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Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent *Mycoplasma* Strain

Joerg Jores^{1,2*}, Li Ma^{3†}, Paul Ssajakambwe^{2,4†}, Elise Schieck², Anne Liljander², Suchismita Chandran³, Michael H. Stoffel⁵, Valentina Cippa¹, Yonathan Arfi^{6,7}, Nacyra Assad-Garcia³, Laurent Falquet⁸, Pascal Sirand-Pugnet^{6,7}, Alain Blanchard^{6,7}, Carole Lartigue^{6,7}, Horst Posthaus⁹, Fabien Labrousseau¹ and Sanjay Vashee³

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*Correspondence:

Joerg Jores
joerg.jores@vetsuisse.unibe.ch

† These authors have contributed
equally to this work

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¹ Department of Infectious Diseases and Pathobiology, Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland, ² International Livestock Research Institute, Nairobi, Kenya, ³ J. Craig Venter Institute, Rockville, MD, United States, ⁴ College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda, ⁵ Division of Veterinary Anatomy, Department of Clinical Research and Veterinary Public Health, University of Bern, Bern, Switzerland, ⁶ UMR 1332 – Biologie du Fruit et Pathologie, Institut National de la Recherche Agronomique, Villenave-d'Ornon, France, ⁷ UMR 1332 – Biologie du Fruit et Pathologie, Université de Bordeaux, Villenave-d'Ornon, France, ⁸ Biochemistry Unit, Swiss Institute of Bioinformatics, University of Fribourg, Fribourg, Switzerland, ⁹ Department for Infectious Diseases and Pathobiology, Institute of Animal Pathology (COMPAT), University of Bern, Bern, Switzerland

Mycoplasmas are the smallest free-living organisms and cause a number of economically important diseases affecting humans, animals, insects, and plants. Here, we demonstrate that highly virulent *Mycoplasma mycoides* subspecies *capri* (*Mmc*) can be fully attenuated *via* targeted deletion of non-essential genes encoding, among others, potential virulence traits. Five genomic regions, representing approximately 10% of the original *Mmc* genome, were successively deleted using *Saccharomyces cerevisiae* as an engineering platform. Specifically, a total of 68 genes out of the 432 genes verified to be individually non-essential in the JCVI-Syn3.0 minimal cell, were excised from the genome. *In vitro* characterization showed that this mutant was similar to its parental strain in terms of its doubling time, even though 10% of the genome content were removed. A novel *in vivo* challenge model in goats revealed that the wild-type parental strain caused marked necrotizing inflammation at the site of inoculation, septicemia and all animals reached endpoint criteria within 6 days after experimental infection. This is in contrast to the mutant strain, which caused no clinical signs nor pathomorphological lesions. These results highlight, for the first time, the rational design, construction and complete attenuation of a *Mycoplasma* strain via synthetic genomics tools. Trait addition using the yeast-based genome engineering platform and subsequent *in vitro* or *in vivo* trials employing the *Mycoplasma* chassis will allow us to dissect the role of individual candidate *Mycoplasma* virulence factors and lead the way for the development of an attenuated designer vaccine.

Keywords: *Mycoplasma mycoides* subsp. *capri*, attenuation, genome engineering, *in vivo* challenge, virulence traits

INTRODUCTION

Bacteria belonging to the genus *Mycoplasma* are wall-less bacteria that cause massive economic losses in the livestock sector (chickens, ruminants and pigs) and are responsible for human pneumonia and sexually transmitted diseases (STDs). Currently, there is an absence of commercial vaccines against infections with the human pathogens *Mycoplasma pneumoniae* and *Mycoplasma genitalium* (Linchevski et al., 2009). In contrast, many livestock vaccines are commercialized, which rely either on adjuvanted killed bacteria or on attenuated strains obtained after successive rounds of sub-culturing or chemical mutagenesis (Browning et al., 2005). Due to these empirical approaches, the exact mechanism triggering the attenuation is unknown for many of the previously developed live attenuated *Mycoplasma* vaccines. Strikingly, these vaccines are far from being optimal since they often display short durations of immunity and limited efficacy (Maes et al., 2008; Nicholas and Churchward, 2012; Jores et al., 2013). A better understanding of pathogenicity and the identification of virulence traits would foster next generation vaccines.

For many years, the lack of genetic tools has limited our basic understanding of *Mycoplasma* pathogenicity. Due to their regressive evolution by gene loss, mycoplasmas appear to lack many of the common bacterial effectors and toxins used to interact with their hosts or to escape the hosts' immune systems (Citti et al., 2010; Chopra-Dewasthaly et al., 2017). Lipoproteins have been proposed to be involved in both aspects by using their cytoadherent properties and allowing antigenic variability through phase or sequence variation (Chambaud et al., 1999). Other candidate virulence traits, such as the *Mycoplasma* Ig binding protein-*Mycoplasma* Ig protease (MIB-MIP) system (Arfi et al., 2016) and the hydrogen peroxide production system (Blotz and Stulke, 2017) have been suggested, but not confirmed *in vivo*.

The availability of a genome engineering platform that allows directed and precise mutagenesis for *Mycoplasma mycoides* is undoubtedly a new starting point toward better understanding of host-pathogen interactions. The species *M. mycoides* consists of the two subspecies *M. mycoides* subsp. *mycoides* (*Mmm*) and *M. mycoides* subsp. *capri* (*Mmc*), which are the causative agents of contagious bovine pleuropneumonia and a caprine MAKEPS syndrome (comprising mastitis, arthritis, keratitis, pneumonia, and septicemia), respectively. In this work, we engineered a *Mmc* strain by deleting approximately one tenth of the genome, including candidate virulence traits. The resulting mutant retains almost wild-type like growth characteristics and was attenuated both *in vitro* and *in vivo*. The construction of this fully attenuated and safe laboratory *Mycoplasma* strain paves the way for research into host-pathogen interactions and is a good starting point to revisit the actual role of suggested virulence determinants in *Mycoplasma*.

MATERIALS AND METHODS

Mycoplasma Strains

The *M. mycoides* subsp. *capri* outbreak strain GM12 was used as positive control in the *in vivo* experiment (DaMassa et al., 1983). A modified *Mycoplasma capricolum* subsp. *capricolum* strain CK was used as recipient strain in genome transplantation protocols (Lartigue et al., 2009).

Yeast Strain and Media

The yeast *Saccharomyces cerevisiae*, strain VL6-48N (MAT α his3- Δ 200 trp1- Δ 1 KIURA3- Δ 1 lys2 ade2-101 met14) containing the 1.08 Mb genome of *M. mycoides* subsp. *capri* (*Mmc*) strain GM12 with an integrated yeast centromeric plasmid (YCp) (Lartigue et al., 2009) was used for construction of the mutants. Yeast cells were grown and maintained in either synthetic minimal medium containing dextrose (SD, Takara Bio) (Lartigue et al., 2009), or in standard rich medium containing glucose (YPD, Takara Bio) or galactose (YPG, Takara Bio) (Noskov et al., 2010). SD medium was supplemented with 5-fluoroorotic acid (5-FOA), for KIURA3 counter-selection (Boeke et al., 1984; Lartigue et al., 2009).

Preparation of Mutagenesis Cassettes

Sixty eight genes that encode candidate virulence traits were seamlessly deleted from the genome of *Mmc* GM12::YCpMmyc1.1 in five consecutive cycles (D1, D3, D4, and D5) using Tandem Repeat coupled with Endonuclease Cleavage [TREC] as described (Noskov et al., 2010; Chandran et al., 2014) or a variation of TREC involving the Cre-lox system for the D2 deletion, see below. Primer sequences to target and confirm the insertion of the mutagenesis cassette into each target site and to verify seamless deletion of the targeted genes are shown in **Supplementary Table S1**.

The *gts* gene cluster (D2) was targeted and deleted in the *Mmc* genome in the back-ground of the *glpFKO* deletion strain by employing a derivative of the *Mmc* synthetic cell JCVI-syn1.0 (Gibson et al., 2010). Primers RC0905 and RC0906 (**Supplementary Table S1**) were used to amplify the mutagenesis cassette from the synthetic cell derivative and was targeted to the *gts* region. Specific primers were used to confirm correct insertion at the target site by amplifying the junctions between the GM12::YCpMmyc1.1 genome and the inserted cassette. Galactose induction resulted in the Cre-mediated deletion of the *gts* region, leaving 13 bp of the 5' end of the *gtsA* region, the 34 bp *loxP* site, and 27 bp of the 3' end of the *lppB* gene. Specific primers were used to verify the knock-out.

Transformation and PCR Analysis

Transformation of the CORE3 cassette was performed by the lithium acetate method as described previously (Gietz et al., 1992). Transformed yeast were plated on appropriate selection media [SD medium minus His (Teknova, CA) or SD medium (minus His and minus Ura)] and incubated at 30°C for 48 h. Yeast colonies were patched on appropriate selective media and total DNA was isolated for PCR screening (Noskov et al., 2002). The correct insertion of the mutagenesis cassette was

verified by PCR amplification using upstream and downstream specific primers (Integrated DNA Technologies, Coralville, IA, United States) (**Supplementary Table S1**).

Transplantation

The modified GM12::YCpMmyc1.1 genomes (D1–D5) were transplanted into *M. capricolum* subsp. *capricolum* (*Mcc*) recipient cells with polyethylene glycol and selected for tetracycline resistance as described previously (Noskov et al., 2002; Lartigue et al., 2009). The resulting mutant strains were subjected to multiplex PCRs and pulsed-field gel electrophoresis as described elsewhere (Labroussaa et al., 2016) to confirm integrity of the genome.

Confirmation of the Mutants Using Next Generation Sequencing and Mapping Assembly

Total DNA of the strains GM12, GM12::YCpMmyc1.1 and GM12::YCpMmyc1.1-Δ68 was isolated as described before (Fischer et al., 2015). DNA was sheared using sonication and subjected to Illumina sequencing using a MiSeq machine by University of California Santa Cruz, Santa Cruz, CA (United States). Reads were mapped to the designed genome sequences based on the parental strains GM12 and GM12::YCpMmyc1.1 and GM12::YCpMmyc1.1-Δ68. The raw reads (300 bp PE) were QC with FastQC¹. The corrected reads were mapped onto the reference genome WT-YCP.fa with bwa mem (Li and Durbin, 2010) and converted to sorted bam with samtools (Li, 2011). The bam files were analyzed for deletions using Delly2 (Rausch et al., 2012) and Sprites (Zhang et al., 2016), and the predictions validated visually using IGV (Thorvaldsdottir et al., 2013). The list of strains and their deleted regions is summarized in **Table 1**.

Scanning Electron Microscopy of *Mycoplasma*

Unless stated otherwise, chemicals were obtained from Merck (Schaffhausen, Switzerland). *Mycoplasmas* were washed with distilled water (dH₂O) and fixed with 4% para-formaldehyde (Life Technologies, Thermo Fisher, Zug, Switzerland; Cat. No. 28906) in dH₂O for 5 days at 4°C. Thereafter, samples of 40 μl of cell suspension were centrifuged onto gold-sputtered poly-L-lysine coated coverslips (high molecular poly-L-Lysine hydrobromide) at 125 *rcf* for 4 min. Coverslips were washed once with PBS and twice with 0.1% bovine serum albumin in PBS (BSA/PBS). Free aldehydes were blocked with 0.05 M glycine in 0.1% BSA-c/PBS (Aurion, ANAWA Trading, Wangen, Switzerland) for 15 min at room temperature. After 3 washes with 0.1% BSA/PBS, cells were fixed with 2.5% glutaraldehyde (Merck 104239) in 0.1 M cacodylate buffer (dimethylarsinic acid sodium salt trihydrate), washed 3 times with dH₂O and postfixed with 1% OsO₄ (Polysciences, Warrington, PA, United States) in 0.1 M cacodylate buffer for 15 min. at room temperature. Five additional washes with dH₂O were followed by dehydration

in an ascending ethanol series. Samples were then transferred to hexamethyldisilazane (Merck 814051) for 10 min, air-dried, mounted onto aluminum stubs with carbon conductive adhesive tabs (Ted Pella Inc., Redding, CA, United States) and coated with approximately 25 nm of gold in an SCD004 (Leica Microsystems, Heerbrugg, Switzerland). Secondary electron micrographs and corresponding backscattered images were obtained with a fully digital field emission scanning electron microscope DSM 982 Gemini (Zeiss, Oberkochen, Germany) at an accelerating voltage of 5 kV, a working distance of 6–8 mm and primary magnifications ranging from 30,000 to 50,000×.

Growth Assay

Overnight cultures of *Mycoplasma* strains were grown at 37°C in SP4 medium containing streptomycin (GM12) or tetracycline (GM12::YCpMmyc1.1, GM12::YCpMmyc1.1-Δ68) for about 16 h. Doubling times of the *Mycoplasma* strains were then determined as described elsewhere (Hutchison et al., 2016), except that time interval samples were collected and processed at 0, 1, 2, 3, 4, 5, 6, 7, 9, 12, 15, and 24 h.

In vitro Hydrogen Peroxide Assay

Overnight cultures of *Mycoplasma* strains were grown as described above. When the pH of overnight cultures reached 6.0–6.5, they were inoculated into fresh SP4 medium at 1:200 dilution and incubated at 37°C for different time intervals of 0, 5, 7, and 24 h. At each time interval, an aliquot of culture was taken for DNA extraction (Hutchison et al., 2016) and another aliquot was taken to determine hydrogen peroxide levels.

To determine hydrogen peroxide levels, the aliquots were spun at 14,000 rpm for 10 min at 4°C. The pellets were washed with 1 ml of cold PBS, pH 7.5 to remove traces of media, then resuspended in 400 μl of cold PBS and stored at 4°C. Hydrogen peroxide levels were determined using the Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies, NY) according to the manufacturer's instructions. Briefly, 50 μl of diluted samples (1:5 in PBS) was aliquoted onto 96-well plates and warmed to 37°C for 1 h prior to starting the assay. 100 μM final concentration of glycerol (Sigma-Aldrich, MO) or GPC (Sigma-Aldrich, MO) was then added to the diluted sample and incubated at 37°C for 1 h. 50 μl of the Amplex Red reagent was added to the samples, incubated at room temperature in the dark for 30 min and fluorescence was measured using a spectrophotometer (SpectraMax M5, Molecular Devices, CA). Three technical replicates were performed for each sample and normalized to their respective DNA concentrations.

Detection of Immunoglobulin Degradation by *Mycoplasma in vitro* and *in vivo*

In vitro functionality of the MIB-MIP system was tested using the strains GM12, GM12::YCpMmyc1.1 and GM12::YCpMmyc1.1-Δ68. Each strain was grown overnight at 37°C in 3 mL modified SP5 medium (containing 5% FBS). 250 μL of each culture was harvested and centrifuged for 10 min at 4,000 g. The cells were

¹<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

TABLE 1 | Table showing the results of the Illumina sequencing-based mapping assemble of the strains used in this study.

Region (size in bp)	YCP (9,192)	D1 (2,984 bp)	D2 (4,950 bp)	D3 (4,677 bp)	D4 (69,220 bp)	D5 (24,906 bp)
GM12	Not present	Present (I)	Present (I)	Present (I)	Present (I)	Present (I)
GM12::YCPMmyc1.1 (position)	Present (25,426– 34,618)	Present (283,950– 286,934)	Present (569,098– 574,048)	Present (18,716– 23,393)	Present (665,418– 734,638)	Present (1,055,169– 1,080,075)
GM12::YCPMmyc1.1-Δ68	Present (I)	Deleted (DIS)	Deleted (IS)	Deleted (DIS)	Deleted (DIS)	Deleted (DIS)

D, detected by Delley; I, validated visually by IGV; S, detected by sprites.

then resuspended in 15 μ L modified SP5 medium (containing 5% FBS) and incubated with 5 μ g of purified caprine IgG (Sigma) at 37°C for 3 h. Bacterial CFUs were estimated for each strain by serial dilutions and were 3.2×10^9 CFUs *Mmc* GM12, 8.5×10^8 CFUs *Mmc* GM12::YCPMmyc1.1, and 3.5×10^8 CFUs *Mmc* GM12::YCPMmyc1.1-Δ68. A sample consisting of 5 μ g caprine IgG in dH₂O was included as a control. The incubated samples were mixed with 2 \times Laemmli Sample Buffer (Bio-Rad) at a 1:1 ratio, boiled for 10 min at 98°C and separated onto a 12% SDS-PAGE gel. They were subsequently transferred onto a 0.2 μ m nitrocellulose membrane (Bio-Rad) using a Bio-Rad Trans-Blot[®] Turbo[™] Transfer System (25 volts, 1.0 A, 30 min). Next, a Western Blot was performed using PBS supplemented with 0.1% Tween-20 and 2% BSA as a blocking buffer, and mouse anti-goat IgG (H+L) (Jackson ImmunoResearch, 205-005-108) and goat anti-mouse (Fc) labeled with horseradish peroxidase (Sigma, A0168) as primary and secondary antibodies. The antibodies were diluted in blocking buffer at 1:2000 and 1:70,000, respectively, and incubated with the membrane for 1 h each. In between the antibody incubations, the membrane was washed once with PBS – 0.1% Tween-20 + 3.2% NaCl and twice with PBS – 0.1% Tween-20, for 10 min each time. The results were visualized using the Fujifilm LAS-3000 Luminescent Image Analyzer.

Serum samples derived from the *in vivo* challenge were diluted in water to achieve a total load of 10–20 μ g of protein per sample. Western Blots were performed and analyzed using the same protocol as described above.

Animal Experiment Setup

All protocols of this study were designed and performed in strict accordance with the Kenyan and United States American legislation for animal experimentation and were approved by the institutional animal care and use committees at both institutions (JCVI and ILRI, IACUC reference number 2014.08).

Sixteen male outbred goats (*Capra aegagrus hircus*), 1–2 years of age and randomly selected in Naivasha, were transferred to the ILRI campus in Nairobi and kept under quarantine for 6 months. After arrival at the campus, all animals were dewormed twice using levamisole and treated prophylactically against babesiosis and anaplasmosis using imidocarb. Upon entry to ILRI, the goats were vaccinated against anthrax and blackleg (Blanthax[®], Cooper), Foot and Mouth Disease (FOTIVAX[®]) and Peste des Petits Ruminants (Live attenuated strain Nig. 75/1). All animals were tested negative for presence of antibodies against contagious caprine pleuropneumoniae (CCPP), using a

competitive ELISA (IDEXX). Two weeks before experimental infection, all animals were transferred to the animal biosafety level two (ABSL2) unit. *Mycoplasma* cells were cultivated in PPLO medium supplemented with horse serum (Sacchini et al., 2011) to early logarithmic phase, aliquoted and stored at –80°C. Afterwards, we determined the CFU using two aliquots. Just before infection we thawed the vials and adjusted the concentration of *Mycoplasma* to 10^9 CFU per mL^{–1} using broth. All 16 goats were infected transtracheally by needle puncture 5–10 cm distal to the larynx. Each animal received 1 mL of *Mmc* GM12 or GM12::YCPMmyc1.1-Δ68 liquid culture (equivalent to 10^9 colony forming units per animal), followed by 5 mL of phosphate buffered saline (PBS). The animals were allowed to move freely within the ABSL2 unit and had *ad libitum* access to water. They were fed *ad libitum* with hay and received pellets each morning. Three veterinarians monitored the health status of the animals throughout the experiment. Rectal temperature, oxygen blood saturation, heart rate and breathing frequency were measured daily in the morning hours using the GLA M750 thermometer (GLA Agricultural Electronics, United States), VE-H100B oximeter (Edan, United States), and a stethoscope classic II (Littmann, United States) with a water-resistant wrist watch Seamaster (Omega, Switzerland), respectively. Blood samples for subsequent analysis were taken twice a week by jugular vein puncture. Goats were euthanized when they developed severe disease associated with unwarranted moderate to severe pain. Therefore, they received an intravenous injection of Lethobarb Euthanasia Injection (Virbac, United States) of 200 mg.kg^{–1} body weight. Severe disease and pain were determined by a fever of $\geq 41^\circ\text{C}$ for >3 consecutive days, an oxygen saturation of $\leq 92\%$ and a lateral recumbency of ≥ 1 day without the ability to feed or intake water. Goats that were not put down because of ethical reasons were euthanized on 28 dpi.

Pathomorphological and Histology Analysis

A complete necropsy was performed on all animals. Tissue samples of the neck region around the inoculation site and all internal organs were fixed in 10% buffered formalin for 72 h and subsequently routinely processed for paraffin embedding. Tissue sections were cut at 3 μ m and stained routinely with hematoxylin and eosin (H&E) and evaluated by a board-certified pathologist.

Microbiology

Venous blood samples, lung samples, carpal joint fluid, and pleural fluid specimens taken at necropsy were used for isolation

of *Mmc* as described elsewhere (Liljander et al., 2015) using *Mycoplasma* liquid medium (Mycoplasma Experience Ltd., United Kingdom). Lung samples and pleural fluid were used for screening of *Pasteurella* and *Mannheimia* spp. using standard methods (Carter and Cole, 1990).

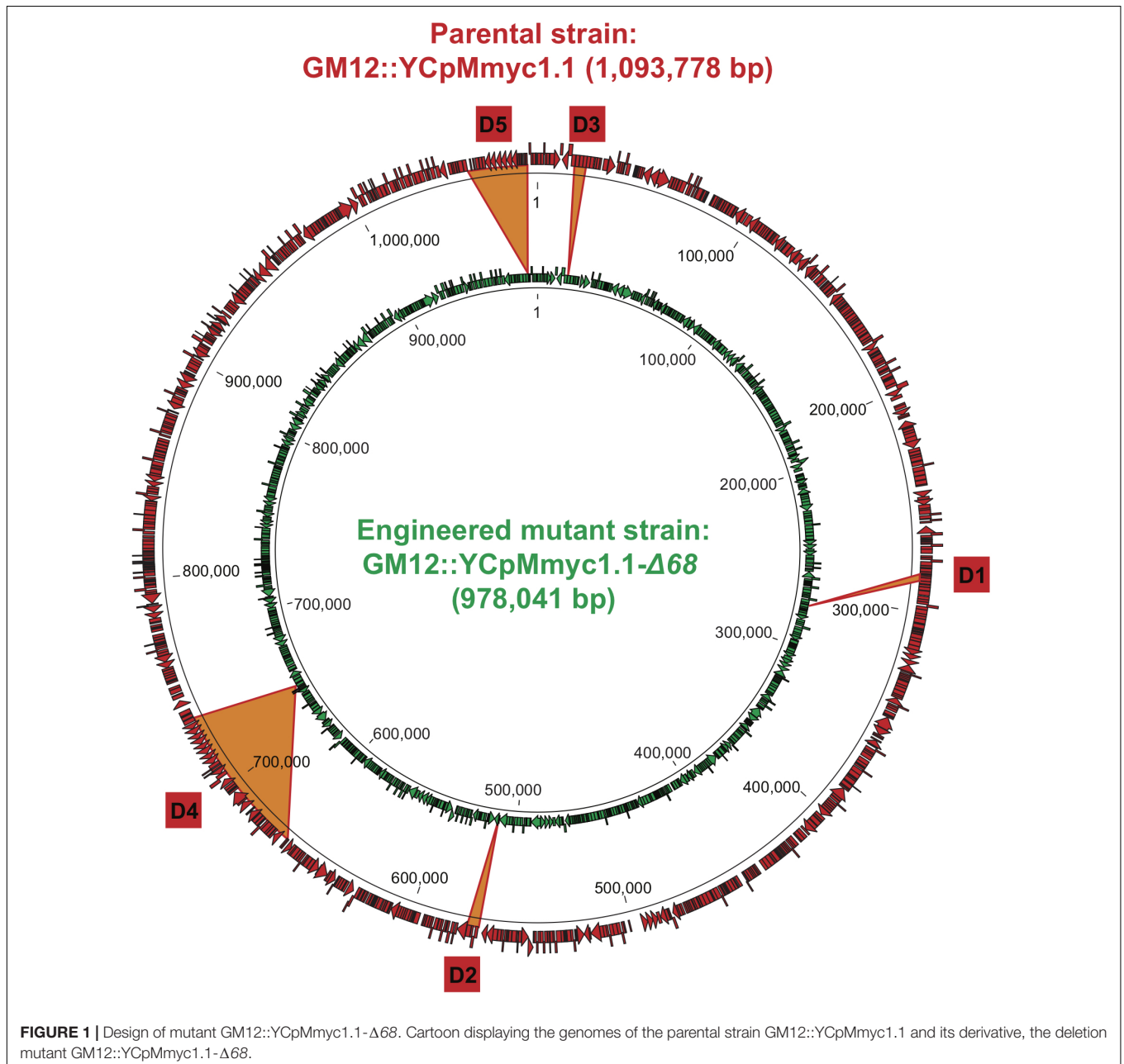
Statistical Analysis

Exact and normal approximation binomial tests were used to compare the two groups using GenStat 12th Edition (Payne et al., 2012). *P*-values for differences in parameters were estimated using a 2-sided 2-sample *t*-test comparing average levels between both groups at 5% level of significance.

RESULTS

Generation of the Mutant Strain GM12::YCpMmyc1.1-Δ68

To demonstrate attenuation of *Mmc* by rational design, five genomic regions were targeted in this study. These modifications were done on the genome GM12::YCpMmyc1.1 cloned in *S. cerevisiae*. This genome has been obtained after the insertion of genetic elements (i.e., ARSH4, CEN6, and HIS3) in the genome of *Mmc* GM12 allowing its maintenance and selection in the yeast (Lartigue et al., 2009). The precise localizations of each deletion are shown in **Figure 1**. The first two target deletion regions



contained genes encoding the glycerol-dependent hydrogen peroxide metabolic pathway and its suggested ABC transporter encoded by the *gtsABCD* operon (Pilo et al., 2007). This pathway has been suggested to be a main virulence mechanism for *M. mycoides* (Pilo et al., 2007), but *in vivo* confirmation is still missing and in *Mycoplasma gallisepticum* the pathway does not seem to be linked to virulence (Szczepanek et al., 2014). Thus, the genes *glpF*, *glpK*, and *glpO* (MMCAP2_0217-0219; 2,984-bp region; D1) and the *gts* gene region that includes the gene *lppB* (MMCAP2_0456-0459; 4,950-bp region; D2) were deleted in the *Mmc* genome by the yeast-based engineering method (Lartigue et al., 2009). As previously mentioned, lipoproteins were another target of interest since they likely trigger not only host–pathogen interactions but also, overwhelming immune reactions that result in inflammation (Browning et al., 2011). Three lipoproteins encoded in the D3 region (MMCAP2_0014-0016; 4,677-bp) as well as six lipoproteins in the D5 region (*lppQ*, MMCAP2_0889-0904; 24,906-bp) were also excised employing again the yeast-based engineering method. On top of that we deleted a large genomic region that encoded the *Mycoplasma*-specific F1-like X0 ATPase (Beven et al., 2012), the MIB-MIP system (Arfi et al., 2016), an integrative and conjugative element (ICE) (Tardy et al., 2015) and eight lipoproteins. The ICE was targeted in an effort to reduce mobile elements from the *Mmc* genome. In this case, about 70 kbp (MMCAP2_0550-0591; 69,220-bp region; D4) were targeted and deleted from the *Mmc* genome using the yeast-based engineering method in one stretch.

After each cycle of deletions, the modified *Mmc* genome was isolated from yeast cells and transplanted back into *M. capricolum* subsp. *capricolum* (*Mcc*) recipient cells to confirm the viability of each mutant *Mmc* strain. Overall, the final mutant strain, named *Mmc* GM12::YCPMmyc1.1-Δ68, was generated in five sequential deletion cycles (Figure 1). The gene knock-outs were verified by amplifying across each

deleted region (Supplementary Figure S1). Genomic DNA from the GM12::YCPMmyc1.1-Δ68 was isolated and analyzed by sequencing to confirm the deletions (Table 2). The genome sequence of GM12::YCPMmyc1.1-Δ68 was deposited at the ENA database under the accession number LS483503.

The Mutant Strain GM12::YCPMmyc1.1-Δ68 Is Viable and Unaffected in Its Morphology or Growth in Axenic Medium

The colonies of *Mmc* GM12::YCPMmyc1.1-Δ68 were of similar size to those of GM12::YCPMmyc1.1 and GM12. Cell morphology of the GM12, the isogenic parental strain GM12::YCPMmyc1.1 and GM12::YCPMmyc1.1-Δ68 strains was evaluated using scanning electron microscopy (Figure 2A). All strains tested were globular in shape and lacked any special morphological features. The diameter of the microorganisms was in the range of 500 nm, as expected for a *Mycoplasma* cell. The mutant GM12::YCPMmyc1.1-Δ68 grew with a doubling time somewhat similar to that of the parental strains GM12 and GM12::YCPMmyc1.1 (Figure 2B). Together, these results strongly suggest that the deletion of approximately 100 kbp of genomic content from the *Mmc* genome did not adversely affect structural integrity or *in vitro* growth of the mutant GM12::YCPMmyc1.1-Δ68.

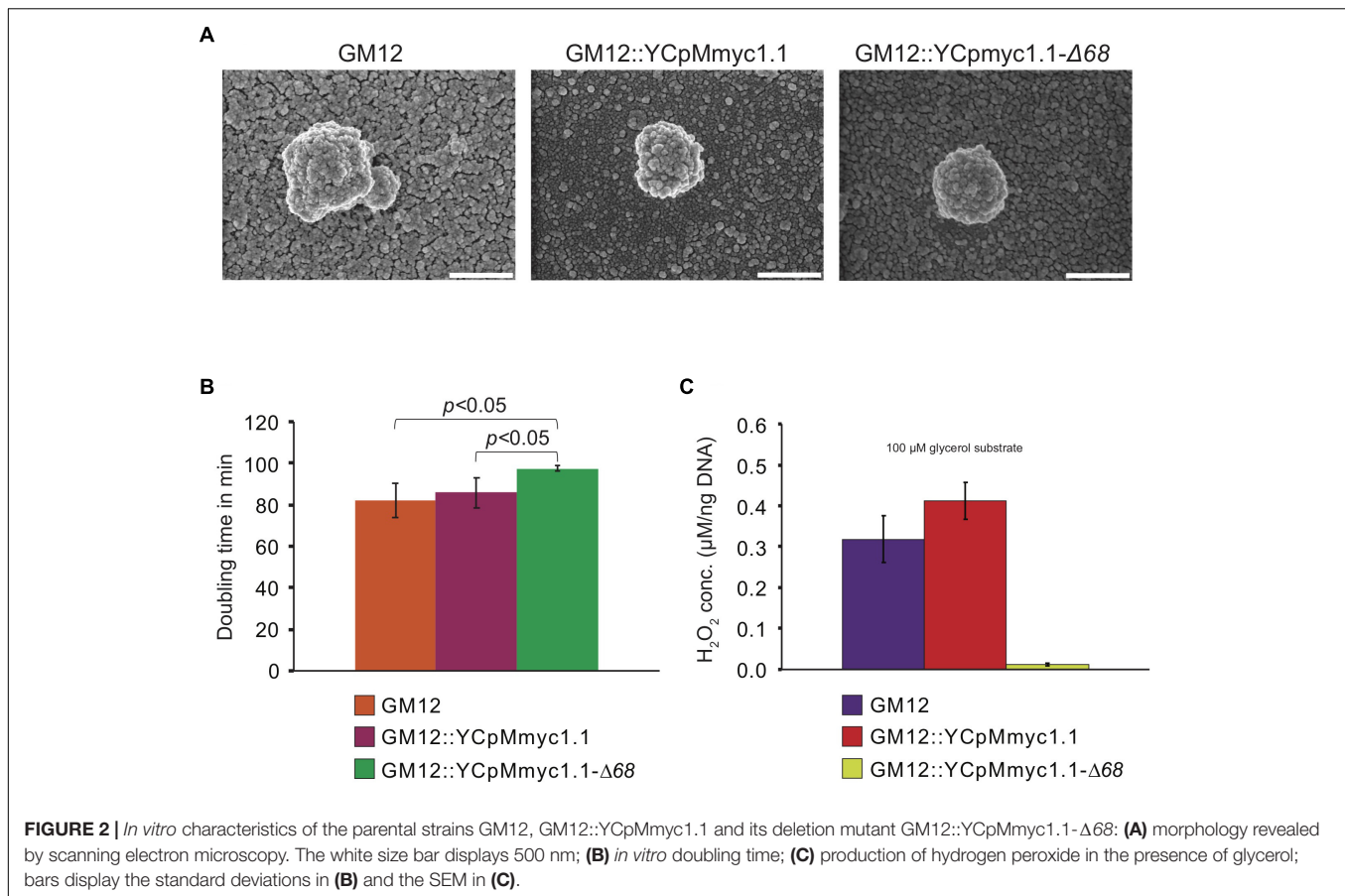
Inability of the Mutant Strain GM12::YCPMmyc1.1-Δ68 to Produce Hydrogen Peroxide in the Presence of Glycerol *in vitro*

This pathway was completely deleted in the construction of the mutant strain GM12::YCPMmyc1.1-Δ68. Therefore to phenotypically confirm the deletion, we measured and

TABLE 2 | Summary of post mortem records of goats.

Animal ID.	Date of euthanasia	Bacteremia ccu/ml	Inflammation of the neck around the injection site	Pulmonary congestion	Pulmonary oedema	Congested kidneys	Mucoid enteritis and congestion	Pleural fluid in thoracic cavity	Adherent lung to rib cage	Liver abscess
CK032	6 dpi	10 ⁷	X	X	X	X	X	X		
CK034	5 dpi	10 ⁷	X	X	X	X	X			
CK040	5 dpi	10 ⁸	X	X	X	X	X			X
CK043	5 dpi	10 ⁸	X	X	X	X	X			
CK046	5 dpi	10 ⁸	X	X	X	X	X			
CK048	5 dpi	10 ⁹	X	X	X	X	X			
CK051	5 dpi	10 ⁹	X	X	X	X	X			
CL002	5 dpi	10 ⁸	X	X	X	X	X		X	
CK035	29 dpi									
CK045	28 dpi									
CK047	29 dpi									
CK049	28 dpi									
CL001	28 dpi									
CL003	28 dpi									

Animals displayed in bold were infected with GM12::YCPMmyc1.1-Δ68, the other animals were infected with GM12; dpi, days post infection; ccu, color changing unit.



compared hydrogen peroxide production levels between the control GM12, GM12::YCpMmyc1.1 and GM12::YCpmyc1.1-Δ68 *in vitro*. In the presence of the glycerol substrate, the mutant GM12::YCpmyc1.1-Δ68 shows a significant decrease in hydrogen peroxide production when compared to its parental strains (Figure 2C). Indeed, while GM12 and GM12::YCpMmyc1.1 produced >0.3 μM of H₂O₂, the mutant strain produced very low amounts of H₂O₂ (0.01 μM), at least 30-fold lower under these conditions.

Inability of the Mutant Strain GM12::YCpMmyc1.1-Δ68 to Degrade Immunoglobulin *in vitro*

Another potential virulence trait encoded by mycoplasmas is the MIB-MIP system, which may play a role in immune evasion by cleavage of immunoglobulins (Figure 3A) (Arfi et al., 2016). Incubation of caprine IgG with GM12, GM12::YCpMmyc1.1 and GM12::YCpmyc1.1-Δ68 showed a clear difference in the strains' abilities to degrade IgG (Figure 3B). The two bands at 26 and 55 kDa corresponds to the IgG light and heavy chains. The mutant strain GM12::YCpmyc1.1-Δ68 exhibited no degradation of IgG, as noted by the lack of the 44 kDa band (Lane 3 of Figure 3B, black asterisk). This band, clearly visible in the other two strains, is indicative of proteolytic cleavage of the IgG heavy chain. Another pattern of degradation, with a

band at a size of about 30 kDa, is visible in the three strains. It was previously reported that this IgG cleavage is not specific or directly linked to the MIB-MIP system (Arfi et al., 2016).

The Mutant Strain GM12::YCpMmyc1.1-Δ68 Is Fully Attenuated *in vivo*

We next tested whether GM12::YCpmyc1.1-Δ68 was able to cause disease in its native host. Sixteen male outbred goats (*Capra aegagrus hircus*) were used in this animal infection trial. The animals were separated into two groups of equal numbers. After the infection, no immediate clinical signs of disease were observed. Two animals in the GM12::YCpmyc1.1-Δ68 group had to be removed from the experiment, because of acquired wounds unrelated to the infectious agent. Animal euthanasia was planned 28 days post infection (dpi). However, all eight animals inoculated with the GM12 strain developed severe clinical signs, with pyrexia starting 2–3 dpi (Figure 4B). Their body temperature continued to increase, up to 41–41.5°C, during the following days (Supplementary Table S2). The animals stopped feeding, were apathetic and showed signs of pain. According to the endpoint criteria stated in the animal experiment protocol, they had to be euthanized between 5–6 dpi (Figure 4A, red line). In sharp contrast, animals inoculated with the GM12::YCpmyc1.1-Δ68 mutant strain did not develop any

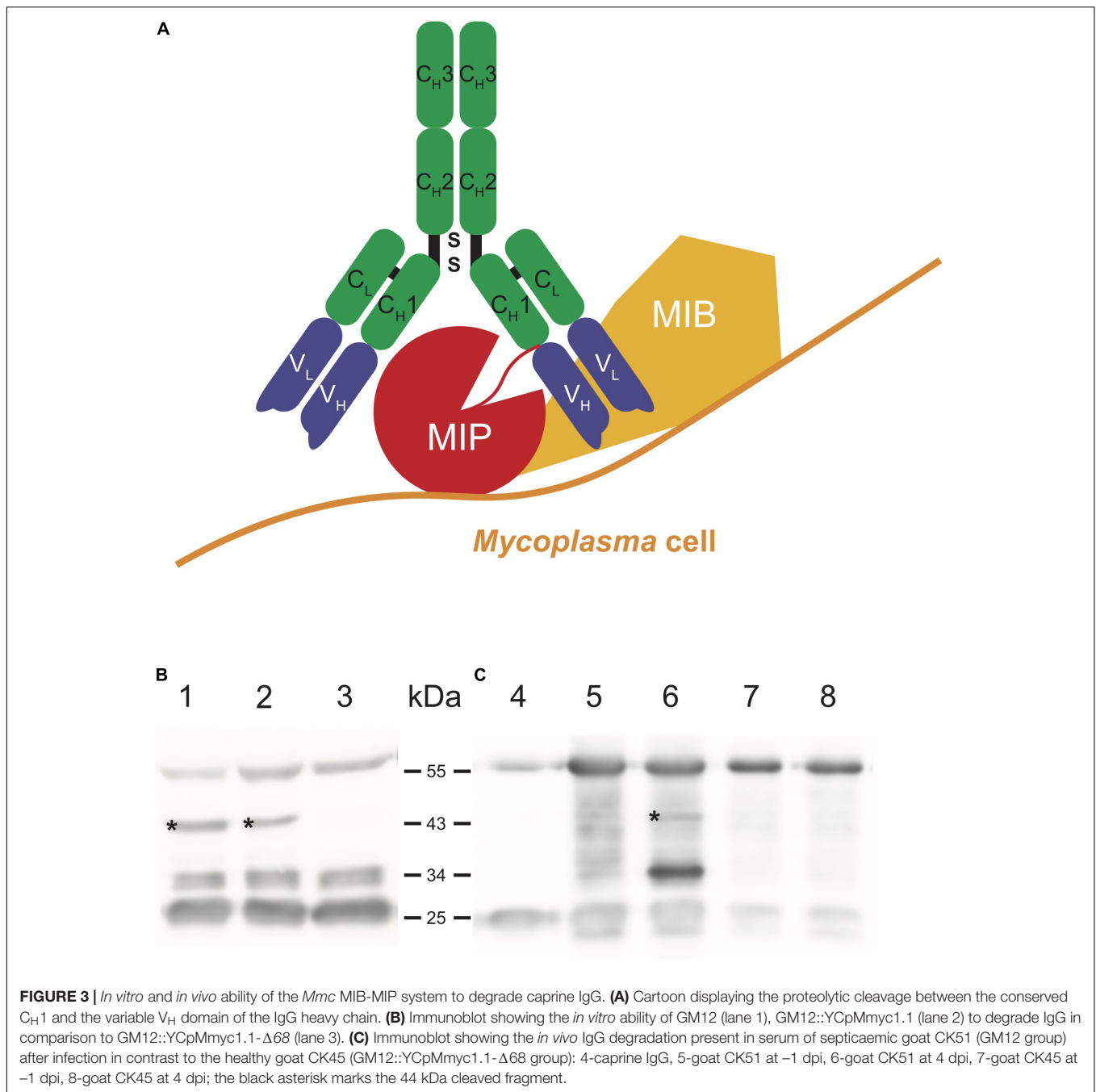
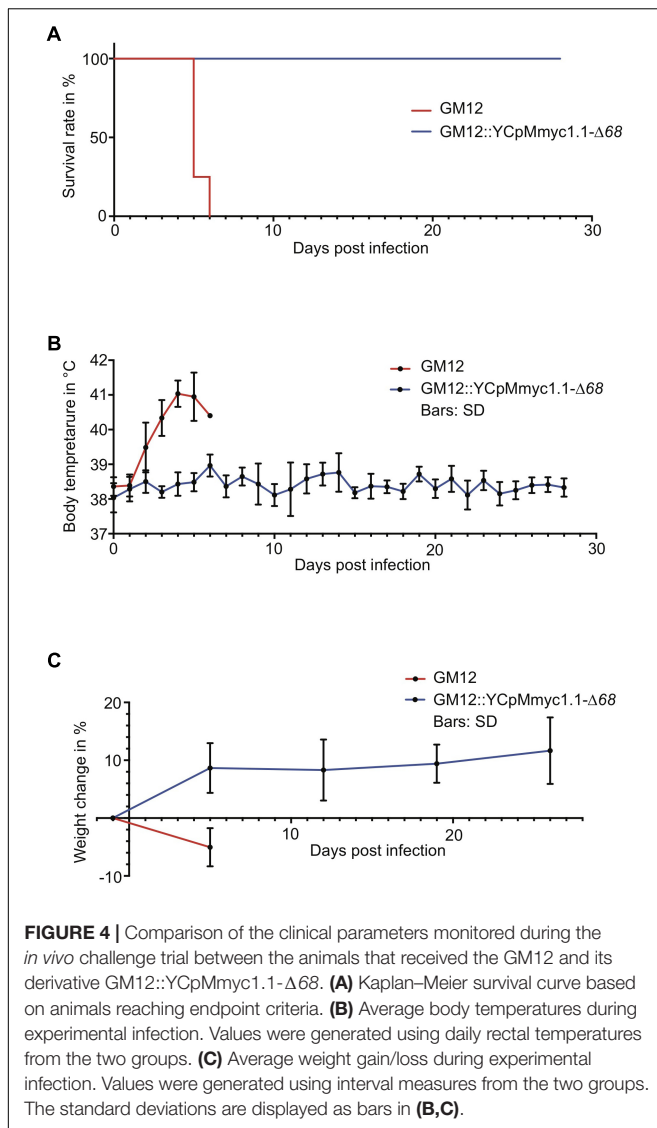


FIGURE 3 | *In vitro* and *in vivo* ability of the *Mmc* MIB-MIP system to degrade caprine IgG. **(A)** Cartoon displaying the proteolytic cleavage between the conserved C_H1 and the variable V_H domain of the IgG heavy chain. **(B)** Immunoblot showing the *in vitro* ability of GM12 (lane 1), GM12::YCpMmyc1.1 (lane 2) to degrade IgG in comparison to GM12::YCpMmyc1.1-Δ68 (lane 3). **(C)** Immunoblot showing the *in vivo* IgG degradation present in serum of septicemic goat CK51 (GM12 group) after infection in contrast to the healthy goat CK45 (GM12::YCpMmyc1.1-Δ68 group): 4-caprine IgG, 5-goat CK51 at -1 dpi, 6-goat CK51 at 4 dpi, 7-goat CK45 at -1 dpi, 8-goat CK45 at 4 dpi; the black asterisk marks the 44 kDa cleaved fragment.

clinical signs of disease and were all monitored until the end of the trial (Figure 4A). Their body temperature fluctuated within the normal physiological range throughout the study period (Figure 4B and Supplementary Table S2). The animals remained healthy and gained weight during the experiment (Figure 4C). Their heartbeat and respiratory rates, between 80–110 beats/min and 20–30 breaths/min, respectively, remained constant over the course of experimentation.

In all animals infected with the GM12 strain, the main pathological lesions were similar, with a severe and extensive inflammation of the soft tissues of the neck around the

site of inoculation. Additional macroscopic findings were severe pulmonary edema and congestion. Histologically, there was extensive coagulation necrosis of the connective tissue (Figure 5B, thick arrows) and musculature surrounding the trachea, in the vicinity of the inoculation site (Figures 5B,D, diamonds). A marked infiltration of mainly degenerate neutrophilic granulocytes (Figures 5B,D, asterisks) was always found associated with the necrosis. The necrotizing process extended to the trachea, the subcutis and skin. In addition, all animals showed multifocal acute necrosis with infiltration of neutrophilic granulocytes in liver and



kidney and for three animals, in the lung. These lesions were indicative of an acute septicemia. Among all animals infected with the mutant strain GM12::YCpMmyc1.1-Δ68, and upon euthanasia 28–29 dpi, no pathological lesions were found around the inoculation site. The soft tissue around the inoculation site was within normal limits. Additionally, neither inflammation nor necrosis associated with the epithelium, the submucosa or any cartilage tissues was histologically observed (**Figures 5A,C**).

Mmc GM12 was re-isolated from the blood of all animals experimentally infected with the wild-type parental strain. Bacteremia was characterized by 10^6 up to 10^9 CCU.ml⁻¹ of blood, as measured by serial dilutions (**Table 2**). We have not been able to re-isolate the deletion mutant from all blood samples collected from goats infected with GM12::YCpMmyc1.1-Δ68. Additionally, we were not able to re-isolate the mutant strain from tissue samples collected post mortem.

The MIB-MIP System in *Mmc* GM12 Is Functional *in vivo*

The MIB-MIP system was shown to be active *in vitro*, and to be present in large amounts at the cell surface (Krasteva et al., 2014) during infection (Weldearegay et al., 2016). Two animal sera were selected from the *in vivo* infection trial: CK51 from the GM12 group and CK45 from the GM12::YCpMmyc1.1-Δ68 group and tested for cleavage of IgG. The pre-infection sera from both animals demonstrated no proteolytic cleavage of IgG when compared to the IgG control (**Figure 3C**). Conversely, the post-infection serum of CK51, which had succumbed to disease and had a high titer of bacteria in the venous blood, clearly exhibited the typical band at 44 kDa, consistent with the size of a cleaved IgG heavy chain by the MIB-MIP system. As expected, no band at 44 kDa was seen in the post-infection serum of CK45. This clearly demonstrates, for the first time, that the MIB-MIP system is functional within the caprine host and that its proteolytic activity is triggered during an *Mmc* infection.

DISCUSSION

The first aim of this work was to fully attenuate a highly pathogenic strain of *M. mycoides* following a rational deletion design. The second aim was to verify this attenuation *in vivo* using the native host, since no rodent animal models for highly virulent *M. mycoides* exist (Jores et al., 2013). Many candidate virulence factors of *M. mycoides* have been suggested but, none except the capsular polysaccharide (Jores et al., 2018) have ever been confirmed according to Falkow's postulates *in vivo* (Falkow, 1988).

In order to generate an attenuated strain, we relied on previous knowledge and selected five genomic regions that encode candidate virulence traits. These regions, distributed around the *Mmc* genome, comprised of 68 genes. The first two regions (D1 and D2) encode enzymes and putative glycerol transporters involved in the production of hydrogen peroxide using the glycerol-dependent metabolism of mycoplasmas (Blotz and Stulke, 2017). The region D3 encodes major antigens (LppA/P72) in *M. mycoides* (Monnerat et al., 1999) that induced T cell responses early in infection (Dedieu et al., 2010). The region D4 includes an integrative conjugative element (ICE), the MIB-MIP system (Arfi et al., 2016) and the F1-likeX0 ATPase (Beven et al., 2012). The last region (D5) encodes six lipoproteins including the lipoprotein Q (LppQ), which has been suspected to be involved in exacerbating immune responses and other virulence determinants (Mulongo et al., 2015). Interestingly, among these 68 genes deleted in our mutant genome, initially comprising 944 genes and annotated RNAs, 67 were defined as non-essential in the JCVI-Syn3.0 minimal cell, a study in which 432 genes were classified as non-essential to sustain the life of a minimal *Mycoplasma* cell only supported at the end by 438 proteins and 35 annotated RNAs (Hutchison et al., 2016). Only one gene encoding the glycerol phosphate kinase (GlpK) (MMCAP2_0218, region D1) was retained in the minimal JCVI-Syn3.0 cell and classified as a “quasi-essential” gene. This means that the function encoded by the glycerol kinase, i.e., the transfer of a phosphate

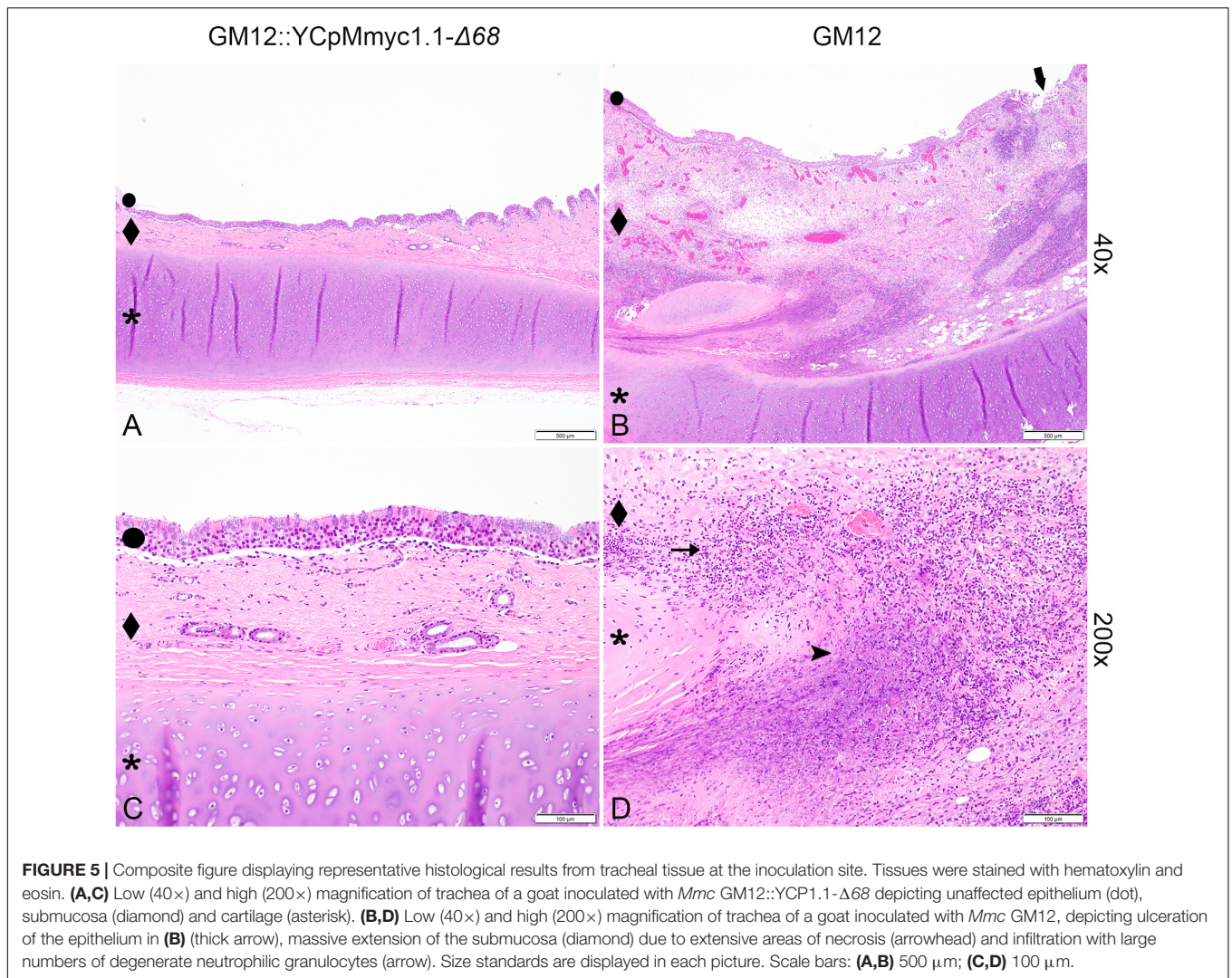


FIGURE 5 | Composite figure displaying representative histological results from tracheal tissue at the inoculation site. Tissues were stained with hematoxylin and eosin. **(A,C)** Low (40 \times) and high (200 \times) magnification of trachea of a goat inoculated with *Mmc* GM12::YCP1.1- Δ 68 depicting unaffected epithelium (dot), submucosa (diamond) and cartilage (asterisk). **(B,D)** Low (40 \times) and high (200 \times) magnification of trachea of a goat inoculated with *Mmc* GM12, depicting ulceration of the epithelium in **(B)** (thick arrow), massive extension of the submucosa (diamond) due to extensive areas of necrosis (arrowhead) and infiltration with large numbers of degenerate neutrophilic granulocytes (arrow). Size standards are displayed in each picture. Scale bars: **(A,B)** 500 μ m; **(C,D)** 100 μ m.

group on the glycerol molecule to produce glycerol-3-phosphate, could be compensated for by another gene which encodes a similar function in the full-length genome. The compensatory gene for *glpK* is probably absent in the minimal JCVI-Syn3.0 cell, but present in our mutant GM12::YCP1.1- Δ 68, allowing the deletion of *glpK* without any defect in growth. Therefore, our results are consistent with the quasi-essentiality of *glpK*. Altogether we retained 69 out of the 87 lipoproteins present in GM12 by deleting 1, 3, 8, and 6 lipoproteins in D2, D3, D4, and D5, respectively, while the minimal cell retained only 15 lipoproteins (Hutchison et al., 2016).

It was paramount for us that the mutant strain GM12::YCP1.1- Δ 68 maintains a doubling time similar to its parental strain, since we wanted to create a 'K12-like' strain that can be further used as a cellular platform to introduce antigen-encoding genes or large stretches of DNA. It was interesting for us to observe that, despite complete removal of the glycerol pathway, which may be important for cell metabolism, there was no compelling impact on *in vitro* growth. It is known that there is a trade-off between genome size and growth rate.

The drastic deletions in the genome of JCVI-syn3.0 strain led to a substantial increase in the generation time, from \sim 60 to \sim 180 min (Hutchison et al., 2016). Recently, we also have shown that the deletion of a gene encoding an enzyme important for synthesis of carbohydrates can subsequently lead to an increase in the generation time (Schieck et al., 2016). However, in this work, we significantly reduced the genome of GM12 by more than 100 kbp (i.e., 106,737 bp) without seeing any compelling difference in the growth rate of GM12::YCP1.1- Δ 68 in comparison to the wild-type strain. Still, this reduction represents \sim 10% of the initial genome size confirming that, in addition to the size of the deletions, the nature of the genes deleted is also very likely to influence the generation time of mycoplasmas.

The main goal of this work was to construct a fully attenuated *Mmc* strain, that is safe to handle in the laboratory. The first confirmation of the attenuation of the GM12::YCP1.1- Δ 68 strain was obtained *in vitro*. The production of hydrogen peroxide was almost completely abolished in the mutant strain, confirming the participation of the *glpFKO* and/or

gtsABCD pathways in the metabolism of glycerol. In addition, the loss of the IgG specific cleavage band at 44 kDa in GM12::YCpMmyc1.1-Δ68 confirmed the role of the MIB-MIP system in the degradation of the host immunoglobulins. The mutant GM12::YCpMmyc1.1-Δ68 is essentially a quasi-intermediate to the minimal cell for which a high degree of genome stability was reported. The stable reproduction of the *in vitro* characteristics of GM12::YCpMmyc1.1-Δ68 also strongly supports stability of this mutant.

To confirm the mutant strain's attenuation *in vivo*, we developed an animal challenge model using Kenyan goats, outbred animals derived from different herds. The use of such animals increased variability to get a better idea of reproducibility and significance of the results (Richter et al., 2010). Similar challenge models using either 7.3×10^7 or 10^{12} CFUs of *M. mycoides* subsp. *capri* have been reported from previous challenge experiments (Sunder et al., 2002; Manimaran et al., 2006).

Animals infected with the GM12 strain developed specific clinical signs (fever, heavy breathing, septicemia, etc.) and were all euthanized by 6 dpi. Strikingly, none of the goats infected with GM12::YCpMmyc1.1-Δ68 developed such signs and were healthy for the entire course of the experimentation. These results exceeded our expectations and confirmed the complete abolishment of pathogenicity of the mutant strain GM12::YCpMmyc1.1-Δ68. In addition, the massive septicemia associated with very high titers of *Mycoplasma* observed in animals infected with the GM12 strain prompted us to investigate whether the MIB-MIP system would leave signatures of its action on immunoglobulins (Ig) in the serum of an animal (CK51) that had a titer of 10^9 CFU/ml. Specific IgG cleavage, characteristic of the MIB-MIP system (Arfi et al., 2016), was observed. No such cleavage was observed in the serum of animals infected with the mutant strain. This work shows, for the first time, that the MIB-MIP system of *M. mycoides* is functional *in vivo*. *Mycoplasmas* have been viewed as stealth pathogenic organisms because they lack most of the immune activators or PAMPs found in other bacteria (Mogensen, 2009). Indeed, the lack of a cell wall or the capacity to produce either LPS or flagellins likely contribute to the chronicity of infection. The only PAMP that has been described for several *Mycoplasma* species is the surface lipoproteins, abundant components of their membrane (Chambaud et al., 1999). In the present study, we suggest another mechanism that could contribute to activating the immune system. Indeed, Ig cleaved by several bacteria, including those generated by *Mycoplasma hyorhinitis*, have been described as ligands of the innate immune receptor LILRA2 (Hirayasu et al., 2016). Once bound to this receptor, it triggers the activation of the innate immune system. It is also possible that this cleavage is in line with the 'nutritional virulence' of the parasite (Abu Kwaik and Bumann, 2013). The exact significance of the Ig cleavage regarding *Mycoplasma* infection of mucosal surfaces remains to be studied. We did not include a Sham group in our study but recent results obtained from a transtracheal challenge of goats with *M. capricolum* subsp. *capripneumoniae* did clearly show no effect of *Mycoplasma* medium and PBS injected transtracheally as expected (Liljander et al., 2019).

Interestingly, we observed severe inflammation around the site of injection in the animals that received GM12 whereas animals that were injected with the strain GM12::YCpMmyc1.1-Δ68 developed no such pathomorphological lesions. Overwhelming immune reactions at the site of vaccination have been reported from immunizations against contagious bovine pleuropneumonia using live *M. mycoides* subsp. *mycoides* based vaccines such as T1/44, which is the closest relative of *Mmc* (Fischer et al., 2012). Therefore, it is likely that any one or several of the deleted genes encode proteins that drive this overwhelming immune reaction in the GM12.

To conclude, we confirmed, *in vitro* and *in vivo*, our ability to design a fully attenuated strain *via* the precise reduction of ~10% of the *Mmc* genome. However, we cannot currently pinpoint the weight of each deletion on the observed attenuation. The total clearance of the pathogen and the absence of a compelling humoral immune response, even at the inoculation point, is surprising and supports the total abolishment of pathogenicity. Now it is necessary to test more defined mutants such as a *glpOKF* mutant strain to get clarity about its real role in pathogenicity.

In addition, the design of next generation vaccines for, but not restricted to, *Mycoplasma* diseases will benefit from this study since a chassis that is fully attenuated and able to accommodate antigens for vaccine delivery that can be constructed based on our deletion mutant is now within reach. To induce a proper immune response via such a chassis, we have the option to add genes that appropriately stimulate an inflammatory immune response or alternatively, we can construct different chassis that direct responses toward Th1 or Th2 using TLR agonists. We consider a genetically modified *Mycoplasma* less problematic than other potential chassis since the survival time of *Mycoplasma* in general in the environment is very short. In addition, the unconventional codon usage (where UGA encodes tryptophan) and high AT content of *Mycoplasma* minimizes the risk of spread of genes to other bacteria. Additional experiments are necessary to decipher the role of individual virulence traits to understand these minimal bacterial pathogens better and to develop next generation rationale vaccines. Regardless, this study provides an attractive blueprint toward these goals, especially for those that are needed in low and middle-income countries.

AUTHOR CONTRIBUTIONS

JJ and SV designed the research. LM, NA-G, SC, JJ, AL, PS, MS, ES, VC, YA, HP, and FL performed the research. JJ, FL, SC, YA, LF, PS-P, CL, AB, and SV analyzed the data. JJ and SV wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00664/full#supplementary-material>

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