Mycoplasma gallisepticum and *Mycoplasma synoviae* express a cysteine protease CysP, which can cleave chicken IgG into Fab and Fc

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Major poultry pathogens *M. gallisepticum* and *M. synoviae* share a gene encoding a putative cysteine protease CysP similar to papain cysteine protease (C1A subfamily). Comparison of the *cysP* gene sequences of 18 *M. synoviae* and 10 *M. gallisepticum* strains sequenced in this study showed polymorphisms, including deletions. Seven *M. synoviae* strains, including the type strain WVU 1853, had a 39 bp deletion in the 3' end of the *cysP* gene. In the same *cysP* region, all *M. gallisepticum* strains showed a deletion of 66 bp. Immunoblot analysis with specific antibodies demonstrated that *M. synoviae* strains expressed CysP, which was approximately 65 kDa. Both *M. synoviae* and *M. gallisepticum* were able to digest chicken IgG (clgG). Incubation of clgG (~170 kDa) with *M. synoviae* or *M. gallisepticum* cells (~15 h at 37 °C) resulted in a papain-like cleavage pattern of clgG and fragments corresponding to the antigen-binding fragment of IgG (Fab, ~45 kDa) and the crystallizable region fragment (Fc) of the IgG heavy chain (dimer of ~60 kDa). Iodoacetamide (50 mM) prevented cleavage of clgG by both *Mycoplasma* species. Following site-directed mutagenesis (eight TGA codons were changed to TGG) the *cysP* gene of *M. synoviae* ULB 925 was expressed as a His-tagged protein in a cell-free system. Purified recombinant CysP (rCysP; ~67 kDa, pI~8) cleaved clgG into Fab and Fc fragments. This indicates that CysP is responsible for the clgG cleavage caused by *M. synoviae* and, probably, by *M. gallisepticum*. This is the first evidence to our knowledge that mycoplasmas have enzymes that can cleave the host IgG and indicates a novel strategy used by *M. gallisepticum* and *M. synoviae* for prolonged survival despite the antibody response of their host.

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INTRODUCTION

Major poultry pathogens *Mycoplasma gallisepticum* and *Mycoplasma synoviae* share hosts, tissue tropism, transmission pathways and immunogenic proteins, including haemagglutinin (Noormohammadi *et al.*, 1998; Benčina, 2002; Berčič *et al.*, 2008a). *M. gallisepticum* and *M. synoviae* belong to different phylogenetic groups, but comparison of

their genomes identified 14 regions that contain a number of horizontally transferred genes (Papazisi *et al.*, 2003; Vasconcelos *et al.*, 2005). Some of these genes are expressed and encode proteins (haemagglutinin VlhA and neuraminidase NanH) that play important roles in interactions with host cells, as well as in their adaptation to avian hosts (Noormohammadi *et al.*, 2000; Berčič *et al.*, 2008a, b; Sirand-Pugnet *et al.*, 2007).

M. gallisepticum and *M. synoviae* also share a gene for a putative cysteine protease of the papain CA1 subfamily (Vasconcelos *et al.*, 2005; Szczepanek *et al.*, 2010). *M. gallisepticum* gene MGA_1153 shares ~94% sequence identity with the *M. synoviae* gene (MS53_0590) considered to encode cysteine protease (Staats *et al.*, 2007). Recently, it has been reported that *M. gallisepticum* strain S6 expressed a gene homologous to MGA_1153 and the expressed protein was reported as a protease (Demina *et al.*, 2009). Whereas most mycoplasma genomes lack genes

Abbreviations: clgG, chicken IgG; Fab, antigen-binding fragment of IgG; Fc, crystallizable region fragment of IgG; HC, heavy chain of IgG; HRP, horseradish peroxidase; IIPA, indirect immunoperoxidase assay; IIF, indirect immunofluorescence; LC, light chain of IgG; rCysP, recombinant CysP; SNA, *Sambucus nigra* agglutinin.

The GenBank/EMBL/DDBJ accession numbers for the *cysP* gene sequences of *M. gallisepticum* and *M. synoviae* strains isolated in this study are listed in Table 1.

Three supplementary figures and one supplementary table are available with the online version of this paper.

encoding cysteine proteases (Staats *et al.*, 2007), recently they have been annotated in the genomes of *M. arthritidis*, *M. mycoides* subs. *capri* and *M. capricolum* (GenBank accession no. CP000123) (Dybvig *et al.*, 2008; Lartigue *et al.*, 2009).

The possibility that mycoplasmas pathogenicity might be linked to their enzymes, particularly proteases, has been postulated long ago (Gabridge, 1984). It has been reported that *Mycoplasma arthritidis* and *Mycoplasma capricolum* had gelatinolytic enzymes (Czekalowski *et al.*, 1973; Voros *et al.*, 2009), but their roles in pathogenic processes are not clear. Bacterial proteases may play important roles in pathogenicity and nutrition. Among them are cysteine proteases: exotoxin B (SpeB) and streptolysin from *Streptococcus pyogenes*, interpain A from *Prevotella intermedia*, gingipains from *Porphyromonas gingivalis* and staphopains from *Staphylococcus aureus* (Kapur *et al.*, 1993; Potempa *et al.*, 2009; Curtis *et al.*, 2001; Shaw *et al.*, 2004).

In addition, several pathogenic bacteria synthesize proteases that can cleave the host's immunoglobulins IgA and IgG. Cysteine proteases SpeB and IdeS of *S. pyogenes* cleave human IgG (Collin & Olsen, 2001). Proteases from *Avibacterium paragallinarium*, *Gallibacterium anatis* and *Pasteurella multocida* that have been reported to digest chicken IgG (cIgG) were not cysteine proteases (Rivero-García *et al.*, 2005; García-Gómez *et al.*, 2005; Negrete-Abascal *et al.*, 1999).

M. gallisepticum and *M. synoviae* have proteins that can bind cIgG 'non-immunologically' via the crystallizable region fragment of IgG (Fc) (Lauerman *et al.*, 1993) but to date and to our knowledge there is no evidence that any *Mycoplasma* species can cleave or degrade host immunoglobulins.

In the present study, we demonstrate that *M. gallisepticum* and *M. synoviae* synthesize homologous cysteine proteases, termed hereafter CysP. Their *cysP* genes have a considerable intraspecies variation, particularly in *M. synoviae*. We also provide the first evidence that both *M. gallisepticum* and *M. synoviae* cleave cIgG into the antigen-binding fragment of IgG (Fab) and Fc fragments. Assays with the purified recombinant CysP (rCysP) showed that it cleaves cIgG indicating that CysP is involved in such a cleavage.

METHODS

***M. synoviae* and *M. gallisepticum* strains and their cultures.** Strains used in this study are presented in Table 1. Strains were grown at 37–38 °C in modified Frey's broth containing 12 % porcine serum, 0.1 g NAD I⁻¹ and 0.1 g cysteine hydrochloride I⁻¹ (Dušanić *et al.*, 2009). Mycoplasma cells were harvested by centrifugation during mid-exponential phase and washed by PBS, pH 7.4. The number of c.f.u. ml⁻¹ was determined as described elsewhere (Rodwell & Whitcomb, 1983).

Antibodies to *M. synoviae* cysteine protease CysP and neuraminidase NanH. Antibodies to CysP and NanH were raised

in chickens (GenWay). Two synthetic peptides representing highly immunogenic regions of CysP (102DIDEHAIDYVSNS) or of neuraminidase NanH (464DKRVNNKDDRGHLEQD) were coupled to keyhole limpet haemocyanin and used as antigens. Two chickens were immunized with CysP or NanH peptide and IgG was isolated from their egg yolks. In addition, affinity-purified cIgG was isolated using immobilized CysP or NanH peptide as specific ligands (isolated by GenWay). These cIgG antibodies were used to study the expression and localization of CysP and NanH. They were received (1 mg ml⁻¹) together with relevant pre-immune cIgG, CysP and NanH peptides.

Monoclonal antibodies (mAbs) to cIgG heavy chain (HC) and light chain (LC). mAbs 1F5 and 4E4 to HC of cIgG have already been used in our previous studies of avian IgG (Narat *et al.*, 2004; Benčina *et al.*, 2005). mAbs CH31 (C7910, Sigma) detecting LC of chicken, turkey and pheasant IgG (Narat *et al.*, 2004) were used to detect the cIgG fragment(s) containing LC, including putative Fab.

Cleavage of cIgG by *M. synoviae* and *M. gallisepticum* or by rCysP. Samples with determined concentrations of pure cIgG were purchased (GenWay) or were isolated from the yolks of eggs laid by specific pathogen-free chickens (Narat *et al.*, 2004). The Fc fragment of cIgG (ChromPure Chicken IgY, Fc; 2.2 mg ml⁻¹) was purchased from Jackson ImmunoResearch Laboratories.

In the cleavage assays, samples usually contained 0.1 or 0.2 mg cIgG ml⁻¹ (or 0.05 mg Fc ml⁻¹) diluted in sterile PBS, pH 7.4. In most assays, mycoplasma cells were tested for the ability to cleave cIgG soon (2–8 h) after their harvest by centrifugation. In some assays frozen (–20 °C) samples of pelleted mycoplasma cells were tested. These were thawed at 37 °C, suspended in PBS, transferred to microtubes containing cIgG and tested as for other samples of mycoplasma cells. Usually, 50 or 100 µl samples (final volume) contained 5 or 10 µl cIgG mixed with *M. gallisepticum* or *M. synoviae* cells (~1–5 × 10⁹ c.f.u. per sample). Samples were incubated at 37–38 °C overnight, but usually the incubation time was extended. In several experiments, the dynamics of the cIgG cleavage was assessed at different time points, typically after 15, 24, 48, 72, 96 and, sometimes, 120 h of incubation. Samples were centrifuged (20 000 g for 10 min), supernatants were collected and analysed by SDS-PAGE. Papain (Sigma), which cleaves cIgG into Fab (~45 kDa) and Fc (~54 kDa, dimer), was used as a positive control under the conditions described elsewhere (Suzuki & Lee, 2004). The cleavage by papain was terminated after 4 or 5 h, because longer incubations caused degradation of Fab and Fc into small fragments. The negative control was cIgG with PBS alone. The capability of rCysP to cleave cIgG was investigated in four independent assays. The rCysP:cIgG ratio was 1:50 or 1:100 (molar ratio 1:19 or 1:38). Incubation took place at 37–38 °C overnight (15 h) and for 24, 54, 72, 96 and 120 h. Buffers were PBS, pH 6.0 or 7.2, and in one experiment, 'digestion buffer', containing 50 mM sodium phosphate (pH 7.0), 1 mM EDTA and 10 mM cysteine hydrochloride (Suzuki & Lee, 2004), was used.

Protease inhibitors which act on cysteine proteases or on metalloproteases were included in appropriate cIgG cleavage assays. They were iodoacetamide (final concentration 20–100 mM), EDTA (20 mM) and E64 (22 µM), all from Sigma.

SDS-PAGE, IEF and immunoblots. Most SDS-PAGE analyses were done by using the PhastSystem (Pharmacia) using PhastGel Gradient (8–25) gels and 1 µl samples as described previously (Benčina *et al.*, 1994, 2005). Molecular mass markers were prestained proteins with *M_r* between 10 and 170 kDa (PageRuler no. SM0671, Fermentas). IEF by PhastGel IEF (3–9) gels and immunoblots with antibodies to CysP (diluted 1:500), rabbit antibodies (to cIgG) conjugated with horseradish peroxidase (HRP) (A9046, Sigma) and TrueBlue peroxidase substrate (KPL) were performed as described previously (Milošević

Table 1. *M. synoviae* and *M. gallisepticum* strains, their *cysP* genes, CysP characterization and expression and their cleavage of cIgG

CysP protein expression was proven by specific antibodies using DIBA, immunoblotting and/or IIPA, and was positive for all strains. Cleavage of cIgG was positive for all strains except *M. synoviae* K4463B, K1723, FMT, K1352 and K3009/37, for which this was not determined.

Strain*	<i>cysP</i> GenBank accession no.†	CysP protein			Reference
		Length (aa)	Relative molecular mass (Da)	pI	
<i>M. synoviae</i>					
WVU 1853	EU999136	572	64269.63	8.56	Berčič <i>et al.</i> (2008b)
F10-2AS	(EU999136)	572	64269.63	8.56	Benčina <i>et al.</i> (2001)
ULB 02/T6	EU999139	572	64277.88	9.11	Berčič <i>et al.</i> (2008a)
ULB 02/OV6	(EU999139)	572	64277.88	9.11	Berčič <i>et al.</i> (2008a)
K4463B	EU999135	572	64267.71	8.88	Benčina <i>et al.</i> (2001)
ULB 9122	EU999130	585	65725.27	8.68	Benčina <i>et al.</i> (2005)
ULB 07/P5	EU999138	585	65725.27	8.68	This study
K1968	HM138508	585	65865.50	8.86	Benčina <i>et al.</i> (2001)
K1723	EU999131	585	65690.35	8.78	Benčina <i>et al.</i> (2001)
K2581	EU999129	585	65686.27	8.12	Benčina <i>et al.</i> (2001)
FMT	EU999132	585	65686.27	8.12	Benčina <i>et al.</i> (2001)
K2426D	EU999128	585	65815.32	8.12	Benčina <i>et al.</i> (2001)
K1352	(EU999129)	585	65686.27	8.12	This study
ULB 925	HQ174250	585	65642.27	8.38	Benčina <i>et al.</i> (1999)
K3009/37	EU999133	585	65815.32	8.12	Benčina <i>et al.</i> (2001)
<i>M. gallisepticum</i>					
R _{LOW} (R25)	(FJ799760)	564	63422.82	8.80	Dušanić <i>et al.</i> (2009)
R _{HIGH}	CP001872 (MGAH_1153)‡	564	63422.82	8.80	Szczepanek <i>et al.</i> (2010)
S6	FJ799758	564	63366.83	8.80	Benčina <i>et al.</i> (1994)
F	CP001873 (MGF_3970)‡	564	63251.61	8.57	Szczepanek <i>et al.</i> (2010)
<i>rCysP</i>		593	66711.37	8.38	This study

*Strains with sequenced *cysP* genes that are not presented in Table 1: *M. synoviae* strains PAA2 (*cysP* gene, EU999141), B27/00 (EU999137), K3344 (EU999134) and *M. gallisepticum* strains ts 11 (FJ799761), 1226 (FJ799762), ULB 931 (FJ799759), ULB 02/S2 (HQ174251), ULB 02/S1 (HQ174252), ULB 992 (HQ174253), PG31 (HQ174254).

†GenBank accession nos in parentheses indicate sequences that were not deposited in GenBank because they had an identical sequence to another strain.

‡*cysP* gene sequences for *M. gallisepticum* strains R_{HIGH} and F determined in this study are identical to those submitted by Szczepanek *et al.* (2010).

Berlič *et al.*, 2000). Isoelectric point (pI) measurement was done using the broad pI calibration kit (10 markers, pI interval 3.50–8.65) as recommended by the manufacturer for the PhastGel IEF 3-9 (PhastSystem, Pharmacia LKB Biotechnology). The cleavage of cIgG was analysed in a PhastGel gradient (8-25), by staining proteins with PhastGel Blue R (Coomassie blue) and by transferring cIgG and its fragments to a PVDF membrane (Immobilon-P; Millipore) and immunoblot detection. The membrane was incubated for 1 h with antibodies (to cIgG) conjugated with HRP (A9046, diluted 1:2000). After washing, cIgG and its fragments were stained and developed by using TrueBlue. In other immunoblot analyses, the membrane was incubated for 1 h with mAbs 1F5 and 4E4 (diluted 1:10), or with mAb CH31 (ascites fluid) diluted 1:1000–1:5000. Following incubation with secondary antibodies, goat anti-mouse IgG conjugated with HRP (A4416, Sigma), diaminobenzidine tetrahydrochloride (DAB; Sigma) or TrueBlue were used to develop reactions. In parallel immunoblots, mAb 1F5, HRP-conjugated secondary antibodies and DAB were used to show the position of a cleaved cIgG HC (putative Fc) in the first reaction. Then, mAb CH31, HRP conjugate and TrueBlue were used to show fragments that contained LC. β -Mercaptoethanol (5%, v/v) was added to reduce cIgG and its separation into HC and LC by SDS-PAGE, as well as to reduce cIgG fragments.

Selected samples were analysed by SDS-PAGE performed in larger gels (10% polyacrylamide gel, 8.2 × 5.5 cm) using 5–10 µl samples which were heated (95 °C, 5 min) and processed under reducing or non-reducing conditions. For immunoblot analyses, the resolved proteins were electroblotted to Immobilon-P membranes, blocked for 45 min in PBS with 0.5% (v/v) Tween 20 (PBS-T20) and assayed with appropriate antibodies as described above.

In an attempt to determine the N-terminal sequence of the approximately 60 kDa cIgG fragment (a putative Fc dimer) generated after incubation of cIgG with *M. synoviae* WVU 1853, this fragment was processed as described previously (Berčič *et al.*, 2008a). Sialylation of cIgG and of its cleavage fragments containing sialic acid (2-6)gal moiety was detected by *Sambucus nigra* agglutinin (SNA) lectin (DIG glycan differentiation kit, Roche) as described elsewhere (Dušanić *et al.*, 2009).

Assays to detect surface-exposed CysP. In order to determine the surface-exposed expression of CysP we used intact colonies of *M. synoviae* and *M. gallisepticum* strains, affinity-purified antibodies to CysP and the indirect immunoperoxidase (IIPA) or indirect immunofluorescence (IIF) assay. The methods were described in detail elsewhere (Benčina & Bradbury, 1991, 1992). Briefly, agar

blocks with mycoplasma colonies were treated with cIgG to CysP ($10 \mu\text{g ml}^{-1}$) for 1 h, washed in PBS and then incubated with rabbit IgG (to cIgG) labelled with HRP or FITC (A9046 or F8888, Sigma). Substrates for IIPA were either DAB or TrueBlue.

Immunoblot analyses of CysP expression. Immunoblots were used to determine the expression of CysP in *M. synoviae* and *M. gallisepticum* strains. The membrane was incubated for 1 h with affinity-purified cIgG antibodies to CysP ($2\text{--}8 \mu\text{g ml}^{-1}$) and then for 45 min with rabbit IgG (to cIgG) labelled with HRP (Sigma) used at 1:2000. NanH expression was detected by using specific antibodies (1 h incubation, $8 \mu\text{g ml}^{-1}$), HRP conjugate (A9046, Sigma) and TrueBlue.

PCR, purification of PCR products, nucleotide sequencing and sequence analysis. DNA of *M. synoviae* and *M. gallisepticum* strains was isolated from their pelleted cells and harvested from 20–50 ml broth cultures by using the RTP spin bacteria DNA mini kit (Invitex). PCRs were performed in a GeneAmp System 2720 thermal cycler (Applied Biosystems) in a total volume of 30 μl , consisting of 1 μl mycoplasma DNA, 10 pmol each primer, 20 mM each dNTP, 3 μl 10-fold-concentrated *Taq* DNA polymerase synthesis buffer, 2.4 μl 25 mM MgCl_2 stock solution and 1.5 U *Taq* DNA polymerase (Fermentas). The sequences of nucleotide primers were based on the relevant sequences from *M. synoviae* strain 53 and *M. gallisepticum* strain R (GenBank accession nos AE017245 and NC004829). PCR primers and conditions are described in Supplementary Table S1. PCR products were analyzed by using horizontal agarose gel electrophoresis in 1% (w/v) agarose gels in Tris/Borate/EDTA buffer. Gels were stained for 15 min in 1 μg ethidium bromide ml^{-1} , washed for 15 min in Tris/Borate/EDTA buffer and analysed and photographed under UV light (312 nm).

Purification and sequencing of PCR amplicons was done by MacroGen. For all *cysP* genes, sequences of both DNA strands were determined. The sequence data were analysed using the BLAST algorithm at the NCBI server (<http://www.ncbi.nlm.nih.gov>), compared with data in GenBank and aligned by using CLUSTAL W (Chenna *et al.*, 2003). Nucleotide sequences were translated into amino acid sequences by using the EMBOSS Transeq at the EBI server (<http://www.ebi.ac.uk/>). The sequence similarities and differences were calculated using BioEdit v7.0.7 (Hall, 1999). Complete sequence data reported in this paper were deposited in GenBank (Table 1).

Site-directed PCR-mediated mutagenesis and expression of recombinant rCysP. The *cysP* gene contains eight UGA codons encoding tryptophans. In heterologous expression systems UGA is a stop codon that would prevent the expression of *cysP*. Therefore, eight point mutations were introduced to convert UGA into UGG, using overlap extension PCR as described by Villarroel & Regalado (1997). Briefly, nine parallel PCRs were performed to generate nine overlapping fragments which were joined in further runs using the most 3' and 5' primers. The list of primers is shown in Supplementary Table S2 and the method's strategy is schematically presented in Supplementary Fig. S1 (both available with the online version of this paper). The PCRs (50 μl) contained 1 U *Taq* DNA polymerase and 1 $\times$ buffer in the presence of 200 mM dNTP, 50 pmol each primer (Supplementary Table S2) and 10 ng *M. synoviae* strain ULB 925 genomic DNA in the first run and similar concentrations of isolated DNA fragments in subsequent runs. The PCR program was optimized as follows: 94 °C for 5 min, 45 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 7 min. Amplicons were loaded on a 1% agarose gel and purified by using a gel extraction kit (Qiagen). Overlapping fragments were combined into four groups containing either two or three sequential fragments from the previous reaction to perform the second PCR using the most 5' and 3' primers of each group. Amplification was performed in 35 cycles of 94 °C for 1 min, 60 °C

for 1 min and 72 °C for 90 s. Gel-purified fragments from the second PCR were combined into two groups to perform the third PCR under the same conditions. The procedure was repeated and the final two overlapping fragments were joined using the most 5' and 3' primers allowing the full-length *in vitro* synthesis of the gene encoding the CysP protein. Sequencing of the final PCR product, which has been used as a template for protein expression, was performed by MacroGen.

rCysP was expressed by using the EasyXpress protein synthesis kit (Qiagen) using a PCR product with eight introduced point mutations as a template. The PCR product was amplified to the proper concentration using EasyXpress linear template kit plus (Qiagen) and was added directly to the cell-free expression reaction. The expressed protein had a His tag at the C terminus and was purified by using Dynabeads (Invitrogen). rCysP contained antigenic determinants recognized by antibodies to CysP (GenWay). These antibodies were used to determine the M_r and pI of rCysP by immunoblotting following SDS-PAGE or IEF in Phast IEF (3–9). In addition, the ability of rCysP to cleave cIgG was investigated (see above).

RESULTS

Deletions in the *cysP* gene sequence

Comparison of the *cysP* gene sequence of *M. synoviae* (MS53_0590) and that of *M. gallisepticum* R_{LOW} (MGA_1153) showed deletions in two different regions at the 3' end of the *cysP* gene. In this study, the *cysP* sequences of 18 *M. synoviae* and 10 *M. gallisepticum* strains were determined (Table 1). Analysis of *M. synoviae* sequences indicated three types of *cysP* gene with reference to the length(s) of its sequence. Strains ULB 9122, ULB 07/P5, K1968, K1723, K1352, FMT, K2581, K3344, K2426D, K30009/37 and ULB 925 have a *cysP* gene of 1758 bp which encodes CysP containing 585 residues (Table 1). The *M. synoviae* type strain, WVU 1853, and strains F10-2AS, ULB 02/OV6, ULB 02/T6, PAA2, K4463B and B27/00 have shorter sequences due to a 39 bp deletion in the 3' end of the *cysP* gene (Fig. 1). Their predicted CysP protein contains only 572 residues. Notably, the *cysP* gene in the genome of strain 53 (MS53_0590) is unique among *M. synoviae* strains. It has an unusual 30 bp deletion upstream of a deletion found in seven *M. synoviae* strains, as well as a stop codon UAA (TAA) indicating a premature termination of translation (Fig. 1). All *M. gallisepticum* strains sequenced showed a 66 bp deletion in the same *cysP* gene region as WVU 1853 (Fig. 1). In comparison with *M. synoviae* strains, all *M. gallisepticum* strains have an insertion (codon 272 for lysine) in the *cysP* gene which encodes a 564 aa CysP.

In all, the CysP protease sequence diversification is higher in *M. synoviae* than in *M. gallisepticum*. However, many parts of CysP retained a conserved sequence in *M. synoviae* and this was found also for ₁₀₂DIDEHAIDYVSNS, identical to that of peptide used to raise CysP-specific antibodies.

M. synoviae expresses CysP

In immunoblot analyses using specific antibodies, CysP was detected in all *M. synoviae* strains examined, as well as

K4463B, FAA2, B27/00	479	NPISKESNTSETESNATLTSKSQTNNSVKTKGQTNSNTTDESSVTKNNNS-----	-----KKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK	572
ULB02/T6, ULB02/OV6	479	NPISKESNTSETKS NATLTSKSQTNNSVKTKGQTNSNTTDESSVTKNNNS-----	-----KKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK	572
WU01853, F10/2AS	479	NPISKESNTSETSNATLTSKSQTNNSVKTKGQTNSNTTDESSVTKNNNS-----	-----KKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK	572
K3344, FMT, K2581, K1352, ULB925	479	NPISKESNTSETESNATLTSKSQTNNSVKTKGQINSNTTNESSVTENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK		585
K1723	479	NPISKKSNTSETESNATLTSKSQTNNSVKTKGQTNSNTTNESSVIENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK		585
K1968	479	NPISKKSNTSETKS NATLTSKSQTNNSVKTKGQTNSNTTNESSVTENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGISTFGMATFFIVKLMKKRVKK		585
ULB9122, ULB07/P5	479	NPISKKSNTSETKS NATLTSKSQTNNSVKTKGQTNSNTTNESSVTENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKRVKK		585
K2426D, K3009/37	479	NPISKQSNSETKS NATLTSKSQTNNSVKTKGQTNSNTTNESSLVTENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGISTFGMATFFIVKLMKKRVKK		585
<i>M. synoviae</i> 53	479	NPISKESNTSE-----SQNNSVKTKGQTNSNTTDESSVTENNNSNNEKELEDVLNSKKPKTNKTNVILWSILGALVGIAFGMATFFIVKLMKKKS--		573
<i>M. gallisepticum</i> S6	480	NPISKESSTSETKSNTAPSSSETNNSVKTKGQTNSNTTTHESSVTE-----	-----NKTNVILWSILGALAGIAFGVTTFFIVKLMKKRVKK	564
R25, TS11, 1226, ULB931, PG31,	480	NPISKESNTSETKSNTTPSSSQNNSVKTKGQTNSNTATHESSVTE-----	-----NKTNVILWSILGALAGIAFGVTTFFIVKLMKKRVKK	564
ULB02/S16, ULB02/S2, ULB992		*****.*.***	*****.*.***	

Fig. 1. Multiple sequence alignment, by using CLUSTAL W, of the deduced amino acid sequences in the C-terminal regions of CysP proteins. Sequences were predicted from the *cysP* gene of *M. synoviae* and *M. gallisepticum* strains. Dashes show deletions. The number of CysP residues is indicated at the right side of each sequence.

in *M. gallisepticum* strains (Table 1 and data not shown). An example of analyses of the CysP protein synthesis is shown in Fig. 2(b). In *M. synoviae* strains WVU 1853, ULB 02/T6 and ULB 925/KF, antibodies to CysP peptide reacted with an approximately 65 kDa protein which is consistent with the M_r of their predicted CysP sequences. In WVU 1853 and ULB 02/T6, antibodies to NanH neuraminidase recognized a protein of approximately 110 kDa, whereas in ULB 925/KF lacking neuraminidase activity (Berčič *et al.*, 2008b), antibodies did not react (Fig. 2a). Pre-immune IgG did not react with either CysP or NanH.

Immunoblot analysis using IEF to separate proteins showed that the pIs of *M. synoviae* strains CysP proteins were in the range of ~8 to ~9, which corresponds with the predicted pIs (Table 1).

We also used IIPA and/or IIF and antibodies to CysP to determine whether CysP is surface-exposed on intact colonies of *M. synoviae* and *M. gallisepticum* strains. Examinations showed that CysP or at least its relevant region (aa 102–114) is accessible for CysP antibodies and thus, surface exposed in *M. synoviae* and *M. gallisepticum* (Supplementary Fig. S2a, b). Pre-immune cIgG did not react with colonies of *M. synoviae* or *M. gallisepticum*. Colonies of *Mycoplasma imitans* (strains 4229 and B2/85), which shares a number of antigens with *M. gallisepticum*, did not react with antibodies to CysP.

***M. synoviae* and *M. gallisepticum* cleave chicken IgG**

Initial analyses indicated that *M. synoviae* cells incubated overnight (~15 h) in samples containing chicken serum could cleave several serum proteins including IgG (not shown). To confirm this, purified cIgG was used as a substrate and a number of different *M. synoviae* strains were tested for the ability to cleave cIgG at 37–38 °C. All strains assayed apparently cleaved cIgG (M_r ~170 kDa) within 24 h (Table 1). Immunoblot analyses using HRP-conjugated antibodies to cIgG demonstrated that cIgG was cleaved into an abundant fragment of about 60 kDa and fragments of about 40–45 kDa (Fig. 3). Notably, the cleavage of cIgG by papain also generated an approximately 43 kDa fragment, whereas the second fragment (~50 kDa) was shorter than the approximately 60 kDa fragment generated by *M. synoviae* (Fig. 3b). When the cleavage of cIgG with *M. synoviae* was less efficient, fragments of approximately 100 kDa or larger were seen. On the other hand, cIgG fragments of approximately 60 and 45 kDa did not cleave further even if incubation with *M. synoviae* was extended for 3 days. This is consistent with our observation that *M. synoviae* did not cleave purified Fc of cIgG (~50 kDa Fc; Jackson ImmunoResearch Laboratories) after 4 days of incubation under conditions used in assays with the whole cIgG molecule (not shown). The cleavage of cIgG into approximately 60 and 45 kDa fragments was repro-

Fig. 2. Western blot analysis of NanH and CysP expression in *M. synoviae* strains WVU 1853, ULB 02/T6 and ULB 925. Whole-cell proteins separated by SDS-PAGE in PhastGel Gradient (8-25) were transferred to the Immobilon-P membrane and incubated with antibodies to NanH (8 $\mu\text{g ml}^{-1}$ for 1 h) (a) or antibodies to CysP (8 $\mu\text{g ml}^{-1}$ for 1 h) (b). Positions of molecular mass markers (PageRuler, Fermentas) are indicated.

Fig. 3. Cleavage of clgG by four *M. synoviae* strains and by papain. (a) clgG (0.1 mg ml^{-1}) was incubated at 38°C for 15 h with PBS alone (PBS) or with cells ($\sim 10^9$ c.f.u.) of strains ULB 07/P5, ULB 02/T6, WVU 1853 or ULB 925. The supernatant ($1 \mu\text{l}$ per lane) proteins separated in the PhastGel Gradient (8-25) were transferred to Immobilon-P and incubated for 45 min with HRP-conjugated clgG antibodies (Sigma; diluted 1:2000). TrueBlue was used to develop staining. (b) clgG (0.1 mg ml^{-1}) was incubated with papain ($2 \mu\text{g ml}^{-1}$) for 5 h at 38°C . clgG fragments were separated in PhastGel Gradient (8-25) and stained with PhastGel Blue R. Positions of molecular mass markers are indicated.

ducible and was demonstrated in 15 separate assays with cells of WVU 1853 cultures.

Several *M. gallisepticum* strains cleaved clgG, whereas the type strain (4229) of *M. imitans* did not (Table 1 and not shown). The cleavage pattern of clgG by *M. gallisepticum* was very similar to that observed for *M. synoviae* (Fig. 4). Thus, the clgG molecule ($\sim 170 \text{ kDa}$) was cleaved into approximately 60 and 40–45 kDa fragments by the *M. gallisepticum* strain R cultures at a low (R_{LOW}) and high (R_{HIGH}) level of passages. In the same assay, strains F and S6 (culture K1) revealed less efficient cleavage of clgG, but before being assayed, their pelleted cells were kept at -20°C . However, another culture of S6 culture K19 (isogenic to S6 K1) cleaved clgG into 60 and 40–45 kDa fragments within 15 h, but its cells were assayed

immediately after harvest (not shown). This suggested that freezing could have a negative effect on the capacity of *M. gallisepticum* to cleave clgG but further studies are required to confirm this.

Iodoacetamide (50 mM) prevented cleavage of clgG by *M. synoviae* and by *M. gallisepticum*, whereas EDTA did not. Under the conditions used, the E64 inhibitor of many cysteine proteases did not inhibit clgG cleavage by *M. synoviae* WVU 1853.

M. synoviae and *M. gallisepticum* cleave clgG into Fab and Fc

In a previous study, papain cleaved clgG into two 45 kDa Fab monomers and an approximately 54 kDa Fc dimer

Fig. 4. Cleavage of clgG by different *M. gallisepticum* strains and by *M. synoviae* WVU 1853. clgG (0.1 mg ml^{-1}) was incubated at 38°C for 15 h with PBS alone, with *M. gallisepticum* strains ($\sim 10^9$ c.f.u.) or *M. synoviae* WVU 1853 ($\sim 5 \times 10^8$ c.f.u.). The supernatant ($1 \mu\text{l}$ per lane) proteins were separated in PhastGel Gradient (8-25), transferred to Immobilon-P which was incubated for 45 min in HRP-conjugated antibodies to clgG (A9046, Sigma). Staining was developed by using TrueBlue. Lanes with *M. gallisepticum* strains are: RL1 and RL2, R_{LOW} cultures; RH, R_{HIGH} culture; F, strain F; S6K1, strain S6, culture K1; MS, *M. synoviae* strain WVU 1853. Positions of molecular mass markers are indicated. The arrowheads indicate positions of major clgG fragments of approximately 45 and approximately 60 kDa. Note, cells of cultures RL2, F and S6K1 were kept frozen (-20°C) before testing for clgG cleavage.

containing CH3 and CH4 regions of HC (Suzuki & Lee, 2004). In this study, both *M. synoviae* and *M. gallisepticum* cleaved cIgG into approximately 60 kDa and 40–45 kDa fragments (Figs 3 and 4). An approximately 60 kDa fragment was recognized by mAb 1F5 to HC of cIgG, whereas this mAb did not bind to the approximately 45 kDa fragments (Fig. 5a). On the other hand, the mAb to LC, CH31, reacted with the approximately 45 kDa fragments (Fig. 5b) but did not react with the approximately 60 kDa fragment. Moreover, approximately 45 kDa fragments of cIgG also reacted with mAb 4E4 (to HC), which did not react with the 60 kDa fragment (not shown). Papain also cleaved cIgG into 40–45 kDa fragments which reacted with mAbs CH31 and 4E4, but not with mAb 1F5. Reduction of the approximately 45 kDa fragment (generated by *M. synoviae*) by β -mercaptoethanol yielded a LC of approximately 22 kDa which reacted with mAb CH31. So, the approximately 45 kDa fragment corresponds to Fab and the approximately 60 kDa fragment to Fc.

In non-reducing conditions, we observed a longer, approximately 60 kDa, fragment of cIgG generated by *M. synoviae* or *M. gallisepticum* and recognized by mAb 1F5. On the other hand, under reducing conditions, mAb 1F5 recognized a considerably smaller protein of approximately 45 kDa (Supplementary Fig. S3b), suggesting that the approximately 60 kDa protein contained two Fc molecules. The 60 kDa fragment reacted with the lectin SNA probe, which binds to sialic acid [sia α (2-6)gal moiety] which is present on HC of cIgG only in the *N*-glycosylation site at Asn₃₀₈ (Suzuki & Lee, 2004). This demonstrated that *M. synoviae* WVU 1853 cleaved HC of cIgG upstream of Asn₃₀₈. The exact cleavage site could not be determined because the N-terminal amino acid sequence of the approximately 60 kDa HC fragment was blocked.

Recombinant CysP cleaved cIgG into Fab and Fc

In order to prove our hypothesis that CysP cleaves cIgG we expressed the *cysP* gene of *M. synoviae* ULB 925. To accomplish this it was necessary to change eight TGA codons into TGG codons in the *cysP* gene. The purified rCysP protein reacted with antibodies to CysP and was approximately 67 kDa and had a pI of approximately 8, consistent with the predicted values (Fig. 6 and not shown).

Under the conditions used, rCysP showed a convincing ability to cleave a part of cIgG. At a molar ratio of approximately 1:40 (rCysP:cIgG), rCysP cleaved cIgG into approximately 100 kDa fragments within 15 h. The amount of the cIgG fragments increased with the increasing time of incubation with rCysP. The cleavage pattern was very similar to that with *M. synoviae* cells (Figs 7 and 3). Indeed, cIgG (~170 kDa) was cleaved by rCysP into approximately 45 kDa fragments recognized by the mAb CH31 (to LC), as well as by mAb 4E4 reacting with the first part of HC (Fig. 7). On the other hand, an approximately 60 kDa fragment was recognized by mAb 1F5 (not shown). Thus, rCysP cleaved cIgG into Fab and Fc, which suggests that CysP is the enzyme used by *M. synoviae* and very probably by *M. gallisepticum* in the cleavage of the host's IgG.

DISCUSSION

cysP, encoding cysteine protease CysP, which was investigated in this study, is among the horizontally transferred genes of *M. synoviae* and *M. gallisepticum*. Its expression in *M. gallisepticum* has already been reported for its strain S6

Fig. 5. *M. synoviae* WVU 1853 cleaved cIgG into Fab and Fc. cIgG (80 μ g ml⁻¹) was incubated for 15 h at 38 °C with WVU 1853 (5 $\times$ 10⁸ c.f.u.). The supernatant proteins of cIgG, incubated in PBS alone (lanes PBS) or with WVU 1853 cells (lanes MS) were separated by using PhastGel Gradient (8-25), transferred to Immobilon-P and probed with mAb 1F5 to HC of cIgG (a), mAb CH31 to LC of cIgG (b) or a mixture of mAbs 1F5 and CH31 (c). Secondary antibodies were goat IgG (to mouse IgG) conjugated with HRP (A4416, Sigma). Positions of molecular mass markers are indicated.

Fig. 6. Characterization of rCysP by Western blot using CysP-specific antibodies. Lanes: ULB 925, *M. synoviae* ULB 925 (total protein $\sim 10 \mu\text{g ml}^{-1}$); rCysP, recombinant CysP ($1 \mu\text{g ml}^{-1}$); and EF-Ts, 32 kDa elongation factor with a C-terminal 6 $\times$ His tag from EasyXpress protein synthesis kit (Qiagen) ($10 \mu\text{g ml}^{-1}$). Samples ($5 \mu\text{l}$) were resolved by using 10% SDS-PAGE and electro-transferred to Immobilon-P membrane. The membrane was incubated for 1 h in a solution containing affinity-purified antibodies to CysP ($2 \mu\text{g ml}^{-1}$). Following washing, the membrane was incubated with HRP-conjugated antibodies to cIgG (Sigma; diluted 1:2500) and visualized by using TrueBlue. Positions of molecular mass markers are indicated.

(Demina *et al.*, 2009). To our knowledge, our study is the first to demonstrate synthesis of the CysP cysteine protease in *M. synoviae* and to report differences in the CysP structure in *M. synoviae* strains.

It is notable that the *cysP* gene of the *M. synoviae* strain 53 (MS53_0590, Vasconcelos *et al.*, 2005) has a very unusual structure. It has a unique deletion of 30 bases, as well as a premature stop codon (Fig. 1). Most *M. synoviae* strains examined have a *cysP* sequence which encodes a 585-residue CysP. Other strains have a deletion in *cysP* and their predicted CysP protein would have only 572 residues. Their CysP proteins were expressed at similar levels as in strains with longer CysP proteins, and all examined strains efficiently cleaved cIgG (Table 1). Interestingly, immediately upstream of the *cysP* gene is a gene encoding a putative transposase (homologous to MS53_0283) but its association with deletions in the *cysP* gene is not clear.

An important novel finding provided by our study is the cleavage of cIgG by *M. gallisepticum* and *M. synoviae*, both of which are major poultry pathogens. The cleavage pattern of cIgG is similar to that of papain which cleaves cIgG into Fab and Fc. Cleavage of cIgG (~ 170 kDa) with papain generated two Fab fragments of about 45 kDa and Fc (dimerized CH3 + CH4) of approximately 54 kDa (Suzuki & Lee, 2004). Notably, the structure of *M. synoviae* CysP is similar (residues 53–295) to that of papain which is a cysteine protease (type C1) as predicted by Pfam HMM search (Finn *et al.*, 2010) and SMART HMM (Schultz *et al.*, 1998).

The cleavage of cIgG by *M. gallisepticum* and *M. synoviae* also generated fragments of approximately 45 kDa which includes LC and corresponds to Fab fragments of IgG. Purified 45 kDa fragments, generated from cIgG (specific for NanH) by *M. synoviae* WVU 1853, reacted with NanH, indicating that they are functional Fab fragments (our unpublished data). The second cIgG fragment of approximately 60 kDa, recognized by mAb 1F5 to HC seems to be a dimerized Fc containing a part of CH2, CH3 and CH4. The epitope for mAb 1F5 includes CH2 since this mAb reacted with recombinant Fc containing CH2, CH3 and CH4 (Taylor *et al.*, 2008), but did not react with recombinant Fc or commercial Fc lacking CH2 domain (D. Benčina, unpublished data). The reduced form of Fc, generated from cIgG by *M. synoviae*, which reacted with mAb 1F5 had a relative molecular mass >45 kDa (Supplementary Fig. S3). For comparison, the relative molecular mass of the reduced recombinant Fc containing CH2–CH4 domains (HC residues 230–568) was approximately 50 kDa (Taylor *et al.*, 2008). This reduced Fc also reacted with mAb 1F5. It seems that cleavage of cIgG by *M. synoviae* occurred upstream of the first N-glycosylation site on HC (on Asn₃₀₈) because the Fc fragment included a sialic acid $\alpha(2-6)$ gal moiety which is present only in the N-glycosylation site at Asn₃₀₈ (Suzuki & Lee, 2004). It is possible that cIgG cleavage occurred just upstream of Asn₃₀₈ because the N-terminal sequence of the 60 kDa HC fragment was blocked. *M. synoviae* can also cleave other chicken serum proteins of, for example, approximately 20, 75, 85 and 200 kDa, but they remain to be identified molecularly. Additional investigations are required to find out whether CysP itself is involved in the cleavage of these proteins.

M. synoviae has proteins (~ 80 and ~ 90 kDa) which bind cIgG via the Fc part (Lauerman *et al.*, 1993). Association between binding and cleavage of cIgG is not clear yet. We observed, in cIgG cleavage experiments with WVU 1853 cells, that about 5 % of IgG from the sample bound to *M. synoviae* cells, whereas most of the IgG remained free, predominantly as Fab and Fc fragments. In addition to binding and cleavage of cIgG, *M. synoviae* is also capable of desialylating cIgG (R. L. Berčič and others, unpublished data). These properties might contribute to the survival of *M. synoviae* in the bloodstream from which it has been recovered as late as 54 days after the experi-

Fig. 7. Comparative analysis of cIgG cleavage by rCysP and *M. synoviae* cells. Samples of cIgG in PBS, pH 6 ($100 \mu\text{g ml}^{-1}$), were incubated at 38°C for 48 h with rCysP ($1 \mu\text{g ml}^{-1}$) or *M. synoviae* WVU 1853 (equivalent to 10^8 c.f.u. per sample) or PBS alone. cIgG and its fragments containing LC were detected by mAb CH31, HRP-conjugated antibodies to mouse IgG (Sigma) and TrueBlue. (a) Samples ($5 \mu\text{l}$) separated by SDS-PAGE (10% gel, $10 \times 8.5 \text{ cm}$) were electrotransferred to Immobilon-P and probed as described above. (b) Samples ($1 \mu\text{l}$) separated in PhastGel Gradient (8-25) were blotted onto Immobilon-P and probed as described above. Positions of molecular mass markers are indicated.

mental infection of chickens (Kerr & Olson, 1970). Cleavage of cIgG into Fab and Fc may disable antibodies since several important effector functions of IgG depend on its Fc part, including binding of complement and binding to Fc receptors on phagocytes. It is well known that *M. synoviae* infection induces the appearance of rheumatoid factor in sera of chickens. Autoantibodies, mainly IgM, are primarily directed against the Fc part of their IgG (D. Benčina and others, unpublished data). It is very likely that 'modification' of cIgG by *M. synoviae* (binding, cleavage, desialylation) is important in the induction of autoantibodies (rheumatoid factor) to the Fc part of IgG.

The members of the genus *Mycoplasma* infect a variety of different hosts and except for *M. gallisepticum* and *M. synoviae*, there is no evidence regarding cleavage of the IgG of their hosts. Few bacteria have been reported to degrade cIgG – among them are *A. paragallinarum*, *G. anatis* and *P. multocida* (Rivero-García *et al.*, 2005; García-Gómez *et al.*, 2005; Negrete-Abascal *et al.*, 1999). Compared with degradation of cIgG by *G. anatis* metalloproteases (García-Gómez *et al.*, 2005), cleavage of the cIgG by *M. synoviae* and *M. gallisepticum* targets only one region of IgG and resembles cleavage of human IgG by the cysteine protease SpeB of *S. pyogenes* (Collin & Olsen, 2001). SpeB cleaves human IgG in the hinge region, whereas CysP of *M. synoviae* cleaves cIgG within CH2, which corresponds to the hinge region of mammalian IgGs.

Several lines of evidence indicate that CysP cysteine protease is involved in the cleavage of cIgG by *M. synoviae* and *M. gallisepticum*. The cleavage pattern of cIgG is similar to that of papain, which is a cysteine protease, and was the same as that for *M. synoviae* and *M. gallisepticum*, which share high CysP sequence identity. Iodoacetamide, which inactivates the catalytic cysteine residue of papain-like cysteine proteases, inhibited cleavage of cIgG by *M. gallisepticum* and *M. synoviae*. Finally, rCysP also cleaved

cIgG into Fab and Fc, confirming our hypothesis that CysP is the enzyme that cleaves cIgG.

In conclusion, this study provides the first evidence that the major poultry pathogens *M. gallisepticum* and *M. synoviae* have the capacity to cleave IgG of their natural hosts. Results with rCysP showed that this cysteine protease is involved in the cleavage of cIgG into Fab and Fc. This supports a previous hypothesis, that proteins encoded by horizontally transferred genes play important roles in interactions of *M. gallisepticum* and *M. synoviae* with their avian hosts.

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