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Secreted Sialidase Activity of Canine Mycoplasmas

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Abstract

Through a survey of the phylogenetic distribution of sialidase among mycoplasmas, we detected activity secreted by the type strains of three of eleven species frequently or first isolated from dogs. The specific activity of washed cells of the type strains of *Mycoplasma canis*, *Mycoplasma cynos*, and *Mycoplasma molare* ranged from $5.2 \pm 0.8 \times 10^{-6}$ to $1.1 \pm 0.3 \times 10^{-5}$ enzymatic units per colony-forming unit (U/CFU). Cells of *M. molare* strain H542^T had twice the specific activity ($P < 0.05$) of *M. canis* strain PG14^T or *M. cynos* strain H831^T. Significant differences in sialidase activity existed among nine clinical isolates of *M. canis*, ranging from not detectable to $2.1 \pm 0.1 \times 10^{-5}$ U/CFU. The type strains of other species previously isolated from dogs (*Mycoplasma arginini*, *Mycoplasma bovis*, *Mycoplasma edwardsii*, *Mycoplasma felis*, *Mycoplasma gatae*, *Mycoplasma maculosum*, *Mycoplasma opalescens*, and *Mycoplasma spumans*) did not exhibit either secreted or cell-associated sialidase activity. Neither specific nor degenerate PCR primers complementary to the three known mycoplasmal sialidase alleles were able to amplify orthologs in *M. canis*, *M. cynos*, or *M. molare*, further evidence that the secreted sialidase of those species is distinct from the strictly cell-associated sialidases of *Mycoplasma alligatoris*, *Mycoplasma synoviae*, and *Mycoplasma gallisepticum*. This is the first report of a well-known bacterial virulence factor whose expression varies among strains of certain *Mycoplasma* species that infect dogs.

1. Introduction

Canine mycoplasmosis is associated with multiple species of *Mycoplasma*. The most firmly established pathogen is *Mycoplasma cynos* (Røsendal, 1973), which was isolated from fatal infections of puppies, and proven to be a primary respiratory pathogen by experimental infection studies (Rosendal, 1982; Zeugswetter et al., 2007). *Mycoplasma canis* (Edward, 1955) is best known as an opportunistic pathogen of dogs, and has been recovered from their respiratory, urinary and reproductive tracts. Unlike *M. cynos*, *M. canis* often infects other mammalian hosts. For example, *M. canis* was isolated from the lower respiratory tracts of humans with pneumonia (Armstrong et al., 1971) and both healthy and pneumonic cattle (Ayling et al., 2004), although studies of *M. canis* strain C3b as a primary pathogen of cattle had equivocal results (ter Laak et al., 1993). *Mycoplasma molare* (Røsendal, 1974) and several other species isolated from dogs are also potential pathogens (Chalker, 2005).

Sialidase is a virulence factor of diverse microorganisms. It promotes microbial colonization, tissue invasion, and damage to sialylated host molecules, cell surfaces and the extracellular

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matrix (Corfield, 1992). Although *Mycoplasma alligatoris* strain A21JP2^T, virulent strains of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, and some strains of other avian mycoplasmas produce cell-associated sialidases (Brown et al., 2004; Berčič et al., 2008a; May and Brown, 2008), the distribution of sialidase among other species of *Mycoplasma* has not been investigated systematically. As part of a broader survey, we detected secreted sialidase activity in three of eleven species isolated from dogs. This is the first report of a well-known bacterial virulence factor occurring in pathogenic species of *Mycoplasma* that infect dogs.

2. Materials and Methods

2.1 Mycoplasma isolates and culture techniques

The type strains *Mycoplasma arginini* G230^T, *Mycoplasma bovis* PG11^T, *M. canis* PG14^T, *M. cynos* H831^T, *Mycoplasma edwardii* PG24^T, *Mycoplasma felis* CO^T, *Mycoplasma gatae* CS^T, *Mycoplasma maculosum* PG15^T, *M. molare* H542^T, *Mycoplasma opalescens* MH5408^T, and *Mycoplasma spumans* PG13^T were obtained from the American Type Culture Collection. Five isolates collected from Shetland sheepdogs with various reproductive disorders, and four isolates from dogs with unknown clinical history, which were obtained during a prior survey (M.B. Brown, unpublished), were identified as *M. canis* by PCR-RFLP screening (Spergser and Rosengarten, 2007) confirmed by direct 16S rRNA gene sequencing (May et al., 2007).

M. canis, *M. cynos*, *M. edwardii*, *M. felis*, and *M. molare* were propagated in SP-4 medium containing 0.5% w/v glucose. *M. arginini*, *M. bovis*, *M. gatae*, *M. maculosum*, *M. opalescens*, and *M. spumans* were propagated in SP-4 medium containing 0.5% w/v glucose plus 0.21% w/v L-arginine. All cultures were incubated at 37 °C under ambient atmospheric conditions. Culture concentrations were determined by inoculating serial 10-fold dilutions in SP-4 broth onto agar. Colonies were counted after 5 days of incubation.

2.2 Measurement of sialidase activity

Sialidase activity of washed cells was quantitated using the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUAN; Sigma-Aldrich, St. Louis MO) and the sialidase inhibitor 2-deoxy-2,3-didehydro-N-acetylneuraminic acid (DANA; Sigma-Aldrich) as previously described (May et al., 2007). The positive control was *M. alligatoris*, which expresses cell-associated but not secreted sialidase. The negative controls were *Mycoplasma crocodyli* strain MP145^T, which does not express any sialidase (Brown et al., 2004), and fresh culture medium. Secreted sialidase activity was detected qualitatively in late log-phase broth cultures, after the cells were harvested, by diluting the cell-free conditioned supernatant medium 1:1 with 0.35% w/v MUAN, then observing the specimen under shortwave ultraviolet illumination. The positive control was Type VI purified *Clostridium perfringens* sialidase in cell-free *M. crocodyli*-conditioned medium, and the negative control was plain cell-free *M. crocodyli*-conditioned medium.

2.3 PCR amplification of sialidase genes

PCR primers complementary to the cell-associated sialidase gene *nanI* sequences of *M. alligatoris* (GenBank AY515695; 5'-TGA CAA AAT GCG CTG AAA AA-3' and 5'-GCG CCA AAT TTA CAT CCT ACA-3'), *M. synoviae* (GenBank NC_007294; 5'-TCT CTT CCT TTT TGA GGG CTA-3' and 5'-GCA AAT CAT CTT AAG AAA AGT CAT T-3'), and *M. gallisepticum* (GenBank NC_004829; 5'-TCA GAT CAT TAA ACT AGC GCC TAA-3' and 5'-CGC ATG ATA CGA TAA CGA AAT G-3'), and degenerate primers designed from consensus sequences among those alleles (5'-GA(CT) G(AG)I GGI (ACT)(AT)I (AT)(GC)I TG(AG)-3' and 5'-CAI (GCT)(AT)I (AGT)(CT)I CCI CC(AG) TC-3') were used in attempts to detect the sialidase genes in *M. canis*, *M. cynos*, and *M. molare*. Genomic DNA templates

were purified using EasyDNA reagents (Invitrogen, Carlsbad CA). The 50- μ l reactions included 1 μ mol of each primer and 100 ng of template DNA, and consisted of initial template denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 20 sec, primer annealing at 48 °C for 20 sec, and extension at 72 °C for 3 min, with a final extension at 72 °C for 10 min. Reactions using degenerate primers were performed with an annealing temperature of 38 °C. The positive control template was *M. gallisepticum* strain R_{low} genomic DNA.

2.4 Statistical analysis

The effect of species on the specific sialidase activity of washed *M. canis*, *M. cynos*, and *M. molare* cells, and the effect of strain within *M. canis*, were analyzed by analyses of variance ($n = 3$ independent replications each), and by Fisher's Protected Least Significant Difference test for posthoc comparisons among species or strains when main effects were significant (May and Brown, 2008). P values < 0.05 were considered significant.

3. Results

As part of a survey of the genus *Mycoplasma*, this study sought to detect the presence of sialidase in primary and opportunistic mycoplasmal pathogens that infect dogs. Sialidase activity was evident in washed cells of the type strains of *M. canis*, *M. cynos*, and *M. molare*. The specific activity ranged from $5.2 \pm 0.8 \times 10^{-6}$ to $1.1 \pm 0.3 \times 10^{-5}$ U/CFU (Table 1). *M. molare* strain H542^T had twice as much activity ($P < 0.05$) as *M. canis* strain PG14^T or *M. cynos* strain H831^T. Nine clinical isolates of *M. canis* were subsequently examined to sample the extent of intraspecific variation. Washed cells of those isolates ranged in specific activity ($P < 0.0001$) from 0 (undetectable) for strain LV, to $2.1 \pm 0.1 \times 10^{-5}$ U/CFU for strain 31 (Fig. 1).

Sialidase activity was also present unexpectedly in cell-free conditioned supernatant broth from cultures of *M. cynos* H831^T, *M. molare* H542^T (Fig. 2A), and all strains of *M. canis* except LV (Fig. 2B). To investigate whether the secreted activity is conferred by an alternative form of the known mycoplasmal sialidase alleles, conserved and degenerate PCR primers were used in attempts to detect orthologous genes, but no amplification was successful from the genomic DNA of *M. cynos*, *M. molare*, or any strain of *M. canis*. No sialidase activity was detected in the other species examined.

4. Discussion and conclusion

Sialidase was formerly considered to be rare among mycoplasmas (Kahane et al., 1990), but its presence has now been documented in eleven species. All mycoplasmas currently known to produce sialidase either are affiliated with the *M. synoviae* phylogenetic cluster on the basis of 16S rRNA gene similarity (Johansson and Pettersson, 2002), or they share a common host with one or more sialidase-positive species in that cluster (Table 1). Consistent with that observation, *M. canis* and *M. cynos* of the *M. synoviae* cluster share the secreted form of sialidase with *M. molare*, a species affiliated with the phylogenetically distant *Mycoplasma neurolyticum* cluster.

We interpret the activity in washed cells of *M. canis*, *M. cynos* and *M. molare* to reflect newly-synthesized enzyme with the potential to be secreted (Fig. 2), although it seems possible that distinct secreted and cell-associated sialidases occur together in some instances. The finding that sialidase was secreted into the culture medium by nine of the ten strains of *M. canis* examined is remarkable because the one unnamed *M. canis* isolate examined by Zakrajšek (2008) had only membrane-associated activity. In a recent abstract, Berčič et al. (2008b) reported that sialidase activity was present in conditioned supernatant medium from a culture

of an unspecified *Mycoplasma corogypsi* strain, another species from birds that is affiliated with the *M. synoviae* phylogenetic cluster.

The four-fold variation among *M. canis* strains in specific sialidase activity of washed cells was statistically significant but much less than the 65-fold variation between high- and low-virulence strains of *M. synoviae* (May et al., 2007). The absence of activity in *M. canis* isolate LV underscores the necessity to assess multiple strains of each species to characterize the phylogenetic distribution of sialidases. This may also explain the conflict between earlier reports on the occurrence of sialidase in *Mycoplasma* when only single strains were examined (Roberts, 1967; Glasgow and Hill, 1980), and the equivocal results regarding *M. canis* strain C3b as a primary pathogen of cattle (ter Laak et al., 1993) if that strain lacked sufficient activity to contribute to virulence.

M. cynos and *M. canis* are the species most consistently associated with canine mycoplasmosis (Chalker, 2005), but to date no specific virulence factor had been proposed as a basis of their pathogenicity. Dogs inoculated with *M. canis* strains S6/L42 or SL1/L45 developed urethritis and prostatitis or metritis (Laber and Holzmann, 1977; Holzmann et al., 1979). *M. canis* strain A56Hzkl, isolated from the pericardium of a dog, colonized the lung, liver and spleen of mice inoculated intraperitoneally, but did not generate lesions (Eberle et al., 1977). Erythrocyte surface desialylation reduced hemadsorption by *M. canis* strain PG14^T (Manchee and Taylor-Robinson, 1969), suggesting that the secreted sialidase might modulate mycoplasmal cytoadherence, colonization and transmission (May and Brown, 2008).

In conclusion, we propose that a secreted sialidase is a candidate virulence factor of *M. cynos* and *M. canis*. Though *M. molare* is not a definitive canine pathogen, its ability to secrete sialidase in the urogenital tract may allow it also to act as a primary or co-factor in disease. Further studies will be required to identify the sialidase genes in these mycoplasmas, and to examine a possible link between sialidase expression and pathogenesis during canine mycoplasmosis.

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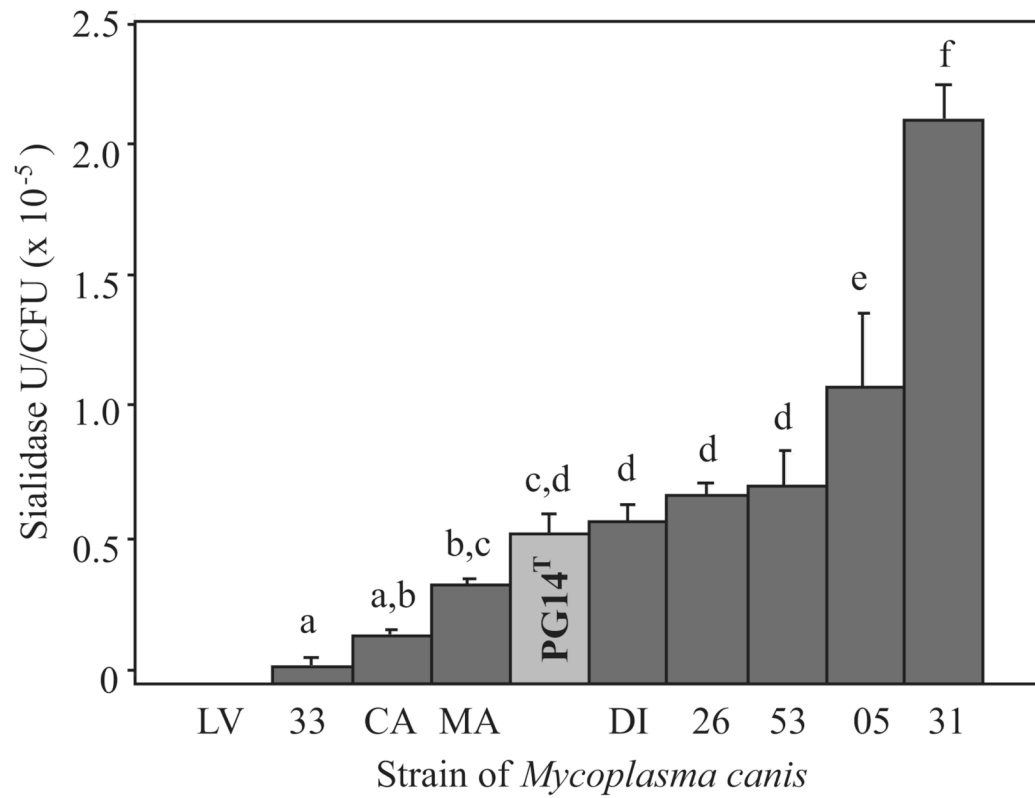
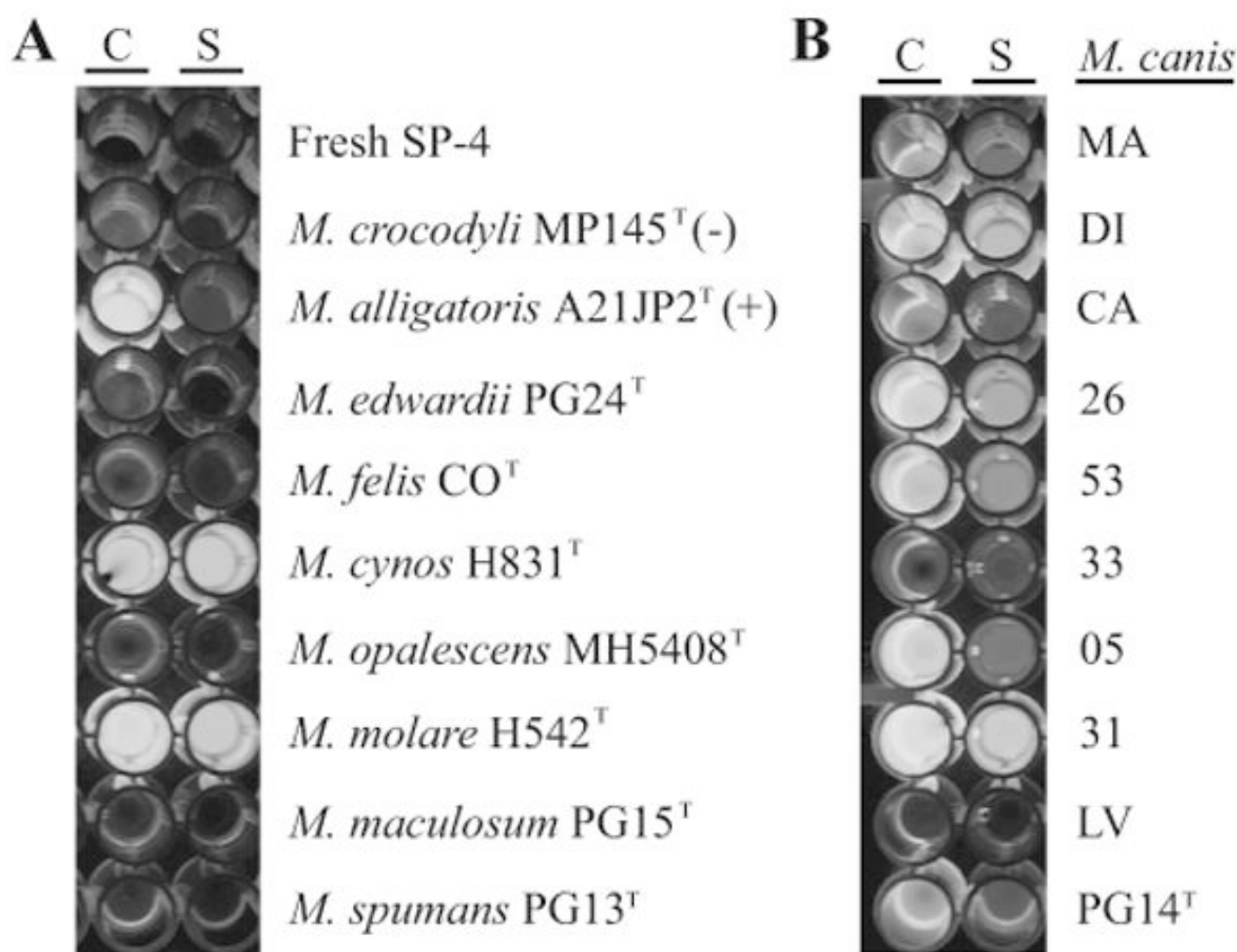


Fig. 1.

Intraspecific variation in sialidase activity of washed *Mycoplasma canis* cells. Bars depict mean \pm standard error of enzyme units (U) per colony-forming unit (CFU) of clinical isolates and the type strain PG14^T. Means with a different letter are different ($P < 0.05$ to $P < 0.001$) by Fisher's Protected Least Significant Difference test ($n = 3$ independent replications each). The clinical isolate LV had no activity.

**Fig. 2.**

Secreted sialidase activity of canine mycoplasmas. **(A)** Sialidase was detected by release of fluorescent 4-methylumbelliferone from labeled *N*-acetylneuraminic acid. Activity was present in washed cells (C) and cell-free conditioned supernatant SP-4 broth (S) from cultures of *Mycoplasma cynos* H831^T and *Mycoplasma molare* H542^T. No activity was detected in the type strains of other species isolated from dogs. *Mycoplasma alligatoris* A21JP2^T was the positive control for strictly cell-associated activity; *Mycoplasma crocodyli* MP145^T and fresh SP-4 broth were negative controls. **(B)** Sialidase activity was present in *Mycoplasma canis* PG14^T and eight clinical isolates of *M. canis*. Isolate LV had no activity.

Table 1

Sialidase-positive mycoplasmas.

<i>Mycoplasma</i> Species	Strain	Sialidase Activity ^a	Host Class	Phylogenetic Cluster	Reference
<i>M. alligatoris</i>	A21JP2 ^T	10 ⁻⁹ U/CFU	reptile	<i>M. synoviae</i>	Brown et al., 2004
<i>M. anseris</i>	1219 ^T	moderate	bird	<i>M. hominis</i>	Berčič et al., 2008a
<i>M. canis</i> ^{b,c}	PG14 ^T	10 ⁻⁶ U/CFU	mammal	<i>M. synoviae</i>	current report
<i>M. cloacale</i>	383	weak	bird	<i>M. hominis</i>	Berčič et al., 2008a
<i>M. coronypsi</i>	BV1 ^T	very strong	bird	<i>M. synoviae</i>	Berčič et al., 2008a
<i>M. cynos</i>	H831 ^T	10 ⁻⁶ U/CFU	mammal	<i>M. synoviae</i>	current report
<i>M. gallisepticum</i> ^{b,c}	R	10 ⁻⁸ U/CFU	bird	<i>M. pneumoniae</i>	May and Brown, 2008
<i>M. iowae</i> serovar I	695 ^T	weak	bird	<i>M. muris</i>	Berčič et al., 2008a
<i>M. meleagridis</i> ^c	17529 ^T	strong	bird	<i>M. bovis</i>	Berčič et al., 2008a
<i>M. molare</i>	H542 ^T	10 ⁻⁵ U/CFU	mammal	<i>M. neurolyticum</i>	current report
<i>M. synoviae</i> ^{b,c}	WVU1853 ^T	10 ⁻⁷ U/CFU	bird	<i>M. synoviae</i>	May et al., 2007

^a Highest reported specific activity (enzymatic units per colony-forming unit) or semi-quantitative estimate for washed cells.

^b Species with multiple strains documented to exhibit sialidase activity.

^c Species with some strains documented to lack sialidase activity.