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Effects of sialidase knockout and complementation on virulence of *Mycoplasma gallisepticum*

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ABSTRACT

Reannotation of the pathogenic *Mycoplasma gallisepticum* strain R_{low} genome identified the hypothetical gene MGA_0329 as a homolog of the sialidase gene MS53_0199 of *Mycoplasma synoviae* strain MS53. Potent sialidase activity was subsequently quantitated in several *M. gallisepticum* strains. Because sialidase activity levels correlate significantly with differing *M. synoviae* strain virulence, we hypothesized this enzyme may also influence the virulence of *M. gallisepticum*. MGA_0329 was disrupted in strain R_{low} to create mutants 6, 358 and P1C5, which resulted in the loss of sialidase activity in all three mutants. Chickens infected with the knockout mutants had significantly less severe ($P < 0.05$) tracheal lesions and tracheal mucosal thickening than chickens infected with equal doses of strain R_{low}. Significantly fewer ($P < 0.05$) CCU especially of strains 6 and P1C5 were recovered at necropsy. Mini-Tn*4001*tet plasmid pTF20 carrying a wild-type copy of MGA_0329 with its native promoter was used to complement the genetic lesion in strain P1C5. Three clones derived from P1C5, each having one copy of MGA_0329 stably transposed into a different site in its genome, expressed sialidase restored to wild-type activity levels (1.58×10^{-8} U/CFU). Complementation of P1C5 with MGA_0329 did not restore it to wild-type levels of virulence, indicating that the contribution of sialidase to *M. gallisepticum* virulence is not straightforward.

1. Introduction

Mycoplasma gallisepticum is associated with chronic respiratory disease in chickens, infectious sinusitis in turkeys, and conjunctivitis in certain passerine species, e.g. the house finch (reviewed by Levisohn and Kleven, 2000). Mucosal lesions in infected birds are characterized by deciliation of epithelial cells, epithelial hyperplasia, and pronounced lymphocytic infiltration with follicular lymphoid hyperplasia. Co-infection by other bacterial and viral pathogens is associated with markedly increased morbidity and mortality.

The economic impact of *M. gallisepticum* on the poultry industry is substantial, and consequently studies have been undertaken to characterize its virulence. The primary mechanism for host cell attachment has been described, and its importance *in vivo* has been demonstrated (Goh et al., 1998; Papazisi et al., 2000, 2002). Multiple additional features have been implicated in the pathogenicity of *M. gallisepticum* including antigenically variable hemagglutinins (VlhA family and PvpA) with a secondary role in cytadherence (Boguslavsky et al., 2000; Markham et al., 1992, 1994), extracellular matrix-binding (Jenkins et al., 2008; May et al., 2006), hydrogen peroxide resistance

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(Jenkins et al., 2007), dihydrolipoamide dehydrogenase (Lpd) (Hudson et al., 2006), and *Mycoplasma*-specific lipoprotein A (MslA) (Szczepanek et al., 2010a,b).

Examining the complete genome sequence of Strain R_{low} (Papazisi et al., 2003; Szczepanek et al., 2010a,b) led to the recognition of the hypothetical open reading frame MGA_0329 (GenBank Accesion # NP_853343) as a putative sialidase gene. Sialidase activity (EC 3.2.1.18) has been documented in a variety of bacteria including *Mycoplasma alligatoris* and *Streptococcus pneumoniae*, and is involved in bacterial colonization and dissemination, extracellular matrix (ECM) degradation, nutrient catabolism, and induced host-cell death (reviewed by Corfield, 1992; Hunt and Brown, 2007; King et al., 2006; reviewed by Vimr and Lichtensteiger, 2002). Because it is frequently associated with pathogenesis in other bacteria, we tested the hypothesis that MGA_0329 encodes a functional sialidase involved in the virulence of *M. gallisepticum*, offering insight into a previously unexplored contributor to the pathogenicity of this species.

2. Materials and methods

2.1. Strains and sialidase activity assay

Filter-cloned stocks of *M. gallisepticum* strain R_{low} was maintained in Hayflick's broth containing 10% (v/v) horse serum. The sialidase activity of washed cells was quantitated using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN) as previously described (May et al., 2007).

2.2. Insertional inactivation and genetic complementation of sialidase activity

Random transposon insertional mutagenesis was used to disrupt MGA_0329 in *M. gallisepticum* strain R_{low} by electroporation with the suicide plasmid vector pISM2062 as described by Hudson et al. (2006). The plasmid carries the transposon Tn*4001*mod encoding a gentamicin resistance element. Additional mutants were generated using the suicide plasmid vector pMT85, which carries a derivative of Tn*4001* (gent miniTn) (Zimmerman and Herrmann, 2005). Transformed gentamicin resistant (gm^R) colonies were titered in Hayflick's broth plus gentamicin and stored at −80 °C. To map the sites of insertion precisely, genomic DNA was extracted from individual isogenic mutants and sequenced directly using the outward primer SG857 complementary to the transposon.

The disrupted MGA_0329 gene of strain P1C5 was complemented by random transposon insertion using the mini-Tn*4001*tetM plasmid vector pTF20 (French et al., 2008). Wild-type MGA_0329 with its native promoter was amplified from strain R_{low} using primers complementary to the flanking open reading frames MGA_0324 (5′-GTC GAC TCA GAT CAT TAA ACT AGC GCC TAA-3′) and MGA_0330 (5′-GTC GAC CGC ATG ATA CGA TAA CGA AAT G-3′). The 3.13 kb PCR product was cloned into the *Sal*I site of pTF20 to create the plasmid pM329, which was electroporated into strain P1C5. Individual gm^R/tet^R colonies were titered in Hayflick's broth plus gentamicin and tetracycline and stored at −80 °C. The site of MGA_0329 insertion was precisely mapped by direct genomic DNA sequencing using outward-directed primers complementary to the mini-tetM transposon (5′-GCT GAA ACT AAG CCC TAA AAG TAC CC-3′ and 5′-TGA GCG AGG AAG CGG AAG AG-3′). Three complemented clones named GSR1, GSR2 and GSR3 were selected for further study.

2.3. In vivo experimental infection studies

The effects of sialidase knockout and complementation on virulence of *M. gallisepticum* were assessed in three trials using methods previously described (Papazisi et al., 2002). White Leghorn chickens ($n = 6$ per group) were inoculated intratracheally with 1.5×10^7 CFU of the strains tested in accordance with United States law, as approved by the University of Connecticut's institutional animal care and use committee. In each trial the positive control group was challenged with wild-type strain R_{low} and the negative control (sham-infected) group received sterile Hayflick's medium. All groups were challenged on day 0 and again on day 2. Briefly, in trial 1 chickens were inoculated with sialidase-negative mutant strain P1C5, and tissues were collected for histopathologic evaluation. In trial 2 the chickens were inoculated with strain P1C5 and complemented strains GSR1, GSR2 and GSR3, and tissues were collected for histopathologic evaluation and quantitative *M. gallisepticum* recovery by culture. In trial 3 chickens were inoculated with sialidase-negative mutant strains 6, 358, and tissues were processed as in trial 2.

Chickens were necropsied 2 weeks post-challenge. Samples of trachea were processed for *M. gallisepticum* recovery by culture, quantitated as color-change units (CCU) in Hayflick's medium. Histopathological evaluations were performed in a blinded fashion and lesions were scored based on criteria adapted from Nunoya et al. (1987) and used in previous studies (Gates et al., 2008; Hudson et al., 2006; Papazisi et al., 2002). Tracheal mucosal thickness was measured using an ocular micrometer, as previously described (Gates et al., 2008; Hudson et al., 2006).

2.4. Statistical procedures

The effects of *M. gallisepticum* strain on sialidase activity, lesion scores and tracheal thickening were analyzed by ANOVA, and by Fisher's Protected Least Significant Difference (PLSD), Tukey's Honestly Significant Difference (HSD) or Dunnett's comparisons to the strain R_{low} control post hoc when the main effect was significant. Quantitative recovery by culture was analyzed by the non-parametric Wilcoxon rank sum test. The statistical analyses were performed using StatView v5.0.1 and JMP v7.02 (SAS), and P values < 0.05 were considered statistically significant.

3. Results

3.1. Insertional inactivation of sialidase activity

The quantitative assay demonstrated sialidase activity in R_{low}, and this activity was abolished in the strain R_{low}-derived gm^R mutants 6, 358, and P1C5. Mutants 6, 358, and

P1C5 had Tn*4001*mod (P1C5) or gent miniTn (6 and 358) insertions in distinct locations in the 2841-base open reading frame of MGA_0329. Irrelevant transposon insertions into the strain R_{low} genome either adjacent upstream or downstream, or comparatively distant from MGA_0329 (gm^R clones named P1H1, P2G1 and P2H12, respectively) had no effect on wild-type sialidase activity.

3.2. Attenuated virulence of sialidase knockout mutants

Across strains, chickens inoculated with the sialidase knockout mutants developed less severe tracheal lesions ($P < 0.05$) and less severe tracheal mucosal thickening ($P < 0.05$) than chickens inoculated with an equal dose of strain R_{low} (Fig. 2). The majority inoculated with knockout strains developed only mild tracheitis (lesion scores of 0, 0.5, or 1), but a minority remained severely affected (lesion scores of 1.5 or higher). Knockout strains 6 and 358 caused tracheal lesion scores intermediate between strains R_{low} and P1C5, but tracheal mucosal thickness of knockout-inoculated chickens did not differ from that of sham-infected controls ($P < 0.05$). *M. gallisepticum* recovery was highly variable but fewer CCU, especially of strains 6 and P1C5 (Wilcoxon rank sum $P < 0.05$), were cultured at necropsy from the trachea (Fig. 3), although recovery by culture approached wild-type levels in the minority of birds with severe tracheitis (data not shown).

3.3. Genetic complementation of sialidase activity and effects on virulence

Three gm^R/tet^R clones having one copy of the wild-type MGA_0329 gene stably inserted into separate locations in the strain P1C5 genome (in the pseudogene *vlhA* 1.05 at R_{low} nt 587148; at R_{low} intergenic nt 257873; and in *apoD* at R_{low} nt 950476 for GSR1, GSR2 and GSR3, respectively) expressed sialidase restored to parent strain R_{low} activity levels ($1.44–1.85 \times 10^{-8} \pm 6.37 \times 10^{-10}$ U/CFU) (Fig. 1). These results formally validated the functional annotation of MGA_0329 as being a homolog of the sialidase in *Mycoplasma synoviae* (May and Brown, 2009). However, complementation of MGA_0329 and restoration of wild-type sialidase activity did not restore P1C5 to wild-type levels of virulence. Chickens inoculated with strain GSR1, GSR2 or GSR3 had less severe ($P < 0.0001$) tracheal lesions and tracheal thickening ($P < 0.01$) than chickens inoculated with strain R_{low} (Fig. 2). Recovery of strains GSR1, GSR2 or GSR3 by

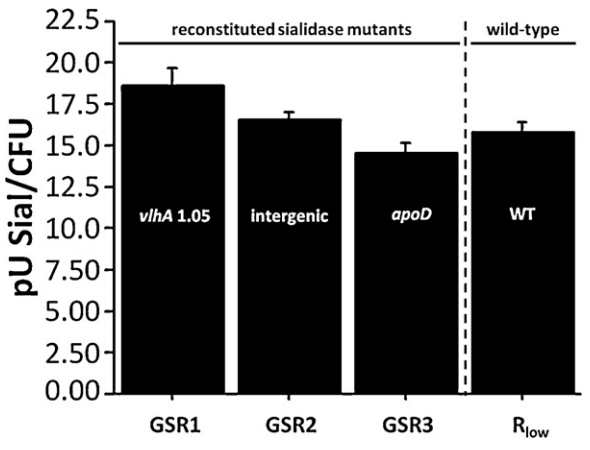

Fig. 1. Complementation of sialidase. MGA_0329 was disrupted by insertional mutagenesis to yield the sialidase-negative mutant P1C5. MGA_0329 was complemented in *cis* using an alternate transposon, and three clonal lineages (GSR1-3) were assessed for sialidase activity using the MUAN assay. Sialidase activity of GSR1-3 was quantified; all exhibited activity at wild-type (strain R_{low}) levels. The insertion site of Tn*4001*Tet/329 is displayed within the bar for each GSR clone. Mutant P1C5 is not shown due to a complete lack of fluorescent signal.

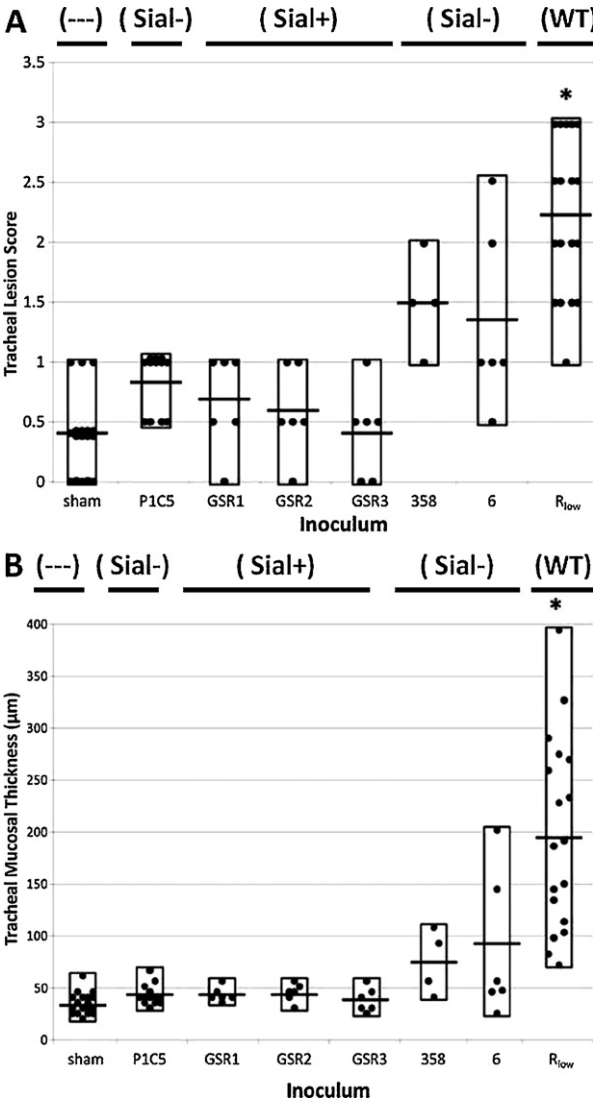

Fig. 2. Pathogenesis of infection with sialidase knockout and complemented strains of *Mycoplasma gallisepticum*. White Leghorn chickens were infected with sialidase knockout mutants P1C5 ($n = 12$), 6 ($n = 6$), and 358 ($n = 4$), and reconstituted P1C5 clones GSR1-3 ($n = 6$ each). Wild-type R_{low} and sterile medium ($n = 18$ each) served as positive and negative controls, respectively. Pooled data collected from three infection studies were analyzed (Dunnett's post-ANOVA $P < 0.05$ or less). All sialidase mutants and recombinants infections resulted in tracheal lesion scores (A) that were significantly lower and tracheal mucosa measurements (B) that were significantly less than those of R_{low}-infected animals. Significant differences are indicated (*).

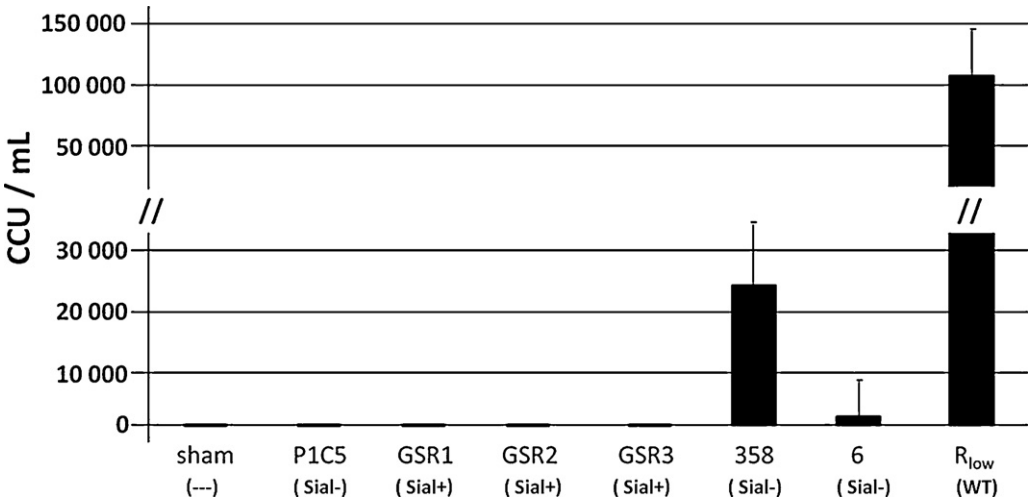

Fig. 3. Recovery of *M. gallisepticum* from Infected Animals. Tracheae from *M. gallisepticum*- or sham-infected animals were assessed for mycoplasma presence by filtration, serial dilution, and CCU determination. Significantly ($P < 0.05$) fewer CCUs of strains 6 and P1C5 were recovered from the trachea than from the tracheae of R_{low}-infected chickens. No viable *M. gallisepticum* cells were recovered from sham-inoculated animals or animals infected with P1C5 or its recombinants.

did not differ significantly from sham-infected controls (Fig. 3).

4. Discussion

Sialidase has been associated with virulence of numerous pathogens, and it has recently been implicated in the pathogenicity of *M. alligatoris* and *M. synoviae* (Hunt and Brown, 2007; May et al., 2007). Because *M. gallisepticum* shares a sialidase allele with *M. synoviae*, we utilized an established system for genetic manipulation to generate sialidase mutants in this species, and performed prospective infection studies to evaluate the potential for a role in virulence of *M. gallisepticum*. Transposon disruption of the sialidase gene MGA_0329 resulted in a loss of enzyme activity, which was restored upon introduction of an exogenous copy of MGA_0329. A gene fragment rather than a complete open reading frame is located immediately downstream of MGA_0329, making it unlikely that another gene is transcriptionally coupled to the sialidase gene. Disruption of MGA_0329 resulted in partial attenuation of *M. gallisepticum*, where a majority of chickens demonstrated milder lesions compared to those that received wild-type while a minority were as severely affected as those that received wild-type. The lack of restoration of *in vivo* virulence of the P1C5 recombinants remains an area of further study, and may be due to an unknown secondary mutation in both the recombinants and their parent mutant. The disruption of sialidase was associated with the loss of virulence in two additional mutants, however, indicating that its absence has an attenuating effect. Numerous studies have reported attenuation of bacterial, viral, and parasitic pathogens *in vivo* following mutagenesis of a single gene, and concluded that the encoded factor is critical to virulence without any confirmatory evidence based on genetic complementation of the mutants (Fittipaldi et al., 2008; Luo et al., 2008; Stewart et al., 2005; Yamamoto et al., 2008). Our observations underscore the importance of including complemented mutants in infection studies to confirm that any observed attenuation is due to a known, mapped disruption, so that the effect of that mutation on virulence may be interpreted with greater accuracy.

Association of sialidase with virulence is common in mucosal pathogens and its detection in a bacterial parasite warrants examination. The mechanism by which sialidase impacts virulence in any *Mycoplasma* species is unclear, with the exceptions of an impact on host cell apoptosis by *M. alligatoris* (Hunt and Brown, 2007) and a complex interaction with cytadherence in the avian mucosal pathogen *M. synoviae* (May and Brown, 2011). Like several other mycoplasmas *M. gallisepticum* uses sialic acid as the primary host cell ligand mediating cytadherence (Gesner and Thomas, 1966; Manchee and Taylor-Robinson, 1969). Host cell desialylation reduces mycoplasmal cytadherence in these species (Feldner et al., 1979; Glasgow and Hill, 1980; May and Brown, 2011), making the detection of an extracellular sialidase somewhat counterintuitive. Attachment to sialic acid moieties and detachment via sialic acid cleavage being functionally balanced is critical to successful infection and reproduction for influenza virus (reviewed by Wagner et al., 2002). Recently, these activities were also demonstrated to have a statistically significant correlation in *M. synoviae*, suggesting that the two functions dynamically interact (May and Brown, 2011). The observed trend toward attenuation of virulence in the absence of sialidase in *M. gallisepticum*, but lack of an absolute correlation, may reflect either subtle differences among individual host animals, or the selective effect that a change in sialidase activity level may have on host cell attachment or other facets of pathogenicity.

5. Conclusion

The present report establishes that sialidase likely contributes to the virulence of *M. gallisepticum*, but that

this contribution is potentially complex. Because sialidase inhibitors used during viral infection have been found to prevent or treat bacterial superinfection (reviewed by McCullers, 2011), we propose that the link between sialidase and *M. gallisepticum* pathogenicity could be relevant to future studies designed to understand therapeutic regimes to treat or prevent bacterial superinfections.

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