Identification of Gene Products Involved in the Oxidative Stress Response of *Moraxella catarrhalis*[†]

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Moraxella catarrhalis is subjected to oxidative stress from both internal and environmental sources. A previous study (C. D. Pericone, K. Overweg, P. W. Hermans, and J. N. Weiser, *Infect. Immun.* 68:3990–3997, 2000) indicated that a wild-type strain of *M. catarrhalis* was very resistant to killing by exogenous hydrogen peroxide (H₂O₂). The gene encoding OxyR, a LysR family transcriptional regulator, was identified and inactivated in *M. catarrhalis* strain O35E, resulting in an increase in sensitivity to killing by H₂O₂ in disk diffusion assays and a concomitant aerobic serial dilution effect. Genes encoding a predicted catalase (KatA) and an alkyl hydroperoxidase (AhpCF) showed dose-dependent upregulation in wild-type cells exposed to H₂O₂. DNA microarray and real-time reverse transcription-PCR (RT-PCR) analyses identified *M. catarrhalis* genes whose expression was affected by oxidative stress in an OxyR-dependent manner. Testing of *M. catarrhalis* O35E *katA* and *ahpC* mutants for their abilities to scavenge exogenous H₂O₂ showed that the KatA catalase was responsible for most of this activity in the wild-type parent strain. The introduction of the same mutations into *M. catarrhalis* strain ETSU-4 showed that the growth of a ETSU-4 *katA* mutant was markedly inhibited by the addition of 50 mM H₂O₂ but that this mutant could still form a biofilm equivalent to that produced by its wild-type parent strain.

Moraxella catarrhalis is an aerobic, Gram-negative coccobacillus that has been shown to be an important cause both of otitis media (i.e., middle ear disease) in infants and young children and of infectious exacerbations of chronic obstructive pulmonary disease (COPD) in adults (18, 31, 46). The mucosa of the human nasopharynx is the niche normally colonized by *M. catarrhalis*. This colonization event provides *M. catarrhalis* with a foothold in the human body from which it can spread to other anatomic sites (i.e., the middle ear and lower respiratory tract) but also exposes this bacterium to negative selective pressures exerted both by the host (25, 32) and by the normal flora of the nasopharynx (38, 55). Among these, oxidative stress is a formidable obstacle both to initial colonization and to persistence in the respiratory tract (for reviews, see references 15 and 52).

While there are different types of reactive oxygen species (e.g., H₂O₂, O₂^{•−}, peroxynitrite) that can cause oxidative stress (15, 29), the ability of bacterial pathogens to resist killing by H₂O₂ has been the subject of considerable attention for some years. The mechanisms by which H₂O₂ can kill bacteria include, but are not limited to, damage to proteins and DNA related to the Fenton reaction, peroxidation of membrane lipids, and oxidation of iron-sulfur clusters in proteins (15). The biologically relevant sources of H₂O₂ include phagocytic cells (40, 41), some members of the normal flora of the host

(38, 54), and endogenous production by the bacterium itself during aerobic growth (i.e., from sources outside the bacterial respiratory chain) (44; for a review, see reference 15).

The ability of bacteria to respond to the presence of H₂O₂ and to increase their resistance to killing by this compound through the induction or upregulation of antioxidant proteins was first studied in *Salmonella enterica* serovar Typhimurium (6, 30) and then in *Escherichia coli* (58) more than 2 decades ago. These studies included the identification of OxyR as a LysR-like positive regulatory element that controlled the expression of some of these H₂O₂-inducible genes (6). The genes positively controlled by OxyR in *E. coli* include those encoding a catalase (*katG*), an alkyl hydroperoxide reductase system (*ahpCF*), glutathione reductase (*gorA*), glutaredoxin (*grxA*), and thioredoxin (*trxC*) (50). *E. coli* OxyR can exist in either an oxidized or a reduced state and can form an intramolecular disulfide bond between C199 and C208 that is eliminated (by reduction) when the source of oxidative stress is removed. While both the oxidized and the reduced forms of OxyR have DNA binding activity, the oxidized form of OxyR typically functions as a transcriptional activator in *E. coli*. In other bacteria, including *Neisseria gonorrhoeae* (56) and *Pseudomonas putida* (12), however, OxyR can function as a repressor for some target genes. Mutants lacking the ability to express OxyR are usually more susceptible to killing by H₂O₂ than are their wild-type parent strains (6, 11, 27), but there are exceptions to this rule (e.g., *N. gonorrhoeae* [56]).

Most previous studies on the interaction of *M. catarrhalis* with other bacteria that can colonize the mucosa of the human upper respiratory tract focused mainly on various alpha-hemolytic streptococci and their ability to affect either the growth or the viability of *M. catarrhalis* strains *in vitro* (4, 38, 54, 55). Two

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
<i>M. catarrhalis</i>		
O35E	Wild-type disease isolate	57
O35E Δ oxyR	oxyR deletion mutant; Kan ^r	This study
O35E Δ oxyR (repaired)	Δ oxyR deletion mutant with wild-type oxyR gene	This study
O35E Δ katA	katA deletion mutant; Kan ^r	This study
O35E Δ ahpC	ahpC deletion mutant; Kan ^r	This study
ETSU-4	Wild-type disease isolate	Steven Berk
ETSU-4 Δ oxyR	oxyR deletion mutant; Kan ^r	This study
ETSU-4 Δ katA	katA deletion mutant; Kan ^r	This study
ETSU-4 Δ ahpC	ahpC deletion mutant; Kan ^r	This study
ETSU-9	Wild-type disease isolate	Steven Berk
V1120	Nasopharyngeal isolate	Frederick Henderson
7169	Wild-type disease isolate	26
ATCC 43617	Wild-type disease isolate	American Type Culture Collection
<i>E. coli</i> M15(pREP4)	Host for pPROEX HTb-derived plasmids	Qiagen
Plasmids		
pWW115	<i>M. catarrhalis</i> cloning vector; Spec ^r Chlor ^r	59
pWL01	pWW115 containing the <i>M. catarrhalis</i> O35E oxyR gene; Spec ^r	This study
pTH61-1	pWW115 containing the <i>M. catarrhalis</i> O35E katA gene; Spec ^r	This study
pWL100E-KanC	Derivative of pACYC177 containing a nonpolar kanamycin resistance cartridge	19
pPROEX Htb	6 $\times$ His expression vector; Amp ^r	Invitrogen
pHIS-OxyR	pPROEX HTb expressing <i>M. catarrhalis</i> O35E OxyR; Amp ^r	This study

of these studies focused on the inhibitory effect of H₂O₂ produced by these streptococci (38, 54). One study showed that a wild-type *M. catarrhalis* strain was much more resistant to killing by exogenous H₂O₂ than the other bacteria tested (38), and another suggested that *M. catarrhalis* could somehow increase its resistance to killing by what was presumed to be H₂O₂ (55). In the present study, inactivation of the *M. catarrhalis* oxyR gene was shown to increase the sensitivity of this organism to killing by H₂O₂. DNA microarray analysis of *M. catarrhalis* gene products whose expression was upregulated under conditions of oxidative stress identified several genes encoding antioxidant proteins, including KatA and AhpCF. Inactivation of these different genes revealed that expression of the *M. catarrhalis* KatA catalase was essential for effective resistance to killing by exogenous H₂O₂.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. *M. catarrhalis* strains were grown using brain heart infusion (BHI)-based broth (Difco/Becton Dickinson, Sparks, MD) or BHI agar (2); these media were supplemented with spectinomycin (15 μ g/ml) or kanamycin (15 μ g/ml) when appropriate. *Escherichia coli* strains were grown on Luria-Bertani (LB) medium as described previously (42).

Construction of *M. catarrhalis* oxyR, ahpC, and katA deletion mutants. The overlapping extension PCR mixture (13) for each mutagenesis construct contained 3 separate amplicons mixed in equal amounts. For amplicon A, the primers (Table 2) were 162-p4 and 162-p3 (oxyR), TH287 and TH288 (ahpC), and katAP1 and katAP2 (katA). Amplicon B was the same for all three constructs and consisted of the nonpolar kan cartridge and its promoter as amplified from pWL100E-KanC (19) with primers KanPro-5' and NPK3-3'. For amplicon C, the primers were 162-p2 and 162-p1 (oxyR), TH289 and TH286 (ahpC), and katAP3 and katAP4 (katA). The final overlapping extension PCR mixture contained equal amounts of amplicons A, B, and C together with the following primer pairs: 162-p4 and 162-p1 (oxyR), TH287 and TH286 (ahpC), and katAP1 and katAP4 (katA). The final amplicons were used to transform *M. catarrhalis* O35E via a plate-based transformation method (36). Kanamycin-resistant mu-

nants were selected, and nucleotide sequence analysis confirmed the deletion of the oxyR, ahpC, or katA gene, respectively. The same strategy was utilized to generate oxyR and ahpC mutations in *M. catarrhalis* ETSU-4. The katA deletion mutant constructed in *M. catarrhalis* ETSU-4 required the usage of primers EU4KATAP3-1 and EU4KATAP4 for the generation of amplicon C.

Repair of the oxyR mutation. Primers 162-P1 and 162-P4 were used to PCR amplify a 2.3-kb fragment comprising the wild-type O35E oxyR open reading frame (ORF) together with ~0.7 kb of both the upstream and downstream flanking regions. The amplicon was utilized to transform the O35E Δ oxyR mutant. The repaired strain containing the wild-type oxyR gene was confirmed by nucleotide sequence analysis and was designated O35E Δ oxyR (repaired).

Complementation of the oxyR and katA mutations. Primers 162comp-3B and 162comp-5B were used to PCR amplify a 1.3-kb fragment of O35E chromosomal DNA containing the oxyR ORF and some flanking DNA. This amplicon was ligated into the BamHI site of the *M. catarrhalis* cloning vector pWW115 (59) and was used for the transformation of *M. catarrhalis* O35E Δ oxyR. The recombinant plasmid containing the *M. catarrhalis* O35E oxyR ORF was designated pWL01. The wild-type katA gene from *M. catarrhalis* strain O35E was amplified with primers TH284 and TH285, ligated into pWW115, and used to transform *M. catarrhalis* O35E Δ katA so as to yield pTH61-1. The latter plasmid was also used to transform *M. catarrhalis* ETSU-4 Δ katA.

Determination of the MBC of H₂O₂ for *M. catarrhalis*. Minimum bactericidal concentrations (MBC) were determined by using a modification of the method described by Weiser and colleagues (38). Portions (1.0 ml) of mid-logarithmic-phase (optical density at 600 nm [OD₆₀₀], ~0.7) cultures were transferred to a 24-well polystyrene tissue culture plate, and H₂O₂ (8.8 M; Fisher Scientific) was added to each well to achieve the range of desired final concentrations. The suspension was incubated at 37°C for 15 min without shaking, and then the contents of the wells were serially diluted and plated onto BHI agar. The MBC was defined as the concentration of H₂O₂ required to cause at least a 99.9% reduction in the number of viable bacteria relative to the number for a control in which water was used instead of H₂O₂.

Disk diffusion assays. Bacterial cells grown to mid-logarithmic phase (OD₆₀₀, 0.7) were added (at a 1:1,000 ratio) to 25 ml of molten 1.5% (wt/vol) BHI agar at 45°C. This suspension was gently mixed with agitation for 30 s; then a 10-ml portion of this mixture was added on top of 20 ml of solidified BHI agar and was allowed to cool until solidified. A sterile disk (diameter, 6 mm) cut from thin chromatography paper was saturated with 10 μ l of 88 mM H₂O₂ and was then applied to the top of the solidified agar. The final size of the zone of growth

TABLE 2. Oligonucleotide primers utilized in this study

Primer	Sequence (5'-3')
TH286	GATACTAAAATCAGCCACGCA
TH287	GGATATGTTTCATCTTTTCA
TH288	GGTCGACGGATCCGGGGGATTATTGATTAT ACACCATAAA
TH289	TGGAGGGAATAATGCGACCCAAGCCAAGC TTGGATTGGTA
kanPro-5'	CCCCGGATCCGTCGACC
NPK3-3'	GGGTGCGATTATTCCTCCCA
TH284	ACGCGGATCCACTTTAATGGCGATGATGT ^a
TH285	ACGCGGATCCCTGCACCAGAAGATTGTGA ^a
162-p1	TCACGCACATCTGAGCTGC
162-p2	AGGTCGACGGATCCGGGGTTGGCGTAGTG TGATCAT
162-p3	GAGGGAATAATGCGACCCCAAGCACTTGC ATCTCA
162-P4	ATCCATCAAGAAATACTCCAA
162comp-5B	ATCGGATCCCATTGAATGTGTCTATCTG ^a
162comp-3B	ACGGATCCGATTAAATATTGCTGCCA ^a
HIS162-B5'	ATCGGATCCATGATCACACTACGCCAA ^a
HIS162-B3'	ATCGAAGCTTTTATGCTTCATTTTCTGAGG ^b
katAP1	CGATGCTGATGCTAACAGGC
katAP2	AGGTCGACGGATCCGGGGAGGGCATTTC TTTCACTAT
katAP3	GAGGGAATAATGCGACCCGCGTATGAGAG CGACCT
katAP4	AACCAAGGCGATAAGATTACCG
EU4katAP3-1	GAGGGAATAATGCGACCCGACCTGCTCG CCAT
EU4katAP4	TGTCATCTTATGTATTTCAGCC
1139rt1	CCACGCTGCTCTGTGTACTC
1139rt2	ACCGCCATAGTTATCGCCC
163rt1	GCGTCACCGACCATTTGGTA
163rt2	AAGCCACGAGTGATTTTGGC
323fw	AAGAAGCCCAAGTGGCTGC
323rv	CCATGATTGCTGCTTTTTTG
164fw	TCGTGAACCTTATGACCAAACTCAAA
164rv	AGGTGACGGGATCTTTTCAAAA
880fw	GGGCTACAGCTACGATGCACT
880rv	ATGGTGGCGTGAATGGTGA
2fw	AACATGGTTGCCCTGGTAC
2rv	GCAGCATATCCCATTTGCAAAA
1249fw	CCAATCAATCTAACCGCATG
1249rv	GACCAAAATTGAGCCAGTAAAGTCA
400fw	CCGATACCAATGTGGATGCA
400rv	GGCGGAGTGAATTTGTAAGCA
1202fw	TGCCAAAATTGCCATATAAAACAAC
1202rv	TCGTTACTATCATTTGGAATGATTGC
425fw	CTGGAAGCGTGAGCCACAC
425rv	GGTAAAGCGTGCAATTGGCA
1579fw	TTGGTTTATGGTGGTGCAAT
1579rv	CTTTTTCCACCATTGTGCGATG
1398fw	GTTAAGCCTGCCGCTGTCA
1398rv	TTTTGGGAGCAGGGACGAC
142fw	ACAGTGATAGCGGCCATTGTT
142rv	CTGTTTTGAGCATAGTTCACAGGTAC
516fw	ACCCAGATTGACACTGCCAG
516rv	AAGGTCGTGCTGAACCGT
701fw	CGTTGTGCCAGGCATGC
701rv	CGTGAGCTTCTTGCCCAAA
165rt1	ACACTGCGATGGCCCATTT
165rt2	CGCTCAACACCTGAGTTACC
1128fw	GAATTGATGGGCGTCTCTGC
1128rv	AAAGGGATAAGCCAGTGCTG
1373fw	TTAGAGCACAGGGCGAAG
1373rv	GCTTGCTCAAACCTGGGTCAAAA
1452rt3	GCCTACTTTGGGTGAGCGTG
1452rt4	GGTAATTGGTGTACCGCCCTC
903fw	TGCTTATTGGATTGCGCTGT
903rv	GGCGTTTGGCAATGTCTGA

^a Underlining indicates a BamHI restriction site.^b Underlining indicates a HindIII restriction site.

inhibition around each disk represents the mean of four axial measurements for each disk.

Use of growth curves to assess the response to exogenous H₂O₂. *M. catarrhalis* strains ETSU-4, ETSU-4ΔoxyR, ETSU-4ΔkatA, ETSU-4ΔkatA(pTH61-1), ETSU-4ΔkatA(pWW115), and ETSU-4ΔahpC were grown in BHI broth to mid-

logarithmic phase (OD₆₀₀, 0.7). H₂O₂ was then added to a final concentration of 50 mM; the flask was incubated at 37°C with agitation; and turbidity measurements (OD₆₀₀) were recorded over time.

Purification of a recombinant *M. catarrhalis* OxyR protein. Primers HIS-162B5' and HIS-162B3' were used to PCR amplify the oxyR coding region from *M. catarrhalis* O35E genomic DNA. This amplicon was sequentially digested with BamHI and HindIII (New England Biolabs, Ipswich, MA) and was then ligated into the pPROEX HTb vector (Invitrogen, Carlsbad, CA). The reaction mixture was used to transform electrocompetent *E. coli* M15 cells, and transformants containing the oxyR gene were selected on LB agar containing ampicillin. The plasmid was designated pHIS-OxyR. The His-tagged OxyR protein was purified from sonicated lysates (cells induced according to the manufacturer's instructions) using Ni²⁺-chelate chromatography. This purified recombinant protein was used by Rockland Immunochemicals (Boyertown, PA) to develop a polyclonal rat OxyR antiserum. Western blot analysis was performed as described elsewhere using appropriate secondary antibodies (22).

Evaluation of the ability of *M. catarrhalis* O35EΔoxyR to form isolated colonies. Strains O35E and O35EΔoxyR were grown to mid-logarithmic phase and were then serially diluted in BHI broth. A 2-μl portion of each dilution was spotted onto BHI agar alone or onto BHI agar containing bovine liver catalase (200 U/ml agar; Worthington Biochemical, Lakewood, NJ), and these plates were incubated overnight.

H₂O₂ scavenging assay. The Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen) was used according to the manufacturer's instructions to measure the rate of H₂O₂ degradation by bacterial cells. The standard assay mixture contained 25 μl of broth-grown bacterial cells (approximately 1.6 × 10⁷ CFU per well), 25 μl of 40 μM H₂O₂ (final concentration, 10 μM), and 50 μl of the horseradish peroxidase-Amplex Red solution. Fluorescence was measured after 1 min by using an excitation wavelength of 530 nm and an emission wavelength of 595 nm in a SpectraFluor Plus microplate reader (Tecan, Research Triangle Park, NC).

RNA isolation for DNA microarray and real-time reverse transcription-PCR (RT-PCR) analyses. Bacterial cells were grown in BHI at 37°C with agitation to an OD₆₀₀ of 0.7. A portion of this culture was diluted 1:100 in fresh BHI, and 2-ml portions of this suspension were added to each of four wells in a 24-well tissue culture plate (Corning, Corning, NY). The tissue culture plate was then placed at 37°C under an atmosphere of 95% air–5% CO₂ without agitation for 19 h. After 19 h, H₂O₂ was added to a final concentration of 50 mM. The contents of each well were mixed and allowed to incubate at 37°C under 95% air–5% CO₂ without agitation for 15 min. Total RNA was extracted from these cells with the RiboPure Bacteria kit (Ambion, Woodlands, TX) according to the manufacturer's instructions. The RNA samples were treated with DNase I by use of a Message Clean kit (GenHunter Corp., Nashville, TN) according to the manufacturer's instructions. The integrity of the RNA samples was measured with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). For the DNA microarray experiments involving (i) analysis of the *M. catarrhalis* O35E response to H₂O₂ and (ii) the evaluation of *M. catarrhalis* O35E versus *M. catarrhalis* O35EΔoxyR in the presence of 50 mM H₂O₂, three independent experiments and accompanying RNA isolations were performed.

cDNA preparation, DNA microarray hybridization, and data analysis. cDNA was prepared by a method similar to that described previously (60). Briefly, 5 μg of each of the RNA samples mentioned above was mixed with 3 μg of *M. catarrhalis* genome-directed primers (60). The individual cDNA samples were labeled with either Cy3-dCTP or Cy5-dCTP as described previously (22). The *M. catarrhalis* DNA microarrays utilized in this study have been described previously (60). Equivalent amounts of the Cy3- and Cy5-labeled cDNAs were mixed thoroughly and were hybridized to the *M. catarrhalis* DNA microarray slides as described previously (60). After hybridization, the slides were washed, dried, and scanned as described previously (22). For both sets of conditions described immediately above, one "dye swap" experiment was performed. The Acuity 4.0 software package (Molecular Devices) was used to analyze the microarray data as described previously (22). To identify genes that exhibited altered expression profiles in the presence of H₂O₂, a threshold of 2-fold increased or decreased expression relative to expression by nonexposed cells was utilized. The gene of interest had to demonstrate the altered expression profile in at least three of the four microarray analyses, and the *P* value for the difference had to be ≤0.05 as calculated by the one-sample *t* test.

Real-time RT-PCR. Oligonucleotide primer pairs were designed for use in real-time RT-PCR (Table 2) with PrimerExpress software (Applied Biosystems, Foster City, CA). Real-time RT-PCRs were prepared as described previously (60), and one-step relative real-time RT-PCR was performed on these samples utilizing the 7500 Fast real-time PCR system (Applied Biosystems). DNA contamination and the presence of "primer dimers" were assessed as described

FIG. 1. Schematic representation of the *M. catarrhalis* *oxyR*, *ahpC*, *ahpF*, and *katA* loci in wild-type and mutant strains. (A) The *oxyR*, *ahpC*, and *ahpF* genes in wild-type *M. catarrhalis* O35E; (B) the *katA* locus in wild-type *M. catarrhalis* O35E; (C) the *M. catarrhalis* O35E Δ *oxyR* mutant; (D) the O35E Δ *ahpC* mutant; (E) the O35E Δ *katA* mutant. Bent arrows represent relevant oligonucleotide primers.

previously (60). All of the relative real-time RT-PCR experiments involved the use of at least two independently isolated RNA samples. The endogenous control was MC1452, encoding a predicted gyrase B (60). The results were analyzed (by the $\Delta\Delta C_T$ method) with Relative Quantification Study software (Applied Biosystems).

Crystal violet-based assay for biofilm measurements. *M. catarrhalis* strains were evaluated for their abilities to form biofilms under *in vitro* conditions by utilizing a method adapted from that previously described (35). Briefly, *M. catarrhalis* cells were grown in BHI broth to an OD₆₀₀ of 0.7. A 2-ml portion of this bacterial suspension was used to inoculate a 24-well tissue culture plate in triplicate. The tissue culture plate was incubated at 37°C in a 95% air–5% CO₂ incubator for 24 h. The plate was then stained with crystal violet to detect biofilm formation as previously described (35).

Microarray data accession numbers. The raw data from the DNA microarray experiments performed in this study have been deposited at the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE24331 and GSE24351.

RESULTS

Sensitivities of wild-type *M. catarrhalis* strains to killing by H₂O₂. A previous study that focused on the ability of *Streptococcus pneumoniae* to inhibit the growth of pathogens that colonize the upper respiratory tract indicated that a strain of *M. catarrhalis* (i.e., Bc1) was markedly more resistant to killing by H₂O₂ than were the other bacterial species tested (38). Using an assay similar to that described previously (38), the MBC of H₂O₂ for *M. catarrhalis* O35E was shown to be between 400 and 500 mM (data not shown). Subsequent testing of three additional wild-type *M. catarrhalis* strains (7169, V1120, and ETSU-4) in a disk diffusion-based system indicated that all three of these strains had essentially the same level of

resistance to killing by H₂O₂ as O35E (data not shown). It was also determined that freshly prepared and filter-sterilized BHI medium was required in order to obtain reproducible and accurate results in these and all subsequent experiments.

Identification of the *M. catarrhalis* *oxyR* gene. In other Gram-negative pathogens that can colonize the upper respiratory tract, including nontypeable *Haemophilus influenzae* (9) and *Neisseria meningitidis* (14), the OxyR protein has been shown to be a global regulator of the oxidative stress response. Inspection of the nucleotide sequence of the genome of *M. catarrhalis* ATCC 43617 (60) indicated that MC162 encoded a LysR family transcriptional regulator that had been annotated as an OxyR protein in several different bacterial species. Located on the opposite strand (Fig. 1A) were two other ORFs (*ahpC* and *ahpF*) encoding a predicted peroxidase (AhpC) and its cognate flavoprotein disulfide reductase (AhpF). AhpF functions to reduce the AhpC protein, which can degrade both H₂O₂ and organic peroxides (for a review, see reference 39). Nucleotide sequence analysis of the *oxyR* ORF from four additional *M. catarrhalis* strains (i.e., O35E, ETSU-9, 7169, and ETSU-4) indicated that the *oxyR* genes in these strains were identical except for a single nucleotide change (G238A) in the ETSU-4 and ETSU-9 *oxyR* genes (data not shown).

Structure of the *M. catarrhalis* OxyR protein. When a simple BLAST search (1) was limited to the *E. coli* genome, the *E. coli* OxyR protein was found to be fairly homologous to the *M. catarrhalis* OxyR protein (E value, 2×10^{-41}). Alignment of these two sequences showed that these proteins share approx-

FIG. 2. Structure-based sequence alignment of *M. catarrhalis* OxyR with OxyR proteins from both *E. coli* and *P. aeruginosa*. The numbering is given on each side of the sequences. Areas of predicted helices are shown in red, while areas of predicted β -strands are blue. (A) The secondary structural elements of *E. coli* OxyR, as assigned by DSSP (17) from PDB entry 1I69, are shown above the sequences as red cylinders (helices) and blue arrows (β -strands). Areas of identity between the two structures are marked with asterisks. Where no structural information is available, predictions from a separate PSIPRED (16) prediction are shown in the color code described above. Clustal W (23) was used for the DNA-binding domain alignment. PROMALS3D (37) was used for the remainder of the alignment. Residues shown to be important for DNA binding in *E. coli* OxyR are boxed. The vertical black arrows indicate the conserved cysteine residues critical to the function of *E. coli* OxyR. (B) Alignment with the PA0218 OxyR protein. PROMALS3D (37) was used for the entire alignment. All coloring and notation conventions are the same as for panel A.

imately 30% identity (Fig. 2A). There are two cysteine residues (C199 and C208 in *E. coli* OxyR) that are critical for the function of the protein. These two cysteines (C200 and C209 in *M. catarrhalis* OxyR) are conserved between these two proteins. The homology between these proteins is particularly pronounced in the N-terminal putative DNA-binding domain (Fig. 2A). In *E. coli* OxyR, residues T31, L32, and S33 have been shown to be important for DNA binding (21). The latter two residues are identical in the *M. catarrhalis* protein: L34 and S35. It is not known whether this implies that the *M. catarrhalis* protein binds to similar DNA targets or that these residues are important for the conformation of the N-terminal domain. Given the high overall sequence identity and the conservation of the critical cysteines, it is very likely that *M. catarrhalis* OxyR shares structure and function with *E. coli* OxyR (see Fig. S1 in the supplemental material). Indeed, the structure is likely to be similar to that of the entire LysR family of proteins.

No crystal structure is available for any full-length OxyR protein. Therefore, a hidden Markov model search (48, 49) was performed against the Protein Data Bank (PDB) for proteins of known structure that are similar in sequence to *M. catarrhalis* OxyR. A cluster of proteins was found with nearly identical statistics (match probability, 100%; E value, 0.0). *M. catarrhalis* OxyR was compared to the PA0218 protein from *Pseudomonas aeruginosa* (unpublished; PDB accession code 3FZV). A sequence alignment demonstrates that the proteins share approximately 21% sequence identity, with no major insertions or deletions (Fig. 2B). The N-terminal, DNA-binding domain is dominated by four α -helices (Fig. 2B), the third of which harbors residues critical for DNA binding. The C-terminal domain adopts a fold similar to that of *E. coli* OxyR (see Fig. S1 in the supplemental material). It should be noted that the relative positions of the DNA-binding domain and the

regulatory domain for *M. catarrhalis* OxyR could be different from those shown here.

Construction and characterization of an *M. catarrhalis* oxyR mutant. The majority of the *M. catarrhalis* O35E oxyR ORF was deleted and replaced with a kanamycin resistance cassette (Fig. 1C) as described in Materials and Methods. This oxyR mutant did not express any detectable OxyR protein as determined by Western blot analysis (Fig. 3A, lane 2). When grown in BHI broth, this oxyR mutant had a longer lag phase than its wild-type parent strain (Fig. 3B). Provision of a wild-type oxyR gene in *trans* in this oxyR mutant resulted in relatively high level expression of OxyR (Fig. 3A, lane 3), but this large amount of OxyR protein did not completely eliminate the mutant's growth defect (Fig. 3B). This inability of pWL01 to restore wild-type growth characteristics to the oxyR mutant may have been the result of the presence of the pWW115 plasmid backbone, because the presence of this vector alone in the oxyR mutant resulted in a slightly greater growth defect than that observed with the mutant alone (Fig. 3B). Alternatively, overexpression of OxyR may have had a negative effect on the growth profile. Replacement (i.e., repair) of the mutated oxyR gene with a wild-type oxyR gene resulted in wild-type expression of OxyR (Fig. 3A, lane 5) and a growth phenotype very similar to that of the wild-type parent strain (Fig. 3B).

Sensitivities of *M. catarrhalis* oxyR mutant constructs to H_2O_2 . The *M. catarrhalis* O35E oxyR mutant (Fig. 3C2) was more sensitive to killing by H_2O_2 than was the wild-type parent strain (Fig. 3C1), as determined by the disk diffusion method. Similarly, the oxyR mutant containing the empty plasmid vector (Fig. 3C4) was also more sensitive to killing by H_2O_2 . These differences were significant as determined by two-way ANOVA (Fig. 3D) ($P, \leq 0.05$). The zones of growth inhibi-

FIG. 3. Characterization of the *M. catarrhalis* O35E Δ oxyR mutant and related strains. (A) Western blot analysis of whole-cell lysates of *M. catarrhalis* O35E (lane 1), O35E Δ oxyR (lane 2), O35E Δ oxyR(pWL01) (lane 3), O35E Δ oxyR(pWW115) (lane 4), and O35E Δ oxyR (repaired) (lane 5) using polyclonal rat OxyR antiserum as the primary antibody. The black arrow on the right indicates the position of the OxyR protein. (B) Comparison of the growth rates of *M. catarrhalis* O35E (■), O35E Δ oxyR (●), O35E Δ oxyR(pWL01) (□), O35E Δ oxyR(pWW115) (○), and O35E Δ oxyR (repaired) (▼). Results of a representative experiment are shown. (C) Sensitivities of the five strains for which results are shown in panels A and B to 88 mM H₂O₂ in disk diffusion assays. (D) Diameter of the zone of growth inhibition for each strain for which results are shown in panel C. Statistical analysis utilizing two-way ANOVA was utilized to determine the significance of each strain's zone of growth inhibition relative to that of wild-type *M. catarrhalis* O35E. Significant differences from the diameter of the zone of growth inhibition of *M. catarrhalis* O35E (bar 1) were observed for the zones of growth inhibition obtained with *M. catarrhalis* O35E Δ oxyR (bar 2) and O35E Δ oxyR(pWW115) (bar 4).

FIG. 4. Catalase reverses the aerobic serial dilution defect of the *M. catarrhalis* O35E Δ oxyR mutant. Serial dilutions of the wild-type strain O35E (A and B) and the O35E Δ oxyR mutant (C and D) were spotted onto BHI agar either lacking catalase (-) or containing catalase (+) and were then incubated overnight.

tion caused by H₂O₂ with the complemented oxyR mutant (Fig. 3C3) and the repaired mutant (Fig. 3C5) were very similar to that obtained with the wild-type parent strain; the differences were not significant.

Aerobic serial dilution defect of the *M. catarrhalis* O35E Δ oxyR mutant. When spotted onto BHI agar, the *M. catarrhalis* O35E Δ oxyR mutant was unable to form single colonies below a density of 10⁶ CFU/ml (Fig. 4C). In contrast, the wild-type *M. catarrhalis* strain O35E was capable of single-colony formation at a cellular density approximately 1,000-fold less (Fig. 4A). This serial dilution defect seen with the oxyR mutant was eliminated by the addition of exogenous catalase to the medium at the time of plating, such that oxyR mutant cells demonstrated the ability to form single colonies at a cellular density of 10³ CFU/ml (Fig. 4D). The provision of catalase to wild-type cells (Fig. 4B) did not increase their ability to form individual colonies beyond that observed in the absence of exogenously supplied catalase (Fig. 4A).

Transcriptional response of the *M. catarrhalis* O35E *kata*, *ahpC*, and *ahpF* genes to increasing concentrations of exogenous H₂O₂ as measured by real-time RT-PCR. Inspection of the annotation of the *M. catarrhalis* ATCC 43617 genome (60) identified a gene (Fig. 1B) encoding a predicted protein that was 83% identical (E value, 0.0) to the KatA catalase (GenBank accession number YP_208798.1) of *N. gonorrhoeae*. Because bacterial catalases (e.g., KatA) and peroxidases (e.g., AhpCF) are involved in the ability to resist killing by H₂O₂, it was of interest to determine whether the expression of these genes in *M. catarrhalis* would be affected by exposure of the bacterium to exogenous H₂O₂. Wild-type *M. catarrhalis* O35E cells were exposed to increasing concentrations of H₂O₂, and the transcriptional responses of these three genes were measured by real-time RT-PCR. All three of the genes tested demonstrated a dose-dependent transcriptional response to the increasing concentrations of H₂O₂, with *kata* exhibiting the greatest response (Fig. 5).

Oxidative stress response of *M. catarrhalis* O35E as measured by DNA microarray analysis. DNA microarray analysis

FIG. 5. Transcriptional responses of the *M. catarrhalis* O35E *ahpC*, *ahpF*, and *katA* genes to increasing concentrations of exogenous H₂O₂ as measured by real-time RT-PCR. Cultures of wild-type *M. catarrhalis* O35E growing in BHI broth were exposed to increasing concentrations of H₂O₂ (10, 20, and 50 mM), and total RNA was extracted and utilized in real-time RT-PCRs with primer pairs specific for the *M. catarrhalis* *ahpC* (filled bars), *ahpF* (open bars), or *katA* (shaded bars) gene. These data are reported as the fold change in transcription from that of *M. catarrhalis* cells that were exposed to water instead of H₂O₂.

was utilized to measure the relative changes in the transcriptome of strain O35E after exposure to 50 mM H₂O₂. The 30 genes most up- or downregulated in response to H₂O₂ exposure are listed in Table S1 in the supplemental material. Real-time RT-PCR was utilized to measure the expression patterns of a selected group of genes that DNA microarray analysis indicated were affected by exposure to H₂O₂ (see Fig. S2 in the supplemental material). There was good correlation between the values obtained by DNA microarray analysis and those derived from real-time RT-PCR (R^2 , 0.93).

The genes most upregulated by H₂O₂ in these wild-type cells included those encoding a hypothetical protein (MC701), isocitrate lyase, and malate synthase, the KatA catalase, the alkyl hydroperoxide reductase CF, and aconitate hydratase (see Table S1 in the supplemental material). Among those genes most downregulated by H₂O₂ were two encoding proteins involved in phosphate uptake, three encoding hypothetical proteins, and one encoding bacterioferritin (see Table S1 in the supplemental material).

In an additional set of DNA microarray experiments, both the wild-type strain O35E and the O35EΔoxyR mutant were exposed to H₂O₂ in order to identify genes whose regulation was dependent on the presence of a functional OxyR protein. Expression of OxyR was found to be essential for the pronounced upregulation of genes (MC701 and MC1128) encoding two hypothetical proteins, as well as for that of the genes encoding *ahpC*, *ahpF*, and *katA* (Table 3). Real-time RT-PCR analysis of selected genes (Fig. 6) identified in this second set of DNA microarray experiments again showed good correlation between the two sets of data (R^2 , 0.92). Interestingly, no genes were found to be downregulated by OxyR under these oxidative stress conditions.

Construction and characterization of mutations in ORFs MC701 and MC1128. The very marked increase in the expression of MC701 and MC1128 under conditions of oxidative stress (Table 3) prompted the construction of O35E deletion mutants unable to express the proteins encoded by these two genes. However, when tested in the disk diffusion assay for their sensitivity to killing by H₂O₂, these two mutants exhibited

TABLE 3. OxyR-dependent *M. catarrhalis* gene expression under conditions of oxidative stress^a

ORF	Description	Median log ₂ ratio of expression with/without oxyR ^b	SD
MC701	Hypothetical protein	7.89	0.82
MC1128	Protein of unknown function	6.25	0.07
MC165	Probable alkyl hydroperoxide reductase subunit F	5.46	0.43
MC163	Putative alkyl hydroperoxide reductase, subunit C	4.97	0.14
MC1139	KatA	4.22	0.17
MC702	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	3.01	0.12
MC286	Probable UDP-3-O-acetyl N-acetylglucosamine deacetylase	2.55	0.21
MC1373	Ferric uptake regulation protein	2.33	0.13
MC1390	GTP diphosphokinase	2.22	0.07
MC351	OsmC-like protein	1.98	0.12
MC1362	Ferrochelatase	1.54	0.18
MC734	Probable glutamyl-tRNA reductase	1.48	0.09
MC903	Transcriptional regulator, BadM/Rrf2 family	1.29	0.09
MC1922	Glutamate synthase (NADPH)	1.26	0.10
MC907	Cochaperone Hsc20	1.26	0.09
MC906	Iron-sulfur cluster assembly protein IscA	1.23	0.08
MC905	FeS cluster assembly scaffold IscU	1.15	0.05
MC904	Cysteine desulfurase IscS	1.15	0.10

^a This table includes all of the ORFs with altered expression after exposure to 50 mM H₂O₂.

^b Median log₂ ratio of expression in *M. catarrhalis* O35E to expression in *M. catarrhalis* O35EΔoxyR when both strains were exposed to 50 mM H₂O₂. The data represent three independent experiments ($P < 0.05$).

levels of resistance equivalent to that of the wild-type O35E strain (data not shown).

H₂O₂ decomposition by wild-type, mutant, and recombinant strains of *M. catarrhalis*. Incubation of cell suspensions of wild-type *M. catarrhalis* O35E (Fig. 7, bar 1) and the O35EΔoxyR mutant (Fig. 7, bar 2) with 10 μM H₂O₂ resulted in degradation of H₂O₂ to levels that did not differ between these two strains. In contrast, the O35EΔkatA mutant (Fig. 7, bar 3)

FIG. 6. Comparison of selected DNA microarray data with real-time RT-PCR results for gene expression affected by the presence of H₂O₂ and OxyR. Shown are the effects of the presence of 50 mM H₂O₂ on the expression of selected genes by strain O35E in the presence and absence of the oxyR gene product, as measured by DNA microarray analysis (filled bars) and real-time RT-PCR (open bars).

FIG. 7. Abilities of different *M. catarrhalis* strains and constructs to scavenge H₂O₂. H₂O₂ was added to a final concentration of 10 μM to $\sim 1.6 \times 10^7$ CFU of bacteria and was incubated for 1 min at room temperature. A nonsignificant difference in the scavenging of H₂O₂ was seen between *M. catarrhalis* O35EΔoxyR (bar 2) and the wild-type strain O35E (bar 1). A statistically significant reduction in the scavenging of H₂O₂ was observed for O35EΔkatA (*, $P = 0.01$) (bar 3). Significant increases in the scavenging of H₂O₂ by O35EΔahpC (#, $P = 0.002$) (bar 4) and O35EΔkatA(pTH61-1) (bar 5) (##, $P = 0.05$) over that by the wild-type strain O35E were observed. O35EΔkatA(pWW115) (bar 6) degraded H₂O₂ to about the same extent as the ΔkatA mutant.

scavenged little or no H₂O₂ in the same time period. Provision of a wild-type *katA* gene in *trans* allowed the recombinant strain O35EΔkatA(pTH61-1) (Fig. 7, bar 5) to degrade H₂O₂ more rapidly (P , 0.05) than its wild-type parent strain (Fig. 7, bar 1). The presence of the pWW115 cloning vector in the O35EΔkatA mutant (Fig. 7, bar 6) did not significantly alter the ability of this mutant to scavenge H₂O₂. The O35EΔahpC mutant (Fig. 7, bar 4) exhibited an ability to scavenge H₂O₂ that was greater (P , 0.002) than that of the wild-type parent strain (Fig. 7, bar 1) and equivalent to that of the recombinant strain O35EΔkatA(pTH61-1) (Fig. 7, bar 5).

Effects of the *oxyR*, *ahpC*, and *katA* mutations on the growth response to exogenous H₂O₂ and on biofilm formation. *M. catarrhalis* O35E forms very poor biofilms in a crystal violet-based biofilm assay system (36). To determine whether any of the mutations described above had an effect on biofilm development, ΔoxyR, ΔahpC, and ΔkatA mutants were constructed in *M. catarrhalis* ETSU-4, which exhibits robust biofilm development in this assay system. The effect of the addition of 50 mM H₂O₂ (final concentration) to cells of these strains growing in broth was examined first, and it was found that only the growth of the ETSU-4ΔkatA mutant (Fig. 8A) was markedly inhibited by this concentration of H₂O₂. After an additional 3.5 h of growth, a portion of this ETSU-4ΔkatA broth culture was plated on BHI agar; no viable cells were present (data not shown). Both the wild-type ETSU-4 strain and the ETSU-4ΔahpC mutant exhibited little or no change in the growth rate after the addition of H₂O₂, while the ETSU-4ΔoxyR mutant showed only a modest decrease in its rate of growth (Fig. 8A). Provision of a wild-type O35E *katA* gene (in pTH61-1) in *trans* in the ETSU-4ΔkatA mutant resulted in a wild-type-like re-

FIG. 8. Effects of *oxyR*, *ahpC*, and *katA* mutations on the growth response to exogenous H₂O₂ and on biofilm formation. (A) Growth response of wild-type *M. catarrhalis* ETSU-4 (●), the ETSU-4ΔoxyR mutant (□), the ETSU-4ΔahpC mutant (*), the ETSU-4ΔkatA mutant (Δ), ETSU-4ΔkatA(pTH61-1) (■), and ETSU-4ΔkatA(pWW115) (○) after the addition of H₂O₂ to a final concentration of 50 mM. H₂O₂ was added to each culture when it reached an OD₆₀₀ of 0.7. No viable *M. catarrhalis* ETSU-4ΔkatA cells were isolated from the culture at the end of the experiment. (B) Measurement of biofilm formation by wild-type *M. catarrhalis* ETSU-4 (bar 1), *M. catarrhalis* ETSU-4ΔoxyR (bar 2), *M. catarrhalis* ETSU-4ΔkatA (bar 3), and *M. catarrhalis* ETSU-4ΔahpC (bar 4) in the crystal violet-based biofilm assay. Bar 5 shows the result obtained from a mock (uninoculated) well.

sponse to H₂O₂, whereas the presence of the plasmid vector pWW115 in the same mutant did not change its response to H₂O₂ (Fig. 8A). When the ETSU-4 wild-type strain and its *oxyR*, *katA*, and *ahpC* mutants were used in the crystal violet-based biofilm system, there was no apparent difference between the wild-type strain and any of these three mutants (Fig. 8B).

DISCUSSION

Evaluation of the sensitivity of *M. catarrhalis* O35E to oxidative stress revealed remarkable resistance to killing by exogenous H₂O₂, a result reinforcing that reported by Weiser and colleagues (38), who indicated that an isolate of *M. catarrhalis* (strain Bc1) was more resistant to killing by H₂O₂ than either *S. pneumoniae* or *H. influenzae*. The levels of resistance to killing by H₂O₂ were very similar among the four *M. catarrhalis* wild-type strains tested in the present study, indicating that *M. catarrhalis* possesses a well-conserved and robust set of factors for dealing with oxidative stress. This relatively high innate level of resistance to exogenous oxidative stress may be a reflection of the environmental niche in which *M. catarrhalis* typically resides, specifically the nasopharyngeal mucosa and,

during times of exacerbation, the lungs of adult patients with COPD. A recent study of patients with COPD demonstrated that at baseline, these patients exhaled a larger amount of H_2O_2 than healthy controls, and the level of exhaled H_2O_2 rose significantly at the time of an exacerbation (3). It should also be noted that freshly prepared BHI liquid medium was utilized for all of the experiments in the present study. Bacteriological media can undergo oxidation during storage, resulting in the endogenous production of 1 to 20 μM H_2O_2 (10, 15). This finding raises the possibility that H_2O_2 in the culture medium could induce the bacterial oxidative stress response prior to the addition of exogenous H_2O_2 . This, in turn, could result in an artificially increased level of resistance to killing by H_2O_2 .

This high level of H_2O_2 resistance among different *M. catarrhalis* strains led to genome-wide screening for gene products relevant to oxidative stress. BLAST analysis of the *M. catarrhalis* ATCC 43617 (60) genome first identified an ORF encoding a protein with similarity to OxyR, a LysR family transcriptional regulator first studied in *E. coli* (7, 51). In both this enteric organism and other Gram-negative bacterial pathogens, OxyR has been shown to be an important regulator of the bacterial response to oxidative stress (9, 11, 14, 24). OxyR is a redox-sensitive transcriptional regulator in *E. coli* that has two functional domains (28, 33). The N-terminal domain contains a helix-turn-helix motif that is responsible for binding DNA (21). The C-terminal domain (i.e., the “regulatory domain”) resembles periplasmic binding components of ABC transporters. In this part, there are two cysteine residues that are critical for the function of the protein. The sulfhydryl group of one of the cysteines (C199 in *E. coli* OxyR) is susceptible to oxidation under oxidative stress (61). Oxidation of this moiety precipitates the formation of a disulfide bridge between the oxidized residue and the side chain of the second cysteine (C208 in *E. coli* OxyR). This conformational rearrangement is presumably the structural signal that changes the DNA-binding characteristics of the protein (5, 61). The two cysteines (C200 and C209 in *M. catarrhalis* OxyR) are conserved between these two proteins.

Additional analysis of the *oxyR* locus in *M. catarrhalis* O35E revealed two genes with homology to *ahpF* and *ahpC*, in close proximity to *oxyR* (Fig. 1). In some other bacteria, these two genes encode proteins that constitute an alkyl hydroperoxide reductase system (39) shown to be important for the handling of oxidative stress (34, 43, 53). Finally, a gene (*katA*) encoding a catalase homolog was identified via genome screening, providing additional evidence that *M. catarrhalis* has the potential to mount an effective response to oxidative stress. The genome of *M. catarrhalis* strain RH4 was reported not to contain an *oxyR* homolog (8), but our BLAST analysis indicated that RH4 contains an ORF encoding a protein that is 99.6% identical to the O35E OxyR protein. The studies described here demonstrate that there is an OxyR protein encoded by all of the strains of *M. catarrhalis* utilized in this study and that it is involved in the regulation of a coordinated gene response to combat oxidative stress.

The growth defect seen with the *M. catarrhalis oxyR* mutant (Fig. 3B) indicates that OxyR is also important for the bacterial response to physiologic levels of H_2O_2 that are generated by the bacterium during normal aerobic growth. Rapid cellular metabolism occurs during logarithmic bacterial growth, and it

is possible that significant amounts of endogenous oxidative stressors are generated, although the predominant source of these remains to be determined (44). It is likely that the growth of the *oxyR* mutant in broth (Fig. 3B) is diminished due to its decreased ability to deal with this endogenously generated oxidative stress. While it is likely that this growth defect is caused by the altered ability of the *oxyR* mutant to degrade H_2O_2 , the studies reported here do not exclude the possibility that the lack of OxyR could have other deleterious effects on the growth of *M. catarrhalis*. Additionally, the increased susceptibility of the *oxyR* mutant to exogenously provided H_2O_2 in the disk diffusion studies (Fig. 3C) shows that the expression of *oxyR* is important for the bacterial response to exogenous H_2O_2 .

The *M. catarrhalis oxyR* mutant was also unable to form individual, isolated colonies on agar medium when plated at a density less than 1×10^6 CFU/ml (Fig. 4). A similar phenotype has been reported for *oxyR* mutants of both *P. aeruginosa* (10) and *Klebsiella pneumoniae* (11). The addition of exogenous catalase to the medium allowed the *M. catarrhalis oxyR* mutant to form single colonies at levels equivalent to those obtained with the wild-type parent strain. While several theories have been proposed for this cellular density threshold effect (10, 11), the ability of exogenous catalase to abrogate this particular mutant phenotype indicates that the ability to increase expression of the KatA catalase (or other antioxidant factors) is necessary for individual colony formation by *M. catarrhalis* below a certain cell density. As suggested by Hassett et al. (10), catalase could be released from lysed bacteria and could then protect other bacterial cells from H_2O_2 in the growth environment.

Increasing levels of exogenous H_2O_2 resulted in an apparent dose-dependent increase in the transcription of three *M. catarrhalis* genes predicted to encode proteins involved in the decomposition of H_2O_2 : *katA*, *ahpC*, and *ahpF*. The ability to modulate the expression of genes encoding products involved in resistance to oxidative stress is likely the result of a need for *M. catarrhalis* to respond to rapidly changing environmental conditions, including the influx of immune cells, increases in the numbers of competing bacteria that can produce H_2O_2 , and elevated levels of exhaled oxidants in the lungs of COPD patients. While the genes described above are known to be important for the bacterial response to oxidative stress in other bacterial systems, it was reasonable to assume that *M. catarrhalis* encoded additional genes that are involved with the coordinated effort to resolve oxidative stress. Therefore, DNA microarray technology was utilized to evaluate the transcriptome of *M. catarrhalis* in response to exogenous H_2O_2 . The use of H_2O_2 as a source of exogenous oxidative stress has been utilized previously to study the transcriptomes of *E. coli* (62), *N. gonorrhoeae* (45), and *H. influenzae* (9).

DNA microarray analysis indicated that several types of genes were upregulated by wild-type *M. catarrhalis* in the presence of oxidative stress. Two primary groups of genes were identified as being highly upregulated in the presence of a functional OxyR protein: (i) those encoding products involved with the direct breakdown of oxidative stress factors (e.g., *ahpCF*, *katA*, and MC2, encoding a probable organic hydroperoxide resistance protein) and (ii) those encoding products important for the regulation and uptake of iron and the subse-

quent bacterial management of iron stores (e.g., MC905 and MC906, encoding proteins involved in iron-sulfur cluster assembly; MC1362, encoding a ferrochelatase; and MC1373, encoding Fur). The upregulation of genes involved with oxidative stress metabolism and iron-sulfur handling has been seen previously in the oxidative stress responses of other bacteria, including *H. influenzae* (9), *N. gonorrhoeae* (45), and *E. coli* (62). In general, the degree of downregulation of genes by addition of H_2O_2 was not as pronounced as that seen with upregulated genes in the wild-type strain (see Table S1 in the supplemental material).

Interestingly, the *M. catarrhalis* ORFs encoding isocitrate lyase and malate synthase were among the genes most upregulated by oxidative stress (see Table S1 in the supplemental material). These two enzymes constitute the glyoxylate bypass of the tricarboxylic acid cycle (20), which functions in an anaplerotic manner. A recent study of the ability of *Pseudomonas fluorescens* to resist oxidative stress indicated that these two enzymes formed part of a metabolic network that produces NADPH at the expense of NADH, a prooxidant (47). Whether the increased transcription of these two genes in *M. catarrhalis* cells undergoing oxidative stress indicates similar roles for these enzymes in *M. catarrhalis* is not known. Additionally, two other unlinked genes (MC701 and MC1128) encoding hypothetical proteins were very highly upregulated, but inactivation of these two genes did not detectably affect the ability of *M. catarrhalis* to resist killing by exogenous H_2O_2 (data not shown).

The studies described above provide the first description of gene products involved in the robust ability of *M. catarrhalis* to deal with oxidative stress. Why *M. catarrhalis* is so much more resistant to killing by H_2O_2 than other pathogens that colonize the nasopharynx is not apparent but could involve either more catalytically active antioxidant enzymes or a much higher level of expression of one or more antioxidant enzymes. The clinical relevance of this finding is likely based on the need for this bacterium to colonize and survive in several hostile *in vivo* environments, including the nasopharynx and the respiratory tracts of adult patients with COPD. Further studies will be necessary to elucidate the extent of the OxyR regulon and to determine the molecular basis for the impressive ability of *M. catarrhalis* to resist killing by H_2O_2 .

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