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MOLECULAR PATHOGENESIS

Variable Lipoprotein Hemagglutinin A Gene (*vlhA*) Expression in Variant *Mycoplasma gallisepticum* Strains *In Vivo*

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ABSTRACT *Mycoplasma gallisepticum*, the primary etiologic agent of chronic respiratory disease, is a significant poultry pathogen, causing severe inflammation and leading to economic losses worldwide. Immunodominant proteins encoded by the variable lipoprotein and hemagglutinin (*vlhA*) gene family are thought to be important for *M. gallisepticum*-host interaction, pathogenesis, and immune evasion, but their exact role remains unknown. Previous work has demonstrated that *vlhA* phase variation is dynamic throughout the earliest stages of infection, with *vlhA* 3.03 being the predominant *vlhA* expressed during the initial infection, and that the pattern of dominant *vlhA* expression may be nonrandom and regulated by previously unrecognized mechanisms. To further investigate this gene family, we assessed the *vlhA* profile of two well-characterized vaccine strains, GT5 and Mg7, a *vlhA* 3.03 mutant strain, and an *M. gallisepticum* population expressing an alternative immunodominant *vlhA*. Here, we report that two *M. gallisepticum* vaccine strains show different *vlhA* profiles over the first 2 days of infection compared to that of wild-type R_{low}, while the population expressing an alternative immunodominant *vlhA* gene reverted to a profile indistinguishable from that of wild-type R_{low}. Additionally, we observed a slight shift in the *vlhA* gene expression profile but no reduction in virulence in a *vlhA* 3.03 mutant. Taken together, these data further support the hypothesis that *M. gallisepticum vlhA* genes change in a nonstochastic temporal progression of expression and that *vlhA* 3.03, while preferred, is not required for virulence. Collectively, these data may be important in elucidating mechanisms of colonization and overall pathogenesis of *M. gallisepticum*.

KEYWORDS *Mycoplasma gallisepticum*, RNA-seq, phase variable, *vlhA*

Mycoplasma gallisepticum, the primary etiologic agent of chronic respiratory disease (CRD), causes significant disease and monetary loss throughout the poultry industry worldwide. This highly transmissible pathogen affects the respiratory tract, causing significant inflammation of the air sacs, lungs, and trachea, as well as the reproductive tract, resulting in decreased weight gain and egg production. *Mycoplasma gallisepticum* is also pathogenic in other avian species, causing infectious sinusitis in turkeys and severe conjunctivitis in house finch (1, 2).

Despite much effort, little is understood about the mechanisms of survival and persistence employed by *M. gallisepticum*. It has been established that the primary attachment proteins, GapA and CrmA (3), fibronectin binding proteins, PlpA and Hlp3 (4), sugar transport permease, MalF (5), and dihydrolipoamide dehydrogenase, Lpd (6), all play key roles in the survival and persistence of *M. gallisepticum* in the host. Additionally, Ron et al. identified 13 proteins preferentially expressed during *in vivo* infection, including GapA, PlpA, and Hlp3 (7).

Likely important in the virulence of *M. gallisepticum* are the members of the variable lipoprotein and hemagglutinin (*vlhA*) gene family, consisting of 43 closely related genes

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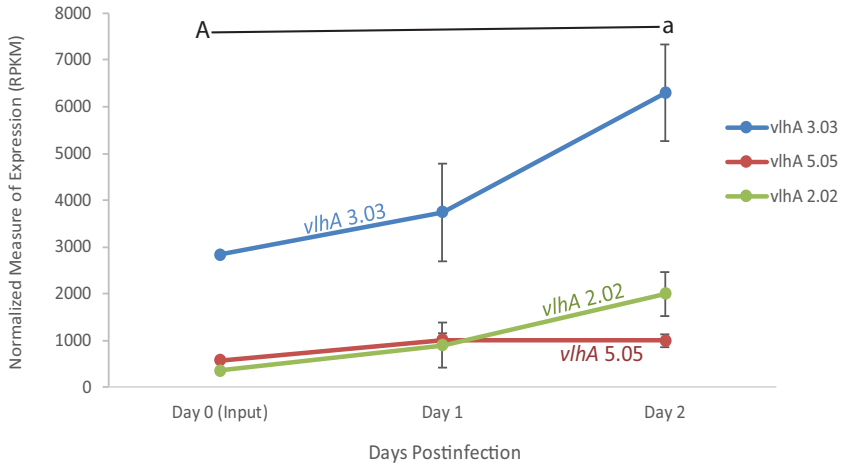

FIG 1 *vlhA* expression profile of *M. gallisepticum* Mg7 extracted directly from tracheas of experimentally inoculated birds over the course of the 2-day infection, as determined by RNA sequencing. Only *vlhA* genes discussed are displayed. Each data point represents an average RPKM value from the results determined for five animals (with the exception of broth). 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 *vlhA* 3.03 (A/a).

distributed across 5 loci and comprising just over 10% of the entire genome in strain R_{low} (8–10). While the function of these gene products remains unknown, it has been speculated to be related to evasion of the host adaptive immune response during infection (11), and variation in *vlhA* gene complement has been observed among *M. gallisepticum* strains differing in virulence (12). These gene products have been hypothesized to be involved in attachment and have been shown to bind host red blood cells (13) and host apolipoprotein A1 (14).

Previous work from our laboratory has demonstrated that *M. gallisepticum* strain R_{low} *vlhA* gene expression changes in a nonstochastic temporal progression in the initial stages of infection in the natural host and may be regulated, at least in part, by previously unrecognized mechanisms (15). *M. gallisepticum vlhA* 3.03 gene expression is dominant during the very initial stages of infection and increases in expression through 1 day postinfection before rapidly decreasing in expression during the remaining infection.

Here, we expand on this work, examining the early *vlhA* gene expression profiles from four different *M. gallisepticum* strains from experimentally inoculated chickens over the earliest stages of infection. We hypothesize that perturbation to the established model will result in changes in the ordered pattern of *M. gallisepticum vlhA* gene expression, providing valuable information about what may be contributing to the changes in *vlhA* gene expression.

We report the *vlhA* profiles over 2 days for two well-characterized vaccine strains, GT5 (16) and Mg7 (6), that produce no significant tracheal lesions or changes to the tracheal architecture, in addition to an *M. gallisepticum* population stably expressing an alternative dominant *vlhA* (15). Additionally, we report the virulence and *vlhA* gene expression profile of an *M. gallisepticum* strain harboring a transposon insertion mutation in the primary immunodominant *vlhA* 3.03 gene.

RESULTS

Mg7 *vlhA* gene expression. The pattern of the *M. gallisepticum* vaccine strain, Mg7, over the course of the 2-day infection showed that the expression of *vlhA* 3.03 increased (Fig. 1; see also Fig. S1 in the supplemental material), with a 1.31-fold increase at day one, compared to that of the broth-grown input culture used for inoculation. Even more dramatic was the increase in expression of *vlhA* 3.03 (1.68-fold) between days one and two postinfection. This pattern of *vlhA* gene expression observed in Mg7

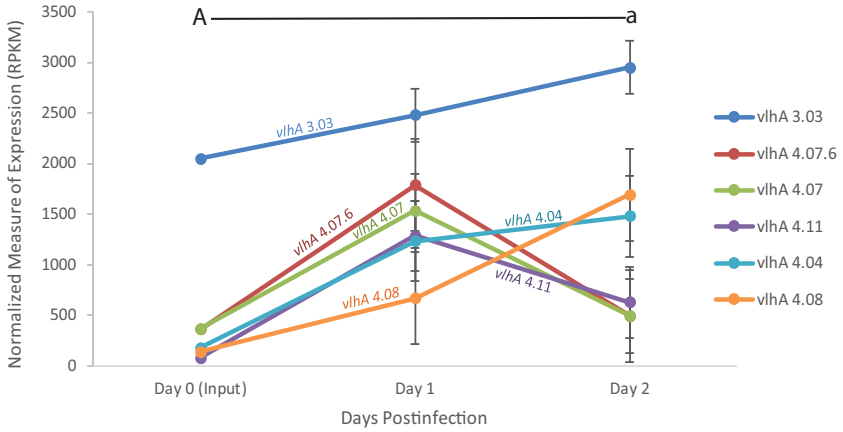

FIG 2 *vlhA* expression profile of *M. gallisepticum* GT5 extracted directly from tracheas of experimentally inoculated birds over the course of the 2-day infection, as determined through RNA sequencing. Only *vlhA* genes discussed are displayed. Each data point represents an average RPKM value from the results determined for five animals (with the exception of broth). Error bars show SEM. Key statistically significant changes between two time points are indicated by paired upper- and lowercase letters for *vlhA* 3.03 (A/a).

is strikingly different from that of wild-type R_{low} *in vivo*, as reported by Pflaum et al. (15), where a dramatic drop in the expression of initially dominant *vlhA* 3.03 is observed to begin between days one and two postinfection.

Interestingly, an increase in *vlhA* 2.02 was observed at days one and two postinfection, an observation that was not seen with the wild-type R_{low} strain. Furthermore, we did not see an increase in the expression of *vlhA* 4.07 and 4.07.6 at days one and two postinfection, as was observed previously in wild-type virulent R_{low}.

Most notable is the continued increase in the expression of *vlhA* 3.03 over the earliest stages of infection in this live attenuated vaccine strain, which does not result in any significant tracheal lesions at 2 weeks postinfection (6) and which is in contrast to the previous *vlhA* pattern observed with virulent strain R_{low} (15).

GT5 *vlhA* gene expression. Similar to the *vlhA* gene expression pattern observed in the Mg7 vaccine strain, *M. gallisepticum* recovered from chickens inoculated with GT5 showed a continued increase in expression of *vlhA* 3.03 over the course of the 2-day infection compared to the broth-grown inoculum, with an increase at day one postinfection continuing through day two postinfection (Fig. 2 and Fig. S2).

We observed an increase in the expression of *vlhA* 4.07 and 4.07.6 at day one postinfection, as was previously observed in the wild-type R_{low} strain. Interestingly, we saw an increase in the expression of *vlhA* 4.08 at days one and two postinfection, a pattern we did not previously observe until much later time points with wild-type virulent R_{low}. Like Mg7, the *vlhA* expression pattern of this vaccine strain, which does not result in any significant tracheal lesions at 2 weeks postinfection, differs from that of virulent R_{low}, in that the expression of *vlhA* 3.03 increased significantly over the earliest days of infection.

***vlhA* 3.03 mutant induced host pathology and recovery.** Surprisingly, the transposon insertion in the *vlhA* 3.03 gene did not cause a significant reduction in virulence. There was a statistically indistinguishable difference in tracheal thickness measurements between chickens challenged with wild-type R_{low} and the *vlhA* 3.03 mutant, both demonstrating moderate mucosal thickening (Fig. 3C), suggesting that this mutant displayed virulence at a level comparable to that of wild-type R_{low} in the chicken respiratory tract. The tracheal thicknesses induced by both wild-type R_{low} and *vlhA* 3.03, while indistinguishable from each other, were significantly higher than those previously observed from a medium control-inoculated chicken (17).

M. gallisepticum was recovered from the trachea of chickens challenged with the *vlhA* 3.03 mutant in at least 4 out of 5 birds at each day throughout the 7-day infection

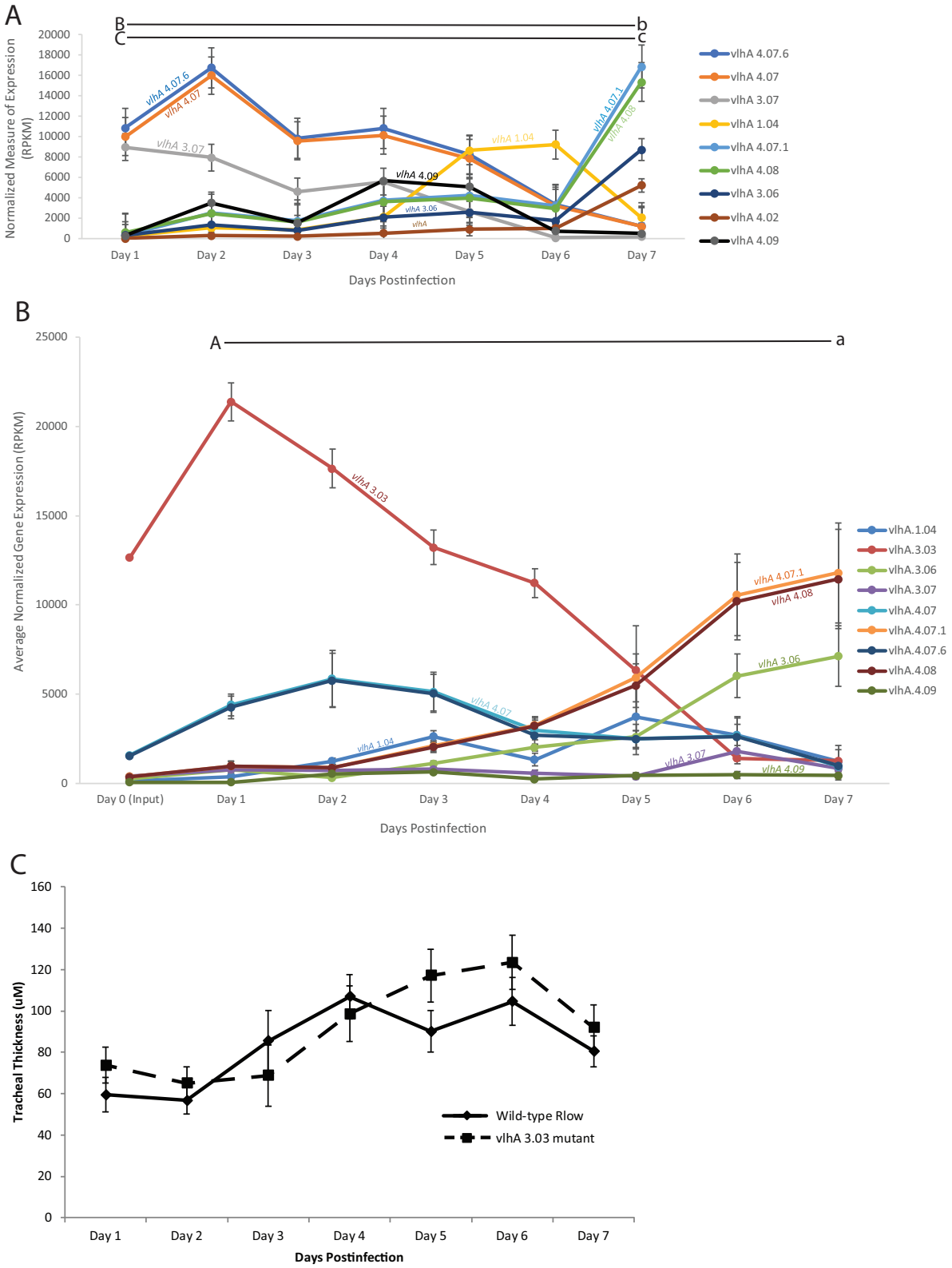

FIG 3 *vlhA* expression profile of the *M. gallisepticum vlhA* 3.03 mutant (A) or wild-type *M. gallisepticum* R_{low} (B) extracted directly from tracheas of experimentally inoculated birds over the course of the 7-day infection, as determined by RNA sequencing. Only *vlhA* genes discussed are shown. Each data point represents an average RPKM value from the results determined for five animals. Error bars show SEM. Key statistically significant changes between two time points are indicated by paired upper- and lowercase letters for the genes *vlhA* 3.03 (A/a), *vlhA* 4.07.6 and *vlhA* 4.07 (B/b), and *vlhA* 4.07.1 and *vlhA* 4.08 (C/c). (C) Tracheal thickness of chickens challenged with wild-type *M. gallisepticum* (solid line) or the *M. gallisepticum* 3.03 mutant (dashed line). Each data point represents an average thickness measure of 4 measurements from 5 animals each. Error bars show SEM.

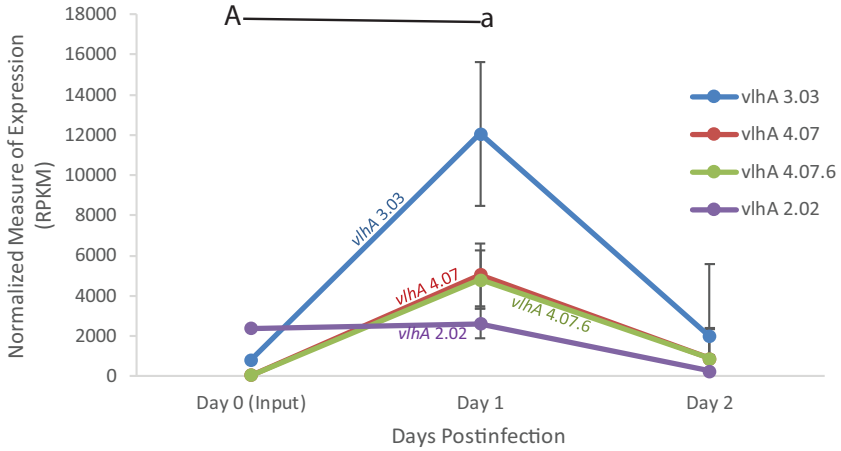

FIG 4 *vlhA* expression profile of the *M. gallisepticum* population expressing *vlhA* 2.02 as the predominant *vlhA* extracted directly from tracheas of experimentally inoculated birds over the course of the 2-day infection, as determined by RNA sequencing. Only *vlhA* genes discussed are displayed. Each data point represents an average RPKM value from the results determined for five animals (with the exception of broth). Error bars show SEM. Key statistically significant changes between two time points are indicated by paired upper- and lowercase letters for *vlhA* 3.03 (A/a).

time course (data not shown). This rate of *M. gallisepticum* recovery was indistinguishable from that of chickens challenged with the virulent wild-type R_{low} strain, suggesting that the mutation in the immunodominant *vlhA* 3.03 does not negatively impact the ability of *M. gallisepticum* to colonize and survive in the respiratory tract of chickens.

Taken together, these data suggest that the *M. gallisepticum vlhA* 3.03 mutation did not have a negative effect on the colonization or virulence of the pathogen in the chicken respiratory tract.

***vlhA* 3.03 mutant *vlhA* gene expression.** The *vlhA* 3.03 mutant of R_{low} demonstrated an *in vivo vlhA* gene expression pattern that was similar to that previously demonstrated for wild-type R_{low}. In both strains, *vlhA* 4.07 and 4.07.6 peaked at day two, albeit at higher expression levels and as the predominant *vlhA* in the *vlhA* 3.03 mutant and as subdominant to *vlhA* 3.03 in wild-type R_{low}. Both the *vlhA* 3.03 mutant and wild-type R_{low} demonstrated peak expression of *vlhA* 1.04 at days 5 and 6 (Fig. 3A and B and Fig. S3). Notably, the *vlhA* 3.03 mutant demonstrated increased expression of *vlhA* genes that were not expressed above baseline in wild-type R_{low}, specifically *vlhA* 3.07, which peaked at day 1, and *vlhA* 4.09, which peaked at day 4. Overall, *vlhA* gene expression in the *vlhA* 3.03 mutant resembled that of wild-type *M. gallisepticum* R_{low} temporally and in genes expressed, save the lack of v*lhA* 3.03 and the additional genes expressed above background.

***vlhA* expression pattern of a population with an alternative dominant *vlhA* gene.** We observed an immediate switch back to a *vlhA* expression pattern highly similar to that of wild-type R_{low}, with a 25-fold increase of *vlhA* 3.03 at day one postinfection (Fig. 4A and Fig. S4). *vlhA* 4.07.6 and 4.07 showed a 65-fold increase in expression by day one postinfection, a pattern similar to that for wild-type R_{low}. By day 1 postinfection the *vlhA* pattern was statistically indistinguishable (with the exception of minor *vlhA* genes) from previous wild-type R_{low} *vlhA* profile patterns (data not shown). These data demonstrate that challenging chickens with an *M. gallisepticum* population that is expressing a non-*vlhA* 3.03 dominant *vlhA* gene does not affect the overall progression of *vlhA* expression and that *vlhA* 3.03 expression immediately and dramatically increases on day one.

DISCUSSION

This study has compared the *vlhA* gene expression profiles of populations of four different strains of *M. gallisepticum* when RNA is collected directly from the tracheal mucosa of experimentally inoculated chickens over the earliest stages of infection. We

hypothesize that *vlhA* switching within the first week of infection may be driven, at least in part, by changes in the host cellular architecture as the disease pathology progresses over time. To test this hypothesis, we exposed chickens intratracheally to live attenuated vaccine strains GT5 and Mg7. Since these two vaccine strains do not cause the tracheal changes that are traditionally associated with wild-type virulent R_{low}, such as deciliation of host cells and squamous cell metaplasia, we expect to see a dramatically different *vlhA* gene expression profile over the earliest stages of infection.

The vaccine strain Mg7 showed very dramatic changes in *vlhA* expression compared to that of wild-type virulent R_{low}. The predominant *vlhA* expressed in the broth (*vlhA* 3.03) continues to increase in expression at day one and day two postinfection, in contrast to wild-type R_{low}. Several other *vlhA* genes, such as *vlhA* 4.07 and *vlhA* 4.07.6, did not show any significant increase or change in expression over the 2-day time course. This finding was particularly significant, as the Mg7 vaccine strain does not cause significant pathology (6), and supports our hypothesis. Additionally, the vaccine strain GT5 showed a pattern of *vlhA* gene expression different from that of wild-type R_{low}. An increase in the expression of *vlhA* 3.03 at days one and two postinfection similar to that of Mg7 was observed and was consistent with our hypothesis.

Since we see a different *vlhA* gene expression profile in the populations of *M. gallisepticum* strains that do not cause significant pathology in the host, it is possible that the changes in tracheal architecture associated with disease pathology could play a role, at least in part, in driving the changes in *M. gallisepticum vlhA* gene expression. The previously reported nonstochastic patterned progression of *vlhA* expression (15) is seen when there are significant pathological changes in the trachea associated with *M. gallisepticum* infection. This same ordered pattern of *vlhA* expression is not seen when there are not significant cellular changes in the trachea (e.g., inoculation with live attenuated vaccine strains); the *vlhA* gene expression pattern appears to be altered where *vlhA* 3.03 increases in expression at day one postinfection and continues to increase through day two postinfection.

Pathogenesis of the *vlhA* 3.03 negative mutant was not reduced relative to that of R_{low} in the chicken respiratory tract during the first week of infection, indicating that *vlhA* 3.03 is not essential for the early stages of infection and colonization. However, it is possible that another *vlhA* gene, such as *vlhA* 4.07, which shows an increase in expression at day one postinfection, could be similar enough in function to serve the same role as *vlhA* 3.03 *in vivo*, highlighting the potential importance of the redundancy within this unique gene family.

While *vlhA* 3.03 may be dispensable for initial colonization and virulence of *M. gallisepticum* in the chicken trachea, *vlhA* 3.03 may play an essential role in a niche within the host that has not yet been explored.

The *vlhA* 3.03 mutant showed a slight shift in the patterned progression of *vlhA* gene expression over the 7-day time course, with some *vlhA* genes (*vlhA* 4.07 and *vlhA* 4.07.6) peaking in expression later than was observed with wild-type virulent R_{low}. This demonstrates resilience and plasticity in the ordered expression pattern among *vlhA* gene paralogs. When there is a mutation in the preferred dominant *vlhA* gene (*vlhA* 3.03), the progression of *vlhA* gene expression is slightly shifted, but the overall pattern was still maintained over the course of a 7-day infection.

Challenging chickens with a population of *M. gallisepticum* expressing an alternative dominant *vlhA* (*vlhA* 2.02) does not alter the pattern progression of *vlhA* expression. In fact, by day one postinfection, the *vlhA* expression pattern is virtually indistinguishable from that of the wild-type virulent R_{low} strain predominantly expressing *vlhA* 3.03, suggesting that this progression of *vlhA* expression is important during the initial stages of the infection process. These data, along with the *vlhA* 3.03 mutant data, also suggest that while the dominant expression of *vlhA* 3.03 at the very earliest stages of the infection process is favored, it may not be essential for the colonization and virulence of *M. gallisepticum* in the chicken trachea.

Additionally, these data further support the hypothesis that *vlhA* gene expression is changing, at least in part, as a response to the changing cellular environment of the

trachea. The vaccine strains Mg7 and GT5 show a significant reduction in virulence (6, 16) in the host and do not show the same initial progression of *vlhA* gene expression as that shown by wild-type virulent R_{low}. Additionally, *M. gallisepticum* GT5 and Mg7 do not cause the same host cell stress response or immune dysregulation (J. Beaudet, unpublished data) that is observed in chickens experimentally challenged with virulent wild-type R_{low} (18). It is possible that since the trachea is not being disrupted or damaged by the vaccine strains, the *vlhA* genes are not forced to change in response. These data also suggest that *M. gallisepticum* colonizing other organs, such as the lungs and air sacs, expresses different suites of *vlhA* genes best suited for *M. gallisepticum* survival in their current environments.

Collectively, these data further support the hypothesis that the *vlhA* genes change in a nonstochastic temporal progression of expression and that *vlhA* 3.03, while preferred, is not required to be the initially predominant *vlhA* expressed at the time of infection for colonization and virulence. These results, revealing global *vlhA* expression changes over the course of early infection in different strains of *M. gallisepticum*, are important in elucidating mechanisms of colonization, persistence, and overall pathogenesis of *M. gallisepticum* in the natural host. While these data provide detailed insights into the *vlhA* gene family of *M. gallisepticum*, it is not clear, at this time, exactly what molecules or mechanisms are driving the change in *vlhA* gene expression seen here. It is also important to note that all *vlhA* expression observations were made on a population level and do not exclude the possibility that the changes observed were due to the survival of members of the population expressing the suite of *vlhA* genes best suited for the current microenvironment in the host airway. Full and complete understanding of the phase variation of *M. gallisepticum vlhA* genes may play a pivotal role in the understanding of the earliest stages of the infection process of *M. gallisepticum* and how the pathogen responds to changes of the microenvironment within the host. To our knowledge, the current study, combined with our previous work, is the first time phase variation has been assessed in a bacterial pathogen at the earliest stages of infection. These findings may be directly relevant to other important bacterial pathogens that possess phase-variable gene families expressing variable surface proteins.

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 prior to the start of the study. Nonmedicated feed and water were provided *ad libitum* throughout the experiment. All animal studies were performed in accordance with approved UConn IACUC protocol number A15-056.

Chicken study. Stocks of *M. gallisepticum* strain R_{low} (passage 17) were grown overnight at 37°C in Hayflick's complete 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, and 10-fold serial dilutions were conducted to confirm viable color-changing unit titers as previously described (4, 6, 16). Bacteria were pelleted by centrifugation at 10,000 $\times$ *g* for 10 min and resuspended in Hayflick's complete medium. A previously identified population of *M. gallisepticum* stably expressing *vlhA* 2.02 as the predominant *vlhA* (15) was verified for predominant *vlhA* 2.02 expression by RNA sequencing (RNA-seq), quantitated, and frozen at −80°C. At the time of infection, the stocks were thawed and immediately used for infection to ensure there were no changes in *vlhA* expression.

Previously characterized *M. gallisepticum* vaccine strains GT5 (16) and Mg7 (6), in addition to the *vlhA* 3.03 mutant (described in detail below), were grown as described above in Hayflick's medium supplemented with 150 μg/ml gentamicin to maintain the transposon insertions. Chickens were inoculated intratracheally as previously described (6) with 1×10^8 CFU/200 μl of the respective cultures, and RNA was collected directly from the tracheal mucosa daily, as described below.

Two-day time course studies were conducted with *M. gallisepticum* strains GT5 and Mg7 to assess the very early changes in *vlhA* expression, as the virulence of these strains has been previously reported (6, 16), in addition to the population expressing *vlhA* 2.02 as the predominant *vlhA*. A 7-day time course study was conducted on the *vlhA* 3.03 mutant to assess the virulence of the mutant in addition to the very early changes in the *vlhA* gene expression profile.

RNA extraction. Five infected chickens per group were humanely sacrificed by cervical dislocation daily for a total of 2 (GT5, Mg7, and the *vlhA* 2.02 predominant population) or 7 (*vlhA* 3.03 mutant) 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 the 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 the NEBNext poly(A) mRNA magnetic isolation module (New England BioLabs, Ispwich, MA, USA) as previously described (15). Briefly, 5 μg of total RNA combined with an equal volume of bead binding buffer was bound to the poly(T) oligonucleotide-attached magnetic beads at 65°C for 5 min. The remaining supernatant was collected, cleaned, and concentrated using the Zymo DNA Clean & Concentrator 25 kit (Zymo Research Corporation) and eluted in 25 μl RNase-free water.

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

Illumina sequencing and RNA-seq analysis. The cDNA libraries were created using the Illumina TruSeq stranded mRNA library preparation kit (Illumina Inc., San Diego, CA, USA) and sequenced on a NextSeq500 sequencing platform (Illumina Inc.) as previously described (15).

RNA-seq analysis was performed as previously described (15). Briefly, Fastq data were assembled and mapped, and differential gene expression was assessed using Rockhopper with Bowtie2 parameters allowing zero mismatches (19, 20). 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 $\log_2$ 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 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 (20).

Identification of the *vlhA* 3.03 mutant. A library of 3,600 R_{low} transposon (Tn) mutants was generated via electroporation using plasmid pMT85, as described previously (6). The plasmid carries mini-Tn*4001*-gentamicin, which encodes a gentamicin resistance gene. Pools of 30 mutants were grown, and genomic DNAs were extracted. PCR screening of mutant pools for transposon insertions in the *vlhA* 3.03 gene was performed in 96-well plates by using a gene-specific primer in conjunction with a 5′ or 3′ transposon-specific primer. PCR was run on positive pools in the reverse orientation (reverse gene primer with the opposite-end transposon primer to ensure the transposon was located within the gene). Pools with positive PCR products that exhibited the correct gene size for a transposon insertion were selected for a second round of screening. DNA was extracted from each individual pool member and screened again as described above. Sanger sequencing was used to identify the Tn insertion site and confirm there was only a single Tn insertion, as described previously (6, 21).

A reverse transcription-quantitative PCR (RT-qPCR) assay was used to verify that the transposon insertion was sufficient to disrupt the expression of *vlhA* 3.03 using the QuantiTect reverse transcription kit (Qiagen) with the following PCR conditions: 50°C for 30 min, 95°C for 15 min, and then 35 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 31 s with 20 pmol of each forward primer 5′-ACGACCAAGCA AAACCTAATGA-3′ and reverse primer 5′-ACTCCTGAAGCGAACACTCC-3′. The Sanger sequencing analysis of the *vlhA* 3.03 mutant showed that the transposon was inserted at base pair 75. RT-qPCR analysis showed a 100-fold reduction in the expression of the gene, which was further confirmed by our RNA-seq gene expression data (data not shown).

Tracheal thickness measurements and bacterial recovery. As an established objective measure of virulence, tracheal thickness was assessed for the *M. gallisepticum vlhA* 3.03 mutant daily over the course of 7 days. Tracheal thickness measurements on a distal ring of the trachea were determined for all chickens, as previously described (16). Histological data were subjected to nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) in the ranks test, in which all pairwise multiple-comparison procedures were performed using the Student-Newman-Keuls method for groups of equal sizes. Statistical tests were conducted using SigmaPlot 11.0 (Systat Software, San Jose, CA). To assess the recovery of viable *M. gallisepticum* from the trachea of infected animals, a ring from the distal portion of the trachea was collected directly into Hayflick's complete medium (with gentamicin for the GT5, Mg7, and *vlhA* 3.03 mutant strains) and incubated for 5 h at 37°C. After the incubation period, cultures were passed through 0.45-μm-pore-size filters to remove non-*Mycoplasma* contaminates, adjusted to pH 7.4, and reincubated at 37°C. Samples were considered positive for *M. gallisepticum* recovery if the color shifted to yellow within 30 days.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00524-18.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.
SUPPLEMENTAL FILE 2, PDF file, 1.0 MB.
SUPPLEMENTAL FILE 3, PDF file, 1.2 MB.
SUPPLEMENTAL FILE 4, PDF file, 1.0 MB.

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