac injection of the Euthasol solution was performed and the animal was monitored for cessation of spontaneous respiratory and cardiac activity. Once the chinchilla was determined to be deceased, decapitation was performed between the second and the third cervical vertebrae, and the head was bisected along the nasal septum, thus exposing the interior structures of the nasal and nasopharyngeal passageways. Once accessible, the turbinates (nasal and ethmoid) (32) were removed with standard dissection techniques and placed in 2.0 ml of sterile PBS-G. The tissues were subsequently homogenized on ice with a Sorvall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, CT) at 5,000 rpm for 30 s. Portions of the homogenized suspension were serially diluted in BHI broth and plated on BHI-VTS agar.

Histopathologic analysis of chinchilla tissue. Chinchillas were inoculated with either *M. catarrhalis* strain O35E.118.rpsL suspended in PBS-G or with PBS-G (as a sham control) via the intranasal method described above. Twenty-four hours later, the animals were anesthetized and then euthanized as described above. After decapitation, the eyes, mandible, and accessory muscles of mastication were removed. Dissec-

tion techniques were employed to excise the additional soft tissues (i.e., skin, muscle, fat, and collagenous material) overlying the bones of each skull. After preparation, the skulls were immediately immersed in formalin and placed on a shaker at room temperature for 24 h. The skulls were transferred to Cal-Rite (Richard-Allen Scientific, Kalamazoo, MI) and returned to the shaker at room temperature until decalcification was complete. The heads were sectioned in the coronal plane, and samples were processed and embedded in paraffin by routine methods. Five-micrometer sections through the region just caudal to the previously described level II in the chinchilla skull (32) were stained with hematoxylin and eosin.

DNA:DNA hybridization. Genomic DNA was prepared from *M. catarrhalis* ATCC 43617 and O35E Δ mapA with the Easy-DNA isolation kit. Each DNA sample was sheared by passing it through a 30-gauge needle 65 times, and a 4- μ g portion of each sheared DNA sample was added to a PCR tube containing 3 μ g of *M. catarrhalis* genome-directed primers (65) in 20 μ l of buffer 2 (New England BioLabs, Ipswich, MA). This mixture was heated to 97°C for 3 min and 50 s and then immediately plunged into an ice water bath for 3 min. A 3- μ l portion of Cy3-dCTP (1 mM) (GE Healthcare, Pittsburgh, PA) was added to 5 μ l of a deoxynucleoside triphosphate (dNTP) mix containing 2 mM (each) dATP, dTTP, and dGTP combined with 1 mM dCTP. The final reaction volume was brought to 49 μ l with water and allowed to incubate at room temperature for 2 min. Klenow enzyme (1 μ l) (NEB) was added and incubated at room temperature for 5 min. Each reaction tube was placed at 37°C for 2.5 h, and then an additional 1 μ l of Klenow enzyme was added before undergoing a 2-h incubation at 37°C. Each of the Cy3-labeled DNA samples was cleaned as described below and hybridized to individual DNA microarray slides as previously described (65). The Cy3 labeling and hybridization was performed on two independent DNA samples. After hybridization, the number of hybridized oligonucleotide spots obtained with the Cy3-labeled ATCC 43617 DNA was compared to that obtained with the Cy3-labeled O35E Δ mapA DNA.

RNA isolation from chinchilla tissue for DNA microarray analysis. Total RNA intended for subsequent DNA microarray analysis was extracted from chinchilla tissue in the following manner. At 24 h postinfection, animals that had been intranasally inoculated with O35E.118.rpsL (10^9 CFU) were euthanized and decapitated. The nasal and ethmoid turbinates from each animal together with their overlying mucosa were removed via standard dissection techniques and placed immediately in 10 ml of ice-cold 50 mM sodium azide (7, 8, 60) and kept on ice. A 10-ml portion of 2% (wt/vol) saponin (23) in TE buffer was added, and the samples were vigorously vortexed for 20 s (5 cycles) and placed at 37°C for 10 min to lyse the superficial epithelial cell layer and release adherent bacteria. After incubation, the tissue suspension was centrifuged at $200 \times g$ for 1 min to remove the large tissue fragments. The supernatant was transferred to a clean, RNase-free 50-ml conical tube and centrifuged at $3,100 \times g$ for 5 min. The resultant pellet was processed for total RNA with the Qiagen RNeasy Midi kit (Qiagen, Valencia, CA).

Four independent sets of animal samples (3 or 4 animals per set) together with simultaneously broth-grown *M. catarrhalis* cells underwent RNA extraction for use in DNA microarray analysis. The Qiagen RNeasy Midi kit was used to isolate total RNA from mid-logarithmic-phase, broth-grown *M. catarrhalis* O35E.118.rpsL cells. Bacteria were grown to a density of 10^9 CFU/ml in 10 ml of BHI broth, and then 5 ml of 50 mM sodium azide was added. These bacterial suspensions in azide were held on ice for the same length of time as the chinchilla tissue samples (described above). Next, 15 ml of 2% (wt/vol) saponin in TE buffer was added, and the suspension was vortexed vigorously for 20 s (5 cycles). This suspension was placed at 37°C for 10 min and then subjected to centrifugation at $3,100 \times g$ for 5 min. The resultant cell pellet was processed for total RNA with the Qiagen RNeasy Midi kit.

Both the *in vivo*-derived and *in vitro*-derived RNA preparations were subjected to DNase I treatment with the Message Clean kit (GenHunter Corp., Nashville, TN) per manufacturer's instructions. Quantitative mea-

surements of total RNA were performed, and the RNA was subsequently stored at -80°C until further use. PCR amplification with ExTaq (Takara Bio Inc., Otsu, Shiga, Japan) was utilized to test freshly isolated RNA preparations for *M. catarrhalis* DNA contamination. Standard controls were performed, using *M. catarrhalis* O35E.118.rpsL chromosomal DNA as a positive control and water only as a negative control.

DNA microarray analysis. Twenty micrograms of total RNA from either the broth-grown bacterial cells or the homogenized chinchilla nasopharyngeal tissues was mixed with 3 μ g of *M. catarrhalis* genome-directed primers (65) and dried by using a Speed-Vac (Savant Instruments, Inc., Farmingdale, NY). The DNA microarrays used in this study were derived from the genome of *M. catarrhalis* ATCC 43617 (65) and were previously used for DNA microarray analysis of both the NsrR regulon (66) and the oxidative stress response (28) of *M. catarrhalis* strain O35E. cDNA preparation, labeling of the cDNAs, hybridization, and statistical analyses were performed as described previously (28). Two "dye swap" experiments were performed.

The RNA isolated from the *in vitro* broth-grown O35E.118.rpsL cells was utilized to determine baseline gene expression. To identify those genes that exhibited an altered expression profile in the chinchilla nasopharynx relative to the *in vitro* broth-grown cells, a threshold of 2-fold-increased or -decreased expression over the *in vitro* baseline was utilized. Two additional constraints were applied to the data: (i) demonstration by the gene of interest of the altered expression profile in at least four of the five DNA microarrays and (ii) a *P* value of <0.05 as calculated by the one-sample *t* test.

RNA extraction for use in real-time reverse transcriptase PCR (RT-PCR). After euthanasia, the nasopharyngeal tissue and nasal turbinates were removed, immediately placed into room temperature RNAlater (Ambion, Austin, TX), and then maintained on ice. Upon completion of the tissue dissection, saponin in PBS-G was added to a final concentration of 1% (wt/vol). Each sample was then vortexed briefly for 15 s and then placed at 37°C for 10 min. After incubation, the samples were vortexed again for 15 s and then subjected to centrifugation for 5 min at $50 \times g$ at 4°C to pellet large pieces of tissue. The supernatant fluid was removed and subjected to centrifugation at $3,220 \times g$ for 15 min at 4°C to pellet bacteria. This new supernatant fluid was discarded, and the pellet was resuspended in 2 ml of RNA Wiz (Ambion, Austin, TX). RNA extraction was then performed as per the manufacturer's instructions with the RiboPure bacteria kit (Ambion, Austin, TX). Bacterial control samples grown in BHI broth underwent identical RNAlater-based preservation and saponin-based extraction protocols. All RNA samples subsequently underwent DNase I treatment as described above. The Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) was utilized to measure the RNA integrity of these samples.

Real-time RT-PCR. At least two independent sets of broth-grown *M. catarrhalis* cells and simultaneously prepared chinchilla-derived samples were used to obtain RNA for real-time RT-PCR analysis. Oligonucleotide primer pairs (Table 2) were designed for use in real-time RT-PCR with either PrimerExpress software (Applied Biosystems, Foster City, CA) or Primer 3 (51). Each 25- μ l real-time RT-PCR mixture consisted of 0.5 μ l of each of two oligonucleotide primers (from 2.5 μ M stock solutions), 12.5 μ l of SYBR green PCR master mix (Applied Biosystems), 5 μ l of total RNA (5 ng/ μ l), 0.125 μ l of Multiscribe reverse transcriptase (Applied Biosystems), and 6.375 μ l of distilled water (dH₂O). One-step relative real-time RT-PCR was performed on these samples utilizing the 7500 Fast real-time PCR system (Applied Biosystems). All of the relative real-time RT-PCR experiments involved the use of at least two independently isolated RNA preparations from two different groups of chinchillas (3 or 4 animals per group). The results were analyzed ($\Delta\Delta C_T$) with Relative Quantification Study software (Applied Biosystems). To test for DNA contamination of the RNA samples, standard PCR was performed with ExTaq and oligonucleotide primers specific for the *M. catarrhalis* *uspA1* ORF.

Real-time RT-PCR analysis of the relative transcript levels for selected

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5'–3')
ORF573FW	ATAACCGAGCCGTACCAATG
ORF573RV	CATTTTGGCAGAGCATTTCA
ORF516FW	ACCCAGATTTGACACTGCCAG
ORF516RV	AAGGTCGTGCCTGAACCGT
ORF323FW	TTAGAAAACGCAAGGCGTGAG
ORF323RV	CCCCATGATTGCCTGCTTT
ORF165FW	GCACACAGCCACCAAATC
ORF165RV	GTCGGAGAATGGTCGGA
ORF1411FW	CGACATATCAGCTATGTGCCCTC
ORF1411RV	TGGCCTGTCTGAGCAAATC
ORF1413FW	ACGCCACTGCGTGAAGAG
ORF1413RV	TGCGCTGATTAAGTCATATGC
ORF1573FW	AGAGGTGTTCCTGAGGTGAA
ORF1573RV	CTGATGCTGAGGCAATGACTG
ORF222FW	AGAAGCTTTGTGAAAAGCGG
ORF222RV	TTTCGTTGCTGGACACGGT
ORF734FW	TTCGCCCAAAACGATACTTG
ORF734RV	CGGCAGTCTGAATGGCTGATT
ORF990FW	GCACAAACTGTCGGTGAGGAA
ORF990RV	TGCCATTCTGCACCATAGCCT
ORF184FW	CGTCAAAACCAACCAACAACG
ORF184RV	ATCAACGCCAGCATCAGCA
ORF903FW	TGCCCTTATTGATTTGGCTGT
ORF903RV	GGCGTTTGGCAATGTCTGA
ORF1550FW	GCAGGCTTGTCTCGCATTTG
ORF1550RV	CCTTCATTGCTTCTACTTGCTCT
ORF681FW	GCCAAAAAGCAGCACGGTA
ORF681RV	GTTCTTGTGAGGCAGCATTATCTG
ORF763FW	TTACAGGCATGATGGGCGT
ORF763RV	ACTGTTTCGGCAGGCGATT
ORF1131FW	CCAAGTGTGTGACAGAACCAGTT
ORF1131RV	CGGGCAGTTGGTGTTTTGAC
ORF1031FW	GTTGCTTATGATGAGATTAGC
ORF1031RV	GACAGTACGATAGCCATTGGT
ORF622FW	AAGATGAACCTAAACGCTACTTGCG
ORF622RV	AGAAATGGTCAAGCAGGACAGG
ORF1300FW	AAAACGCCATCGCAGCA
ORF1300RV	GTTTGAACTGCAAGGTCAGC
ORF1686FW	GTGCGTTTCATGATTCAATTA
ORF1686RV	ATCCACACGCCAGTCAA
ORF1405FW	TACCCCATTTTATGCCAAGG
ORF1405RV	CCTGCTAGGCTGGTTTGTGTC
ORF1432FW	GCTAAGCGTAACCGTGGAATAAA
ORF1432RV	ATGCACGCTCAACGCCA
ORF275FW	AGGCGGTTAGTAATCCAGCAG
ORF275RV	TGGTTTGCATGCTAATGATGATA
ORF987FW	CCTATTGTGGGCGATGCT
ORF987RV	GCGGTGCTGTTCACAAAGTCA
ORF897FW	ACAAGTGGGCGTGGATAAAGA
ORF897RV	AGTTGGTCTGACCTGCGGC
ORF1367FW	AGCAGGTGACACAATCATTC
ORF1367RV	AACTGTTTCTACTTTACCATCCTTG
ORF1045FW	TGTCGATGCTCAAGCAACT
ORF1045RV	GCCTTCTTTGATGCCTGAAC
Pr930	TATTGCCAAAATCCGTTG
Pr947	AAAACGCATCTTGACGCTG
P1	AGCTTTGAGCATGTGCCTC
P2	AATCATGCAAAATGATACGCCCA
P3	TTATTCTCTAGTTAGTACCCGGGTAGCTTCAATGT CATACAATCGTAC
P4	TGTTTTAGTACCTGGAGGGAATAGTACGCCGTGCTCGT
PFkn3	CGGGTGACTAACTAGGAGGAATAA
PR2kn3	TATTCCTCCAGGTAATAAAACA
1550-F	AAGAAGAGCATGCCACATTG
1550-R	CCCGAATCATTAAATACGGCG
Hyp-F	CGCATCAGTCAAAACATCCAA
Hyp-R	TGGATTAAATCAAAACCCAGCTT
1549-F	ATGATGCCTCTTTGGCAACT
1549-R	TGGTAATTTTGGCGTGTGTG
1550 FRT	GAAACCATTCAGCGTGAAGAGCT
HYP RRT	GGATTAAATCAAAACCCAGCTTTTTCAGTATTGA
HYP FRT	CAGGGCAATGCTCTGGC
1549 RRT	CAAGATGTTCTTCAAGGTGATGGG

genes in the wild-type O35E::spec surrogate strain and the O35EΔ1550 mutant was accomplished using RNA prepared from broth-grown cells. Primer pairs were 1550-F and 1550-R for MC ORF 1550; Hyp-F and Hyp-R for the small, hypothetical ORF located between MC ORF 1550 and MC ORF 1549; and 1549-F and 1549-R for MC ORF 1549.

RT-PCR-based analysis of transcriptional linkage. The ImProm-II reverse transcription system (Promega, Madison, WI) was used to synthesize cDNA from 1 μg RNA obtained from broth-grown O35E::spec cells. Controls containing no reverse transcriptase (NRT) were included with the reactions. A 1-μl portion of cDNA or NRT control or 40 ng *M. catarrhalis* O35E genomic DNA was added to a PCR mix containing 5× HF buffer (Bio-Rad Laboratories, Hercules, CA), 0.2 mM dNTPs, 200 nM forward and reverse primers, and 0.25 U iProof DNA polymerase (Bio-Rad Laboratories) for a total volume of 25 μl. The PCR was performed as per the manufacturer's protocol, using an annealing temperature of 57°C and an extension time of 20 s. Primers 1550 FRT and HYP RRT were used for the intergenic region between MC ORF 1550 and the small, hypothetical ORF. Primers HYP FRT and 1549 RRT were used for the intergenic region between the small, hypothetical ORF and MC ORF 1549. The expected length of the PCR product for primers 1550 FRT and HYP RRT was 248 bp, and that for primers HYP FRT and 1549 RRT was 280 bp.

Construction of a spectinomycin-resistant *M. catarrhalis* O35E strain as a wild-type surrogate for competitive index experiments. The *M. catarrhalis* *galE* and *glmS* genes encode a UDP-glucose 4-epimerase and a glucosamine-fructose-6-phosphate aminotransferase, respectively. These genes and their intergenic region have 98% nucleotide identity in strains 7169 and O35E (Nicole Luke, personal communication). Chromosomal DNA from *M. catarrhalis* strain 7169 containing a spectinomycin resistance (*spec*) cassette inserted into the *galE*-*glmS* intergenic region was used as the template for a PCR with nucleotide primers Pr947 and Pr930 (Table 2) and iProof High Fidelity DNA polymerase. The resultant 2.7-kb amplicon containing the *spec* cartridge and flanking chromosomal DNA was gel purified and used to transform wild-type O35E. A spectinomycin-resistant transformant, designated the O35E::spec strain, was passaged twice and then subjected to PCR and nucleotide sequence analysis to confirm the insertion of the *spec* cartridge between *galE* and *glmS*.

Construction of an MC ORF 1550 deletion mutant. The majority of MC ORF 1550 (66) was deleted in *M. catarrhalis* O35E and replaced with the kanamycin resistance cartridge from pUC18K (40). Primers P1 and P3 (Table 2) were used to amplify a 0.8-kb fragment containing the extreme 5' end of the O35E MC ORF 1550 gene and its upstream flanking DNA, whereas primers P2 and P4 were used to obtain a 0.9-kb fragment containing the extreme 3' end of the O35E MC ORF 1550 gene and its downstream flanking DNA. Additionally, primers PFkn3 and PR2kn3 were used to amplify the Kan cartridge from pUC18K (40). These three amplicons were mixed in equal amounts in one reaction tube and used as the templates for overlapping extension PCR (30) with iProof High Fidelity DNA polymerase (Bio-Rad Laboratories, Hercules, CA) and primers P1 and P2. The resultant 2.5-kb amplicon was used to transform wild-type O35E. A kanamycin-resistant transformant was passaged twice and then subjected to PCR and nucleotide sequence analysis to confirm the successful deletion of the majority of MC ORF 1550 and the presence of the Kan cartridge. This mutant was designated as O35EΔ1550.

Competitive index experiments. *M. catarrhalis* O35E::spec and O35EΔ1550 strains were grown separately in BHI broth to an OD₆₀₀ of 0.900. Equal volumes (5 ml) of each culture were mixed, harvested by centrifugation, and suspended in 10 ml of PBS-G. A 150-μl portion of this bacterial suspension was utilized to atraumatically inoculate the nares of an anesthetized chinchilla as described above. The total inoculum that was utilized for each competitive index experiment ranged from 1.92×10^8 CFU/animal to 5.02×10^8 CFU/animal. Seventy-two hours after inoculation, the animals were euthanized and the nasopharyngeal tissues were extracted and homogenized as described above. These homogenized samples were plated in triplicate on BHI agar plates containing either vanco-

mycin, trimethoprim, and spectinomycin or vancomycin, trimethoprim, and kanamycin to allow the differential enumeration of each surviving strain. Further confirmation that the recovered organisms were *M. catarrhalis* was accomplished by screening colonies for their resistance to carbenicillin; *M. catarrhalis* O35E is resistant to this antimicrobial (E. Lafontaine, personal communication). A competitive index was calculated and was defined as follows: [output CFU O35E::spec/output CFU O35EΔ1550: input CFU O35E::spec/input CFU O35EΔ1550]. The competitive index experiments were comprised of five separate experiments involving a total of 14 animals. Statistical analysis was performed by means of a sign test (67).

Microarray data accession number. The raw data from these DNA microarray experiments were deposited at the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE13559.

RESULTS

Measurement of *M. catarrhalis* survival in the chinchilla nasopharynx. *M. catarrhalis* expresses a number of phase-variable surface proteins, including the UspA1 protein, which is a well-studied adhesin (2, 12, 16, 27). To ensure maximal (wild-type) expression of UspA1, we elected to use the O35E.118 strain, which was shown to express UspA1 at wild-type levels (33). In addition, we selected a spontaneous streptomycin-resistant (*rpsL*) mutant of *M. catarrhalis* O35E.118 for use in these challenge experiments to enhance the ability to recover viable *M. catarrhalis* in the presence of the normal flora of the chinchilla nasopharynx and accurately enumerate the number of viable *M. catarrhalis*. This *rpsL* mutant had *in vitro* growth characteristics in broth (Fig. 1A) and an outer membrane protein profile (Fig. 1B) that were similar if not identical to those of the O35E.118 parent strain.

After inoculation of 5×10^8 to 9×10^8 CFU of *M. catarrhalis* strain O35E.118.*rpsL* per animal, recovery was performed 24 and 72 h later on separate animals. Viable *M. catarrhalis* organisms were recovered from the nasopharynx of the chinchillas by either nasopharyngeal lavage or by homogenization of the nasal and ethmoid turbinates. A mean of approximately 10^8 CFU of *M. catarrhalis* per ml of lavage fluid was recovered from the nasopharynx after 24 h, and a mean of approximately 10^5 CFU/ml was recovered at 72 h (data not shown). Utilizing the tissue homogenization method, a mean of approximately 10^7 viable *M. catarrhalis* cells were recovered at the 24-h time point, with a mean of approximately 10^4 viable *M. catarrhalis* cells being recovered from each animal at the 72-h time point (data not shown). Beyond the 72-h time point, the number of viable *M. catarrhalis* cells recovered from the nasopharynx declined significantly, and no viable *M. catarrhalis* organisms could be isolated by 168 h after inoculation (data not shown).

Histopathologic analysis of the chinchilla nasal cavity after inoculation with *M. catarrhalis*. Acute suppurative rhinitis developed in the chinchillas 24 h after inoculation with *M. catarrhalis*. Edema was present, and neutrophils were evident beneath the epithelium and were seen migrating between the epithelial cells (Fig. 2D). These neutrophils also accumulated on the mucosal surface to form a purulent exudate (Fig. 2B and D). There was multifocal necrosis of epithelial cells, but there was no ulceration. There were no lesions or exudate seen in the sham-inoculated animals (Fig. 2A and C).

DNA microarray analysis of *M. catarrhalis* genes affected by exposure to the chinchilla nasopharynx. The DNA microarrays used for this study were derived from the nucleotide sequence of

FIG 1 Phenotypic comparison of strains O35E.118 and O35E.118.*rpsL*. (A) Growth of O35E.118 (closed circles) and O35E.118.*rpsL* (open circles) in BHI broth. (B) Outer membrane protein profiles of O35E.118 (lane 1) and O35E.118.*rpsL* (lane 2) as determined by SDS-PAGE and Coomassie blue staining.

the genome of *M. catarrhalis* ATCC 43617, whose genome (1.91 Mbp) is slightly larger than that of O35E (1.78 Mbp) (18). A DNA:DNA hybridization study was performed to determine the extent to which these ATCC 43617-derived probes would bind O35E-derived cDNAs. For this purpose, the O35EΔ*mapA* mutant (29) was used as a representative O35E-derived strain because this strain was a potential candidate for use in competitive index experiments in the chinchilla model. This O35EΔ*mapA* mutant differs from the O35E.118.*rpsL* strain only by the presence of a spectinomycin resistance cartridge inserted into its *mapA* gene (29) and the absence of the single nucleotide change in the *rpsL* gene. When probed in DNA:DNA hybridization experiments with chromosomal DNA fragments from the homologous ATCC 43617 strain, a hybridization frequency of 99.1% was obtained. When O35EΔ*mapA*-derived chromosomal DNA fragments were used, a hybridization rate of 92.5% was obtained.

DNA microarray analysis of RNA samples extracted from either broth-grown *M. catarrhalis* cells or *M. catarrhalis*-infected nasopharyngeal tissues revealed several interesting findings. Three

FIG 2 Histopathologic analysis of the chinchilla response to intranasal inoculation with *M. catarrhalis* O35E.118.rpsL. (A and B) Histologic sections of the chinchilla nasal cavity in the sham-inoculated animal (A) and the *M. catarrhalis* strain O35E.118.rpsL-inoculated animal (B) 24 h following inoculation. These images depict the dorsal meatus from the region just caudal to the previously described level II in the chinchilla skull (32). Purulent exudate is evident within the nasal cavity overlying the intact epithelium (indicated by black arrows in B). (C and D) Higher magnification of the same mucosal regions in the sham-inoculated animal (C) and the *M. catarrhalis* O35E.118.rpsL-inoculated animal (D). Neutrophils are evident in migration between the epithelial cells in D (white arrow). Bars, 60 μ m.

hundred thirteen bacterial genes demonstrated at least a 2-fold change in expression in the chinchilla nasopharynx relative to the *in vitro* state. There were more than 100 genes whose expression profile was increased in the *in vivo* environment (see Table S1 in the supplemental material), whereas 200 genes showed decreased expression in the chinchilla nasopharynx (see Table S1). The three genes most upregulated in *M. catarrhalis* were those encoding a predicted glycosyl transferase, a predicted acriflavin resistance protein, and a protein predicted to be involved in phosphate uptake (Table 3). Table 3 lists the 50 most upregulated and the 50 most downregulated genes from the DNA microarray experiments.

At least five of the upregulated genes were predicted to encode regulatory proteins. MC ORF 903 (Table 3) encoded a protein most similar to a transcriptional regulator from the BadM/Rrf2 family of proteins identified in *Psychrobacter cryohalolentis* K5 (GenBank accession number YP_580916). This *M. catarrhalis* protein also has homology to an *Acinetobacter baumannii* protein involved with Fe-S cluster assembly (GenBank accession number YP_001084663). The protein encoded by MC ORF 1413 (Table 3) is described as a transcriptional regulator belonging to either the Fur (GenBank accession number YP_265314) or the Zur family (GenBank accession number YP_001202725). The other three upregulated *M. catarrhalis* genes included MC ORF 1895 (Table 3) encoding a protein with a cyclic AMP (cAMP)-binding domain (GenBank accession number ZP_01059129), MC ORF 1461 (Table 3) encoding a protein involved in sensory transduction (GenBank accession number YP_355528.1), and MC ORF 839

(see Table S1 in the supplemental material), encoding a protein with homology to a LuxR-family transcriptional regulator in *Psychrobacter* sp. PRwf-1 (GenBank accession number YP_001280324).

Review of the DNA microarray data also revealed that *M. catarrhalis* MC ORF 681 (Table 3) was upregulated. This ORF encodes the AniA nitrite reductase (66), which was one of the most highly upregulated genes in *M. catarrhalis* cells growing in a biofilm *in vitro* (65). The AniA protein (GenBank accession number ACJ68080) is a component of the truncated denitrification pathway in *M. catarrhalis* and has been shown to be coregulated with *norB* (66). While the *norB* expression levels in the chinchilla nasopharynx did not meet the statistical requirements for the DNA microarray analysis, subsequent real-time RT-PCR analysis demonstrated that both of these genes are significantly upregulated in the chinchilla nasopharynx (data not shown). Additionally, real-time RT-PCR analysis showed that two components of the *narGHJI* operon (i.e., *narH* [MC ORF 924] and *narJ* [MC ORF 925]) were also at least 16-fold upregulated in the chinchilla nasopharynx (data not shown). This operon encodes the nitrate reductase complex that forms the first component of the truncated denitrification pathway and was the most highly upregulated of all *M. catarrhalis* genes during biofilm growth *in vitro* (65).

DNA microarray analysis of *M. catarrhalis* transcripts from the *in vivo* environment also demonstrated upregulation of MC ORF 1411 (Table 3), which encodes a protein most similar to ZnuC, a component of the ATP-binding cassette (ABC) transporter involved with zinc uptake in *Psychrobacter cryohalolentis* K5 (GenBank accession

TABLE 3 Genes in *M. catarrhalis* O35E.118.rpsL whose expression was maximally affected by exposure to the chinchilla nasopharynx

ORF	Description ^d	Median log ₂ (chinchilla/broth ratio ^a)	SD
Genes whose expression was increased			
MCORF717	Glycosyl transferase, group 1	6.07	0.31
MCORF1903	Acriflavin resistance protein	5.81	0.69
MCORF516 ^b	Periplasmic phosphate binding protein	5.29	0.58
MCORF593	Protein of unknown function	5.23	0.67
MCORF944	Probable molybdopterin biosynthesis enzyme	4.70	0.33
MCORF521	ATPase-like:histidine kinase A-like	4.67	0.15
MCORF424	Lytic transglycosylase, catalytic	4.54	0.25
MCORF948	Hypothetical protein	4.27	0.69
MCORF481	Conserved hypothetical protein	4.00	0.51
MCORF1023	Hypothetical protein	4.00	0.33
MCORF323 ^b	TonB-like	3.86	0.10
MCORF1894	ATP-dependent DNA helicase	3.77	0.17
MCORF1082	Probable DNA/RNA nonspecific endonuclease	3.57	0.38
MCORF573 ^b	Hypothetical protein	3.56	0.06
MCORF1835	Hypothetical protein	3.50	0.17
MCORF1916	Periplasmic lipoprotein involved in iron transport	3.39	0.22
MCORF1703	Glycyl-tRNA synthetase alpha chain	3.29	0.12
MCORF1361	Lipoate synthase	3.26	0.23
MCORF288	Hypothetical protein	3.22	0.15
MCORF903 ^b	Transcriptional regulator, BadM/Rrf2 family	3.16	0.16
MCORF1411 ^b	Zinc import ATP-binding protein ZnuC	3.16	0.18
MCORF828	Hypothetical protein	3.07	0.12
MCORF928	Molybdopterin binding domain:MoeA	2.86	0.41
MCORF1192	ATPase AAA-2	2.75	0.25
MCORF1715	Acetoin reductase	2.74	0.10
MCORF1550 ^b	Conserved hypothetical protein	2.73	0.22
MCORF1413 ^b	Transcriptional regulator, probable Fur family	2.66	0.28
MCORF318	Hypothetical protein	2.63	0.52
MCORF54	Hypothetical protein	2.52	0.45
MCORF681 ^b	Nitrite reductase AniA	2.51	0.22
MCORF439	Glycosyltransferase	2.49	0.21
MCORF937	Molybdopterin binding domain	2.35	0.44
MCORF836	Hypothetical protein	2.34	0.48
MCORF977	Hypothetical protein	2.21	0.12
MCORF1895	cAMP-binding domain-containing regulatory protein	2.21	0.09
MCORF718	No annotation	2.17	0.24
MCORF335	Transcription antitermination protein NusG	2.14	0.12
MCORF860	Periplasmic protein	2.11	0.27
MCORF1849	Hypothetical protein	2.05	0.30
MCORF994	Conserved hypothetical protein	2.04	0.12
MCORF510	Thioredoxin	2.04	0.35
MCORF16	Succinyl-CoA:3-ketoacid-CoA transferase	1.99	0.32
MCORF635	Hypothetical protein	1.95	0.31
MCORF746	GTP-binding protein, HSR1-related	1.94	0.06
MCORF1785	Transglycosylase-associated protein	1.89	0.18
MCORF1461	Sensory transduction system, regulatory protein	1.89	0.18
MCORF763 ^b	Outer membrane lipoprotein carrier protein LolA	1.83	0.14
MCORF503	Possible thiamine monophosphate synthase	1.83	0.15
MCORF1696	Malate synthase	1.82	0.28
MCORF1123	Phage integrase, N-terminal SAM-like	1.81	0.19
Genes whose expression was decreased			
MCORF184 ^b	Probable tryptophan synthase, alpha chain	-3.41	0.28
MCORF975	MchA1	-3.40	0.27
MCORF1578	Acetyl-CoA carboxylase	-3.35	0.43
MCORF990 ^b	Oligopeptide permease	-3.01	0.20
MCORF1855	Hypothetical protein	-2.93	0.34
MCORF1509	UDP-glucose 4-epimerase	-2.82	0.30
MCORF1761	GTP-binding protein LepA	-2.80	0.43
MCORF419	Preprotein translocase, subunit YajC	-2.68	0.31

(Continued on following page)

TABLE 3 (Continued)

ORF	Description ^d	Median log ₂ (chinchilla/broth ratio ^a)	SD
MCORF1686 ^b	Protein of unknown function YGGT	-2.63	0.24
MCORF752	Transferrin binding protein A; TbpA	-2.62	0.17
MCORF154	3-oxoacyl-(acyl-carrier-protein) reductase	-2.57	0.48
MCORF1311	Conserved hypothetical protein	-2.56	0.08
MCORF425	Phosphoenolpyruvate carboxykinase	-2.46	0.32
MCORF1569	Fumarate hydratase	-2.40	0.07
MCORF1329	Restriction endonuclease S subunits	-2.39	0.35
MCORF1012	Carbon starvation protein A	-2.31	0.17
MCORF1213	Signal recognition particle protein	-2.28	0.18
MCORF1153	Threonyl-tRNA synthetase	-2.24	0.22
MCORF1405 ^b	Outer membrane protein J	-2.23	0.32
MCORF418	Protein export protein SecD	-2.20	0.17
MCORF997	Hypothetical protein	-2.18	0.15
MCORF255	Hypothetical protein	-2.11	0.09
MCORF1307	Surface antigen (D15)	-2.09	0.15
MCORF1432 ^b	Ribosomal protein L19	-2.08	0.24
MCORF338	Ribosomal protein L10	-2.04	0.23
MCORF1865	Succinate dehydrogenase/fumarate reductase	-2.02	0.17
MCORF433	ATP synthase gamma chain	-2.01	0.17
MCORF118	Glutamyl-tRNA(Gln) amidotransferase A subunit	-2.00	0.30
MCORF1246	AdeJ	-1.93	0.12
MCORF116	BRO-2	-1.93	0.13
MCORF275 ^b	McaP	-1.93	0.29
MCORF1418	ATP synthase F0, C subunit	-1.92	0.06
MCORF1245	Secretion protein HlyD	-1.91	0.27
MCORF1863	Succinate dehydrogenase, hydrophobic subunit	-1.89	0.16
MCORF38	Acetyl-CoA hydrolase	-1.89	0.19
MCORF1127	Phosphate acetyltransferase	-1.88	0.15
MCORF1261	Major outer membrane protein	-1.86	0.20
MCORF1873	Serine-rich adhesin for platelets precursor	-1.86	0.12
MCORF1338	Ribosomal protein L21	-1.85	0.25
MCORF1398	Aminobutyrate aminotransferase	-1.84	0.34
MCORF1419	ATP synthase Fo, B subunit	-1.82	0.16
MCORF261	FOG: TPR repeat, SEL1 subfamily	-1.82	0.19
MCORF132	Ribosomal protein S17	-1.81	0.09
MCORF987 ^b	Oligopeptidepermease (ATP-binding protein)	-1.80	0.28
MCORF57	Amino acid/peptide transporter	-1.77	0.12
MCORF1605	Cytidylate kinase	-1.77	0.17
MCORF126	Ribosomal L23 protein	-1.76	0.09
MCORF685	Phenylalanyl-tRNA synthetase, beta subunit	-1.75	0.10
MCORF882	Ubi/menaquinone biosynthesis methyltransferase	-1.73	0.44
MCORF1829	CRISPR-associated protein, Csy4 family	-1.72	0.26
Genes whose expression was not affected			
MCORF622 ^{b,c}	Carbamoyl phosphate synthase	-0.27	0.20
MCORF1045 ^b	Hypothetical protein	0.29	0.30
MCORF1300 ^{b,c}	Transketolase	0.41	0.38
MCORF222 ^b	Phosphoglycerate mutase	0.34	0.05
MCORF734 ^b	Probable glutamyl-tRNA reductase	-0.48	0.14
MCORF1573 ^b	Glycerol-3-phosphate dehydrogenase	0.44	0.07

^a Median log₂ ratio of expression in the chinchilla nasopharynx relative to expression in broth from five experiments ($P < 0.05$).

^b ORF whose expression was also measured by real-time RT-PCR.

^c $P > 0.05$.

^d CoA, coenzyme A.

number YP_581598). This *M. catarrhalis* protein also had homology with the ZnuC proteins found in several other Gram-negative organisms including *Salmonella enterica* (GenBank accession number ZP_03217112) and *Escherichia coli* HS (GenBank accession number YP_001458645). A putative Zur regulatory protein encoded by MC ORF 1413 (described above) is located immediately upstream from

MC ORF 1411 and is transcribed in the same direction. Real-time RT-PCR confirmed that both of these two *M. catarrhalis* genes are significantly upregulated in the *in vivo* environment (Fig. 3).

Additionally, the DNA microarray experiments demonstrated the upregulation of eight *M. catarrhalis* genes recently reported to also be upregulated after exposure to oxidative stress *in vitro* (28).

FIG 3 Relative expression levels of selected *M. catarrhalis* genes in the chinchilla nasopharynx as measured by real-time RT-PCR and DNA microarray methods. Expression of 26 selected genes in *M. catarrhalis* O35E.118.rpsL cells grown in broth or harvested from the chinchilla nasopharynx was measured by real-time RT-PCR analysis as described in Materials and Methods. The log₂ of the fold difference in gene expression between *in vivo* and *in vitro* expression as determined by real-time RT-PCR (white columns) is plotted adjacent to the results obtained in DNA microarray analysis (black columns). MC ORF 1045 was chosen as an internal control for real-time RT-PCR analysis because this gene apparently did not alter its level of expression between *in vitro* and *in vivo* conditions as measured by DNA microarray analysis.

These genes included ones encoding predicted homologs of malate synthase, alkyl hydroperoxidase subunit F, the previously mentioned transcriptional regulator of the BadM/Rrf2 family, and several proteins that appear to be involved in iron and sulfur uptake by bacterial cells (Table 4). Five of these genes (Table 4) were previously shown to be upregulated under conditions of oxidative stress in an OxyR-dependent manner (28).

Expression of several well-characterized *M. catarrhalis* surface proteins appeared to be affected by this *in vivo* environment. Interestingly, all of these appeared to be downregulated to some extent. The gene encoding MchA1 (MC ORF 975) (49) was downregulated in DNA microarray analysis (Table 3) and found to be approximately 2-fold downregulated when measured by real-time RT-PCR analysis. MchA1 is the same protein as that designated as MhaB2 by Balder and colleagues (6). The genes encoding OmpJ (MC ORF 1405) (26) and McaP (MC ORF 275) (59) were also found to be downregulated in both the DNA microarray (Table 3) and the real-time RT-PCR analyses (Fig. 3). Finally, the *hag* gene (MC ORF 897) (21, 46) was found to be at least 2-fold downregulated in the DNA microarray (see Table S1 in the supplemental material), with real-time RT-PCR analysis yielding similar values (Fig. 3). In addition, *uspA2* (MC ORF 1367) (Fig. 3) transcript levels were slightly downregulated *in vivo*.

Negative control experiment. The usage of genome-directed primers (56) for *M. catarrhalis* was intended to limit the detection of other bacterial transcripts beyond those derived from the *M. catarrhalis* genome. As a stringent negative control, three animals were inoculated in a sham fashion with PBS-G only, and total

RNA was extracted from the nasopharyngeal tissues 24 h later as described for the *M. catarrhalis*-inoculated animals. This RNA then was used for cDNA synthesis and subsequent DNA microarray analysis as described above. Only one probe in the DNA microarray bound a target from this cDNA, indicating that the genome-directed primers used here were very specific for *M. catarrhalis* mRNA transcripts (data not shown).

Validation of DNA microarray data. Real-time RT-PCR analysis was performed on a set of *M. catarrhalis* genes that had been identified as being either up- or downregulated at least 2-fold *in vivo* (by DNA microarray analysis) to confirm the validity of these findings. The RNA preparations used for real-time RT-PCR analysis were obtained from two sets of additional animals, were processed using RNeasy to optimize RNA recovery, and were not used for DNA microarray analysis. This analysis included 12 genes whose expression appeared to be upregulated and nine genes which appeared to be downregulated in the *in vivo* environment relative to the *in vitro* state (Table 3 and Fig. 3; also see Table S1 in the supplemental material). Additionally, five genes whose expression was not significantly affected by growth *in vivo* underwent this same analysis (Table 3 and Fig. 3). The expression profile of all of the selected genes as measured by real-time RT-PCR correlated very well (Pearson's $r = 0.85$; $P < 0.0001$) compared with the data acquired by DNA microarray analysis (Fig. 3).

Construction and testing of an MC ORF 1550 mutant. MC ORF 1550 was one of the genes that was upregulated by exposure to the chinchilla nasopharynx (Table 3) and was previously shown to be a member of the regulon controlled by the NsrR regulatory

TABLE 4 *M. catarrhalis* genes upregulated both in the chinchilla nasopharynx and under conditions of oxidative stress *in vitro*

ORF	Description	Median log ₂ (chinchilla/broth ratio ^a)	SD
MCORF323	TonB-like	3.86	0.10
MCORF903 ^b	Transcriptional regulator, BadM/Rrf2 family	3.16	0.16
MCORF1696	Malate synthase	1.82	0.28
MCORF702 ^b	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	1.80	0.37
MCORF165 ^b	Probable alkyl hydroperoxide reductase, subunit F	1.67	0.29
MCORF325	Iopolymer transport protein ExbD/TolR	1.40	0.08
MCORF905 ^b	FeS cluster assembly scaffold IscU	1.30	0.13
MCORF906 ^b	Iron-sulfur cluster assembly protein IscA	1.26	0.11

^a Median log₂ ratio of expression in the chinchilla nasopharynx relative to expression in broth ($P < 0.05$).

^b Genes whose expression has been shown to be OxyR dependent in *M. catarrhalis* (29).

protein (66). MC ORF 1550 from *M. catarrhalis* ATCC 43617 encodes a hypothetical protein containing 295 aa which is highly conserved (94 to 96% identity) among *M. catarrhalis* strains O35E, BC8, RH4, 7169, and 103P14B1 (data not shown). This protein has no apparent signal peptide, as determined by bioinformatics analysis, but does possess a domain which has similarity to a photosystem reaction center subunit H (pfam05239) of purple bacteria. In *M. catarrhalis* O35E, this gene is flanked upstream by an ORF encoding a predicted ribosome binding factor A (*rbfA*) and downstream by a very small ORF encoding a hypothetical 43 aa protein, followed immediately by a larger ORF (MC ORF 1549) that also encodes a hypothetical protein (Fig. 4A). Reverse transcriptase PCR analysis indicated that the very small ORF and MC ORF 1549 were transcriptionally linked to MC ORF 1550 (Fig. 4C).

A mutant unable to express the protein encoded by MC ORF 1550 was constructed to determine whether the lack of expression of this protein would affect the survival of *M. catarrhalis* in the nasopharynx (Fig. 4B). Real-time RT-PCR analysis showed that transcription of the small ORF immediately downstream from MC ORF 1550 was not reduced in the O35EΔ1550 mutant ($P < 0.463$), whereas transcription of MC ORF 1549 was decreased to a degree that barely achieved significance ($P < 0.0498$) (Fig. 4D). The O35EΔ1550 mutant grew in BHI broth at a rate that was essentially identical to that of the spectinomycin-resistant O35E::spec strain used here as a surrogate for the wild-type O35E strain (Fig. 4E). This O35E::spec strain (with a spec cartridge inserted in the intergenic space between *glmS* and *galE*) was used here as a wild-type surrogate instead of the O35E.118.rpsL mutant because we could not exclude the possibility that the *rpsL* mutation had an undetected effect on fitness that would be expressed only *in vivo* (45). Because we did not want to use a true mutant as a surrogate wild-type strain, we elected not to use the O35EΔmapA mutant in these chinchilla experiments.

A competitive index experiment was performed in which a suspension containing approximately equivalent numbers of the O35E::spec strain and the O35EΔ1550 mutant was inoculated into the chinchilla nasopharynx. Samples of this inoculum were then plated on BHI agar containing appropriate antimicrobials to determine the input ratio (CFU O35E::spec/CFU O35EΔ1550). Three days later, these animals were euthanized, the relevant tissues were homogenized and cultured for both *M. catarrhalis* strains, and the output ratio was calculated. In 12 of 14 animals, the surrogate wild-type O35E::spec strain outcompeted the O35EΔ1550 mutant (Fig. 5) ($P < 0.0129$).

DISCUSSION

The chinchilla has emerged as a useful tool for studying the interaction of *M. catarrhalis* with the *in vivo* environment (3, 5, 36). By using DNA microarray analysis in conjunction with this animal model, we were able to evaluate the global transcriptome response of *M. catarrhalis* to the *in vivo* environment. Paramount to the success of this study was the ability to recover viable *M. catarrhalis* organisms from the nasopharynx of the chinchilla. At 24 h postinfection, we were able to recover at least 10^7 viable *M. catarrhalis* bacteria per animal. Recovery of this number of organisms facilitated the extraction of a quantity of total RNA sufficient for successful DNA microarray analysis of the *M. catarrhalis* transcriptome. The histopathologic sections demonstrated an inflammatory response in the

chinchilla nasopharynx as a consequence of inoculation with *M. catarrhalis*. The animals which received the *M. catarrhalis* inoculum showed edema of the mucosa and submucosa, neutrophilic infiltration of the epithelium and submucosa, and a mucopurulent exudate in the nasal cavity. This inflammatory response was not seen in the animals which had been inoculated with the carrier vehicle only. Similar to humans colonized in the nasopharynx by *M. catarrhalis*, these chinchillas did not exhibit any overt signs of systemic infection or inflammation. Nonetheless, our demonstration of inflammation at the microscopic level in these infected animals (Fig. 2B and D) indicates that the *M. catarrhalis* bacteria in this environment would be exposed to elements of the innate immune response and likely respond with changes in gene expression.

It is important to note that the chinchilla nasopharynx is not a sterile environment. The polymicrobial nature of the chinchilla nasopharynx, while clearly not identical to the human situation, still places a burden on *M. catarrhalis* to survive in this niche. Recent data have indicated that *M. catarrhalis* is often cultured from patients with otitis media or an exacerbation of COPD together with other respiratory tract pathogens including, but not limited to, *H. influenzae* and *Streptococcus pneumoniae* (48, 63). In fact, there is recent evidence for interspecies signaling between *H. influenzae* and *M. catarrhalis* in a polymicrobial infection model of otitis media (3).

While the total RNA isolated from the chinchilla tissue homogenates in the present study originated from both bacterial and eukaryotic sources, the utilization of *M. catarrhalis* genome-derived primers (28, 56, 66) allowed the selective amplification from mRNA products specific to *M. catarrhalis*. This approach was based on the studies of Talaat et al. (50, 56, 57), in which 37 genome-directed primers were used for the amplification of *M. tuberculosis* mRNA transcripts in a mixture which also contained mammalian RNA. In our study, the use of the *M. catarrhalis* GDPs to amplify mRNA isolated from sham-inoculated animals failed to appreciably amplify cDNA products that would hybridize to the *M. catarrhalis* DNA microarray. This particular control demonstrated the specificity of our DNA microarray method for *M. catarrhalis* mRNA transcripts.

Review of the *M. catarrhalis* genes whose expression was affected, positively or negatively, by exposure to the chinchilla nasopharynx showed a number of previously described *M. catarrhalis* ORFs. While an exhaustive analysis of all these gene products is beyond the scope of this study, there are several sets that warrant further comment. First, the genes (*narH*, *narJ*, *aniA*, and *norB*) encoding proteins involved in the truncated denitrification pathway in *M. catarrhalis* (66) were substantially upregulated. This same set of genes was first recognized in a DNA microarray-based study of the *M. catarrhalis* transcriptome in biofilm-grown cells *in vitro* (65), and subsequently two of these genes (*aniA* and *norB*) were shown to encode the enzymes necessary to reduce nitrite to the level of nitrous oxide (64, 66). The upregulation of these same denitrification genes in the chinchilla nasopharynx may indicate that there is limited oxygen availability in this environment. It is relevant to note here that a recent study from Murphy and colleagues (52) involving COPD patients indicated that *aniA* was expressed by *M. catarrhalis* during human infection.

The *aniA* and *norB* genes are two members of the NsrR regulon first described by Wang and colleagues (66). This recently described regulon also includes MC ORF 1550, which encodes a

FIG 5 Competitive index values from mixed infections of the chinchilla nasopharynx. Mixtures of the O35E::spec surrogate wild-type strain and the O35EΔ1550 mutant were inoculated into the chinchilla nasopharynx. The input ratios varied from 0.70 to 0.90 in five independent experiments involving a total of 14 animals. After 72 h, the animals were euthanized and the relevant tissues were homogenized and plated on selective media to allow enumeration of each strain. The output ratios varied from 0.93 to 2.83. Competitive indices greater than 1.0 are indicated by black circles, and those less than 1.0 are indicated by open squares. The horizontal bar represents the mean of the competitive indices. A competitive index greater than 1.0 indicated that the wild-type surrogate strain outcompeted the mutant in that animal.

hypothetical protein of unknown function that was upregulated by exposure to the chinchilla nasopharynx (Table 3). Inactivation of this same gene did not affect the ability of *M. catarrhalis* O35E to grow in broth *in vitro* but did result in a decrease in the ability of this strain to persist in the chinchilla nasopharynx (Fig. 5). This is only the second report of an *M. catarrhalis* gene whose inactivation has a measurable effect on the ability of this bacterium to persist in the chinchilla nasopharynx, the first being a gene (i.e., *pilA*) required for type IV pilus production (36). Efforts are under way to elucidate the function of the MC ORF 1550 gene product.

The identification of at least eight *M. catarrhalis* genes that are significantly upregulated both in the chinchilla nasopharynx and under conditions of oxidative stress *in vitro* (Table 4) indicates that the *in vivo* environment in this study contains elements that can trigger the oxidative stress response in this bacterium. It is possible that this oxidative stress is due to the presence of inflammatory cell infiltrates (Fig. 2B and D), or this could also be a consequence of altered oxygen tension in the chinchilla nasopharynx. The detection of these particular upregulated genes suggests that the inoculated bacteria must be able to cope with and detoxify oxidative stressors in order to survive in this *in vivo* environment. Five of these genes were previously found to be upregulated in an OxyR-dependent manner (28) and suggest that the OxyR global regulator may be important for the *in vivo* survival of *M. catarrhalis*.

The third set of genes had in common the facts that their expression was downregulated by exposure to the environment in the chinchilla nasopharynx and that they encoded outer membrane or surface-expressed proteins. The encoded gene products

included UspA2, McaP, Hag, OmpJ, and MchA1 (MhaB2). Most of these proteins have been shown to either be potential virulence factors involved in resistance to complement-mediated serum killing (4) or adherence to epithelial cells (6, 13, 20, 49) or to be targets for potentially protective antibodies (14, 58). In addition, at least UspA2 and Hag have been shown to be expressed by *M. catarrhalis* in humans (39, 41), based on the development of an antibody response in patients to these different proteins. Because no quantitative data on *M. catarrhalis* protein expression in humans has been reported, it is uncertain whether the level of these genes' expression in the chinchilla nasopharynx is higher or lower than that in humans. It is possible that abundant surface expression of these proteins may make the bacterium more susceptible to host defense mechanisms and therefore that downregulation of these genes' expression may help to minimize recognition by the host. With another pathogen (i.e., nontypeable *H. influenzae*) of the upper respiratory tract, *in vivo* colonization has been shown to select for variants with downregulated expression of at least one surface protein (15).

We used real-time RT-PCR to verify gene expression patterns identified with our DNA microarrays (Fig. 2). This was especially important in view of the fact that the genome sequence from *M. catarrhalis* ATCC 43617 was used to design the DNA microarray, whereas our cDNAs were derived from an *M. catarrhalis* O35E-derived strain. We did not use the former strain in these *in vivo* experiments because, in our hands, *M. catarrhalis* ATCC 43617 is genetically intractable and would not be suitable for subsequent construction of mutants. In contrast, *M. catarrhalis* O35E can be readily mutagenized by different methods (1, 35, 47, 59). While the results of the present study are limited to a single strain (i.e., one derived from O35E), the recent description of a DNA microarray system for another *M. catarrhalis* strain (i.e., RH4) (19) would allow investigation of gene expression by this latter strain in this animal model.

The successful application of DNA microarray technology to study gene expression by *M. catarrhalis* in the chinchilla nasopharynx expands the experimental armamentarium available to workers in this field. It should be noted that the use of a chinchilla model to study the interaction of nontypeable *H. influenzae* with the host environment has allowed the identification of virulence factors of this pathogen which function in either colonization or survival (31, 37, 38). One significant difference, however, between nontypeable *H. influenzae* and *M. catarrhalis* with respect to the chinchilla model is that the latter organism has demonstrated little or no ability to produce otitis media in this animal model. Interpretation of data obtained from this model system must be tempered by the fact that *M. catarrhalis* is a strict human pathogen. However, at the very least, the data presented herein indicate that the chinchilla model can be used for analysis of *in vivo* gene expression by *M. catarrhalis*. A recent study from Luke and col-

FIG 4 Characterization of *M. catarrhalis* O35EΔ1550. (A) Wild-type O35E locus containing MC ORF 1550. (B) Insertion of the Kan cartridge into a deletion within MC ORF 1550 in O35EΔ1550. (C) Reverse transcriptase PCR (RT-PCR)-based analysis of transcriptional linkage between selected ORFs downstream from MC ORF 1550 in the O35E::spec strain. The primer pair (Table 2) used for each experiment is specified at the top of the figure, as is the relevant intergenic region. Lanes: 1 and 4, samples from RT-PCR with RNA as the template but no added RT; 2 and 5, samples from RT-PCR with RNA as the template and RT added; 3 and 6, samples from RT-PCR with chromosomal DNA. Size position markers (in base pairs) are present on the left. A representative experiment is shown. (D) Real-time RT-PCR analysis of relative transcript levels for MC ORF 1550, the small hypothetical ORF (hyp. ORF), and MC ORF 1549 in O35EΔ1550 relative to the surrogate wild-type O35E::spec strain. Three independently isolated sets of RNA samples (#1, #2, and #3) were used for these experiments. Statistical analysis was performed using a one-sample *t* test with Bonferroni's correction. (E) Growth of the O35E::spec (closed circles) and O35EΔ1550 (open circles) strains in BHI broth. These data represent the mean from two independent experiments.

leagues (36) first demonstrated the utility of this animal model for mutant analysis with *M. catarrhalis*. While this chinchilla model in its present form does not allow overt disease production by this pathogen, the ability to collect *M. catarrhalis* bacteria subjected to the physiologic stresses and innate immune response system present in the nasopharynx will allow more detailed analyses of bacterial gene expression *in vivo*. It is hoped that the eventual development of appropriate transcriptional reporter systems for use in *M. catarrhalis* will allow additional studies in this animal model that will complement the use of DNA microarray analysis to detect or measure bacterial gene expression *in vivo*. Finally, recent efforts at mining the genome of *M. catarrhalis* have revealed the presence of several hundred bacterial genes encoding proteins with predicted signal sequences (52), many of which could be exposed on the bacterial cell surface or even secreted into the environment. The use of the chinchilla model to provide *M. catarrhalis* bacteria isolated directly from the relevant *in vivo* environment would greatly facilitate experimental determination of which of these different bacterial proteins might be expressed *in vivo*.

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