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Sialidase Activity in *Mycoplasma synoviae*

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SUMMARY

Eleven strains of the avian pathogen *Mycoplasma synoviae* were evaluated for the presence of sialidase activity by using the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid and the sialidase inhibitor 2-deoxy-2,3-didehydro-N-acetylneuraminic acid. The kinetics of *in vitro* growth in modified Frey's medium were also assessed for each strain. Five strains had been isolated from clinically symptomatic chickens, and strains WVU1853^T and K3344 have been demonstrated to be capable of reproducing disease in specific pathogen-free chickens. All strains exhibited sialidase activity, although the amount varied 65 fold ($P < 0.0001$) from 1.3×10^{-7} to 2.0×10^{-9} activity units/colony-forming unit among strains. Strains originally isolated from clinically symptomatic birds had more ($P < 0.05$) sialidase activity than strains from asymptomatic birds. Strain WVU1853^T exhibited the most sialidase activity ($P < 0.0001$) and grew to the highest culture density ($P < 0.0001$) among strains, but across strains the rank correlation of growth rate with sialidase activity was not significant. Negligible activity was detected in conditioned culture supernatant fluid. This is the first report of sialidase activity in pathogenic strains of *M. synoviae*, which suggests a potential enzymatic basis for virulence of the organism.

Keywords

2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid; 2-deoxy-2,3-didehydro-N-acetylneuraminic acid; growth kinetics; *Mycoplasma synoviae*; sialidase; virulence

INTRODUCTION

Mycoplasma synoviae causes osteoarthritis, synovitis, and respiratory tract lesions in gallinaceous birds (18,21,22). Infection can produce disease that ranges from subclinical to severe. Clinical manifestations vary by strain, and some appear to have a greater tropism for synovial membranes or the respiratory tract than others (21). Disease is often complicated by co-infection with other pathogens, most notably Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus, *Escherichia coli*, *Mycoplasma gallisepticum* and *Mycoplasma meleagridis* (11,20,32,37,38). The molecular basis of *M. synoviae* virulence is not well-understood.

The recently annotated genome of *M. synoviae* field isolate 53 included six genes associated with sialic acid scavenging and degradation (39). Sialidase (EC 3.2.1.18) activity is common in other pathogens, and has been associated with bacterial colonization, extracellular matrix degradation, nutrition, dissemination, and induction of apoptosis (7,15,19,25,40). The enzyme catalyzes hydrolysis of α -(2–3)-, α -(2–6)-, and α -(2–8)- glycosidic linkages of terminal sialic residues in oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates. Sialidase activity is rare in mycoplasmas, however, possibly due to the utilization of sialic acid residues for attachment to host epithelial cell surfaces by many species of

mycoplasma (10,34,35). An extracellular neuraminidase-like activity was described in an unidentified strain of *M. gallisepticum* (27,33), and cell-surface sialidase activity of *M. gallisepticum* strain TT was characterized in detail (36). The *M. gallisepticum* strain R genome encodes a putative protein (MGA_0329) identical in sequence to MS53_0199, the predicted sialidase from *M. synoviae* (30,39), but sialidase activity was absent from other strains of *M. gallisepticum* (17). The hypervirulent *Mycoplasma alligatoris* A21JP2^T is the only extant isolate in which sialidase activity has been demonstrated (4). The activity is congruous with the invasiveness of *M. alligatoris* which is rapidly fatal to susceptible hosts, in contrast to the chronic epithelial lesions observed during typical mycoplasmal infections (3). Therefore the discovery of a putative sialidase gene in an isolate of *M. synoviae* was remarkable for at least three reasons: *M. synoviae* is phylogenetically distant from *M. gallisepticum* within the class *Mollicutes* (16); it is in the same phylogenetic cluster as *M. alligatoris*, although *M. synoviae* is less virulent; and sialic acid degradation pathways are absent from the other annotated *Mycoplasma* spp. genomes. Although *M. synoviae* field isolate 53, the strain used for determining the genome sequence (39), was not readily available for phenotypic analysis, we assessed the type strain WVU1853^T and ten additional clinical isolates of *M. synoviae* for sialidase activity in order to support the annotation of MS53_0199 as a sialidase in *M. synoviae*, and to examine possible correlations of sialidase activity with *in vitro* growth rate and virulence of the organism.

MATERIALS AND METHODS

M. synoviae strains

Clinical features of the strains examined are summarized in Table 1. Strain WVU1853^T has been most commonly reported to cause airsacculitis and synovitis (21), however, experimental infection studies indicated that this strain is capable of systemic spread and the generation of lesions in multiple tissues (13,22). Strain K3344 was isolated during an outbreak of apparent reproductive disease in a breeder flock in 1992, but was demonstrated to produce respiratory lesions during experimental infections (8). Strains MS117, MS173, and MS178 were isolated during an outbreak of severe synovitis in Argentina (5). Lesions from infected birds involved synovial membranes, bursa of Fabricius, liver, kidney, and the lower respiratory tract in breeders (6). Strain F10-2AS was associated with respiratory lesions, most notably airsacculitis, in experimentally infected birds (21,22). The FMT strain, originally isolated from chicken trachea, induced minor respiratory lesions following experimental infection (22); stocks FMT33 and FMT126 were derived from serial *in vitro* passages ($n = 33$ and 126, respectively) of FMT. Strains K4907A, K5016, K5113, and K5599A were isolated from clinically normal chickens, and are not suspected to cause significant lesions. For the present study, the species identity of each stock culture was confirmed by PCR amplification and partial sequencing of the 16S rDNA gene by methods described previously (2).

Comparative growth rate

The *M. synoviae* strains were grown in MFM including 1% w/v glucose, 15% v/v porcine serum, 0.05% w/v L-cysteine and 0.05% w/v nicotinamide adenine dinucleotide (9). Triplicate cultures were synchronized by dilution with MFM to an initial optical density (A_{600}) of $7 \pm 5 \times 10^{-4}$. All strains were incubated at 37 °C in 5% v/v CO₂ without agitation, and aliquots were withdrawn every 24 hr for 120 hr to measure A_{600} and the concentration of CFUs. To determine CFUs, an aliquot of each culture was serially diluted 10-fold in MFM and inoculated onto modified Frey's agar. The CFUs were counted after 4 d incubation.

Sialidase assays

Whole *M. synoviae* cells from broth cultures in early log-phase growth, as detected by acidification of the MFM, were pelleted by centrifugation for 1 hr at 14,000 X g and washed

with phosphate-buffered saline. Detection of sialidase activity in washed cells and cell-free supernatant fluid was performed as previously described (14) following a 15 min incubation with the fluorogenic substrate MUAN. The intensity of cyan fluorescence at 450 nm, excited at 365 nm with a cutoff filter at 420 nm, was proportional to sialidase activity (41). The specificity of the activity was confirmed by co-incubation of *M. synoviae* cells with 0.35% w/v MUAN plus 5 mg/ml of the sialidase inhibitor DANA. Positive and negative controls for the sialidase assay were *M. alligatoris* and *Mycoplasma crocodyli*, respectively (4). The amount of sialidase activity per CFU was calculated from a standard curve generated by similar incubation of 1 to 100 µg/mL (0.77 to 77 mU/mL) Type VI *Clostridium perfringens* neuraminidase (Sigma-Aldrich, St. Louis, MO) with MUAN.

Statistical procedures

The effect of *M. synoviae* strain on culture density and sialidase activity ($n = 3$ independent replications each) was analyzed by analyses of variance, and by Fisher's Protected Least Significant Difference test for posthoc comparisons when main effects were significant, using StatView version 5.0.1 (Abacus Concepts, Inc., Berkeley, CA). An association between culture density and sialidase activity was tested at each time point by non-parametric Spearman rank correlation analysis. P values < 0.05 were considered significant.

RESULTS

M. synoviae species confirmation

The 16S rDNA sequence of each of the stock cultures examined in the present study was determined and confirmed by BLAST analyses to be identical to that of *M. synoviae* field isolate 53 and the type strain WVU1853^T (GenBank accession numbers AE017245 and X52083, respectively).

Growth rate

The mean initial culture density was 507 ± 21 CFU/mL. In each of 3 independent replications, strains FMT126, K3344, and MS117 reached a higher ($P < 0.0001$) culture density than all other strains by 48 hr, although the culture density of strain WVU1853^T exceeded ($P < 0.0001$) all other strains at 72, 96 and 120 hr. Strains MS173, MS178, FMT33, K4907A, K5016, K5113, and K5599A did not differ significantly from each other at any time point (Figure 1). Across all time points, the correlation ($P < 0.0001$) between mean A_{600} and mean CFU/ml ranged among strains from 0.49 to 0.97, with weakest correlations for the fastest-and slowest-growing strains.

Sialidase activity of clinical isolates of *M. synoviae*

Across strains, MUAN cleavage was inhibited 20 fold ($P < 0.0001$) by the addition of DANA, providing independent evidence that the fluorescence measured was proportional specifically to sialidase activity. All *M. synoviae* strains displayed cell-associated sialidase activity. The activity varied 65 fold ($P < 0.0001$) among strains, ranging from 2.0×10^{-9} to 1.3×10^{-7} U/CFU (Table 1). The group of strains WVU1853^T, F10-2AS, K3344, MS117, MS173, and MS178, originally isolated from clinically symptomatic birds, had more ($P < 0.05$) sialidase activity than the group of strains K4907A, K5016, K5113 and K5599A from asymptomatic birds. The strain that eventually attained the highest culture density, WVU1853^T, also exhibited the most sialidase activity, but across strains the rank correlations of culture density with sialidase activity were not statistically significant. Negligible activity was detected in conditioned culture supernatant fluid.

DISCUSSION

In this study, the type strain WVU1853^T of *M. synoviae* and all additional isolates examined were demonstrated to have sialidase activity. Negligible activity was detectable in conditioned culture supernatant fluid, which is consistent with the conclusion by Roberts (33) that the *M. gallisepticum* sialidase is extracellular surface-associated. Strain WVU1853^T had the most sialidase activity and attained the highest culture density, but although free sialic acid can serve as a nutritional substrate for some bacteria (7,26), it seems unlikely to have been rate-limiting in the rich glucose- and serum-supplemented MFM required to support *in vitro* growth of *M. synoviae*.

This is the first report that sialidase activity is common among strains of *M. synoviae*. The activity was higher among more pathogenic isolates. The highest level of sialidase activity observed was for strain WVU1853^T, which was associated with severe systemic disease in experimentally infected birds (13). Intermediate levels of sialidase activity were observed for F10-2AS, a strain associated with respiratory lesions (22); FMT33 and FMT126, whose parent isolate FMT induced respiratory lesions and embryo lethality in chickens (22,23); MS117 and MS178, which originated from an outbreak of severe synovitis (5,6); and for strain K3344, which was associated with respiratory lesions in experimentally infected chickens (8). Low levels of sialidase activity were observed for strains K4907A, K5016, K5113 and K5599A, which were not associated with pathologic lesions. Neither sialidase activity nor clinical data have been reported for strain 53, a field isolate whose complete genome sequence appears to encode all elements of the canonical bacterial sialic acid scavenging and degradation pathway (7,39). The association suggests a potential enzymatic mechanism for virulence of the organism, because sialylation normally protects against hydrolysis of the glycosidic or peptide bonds of oligosaccharides, glycoproteins, and glycolipids located on host cell surfaces, and against degradation of the host's extracellular matrix. It has also been proposed that bacterial desialylation of host glycoconjugates could expose or form new host antigens to play a role in autoimmune complications of infection (17,19). The majority of studies examining the virulence of *M. synoviae* have focused on cytoadherence and/or hemadsorption, with particular attention paid to antigenically-variable hemagglutinins (1,28). More recently, work by Lockaby *et al.* (24) examining the relationship between virulence of *M. synoviae* isolates and their cytoadherence capabilities indicated that there are likely to be additional virulence factors, because cytoadherence did not completely correlate with pathogenicity. It is also noteworthy that the amount of sialidase activity remained virtually unchanged after 93 *in vitro* passages of *M. synoviae* strain FMT33 (Table 1). Hunt and Brown (15) showed that co-incubation with DANA protected cultured pulmonary fibroblasts from *M. alligatoris*-induced apoptosis. However, incubation with purified *C. perfringens* sialidase alone did not induce apoptosis, suggesting that interaction of sialidase with another virulence factor(s) is necessary to elicit the pro-apoptotic effects of *M. alligatoris* infection (14).

The presence of sialidases in both *M. synoviae* and *M. alligatoris* of the *M. synoviae* phylogenetic cluster indicates that it may be a common characteristic of those related species. However, *M. crocodyli*, a third member of the cluster having comparatively attenuated virulence, does not exhibit sialidase activity (4), so it may be of substantial value to examine additional members of the cluster to elucidate the role of sialidase in pathogenesis that cannot be attributed to cytoadherence. The amount of sialidase activity varied 65 fold among *M. synoviae* strains, suggesting that the enzymes in the sialic acid scavenging pathway may be susceptible to genetic heterogeneity, or variable in copy number. Experimental infection studies employing disruption of the genes required for this activity may be necessary for definitive demonstration of the role of sialidase in the virulence of *M. synoviae*.

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Abbreviations

BLAST	Basic Local Alignment Search Tool
CFU	colony-forming units
DANA	2-deoxy-2,3-didehydro- <i>N</i> -acetylneuraminic acid
EC	Enzyme Commission
MFM	modified Frey's medium
MUAN	2'-(4-methylumbelliferyl)- α -D- <i>N</i> -acetylneuraminic acid
PCR	polymerase chain reaction

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Figure 1. Growth of *Mycoplasma synoviae*

Stocks (left to right: K5016, K4907, FMT33, K5599, K5113, MS173, F10-2AS, FMT126, K3344, MS117, MS178, WVU1853^T) were grown in modified Frey's medium (MFM) at 37 °C in 5% v/v CO₂ without agitation. Triplicate cultures were initially synchronized by dilution with MFM to 507 ± 21 colony-forming units (CFUs) per mL. Aliquots were withdrawn every 24 hr for 120 hr to measure culture density. Strains FMT126, K3344, and MS117 reached a higher (***) = $P < 0.0001$) density than all other strains by 48 hr, although the density of strain WVU1853^T exceeded all other strains at 72, 96 and 120 hr.

Table 1

Clinical features and sialidase activities of *Mycoplasma synoviae* strains^a.

Strain ^b	Pathology at 1° isolation	Respiratory lesions	Synovial lesions	Embryo lethality	Reference	Sialidase U/CFU ^c
WVU1853 ^T [32]	+	+	+	+	13,21,22,23,29	1.3x10 ⁻⁷ ± 2.7x10 ^{-9a}
MS178 [7]	+	+/- ^d	+	N/A	6	3.9x10 ⁻⁸ ± 7.8x10 ^{-9b}
FMT [N/A]	+	+	-	+	22,23	N/A
FMT126 [126] ^e	N/A	N/A	N/A	N/A	N/A	3.6x10 ⁻⁸ ± 9.3x10 ^{-9b} , c
FMT33 [33] ^e	N/A	N/A	N/A	N/A	N/A	2.7x10 ⁻⁸ ± 1.1x10 ^{-8b} , c
MS117 [7]	+	+/- ^d	+	N/A	6	2.1x10 ⁻⁸ ± 2.3x10 ^{-9c,d}
K3344 [8]	+	+	-	N/A	8	1.3x10 ⁻⁸ ± 3.8x10 ^{-9d,e}
F10-2AS [13]	+	+	-	-	21	9.7x10 ⁻⁹ ± 1.2x10 ^{-9d,e}
K5016 [5] ^f	-	N/A	N/A	N/A	N/A	7.2x10 ⁻⁹ ± 7.2x10 ^{-9d,e}
K4907A [7] ^f	-	N/A	N/A	N/A	N/A	6.0x10 ⁻⁹ ± 3.1x10 ^{-9d,e}
MS173 [7]	+	+/- ^d	+	N/A	6	2.9x10 ⁻⁹ ± 1.3x10 ^{-9e}
K5599A [3] ^f	-	N/A	N/A	N/A	N/A	2.7x10 ⁻⁹ ± 1.5x10 ^{-9e}
K5113 [7] ^f	-	N/A	N/A	N/A	N/A	2.0x10 ⁻⁹ ± 1.0x10 ^{-9e}

^aN/A = not published or not determined.
^bPassage number indicated in brackets.
^cMean ± standard error enzyme units (U) per *M. synoviae* colony forming unit (CFU). Means followed by a different letter are different (*P* < 0.001) by Fisher's Protected Least Significant Difference test.
^dLesions in breeder chickens but not broiler chickens.
^eFMT33 and FMT126 were derived from serial *in vitro* passages (*n* = 33 and 126, respectively) of virulent strain FMT.
^fIsolated from clinically asymptomatic chickens; histopathology not assessed.