

Global Changes in *Mycoplasma gallisepticum* Phase-Variable Lipoprotein Gene *vlhA* Expression during *In Vivo* Infection of the Natural Chicken Host

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Mycoplasma gallisepticum is the primary etiologic agent of chronic respiratory disease in poultry, a disease largely affecting the respiratory tract and causing significant economic losses worldwide. Immunodominant proteins encoded by members of the variable lipoprotein and hemagglutinin (*vlhA*) gene family are thought to be important for mechanisms of *M. gallisepticum*-host interaction, pathogenesis, and immune evasion, but their exact role and the overall nature of their phase variation are unknown. To better understand these mechanisms, we assessed global transcriptomic *vlhA* gene expression directly from *M. gallisepticum* populations present on tracheal mucosae during a 7-day experimental infection in the natural chicken host. Here we report differences in both dominant and minor *vlhA* gene expression levels throughout the first week of infection and starting as early as day 1 postinfection, consistent with a functional role not dependent on adaptive immunity for driving phase variation. Notably, data indicated that, at given time points, specific *vlhA* genes were similarly dominant in multiple independent hosts, suggesting a nonstochastic temporal progression of dominant *vlhA* gene expression in the colonizing bacterial population. The dominant expression of a given *vlhA* gene was not dependent on the presence of 12-copy GAA trinucleotide repeats in the promoter region and did not revert to the predominate *vlhA* gene when no longer faced with host pressures. Overall, these data indicate that *vlhA* phase variation is dynamic throughout the earliest stages of infection and that the pattern of dominant *vlhA* expression may be nonrandom and regulated by previously unrecognized mechanisms.

Mycoplasma gallisepticum, the primary etiologic agent of chronic respiratory disease (CRD) in poultry, is highly transmissible and causes severe inflammation of trachea, lungs, and air sacs, ultimately resulting in an overall reduction in weight gain and egg production in infected chickens. *M. gallisepticum* is pathogenic in other species, causing infectious sinusitis in turkeys and conjunctivitis in house finches (1, 2). Aside from primary attachment proteins GapA and CrmA, fibronectin binding proteins PlpA and Hlp3, sugar transport permease MalF, and dihydroipoamide dehydrogenase Lpd, little is understood about specific bacterial factors affecting survival and persistence of this important avian pathogen in its natural host (3–6).

The lack of obvious homologs of traditional bacterial transcriptional regulators and signaling factors had previously led to the thought that mycoplasmas were unable to regulate transcription by conventional mechanisms. However, *M. gallisepticum* exhibits differential levels of gene expression in response to coinfection with MRC-5 human lung fibroblasts (7) and in response to various stress conditions (8, 9). These data suggest that changes in transcription are indeed occurring as mycoplasmas respond to external stimuli and that the regulation is, in fact, more dynamic than originally hypothesized, with genes potentially affected by multiple regulators (10).

The members of the variable lipoprotein and hemagglutinin (*vlhA*) family, consisting of 43 genes distributed across 5 loci and totaling just over 10% of the entire genome in strain R_{low}, are potentially important for *M. gallisepticum* pathogenesis (11). Previous studies have indicated that *M. gallisepticum vlhA* expression is dependent on the presence of exactly 12 GAA trinucleotide repeats upstream of the gene (12–15). It has also been hypothesized that *M. gallisepticum vlhA* phase variation is stochastic, driven by anti-VlhA antibody-mediated negative selection of the initial

population and resulting in random outgrowth of bacterial subpopulations expressing alternative *vlhA* genes (16). If that were correct, we would expect to see various patterns of *vlhA* expression from the individual chickens at each time point.

The function of VlhA proteins is still unknown, but it has been speculated to involve immune evasion during infection (17), and variation in *vlhA* gene content has been loosely associated with virulence (18). While there have been efforts to understand the antigens expressed by *M. gallisepticum in vivo* (19), there has not yet been a comprehensive *in vivo* transcriptome study of *M. gallisepticum* in response to the natural host over the course of early infection at the global level. Previous studies assessed selected *in vivo vlhA* gene expression changes using monoclonal antibodies; however, methods for comprehensive *vlhA* analysis were not yet available (12, 13). Now, using next-generation sequencing, which enables comprehensive gene expression assessment at the nucleotide level, we present results indicating changes in *vlhA* expression of *M. gallisepticum* sampled directly from the tracheas of experimentally infected chickens over the course of a 7-day infection.

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MATERIALS AND METHODS

Animals. Four-week-old female specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, CT, USA) were received and divided randomly into groups, placed in HEPA-filtered isolators, and allowed to acclimate for 1 week. Nonmedicated feed and water were provided *ad libitum* throughout the experiment. All animal studies were performed in accordance with approved University of Connecticut (UConn) IACUC protocol number A13-001.

Chicken infection. Stocks of *M. gallisepticum* strain R_{low} (passage 17) were grown overnight at 37°C in complete Hayflick's medium until mid-log phase was reached as indicated by a color shift from red to orange. Bacterial concentrations were determined by the optical density at 620 nm (OD₆₂₀), and 10-fold serial dilutions were conducted to confirm viable color-changing-unit titers. Bacteria were pelleted by centrifugation at 10,000 × *g* for 10 min and resuspended in Hayflick's complete medium. Chickens were challenged intratracheally as previously described (6) with 1 × 10⁸ CFU/200 μl.

RNA extraction. Five infected chickens were humanely sacrificed each day for a total of 7 days. After sacrifice, tracheas were excised and total RNA was extracted from each individual trachea by washing the lumen with 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then purified using a Zymo Direct-zol RNA Miniprep kit (Zymo Research Corporation, Irvine, CA, USA), and standard PCR was conducted to ensure that the RNA preparations were free of any DNA. RNA was quality-checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and high-quality samples with RNA integrity numbers (RIN) of >8 were utilized to construct cDNA libraries.

To enrich for bacterial RNA, total RNAs were subjected to a poly(A) depletion step to remove the eukaryotic mRNA using a NEBNext poly(A) mRNA magnetic isolation module (New England BioLabs, Ipswich, MA, USA). Briefly, 5 μg of total RNA combined with an equal volume of bead binding buffer was bound to poly(T) oligonucleotide-attached magnetic beads at 65°C for 5 min. The remaining supernatant was collected, cleaned, and concentrated using a Zymo Clean & Concentrator-25 kit (Zymo Research Corporation) and eluted in 25 μl RNase-free water.

Both prokaryotic rRNA and eukaryotic rRNA were removed from 2.5 μg of poly(A)-depleted RNA using a RiboZero Magnetic Gold kit (Epidemiology) (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. Each rRNA-depleted RNA sample obtained after cleaning and concentrating with the Zymo Clean & Concentrator-25 kit (Zymo Research Corporation) was eluted in 25 μl of RNase-free water, and the samples were used to create a cDNA library.

Illumina sequencing. The cDNA libraries were created using an Illumina TruSeq stranded mRNA library preparation kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions starting at "Make RFP" (step 14; p. 56) of the Illumina TruSeq RNA Sample Preparation v2 (HT) protocol. Briefly, 10 to 400 ng of purified mRNA was fragmented and used to synthesize first-strand cDNA using reverse transcriptase and random hexamer primers. Second-strand cDNA synthesis was performed using dUTP, DNA polymerase, and RNase. The products were amplified by PCR and purified after end repair and adaptor ligations.

The cDNA libraries were assessed for quantity using a Qubit 2.0 fluorometer (Invitrogen) and for correct fragment size (~260 bp) using an Agilent TapeStation 2200 instrument (Agilent Technologies). Libraries were then normalized to 2 nM, pooled, denatured, and sequenced on a NextSeq500 Sequencing platform (Illumina Inc.) using a 75-bp paired-end approach.

RNA-seq analysis. Fastq data were assembled and mapped, and differential gene expression levels were assessed, using Rockhopper, an RNA sequencing (RNA-seq) analysis program with algorithms specifically designed for the bacterial gene structures and transcriptomes (<http://cs.wellesley.edu/~btjaden/Rockhopper/>) (20, 21). Bowtie2 parameters allowing 0 mismatches were used to map sequence reads to the R_{low} genomic template (11). The data were normalized by the standard method of determining the ratio of reads per kilobase per million (RPKM)

mapped, allowing for comparisons both within and between samples. The fold change data were determined from the log₂ transformation of the RPKM data between two samples. The differential levels of gene expression were determined by pairwise comparisons between the normalized values of expression of a given *vlhA* gene from two different days. The program-generated *P* value was used to determine the significance of the differential levels of gene expression by calculating *q* values based on the Benjamini-Hochberg correction with a false-discovery rate of <1%. Differences in expression values were considered significant when the *q* value was <0.02 (21).

Sequencing of culture-passaged bacteria. Mycoplasma cultures recovered from tracheas of the experimentally infected birds at day 7 postinfection were passaged 5 or 10 times in Hayflick's complete medium. Cultures were then grown to mid-log phase for DNA extraction using Chelex reagent (Bio-Rad, Hercules, CA, USA) or RNA extraction, enrichment, and sequencing as was described for the trachea samples above.

Targeted sequencing of *vlhA* 2.02 gene promoter sequences to query GAA repeat copy numbers was performed directly on specific PCR amplicons. PCR primers targeting the *vlhA* 2.02 promoter region designed using the *M. gallisepticum* R_{low} genomic sequence (GenBank accession no. AE015450) (11) were as follows: forward, 5'-GATGAGATTATCAAGCTTTTAGATG-3'; reverse, 5'-CTGAACGAATCAAAGAGTTACAGC-3'. PCR was performed using AmpliTaq (Invitrogen), 20 pmol of each primer, and 200 to 300 ng DNA under the following cycling conditions: 94°C for 5 min, followed by 40 cycles at 94°C for 15 s, 54°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 10 min. Amplicons were purified using a MinElute Reaction Cleanup kit (Qiagen, Valencia, CA, USA) to remove excess primers.

Sequencing was performed using 2 μl BigDye Terminator Mix (Invitrogen), 10 pmol of primer, and 300 ng of DNA (22). Excess dye terminators were removed with AutoSeq G-50 spin columns (Amersham Biosciences, Piscataway, NJ, USA), and the sequencing was performed at the University of Connecticut Biotechnology Center. Using Sequencher software (Gene Codes, Ann Arbor, MI, USA), the sequences were compared to the R_{low} genome and the numbers of GAA trinucleotide repeats upstream of the gene start codon were assessed.

RESULTS

***M. gallisepticum* input *vlhA* expression.** In broth-grown *M. gallisepticum* strain R_{low} input cultures, data here indicated that 31 *vlhA* genes were expressed with an average normalized expression value of 719. *vlhA* 3.03 was the predominant *vlhA* expressed in this culture, with an RPKM value of >12,000 (Fig. 1). This *vlhA* is preceded by exactly 12 GAA repeats (11), a characteristic previously thought to be necessary for expression (12–15). *vlhA* 3.03 was not the only *vlhA* expressed, however, as several other *vlhA* genes, including 2.02, 5.06, and 4.07, were expressed at levels (RPKM values of 1,500 to 2,300) above the average RPKM value (1,455) for all genes in the genome that were expressed. This indicated that, while *vlhA* 3.03 expression predominated, additional *vlhA* genes that were not preceded by 12 GAA repeats were expressed at minor levels in the cultured experimental input population.

***M. gallisepticum vlhA* expression over the course of infection.** Over the course of the 7-day infection, the *vlhA* expression profile changed dramatically. Expression of *vlhA* 3.03 showed an initial increase at day 1 postinfection and then decreased daily throughout the experiment (Fig. 2). Over the course of infection, there was a 34-fold total decrease in *vlhA* 3.03 expression (*q* < 0.0001), with the largest change being a 4.5-fold decrease between days 5 and 6 postinfection (*q* < 0.0001).

While expression of *vlhA* 3.03 decreased early in infection, expression of *vlhA* 4.07 and its tandem repeat variant, *vlhA* 4.07.6,

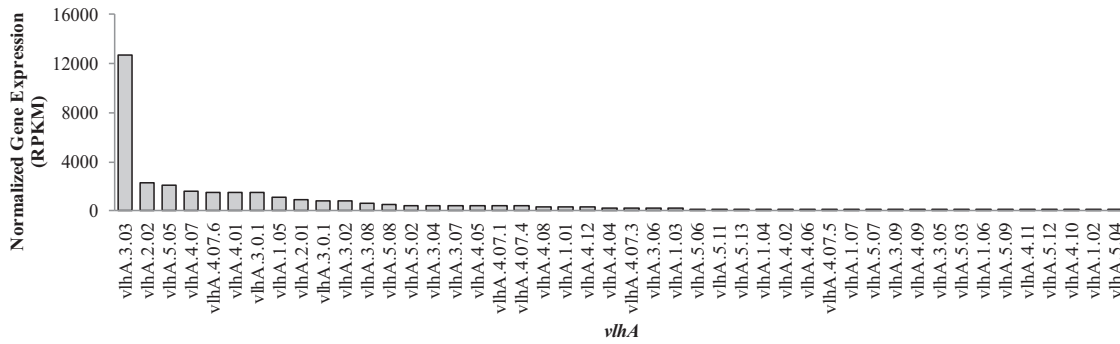


FIG 1 The *vlhA* expression profile of the broth-grown *M. gallisepticum* input cultures as determined from RNA sequencing.

began to increase as early as 1 day postinfection. These expression levels peaked at 2 days postinfection (3.7-fold higher than input culture) before decreasing over the remainder of the 7-day infection (Fig. 2). Similarly, as *vlhA* 4.07 and 4.07.6 decreased in expression, *vlhA* 4.08 and its tandem repeat, *vlhA* 4.07.1, showed increased expression until peaking at 7 days postinfection with 31-fold-higher ($q < 0.0001$) expression than the levels of input cultures (Fig. 2). Finally, there was an initial increase in *vlhA* 5.05 expression at 1 day postinfection, followed by decreasing expres-

sion through the time course. The *vlhA* expression profile observed here at day 7 postinfection was consistent with that of a previous pilot experiment (data not shown). Taking the data together, these changes in *vlhA* gene expression were not completely random and appeared to be relatively coordinated, as similar *vlhA* profiles were seen in five independently infected birds at each time point postinfection.

***M. gallisepticum vlhA* expression in recovery cultures.** From infected birds sacrificed 7 days postinfection, *M. gallisepticum* was

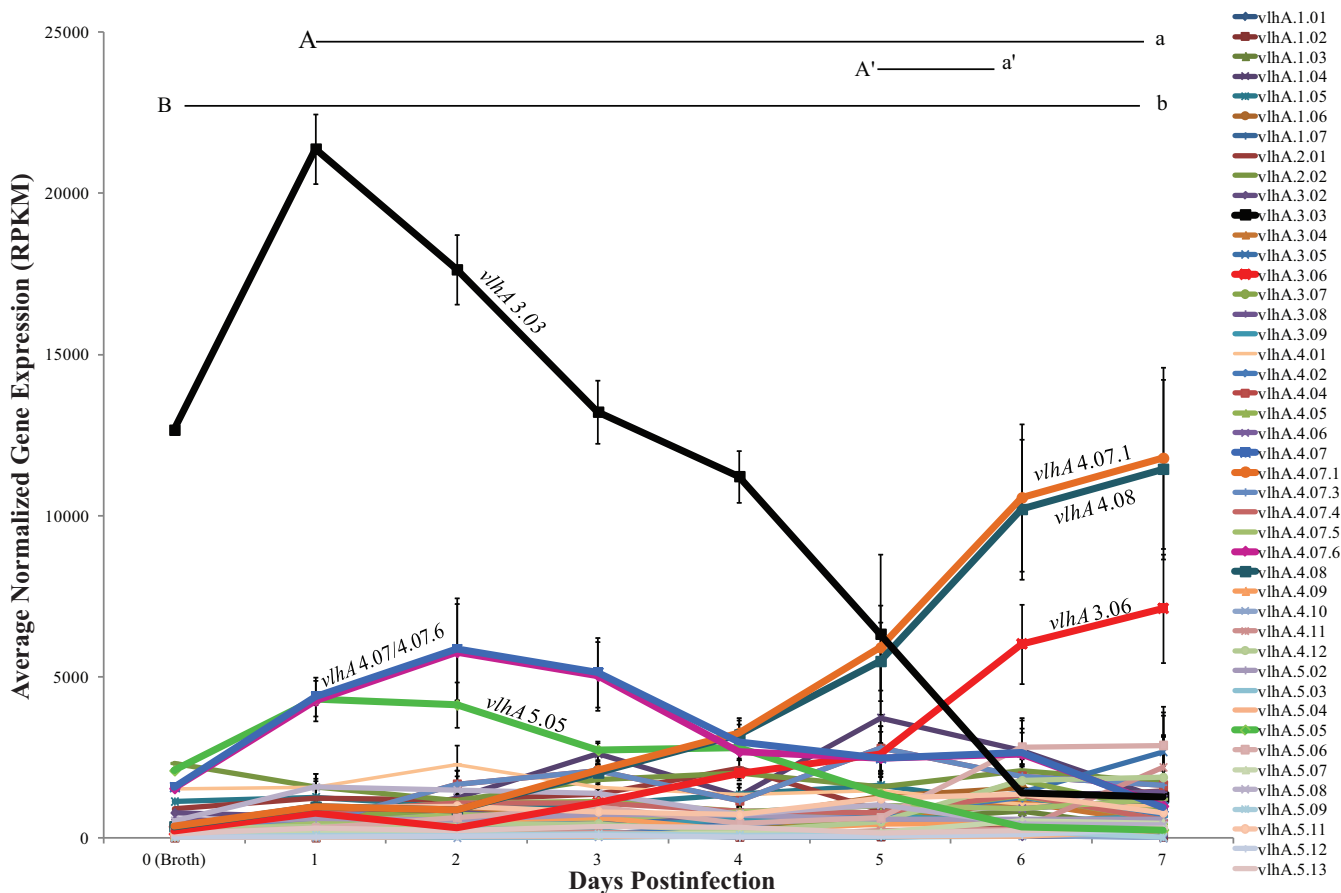


FIG 2 The *vlhA* expression profile of *M. gallisepticum* extracted directly from tracheas of experimentally infected birds over the course of the 7-day infection as determined through RNA sequencing. Each data point represents an average RPKM value from the results determined for five animals (with the exception of the broth sample). Error bars show standard errors of the means (SEM). Key statistically significant changes between two time points are indicated by paired upper- and lowercase letters for the following genes: *vlhA* 3.03 (A/a and A'/a') and *vlhA* 4.07.1/4.08 (B/b).

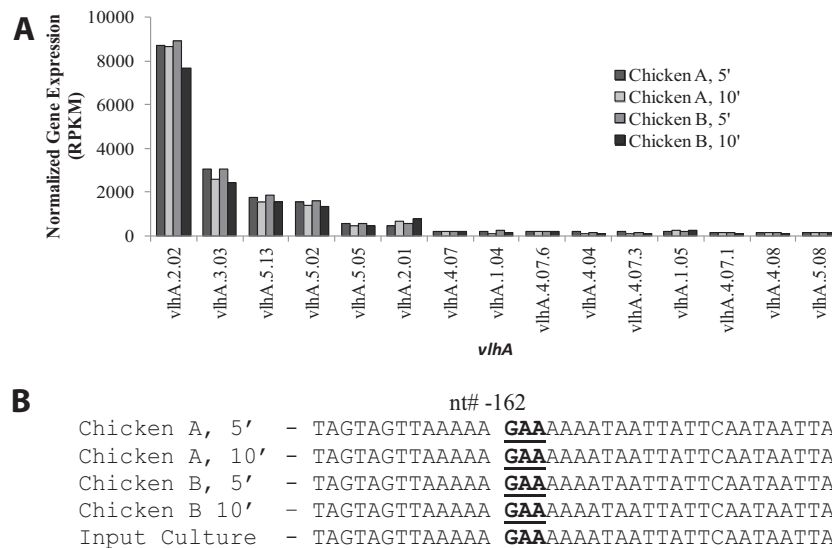


FIG 3 (A) *vlhA* expression of *M. gallisepticum* recovered and cultured at five (5') or 10 (10') passages from the tracheas of the two different infected birds at 7 days postinfection. Only data from *vlhA* genes with an average RPKM value of >100 are displayed. (B) The sequences of the region upstream of the highly expressed *vlhA* 2.02 gene in the cultures recovered from the infected birds. nt, nucleotide.

recovered directly from tracheas into Hayflick's complete medium and serially passaged *in vitro*. After either 5 or 10 passages, *M. gallisepticum* populations were predominantly expressing *vlhA* 2.02 with an average RPKM value of 8,463, a 4.4-fold increase relative to *vlhA* 2.02 expression observed directly on tracheal mucosae at 7 days postinfection ($q < 0.0001$) (Fig. 3A). The *M. gallisepticum* recovered after one passage *in vitro* displayed a transitional *vlhA* profile, with *vlhA* 3.03 and 2.02 increasing in expression and *vlhA* 4.07.1 and 4.08 decreasing in expression (data not shown). These data suggest that expression of *vlhA* 2.02 is favorable for the pathogen after recovery from the host trachea and passage in a nutritive medium. Several other *vlhA* genes, including *vlhA* 3.03, were also expressed in the recovery cultures but at levels much lower than *vlhA* 2.02. Interestingly, *vlhA* 3.03 was expressed at a much (4.5-fold) lower, nondominant level than in the initial input culture (Fig. 3A).

When the promoter region upstream of the highly expressed *vlhA* 2.02 gene from the recovery cultures was PCR amplified and sequenced, data revealed only a single GAA trinucleotide upstream of the start codon, suggesting that 12 GAA repeat copies might not be essential for the expression of a given *vlhA*, at least in the *in vitro* culture following direct recovery from the trachea (Fig. 3B). Furthermore, we observed no changes in the genomic nucleotide sequence upstream of *vlhA* 2.02 in the cultures recovered from the infected animals and passaged *in vitro* compared to the broth-grown input culture.

DISCUSSION

This study has demonstrated the global population changes in *M. gallisepticum vlhA* gene expression assessed directly from tracheas of experimentally infected chickens over the course of a 7-day infection. It was important to recover the *M. gallisepticum* RNA directly from the trachea and introduce it into the TRIzol reagent to minimize the time between collection and extraction, as we observed significant changes in the gene expression profile of *M. gallisepticum* after just a single passage in culture medium (data

not shown). This study design allowed the capture of the most accurate representation of the *vlhA* profile *in vivo* at each time point during the infection process.

These observed changes in expression are likely not antibody mediated, as the decrease in expression of *vlhA* 3.03 is seen as early as 2 days postinfection, well before the formation of specific antibodies (23). This finding is in agreement with a previous study that demonstrated that *vlhA* expression in infected chickens switches prior to the host mounting an antibody response (13). However, this study greatly expanded on those initial observations to include the expression status of all of the *vlhA* genes over the early course of infection.

These findings are significant as they imply that the driving force behind the shift in *vlhA* expression is not controlled entirely by the adaptive immune system. These data suggest that another mechanism is, at least in part, controlling changes in expression of the *vlhA* genes *in vivo*.

It is possible that early phase variation could be driven, in part, by the alterations in the cellular architecture of the tracheas during infection. As the infection progresses, the host cells experience denuding of cilia, squamous cell metaplasia, and eventual destruction of the host cell membranes. It is possible that *M. gallisepticum vlhA* expression is changing in response to these alterations and the pathogen is expressing a specific set of *vlhA* genes to best persist in the current environment. Consistent with the hypothesis that *M. gallisepticum vlhA* expression is nonrandom and follows the progression of pathogenesis in the host, *vlhA* 1.07 and 4.01, reported by Ron et al. (19) to express significant antigens at 2 to 5 weeks postinfection, were not predominantly expressed within 1 week postinfection, as reported here.

Data here indicate that, despite expression of different *vlhA* genes being seen in the host at day 7 postinfection, *vlhA* 2.02 is the predominant *vlhA* gene expressed in the *M. gallisepticum* population following recovery and passage in Hayflick's complete medium. The dominance of *vlhA* 2.02 and the lack of reversion to predominant *vlhA* 3.03 expression observed in the broth-grown

input culture were unanticipated. However, in these recovery cultures, expression of *vlhA* 2.02 was also accompanied by expression (albeit at lower levels) of several other *vlhA* genes in the population, including *vlhA* 3.03 and 5.13, suggesting that two or more *vlhA* genes are expressed at a given time point in the population. These data also indicate that *M. gallisepticum* may favor the expression of several *vlhA* genes (*vlhA* 3.03, 2.02, and 5.13) when no longer faced with the pressures of surviving in the airway of the host.

Data here also demonstrated that 12 GAA trinucleotide repeats are not essential for the expression of a given *vlhA*, since the *vlhA* 2.02 gene that was highly expressed in the recovery samples does not contain 12 GAA repeats in upstream sequences. It had previously been hypothesized that 12 GAA repeats were necessary for expression of a given *vlhA* because it allowed the correct spacing between flanking sequences for necessary accessory factors to bind, as *M. gallisepticum* does not possess a robust consensus sequence at -10 and -35 (15). The observed expression of *vlhA* genes without the 12 GAA repeats upstream suggests that there may be another mechanism at play that is responsible, at least in part, for the regulation of *vlhA* expression in *M. gallisepticum*.

These results, revealing global *vlhA* expression changes over the course of early infection, are important in elucidating mechanisms of persistence, colonization, and overall pathogenesis of *M. gallisepticum* in the natural host. Full and complete understanding of these mechanisms will provide information likely to be critical for development of rationally designed *M. gallisepticum* therapeutics and vaccines.

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