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

Joerg Jores, Li Ma, Paul Ssajjakambwe, Elise Schieck, Anne Liljander, Suchismita Chandran, Michael H. Stoffel, Valentina Cippa, Yonathan Arfi, Nacyra Assad-Garcia, et al.

To cite this version:

Joerg Jores, Li Ma, Paul Ssajjakambwe, Elise Schieck, Anne Liljander, et al.. Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent Mycoplasma Strain. Frontiers in Microbiology, Frontiers Media, 2019, 10, pp.664. 10.3389/fmicb.2019.00664. hal-02629101
Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent Mycoplasma Strain

Joerg Jores1,2*, Li Ma3†, Paul Ssajjakambwe2,7, Elise Schieck2, Anne Liljander2, Suchismita Chandran3, Michael H. Stoffel5, Valentina Cippa1, Yonathan Arfi6,7, Nacyra Assad-Garcia3, Laurent Falquet8, Pascal Sirand-Pugnet6,7, Alain Blanchard6,7, Carole Lartigue6,7, Horst Posthaus9, Fabien Labroussaa1 and Sanjay Vashee3

OPEN ACCESS

Edited by:
Thomas Dandekar,
University of Würzburg, Germany

Reviewed by:
Rajneesh Rana,
Indian Veterinary Research Institute (IVRI), India
Chih-Horng Kuo,
Academia Sinica, Taiwan

*Correspondence:
Joerg Jores
joerg.jores@vetsuisse.unibe.ch

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Microbiology

Received: 22 January 2019
Accepted: 18 March 2019
Published: 03 April 2019

Citation:
Jores J, Ma L, Ssajjakambwe P, Schieck E, Liljander A, Chandran S, Stoffel MH, Cippa V, Arfi Y, Assad-Garcia N, Falquet L, Sirand-Pugnet P, Blanchard A, Lartigue C, Posthaus H, Labroussaa F and Vashee S (2019) Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent Mycoplasma Strain. Front. Microbiol. 10:664. doi: 10.3389/fmicb.2019.00664

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* (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\(\alpha\)his3-\(\Delta200\) trp1-\(\Delta1\) KIURA3-\(\Delta1\) 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 background 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 FastQC\(^1\). 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 (dH\(_2\)O) and fixed with 4% para-formaldehyde (Life Technologies, Thermo Fisher, Zug, Switzerland; Cat. No. 28906) in dH\(_2\)O 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-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 (dithimethylarsinic acid sodium salt trihydrate), washed 3 times with dH\(_2\)O and postfixed with 1% OsO\(_4\) (Polysciences, Warrington, PA, United States) in 0.1 M cacodylate buffer for 15 min. at room temperature. Five additional washes with dH\(_2\)O 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/
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 10⁹ 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::YCpMyc1.1–Δ68 liquid culture (equivalent to 10⁹ 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. Thereafter, they received an intravenous injection of Lethabarb Euthanasia Injection (Virbac, United States) of 200 mg.kg⁻¹ body weight. Severe disease and pain were determined by a fever of ≥41°C for >3 consecutive days, an oxygen saturation of ≤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.

**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 10⁹ 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::YCpMyc1.1–Δ68 liquid culture (equivalent to 10⁹ 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 Lethabarb Euthanasia Injection (Virbac, United States) of 200 mg.kg⁻¹ body weight. Severe disease and pain were determined by a fever of ≥41°C for >3 consecutive days, an oxygen saturation of ≤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 μm 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 \textit{Mmc} as described elsewhere (Liljander et al., 2015) using \textit{Mycoplasma} liquid medium (Mycoplasma Experience Ltd., United Kingdom). Lung samples and pleural fluid were used for screening of \textit{Pasteurella} and \textit{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). \textit{P}-values for differences in parameters were estimated using a 2-sided 2-sample \textit{t}-test comparing average levels between both groups at 5\% level of significance.

RESULTS

Generation of the Mutant Strain

\textbf{GM12::YCpMmyc1.1-Δ68}

To demonstrate attenuation of \textit{Mmc} by rational design, five genomic regions were targeted in this study. These modifications were done on the genome GM12::YCpMmyc1.1 cloned in \textit{S. cerevisiae}. This genome has been obtained after the insertion of genetic elements (i.e., ARSH4, CEN6, and HIS3) in the genome of \textit{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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Design of mutant GM12::YCpMmyc1.1-Δ68. Cartoon displaying the genomes of the parental strain GM12::YCpMmyc1.1 and its derivative, the deletion mutant GM12::YCpMmyc1.1-Δ68.}
\end{figure}
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::Y CpMmyc1.1-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::Y CpMmyc1.1-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

| Animal ID | Date of euthanasia | Bacteremia | Inflammation | 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             | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK034     | 5 dpi             | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK040     | 5 dpi             | 10⁸        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK043     | 5 dpi             | 10⁸        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK046     | 5 dpi             | 10⁸        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK048     | 5 dpi             | 10⁸        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK051     | 5 dpi             | 10⁹        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CL002     | 5 dpi             | 10⁸        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK035     | 29 dpi            | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK045     | 28 dpi            | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK047     | 29 dpi            | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CK049     | 28 dpi            | 10⁷        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CL001     | 28 dpi            | 10⁹        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |
| CL003     | 28 dpi            | 10⁹        | X            | X                   | X               | X                | X                          | X                          | X                         | X           |

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.
FIGURE 2 | In vitro characteristics of the parental strains GM12, GM12::YCpMmyc1.1 and its deletion mutant GM12::YCpMmyc1.1-Δ68: (A) morphology revealed by scanning electron microscopy. The white size bar displays 500 nm; (B) in vitro doubling time; (C) production of hydrogen peroxide in the presence of glycerol; bars display the standard deviations in (B) and the SEM in (C).

compared hydrogen peroxide production levels between the control GM12, GM12::YCpMmyc1.1 and GM12::YCpMmyc1.1-Δ68 in vitro. In the presence of the glycerol substrate, the mutant GM12::YCpMmyc1.1-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::YCpMmyc1.1-Δ68 showed a clear difference in the strains’ abilities to degrade IgG (Figure 3B). The two bands at 26 and 55 kDa correspond to the IgG light and heavy chains. The mutant strain GM12::YCpMmyc1.1-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::YCpMmyc1.1-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::YCpMmyc1.1-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::YCpMmyc1.1-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 CH1 and the variable VH 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 septicaemic 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 introduced into a 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
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::YCpMmyc1.1-\( \Delta 68 \), 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::YCpMmyc1.1-\( \Delta 68 \) 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 \textit{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::YCpMmyc1.1-\( \Delta 68 \) 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::YCpMmyc1.1-\( \Delta 68 \) strain was obtained \textit{in vitro}. The production of hydrogen peroxide was almost completely abolished in the mutant strain, confirming the participation of the \( glpFKO \) and/or
gm12 were euthanized by 6 dpi. Strikingly, none of the goats infected with gm12 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. In addition, the massive septicemia 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 determinants 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 hyorhinis, 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 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.

**FUNDING**

This research was supported by National Science Foundation [Grant Number IOS-1110151 (to SV, CL, and JJ)]. Additional support was received from the University of Bern and the CGIAR research program on Livestock and Fish. ES was supported by the German
Federal Ministry for Economic Cooperation and Development (Project No. 09.7860.1-001.00 Contract No. 81136800). AL was supported by the Centrum for International Migration and Development in Germany.

ACKNOWLEDGMENTS

We thank Ray-Yuan Chuang, Vladimir Noskov, Steven Weber, Pamela Nicholson, Helga Mogel, Elisabeth Cook, and the ILRI animal caretakers for their excellent advice and help. This manuscript has been released as a preprint at bioRxiv (DOI: https://doi.org/10.1101/508978).

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
Manimaran, K., Singh, V. P., Ltu, K., Das, S., Kumar, A. A., and Srivastava, S. K. (2006). Development of lyophilized Mycoplasma mycoides subsp. capri vaccine against caprine pleuropneumonia. *Indian J. Anim. Sci.* 76, 900–902.

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* 22, 240–273. doi: 10.1128/CMR.00046-08

Monnerat, M. P., Thiaucourt, F., Poveda, J. B., Nicolet, J., and Frey, J. (1999). Genetic and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp. mycoides LC and *Mycoplasma mycoides* subsp. capri. *Clin. Diagn. Lab. Immunol.* 6, 224–230.

Mulongo, M., Frey, J., Smith, K., Schnier, C., Wesonga, H., Naessens, J., et al. (2015). Genetic and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp. mycoides LC and *Mycoplasma mycoides* subsp. capri. *Clin. Diagn. Lab. Immunol.* 6, 224–230.

Nicholas, R., and Churchward, C. (2012). Contagious caprine pleuropneumonia: new aspects of an old disease. *Transbound. Emerg. Dis.* 59, 189–196. doi: 10.1111/j.1865-1682.2011.01262.x

Noskov, V., Kourprina, N., Leem, S. H., Koriabine, M., Barrett, J. C., and Larionov, V. (2002). A genetic system for direct selection of gene-positive clones during recombinatorial cloning in yeast. *Nucleic Acids Res.* 30:E8. doi: 10.1093/nar/30.2.e8

Noskov, V. N., Segall-Shapiro, T. H., and Chuang, R. Y. (2010). Tandem repeat coupled with endonuclease cleavage (TREC): a seamless modification tool for genome engineering in yeast. *Nucleic Acids Res.* 38, 2570–2576. doi: 10.1093/nar/gqq099

Payne, R. W., Murray, D. A., Harding, S. A., Baird, D. B., and Soutar, D. M. (2012). *GenStat for Windows* (15th Edition) Introduction. Hemel Hempstead: VSN International.

Pilo, P., Frey, J., and Vilei, E. M. (2007). Molecular mechanisms of pathogenicity of *Mycoplasma mycoides* subsp. mycoides SC. *Vet. J.* 174, 513–521.

Rausch, T., Zichner, T., Schlattl, A., Stutz, A. M., Benes, V., and Korbel, J. O. (2012). DELLY: structural variant discovery by integrated paired-end and split-read analysis. *Bioinformatics* 28, 1333–1339. doi: 10.1093/bioinformatics/bts378

Richter, S. H., Garner, J. P., Auer, C., Kunert, J., and Wurbel, H. (2010). Systematic variation improves reproducibility of animal experiments. *Nat. Methods* 7, 167–168. doi: 10.1038/nmeth0310-167

Sacchini, F., Naessens, J., Awino, E., Heller, M., Hlinak, A., Haider, W., et al. (2011). A minor role of CD4+ T lymphocytes in the control of a primary infection of cattle with *Mycoplasma mycoides* subsp. mycoides. *Vet. Res.* 42:77. doi: 10.1186/1297-9716-42-77

Schieck, E., Lartigue, C., Frey, J., Vozza, N., Hegermann, J., Miller, R. A., et al. (2016). Galactofuranose in *Mycoplasma mycoides* is important for membrane integrity and conceals adhesins but does not contribute to serum resistance. *Mol. Microbiol.* 99, 55–70. doi: 10.1111/mmi.13213

Sunder, J., Srivastava, N. C., and Singh, V. P. (2002). Preliminary Trials on Development of Vaccine Against *Mycoplasma mycoides* subsp. *mycoides* type LC Infection in Goats. *J. Appl. Anim. Res.* 21, 75–80. doi: 10.1080/09712119.2002.9706359

Szczepanek, S. M., Boccaccio, M., Pflaum, K., Liao, X., and Geary, S. J. (2014). Hydrogen peroxide production from glycerol metabolism is dispensable for virulence of *Mycoplasma gallisepticum* in the tracheas of chickens. *Infect. Immun.* 82, 4915–4920. doi: 10.1128/IAI.02208-14

Tardy, F., Mick, V., Dordet-Frisioni, E., Marenda, M. S., Sirand-Pugnet, P., Blanchard, A., et al. (2015). Integrative conjugative elements are widespread in field isolates of *Mycoplasma species* pathogenic for ruminants. *Appl. Environ. Microbiol.* 81, 1634–1643. doi: 10.1128/AEM.03723-14

Thorvaldsdottir, H., Robinson, J. T., and Mesirov, J. P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief. Bioinform.* 14, 178–192. doi: 10.1093/bib/bbs017

Weldearegay, Y. B., Pich, A., Schiek, E., Liljander, A., Gicheru, N., Wesonga, H., et al. (2016). Proteomic characterization of pleural effusion, a specific host niche of *Mycoplasma mycoides* subsp. mycoides from cattle with contagious bovine pleuropneumonia (CBPP). *J. Proteomics* 131, 93–103. doi: 10.1016/j.jprot.2015.10.016

Zhang, Z., Wang, J., Luo, J., Ding, X., Zhong, J., Wang, J., et al. (2016). Sprites: detection of deletions from sequencing data by re-aligning split reads. *Bioinformatics* 32, 1788–1796. doi: 10.1093/bioinformatics/btw053

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Jores, Ma, Saajjakambwe, Schiek, Liljander, Chandran, Staffel, Cippa, Arfi, Assad-Garcia, Falquet, Sirand-Pugnet, Blanchard, Lartigue, Posthaus, Labroussea and Vashee. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.