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Neuraminidase of *Mycoplasma synoviae* desialylates heavy chain of the chicken immunoglobulin G and glycoproteins of chicken tracheal mucus

Rebeka Lucijana Berčič¹, Ivanka Cizelj¹, Daliborka Dušanić¹, Mojca Narat¹, Olga Zorman-Rojs², Peter Dovč¹, Dušan Benčina^{1*}

¹ Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, 1230 Domžale, Slovenia

² Veterinary Faculty, University of Ljubljana, Gerbičeva 60, 1000 Ljubljana, Slovenia

Corresponding author:

Dušan Benčina*

Tel: +38613203809

Fax No.: +38617241005

E-mail: dusan.bencina@bf.uni-lj.si

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Abstract

Major poultry pathogens, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* share several genes, including *nanH* that encodes their sialidases (neuraminidases). Previous studies have shown considerable differences in neuraminidase enzymatic activity (NEAC) in *M. synoviae* strains and NEAC absence in individual cultures of two strains, ULB 925 and ULB 9122. This study showed that their cultures lacking NEAC did not express NanH neuraminidase detectable by specific antibodies. In cultures of *M. synoviae* ULB 925 and ULB 9122, which lacked NEAC and detectable NanH, deletions of a single adenine in different *nanH* regions of each strain created translational frameshifts resulting in TAA (UAA) stop codons and premature termination of translation. ULB 925 and ULB 9122 with such *nanH* mutations did not desialylate reference fetuin and transferrin or chicken glycoproteins which *M. synoviae* strains with NEAC efficiently desialylated. They desialylated several chicken serum glycoproteins with SA α (2-6)gal moieties, including immunoglobulin G heavy chain. Neuraminidase inhibitor 2,3-didehydro-2-deoxy-N-acetylneuraminic acid (DANA) inhibited such desialylation otherwise caused by *M. synoviae* WVU 1853 neuraminidase. WVU 1853 also cleaved sialic acid from SA α (2-3)gal moieties from glycoproteins of mucus from chicken tracheas. This is the first demonstration that *M. synoviae* desialylates glycoproteins of its host.

Introduction

Mycoplasma gallisepticum and *Mycoplasma synoviae* are major poultry pathogens that cause great economic losses in intensively reared chickens and turkeys (Kleven, 2003; Ley, 2003). Although *M. gallisepticum* and *M. synoviae* are phylogenetically distant, they share hosts, tissue tropism, ways of transmission, as well as numerous horizontally transferred genes (Papazisi *et al.*, 2003; Vasconcelos *et al.*, 2005). Some of these genes encode proteins, which are important in interactions with host cells and most likely, in adaptation of *M. gallisepticum* and *M. synoviae* to the poultry hosts (Noormohammadi *et al.*, 1998; Sirand-Pugnet *et al.*, 2007; Vasconcelos *et al.*, 2005). These two species adhere to host cells via receptors containing sialic acid (SA) and share haemagglutinating lipoproteins VlhA involved in their binding to erythrocytes (Benčina, 2002; Markham *et al.*, 1993; Noormohammadi *et al.*, 1997; 1998; Razin, 1985). Sialidases (neuraminidases) are their potential virulence factors. Higher neuraminidase activity has been reported in *M. gallisepticum* and *M. synoviae* strains that are more invasive and/or more pathogenic for chickens and turkeys (Berčič *et al.*, 2008b; May *et al.*, 2007).

Significant differences in NEAC (up to 65-fold) have been reported for *M. synoviae* strains (May *et al.*, 2007). Moreover, *M. synoviae* ULB 925 cultures and one culture of ULB 9122 lacked detectable neuraminidase enzymatic activity (NEAC), but the molecular basis responsible for the absence of NEAC has not been reported (Berčič *et al.*, 2008b). Because desialylation of host receptors can reduce or abolish cytoadherence by both *M. gallisepticum* and *M. synoviae*, a hypothesis has been proposed that a balance between variable sialidase activity (i.e. NEAC) and avidity of adherence to sialylated receptors on host cells is predictable (May & Brown, 2008; 2009a). However, only for *M. gallisepticum* strain TT it has been reported that it cleaved sialic acid from human transferrin, human α 1-

glycoprotein and rabbit serum (Sethi & Müller, 1972). Generally, cultures of *M. synoviae* showed higher levels of NEAC than those of *M. gallisepticum* (Berčič *et al.*, 2008b), but there was no data about *M. synoviae* capability to desialylate glycoproteins of its host or other glycoproteins.

In this study, we attempted to address several aspects related to NEAC of *M. synoviae* strains. With specific antibodies we determined the expression of their NanH neuraminidase. In *M. synoviae* cultures without detectable NEAC and NanH protein the *nanH* gene sequences were determined and this enabled identification of mutations responsible for the absence of NEAC. We also demonstrated for the first time, that *M. synoviae* desialylates several chicken glycoproteins, including those from the upper trachea. This capability could enable an efficient colonization and long persistence of *M. synoviae* in the upper respiratory tract of chickens.

Materials and Methods

***Mycoplasma synoviae* strains and their growth.** *M. synoviae* strains used in this study are shown in Table 1. Except strain ULB 07/P5, they have been characterized in previous studies (Narat *et al.*, 1998; Benčina *et al.*, 1999, 2001, 2005; Berčič *et al.*, 2008 a,b). Cultures of the type strain WVU 1853 were from two *Mycoplasma* culture collections (Berčič *et al.*, 2008b). Those used throughout of this study and designated WVU 1853, were from the WVU 1853 culture provided by S. H. Kleven (Benčina *et al.*, 2001). The second WVU 1853 culture (designated WVU 1853 Aa), donated by Janet M. Bradbury, was from the collection of FAO/WHO collaborating Center for Animal Mycoplasmas, Aarhus, Denmark. Strains without NEAC (Berčič *et al.*, 2008b) and closely related strains are shown in Fig. 1. AAY-4 has been

used to induce infectious synovitis in chickens (Narat *et al.*, 1998). ULB 925 has been isolated from the hock joint of a chicken inoculated with the haemagglutination positive (HA⁺) culture of AAY-4 (Narat *et al.*, 1998; Benčina *et al.*, 1999). ULB 925 HA⁺ clone KF was cloned to obtain HA⁺ subclone KF9 (and its subclones KF9/3 and KF9/4) and HA-negative (HA⁻) subclone KF6 (Benčina *et al.*, 1999, 2001; Berčič *et al.*, 2008b). ULB 9122 was isolated in 1991 from the upper trachea of a hen of brown egg-layer of breed RIR in Slovenia (Benčina *et al.*, 2001, 2005). ULB 9122 culture C1 had NEAC, whereas its another culture (C2) was NEAC- negative (Berčič *et al.*, 2008b). Both cultures, C1 and C2, underwent 20 passages (Fig.1). ULB 9122 culture C3 (also NEAC-negative) derived from C2 after additional 2 passages. Strain ULB 07/P5 was isolated in 2007 from the choanal cleft of a chicken belonging to the same chicken breed as chicken from which ULB 9122 was recovered 16 years earlier. Sequences of highly polymorphic *M. synoviae* genes (5'-*vlhA*, *nanH* and for a putative cysteine protease MS53_0590) of ULB 9122 and ULB 07/P5 were identical, except a single nucleotide polymorphism (SNP) in the *nanH* gene (see Results). *M. synoviae* cultures were grown in Frey's medium at 37-38 °C and were harvested before pH of the broth reached pH 6.7 (Berčič *et al.*, 2008b; Dušanić *et al.*, 2009). The number of colony forming units (CFU) of their broth cultures was determined as recommended (Rodwell & Whitcomb, 1983).

Polymerase chain reaction (PCR) and sequencing. Purified DNA of *M. synoviae* strains was obtained by RTP Spin Bacteria DNA Mini Kit according to the manufacturers' instruction (Invitex). The oligonucleotide primers used for PCR are presented in Table 2. The published *M. synoviae* genome sequence was used to design primers enabling amplification and sequencing the region from the 3'- end of *nanA* gene, the entire *nanH* (neuraminidase)

gene to the 5'-end of the *gapDH* gene i.e. region from nt 221086 to 225216 (Vasconcelos *et al.*, 2005, GenBank accession number AE017245).

The 30- μ l PCR mixture contained 3 μ l of 10 x PCR buffer (MBI Fermentas, Lithuania), 0.5 U of Taq DNA polymerase (MBI Fermentas), 1.5 μ l of each primer at a concentration of 10 pM, 1.5 μ l of mixture of all four deoxynucleoside triphosphates at a concentration of 2mM and 2.4 μ l of 25 mM MgCl₂. The program consisted of initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation for 1 min at 95 °C, annealing for 30 s (for specific set of primers the annealing temperature is described in Table 2), extension for 150 s at 72 °C, and final extension for 10 min at 72 °C. The PCR products were sent for sequencing to Macrogen DNA Sequencing Service. Sequence analyses were performed by EMBL-EBI Tool ClustalW2 (Thompson *et al.*, 1994), BCM Search Launcher (Smith *et al.*, 1996), NCBI BLAST Tools (Zhang *et al.*, 1997).

Evaluation of neuraminidase enzymatic activity (NEAC). *M. synoviae* cells were harvested from their broth cultures (50 or 100 ml) by centrifugation, washed in sterile phosphate buffered saline (PBS, pH 7.4) and their suspension in PBS were prepared for NEAC assays. Ten μ l suspension of *M. synoviae* cells ($\sim 10^8$ or 10^9 CFU) was mixed (in small microfuge tubes with caps) with ten μ l of the chromogenic substrate containing 5 μ g of 5-bromo-4-chloro-3-indolyl- α -D-N-acetylneuraminic acid (called hereafter BIN; B4666, Sigma-Aldrich, St. Louis, USA).

Samples were incubated at room temperature and times of their positive NEAC reactions, colour change from colourless to indigoblue, were recorded. *M. synoviae* cultures without NEAC (see Table 1) were also assayed at higher cell-density (10^{10} CFU) and longer incubation with BIN (at least 24 hours). In each NEAC assay the positive control was

neuraminidase of *Clostridium perfringens* (type V, N2876, Sigma-Aldrich), whereas the negative control was PBS.

Assays using neuraminidase inhibitor. To confirm that neuraminidase is involved in the NEAC reaction and desialylation of glycoproteins by *M. synoviae*, the neuraminidase inhibitor 2,3-didehydro-2deoxy-N-acetylneuraminic acid (DANA; D9050, Sigma-Aldrich) was used. Stock solution of DANA (10 mg/ml) was kept at -20 °C. To demonstrate neuraminidase inhibition of *M. synoviae* cells (10^8 or 10^9 CFU) their samples were incubated with 1 – 10 mg/ml DANA (10 or 100 µg/30 µl sample) and 5 µg of BIN. Parallel NEAC assays were performed with *C. perfringens* neuraminidase (1 or 10 µg) and 10 or 100 µg of DANA. One µg of *C. perfringens* neuraminidase had 1.7 mU if the neuraminidase substrate was mucin (data from Sigma).

Antibodies to the NanH neuraminidase and other antibodies. Chicken antibodies specific for the NanH protein of *M. synoviae* were custom produced by GenWay (San Diego, CA, USA). A synthetic peptide, DKRVNNKDDRGHLEQD, representing theoretically highly immunogenic “epitop” of NanH (residues 464-478) was coupled (via cysteine added to the first residue of this peptide) to keyhole limpets haemocyanin (KHL). Two chickens were immunized, each received ~ 2 mg of the NanH peptide coupled to KLH during the immunization procedure. Immunoglobulin G (IgG) antibodies were purified from their egg yolks. Affinity-purified IgG to NanH were obtained using the NanH peptide as a specific ligand immobilized on CNBr beads (isolated by GenWay). They were received as a solution containing 1 mg of IgG per ml.

Antibodies to bacterial glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were raised in mouse. GAPDH of *Bacillus (Geobacter) stearothermophilus* was purchased (G5892,

Sigma). The BALB/c mouse was immunized with GAPDH (total protein ~ 100 µg) using previously described procedure (Benčina *et al.*, 2001) and finally with the *M. synoviae* cytoplasmic protein fraction obtained following osmotic lysis of WVU 1853 cells (Razin, 1983). In this fraction GAPDH was the most abundant protein. A week later the antiserum to GAPDH was collected. In immunoblots it strongly reacted with GAPDH of *G. stearotermophilus* (at dilution 1:5000), whereas in *M. synoviae* it reacted only with a protein similar in Mw to *M. synoviae* GAPDH (~ 36 kDa).

Monoclonal antibody (mAb) 1F5 was obtained after immunization of a mouse with chicken IgG (cIgG, also termed cIgY) purified from egg yolk (Narat *et al.*, 2004). mAb 1F5 recognizes heavy chain (HC) of cIgG (~ 67 – 70 kDa), as well as HC of turkey and pheasant IgG (Narat *et al.*, 2004).

Analyses of proteins with electrophoresis and immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of *M. synoviae* proteins were performed with the PhastSystem and PhastGel Gradient (8-25) gels (Healthcare Bio-Sciences, Sweden) using ~1 µg of protein per lane (Benčina *et al.*, 1994; Narat *et al.*, 1998). Analyses also were performed in larger gels (10% gel, 12 x 10 cm) using 10 µg of protein per lane (Berčič *et al.*, 2008a). Samples of *M. synoviae* cells were lysed by SDS (final SDS concentration 2%), heated at 95 °C for 5 min and usually analyzed under reducing conditions (5% β-ME). For comparison of NanH expression (see Fig. 2 AB) samples were first analyzed by SDS-PAGE and staining by coomassie blue (PhastGel™ BlueR) to determine dilutions at which samples contained similar amount of major proteins like EF-Tu, enolase and DnaK (Berčič *et al.*, 2008a). Following the SDS-PAGE, proteins were transferred to the Immobilon P membrane (Millipore, USA). NanH was detected by affinity-purified antibodies (2 µg/ml) and incubation for 60 min. After washing the membrane was incubated for 45 min with rabbit

IgG (to cIgG) conjugated with horseradish peroxidase (HRP) (A9046, Sigma). The chromogenic substrate used was either diaminobenzidine tetrahydrochloride (DAB; D5905, Sigma) or TrueBlue™ (Kirkegaard & Perry Laboratories, USA). Mouse antiserum to bacterial GAPDH was used at a dilution of 1:500 for 60 min. As secondary antibodies, goat Ig (to mouse IgG) labeled with HRP (A4416, Sigma) were used at a dilution of 1:2000 for 45 min and then, staining was developed by TrueBlue.

Desialylation of glycoproteins by *M. synoviae*. DIG Glycan Differentiation Kit (Roche, Mannheim, Germany) was used to determine sialylation of glycoproteins, as well as levels of their desialylation with the *M. synoviae* neuraminidase. Sialylation with SA α (2-6)galactose linkage was detected by SNA (Sambucus nigra agglutinin), whereas SA α (2-3)gal linkage was detected by MAA (Maackia amurensis agglutinin) using the recommended reagents and procedure (Roche). The same kit included PNA (Peanut agglutinin) and DSA (*Datura stramonium* agglutinin), used to show cleavage of sialic acid from glycoproteins with distinct galactose and N-acetylglucosamine linkages. Reference glycoproteins (fetuin, asialofetuin, transferrin) from the same kit were used at concentrations from 0.01 to 0.1 mg/ml in PBS (pH 7.4). Ten or 20 μ l samples of glycoproteins were mixed with equal volumes of *M. synoviae* cells (in PBS) in microcentrifuge tubes. Samples of *M. synoviae* strains (from 10^8 to 10^9 CFU) were from their cultures with determined NEAC (see Table 1). Incubations took place at 37-38 °C for 12-18 hours (overnight) () or longer (24-72 hours). Negative controls, consisting of PBS alone (without *M. synoviae*) were incubated with appropriate glycoprotein and included in each assay. In addition, *C. perfringens* neuraminidase (type V, N 2876, Sigma) was used as a positive control (0.1 or 1 μ g/sample) in desialylation assays. Following incubation, samples were centrifuged (30 000 x g for 10 min) and supernatants examined for desialylation of glycoproteins. Samples were assayed with the PhastSystem and PhastGel Gradient (8-25) or

in larger gels (12 x 10 cm). The proteins resolved in gels were transferred to Immobilon P and probed by the DIG glycan differentiation kit according to the manufacturer's instructions (Roche).

Desialylation of chicken serum glycoproteins and chicken IgG. Sera of adult specific pathogen-free (SPF) chickens were diluted from 1:60 to 1:100 (final dilution) in PBS pH 7.4. Initial analyses using SNA and MAA showed that serum glycoproteins contained SA α (2-6)gal, rather than SA α (2-3)gal moiety. In desialylation assays samples of diluted sera (10 or 20 μ l) were mixed with the tested *M. synoviae* cells (10^8 or 10^9 CFU in 10 or 20 μ l PBS) and incubated at 37-38°C overnight or longer. Assays included NEAC-positive, as well as NEAC-negative cultures (shown in Table 1). Positive control was *C. perfringens* neuraminidase (0.5 – 5 μ g per sample of serum glycoproteins), whereas the negative control was PBS alone. The neuraminidase inhibitor DANA was used (up to 100 μ g DANA per sample) in assays in which we tried to confirm that neuraminidase desialylated chicken serum glycoproteins. Following incubation, the samples were centrifuged (30 000 x g, 20 min), supernatants collected, their proteins subjected to SDS-PAGE and transferred to Immobilon P as described above. Levels of SA α (2-6)gal moiety on serum glycoproteins were estimated by visual comparison of staining intensities of bands to which SNA bound (Roche). Samples of purified cIgG from egg yolks or from sera of SPF chickens were prepared as described elsewhere (Narat *et al.*, 2004). Samples of cIgG were analyzed by SDS-PAGE and Coomassie blue staining of proteins. They were used in assays only when they contained pure IgG (~ 170 kDa protein). Desialylation assays with cIgG were conducted as described above for chicken serum glycoproteins. Cleavage of SA α (2-6)gal moiety from cIgG was determined by SNA probe.

In order to show that the heavy chain (HC) of cIgG was desialylated by *M. synoviae* and *C. perfringens* neuraminidases, supernatants of cIgG samples incubated with *M. synoviae* were reduced by 5% β -ME and heated at 95 °C for 5 min. Following SDS-PAGE and transfer of cIgG or its HC to Immobilon P, the membrane was probed with SNA to determine levels of sialylation with SA α (2-6)gal moiety. Then, the membrane was incubated with mAb 1F5 (to HC of cIgG) for 60 min. After washing, the membrane was incubated 45 min with goat IgG (to mouse IgG) conjugated with HRP and developed using TrueBlue.

Desialylation of glycoproteins from chicken tracheal mucus. Samples of tracheal mucus were collected from upper trachea of adult healthy chickens using a syringe and kept at -20 °C in microfuge tubes. These samples were thawed, diluted 1:10 in PBS (pH 7.4), centrifuged (20 000 x g, 20 min) and supernatant was used (final dilution of mucus 1:50 or 1:100) for analyses of glycoproteins with sialic acid linkages detected by SNA or MAA. Three samples with relatively abundant sialylation of glycoproteins with SA α (2-3)gal moiety, recognized by MAA, were used in desialylation assays.

Assays were performed at 37-38 °C in microfuge tubes with caps. Typically, 10 μ l of diluted mucus sample was mixed with suspension of *M. synoviae* cells (10^8 or 10^9 CFU) in 10 μ l PBS, pH 7.4 and incubated overnight or longer (see Table 1 for *M. synoviae* strains tested). Analyses of mucus glycoproteins desialylation were performed by the MAA probe using procedures described above for other glycoproteins.

In some assays, additional consecutive reactions were conducted with the lectin DSA which binds to galactose β (1-4)-N-acetylglucosamine to examine if *M. synoviae* cleaved from mucus glycoproteins only terminal sialic acid linked via α (2-3) to galactose (see Fig. 7 A,B).

Results

***M. synoviae* cultures lacking NEAC did not express NanH neuraminidase.** Immunoblots with antibodies to NanH showed that NEAC-positive *M. synoviae* strains expressed NanH of approximately 110 kDa (Table 1, Fig. 2 and data not shown). In the strain ULB 02/OV6 which expressed 110-kDa protein with the N-terminal amino acid sequence of NanH confirmed by amino acid sequencing (see Berčič *et al.*, 2008a), specific antibodies also recognized 110-kDa NanH. As shown in Fig.2A, two NEAC-positive cultures of WVU 1853 and that of F10 2AS expressed ~ 110-kDa NanH, whereas NEAC-negative cultures of ULB 925 (KF9/3 and KF9/4) and ULB 9122 (culture C2) did not. However, they expressed the enzyme GAPDH which is encoded by two gene copies of which the first is adjacent to the *nanH* gene (Fig. 2B). Strains AAY-4, ULB 925, as well as clones and subclones of ULB 925 lacked NEAC and expression of NanH (Table 1 and Fig 1). On the other hand, in ULB 9122 its cultures C2 and C3 lacked NEAC and NanH, whereas its culture C1 had NEAC and synthesized NanH. In addition, all examined cultures of the strain ULB 07/P5, which is closely related to ULB 9122, expressed NanH and had NEAC (Table 1 and data not shown).

Deletions in the *nanH* gene causing premature terminations of translation. To elucidate the molecular basis for the absence of NanH and NEAC in AAY-4, ULB 925 and ULB 9122 cultures C2 and C3, sequences of their *nanH* gene, its promoter and intergenic region downstream of *nanH* were analysed (see Table 1). Strains AAY-4 and ULB 925 (its clone KF, and subclones KF6 and KF9) had identical sequences (4051 bp) with the same point mutation in the *nanH* gene (Fig 3A). In the codon for the K510 (lysine) one adenine was deleted. This would cause a frameshift introducing a stop codon UAA (TAA) six bases downstream and consequently, a premature termination of translation. Theoretically, the

truncated NanH protein would have only 512 instead 938 residues and would lack domains that are critical for the neuraminidase activity. However, a truncated NanH was not detected by specific antibodies, although they recognize an epitope (residues 464 – 478) within the molecule predicted for such truncated NanH.

Cultures of ULB 9122 had identical sequences except a deletion in the polyA sequence of the *nanH* gene in cultures C2 and C3 lacking NanH. In ULB 9122 C2 and C3 a deletion of one adenine (change 6A to 5A) caused frameshift and introduced stop codon TAA eight bases downstream (Fig. 3B). The predicted NanH polypeptide of C2 and C3 cultures (832 residues) would lack 106 amino acids and would have Mw approximately 97 kDa. Specific antibodies did not detect in their cultures NanH of any size, whereas culture C1 (without deletion in *nanH*) expressed 110-kDa NanH. Strain ULB 07/P5, isolated 16 years after the isolation of ULB 9122, had sequence (comparison of 4063 bp) almost identical to that of ULB 9122, culture C1. In the *nanH* gene of ULB 07/P5 a single nucleotide polymorphism was observed in the codon for residue 740 (change CTT → CCT). Thus, at this position ULB 9122 has leucine, whereas ULB 07/P5 has proline.

Neuraminidase of *M. synoviae* desialylates fetuin and transferring. *M. synoviae* strains cultures expressing NanH cleaved sialic acid from SA α (2-6)gal moiety recognized by SNA on reference transferrin (Table 1). *M. synoviae* cultures lacking NEAC and NanH expression did not desialylate transferrin (Table 1).

M. synoviae cultures having NEAC and expressing NanH cleaved from fetuin sialic acid from SA α (2-6)gal moiety detected by SNA (Fig. 4, Table 1 and data not shown). *M. synoviae* cultures lacking NEAC and NanH did not cleave sialic acid from fetuin (Fig. 4, Table 1) or transferrin in 72 hours. On the other hand, WVU 1853 cleaved in 24 hours from those glycoproteins all sialic acid moieties detectable by SNA and/or MAA. A strong reaction

with a PNA lectin probe, that binds only to asialofetuin confirmed cleavage of sialic acid from fetuin in consecutive tests (not shown).

***M. synoviae* neuraminidase cleaves sialic acid SA α (2-6)gal moiety of chicken serum**

glycoproteins. This showed that *M. synoviae* can cleave sialic acid from SA α (2-6)gal moiety of various chicken serum proteins. Overnight incubation with WVU 1853 cells ($\sim 10^8$ CFU) reduced the quantity of SA α (2-6)gal moiety detected by SNA on serum glycoproteins with Mw ranging from ~ 60 to ~ 200 kDa (Fig. 4, lane 4). Longer incubation with WVU 1853 resulted with the absence of SA α (2-6)gal moiety (Fig. 4, lane 6). However, intensely sialylated serum glycoproteins, of ~ 170 kDa (presumably IgG) and of ~ 80 kDa, lost all detectable SA α (2-6)gal moiety after 15 hours of incubation with $\sim 10^9$ CFU of WVU 1853 (Fig. 5, lane 2). Strains lacking NEAC, like ULB 925/KF9, did not cause desialylation of serum glycoproteins (Fig. 4, lane 5).

Other *M. synoviae* strains expressing NanH and NEAC, including F10 2AS, ULB 07/P5 and ULB 9122 (culture C1), also cleaved SA α (2-6)gal moiety from serum glycoproteins.

To confirm that neuraminidase desialylates chicken serum glycoproteins, the neuraminidase inhibitor DANA was used in assays. DANA obviously inhibited NEAC of WVU 1853 (measured by BIN), but high concentrations of DANA (> 50 μ g of DANA per sample with 10^9 CFU of WVU 1853) were needed to prevent reaction of BIN after incubation of 15 hours. Therefore 100 μ g of DANA was used in assays of desialylation of serum glycoproteins with WVU 1853. As shown in Fig. 5, DANA prevented cleavage of SA α (2-6)gal moiety from serum glycoproteins incubated with WVU 1853 for 40 hours. Complete cleavage of this moiety from glycoproteins of ~ 170 and ~ 80 kDa was observed already after 15-hour incubation with WVU 1853 (Fig. 5, lane 2).

Similar efficiency of desialylation was observed with 0.5 μ g of *C. perfringens* neuraminidase, but DANA (100 μ g/sample) also prevented its neuraminidase activity (Fig. 5, lanes 6 and 7).

***M. synoviae* cleaves SA α (2-6)gal moiety from heavy chain of chicken IgG.** Assays with serum glycoproteins indicated that *M. synoviae* cleaved SA α (2-6)gal moiety from chicken IgG of ~ 170 kDa and IgG purified from chicken serum or egg yolk. *M. synoviae* cultures having NEAC and expressing NanH, were capable of cleaving SA α (2-6)gal moiety from chicken IgG, whereas those without NEAC and NanH were not. This moiety is in the N-glycosylation site (N 308) of the CH2 domain of heavy chain of chicken IgG (Suzuki & Lee, 2004). As shown in Fig. 6, after reduction of IgG (~ 170 kDa) SNA probe bound to ~ 70-kDa heavy chain. Incubation of IgG with neuraminidase of *C. perfringens* or *M. synoviae* strains with NEAC caused cleavage of SA α (2-6)gal moiety from heavy chain (Fig. 6A). Consecutive reaction with mAb 1F5 to heavy chain of chicken IgG revealed the position of desialylated heavy chain (Fig. 6B, lanes 4 and 6). *M. synoviae* cultures lacking NEAC and NanH (ULB 925/KF9 and ULB 9122/C2) did not cleave SA α (2-6)gal moiety from cIgG heavy chain.

***M. synoviae* cleaves sialic acid from SA α (2-3)gal moiety of mucus glycoproteins.** Tests performed with tracheal mucus samples from different chickens showed considerable differences concerning molecular weight of glycoproteins containing sialic acid and level of their sialylation. However, they contained SA α (2-3)gal moieties detected by MAA, rather than SA α (2-6)gal moiety being predominant in serum glycoproteins. WVU 1853 was able to cleave SA α (2-3)gal moiety of ~ 40 kDa mucus glycoprotein and to reduce this moiety of ~ 80- and 110-kDa glycoproteins after incubation of 15 hours. Several other strains also cleaved sialic acid from mucus glycoproteins, including ULB 9122 (culture C1), whereas those

without NEAC and NanH expression did not (Table 1). Fig. 7 A, B shows an example of a test examining cleavage of SA α (2-3)gal moiety from mucus glycoproteins. ULB 925 KF9 lacking NEAC did not cleave this moiety, whereas WVU 1853 visibly reduced SA α (2-3)gal moiety on ~ 80 kDa glycoprotein. Consecutive probing with the lectin DSA probe revealed that WVU 1853 cleaved from ~ 80-kDa glycoprotein sialic acid, but not galactose β (1-4)-N-acetylglucosamine which bound DSA probe (Fig. 7B).

Discussion

NEAC is associated with virulence of *M. gallisepticum* and *M. synoviae* and varies among their strains, as well as among cultures of individual strains (May *et al.*, 2007; May & Brown, 2009a; Berčič *et al.*, 2008b). Among *M. synoviae* strains compared, WVU 1853 had the highest level of NEAC (May *et al.*, 2007; Berčič *et al.*, 2008b). It seems that NEAC is dependent on the NanH neuraminidase expression, at least in strains AAY-4, ULB 925 and ULB 9122 (cultures C2 and C3) which were without NEAC and NanH expression due to deletions in *nanH* gene and premature termination of translation into functional NanH polypeptide.

In *Mycoplasma* species slipped-strand mispairing during chromosomal replication frequently causes insertions or deletions in short polyA tracts and affects gene expression at the level of transcription initiation (mutations in promoters) or at translation due to frameshift and premature termination of translation (Citti *et al.*, 2005). We found deletions in polyadenosine tracts in two different regions of the *nanH* gene (Fig. 3 A,B). In both cases, strains with such deletions did not express NanH (Table 1 and Fig. 2). A similar event has been observed in *Mycoplasma fermentans*. A deletion in a polyA tract causing frameshift

which introduces stop codon TAA in the gene encoding ABC transporter prevented its expression and even a truncated form of this protein was absent (Theiss & Wise, 1997). However, *M. synoviae* strains lacking NanH expressed GAPDH (one copy of its gene is adjacent to *nanH*) like WVU 1853 and other *M. synoviae* strains (Berčič *et al.*, unpublished data). Interestingly, ULB 925, its clones and their subclones that all lacked NEAC, had either HA⁺ or HA⁻ phenotype like clone KF6 (Benčina *et al.*, 1999) showing that there was no association of NEAC with ability to agglutinate chicken erythrocytes (Fig. 1).

Although AAY-4 lacks NEAC, its culture, with the HA⁺ phenotype, induced acute synovitis in chickens (Narat *et al.*, 1998). It replicated in hock joints and was recovered as an isolate ULB 925 which cultures lack NEAC, as well (Benčina *et al.*, 1999; Berčič *et al.*, 2008b). On the other hand, AAY-4 did not colonize tracheas of chicken when it was inoculated into their upper respiratory tract (Benčina, unpublished data). This may be due to its incapability to desialylate glycoproteins present in the respiratory mucus, which represents antiadhesive barrier, particularly for pathogens like *M. synoviae* and *M. gallisepticum* that target and bind to sialic acid moieties of receptors on epithelial cells. This is consistent with a recent report that inactivation of the neuraminidase (i.e. sialidase) gene abolished in *M. gallisepticum* strain R_{LOW} its NEAC (sialidase activity) and its ability to colonize tracheas of chickens (May *et al.*, 2008).

Several lines of evidence indicate that neuraminidase NanH of *M. synoviae* desialylates chicken glycoproteins. *M. synoviae* cultures having NEAC and expressing NanH desialylated glycoproteins, whereas *M. synoviae* cultures lacking NEAC and NanH did not. The neuraminidase inhibitor DANA prevented desialylation of chicken serum glycoproteins with *M. synoviae* WVU 1853 (which caused desialylation in less than 15 hours) for 40 hours. We used relatively high DANA concentrations (up to 100 µg DANA per sample). However, in experiments with the neuraminidase (sialidase) of *Mycoplasma alligatoris* degradation of

substrate (MUAN) was observed after prolonged incubation with as much as 3 mg DANA/ml (Hunt & Brown, 2007). For comparison, sialidase (neuraminidase) activity (enzymatic units per CFU) of *M. synoviae* (WVU 1853) is 100-fold stronger as compared to *M. alligatoris* (May & Brown, 2009b).

M. synoviae and *M. gallisepticum* bind to sialic acid-receptors of hosts cells and must overcome various obstacles, including mucins displaying sialylated glycoproteins, for efficient colonization of host's mucosa. Desialylation of host proteins by the NanA neuraminidase of *Streptococcus pneumoniae* is important for its survival in humans (King *et al.*, 2004). Such events may be relevant to survival and persistence of *M. synoviae* in chickens. We postulate that desialylation of hosts glycoproteins by *M. synoviae* enables colonization of the respiratory tract, as well as its spreading to other tissues and organs. This is consistent with observations that strains of *M. synoviae* and *M. gallisepticum* with high levels of NEAC were more invasive than their strains with weak NEAC (Berčič *et al.*, 2008b; May *et al.*, 2007; Dušanić *et al.*, 2009). *M. synoviae* also is capable of cleaving sialic acid $\alpha(2-6)$ linked to galactose from various chicken serum glycoproteins, including from HC of IgG. This may contribute to its survival in the bloodstream and consequently, to its spreading to various organs including joints. Indeed, WVU 1853 having the strongest NEAC among *M. synoviae* strains examined (May *et al.*, 2007; Berčič *et al.*, 2008b and this study) was recovered from the blood of chickens up to day 54 after infection (Kerr & Olson, 1970). Notably, WVU 1853 showed *in vitro* the highest relative invasion frequency of chicken erythrocytes (Dušanić *et al.*, 2009).

In conclusion, NEAC of *M. synoviae* depends on the expression of the NanH neuraminidase. Mutations in the *nanH* gene causing frameshifts and premature termination of translation eliminate NanH expression and NEAC. *M. synoviae* strains are capable of cleaving sialic acid $\alpha(2-6)$ linked to galactose from various chicken serum glycoproteins, including IgG

heavy chain (CH₂ domains). *M. synoviae* neuraminidase also can cleave sialic acid $\alpha(2-3)$ linked to galactose from glycoproteins of chicken tracheal mucus. Such cleavage could be a factor facilitating colonization of the respiratory tract, as well as spreading of *M. synoviae* to other host organs.

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Legends to Figures:

Fig. 1. Schematic presentation of *M. synoviae* cultures, clones and subclones derived from strain AAY-4 (the left side) or ULB 9122 (the right side). HA denotes haemagglutination; HA⁺ - positive; HA⁻ - negative; NEAC - neuraminidase enzymatic activity; NEAC⁺ - positive; NEAC⁻ - negative.

Fig. 2 A,B: Immunoblot analysis of the NanH neuraminidase and GAPDH expression in *M. synoviae* cultures. They are in lanes: 1, WVU 1853 Aa (NEAC⁺); 2, ULB 9122 C2 (NEAC⁻); 3, WVU 1853 (NEAC⁺); 4, ULB 925 KF 9/4 (NEAC⁻); 5, ULB 925 KF 9/3 (NEAC⁻) and 6, F10 2AS (NEAC⁺). Panel A, Reaction with antibodies to NanH (2µg/ml) showed the position of ~ 110 kDa NanH in cultures with NEAC, but not in cultures lacking NEAC. Panel B, Reaction with antibodies to GAPDH (mouse antiserum diluted 1:500) with strains shown in Panel A (analyzed in parallel gel) revealed abundant GAPDH also in strains without NanH expression. Prestained proteins of the molecular weight marker (Fermentas, Lithuania) are at the right side.

Fig. 3: Mutations in the *nanH* gene associated with the absence of NEAC and NanH protein in cultures of two *M. synoviae* strains. Two regions in which deletions were found are shown on schematically presented NanH. Neuraminidase characteristic motifs, WRIP and so called aspartic acid boxes (Asp) are shown. Region A, deletion of one adenine in the *nanH* gene of ULB 925 (codon 510) causes frameshift and introduces stop codon (TAA), six bases downstream. The same *nanH* mutation was found in its putative ancestral strain AAY 4, as well as in ULB 925 clone KF and subclones KF 9 and KF 6. A truncated NanH protein would

have 512 instead of 938 residues. Region B, in comparison to ULB 9122 culture C1 (upper sequence) in two cultures that lost NEAC, (cultures C2 and C3) a deletion of one adenine (reduction from 6 to 5 adenosines) causes frameshift and introduces downstream a stop codon TAA. Theoretically, their truncated NanH would contain only 832 residues.

Fig. 4: *M. synoviae* cleaves sialic acid from SA α (2-6)gal linkage of glycoproteins. SA α (2-6)gal moiety was detected by SNA. Glycoproteins were resolved in PhastGel Gradient 8-25. Samples of fetuin were incubated overnight with PBS (lane 1), with *M. synoviae* ULB 925 KF9 lacking NEAC (lane 2) or with WVU 1853 with strong NEAC (lane 3). Samples of a chicken serum (final dilution 1:80) were incubated overnight with WVU 1853 (lane 4) or for 24 hours with ULB 925 KF9 (lane 5), WVU 1853 (lane 6) or PBS (lane 7). Prestained proteins weight markers (*M_w*) are indicated in kDa. Note, WVU 1853 reduced the amount of SA α (2-6)gal moiety after overnight incubation (~12 hours) and caused complete desialylation of serum glycoproteins after 24-hour incubation. In contrast, ULB 925 KF lacking NEAC did not cause notable desialylation.

Fig. 5: Neuraminidase inhibitor DANA inhibited desialylation of SA α (2-6)gal moiety of chicken serum glycoproteins by *M. synoviae* and *C. perfringens*. Glycoproteins were separated in PhastGel Gradient 8-25, transferred to Immobilon P and probed by SNA. Chicken serum (diluted 1:60) was incubated with WVU 1853 cells (10^9 CFU) or neuraminidase of *C. perfringens* (0.5 μ g/30 μ l sample). DANA (100 μ g/sample) was added to samples before incubation (at 37 – 38 °C) started. Samples are in lanes: 1, serum alone; 2, serum incubated 15 hours with WVU 1853; 3, serum incubated 15 hours with WVU 1853 plus DANA; 4, serum incubated 40 hours with WVU 1853; 5, serum incubated 40 hours with WVU 1853 plus DANA; 6, serum incubated 15 hours with neuraminidase of *C. perfringens* and 7,

serum incubated 15 hours with neuraminidase of C. perfringens plus DANA. Lane Mw, molecular weight marker. Note that DANA prevented desialylation of glycoproteins recognized by SNA. Consecutive immunoblot with antibodies to cIgG indicated that desialylated glycoprotein of ~ 170 kDa had the same Mw as cIgG (Fig. not shown).

Fig. 6 A,B: Neuraminidase of *M. synoviae* and *C. perfringens* cleave SA α (2-3)gal moiety from heavy chain (HC) of chicken IgG. *Samples (10 μ l) of cIgG (0.1 mg/ml) purified from egg yolks and electroblotted to Immobilon P were probed by SNA. All cIgG samples, except those in lanes 1 and 3, were reduced with 5% β -ME. Panel A, Reaction with SNA. Samples of cIgG are in lanes: 1 and 2, with PBS alone (in lane 1 nonreduced); 3 and 4, incubated 12 hours with 5 μ g neuraminidase of *C. perfringens* (in lane 3 nonreduced); 5, incubated 36 hours with ULB 925/KF9 (10^9 CFU); 6, incubated 36 hours with WVU 1853 (10^9 CFU); 7, incubated 36 hours with ULB 9122 C2 (10^9 CFU); 8, incubated 36 hours with ULB 9122 C1 (10^9 CFU) and lane 9, reference fetuin (0.02 mg/ml). Note that reduction of cIgG generated HC of ~ 70 kDa and that neuraminidases of *C. perfringens*, WVU 1853 and ULB 9122/C1 desialylated HC whereas cultures lacking NEAC and NanH, ULB 925/KF9 and ULB 9122/C2, did not. Panel B, in consecutive assay a part of the same membrane (see Panel A) was incubated with mAb 1F5 to HC of cIgG (Narat et al., 2004), HRP conjugate and TrueBlue to show the position of HC. Note that HC desialylated by *C. perfringens* and WVU 1853 (lanes 4 and 6) reacted by mAb 1F5.*

Fig. 7: *M. synoviae* neuraminidase cleaves sialic acid from SA α (2-3)gal moiety of chicken tracheal mucus glycoproteins. *SA α (2-3)gal moiety was detected by MAA. Panel A, WVU 1853 (10^9 CFU) with NEAC visibly reduced SA α (2-3)gal moiety of ~ 80-kDa glycoprotein after overnight incubation (lane 2) whereas ULB 925 KF9 (10^9 CFU) lacking NEAC did not*

(lane 3). Control (in PBS) was a reference fetuin (lane 1). Panel B, consecutive reaction with DSA (which binds to galactose linked to N-acetylglucosamine moiety) demonstrates that WVU 1853 cleaved from ~ 80-kDa glycoprotein only sialic acid, but not galactose. Prestained proteins of the molecular weight marker and their Mw (in kDa) are shown at the right side of each panel.

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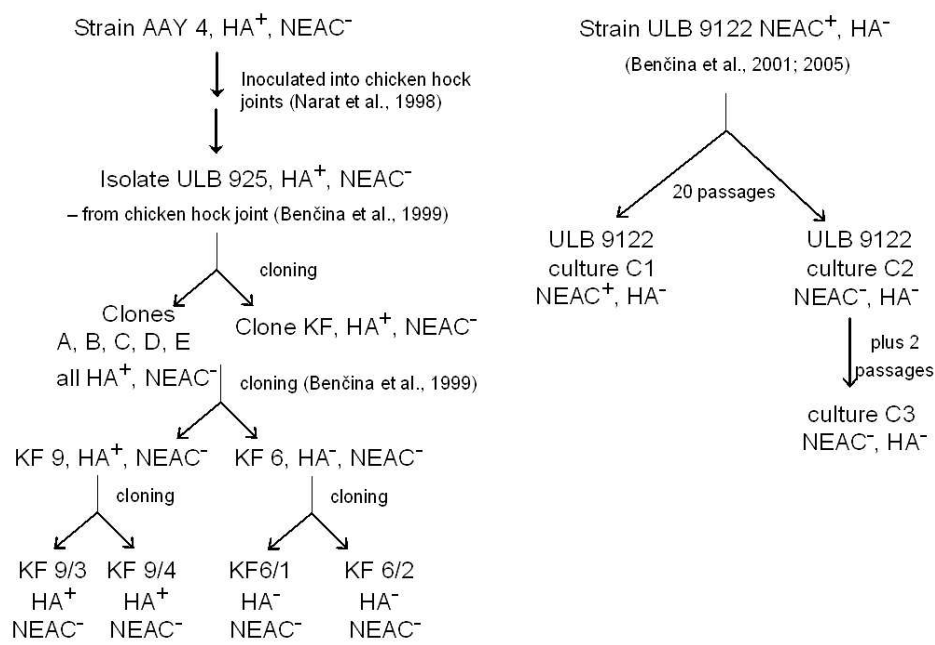


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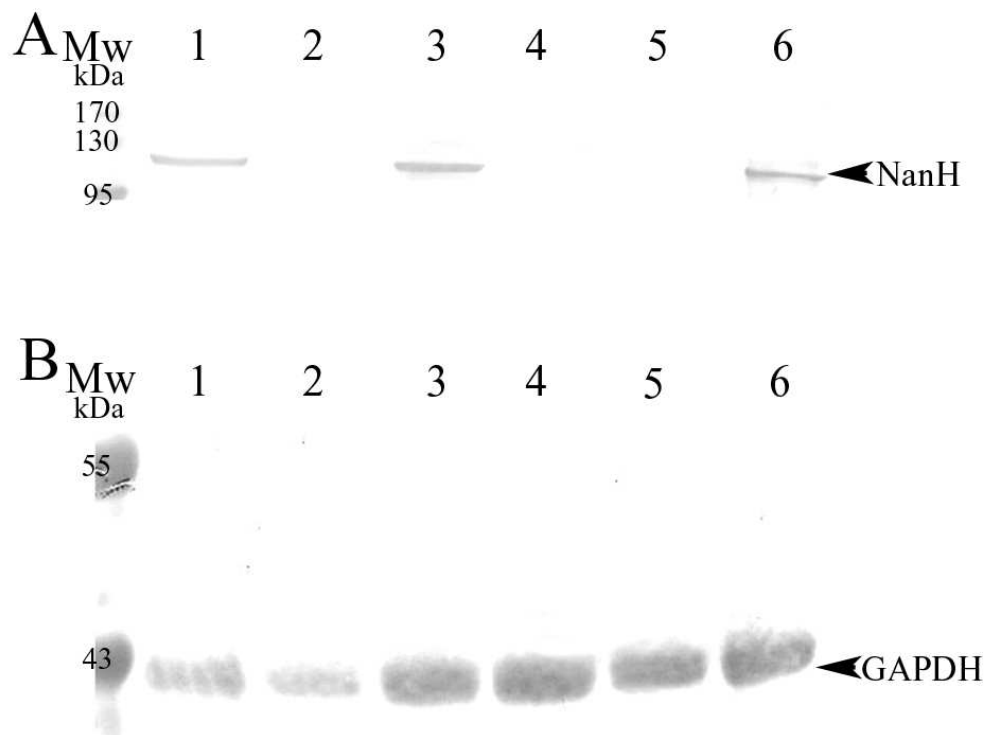


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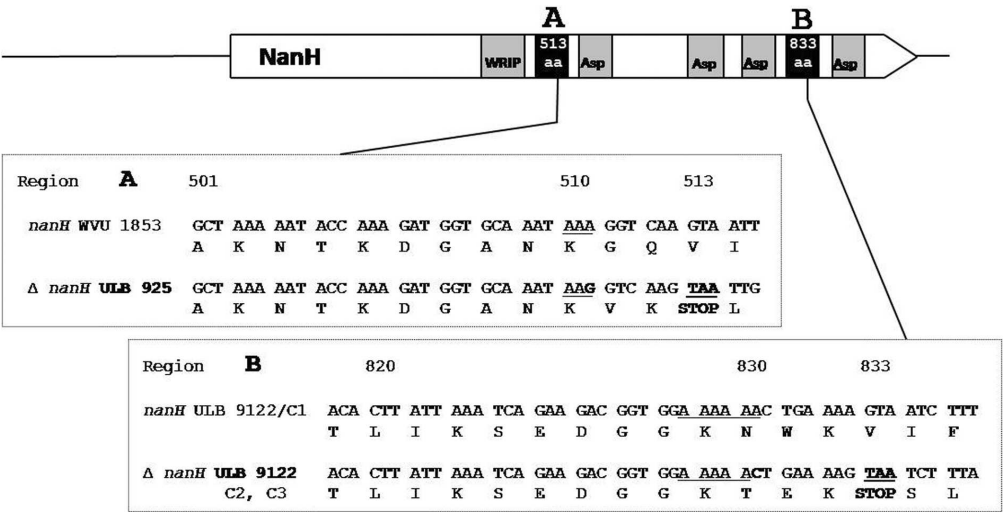


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160x84mm (300 x 300 DPI)

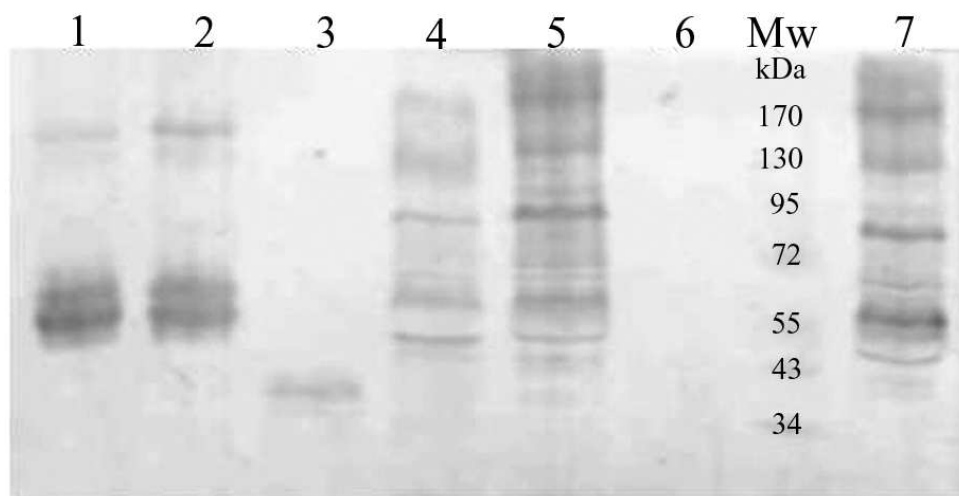


Fig. 4: *M. synoviae* cleaves sialic acid from SA α (2-6)gal linkage of glycoproteins. SA α (2-6)gal moiety was detected by SNA. Glycoproteins were resolved in PhastGel Gradient 8-25. Samples of fetuin were incubated overnight with PBS (lane 1), with *M. synoviae* ULB 925 KF9 lacking NEAC (lane 2) or with WVU 1853 with strong NEAC (lane 3). Samples of a chicken serum (final dilution 1:80) were incubated overnight with WVU 1853 (lane 4) or for 24 hours with ULB 925 KF9 (lane 5), WVU 1853 (lane 6) or PBS (lane 7). Prestained proteins weight markers (Mw) are indicated in kDa. Note, WVU 1853 reduced the amount of SA α (2-6)gal moiety after overnight incubation (~12 hours) and caused complete desialylation of serum glycoproteins after 24-hour incubation. In contrast, ULB 925 KF lacking NEAC did not cause notable desialylation.
80x40mm (300 x 300 DPI)

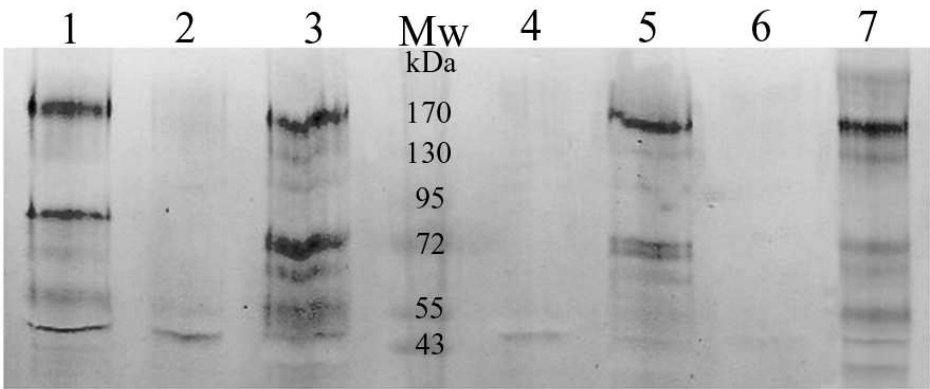


Fig. 5: Neuraminidase inhibitor DANA inhibited desialylation of SAα(2-6)gal moiety of chicken serum glycoproteins by *M. synoviae* and *C. perfringens*. Glycoproteins were separated in PhastGel Gradient 8-25, transferred to Immobilon P and probed by SNA. Chicken serum (diluted 1:60) was incubated with WVU 1853 cells (10^9 CFU) or neuraminidase of *C. perfringens* (0.5 µg/30 µl sample). DANA (100 µg/sample) was added to samples before incubation (at 37 – 38 °C) started. Samples are in lanes: 1, serum alone; 2, serum incubated 15 hours with WVU 1853; 3, serum incubated 15 hours with WVU 1853 plus DANA; 4, serum incubated 40 hours with WVU 1853; 5, serum incubated 40 hours with WVU 1853 plus DANA; 6, serum incubated 15 hours with neuraminidase of *C. perfringens* and 7, serum incubated 15 hours with neuraminidase of *C. perfringens* plus DANA. Lane Mw, molecular weight marker. Note that DANA prevented desialylation of glycoproteins recognized by SNA. Consecutive immunoblot with antibodies to cIgG indicted that desialylated glycoprotein of ~ 170 kDa had the same Mw as cIgG (Fig. not shown).

80x33mm (300 x 300 DPI)

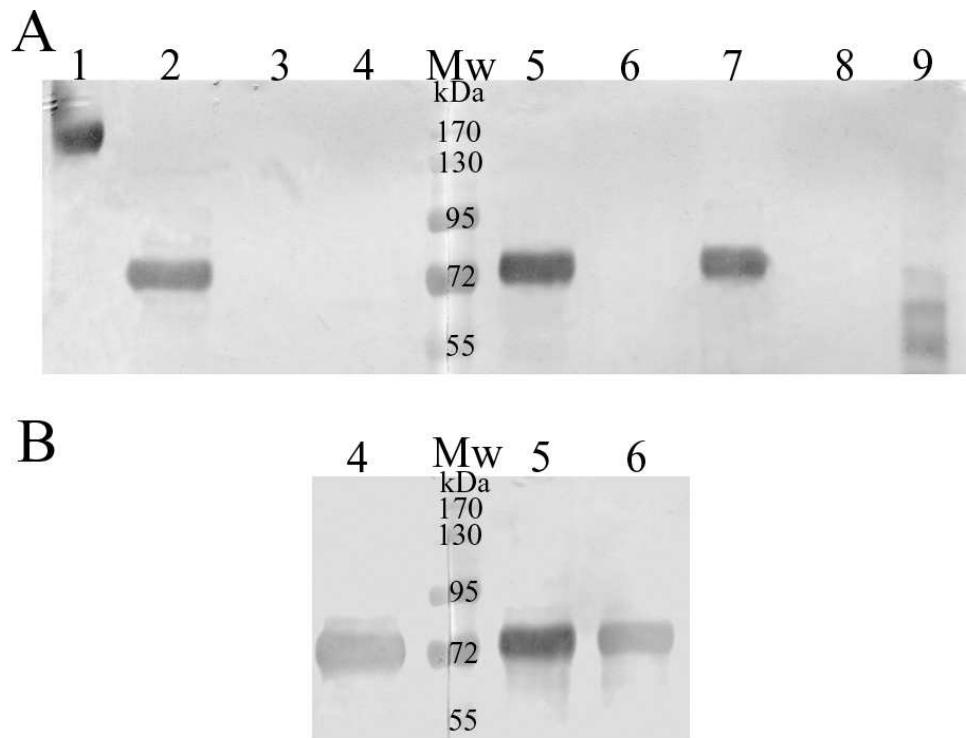


Fig. 6 A,B: Neuraminidase of *M. synoviae* and *C. perfringens* cleave SA α (2-3)gal moiety from heavy chain (HC) of chicken IgG. Samples (10 μ l) of cIgG (0.1 mg/ml) purified from egg yolks and electroblotted to Immobilon P were probed by SNA. All cIgG samples, except those in lanes 1 and 3, were reduced with 5% β -ME. Panel A, Reaction with SNA. Samples of cIgG are in lanes: 1 and 2, with PBS alone (in lane 1 nonreduced); 3 and 4, incubated 12 hours with 5 μ g neuraminidase of *C. perfringens* (in lane 3 nonreduced); 5, incubated 36 hours with ULB 925/KF9 (10^9 CFU); 6, incubated 36 hours with WVU 1853 (10^9 CFU); 7, incubated 36 hours with ULB 9122 C2 (10^9 CFU); 8, incubated 36 hours with ULB 9122 C1 (10^9 CFU) and lane 9, reference fetuin (0.02 mg/ml). Note that reduction of cIgG generated HC of \sim 70 kDa and that neuraminidases of *C. perfringens*, WVU 1853 and ULB 9122/C1 desialylated HC whereas cultures lacking NEAC and NanH, ULB 925/KF9 and ULB 9122/C2, did not. Panel B, in consecutive assay a part of the same membrane (see Panel A) was incubated with mAb 1F5 to HC of cIgG (Narat et al., 2004), HRP conjugate and TrueBlue to show the position of HC. Note that HC desialylated by *C. perfringens* and WVU 1853 (lanes 4 and 6) reacted by mAb 1F5.

80x62mm (300 x 300 DPI)

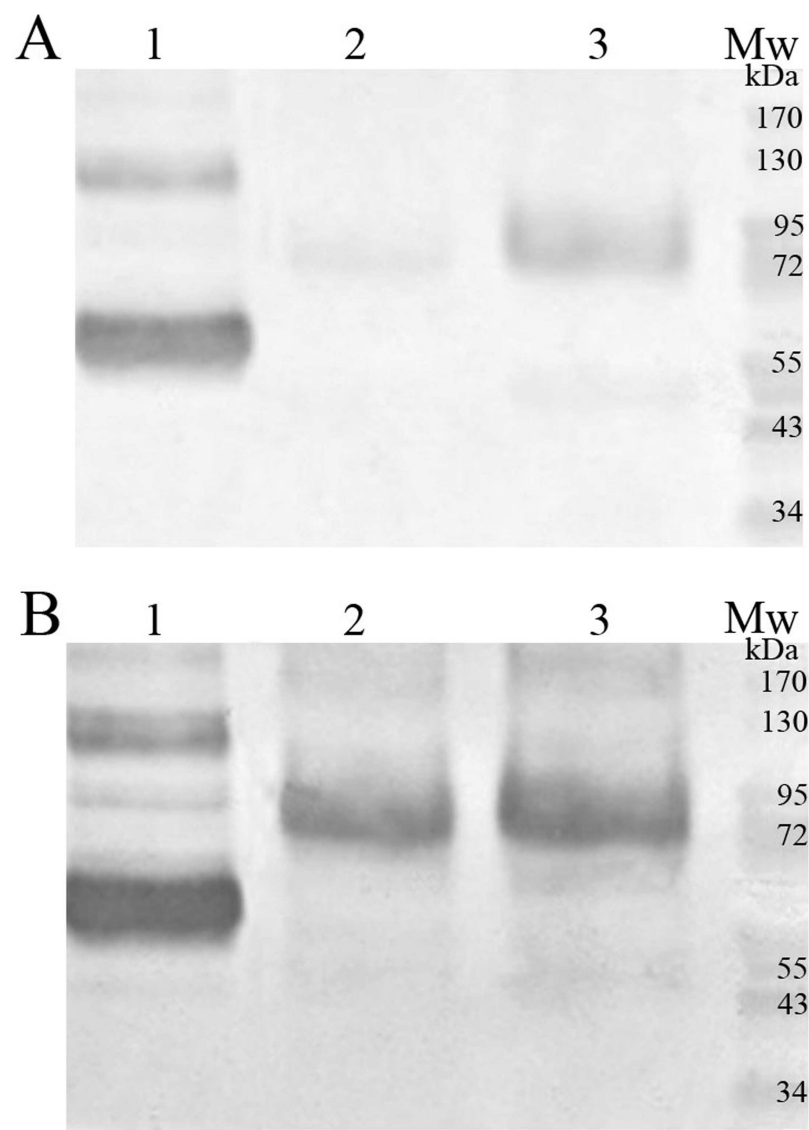


Fig. 7: *M. synoviae* neuraminidase cleaves sialic acid from SA α (2-3)gal moiety of chicken tracheal mucus glycoproteins. SA α (2-3)gal moiety was detected by MAA. Panel A, WVU 1853 (10^9 CFU) with NEAC visibly reduced SA α (2-3)gal moiety of \sim 80-kDa glycoprotein after overnight incubation (lane 2) whereas ULB 925 KF9 (10^9 CFU) lacking NEAC did not (lane 3). Control (in PBS) was a reference fetuin (lane 1). Panel B, consecutive reaction with DSA (which binds to galactose linked to N-acetylglucosamine moiety) demonstrates that WVU 1853 cleaved from \sim 80-kDa glycoprotein only sialic acid, but not galactose. Prestained proteins of the molecular weight marker and their Mw (in kDa) are shown at the right side of each panel.

Table 1. *M. synoviae* strains, cultures, NEAC, NanH synthesis, ability to desialylate chicken glycoproteins and nanH sequences

Strain	Culture	NEAC	Synthesis of NanH	Desialylation of ^a				<i>nanH</i> sequence ^b	Reference
				Fet	Tra	cIgG	TM		
WVU 1853 S ^c	KD2	+	+	+	+	+	+	EU 245026	Berčič et al., 2008b
F10 2AS	C1	+	+	+	ND	+	ND	(EU245026)	Benčina et al., 2001
K1968	ZC3	+	+	ND	ND	+	ND	EU 532615 ^d	Benčina et al., 2001
K 4463B	C5	+	ND	+	ND	+	ND	EU245028 ^d	Benčina et al., 2001
K 1723	C23	+	+	ND	+	+	ND	not deposited	Benčina et al., 2001
K 2581	C2	+	ND	ND	ND	+	ND	EU 245025	Benčina et al., 2001
K 2426D	AA3	+	+	+	ND	+	ND	EU 015400 ^d	Benčina et al., 2001
ULB 02/P4	K4	+	+	+	ND	+	+	EU 245029 ^d	Lavrič et al., 2007
ULB 02/T6	KA1	+	+	ND	+	+	ND	(EU 245029)	Berčič et al., 2008a
ULB 02/OV6	C4	+	+	+	+	+	ND	(EU 245029)	Berčič et al., 2008a
ULB 07/P5	C5	+	+	+	+	+	+	EU 532616 ^d	This study
ULB 9122	C1	+	+	+	+	+	+	not deposited ^c	Benčina et al., 2005
ULB 9122	C2 and C3	-	-	-	-	-	-	EU 245027	Berčič et al., 2008b
AAY 4	K 1/3	-	-	-	ND	ND	ND	(EU 532617)	Narat et al., 1998
ULB 925	KF	-	-	-	-	-	-	EU 532617	Benčina et al., 1999; 2001
ULB 925	KF6	-	-	-	-	-	ND	(EU 532617)	Benčina et al., 1999
ULB 925	KF9	-	-	-	-	-	-	(EU 532617)	Benčina et al., 1999

^a Fet/Tra, reference fetuin and transferrin; cIgG, chicken IgG; TM, glycoproteins of mucus from chicken tracheas; + denotes positive; - denotes negative; ND, not determined.

^b Accession numbers of sequences in GenBank; sequences in parentheses are identical to those with the same designation but were not deposited.

^c Except deletion show in Fig. 2 identical to EU 245027.

^d These sequences were included in comparison in a study reporting diversifying selection of sialidase (neuraminidase) in *M. synoviae* (May & Brown, 2009a – Suppl. Table 1).

^e It was donated by S.H. Kleven. Another culture of the type strain, designated WVU 1853 Aa, had the *nanH* sequence identical to EU245026.

Table 2: *Oligonucleotide primer pairs and their annealing temperatures used in polymerase chain reaction (PCR).*

Primer pairs	Forward (F)/sequence 5'- 3'	Reverse (R)/sequence 5'- 3'	Annealing T
NEUI 1	TGCGTCTTCATGGAGTAGATG	CCGAGCTTGACGAAGCTAAT	57°C
NEUI 2	AGGCTCACCGAAAGTGCTAA	GGAGCTGTAGTTACGCTAAGCAA	57°C
NEUI 3	CATCAACATCACTGTATAAACCTGAA	AACCTTCCAGAGTTTGTTAATGC	57°C
NEU	CTGAAGCTTGAACATAAACTCA	AAATTTGAAACCGATGAAATAG	50°C
GAP 3	CCGTCTTTTGATTCAACAAAGT	GAAACAAGCCTTACAGACAAAG	54°C

The neuraminidase gene was amplified using PCR primers designed to anneal in the flanking *nanA* (NEUI 1 F) and *gapA* (NEU R, GAP 3 F/R) genes as well as inside the neuraminidase gene (NEUI 1 R, NEUI 2 F/R, NEUI 3 F/R, NEU F) open reading frame of *Mycoplasma synoviae* strain 53 (NC 007294).