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Development of a LacZ-Based Transcriptional Reporter System for Use with *Moraxella catarrhalis*

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Abstract

The lack of a transcriptional reporter system for use in *Moraxella catarrhalis* has hindered studies of gene regulation in this pathogen. PCR and recombinant DNA methods were used to insert a multicloning site (MCS) and promoterless full-length *E. coli lacZ* gene, flanked by transcriptional terminators both immediately upstream and downstream, into the *M. catarrhalis* recombinant plasmid pWW115. Insertion into the MCS in the newly constructed plasmid pASE222 of *M. catarrhalis* promoter regions controlled by either a repressor (i.e., NsrR) or activator (i.e., PhoB) yielded transcriptional fusion constructs that were appropriately responsive to signal inputs dependent on the host strain genotype, as measured quantitatively by means of a Miller β -galactosidase assay. The transcriptional reporter plasmid pASE222 should prove to be a useful tool for rapid screening of factors affecting gene expression in *M. catarrhalis*.

Keywords

Moraxella catarrhalis; plasmid; transcriptional reporter

Moraxella catarrhalis can colonize the human nasopharynx asymptotically and is a cause of disease in both the upper and lower respiratory tracts of humans (Murphy, 2005; Verduin et al., 2002). In infants and young children, this gram-negative pathogen is best known for producing acute otitis media (middle ear disease) (Karalus and Campagnari, 2000; Murphy, 2000). In adults, *M. catarrhalis* is likely responsible for 2 million – 4 million infectious exacerbations of chronic obstructive pulmonary disease (Murphy et al., 2005).

Knowledge about the pathogenesis of disease caused by *M. catarrhalis* is still fairly limited. Investigation of the bacterial gene products necessary for colonization of the nasopharyngeal mucosa and disease production has been facilitated by both the introduction of genetic methods for use with *M. catarrhalis* and the utilization of a relevant animal model (i.e., the chinchilla) (Armbruster et al., 2010; Bakaletz et al., 1995; Hoopman et al., 2012; N.R. Luke,

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2007). Mutagenesis of this bacterium has been achieved using either transposons (Timpe et al., 2003) or genetic transformation in conjunction with recombinant DNA methods (Helminen et al., 1993), and the genomes of at least a dozen *M. catarrhalis* strains were sequenced recently (Davie et al., 2011; de Vries et al., 2010). At least two different plasmids capable of replication in *M. catarrhalis* have been described to date (Beaulieu et al., 1988; Hays et al., 2005), and recombinant plasmids (i.e., pWW102B and pWW115) derived from one of these have been used for complementation analysis (Wang et al., 2006; Wang and Hansen, 2006).

Previous studies of gene regulation in *M. catarrhalis* have been mostly limited to data derived from DNA microarray, real-time RT-PCR, Northern blot, and Western blot analyses (de Vries et al., 2010; Furano and Campagnari, 2003; Heiniger et al., 2005; Hoopman et al., 2011; Wang et al., 2007; Wang et al., 2008). No systems have been described to date for rapid screening for regulatory and other mutations affecting gene expression in *M. catarrhalis*. To address this issue, we constructed a transcriptional reporter plasmid using the pWW115 plasmid backbone and a *lacZ* gene derived from *E. coli*.

Wild-type, mutant, and recombinant *M. catarrhalis* strains were routinely grown at 37°C in either brain heart infusion (BHI) broth (Difco/Becton Dickinson) or on BHI agar plates in an atmosphere containing 95% air-5% CO₂. A chemically defined medium (MB4+; manuscript in preparation) derived from that described by Juni and colleagues (Juni et al., 1986) was used for experiments involving phosphate limitation. In this defined medium, the original phosphate-based buffer was replaced with morpholinopropane sulfonate (MOPS) (Neidhardt et al., 1974). The antimicrobial compounds used with *M. catarrhalis*, when appropriate, were kanamycin (15 µg mL⁻¹) and spectinomycin (15 µg mL⁻¹). *E. coli* strains were grown on Luria-Bertani (LB) medium, supplemented with ampicillin (100 µg mL⁻¹) or kanamycin (30 µg mL⁻¹) when appropriate. Broth cultures were grown in a 37°C water bath with shaking at 200 rpm.

Chromosomal DNA was purified using the EasyDNA kit (Invitrogen). PCR for the construction of pASE222 was performed using the Takara ExTaq DNA polymerase (Fisher Scientific). PCR products were purified by using the Qiagen Gel Extraction system (Qiagen). Plasmid DNA and products from restriction endonuclease reactions were purified by using the Qiagen Mini-Prep kit (Qiagen). Primers AE52 and AE60 were used together with chromosomal DNA from *E. coli* MG1655 in a PCR to amplify the entire *lacZ* gene including approximately 100-bp 5' and 3' of the ORF. The forward primer (AE52) included a newly generated 19-bp multi-cloning site (MCS) whereas the reverse primer, AE60, contained the sequence of the *rnnB* T1 transcription terminator. This amplicon was then used in a second PCR with primers AE52 and AE85 to add random DNA sequence to the end of the *rnnB* T1 sequence. This new amplicon was used together with forward primer AE89 (containing restriction sites for *Bam*HI and *Nco*I) and reverse primer AE 86 (containing a restriction site for *Bam*HI) to obtain a third and final PCR product containing the *Nco*I site, MCS, *lacZ* gene, and 3' *rnnB* T1 terminator sequence, flanked on either end by *Bam*HI sites. This ~3.5 kb fragment was purified, sequence-confirmed, digested with *Bam*HI, and ligated into the *Bam*HI site of pWW115. The ligation reaction mixture was used for transformation of *M. catarrhalis* O35E via a BHI agar plate-based method (Pearson et al., 2006), yielding plasmid pASE207.1

The constitutively active kanamycin (*kan*) gene promoter region originally derived from pUCK4 (Pharmacia) was used to allow LacZ expression at this point. This promoter was obtained by PCR using primers AE75 and AE76 (both containing *Sac*II restriction sites). This amplicon was tailed with dATP using *Taq* DNA polymerase, ligated into the pGEM-T Easy vector (Promega), and transformed into *E. coli* Top10 cells. The resultant plasmid

pASE213.2 was digested with *Sac*II to obtain a ~200-bp fragment containing the *kan* promoter which was then ligated into the *Sac*II site within the MCS of pASE207.1 and transformed into *M. catarrhalis* O35. Spectinomycin-resistant colonies were selected on BHI agar plates containing 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal). The plasmid from one of the resultant blue colonies was designated pASE217.Blue4.

To introduce a set of transcription terminators 5' from the MCS, plasmid pRS551 was digested with *Sma*I to obtain a ~1.6 kb fragment containing four *rnnB* T1 transcription terminators. The fragment was used as the template for a PCR with primers AE51 and AE59. The resultant ~800-bp product was used as the template for a second PCR with primers AE90 and AE91, which introduced *Nco*I sites onto the 5' and 3' ends. This blunt PCR product was ligated into the pGEM-T Easy vector and used to transform *E. coli* Top10 cells. The resultant plasmid pASE218 was digested with *Nco*I to yield a ~800-bp fragment containing the four *rnnB* T1 terminators; this fragment was subsequently ligated into the *Nco*I site of pASE217.Blue 4, yielding pASE219.14. This latter plasmid was digested with *Sac*II to excise the *kan* promoter and then re-ligated to obtain pASE222 (GenBank accession number JX964749; Fig. 1A and Fig. 1B). Nucleotide sequence analysis revealed five nucleotide changes in the *lacZ* gene in pASE222, including G354A, A466G, T985C, G1182A, and C2936T. Three of these resulted in amino acid changes including N156D, C329R, and A979V.

To confirm that this plasmid was capable of replication in other *M. catarrhalis* strains, plasmid pASE222 was introduced into strains ETSU-9 and O12E by electroporation and the BHI agar plate-based transformation method (Pearson et al., 2006), respectively. For electroporation, a few colonies of ETSU-9 were suspended in 20 μ l of 10% (v/v) glycerol together with 2 μ l of a pASE222 plasmid preparation and held on ice. The cells were electroporated at 1.6 kV using an *E. coli* Pulser (BioRad). These cells were added to 250 μ l of BHI, incubated with shaking at 37°C for one hr, and then spread on selective media and incubated overnight. Both ETSU-9 and O12E transformants were shown to contain pASE222 (Fig. 1C), indicating the ability of this plasmid to replicate in other *M. catarrhalis* strains.

Two different *M. catarrhalis* promoter regions were used to evaluate the ability of pASE222 to respond to both positive and negative regulatory signals. NsrR is a repressor protein that controls expression of the small NsrR regulon in *M. catarrhalis* (Wang et al., 2008). Genes controlled by NsrR include both *aniA* and *norB*, encoding a nitrite reductase and nitric oxide reductase, respectively, that comprise the terminal two steps in the truncated denitrification pathway found in *M. catarrhalis* (Wang et al., 2011; Wang et al., 2007; Wang et al., 2008). PhoB is the response regulator of a two-component signal transduction system (PhoBR) that positively controls expression of genes encoding proteins involved in the high affinity uptake of inorganic phosphate (Hsieh and Wanner, 2010; Lamarche et al., 2008). Genes activated by PhoB include *pstS* which encodes the periplasmic-binding component of an ABC transporter complex involved in phosphate transport (Hsieh and Wanner, 2010).

Nucleotide fragments containing 500-bp 5' and 100-bp 3' from the ATG translation initiation codon for both *norB* and *pstS* were PCR-amplified and cloned into the MCS site in pASE222. Briefly, the primers (Table 2) used to amplify each promoter from O35E genomic DNA were CP101 and CP102 for *norB*, and CP103 and CP104 for *pstS*. Each amplicon was subcloned into the pGEM-T Easy vector which was subsequently digested with *Sac*II and *Sal*I. These products were ligated to similarly digested pASE222 using Promega T4 DNA ligase (Promega) and transformed into strain O35E using the BHI agar plate-based method (Pearson et al., 2006). Colonies were PCR-screened for the presence of the promoter in the MCS using the forward primer for each promoter region and primer AE84 (located in the

*lacZ*ORF). Plasmids pASE230 (containing the *norB* promoter) and pASE240 (containing the *pstS* promoter) were purified from O35E and introduced into a O35E Δ *nsrR* mutant (Wang et al., 2008) and a O35E Δ *phoB* mutant (manuscript in preparation), respectively, by the plate transformation method. Plasmid pASE222 was also introduced into these same two O35E mutants.

Expression of β -galactosidase by these different constructs was measured quantitatively by means of a Miller assay performed in a manner similar to that described by Zhang and Bremer (Zhang and Bremer, 1995), with some modifications. Briefly, 10-ml broth cultures were grown to mid-exponential phase ($OD_{600} \sim 0.7$). Each culture was harvested via centrifugation and then resuspended in Z buffer [permeabilization solution containing hexadecyltrimethylammonium bromide (0.8 mg mL^{-1}), sodium deoxycholate (0.4 mg mL^{-1}), $100 \text{ mM Na}_2\text{HPO}_4$, 20 mM KCl , 2 mM MgSO_4 , and $5.4 \mu\text{L mL}^{-1}$ β -mercaptoethanol]. An OD_{600} measurement was taken of this suspension. In triplicate, aliquots of the suspension were transferred to microcentrifuge tubes and incubated at 30°C for 20 min. At the start of the reaction, $200 \mu\text{L}$ ONPG buffer (substrate solution containing 4 mg mL^{-1} of o-nitrophenyl- β -D-galactoside (ONPG), $60 \text{ mM Na}_2\text{HPO}_4$, $40 \text{ mM NaH}_2\text{PO}_4$, and $2.7 \mu\text{L mL}^{-1}$ β -mercaptoethanol) was added to each reaction and vortexed briefly. Samples were incubated at 30°C for 30 min. To stop the reaction, $500 \mu\text{L}$ of $1 \text{ M Na}_2\text{HCO}_3$ was added to each reaction followed by vortexing. Each tube was then centrifuged at $16,000 \times g$ for 5 min in a table top centrifuge. The supernatant from each sample was transferred to a cuvette, and the OD_{420} and OD_{500} of this solution were recorded. Miller units of β -galactosidase activity were calculated as described previously (Zhang and Bremer, 1995). Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Bonferroni correction. Comparisons were considered significant at $P < 0.001$.

When introduced into wild-type *M. catarrhalis* O35E which makes readily detectable levels of the NsrR repressor while growing under aerobic conditions in broth (Wang et al., 2008), pASE230 carrying the *norB* promoter region expressed just 63 Miller units of activity (Fig. 2A, column 2). In contrast, when this same reporter construct was present in the O35E Δ *nsrR* mutant, the level of Miller units of activity increased to 227 in the absence of the NsrR repressor protein (Fig. 2A, column 4). The pASE222 plasmid expressed little or no detectable Miller activity when present in either the O35E wild-type strain or the O35E Δ *nsrR* mutant (Fig. 2A, columns 1 and 3, respectively).

Initial experiments involving a reporter (pASE240) containing the *pstS* promoter were conducted using BHI broth which contains relatively high concentrations (i.e., 10 mM) of inorganic phosphate (Nguyen et al., 2009). When present in the wild-type O35E parent strain with a functional PhoB activator protein, the pASE240 reporter expressed a very low but detectable level of 7 Miller units of activity (Fig. 2B, column 2). However, when present in the O35E Δ *phoB* mutant (Fig. 2B, column 4), the level of β -galactosidase activity expressed from pASE240 decreased to that seen with the empty plasmid vector (Fig. 2B, columns 1 and 2). To determine the effect of phosphate limitation on *pstS* promoter activity, wild-type *M. catarrhalis* O35E cells containing either pASE222 or pASE240 were grown in MB4+ medium containing a high concentration of phosphate (10 mM), washed, and used to inoculate this same defined medium containing either high (10 mM , i.e., phosphate-replete) or very low ($10 \mu\text{M}$, i.e., phosphate-limited) phosphate. The level of β -galactosidase activity expressed from pASE222 and pASE240 was then followed over time. Promoter activity of pASE240 showed little or no increase when the cells were re-inoculated into the medium containing high phosphate (Fig. 3, columns 3, 7, 11, and 15). In contrast, when the phosphate concentration was very low, pASE240 promoter activity increased steadily, increasing to a maximum of 917 Miller units of activity over the 3 hr experimental period (Fig. 3, columns 4, 8, 12, and 16).

To determine if pASE222 and *M. catarrhalis* could be used together in a conventional blue/white screening system, all of the strains listed above in Fig. 2 were initially streaked onto BHI containing spectinomycin and then onto BHI containing X-gal. After overnight incubation, the appearance of the colonies on BHI/X-gal agar reflected the Miller assay results in terms of the relative level of intensity of the blue color, or lack thereof (data not shown). Taken together, these quantitative and qualitative results indicate the pASE222 transcriptional reporter system is responsive to both positive and negative *M. catarrhalis* control elements. The availability of pASE22 should further efforts to study transcriptional control elements and other factors affecting gene regulation in *M. catarrhalis*.

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Highlights

1. Construction of a LacZ-based transcriptional reporter system that functions in *Moraxella catarrhalis*.
2. Transcriptional fusion constructs constructed using this system were appropriately responsive to signal inputs dependent on the *M. catarrhalis* host strain genotype and/or environment, as measured quantitatively by means of a Miller β -galactosidase assay.
3. This transcriptional reporter system also has the potential to be used in large-scale, blue/white screens to identify factors affecting gene expression in *M. catarrhalis*.

Figure 1. Characterization of pASE222

(A) Partial restriction map of pASE222 (GenBank Accession No. JX964749). (B) Nucleotide sequence of the region flanking the MCS in pASE222. The location of the five transcriptional terminators is indicated by the stem loop symbols. (C) Detection of pASE222 in different *M. catarrhalis* strains. Plasmids were linearized by digestion with *BglII* and resolved by agarose gel electrophoresis. Columns: 1, pWW115; 2, pASE222 from O35E (positive control); 3 and 4, pASE222 from two *M. catarrhalis* ETSU-9 transformants; columns 5 and 6, pASE222 from two *M. catarrhalis* O12E transformants. Size markers (in kb) are present on the left side of panel C.

Figure 2. Use of pASE222 to measure the effect of host strain background on promoter activity
 Cells were grown to mid-logarithmic phase ($OD_{600} \sim 0.7$) and then processed for use in the Miller assay. (A) Measurement of *norB* promoter activity in pASE230 present in wild-type O35E and a O35E *nsrR* deletion mutant. Columns: 1, O35E(pASE222); 2, O35E(pASE230); 3, O35EΔ*nsrR*(pASE222); 4, O35EΔ*nsrR*(pASE230). Data are the mean from two independent experiments; error bars indicate standard deviation. *, $P < 0.0001$. (B) Measurement of *pstS* promoter activity in pASE240 present in wild-type O35E and a O35E *phoB* deletion mutant. Columns: 1, O35E(pASE222); 2, O35E(pASE240); 3, O35EΔ*phoB*(pASE222); 4, O35EΔ*phoB*(pASE240). Data are the mean from three independent experiments; error bars indicate standard deviation. *, $P < 0.0001$.

Figure 3. Use of pASE240 to measure the effect of phosphate limitation on *pstS* promoter activity
Cells grown in phosphate-replete defined medium were washed and inoculated into phosphate-replete or phosphate-limited media. Cells were harvested for use in the Miller assay over a three hr time period. Columns 1, 5, 9, 13 – O35E(pASE222) under phosphate-replete conditions. Columns 2, 6, 10, 14 – O35E(pASE222) under phosphate-limited conditions. Columns 3, 7, 11, 15 – O35E(ASE240) under phosphate-replete conditions. Columns 4, 8, 12, 16 – O35E(pASE240) under phosphate-limited conditions. The data are the mean from two independent experiments and the error bars indicate the standard deviation. $P < 0.001$ for columns 7 vs. 8, 11 vs. 12, and 15 vs. 16.

Table 1

Bacterial strains and plasmids used in this study.

Strain	Genotype or description	Reference or source
<i>M. catarrhalis</i>		
O35E	Wild-type disease isolate	(Helminen et al., 1993)
O35EΔ <i>nsrR</i>	<i>nsrR</i> deletion mutant	(Wang et al., 2008)
O35EΔ <i>phoB</i>	<i>phoB</i> deletion mutant	This study
ETSU-9	Wild-type disease isolate	Steven Berk
O12E	Wild-type disease isolate	John Nelson
<i>E. coli</i>		
Top10	Host for subcloning	Invitrogen
MG1655	Source of <i>lacZ</i> gene	(Perkins et al., 1993)
<i>E. coli</i> Plasmids		
pRS551	Source of <i>rrnB</i> T1 terminator sequence	(Simons et al., 1987)
pUCK4	Source of <i>kan</i> promoter	Pharmacia
pGEM-T Easy	<i>E. coli</i> cloning vector, amp ^r	Promega
pASE213.2	pGEM-T Easy vector with cloned <i>kan</i> promoter	This study
pASE218	pGEM-T Easy containing a PCR fragment with four <i>rrnB</i> T1 transcription terminators	This study
<i>M. catarrhalis</i> Plasmids		
pWW115	<i>M. catarrhalis</i> cloning vector, spec ^r , chlor ^r	(Wang and Hansen, 2006)
pASE207.1	pWW115 with MCS, <i>lacZ</i> gene, and single <i>rrnB</i> T1 terminator	This study
pASE217.Blue4	pASE207.1 with <i>kan</i> promoter	This study
pASE219.14	pASE217.Blue4 with four <i>rrnB</i> T1 terminators preceding MCS	This study
pASE222	pASE219.14 lacking the <i>kan</i> promoter	This study
pASE230	pASE222 with <i>norB</i> promoter	This study
pASE240	pASE222 with <i>pstS</i> promoter	This study

Table 2

Oligonucleotide primers utilized in this study.

Primer	Sequence (5' → 3')
AE51	GCTAGCGGCCGCGGTCGACGATCCCCAATTCCTGGCAG
AE52	GTCGACCGCGGCCGCTAGCTTGTGTGGAATTGTGAGC
AE59	ACCCGGGACTGATGAATGTTCCGTTGCGCTG
AE60	TCCCGGGAACAGATAAAACGAAAGGCCAGTCTTCGACTGAGCCTTTCGTTTATTTGATGATAATGGATTTCCTTACG
AE75	ACTCCGCGGCCCGGATCCGTCGA ^a
AE76	ACTCCGCGGAACACCCCTTGTATTACTGT ^a
AE84	AGGGTTTTCCAGTCACGACG
AE85	ATCTAGATAATAATTATTTTAGTTAATTTTAACAGATAAAACGAAAGGCCAGTCTT
AE86	CGCGGATCCTCCCCGGGATCTAGATAATAATTATTTA ^b
AE89	CGCGGATCCTCCCATGGGTCGACCGCGCCGCTAGCTTGTGTGGAATTGTGAGC ^{b,c}
AE90	TCCCATGGACTGATGAATGTTCCGTTGCGCTG ^c
AE91	TCCCATGGGCTAGCGGCCGCGGTCGACGATCCCCAATTCCTGGCAG ^c
CP101	AACCGTCGACTGGTCTAAGCTACAGCCGCT ^d
CP102	CCAACCGCGGAATGGGCGGGTGGTAC ^a
CP103	CCAAGTCGACGGCTTGATGAGATAGCTGCATGA ^d
CP104	AATTCCGCGGTGCAGCTGGTTCTGGCT ^a

^a *Sac*II site^b *Bam*HI site^c *Nco*I site^d *Sa*I site