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.

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...
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 paragallinarum*, *Gallibacterium anatis* and *Pasteurella multocida* that have been reported to digest chicken IgG (clG) 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 clG ‘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 clG into the antigen-binding fragment of IgG (Fab) and Fc fragments. Assays with the purified recombinant CysP (rCysP) showed that it cleaves clG 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 1 ° and 0.1 g cysteine hydrochloride 1 ° (Dušanči 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 ° 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 (µ9DIDEHAIDYVYSNS) or of neuraminidase NanH (µ9DKRVNNKDGRHLEQD) 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 clG was isolated using immobilized CysP or NanH peptide as specific ligands (isolated by GenWay). These clG antibodies were used to study the expression and localization of CysP and NanH. They were received (1 mg ml °) together with relevant pre-immune clG, CysP and NanH peptides.

Monoclonal antibodies (mAbs) to clG heavy chain (HC) and light chain (LC). mAbs 1F5 and 4E4 to HC of clG 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 clG fragment(s) containing LC, including putative Fab.

Cleavage of clG by *M. synoviae* and *M. gallisepticum* or by rCysP. Samples with determined concentrations of pure clG 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 clG (ChromPure Chicken IgY, Fc; 2.2 mg ml °) was purchased from Jackson ImmunoResearch Laboratories.

In the cleavage assays, samples usually contained 0.1 or 0.2 mg clG ml ° (or 0.05 mg Fc ml °) diluted in sterile PBS, pH 7.4. In most assays, mycoplasma cells were tested for the ability to cleave clG 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 clG and tested as for other samples of mycoplasma cells. Usually, 50 or 100 µl samples (final volume) contained 5 or 10 µl clG mixed with *M. gallisepticum* or *M. synoviae* cells (~1–5x10 ° 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 clG cleavage was assessed at different time points, typically after 15, 24, 48, 72, 96 and, sometimes, 120 h of incubation. Samples were centrifuged (20000 g for 10 min), supernatants were collected and analysed by SDS-PAGE. Papain (Sigma), which cleaves clG 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 clG with PBS alone. The capability of CysP to cleave clG was investigated in four independent assays. The rCysP:clG 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 clG 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 keyhole limpet haemocyanin and used as antigens. SDS-PAGE gels were stained with Coomassie with or without 1 mM EDTA.

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

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*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 RHIGH and F determined in this study are identical to those submitted by Szczepanek et al. (2010).

Berčić 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 clgG was analysed in a PhastGel gradient (8–25), by staining proteins with PhastGel Blue R (Coomassie blue) and by transferring clgG and its fragments to a PVDF membrane (Immobilon-P; Millipore) and immunoblot detection. The membrane was incubated for 1 h with mAbs 1F5 and 4E4 (diluted 1 : 10), or with mAb CH31, secondary antibodies and DAB were used to show the position of a reactions. In parallel immunoblots, mAb 1F5, HRP-conjugated secondary antibodies and DAB were used to show the position of a cleaved clgG 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 clgG and its separation into HC and LC by SDS-PAGE, as well as to reduce clgG 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 clgG fragment (a putative Fc dimer) generated after incubation of clgG with M. synoviae WVU 1853, this fragment was processed as described previously (Berčić et al., 2008a). Sialylation of clgG and of its cleavage fragments containing sia (2-6)gal moiety was detected by Sambucus nigra agglutinin (SNA) lectin (DIG glycan differentiation kit, Roche) as described elsewhere (Dušanči 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 clglG to CysP (10 μg ml⁻¹) for 1 h, washed in PBS and then incubated with rabbit IgG (to clglG) 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 clglG antibodies to CysP (2–8 μg ml⁻¹) and then for 45 min with rabbit IgG (to clglG) labelled with HRP (Sigma) used at 1:2000. NanH expression was detected by using specific antibodies (1 h incubation, 8 μg 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 (Invitrek). 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 MgCl₂ 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 agrose gel electrophoresis in 1 % (w/v) agarose gels in Tris/Borate/EDTA buffer. 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 EMBOS 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× 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 3' and 5' 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 Mr and pl of rCysP by immunoblotting following SDS-PAGE or IEF in Phast IEF (3-9). In addition, the ability of rCysP to bind clglG 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 07/1P, K1968, K1723, K1352, FMT, K2581, K3344, K2426D, K3009/37 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 10225ID9HAIL003, 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
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*. 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 (Bercˇicˇ 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 clgG 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 clgG was used as a substrate and a number of different *M. synoviae* strains were tested for the ability to cleave clgG at 37–38°C. All strains assayed apparently cleaved clgG (*M*. ~170 kDa) within 24 h (Table 1). Immunoblot analyses using HRP-conjugated antibodies to clgG demonstrated that clgG was cleaved into an abundant fragment of about 60 kDa and fragments of about 40–45 kDa (Fig. 3). Notably, the cleavage of clgG 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 clgG with *M. synoviae* was less efficient, fragments of approximately 100 kDa or larger were seen. On the other hand, clgG 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 clgG (~50 kDa Fc; Jackson ImmunoResearch Laboratories) after 4 days of incubation under conditions used in assays with the whole clgG molecule (not shown). The cleavage of clgG into approximately 60 and 45 kDa fragments was repro-

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**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.

**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 µg ml⁻¹ for 1 h) (a) or antibodies to CysP (8 µg ml⁻¹ for 1 h) (b). Positions of molecular mass markers (PageRuler, Fermentas) 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 (~170 kDa) was cleaved into approximately 60 and 40–45 kDa fragments by the *M. gallisepticum* strain R cultures at a low (RLOW) and high (RHIGH) 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.
containing CH3 and CH4 regions of HC (Suzuki & Lee, 2004). In this study, both *M. synoviae* and *M. gallisepticum* cleaved clgG 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 clgG, 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 clgG also reacted with mAb 4E4 (to HC), which did not react with the 60 kDa fragment (not shown). Papain also cleaved clgG 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 clgG 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 \((2-6)\)gal moiety] which is present on HC of clgG only in the N-glycosylation site at Asn308 (Suzuki & Lee, 2004). This demonstrated that *M. synoviae* WVU 1853 cleaved HC of clgG 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 clgG into Fab and Fc**

In order to prove our hypothesis that CysP cleaves clgG 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 pl 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 clgG. At a molar ratio of approximately 1:40 (rCysP : clgG), rCysP cleaved clgG into approximately 100 kDa fragments within 15 h. The amount of the clgG 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, clgG (~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 clgG 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. 6. Characterization of rCysP by Western blot using CysP-specific antibodies. Lanes: ULB 925, M. synoviae ULB 925 (total protein ~10 μg ml⁻¹); rCysP, recombinant CysP (1 μg ml⁻¹); and EF-Ts, 32 kDa elongation factor with a C-terminal 6×His tag from EasyXpress protein synthesis kit (Qiagen) (10 μg ml⁻¹). Samples (5 μl) 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 μg ml⁻¹). Following washing, the membrane was incubated with HRP-conjugated antibodies to clgG (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 clgG (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 clgG by M. gallisepticum and M. synoviae, both of which are major poultry pathogens. The cleavage pattern of clgG is similar to that of papain which cleaves clgG into Fab and Fc. Cleavage of clgG (~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 clgG 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 clgG (specific for NanH) by M. synoviae WVU 1853, reacted with NanH, indicating that they are functional Fab fragments (our unpublished data). The second clgG 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. Bencina, unpublished data). The reduced form of Fc, generated from clgG 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 clgG by M. synoviae occurred upstream of the first N-glycosylation site on HC (on Asn308) because the Fc fragment included a sia α(2-6)gal moiety which is present only in the N-glycosylation site at Asn308 (Suzuki & Lee, 2004). It is possible that clgG 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 clgG via the Fc part (Lauerman et al., 1993). Association between binding and cleavage of clgG is not clear yet. We observed, in clgG 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 clgG, M. synoviae is also capable of desialylating clgG (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-
M. gallisepticum infect different hosts and except for very likely that ‘modification’ of cIgG by M. synoviae their IgG (D. Bencˇina and others, unpublished data). It is mainly IgM, are primarily directed against the Fc part of rheumatoid factor in sera of chickens. Autoantibodies, 370 Microbiology M. synoviae that binding to Fc receptors on phagocytes. It is well known on its Fc part, including binding of complement and since several important effector functions of IgG depend (binding, cleavage, desialylation) is important in the cleavage of cIgG into Fab and Fc may disable antibodies inactivates the catalytic cysteine residue of papain-like cysteine proteases, inhibited cleavage of cIgG by M. gallisepticum and M. synoviae. Finally, rCysP also cleaved mental infection of chickens (Kerr & Olson, 1970). Cleavage of clgG 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. Bencˇina and others, unpublished data). It is very likely that ‘modification’ of clgG 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 clgG – among them are A. paragallinarium, G. anatis and P. multocida (Rivero-Garcı´a et al., 2005; García-Gómez et al., 2005; Negrete-Abascal et al., 1999). Compared with degradation of clgG by G. anatis metalloproteases (Garcı´a-Gómez et al., 2005), cleavage of the clgG 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 clgG 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 clgG by M. synoviae and M. gallisepticum. The cleavage pattern of clgG 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 clgG by M. gallisepticum and M. synoviae. Finally, rCysP also cleaved clgG into Fab and Fc, confirming our hypothesis that CysP is the enzyme that cleaves clgG.

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 clgG 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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