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Genetic Variation in Sialidase and Linkage to N-acetylneuraminate Catabolism in *Mycoplasma synoviae*

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Abstract

We explored the genetic basis for intraspecific variation in mycoplasmal sialidase activity that correlates with virulence, and its potentially advantageous linkage to nutrient catabolism. Polymorphism in N-acetylneuraminate scavenging and degradation genes (sialidase, N-acetylneuraminate lyase, N-acetylmannosamine kinase, N-acetylmannosamine-6-phosphate epimerase, N-acetylglucosamine-6-phosphate deacetylase, and glucosamine-6-phosphate deaminase) was evident among eight strains of the avian pathogen *Mycoplasma synoviae*. Most differences were single nucleotide polymorphisms, ranging from 0.34 ± 0.04 substitutions per 100 bp for N-acetylmannosamine kinase to 0.65 ± 0.03 for the single-copy sialidase gene *nanI*. Missense mutations were twice as common as silent mutations in *nanI*; 26% resulted in amino acids dissimilar to consensus; and there was a 12-base deletion near the *nanI* promoter in strain WVU1853^T, supporting a complex genetic basis for differences in sialidase activity. Two strains had identical frameshifts in the N-acetylneuraminate lyase gene *nanA*, resulting in nonsense mutations, and both had downstream deletions in *nanA*. Such genetic lesions uncouple extracellular liberation of sialic acid from generation of fructose-6-phosphate and pyruvate via intracellular N-acetylneuraminate degradation. Retention of *nanI* by such strains, but not others in the *M. synoviae* phylogenetic cluster, is evidence that sialidase has an important non-nutritional role in the ecology of *M. synoviae* and certain other mycoplasmas.

Keywords

Mycoplasma synoviae; polymorphism; N-acetylneuraminate catabolism; sialidase; virulence

1. Introduction

Mycoplasma synoviae is a major avian pathogen associated with osteoarthritis, synovitis, and respiratory tract lesions in gallinaceous birds [1,2,3]. Infection can produce disease that ranges from subclinical to severe, and clinical outcome can be influenced by co-infection with other agents [4,5,6,7,8]. The majority of prior studies have focused on cytadherence and/or hemadsorption as pathogenic mechanisms of *M. synoviae*, with particular attention to

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antigenically-variable hemagglutinins, although the molecular basis of *M. synoviae* pathogenicity is still not well-understood [9,10].

The recently annotated genome of *M. synoviae* field isolate 53 includes putative sialidase *nanI* (synonymous with *nanH* of Gram-negative species and strain 53 GenBank accession no. **ABS50356**), N-acetylneuraminate lyase *nanA*, N-acetylmannosamine kinase *nagC*, N-acetylmannosamine-6-phosphate epimerase *nanE*, N-acetylglucosamine-6-phosphate deacetylase *nagA*, and glucosamine-6-phosphate deaminase *nagB* genes in a locus comprising a canonical sialic acid scavenging and degradation pathway (Figure 1A) [11]. This was unexpected, although sialidase activity is common in other pathogenic bacteria [12], because it is very rare in mycoplasmas, having been described previously only in the lethal pathogen of alligators *Mycoplasma alligatoris* [13] and an extinct strain of the avian pathogen *Mycoplasma gallisepticum* [14, 15, 16]. The term sialic acid is the family name covering all derivatives of neuraminic acid [17], the aldol condensation product of D-mannosamine and pyruvic acid, which are potential bacterial nutrients. In vertebrate animals including birds, diverse sialic acid derivatives are involved in recognition processes, cellular connections with extracellular matrix (ECM) components, and intercellular interactions [18]. They protect against hydrolysis of the glycosidic or peptide bonds of oligosaccharides, glycoproteins, glycolipids and gangliosides located on eukaryotic cell surfaces, and against degradation of the ECM. In addition, sialylated lipopolysaccharide and polysialic acid capsules are surface features of certain Gram-negative and Gram-positive bacteria [19].

Exo-α-sialidases (EC 3.2.1.18) catalyze hydrolysis of α-(2–3)-, α-(2–6)-, and/or α-(2–8)-glycosidic linkages of terminal sialic acid residues on oligosaccharides, glycoproteins, glycolipids, colominic acid (a homopolymer of N-acetylneuraminic acid), and synthetic substrates. Synonyms for sialidase include neuraminidase, α-neuraminidase, and N-acylneuraminate glycohydrolase. Most bacterial sialidases preferentially cleave α-(2–3)-linked sialic acids, and are found in species that live in close contact with vertebrate host cells as commensals or facultative pathogens. Sialidase activity is involved in bacterial colonization and dissemination, ECM degradation, and induced host-cell death [12,20,21,22,23]. 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 [24,25].

Most recently, in support of the prediction based on the genomic sequence of strain 53 that a functional sialidase gene occurs in *M. synoviae*, sialidase activity was readily detected in several additional strains [26,27]. This suggested that an ability to desialylate sialoconjugates present in its environment is important in the ecology of *M. synoviae*. Strikingly, strains originally isolated from clinically symptomatic birds had significantly more sialidase activity than strains from asymptomatic birds [27], and certain strains lacked detectable sialidase activity [26], suggesting substantial intraspecies genetic heterogeneity and a role for sialidase activity in the virulence of the organism. In bacterial genomes, including that of *M. synoviae* strain 53, sialidase genes are often part of a locus encoding additional enzymes that enable the import and intracellular catabolism of free sialic acid. This pathway culminates with the production of fructose-6-phosphate for entry to glycolysis [28], constituting a bacterial nutrient stream that might be essential to offset any selective disadvantage of increased virulence attributable to desialylation of host glycoconjugates [20]. In the present study, we explored the genetic basis for the observed intraspecific variation in sialidase activity, and its potentially advantageous linkage to nutrient catabolism, by characterizing the natural DNA sequence diversity within the sialic acid degradation locus among eight strains of *M. synoviae*.

2. Results

2.1. Amplification of the sialic acid degradation locus

An 8.4 kb PCR amplicon, including the 7.9 kb sialic acid degradation locus as predicted from the genomic sequence of strain 53 [11], was amplified from *M. synoviae* strains K3344, K4907A, K5395B, MS173, MS178, and WVU1853^T [27] using the flanking primers described in section 4.2. Passages 33 and 126 of the FMT strain [3] only generated approximately 8 kb amplicons, suggesting the deletion of a portion(s) of the locus. Homologs of *nanA*, *nanE*, *nanI*, *nagA*, *nagB*, and *nagC* were present in each strain in the same orientations as in strain 53 (Figure 1B). The 521 bp gap between *nagC* and *nanE*, annotated for the strain 53 genome as encoding the 151 aa hypothetical protein of unknown function MS53_0196 (GenBank accession no. **AAZ43615**), was also conserved. When Southern blots of their fragmented genomic DNA were probed to determine *nanI* copy number, the strains having the highest (WVU1853^T) and lowest (K4907A and K5395B) amounts of sialidase activity all exhibited the banding pattern predicted from the whole-genome sequence of strain 53, showing that only a single copy of *nanI* was present in those strains despite their having almost 100-fold differences in sialidase activity per colony-forming unit (CFU) [27].

2.2. Nucleotide sequence variability

Numerous point mutations with respect to the consensus sequence were observed throughout the locus in each of the eight strains. The mean (± standard error) number of substitutions per 100 bp across the six genes constituting the locus ranged from 0.34 ± 0.09 for strain MS173 to 0.65 ± 0.08 for strain K4907A (Table 1). The mean number of substitutions per 100 bp within each gene ranged from 0.34 ± 0.04 for the 861 bp N-acetylmannosamine kinase *nagC* to 0.65 ± 0.03 for the 2,817 bp sialidase *nanI* (MS53_0199, GenBank accession no. **YP_278329**). In contrast, the number of substitutions per 100 bp within the two 16S rRNA genes of *M. synoviae* was only 0.04. For comparison to another species of mycoplasma, the number of substitutions per 100 bp in the 1,002 bp signal recognition particle receptor subunit Y gene *ftsY* ranged from 0.14 in *Mycoplasma hyopneumoniae* strain J (GenBank accession no. **AE017243**) to 0.42 in strain 232 (**AE017332**), and in the 1,392 bp transcriptional dual regulator *dnaA* gene ranged from 0.2 in *M. hyopneumoniae* strains J and 232 to 0.3 in strain 7448 (**AE017244**).

2.3. Guanine+cytosine content

The guanine+cytosine content (%G+C) was calculated for the sialic acid degradation genes from each strain, individually and for the consensus sequence of each gene (Table 2). Genes of this locus tended to have a slightly higher %G+C than the 27% calculated for the *M. synoviae* strain 53 genome as a whole [11], ranging from 27.2 ± 0.11% for N-acetylglucosamine-6-phosphate deacetylase *nagA* to 33.1 ± 0.23% for N-acetylmannosamine-6-phosphate epimerase *nanE* (MS53_0197, GenBank accession no. **AAZ43616**).

2.4. Insertions, deletions, frameshift, and nonsense mutations

There were no insertions, deletions, or frameshift mutations, with respect to the consensus sequence, in the protein-coding sequences of the *nanE*, *nanI*, *nagA*, *nagB*, and *nagC* genes of the eight strains examined. However, strain WVU1853^T had a 12 bp deletion in the flanking glyceraldehyde-3-phosphate dehydrogenase *gapDH-nanI* intergenic region, beginning at base minus 41 from the predicted *nanI* start codon (Figure 2A), that substantially altered the predicted nucleic acid stem-loop secondary structure of the putative *nanI* promoter region. The 891 bp *nanA* gene contained a 2 bp insertion, common to strains FMT (passages 33 and 126) and K3344, creating a frameshift with respect to the consensus sequence that resulted in seven

premature stop codons and effectively disabled the gene. Also, deletions of 14 and 462 bp were present in strains K3344 and FMT (both passages), respectively, downstream of the *nanA* nonsense mutations (Figure 2B). The deletion in strain FMT was bracketed by the direct repeats 5′-AAT TTC TTC A-3′, and completely overlapped the deletion in strain K3344 (Figure 2C). The deletions accounted for the notably short PCR amplicon obtained for the locus from both passages of strain FMT. Strain WVU1853^T had a nonsense mutation arising from a single nucleotide substitution in *nanA*, predicted to result in the loss of 17 aa from the carboxyl terminus of the 296 aa NanA of the other strains.

2.5. Missense and silent mutations

Missense mutations with respect to the consensus sequences were approximately twice as common as silent mutations in the *nanI* and *nagA* genes (Figure 3). The number of missense and silent mutations was approximately equal in *nanA* and *nagC*, and *nagB* and *nanE* genes had slightly more silent mutations. For perspective, silent mutations were approximately 2.5 and 15 fold more common than missense mutations in the *M. hyopneumoniae* genes *dnaA* and *ftsY*, respectively.

Missense mutations resulting in dissimilar amino acids were common throughout the sialic acid degradation locus in all eight *M. synoviae* strains. They represented 26%, 28%, and 26% of all mutations in NanI, NagA, and NagB, respectively (Figure 3). Approximately half of the dissimilar amino acid substitutions in NanI were in the functional domain defined by the Conserved Domain Database [29]. The signature Arg-Ile-Pro and two Ser-X-Asp-X-Gly-X-Thr-Trp “Asp box” motifs [30], plus Asp box variants Thr-X-Asp-X-Gly-X-Thr-Trp and Ser-X-Asp-X-Gly-X-Asn-Trp, were conserved in NanI. Candidate equivalents of the highly-conserved Arg_{37}, Asp_{54}, Arg_{56}, Asp_{62}, Asp_{100}, Glu_{230}, Arg_{245}, and Tyr_{347} residues (numbering of the *Clostridium perfringens* NanI; GenBank accession no. **P10481**) were readily identified by inspection of local and global sequence alignments, and were conserved across strains, but no equivalent of the Arg_{312} that is strictly conserved in many other bacterial and eukaryotic sialidases [31] could be recognized in any strain. All of the dissimilar amino acid substitutions of NanA, NanE, NagA, NagB, and NagC were in their broadly-defined functional domains [29]. Dissimilar amino acid changes were least common in NanA, representing just 4% of all mutations in *nanA* across strains. Missense mutations resulting in dissimilar amino acids were not present in either *dnaA* or *ftsY* genes in the three strains of *M. hyopneumoniae* examined.

3. Discussion and conclusions

The recent discovery by Vasconselos et al. [11] of putative genes for sialidase and the N-acetylneuraminate catabolism pathway in *M. synoviae* strain 53, when sialidase activity was believed to be extremely rare among mycoplasmas [25,32], prompted us and others to confirm the annotation by using assays for the enzyme to examine additional strains [26,27]. The activity was present in most, but not all, *M. synoviae* strains examined. Unexpectedly, although strain WVU1853^T had by far the most activity, there was essentially continuous variation in the amount of sialidase activity per CFU among other strains, which also correlated positively with the degree of strain virulence. In this study, we sought to explain the remarkable intraspecific variation in activity of this candidate virulence factor by measuring *nanI* copy number in high- and low-activity strains, and by sequencing the sialidase gene *nanI* and genes constituting the sialic acid degradation locus in multiple strains.

Although the presence and relative spacing of signature motifs and active site residues are conserved in bacterial sialidase catalytic domains, their primary amino acid sequences and lengths are otherwise highly variable [30]. The NanI sialidase of *M. synoviae* has a theoretical and observed [33] molecular weight of approximately 109 kDa. It consists of an approximately

420 aa N-terminal domain that includes a predicted 29 aa transmembrane region near the N-terminus, and an approximately 520 aa C-terminal six-sheet beta-propellor catalytic domain, Pfam 00064 [34]. NanI has been reported to be extracellular surface-localized in *M. synoviae* and *M. gallisepticum* [15,26], consistent with most other bacterial sialidases that are secreted to effect their actions on the surrounding environment [20]. Negligible activity was found in *M. synoviae*-conditioned cell-free broth [26,27]. Since extracellular surface-localization precludes an influence of substrate import on the enzyme's activity *in situ*, the quantitative differences in sialidase activity of the magnitude observed for *M. synoviae* are most simply explained by interstrain variations in the topology of the enzyme. The evidence for a complex genetic basis for the intraspecific variation includes: 1) numerous single nucleotide polymorphisms with respect to their consensus *nanI* DNA sequence; resulting in 2) a comparatively high frequency of dissimilar missense mutations with respect to the consensus NanI amino acid sequence; 3) presence of only a single copy of *nanI* regardless of sialidase activity per CFU; and 4) strict conservation of all but one of the several residues believed to constitute the active site [26,28,30,31]. For perspective, individual site-directed mutagenesis of each of the active site residues other than Arg_{312} reduced *C. perfringens* sialidase specific activity by 100 to 10, 000 fold [31]. It was noteworthy that the sialidase activity of *M. synoviae* strain FMT remained quantitatively unchanged after 93 *in vitro* passages [27]. Since the 12-base deletion in the *nanI* promoter region in strain $WVU1853^T$ was a singular finding among several *M. synoviae* strains examined [26,27], and no distinct TATA or ribosome binding sequences were evident in the *gapDH-nanI* intergenic region adjacent to the putative *nanI* start codon, a causal relationship between the deletion and that strain's comparatively high sialidase activity per CFU remains plausible but speculative. It was also remarkable that virulent strain $WVU1853^T$ had the least sequence variation in *nanI* but the highest activity per CFU, whereas avirulent strain K4907A had the most sequence variation in *nanI* and nearly the least activity per CFU of the strains with quantitated sialidase activity [27].

From a taxonomic standpoint, the capacity to produce sialidase occurs irregularly among bacteria, and sialidases are sometimes produced even by only a single strain within a species [35,36]. Those findings are most readily explained by horizontal transfer of sialidase genes [30]. The hypothetical protein MGA_0329 of *M. gallisepticum* strain R_{low} (GenBank accession no. **NP_853343**) shares 94.5% aa identity and 96.5% aa similarity to *M. synoviae* NanI (hypothetical protein MS53_0199, GenBank accession no. **YP_278329**). We used the methods described in section 4.1 [27] to confirm that *M. gallisepticum* strain R_{low} does express sialidase activity (our unpublished data). Vasconselos et al. [11] hypothesized a history of *nanI* homolog transfer between *M. synoviae* and *M. gallisepticum*, even though the strain R_{low} genome (GenBank accession no. **AE015450**) lacks all of the genes of the N-acetylneuraminate catabolism pathway [37]. The Arg_{312} that is conserved in other sialidases including in *M. gallisepticum*, but missing from *M. synoviae*, provides evidence of the direction of transfer. The corresponding *M. gallisepticum* Arg_{312} codon CGT was substituted in *M. synoviae* with Gly codons GGT in six of eight strains, or GGC in two strains. Thus a first-position C→G transversion mutation accounts for the loss of the Arg_{312} residue in *M. synoviae* and indicates that the direction of transfer was from *M. gallisepticum* to *M. synoviae*. In that case, either 1) *nanI* alone was transferred from *M. gallisepticum* to *M. synoviae* or its ancestor, which independently acquired five N-acetylneuraminate catabolism genes in a cluster precisely adjacent to *nanI* in its chromosome; or, more likely 2) the entire locus was transferred to *M. synoviae* from a strain of *M. gallisepticum* or its ancestor whose descendants later lost all of the N-acetylneuraminate catabolism genes. The *nanI* %G+C, which was similar to the %G+C of the whole genome, did not suggest any other history. Regardless of which history is correct, the implication is that sialidase activity need not remain linked to N-acetylneuraminate catabolism in order to persist in mycoplasmal genomes.

A “neuraminidase-like enzyme” was detected in an unidentified strain of *M. gallisepticum* [15], and sialidase activity of *M. gallisepticum* strain TT was characterized in detail [14,16], but no evidence for sialidase activity was found in *M. gallisepticum* strains S6 [32] or A5969 [25]. Its genomic context suggests that sialidase activity is important in the ecology of *M. gallisepticum* strain R_{low} independently of its potentially advantageous role in nutrient acquisition [20]. The present study provides further evidence of a separate role for extracellular sialidase also in at least some strains of *M. synoviae*. Strains FMT and K3344 had identical frameshifts in the 5′-third of *nanA*, resulting in multiple nonsense mutations, and both strains also had downstream deletions in *nanA*. Such genetic lesions naturally uncouple extracellular liberation of sialic acid, via sialidase, from generation of N-acetylmannosamine, pyruvate, and eventually fructose-6-phosphate via intracellular N-acetylneuraminate catabolism. We interpret the different deletions as subsequent decay of the gene initially disabled by frameshift in a common ancestor, because it seems less likely that exactly the same 2 bp insertion would occur upstream in the gene independently in two strains if the deletions had occurred first. The deletion in strain FMT completely overlapped the deletion in strain K3344, which implies that K3344 may be an ancestor of FMT, or at least that FMT is less similar to a common ancestor than K3344 is at this locus. In a small sample of other species affiliated with the *M. synoviae* phylogenetic cluster [38], which we screened for sialidase activity as described in section 4.1 [27], *Mycoplasma felis* ATCC 23391^T, *Mycoplasma leonicaptivi* ATCC 49890^T, and *Mycoplasma sturni* UC/MF^T were negative, but 12 of 13 canine clinical isolates confirmed by PCR-RFLP typing to be the opportunistic mammalian pathogen *Mycoplasma canis* [39] did express activity (our unpublished data).

An ecological function of sialidase in strains that express activity not linked to N-acetylneuraminate catabolism may be to modulate cytadherence. For example, *M. synoviae* and *M. gallisepticum* both utilize sialylated glycoproteins on eukaryotic cell surface membranes as receptors for cytadherence mediated by adhesins such as the *vlhA* system hemagglutinins [40]. Since receptor desialylation reduces or abolishes cytadherence by *M. synoviae*, *M. gallisepticum*, and *M. canis* [32,41,42], it is predictable that a functional balance between the amount of sialidase activity and receptor binding affinity must be essential to promote both colonization and transmission of these mycoplasmas. Strains with comparatively higher sialidase activity would be expected to possess higher-affinity adhesins [43]. The *vlhA* locus in the *M. synoviae* strain 53 genome is flanked by homologs of *gapDH* adjacent to the sialic acid degradation locus. Hypervariability in the VlhA hemagglutinins expressed within and among strains is generated by site-specific recombinations among a large assemblage of *vlhA* pseudogenes constituting the 69 kb locus [44,45]. In contrast, there are many potentially independently-transcribed *vlhA* genes dispersed throughout the *M. gallisepticum* genome [37,46], supporting the hypothesis that, like *nanI*, *vlhA* also may have been exchanged between these species by horizontal transfer [11,47]. Results of the present study contribute to a foundation for further work to correlate the probably shifting balances among host immune responses to antigenic adhesins such as VlhA, variations in receptor binding affinity and sialidase activity, and their interplay with cytadherence and pathogenicity in *M. synoviae*.

4. Materials ands methods

4.1. Mycoplasma synoviae strains and culture conditions

Mycoplasma synoviae strains FMT (passages 33 and 126), K3344, K4907A, K5395B, MS173, MS178, and WVU1853^T were cultured in modified Frey’s medium as previously described [27]. The FMT strain, originally isolated from chicken trachea, induced minor respiratory lesions following experimental infection [3]; stocks FMT33 and FMT126 were derived from serial *in vitro* passages of FMT. 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 [48]. Strains MS173 and MS178 were isolated during an outbreak of severe synovitis in Argentina [49]. Lesions from infected birds involved synovial membranes, bursa of Fabricius, liver, kidney, and the lower respiratory tract in breeders [50]. Strain WVU1853^T has been most commonly reported to cause airsacculitis and synovitis [2], however, experimental infection studies indicated that this strain is capable of systemic spread and the generation of lesions in multiple tissues [3,51]. Strains K4907A and K5395B were isolated from clinically normal chickens, and are not suspected to cause significant lesions. A quantitative analysis using using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid and the sialidase inhibitor 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid [27] showed that the units (U) of sialidase activity per CFU varied as much as 65 fold (ANOVA $P < 0.0001$) among these strains. The highest (Fisher's Protected Least Significant Difference test $P < 0.001$) activity observed was for strain WVU1853^T (1.3×10^{-7} U/CFU), intermediate amounts were observed for FMT33, FMT126, K3344, and MS178 ($1.3–3.9 \times 10^{-8}$ U/CFU), and low amounts were observed for strains K4907A, K5395B, and MS173 ($2.7–6.0 \times 10^{-9}$ U/CFU). The *M. synoviae* field isolate 53 was not readily available for phenotypic analysis, and neither clinical data nor sialidase activity have been reported for that strain.

4.2. PCR amplification of the sialic acid degradation locus

Genomic DNA was extracted using Easy DNA reagents (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The sialic acid degradation locus (Figure 1B) was amplified from strains FMT33, FMT126, K3344, K4907A, K5395B, MS173, MS178, and WVU1853^T using PCR primers designed to anneal in the flanking *gapDH* (5′-TGT TGA ATC AAA AGA CGG AAG A-3′) and hypothetical gene MS53_0192 (5′-TCA TCG CTT AAT ACT GGG CTT T-3′) open reading frames of strain 53. Amplification reactions were carried out as follows, using the Expand High Fidelity PCR System (Roche Applied Sciences, Indianapolis, Indiana): initial denaturation at 94°C for 2 min, followed by 30 cycles of template denaturation at 94°C for 20 sec, primer annealing at 50°C for 30 sec, and extension at 68°C for 9 min, completed by a final extension at 68°C for 10 min. The expected length of the product was approximately 8.4 kb.

4.3. Nucleotide sequencing and sequence analyses of the sialic acid degradation locus

Nucleotide sequencing for the sialic acid degradation locus of each strain, from the *gapDH-nanI* intergenic region to the *nagB*-MS53_0192 junction, was achieved by primer walking using four-dye fluorescent dideoxy labeling methods and the Model 3130 capillary system (Applied Biosystems, Foster City, California). The uncloned amplified DNA described in section 4.2 served as the cycle-sequencing templates. For each strain, 31 reads were required to assemble a contig of reconciled double-stranded sequences using Sequencher version 4.7 software (Gene Codes, Ann Arbor, Michigan). Open reading frames were identified by BLAST alignments with homologs from the *M. synoviae* strain 53 genome (GenBank accession no. **AE017245**). The nucleotide sequence of each gene was translated using the ExPASy Translate Tool [52]. The secondary structure of the putative *nanI* promoter region and structural features of the NanI protein were investigated using tools of the European Molecular Biology Open Software Suite [53,54]. Nucleotide and amino acid substitutions among strains were mapped using ClustalW alignments [55]. As a benchmark, 1,240 bases of each of the two *M. synoviae* 16S rRNA genes were sequenced from strains WVU1853^T, K3344, MS173, and MS178 using internal primers previously described [56], and compared similarly to the corresponding sequences from strain 53. For another perspective, intraspecies heterogeneity among *M. hyopneumoniae* strains 232, 7448, and J (GenBank accession nos. **AE017332**, **AE017244**, and **AE017243**) in the sequences of housekeeping genes *dnaA* and *ftsY* was also measured.

4.4. Southern blotting to determine nanI copy number

To compare the *nanI* copy number among strains by Southern blotting, a probe consisting of the 3′ 2.8 kb of *nanI* was amplified from WVU1853^T genomic DNA using PCR primers 5′-TCT CTT CCT TTT TGA GGG CTA-3′ and 5′-GCA AAT CAT CTT AAG AAA AGT CAT T-3′. Amplification conditions, using GoTaq reagents (Promega, Madison, Wisconsin), were as described in section 4.2, with the exception of extension steps of 3 min at 72°C. The amplicons were labeled with digoxygenin (DIG Hi prime, Roche Applied Sciences) according to the manufacturer's instructions. Genomic DNA from the strains with the highest (WVU1853^T) and lowest (K4907A and K5395B) levels of sialidase activity was digested with endonuclease *Vsp*I (New England Biolabs, Ipswich, Massachusetts), then separated on a 0.6% agarose gel. The DNA fragments were electrotransferred to nitrocellulose, then cross-linked with shortwave ultraviolet light, using standard methods. Hybridization of the *nanI* probe and detection of the digoxygenin label were carried out using the DIG EasyHyb system (Roche Applied Sciences) according to the manufacturer's instructions.

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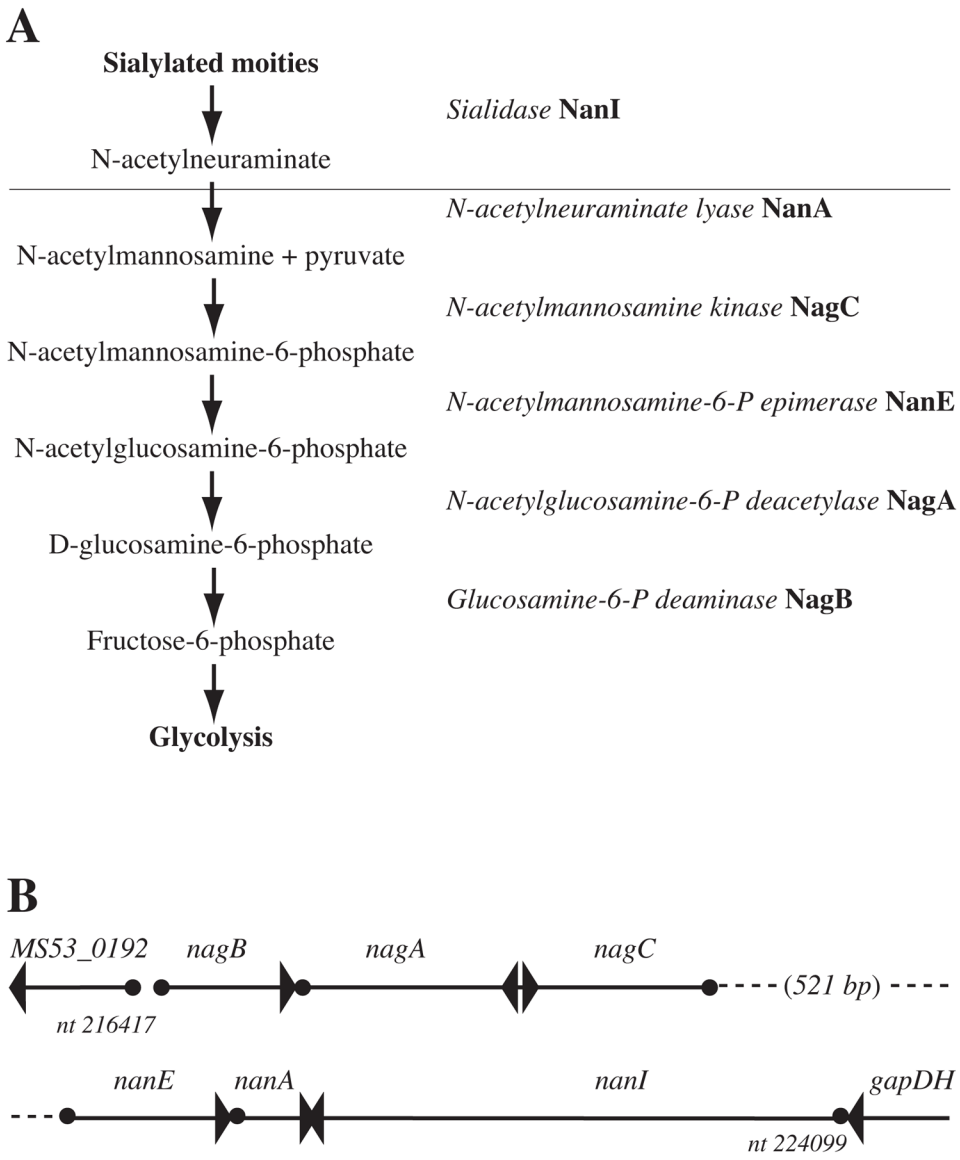

Figure 1.
(A) The canonical sialic acid degradation pathway [28]. The horizontal line represents the interface between extracellular and intracellular processes. (B) Organization of the 7.9 kb sialic acid degradation locus in the *M. synoviae* strain 53 genome [11].

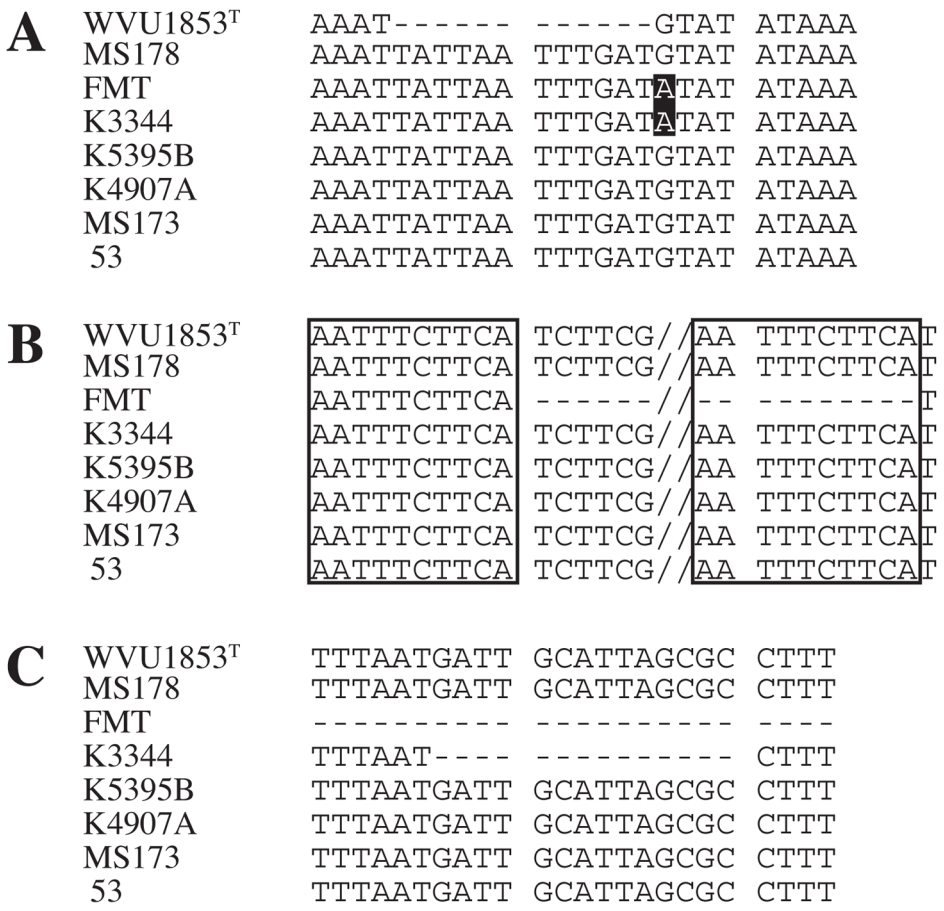

Figure 2.

Deletion mutations in the sialic acid degradation locus. The *M. synoviae* strains examined are listed (left). Nucleotide numbering is from the *M. synoviae* strain 53 genome (GenBank accession no. **NC_007294**) [11]. (A) Nucleotides 224131–224155. A 12 bp deletion occurred in the intergenic sequence immediately upstream of the sialidase gene *nanI* in strain WVU1853^T. (B) Nucleotides 220678–221153. Slashes represent 446 intervening bases. A 462 base deletion occurred in the 891 bp N-acetylneuraminate lyase gene *nanA* of strain FMT. A direct repeat bracketing the deletion is boxed. (C) Nucleotides 220953–220976. A 14 bp deletion, that was overlapped by the larger deletion in strain FMT, occurred in the *nanA* gene of strain K3344.

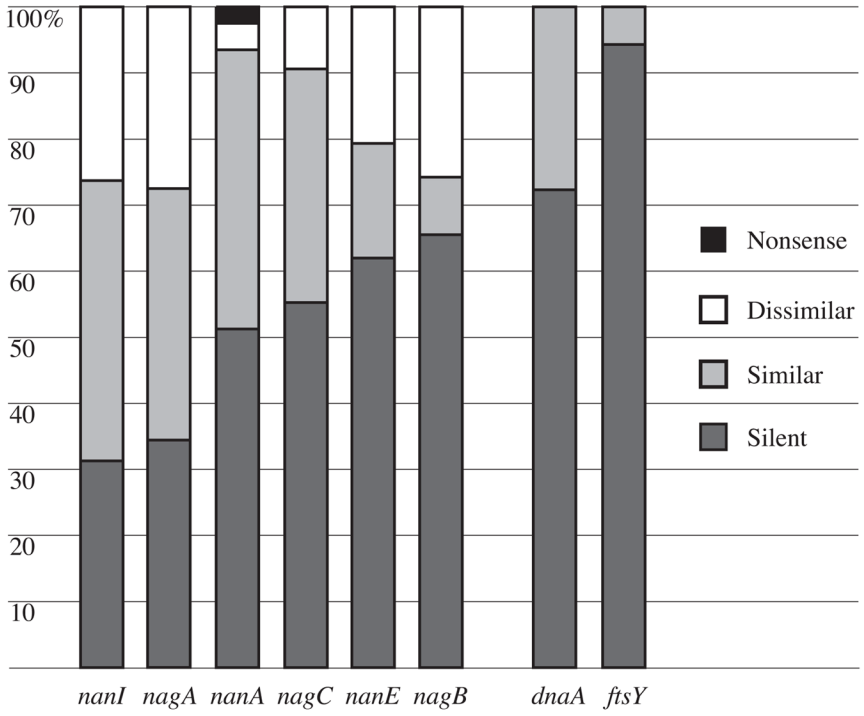

Figure 3.
Composition of substitution mutations with respect to consensus sequences. Percentages represent the mean across eight *M. synoviae* strains for *nanI*, *nagA*, *nanA*, *nagC*, *nanE*, and *nagB* genes of the sialidase locus, and three *M. hyopneumoniae* strains for housekeeping genes *dnaA* and *ftsY*.

Table 1

Genetic heterogeneity in the *Mycoplasma synoviae* sialic acid degradation locus[a].

	Strain								
	K4907A	**K5395B**	**WVU1853^T**	**K3344**	**FMT**	**53**	**MS178**	**MS173**	**Mean ± S.E.**
Gene[b]									
nanI	0.78	0.61	0.57	0.61	0.61	0.82	0.61	0.61	0.65 ± 0.03
nanA	0.92	0.61	0.58	0.38	0.49	0.58	0.46	0.46	0.56 ± 0.06
nagA	0.64	0.55	0.55	0.73	0.55	0.55	0.46	0.46	0.56 ± 0.03
nanE	0.7	0.99	0.7	0.56	0.56	0.56	0	0	0.51 ± 0.12
nagB	0.42	0.42	0.56	0.56	0.71	0.28	0.14	0.14	0.40 ± 0.07
nagC	0.46	0.23	0.34	0.46	0.34	0.11	0.46	0.34	0.34 ± 0.04
Mean ± S.E.	0.65 ± 0.08	0.57 ± 0.10	0.55 ± 0.05	0.55 ± 0.05	0.54 ± 0.05	0.48 ± 0.10	0.36 ± 0.10	0.34 ± 0.09	
16S rDNA[c]	N/D[d]	N/D[d]	0.04	0.04	N/D[d]	0.04	0.04	0.04	0.04

[a] Nucleotide substitutions per 100 bases of consensus sequence.

[b] *nanI* = sialidase MS53_0199 (synonymous with *nanH* of GenBank accession no. **ABS50356**); *nanA* = N-acetylneuraminate lyase; *nagA* = N-acetylglucosamine-6-phosphate deacetylase; *nanE* = N-acetylmannosamine-6-phosphate epimerase MS53_0197 (GenBank accession no. **AAZ43616**); *nagB* = glucosamine-6-phosphate deaminase; *nagC* = N-acetylmannosamine kinase.

[c] Both copies.

[d] N/D = not determined.

Table 2

Guanine+cytosine content of genes in the *Mycoplasma synoviae* sialic acid degradation locus.

	Strain									
	K4907A	**K5395B**	**WVU1853^T**	**K3344**	**FMT**	**53**	**MS178**	**MS173**	**Mean ± S.E.**	**Consensus**
Gene[a]										
nanI	29.3	29.2	29.1	29.0	29.0	29.1	29.1	29.1	29.1 ± 0.04	29.3
nanA	32.4	32.4	32.6	31.9	31.9	33.2	32.8	32.8	32.5 ± 0.05	32.4
nagA	27.2	27.2	27.0	27.3	27.3	27.1	27.3	27.3	27.2 ± 0.11	27.4
nanE	33.1	32.6	32.9	33.2	33.1	33.3	33.2	33.2	33.1 ± 0.23	33.2
nagB	30.4	30.2	30.1	30.2	30.2	30.2	30.6	30.6	30.3 ± 0.20	30.2
nagC	30.7	30.9	30.8	30.9	31.0	31.0	30.9	31.0	30.9 ± 0.04	31.1

[a] *nanI* = sialidase MS53_0199 (synonymous with *nanH* of GenBank accession **ABS50356**); *nanA* = N-acetylneuraminate lyase; *nagA* = N-acetylglucosamine-6-phosphate deacetylase; *nanE* = N-acetylmannosamine-6-phosphate epimerase MS53_0197 (GenBank accession no. **AAZ43616**); *nagB* = glucosamine-6-phosphate deaminase; *nagC* = N-acetylmannosamine kinase. The calculated %G+C for the *M. synoviae* strain 53 genome as a whole was 27% [11].