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Expression of the Oligopeptide Permease Operon of *Moraxella catarrhalis* Is Regulated by Temperature and Nutrient Availability

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***Moraxella catarrhalis* causes otitis media in children and exacerbations of chronic obstructive pulmonary disease in adults. Together, these two conditions contribute to enormous morbidity and mortality worldwide. The oligopeptide permease (*opp*) ABC transport system is a nutritional virulence factor important for the utilization of peptides. The substrate binding protein OppA, which binds peptides for uptake, is a potential vaccine antigen, but little was known about the regulation of gene expression. The five *opp* genes *oppB*, *oppC*, *oppD*, *oppF*, and *oppA* are in the same open reading frame. Sequence analysis predicted two promoters, one located upstream of *oppB* and one within the intergenic region between *oppF* and *oppA*. We have characterized the gene cluster as an operon with two functional promoters and show that cold shock at 26°C for ≤0.5 h and the presence of a peptide substrate increase gene transcript levels. Additionally, the putative promoter upstream of *oppA* contributes to the transcription of *oppA* but is not influenced by the same environmental cues as the promoter upstream of *oppB*. We conclude that temperature and nutrient availability contribute to the regulation of the Opp system, which is an important nutritional virulence factor in *M. catarrhalis*.**

Moraxella catarrhalis is an increasingly important human-specific pathogen contributing to worldwide morbidity and mortality that has transitioned from an emerging to an established pathogen (1–3). Otitis media in children is the primary cause of new antibiotic prescriptions and pediatric office visits, with *M. catarrhalis* accounting for 10% to 20% of acute otitis media episodes (2, 4–6). Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States, with *M. catarrhalis* contributing to at least 10% of exacerbations (7–11). The socioeconomic burden of otitis media and COPD is significant, with an estimated $50 billion dollars annually in health care expenses globally (5, 9, 12). In view of the morbidity and health care cost associated with *M. catarrhalis*, it is important to understand the mechanisms of pathogenesis in order to guide development of novel approaches to treatment and prevention (2, 13, 14). In previous work, we identified OppA as a promising vaccine antigen and a nutritional virulence factor for *M. catarrhalis* (15, 16).

We previously characterized the oligopeptide permease (*opp*) gene cluster as an ABC transport system vital for the utilization of peptide substrates. The gene cluster encodes two permeases, OppB and OppC; two ATPases, OppD and OppF; and a substrate binding protein, OppA (16). We hypothesized that this gene cluster was transcribed as an operon and that environmental factors, temperature and essential nutrients available as peptides, would alter the rate of transcription.

The lower respiratory tract has a normal body temperature of 37°C, while the upper airways, specifically the nasopharynx, where *M. catarrhalis* first colonizes, ranges from 34°C at room temperature (25°C) down to 26°C after a short time of breathing air near 0°C, a temperature which many of the temperate climates throughout the world experience regularly throughout the winter months (2, 17–22). Exposure to cold shock at 26°C has an important impact on virulence factors and transcriptional regulation in *M. catarrhalis* (23–25).

Little is known about the transcriptional regulation of *M. catarrhalis*, particularly in the complex and changing environment of the human respiratory tract, the ecological niche of *M. catarrhalis* (13, 26). Based on analysis of the sequence in the *opp* gene cluster, we hypothesize that a promoter region upstream of *oppB* is responsible for transcription of the putative operon. There was also a predicted promoter region upstream of *oppA*. ABC transport systems often have altered transcription of the substrate binding protein compared to the rest of the genes in the system (27, 28). We hypothesize that this secondary promoter may alter the transcription of *oppA* independently of the other *opp* genes.

The goal of this study was to characterize the gene expression of the *opp* cluster and to determine the effect of temperature and nutrient availability on the expression of these genes. A second promoter within this putative operon that may contribute to gene expression and have secondary regulatory mechanisms would also be a novel observation for *M. catarrhalis* and contribute to the understanding of how this pathogen thrives under the hostile conditions of the human respiratory tract. We show that transcription of the *opp* operon is upregulated during cold shock at 26°C in as little as 30 min and that the secondary promoter upstream of *oppA* is influenced by the presence of a peptide substrate.

MATERIALS AND METHODS

Bacterial strains and growth. Wild-type (WT) *M catarrhalis* strain O35E, a prototype otitis media strain previously isolated from the middle ear

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TABLE 1 Oligonucleotide primer sequences

Primer	Gene(s)[a]	Experiment(s)	Direction	Sequence
oppBRealF	*oppB*	RT-PCR and qRT-PCR	Forward	TTGGGCGTTGGTTGGTTCTG
oppBRealR	*oppB*	RT-PCR and qRT-PCR	Reverse	CCTTGTCCTTGTGTAATCACCTGC
oppCRealF3	*oppC*	RT-PCR and qRT-PCR	Forward	GTTGTTGTAGGTGCGCTGTG
oppCRealR3	*oppC*	RT-PCR and qRT-PCR	Reverse	GAACCCGAATGGTAAAAGCA
oppDRealF	*oppD*	RT-PCR and qRT-PCR	Forward	GCCATCCTTGTACTCGCCTA
oppDRealR	*oppD*	RT-PCR and qRT-PCR	Reverse	GCAAATCACAGATCGCTTCA
oppFRealF	*oppF*	RT-PCR and qRT-PCR	Forward	TTAGTTGGTGAATCAGGCAGTGG
oppFRealR	*oppF*	RT-PCR and qRT-PCR	Reverse	GGCAGGGTCTTGGAAAATCATC
oppARealF	*oppA*	RT-PCR and qRT-PCR	Forward	CCAATAGCACAAAAACGACAGAGC
oppARealR	*oppA*	RT-PCR and qRT-PCR	Reverse	CCATCGGCAGACACAAAAGTTG
gyrBRealF	*gyrB*	qRT-PCR	Forward	TTGCCAAGAAAAAGACCCCG
gyrBRealR	*gyrB*	qRT-PCR	Reverse	TAATCAGTGTCCCCACCTCAGC
BCsmF1	*oppB-C*	RT-PCR	Forward	ATTTACAAAAAGCACTT
BCsmR1	*oppB-C*	RT-PCR	Reverse	CAAATATAGCGCTTAGAA
CDsmF1	*oppC-D*	RT-PCR	Forward	TTGCCAGTTTATATCTCCTA
CDsmR1	*oppC-D*	RT-PCR	Reverse	GGTAAGTTTGGTTAAATCT
DFsmF1	*oppD-F*	RT-PCR	Forward	CCTTGTACTCGCCTATT
DFsmR1	*oppD-F*	RT-PCR	Reverse	TGTTATTAAGTCCGATA
FAsmF1	*oppF-A*	RT-PCR	Forward	AGACCCGATACTTGAGCGTA
FAsmRa	*oppF-A*	RT-PCR	Reverse	AAAGATTTTGGGTCACCTGA
BsmF1	*oppB*	RT-PCR	Forward	CAGCAGGTTGGGCG
BsmR1	*oppB*	RT-PCR	Reverse	CTACCAGCAAAACGGA
AsmF1	*oppA*	RT-PCR	Forward	ATACCGATGGCTCAGATCC
AsmR1	*oppA*	RT-PCR	Reverse	TCACGCCTTGAGCTTCTAA
prmF1.5	*oppB* promoter	Mutant construction	Forward	CCAAATAACGACGAAACCAAAT
prmF1.3	*oppB* promoter	Mutant construction	Reverse	TCTAGATTATCTGTCAACCTTAAATGGTCA
prmF2.5	*oppB* promoter	Mutant construction	Forward	AAGGTTGACAGATAATCTAGAATAAAAT
prmF2.3	*oppB* promoter	Mutant construction	Reverse	ATCATTTTATCTTATAATTTTTTTAATCTGT
prmF3.5	*oppB* promoter	Mutant construction	Forward	AAAATTATAAGATAAAATGATAAAATCTTGC
prmF3.3	*oppB* promoter	Mutant construction	Reverse	CAATCTTAATGGGTTCTCGTCCT

[a] A hyphenated designation refers to the region spanning the two genes.

fluid of a child with otitis media in Dallas, TX, was provided by Eric Hansen. Bacteria were grown on brain heart infusion (BHI) agar plates at 35°C with 5% CO_2 overnight or in BHI broth at 37°C with shaking at 225 rpm, unless otherwise indicated. Chemically defined medium (CDM) is a minimal nutrient medium containing the essential elements that *M. catarrhalis* requires to grow (29, 30). For cold shock growth, bacteria were resuspended in BHI broth to an optical density at 600 nm (OD_{600}) of 0.07 from a plate and grown to an OD_{600} of 0.3 at 37°C with shaking at 225 rpm. Cultures were then shifted to 26°C with the same shaking for 0.5, 1, 2, and 3 h.

For reverse transcriptase PCR (RT-PCR), bacteria were resuspended at an OD_{600} of 0.07 and grown to an OD_{600} of 0.9. For quantitative real-time PCR (qRT-PCR) to assess the influence of temperature and nutrient availability on gene expression, bacteria from a culture grown overnight were washed with phosphate-buffered saline (PBS), resuspended to an OD_{600} of 0.07 in 10 ml of fresh medium (BHI broth, CDM containing free arginine, or CDM with no free arginine and with the peptide VANRP [0.25 mg/ml] as the only source of arginine) (16), and grown at 26°C, 30°C, 34°C, and 37°C to an OD_{600} of 0.8. For all RNA isolations, bacteria were treated with RNAprotect (Qiagen) and frozen at −80°C until needed.

Construction of mutants. A mutant was constructed in which a 500-bp region including 200 bp upstream and 300 bp downstream of the *oppB* start site surrounding the promoter region was knocked out through the use of overlap extension PCR and homologous recombination, as previously described (15, 30). Briefly, the transforming DNA for the promoter mutant was composed of 3 overlapping fragments that included 800 bp upstream of the putative promoter region of *oppB*, a spectinomycin resistance cassette, and 800 bp downstream of the first 300 bp of *oppB*, using the oligonucleotide primers listed in Table 1 (15, 16). The mutant (called *prm* mutant) was verified by PCR and sequencing.

Reverse transcriptase PCR. Thawed bacterial pellets were resuspended in 100 μl of lysozyme (1 mg/ml) and incubated for 10 min at room temperature. RNA was isolated with the addition of RLT buffer (350 μl) (RNeasy Mini-Prep; Qiagen, Valencia, CA), vortexed until clear, applied onto a Qia Shredder column, and centrifuged for 2 min at 14,000 × *g*. The lysate was mixed with 70% ethanol and applied onto a miniprep column (Qiagen RNeasy Mini-Prep). RNA was further purified according to the RNeasy Mini-Prep instructions, beginning at the RPE buffer wash step. DNA contamination was removed according to instructions provided with the Promega DNase kit (Promega, Madison, WI). Clean RNA was frozen at −80°C until use. Reverse transcriptase PCR was performed with the Qiagen One-Step RT-PCR kit according to the manufacturer's instructions, with 50 ng of RNA per reaction mixture.

Quantitative real-time PCR. Thawed bacterial pellets were resuspended in 500 ml RNA Wiz (RiboPure RNA purification kit for bacteria; Ambion-Life Technologies, Grand Island, NY). RNA was further isolated by chloroform extraction (0.2× volume; 100 μl) and centrifuged for 5 min at 4°C at 14,000 × *g*. The top aqueous layer (~200 μl) was mixed with 0.5× 100% ethanol (100 μl), further purified with RiboPure spin columns according to the manufacturer's instructions, and eluted with 40 μl of elution solution (Ribopure kit) heated to 95°C. Residual DNA contamination was eliminated from 10 μg of RNA with the Promega DNase kit according to the manufacturer's instructions, further purified with a Qiagen RNA minikit according to the manufacturer's instructions, and eluted with 40 μl of H_2O. RNA was quantitated with a NanoDrop instrument (Thermo Scientific, Wilmington, DE) and frozen at −80°C in 1-μg aliquots until use. Integrity was assessed by electrophoresis through a 2% agarose denaturing (*N*-morpholino)propanesulfonic acid (MOPS) gel.

RNA (1 μg) was converted to cDNA by using the iScript cDNA con-

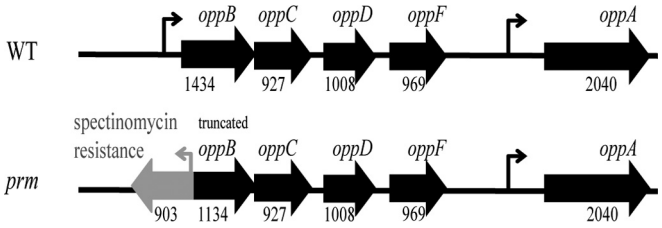

FIG 1 Diagram of the *opp* operon in the wild-type (WT) strain and deleted segments in the *prm* mutant (not to scale). Numbers denote base pairs, and small arrows indicate promoter regions. Black arrows denote open reading frames, and gray arrows denote the drug resistance cassette.

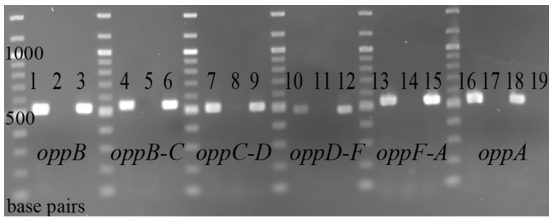

FIG 2 Results of reverse transcriptase PCR showing that the *opp* genes are transcribed as an operon. RNA was isolated from wild-type O35E cultures grown to late log phase in brain heart infusion medium. Primers were designed to span the end of one gene through the beginning of another (intergenic region) or the middle of each gene, as indicated. Lanes 1, 4, 7, 10, 13, and 16 are RNA with reverse transcriptase and polymerase. Lanes 2, 5, 8, 11, 14, and 17 are RNA with no reverse transcriptase and only polymerase. Lanes 3, 6, 9, 12, 15, and 18 are DNA with polymerase. Lane 19 has no template with reverse transcriptase and polymerase.

version kit (Bio-Rad, Berkeley, CA) according to the manufacturer's instructions (25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and a 4-min hold). Immediately following the PCR, the cDNA template (1 μl; 50 ng) was added to 10 μl Sybr green master mix (Bio-Rad) with primers (1 μl of 2.5 μM stock) and H_2O (7 μl) to a final volume of 20 μl according to the manufacturer's instructions for qRT-PCR in a CFX 384 or CFX Connect machine (Bio-Rad). Primers were designed to amplify a 150- to 190-bp product for *oppB*, *oppC*, *oppD*, *oppF*, *oppA*, and the gyrase B housekeeping gene (*gyrB*) (31). The resulting quantification cycle (C_q) values were converted to SQ values based on a standard curve. Transcript quantities were normalized to *gyrB* levels and displayed as fold changes in relation to *gyrB* expression. Statistical significance was determined by performing a *t* test, with a *P* value of <0.5 being considered significant.

Assessment of bacterial growth. Growth curves were performed by using the BioscreenC automated growth curve analysis system (Oy Growth Curves AB Ltd., Helsinki, Finland), as previously described (16). Briefly, growth curves were performed with a 200-μl inoculum of WT and *prm* mutant cultures grown overnight, washed in PBS, and diluted 1:100 in CDM with free arginine or CDM with no free arginine supplemented with the peptide triornithine (VWR, Radnor, PA) at a final concentration of 0.25 mg/ml. The experiment was performed with 5 replicate wells, with OD_{600} measurements being taken at 1-h intervals at 37°C with constant shaking (machine settings, high speed and high amplitude).

RESULTS

The *opp* gene cluster is transcribed as an operon. Five oligopeptide permease (*opp*) genes are present in the same open reading frame and have no other homologues in the *M. catarrhalis* genome (Fig. 1) (15, 32). Reverse transcriptase PCR was used to determine if these genes were being actively transcribed as an operon during growth in rich laboratory medium (BHI broth) at 37°C. The mRNA transcript regions between each gene were amplified from total RNA with primers designed to amplify an ~150-bp product spanning the entire intergenic region (Fig. 2). These primers amplified a product from the transcript including both the region upstream of the stop codon of the first gene and the region downstream of the start codon of the second gene (Table 1). There is no intergenic space between the 3′ end of *oppB* and the start of *oppC*, 73 bp between *oppC* and *oppD*, 58 bp between *oppD* and *oppF*, and 204 bp between *oppF* and *oppA* (Fig. 1). Given the much larger intergenic region between *oppF* and *oppA*, we designed a second primer set with no overlap with the first set to confirm the result. Both sets of primers amplified a PCR product from total RNA, indicating that the five *opp* genes are transcribed as an operon.

Regulation of *oppA* is influenced by a second promoter. Previous studies of ABC transport systems in bacteria have observed that the substrate binding protein genes are sometimes expressed at a higher level than the other genes in the operon (27). We identified a putative promoter region, using SoftBerry BPROM bacterial promoter prediction software (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb), in the 204-bp intergenic region between *oppF* and *oppA*. We hypothesized that this secondary promoter influences the transcription of *oppA* in comparison to the rest of the *opp* genes in the operon. To address this, we created a promoter mutant (*prm*) in which ~500 bp of sequence surrounding the promoter region upstream of *oppB*, including ~300 bp of the *oppB* gene, were replaced with a spectinomycin drug resistance gene with transcription oriented in the opposite direction of the operon (Fig. 1). This mutant was confirmed by PCR and sequencing.

Transcript levels of the *opp* genes in the WT and *prm* mutant grown in BHI broth to late log phase at 37°C were assessed by qRT-PCR and normalized to the level of the housekeeping gene *gyrB*, which had the same amount of transcript present under all conditions tested, including different temperatures (not shown). There was ~1.5- to 2-fold more expression of the first four *opp* genes, *oppB*, *-C*, *-D*, and *-F*, with no significant difference between any of them, but there was significantly more *oppA* transcript than *oppD* and *oppF* transcripts in the WT. The *prm* mutant had no *oppB*, *-C*, and *-D* transcripts, while there were low but detectable levels of *oppF* transcript (Fig. 3). Levels of *oppA* transcript were significantly higher ($P < 0.0014$) than those of all the other *opp* genes in the *prm* mutant. The experiment was also conducted at

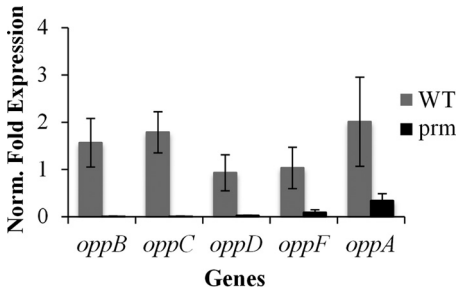

FIG 3 Results of quantitative real-time PCR to determine the levels of the *oppB*, *oppC*, *oppD*, *oppF*, and *oppA* transcripts in the *prm* mutant compared to wild-type levels. RNA was isolated from bacterial cultures grown from an OD_{600} 0.07 to an OD_{600} of 0.8 at 37°C. Transcript quantity was normalized to the level of the housekeeping gene *gyrB*. All results are the averages of results from 3 separate experiments performed in triplicate. Error bars represent standard errors of the means from 3 biological replicates. See the text for statistical analysis.

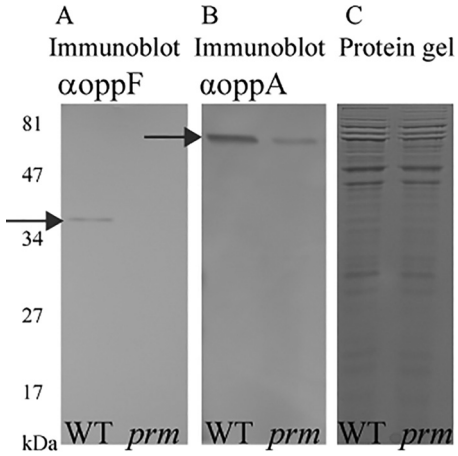

FIG 4 Characterization of mutants. (A) Immunoblot assay of wild-type and *prm* whole bacterial lysates probed with OppF antiserum (1:10,000). OppF is ~36 kDa, as indicated by the arrow. (B) Immunoblot assay of wild-type and *prm* whole bacterial lysates probed with OppA antiserum (1:1,000,000). OppA is ~75 kDa, as indicated by the arrow. A secondary anti-rabbit horseradish peroxidase conjugate was used at a 1:2,000 dilution for detection for both immunoblots. (C) Coomassie blue-stained SDS-PAGE gel of the 2 whole bacterial lysates showing equal protein loading. Molecular mass markers (in kilodaltons) are indicated on the left.

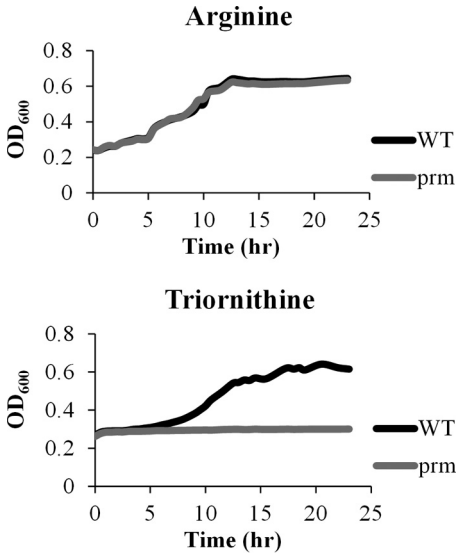

FIG 5 Growth curves of wild-type strain O35E and the *prm* knockout mutant in chemically defined medium broth supplemented with arginine (top) or triornithine (bottom). The *x* axis is time in hours, and the *y* axis is the optical density at 600 nm. Each point is the average of data from 5 wells.

26°C, with the same results (not shown). An immunoblot assay was performed to assess the presence of the OppF and OppA proteins in the WT compared to the *prm* mutant by using polyclonal rabbit antisera previously produced against recombinantly expressed proteins (15, 16). OppA is present in the *prm* mutant but at a visibly lower level than that in the WT, while OppF was undetectable (Fig. 4). Collectively, these data indicate that all *opp* genes are transcribed as an operon and that *oppA* has a separate promoter that influences transcription.

We hypothesized that without active transcription and, therefore, translation, the *prm* mutant would be unable to incorporate the peptide substrate triornithine (16). Arginine is essential for *M. catarrhalis* growth but can be replaced with ornithine, which is a downstream by-product of arginine metabolism (29, 33). Triornithine is toxic to *Escherichia coli* and has been used to screen for peptide uptake mutants (34). This molecule is taken up by the Opp system but is not toxic to *M. catarrhalis* and can be metabolized in place of arginine. A growth curve was performed to compare WT growth to the growth of the *prm* mutant in minimal medium where the only source of arginine was the triornithine peptide. The *prm* mutant did not grow, while the WT grew normally (Fig. 5). Both strains grew similarly when free arginine was present in the minimal medium, as expected. We conclude that the proteins encoded by the *oppB*, *-C*, *-D*, and *-F* genes are required for the utilization of a peptide substrate by *M. catarrhalis* and that the promoter upstream of *oppB* is responsible for their transcription.

Temperature alters *opp* gene transcript levels. The temperature of the human nasopharynx is variable, with measurements ranging from 37°C to 26°C (2, 17–22). Temperature has an important impact on virulence factors and transcriptional regulation in *M. catarrhalis* (23–25). When cultures were grown at 26°C, the transcript levels of all of the *opp* genes were significantly elevated compared to transcript levels at 37°C (Fig. 6). The levels of *oppB*, *-C*, *-D*, and *-F* were all increased ~2-fold, while the transcript levels of *oppA* were increased ~3-fold at 26°C compared to the transcript levels at 37°C. This observation supports our conclusion that the *opp* genes are transcribed as an operon and that *oppA* is influenced by a second promoter.

Time course of cold shock impact on *opp* gene transcript levels. Our previous experiments showed that growth at 26°C caused a significant increase in the transcript levels of all the *opp* genes. We were interested in determining the time course of cold shock causing an increase in transcription. Bacteria were grown to an OD_{600} of 0.3 and then subjected to cold shock at 26°C for 0.5, 1, 2, and 3 h, followed by RNA isolation and qRT-PCR. We examined the transcript levels of *oppA* and chose *oppB* to represent *oppC*, *-D*, and *-F*, since we have seen that these genes are expressed from the same promoter and at similar levels (Fig. 3 and 6). Transcript levels of *oppB* were significantly increased after cold shock at 26°C for 30 min compared to the transcript levels at 37°C. In contrast, *oppA* transcript levels did not increase until 1 h of cold shock (Fig. 7). These data suggest that cold shock for 30 min will increase transcription through the promoter upstream of *oppB*.

We next questioned if the promoter upstream of *oppA* was influenced by temperature in a manner similar to that of the promoter upstream of *oppB*. Based on previous experiments, we chose to examine the transcript level of *oppA* in the *prm* mutant after cold shock for 0.5 and 1 h. We also assayed transcript levels at 3 h to assess delayed responses. In contrast to the WT, the amount of *oppA* transcript did not increase with cold shock at 26°C compared to 37°C in the *prm* mutant, indicating that the promoter upstream of *oppA* is not influenced by temperature (Fig. 8).

Nutrient availability alters gene transcription. Previous work characterized this operon as being vital for the utilization of peptide substrates between 5 and 10 amino acids long (16).We hypothesized that the presence of peptide substrates would increase the transcription of this operon under nutrient-limiting conditions with only peptide substrates as the source of arginine, which

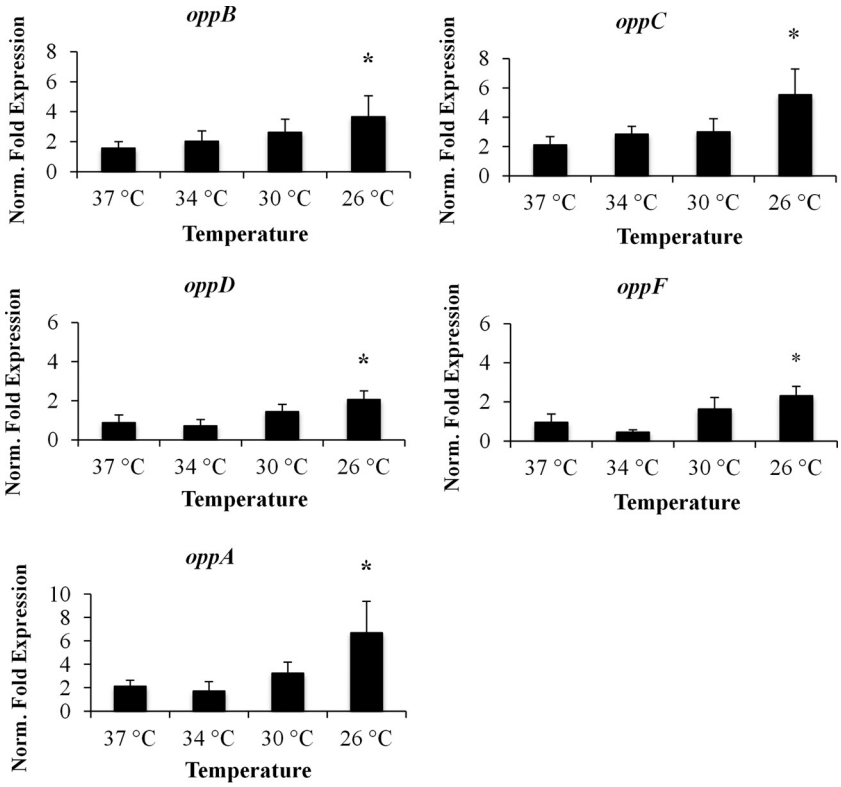

FIG 6 Temperature alters *opp* gene transcription during growth. Quantitative real-time PCR was performed on 50 ng of RNA per sample isolated from WT bacterial cultures grown to an OD_{600} of 0.8 at 37°C in BHI medium. Normalized transcript levels were statistically higher (*), as determined by a *t* test ($P < 0.05$), at 26°C than at 37°C for all the *opp* genes. Results are the averages of data from 3 experiments performed in triplicate, with standard deviations denoted with error bars.

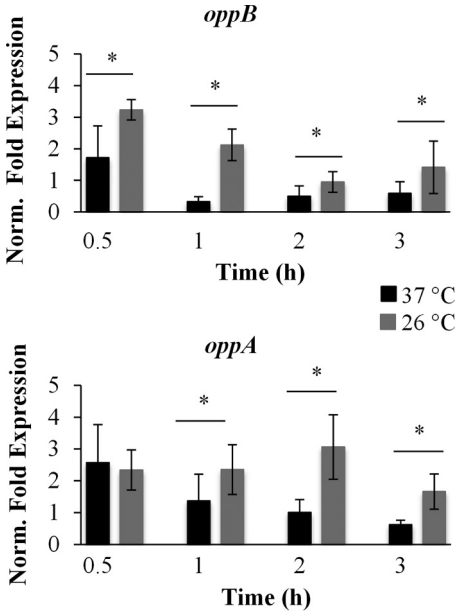

FIG 7 Impact of timed cold shock exposure on *oppB* and *oppA* gene transcription. Quantitative real-time PCR was performed on 50 ng of RNA per sample isolated from WT bacterial cultures grown to an OD_{600} of 0.3 at 37°C and subjected to growth at 26°C for set time intervals of 0.5, 1, 2, and 3 h. Transcript levels were normalized to the level of the housekeeping gene *gyrB*. Transcript levels of *oppB* and *oppA* at 37°C were compared with those at 26°C. Results are the averages of data from 3 experiments performed in triplicate, with standard deviations denoted with error bars, and statistical significance (*) was determined with a *t* test ($P < 0.05$).

is an essential amino acid for *M. catarrhalis* and a limiting nutrient in the respiratory tract (16, 35–38). Total RNA was isolated from WT bacteria grown from an OD_{600} of 0.07 to an OD_{600} of 0.8 at 37°C and 26°C in chemically defined medium containing free arginine (R) and defined medium with no free arginine supplemented with the peptide VANRP. The *opp* gene transcript quantities were evaluated by qRT-PCR. The presence of the peptide substrate as the sole source of arginine significantly increased the

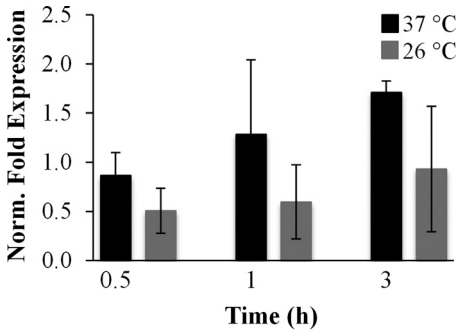

FIG 8 Quantitative real-time PCR results showing the normalized *oppA* transcript levels in the *prm* mutant during timed cold shock intervals. RNA was isolated from bacterial cultures grown to an OD_{600} of 0.3 at 37°C, followed by growth for timed intervals of 0.5, 1, and 3 h at 37°C or cold shock at 26°C. Normalized results are the averages of results from 2 separate experiments performed in triplicate, with standard deviations denoted with error bars, and statistical significance (*) was determined with a *t* test ($P < 0.05$).

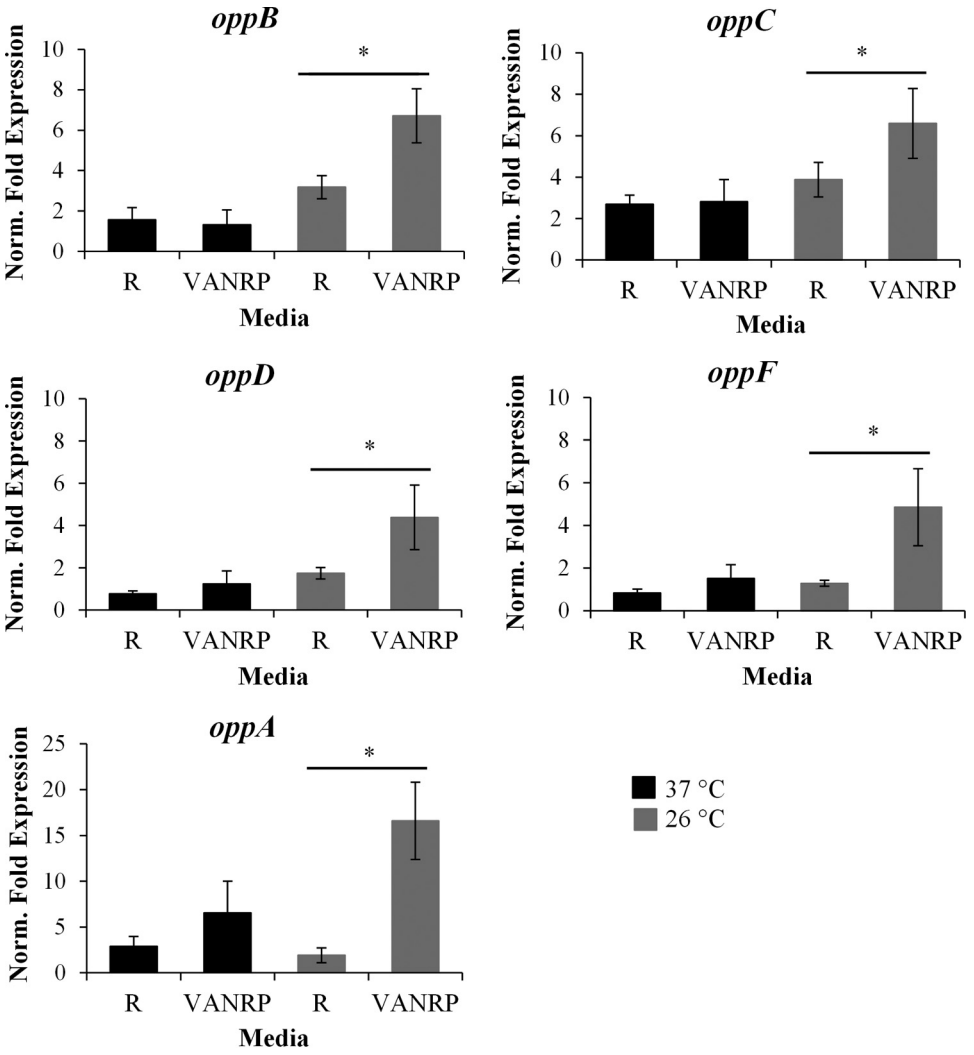

FIG 9 Nutrient availability impacts *opp* gene transcription. Quantitative real-time PCR was performed on 50 ng of RNA per sample isolated from bacterial cultures grown to an OD_{600} of 0.8 at 37°C and 26°C in chemically defined medium supplemented with free arginine (R) or chemically defined medium supplemented with a peptide as the only source of arginine (VANRP). Normalized transcript levels were statistically higher (*), as determined by a *t* test ($P < 0.05$), in medium supplemented with the peptide at 26°C but not at 37°C for all the *opp* genes. Results are the averages of data from 3 experiments performed in triplicate, with standard deviations denoted with error bars.

transcription levels of *oppB*, *-C*, *-D*, and *-F* by ~2.5-fold compared to the transcript levels in medium containing free arginine at 26°C (Fig. 9). The level of *oppA* transcript increased ~15-fold more with the peptide substrate as the sole source of arginine at 26°C than at 37°C. These data indicate that when peptides are the only source of arginine, transcription of the *opp* operon at 26°C is increased compared to when free arginine is available. Also, peptide substrate availability plays an important role in the regulation of the promoter upstream of *oppA*.

DISCUSSION

The *M. catarrhalis* Opp ABC transport system is a nutritional virulence factor, and OppA is a promising vaccine antigen candidate (15, 16). The regulation of this system in response to changing conditions such as cold shock and limiting essential nutrients has not been investigated. An understanding of such systems is necessary for the development of new antimicrobial agents for which ABC transporters are increasingly important targets (15, 27, 39–43). Furthermore, as OppA was developed as a vaccine antigen, it is important to characterize the regulation of its expression. This work advances our understanding of how *M. catarrhalis* increases the transcription of an important virulence factor, the Opp ABC transport system, based on environmental cues of both temperature and nutrient availability.

While a number of operons have been identified in *M. catarrhalis*, little is known about how environmental factors influence transcriptional regulation (13, 27, 44–50). In the present study, we show that the entire *opp* gene cluster is transcribed as a single transcript and is thus an operon (Fig. 2). We were somewhat surprised by this result because the region between *oppF* and *oppA* is 204 bp, compared to gaps of ~60 bp between the other *opp* genes, and because of the presence of a second predicted promoter immediately upstream of *oppA*.

The *prm* mutant lacking the operon promoter upstream of *oppB* was unable to utilize a peptide substrate, indicating the lack

of a functional Opp system (Fig. 5) (16). The *prm* mutant had essentially absent transcription of *oppB*, *-C*, and *-D* but a very low level of *oppF*, which did not translate to any detectable protein (Fig. 3 and 4). We speculate that the recruitment of transcription machinery to the *oppA* promoter and to the spectinomycin drug cassette promoter further upstream predisposes this region of sequence to a low level of background transcription, allowing a detectable amount of *oppF* transcript. On the other hand, *oppA* was transcribed at levels 5-fold higher than those of the upstream *opp* genes in spite of the absence of the operon promoter upstream of *oppB*, indicating that the predicted promoter upstream of *oppA* is active. The reduced level of *oppA* transcript compared to that of the WT correlated with the visibly reduced OppA protein levels as well.

The results indicating that the promoter upstream of *oppA* actively contributes to transcription are consistent with the observation that the substrate binding proteins of ABC transporters of other bacterial species have both secondary transcriptional regulation and increased protein stoichiometry compared to the other genes and proteins in the system (27, 28). We conclude that the promoter upstream of *oppB* is responsible for transcription of the *opp* operon and that the promoter upstream of *oppA* contributes to the additional regulation of *oppA* expression.

The temperature throughout the respiratory tract ranges from 37°C to 26°C, and changes in temperature and environmental factors alter the expression of homologous Opp systems in other pathogenic bacterial species (2, 17–22, 40, 43, 47, 51, 52). Exacerbations of COPD related to the acquisition of *M. catarrhalis* and otitis media increase during the cooler winter months of the year (8, 53–55). The temperature of the nasopharynx, where *M. catarrhalis* first colonizes, is 34°C and decreases to 26°C upon exposure to colder temperatures in winter months in temperate climates (17–23, 56). Cold shock increases the expression of the outer membrane protein UspA1, a known virulence factor, which is important for *M. catarrhalis* binding to host cells as well as immune evasion (23, 25). Thus, time spent outdoors breathing cooler air alters the temperature of the respiratory tract and could have important effects on bacterial homeostasis and regulation of the Opp system.

Similar to these other virulence factors, transcription levels of all *opp* genes were significantly higher at 26°C, and transcription of the operon in *M. catarrhalis* was increased after only 30 min of cold shock exposure (Fig. 6 and 7). We speculate that given the relatively small amount of time required to increase transcription of this operon, the Opp system plays an important role in fitness *in vivo* in the cooler temperatures of the nasopharynx, the natural ecological niche of *M. catarrhalis*. Interestingly, *oppA* transcript levels did not increase upon cold shock in the *prm* mutant. We conclude that exposure to cooler temperature results in increased transcription of the genes in the *opp* operon by increasing transcription of the promoter upstream of *oppB*, while the promoter upstream of *oppA* is not influenced by temperature.

The middle ear space and the lower airways where *M. catarrhalis* causes disease are characterized by limited nutrient availability. For example, much of the free arginine in the human respiratory tract is methylated or sequestered inside cells, particularly under conditions that promote lung inflammation, such as that of a COPD exacerbation (36, 38, 57–60). Thus, the ability of *M. catarrhalis* to utilize peptide substrates is essential for survival *in vivo* (16). Transcript levels of all the *opp* genes were increased in medium containing a peptide as the only source of essential arginine compared to those in medium with free arginine present at 26°C (Fig. 9). Thus, the *opp* operon is expressed at higher levels in media that require the uptake of oligopeptides by *M. catarrhalis*. The increase in the *oppA* transcript level is dramatically higher than the increase seen for the other *opp* genes, indicating that the promoter upstream of *oppA* is sensitive to the amount of available peptide substrate in the surrounding medium. Similar increases in the levels of homologous OppA proteins with the increased availability of substrates in the surrounding environment support these conclusions (40, 60).

M. catarrhalis colonizes a unique niche in the nasopharynx and has adapted to thrive in this environment of changing temperature and nutrient availability. In this study, we have characterized the *opp* gene cluster as an operon and shown that cold shock influences the regulation of this system, which is important for nutrient acquisition. We have made the novel observation of a second promoter within the *opp* operon and shown that it is not regulated by temperature. Additionally, the increased transcription of *oppA* under physiologically relevant conditions further supports the case for OppA as a vaccine antigen target for *M. catarrhalis*. This work advances the field in characterizing the mechanisms of transcriptional control in *M. catarrhalis*. A further understanding of how *M. catarrhalis* regulates gene transcription could provide a greater understanding of virulence mechanisms and provide new targets for antimicrobial development for this increasingly relevant yet understudied human pathogen.

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