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RESEARCH ARTICLE
Synthetic Biology

Attenuation of a Pathogenic *Mycoplasma* Strain by Modification of the *obg* Gene by Using Synthetic Biology Approaches

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ABSTRACT *Mycoplasma* species are responsible for several economically significant livestock diseases for which there is a need for new and improved vaccines. Most of the existing mycoplasma vaccines are attenuated strains that have been empirically obtained by serial passages or by chemical mutagenesis. The recent development of synthetic biology approaches has opened the way for the engineering of live mycoplasma vaccines. Using these tools, the essential GTPase-encoding gene *obg* was modified directly on the *Mycoplasma mycoides* subsp. *capri* genome cloned in yeast, reproducing mutations suspected to induce a temperature-sensitive (TS^+) phenotype. After transplantation of modified genomes into a recipient cell, the phenotype of the resulting *M. mycoides* subsp. *capri* mutants was characterized. Single-point *obg* mutations did not result in a strong TS^+ phenotype in *M. mycoides* subsp. *capri*, but a clone presenting three *obg* mutations was shown to grow with difficulty at temperatures of ≥40°C. This particular mutant was then tested in a caprine septicemia model of *M. mycoides* subsp. *capri* infection. Five out of eight goats infected with the parental strain had to be euthanized, in contrast to one out of eight goats infected with the *obg* mutant, demonstrating an attenuation of virulence in the mutant. Moreover, the strain isolated from the euthanized animal in the group infected with the *obg* mutant was shown to carry a reversion in the *obg* gene associated with the loss of the TS^+ phenotype. This study demonstrates the feasibility of building attenuated strains of mycoplasma that could contribute to the design of novel vaccines with improved safety.

IMPORTANCE Animal diseases due to mycoplasmas are a major cause of morbidity and mortality associated with economic losses for farmers all over the world. Currently used mycoplasma vaccines exhibit several drawbacks, including low efficacy, short time of protection, adverse reactions, and difficulty in differentiating infected from vaccinated animals. Therefore, there is a need for improved vaccines to control animal mycoplasmoses. Here, we used genome engineering tools derived from synthetic biology approaches to produce targeted mutations in the essential GTPase-encoding *obg* gene of *Mycoplasma mycoides* subsp. *capri*. Some of the resulting mutants exhibited a marked temperature-sensitive phenotype. The virulence of one of the *obg* mutants was evaluated in a caprine septicemia model and found to be strongly reduced. Although the *obg* mutant reverted to a virulent phenotype in one infected animal, we believe that these results contribute to a strategy that should help in building new vaccines against animal mycoplasmoses.

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Mycoplasma diseases continue to be a major problem in the livestock sector worldwide. Among the most serious diseases, one can cite contagious bovine and caprine pleuropneumonia, contagious agalactia in small ruminants, enzootic pneumonia in pigs, and chronic respiratory disease in poultry. The socioeconomic cost of these infectious diseases is due mostly to mortality, decreased productivity, trade restrictions, and the costs associated with disease control. The measures currently being pursued for curbing mycoplasma disease mainly rely on antibiotic treatments and prophylaxis, including vaccines if available. Vaccines constitute the most cost-effective measure to control livestock mycoplasma diseases, especially considering increasing antimicrobial resistance, as reported for *Mycoplasma* (1) and other bacteria.

Mycoplasma vaccines have been used for decades, but little progress has been made in developing vaccines that are more efficient and induce long-lasting immunity. Most commercially available mycoplasma vaccines are still made of attenuated mycoplasma strains, obtained after serial passaging of the pathogen, and are generally characterized by their lack of efficacy and the production of adverse reactions (for a review, see reference 2). This is particularly true for ruminant diseases caused by *Mycoplasma* species belonging to the *Mycoplasma mycoides* cluster (3). Recently, temperature-sensitive (TS^+) mutants have been widely used for the control of mycoplasmoses in poultry. Most of these vaccines were initially obtained by empirical chemical mutagenesis. This is the case for the attenuated strain of *Mycoplasma synoviae*, MS-H, that has been used as a commercial poultry vaccine (Vaxsafe MS; Bioproperties Pty. Ltd., Ringwood, Victoria, Australia) in several countries (4). The TS^+ phenotype has been proposed as a general strategy to design live vaccines (for a review, see reference 5). Interestingly, a genome comparison between *M. synoviae* strains has indicated that the temperature sensitivity of MS-H is associated with a single-nucleotide change resulting in a Gly123Arg substitution in the Obg sequence (6). The *obg* gene encodes an essential P-loop GTPase in all bacteria. The Obg protein (*Spo0B*-associated GTP-binding protein) acts as a sensor of the GDP/GTP pools and participates in central processes that affect essential cellular functions (7, 8). Recent studies also suggest a role connecting ribosome assembly with the stress response, since it was shown in *Escherichia coli* that Obg interacts with both the 50S ribosomal subunit and the stringent response alarmone (p)ppGpp (guanosine penta/tetraphosphate) (9).

The direct implication of *obg* mutations in the TS^+ phenotype in *Mycoplasma* species, including in *M. synoviae*, has not yet been completely demonstrated (10), as there is a lack of genetic tools for producing mutants. However, other studies conducted using model organisms such as *Escherichia coli* (8) and *Bacillus subtilis* (11) revealed that mutations of the Obg protein have been associated with a TS^+ phenotype. The Obg protein structure has been determined for *B. subtilis* (12) and for *Thermus thermophilus* (13). Interestingly, almost all mutations identified so far, associated with a TS^+ phenotype, have been located in the N-terminal part of the Obg protein (8, 14).

These results, combined with the universality of Obg in bacteria, reinforce the emergence of new therapeutic strategies targeting this essential bacterial GTPase (15). In this study, we used synthetic biology approaches to generate a series of targeted mutations in the *obg* gene of *Mycoplasma mycoides* subsp. *capri* strain GM12. These changes comprised the mutations known to induce a TS^+ phenotype in both *E. coli* and *B. subtilis* as well as the mutation suspected of being involved in the TS^+ phenotype of the *M. synoviae* vaccine strain. The temperature sensitivity of the resulting *M. mycoides* subsp. *capri* mutants was characterized *in vitro*. A mutant that showed the strongest TS^+ phenotype was selected and finally tested to evaluate its effect on virulence and pathogenicity using a caprine challenge model. Our results strongly suggest that this mutant is attenuated and demonstrate the feasibility of using synthetic biology ap-

proaches to design attenuated next-generation vaccines that express their normal repertoire of antigens and are safe.

RESULTS

Selection of the targeted mutations in the *M. mycoides* subsp. *capri obg* gene. The protein encoded by the *obg* gene is essential for cell viability in all bacterial species tested so far (16). For mycoplasma species, this essentiality was confirmed in *M. pulmonis* (17), *M. genitalium* (18), and *M. pneumoniae* (19). In the synthetic version of the *M. mycoides* subsp. *capri* genome constructed at the J. Craig Venter Institute (JCVI-syn1.0), the *obg* gene was shown to be essential in global transposon mutagenesis assays and was retained in the half-reduced genome of the derivative JCVI-syn3.0 (20). The *obg* gene in the genome of *M. mycoides* subsp. *capri* strain GM12 encodes a 433-amino-acid (aa) protein structured into three domains (see Fig. S1A in the supplemental material). An alignment of the entire *M. mycoides* subsp. *capri* Obg amino acid sequence with other bacterial Obg protein sequences shows only moderate conservation, with identity percentages ranging from 44.9% with *M. synoviae* Obg to 41.8% with *E. coli* and *B. subtilis* Obg and 37.7% with *T. thermophilus* Obg (Fig. S1B). However, the identity scores increase up to ~50% with *M. synoviae* and *B. subtilis* Obg proteins when considering the Obg fold and GTP-binding domains only (Fig. S1B). In *M. synoviae*, the mutation that has been associated with a TS^+ phenotype is a Gly123Arg substitution (6). We chose to produce the same mutation in the *M. mycoides* subsp. *capri obg* gene using two different changes in the nucleotide sequence: either GGG>AGA or GGG>CGT. In addition, we produced the mutations Gly80Glu (GGT>GAG) and Asp85Asn (GAT>AAC) that have been reported to be associated with a TS^+ phenotype in *B. subtilis* (Gly80Glu and Asp85Asn) (11), *E. coli* (Gly80Glu and Asp85Asn) (8), and *Caulobacter crescentus* (Gly80Glu only) (21) (Fig. 1A). We included two nucleotide changes per codon to limit the possibility of reversion during both the *in vitro* and *in vivo* cell propagation. It should be noted that even though the targeted Gly123, Gly80, and Asp85 residues are conserved between *M. mycoides* subsp. *capri*, *M. synoviae*, *B. subtilis*, and *E. coli*, the amino acid numbering slightly differs from one organism to another. Throughout this text, the numbering of the positions of the residues in the *M. mycoides* subsp. *capri* Obg sequence was adopted to avoid confusion.

Generation of *M. mycoides* subsp. *capri obg* mutants. Figure 1B shows the general experimental strategy conducted in this study that led to the generation of the different TS^+ *M. mycoides* subsp. *capri* mutants as well as the different validation methods used at each step. Each of the above-mentioned substitutions was introduced into the parental *M. mycoides* subsp. *capri* genome (strain GM12) formerly cloned into yeast (YCpMmyc1.1) (22), using the TREC-IN (tandem repeat endonuclease cleavage-insertion) method, an approach allowing seamless gene replacement in yeast (Fig. 1B, step 1, and Fig. S2). Yeast transformants were screened by PCR, and the amplicons covering the *obg* region were sequenced to confirm the presence of the designed mutations (Fig. S3). One of the yeast transformants (clone 43 [cl43]), isolated initially to obtain the GGG>AGA (Gly124Arg) mutant, carried additional point mutations. Indeed, this particular clone presented four mutations in total, three in the *obg* gene, GAT>GGT (Asp85Gly), GGG>AGA (Gly124Arg), and TAT>TGT (Ile244Val), and one in the downstream *nadE* gene that encodes a putative NH_3-dependent NAD^+ synthetase (CAA>CGA [Gln7Arg]). Because of the presence of mutations at two targeted positions (Gly124Arg and Asp85Gly), yeast clone 43 was subjected to further analysis. In order to take into account any potential impact of the substitution located on the *nadE* gene, an additional yeast clone (cl19) was constructed by repairing this substitution (Fig. S4). Genome integrity was verified by multiplex PCR and pulsed-field gel electrophoresis (PFGE) (Fig. S3 and S4), and six yeast clones (cl2, cl37, cl80, cl69, cl43, and cl19) carrying each the modified *M. mycoides* subsp. *capri* genomes were selected.

The modified *M. mycoides* subsp. *capri* genomes were isolated from the yeast clones and independently transplanted into McapΔRE recipient cells (Fig. 1B, step 2). The resulting transplants were serially cultured for three passages, and their genomes were

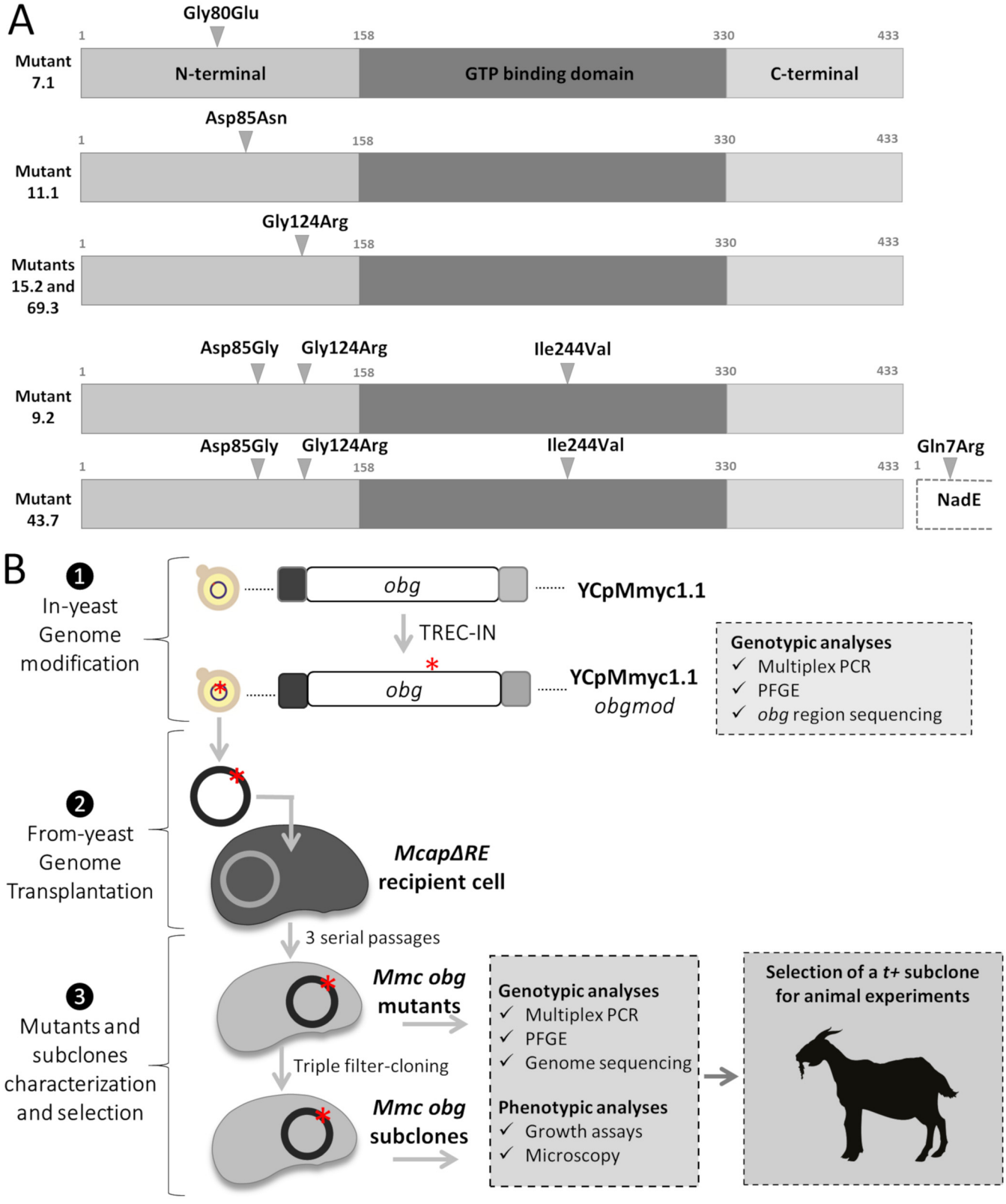

FIG 1 Global strategy used to produce *M. mycoides* subsp. *capri obg* mutants. (A) Residues targeted in the *M. mycoides* subsp. *capri* Obg protein during this study. The amino acid changes targeted in this study (Gly80Glu, Asp85Asn, and Gly124Arg) were all located in the N-terminal end of the Obg protein. The Gly80Glu (mutant 7.1) and Asp85Asn (mutant 11.1) substitutions were produced using the nucleotide changes GGT>GAG and GAT>AAC, respectively. The Gly124Arg substitution was produced using two different changes in the nucleotide sequence, either GGG>AGA (mutant 15.2) or GGG>CGT (mutant 69.3). Unexpectedly, one clone (43.7) from the plate for obtaining the Gly124Arg (GGG>AGA) substitution showed three other mutations, two in the *obg* gene, Ile244Val (TAT>TGT) and Asp85Gly (GAT>GGT), and one in the neighboring *nadE* gene, Gln7Arg (CAA>CGA). The latter mutation was repaired and resulted

(Continued on next page)

TABLE 1 *M. mycoides* subsp. *capri obg* mutants and subclones obtained in this study[c]

M. mycoides subsp. *capri obg* mutant	*obg* nucleotide change(s) (position)[a]	Obg amino acid change(s)[a]	NadE amino acid change	TS phenotype of mutant[b]	*M. mycoides* subsp. *capri obg* subclone	TS phenotype of clone[b]
11.1	GAT>AAC (253/255)	Asp85Asn		None	ND	ND
15.2	GGG>CGT (369/371)	Gly124Arg		None	ND	ND
69.3	GGG>AGA (369/371)	Gly124Arg		None	ND	ND
7.1	GGT>GAG (239/240)	Gly80Glu		+	7.1.2A	+
					7.1.3A	+
43.7	GAT>GGT (254), GGG>AGA (369/371), ATA>GTA (732)	Asp85Gly, Gly124Arg, Ile244Val	Gln7Arg (CAA>CGA)	+++	43.7.3E	+++
					43.7.4B	+++
9.2	GAT>GGT (254), GGG>AGA (369/371), ATA>GTA (732)	Asp85Gly, Gly124Arg, Ile244Val		+++	9.2.1A	+++
					9.2.3B	+++

[a]Numbering refers to the nucleotide and amino acid positions in the *M. mycoides* subsp. *capri obg* gene and protein.
[b]None, no TS^+ phenotype; +, weak TS^+ phenotype; +++, strong TS^+ phenotype (as determined by culture assays).
[c]ND, not determined.

sequenced to confirm the expected substitutions on the *obg* gene. Altogether, six *M. mycoides* subsp. *capri obg* mutants were finally selected for phenotypic characterization (Fig. 1B, step 3), with four presenting a single-amino-acid substitution (mutants 7.1, 11.1, 15.2, and 69.3) and two presenting multiple substitutions (mutants 43.7 and 9.2) (Fig. 1A and Table 1).

Phenotypic characterization of the *M. mycoides* subsp. *capri obg* mutants. The impact of the above-mentioned *obg* substitutions on *M. mycoides* subsp. *capri* growth was evaluated by combining culture assays and quantitative PCR (qPCR) analyses. All *M. mycoides* subsp. *capri obg* mutants were cultivated in standard SP5 mycoplasma medium at 32°C and 40°C together with the parental clone 30.1 (YCpMmyc1.1 with a wild-type [WT] *obg* region) as a control. The color change of the medium was first observed daily during 8 consecutive days (48-h and 144-h time points are shown in Fig. 2). Similarly to WT *M. mycoides* subsp. *capri* strain GM12 (data not shown), the control clone 30.1 grew slightly faster at 40°C than at 32°C and reached a maximum titer ($>10^9$ color-changing units [CCU] · ml^{-1}) after 48 h of incubation at 40°C. Unexpectedly, the *M. mycoides* subsp. *capri obg* mutants 11.1, 15.2, and 69.3 with the

FIG 1 Legend (Continued)
in mutant 9.2. (B) Scheme of the general experimental strategy followed in this study. The YCpMmyc1.1 genome was previously cloned into the yeast strain VL6-48N (7). The replacement of the essential *obg* gene in the YCpMmyc1.1 genome was achieved using TREC-IN, a three-step method allowing the seamless knock-in of the target gene(s) on bacterial genomes cloned into yeast (10). After modification, *M. mycoides* subsp. *capri* (*Mmc*) *obg*-mutated genomes (YCpMmyc1.1-*obgmod*) were isolated from yeast and transplanted into restriction-free *M. capricolum* subsp. *capricolum* recipient cells (McapΔRE) to obtain *M. mycoides* subsp. *capri obg* mutants characterized by mutation at a precise position within the *obg* gene. Mutants and filter-cloned derivatives were characterized *in vitro* (genotypic and phenotypic analyses). The *M. mycoides* subsp. *capri obg* subclone that showed the most temperature-sensitive phenotype (t^+) was selected for animal experiments using goats.

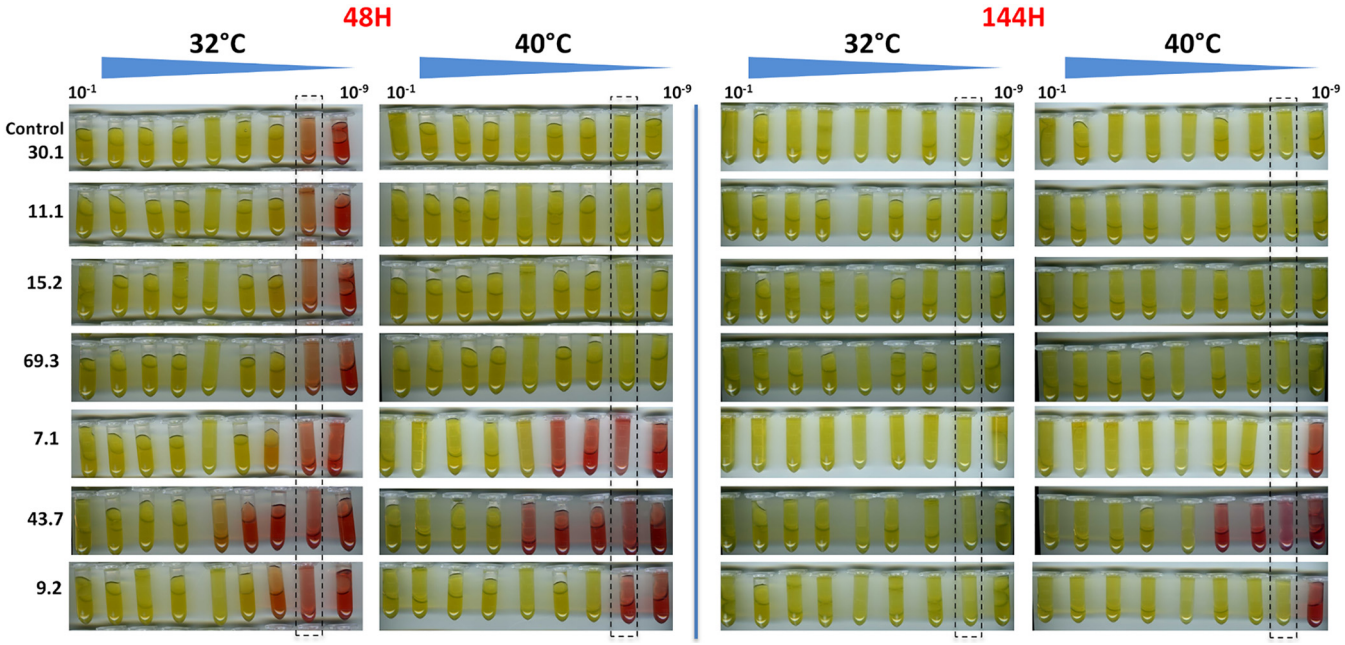

FIG 2 Growth monitoring of *M. mycoides* subsp. *capri obg* mutants at permissive (32°C) and nonpermissive (40°C) temperatures. *M. mycoides* subsp. *capri obg* mutants were first precultured in SP5 medium at 32°C. At pH 6.4, cell cultures were then serially diluted (10^{-1} to 10^{-9}) and incubated at either 32°C or 40°C for 8 consecutive days. SP5 medium color change from red to yellow evidenced cell propagation, since glucose utilization during *M. mycoides* subsp. *capri* propagation results in an acid shift. Pictures taken at 48 h and 144 h are shown, as indicated. Control 30.1 corresponds to YCpMmyc1.1 with an intact *obg* region. The dotted rectangles indicate tubes (dilution, 10^{-8}) from which the 100-μl aliquots were removed every 24 h from 0 h to 144 h to perform the qPCR analysis (Fig. 3A and B).

single-amino-acid changes Asp85Asn (GAT>AAC) and Gly124Arg (GGG>AGA or GGG>CGT) grew as well as the control at both temperatures tested and therefore did not show a TS^+ phenotype (Fig. 2). In contrast, the mutants (7.1, 43.7, and 9.2) with the single mutation Gly80Glu (GGT>GAG) and with the multiple mutations Asp85Gly (GAT>GGT), Gly124Arg (GGG>AGA), and Ile244Val (TAT>TGT), with or without a mutation in *nadE* (Gln7Arg, CAA>CGA), showed a marked TS^+ phenotype. Clone 43.7 was the most affected, with a 4-log lag behind the control clone 30.1 still after 144 h of culture at 40°C (Fig. 2).

As the color change of the medium does not precisely reflect the cell concentration in a mycoplasma culture, we performed 16S rRNA gene qPCR analyses during the *in vitro* culture. While the qPCR curves confirmed the results obtained by the color change culture assay (Fig. 3), they provided a more precise measure of mycoplasma growth. At 32°C, we observed that the curves were very similar between the *M. mycoides* subsp. *capri* control clone 30.1 and the different mutants tested (Fig. 3A). All bacterial cultures reached their stationary phase after 72 h of incubation, except for mutant 43.7, which was slightly delayed and reached its stationary phase after 96 h (24 h later). At 40°C (Fig. 3B), no growth difference was observed between mutants 11.1, 15.2, and 69.3 and the control strain 30.1. They all reached the stationary phase at the 48-h time point (T_{48}), confirming that *M. mycoides* subsp. *capri* grows faster at 40°C than at 32°C, but an ~1-log decrease in the final bacterial concentration was consistently measured (~10^8 cells · ml^{-1} at 40°C versus ~10^9 cells · ml^{-1} at 32°C) (Fig. 3A and B). Interestingly, the growth curves of three other mutants did not follow the trend of the control strain. At 40°C, the doubling time of mutants 7.1 and 9.2 appeared to be greatly affected, since these cells reached the stationary phase at T_{120} and T_{144}, respectively. In addition, mutant 43.7 did not appear to grow at all, since the bacterial concentration remained consistently below 10^3 cells · ml^{-1} (Fig. 3B), even after 144 h of incubation. However, according to Fig. 2, a color change from red to yellow could be observed for this mutant up to a 10^{-5} dilution, indicating a residual ability to grow. Consistent with this result, the qPCR measurements of the *in vitro* culture done with the 10^{-8} dilution did not

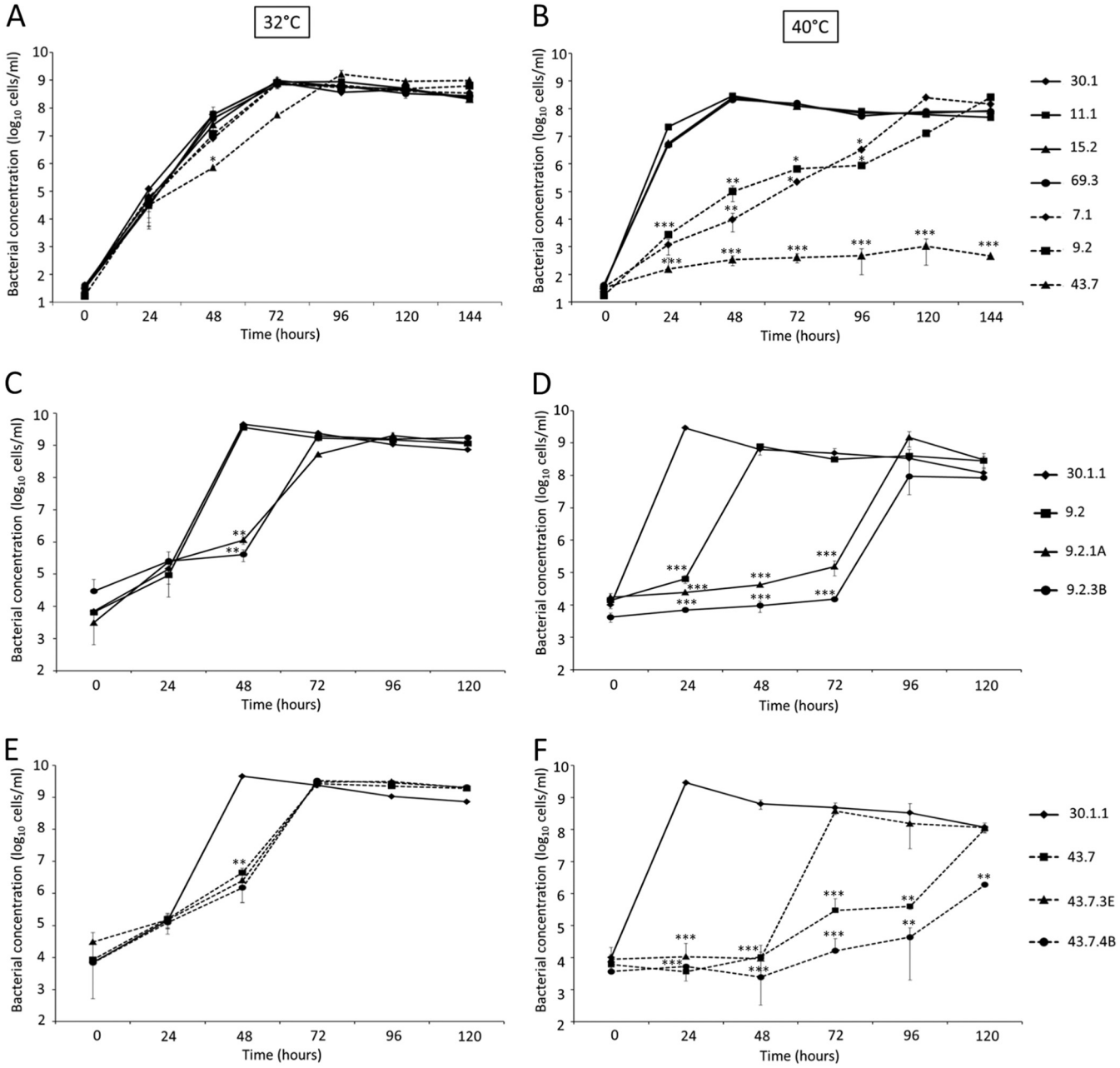

FIG 3 Temperature sensitivity of the different *M. mycoides* subsp. *capri obg* mutants and subclones as measured by qPCR. Bacterial concentrations of the different *M. mycoides* subsp. *capri obg* mutants and subclones were measured at 32°C (A, C, and E) and 40°C (B, D, and F) over time. (A and B) Cultures of the different *M. mycoides* subsp. *capri obg* mutants incubated at 32°C (A) or 40°C (B) were sampled every 24 h for up to 144 h, and bacterial titers were assessed using a qPCR assay targeting the 16S rRNA gene. (C to F) Similar experiments were performed to estimate the growth of individual subclones derived from the initial mutant 9.2 (C and D) or mutant 43.7 (E and F) for up to 120 h. Cultures monitored over time for qPCR assays are surrounded by dotted rectangles (Fig. 2 [for the mutants]; see also Fig. S5 in the supplemental material [for the subclones]). Errors bars represent standard deviations (SD) from three independent replicates. Statistical differences (*t* test) between the *M. mycoides* subsp. *capri* controls (30.1 or 30.1.1) and the different mutants were determined at each time point. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

reveal any growth (Fig. 2, dotted rectangles). Based on these results, mutants 7.1, 9.2, and 43.7 were selected for further analysis.

Genotypic and phenotypic analyses of the selected *M. mycoides* subsp. *capri obg* subclones. In order to eliminate the possibility of having heterogeneous populations, the three mutants (7.1, 9.2, and 43.7) were submitted to three rounds of filter cloning (Fig. 1B). After this procedure, a total of six subclones were selected for each of the three selected mutants and cultured in SP5 medium supplemented with tetracy-

cline prior to genotypic analysis. For mutants 7.1 and 43.7, all subclones showed an *obg* sequence identical to that of the parental clones. However, for mutant 9.2, the sequencing data of the 6 subclones revealed some sequence heterogeneity. While 4 subclones carried the expected the GAT>GGT (Asp85Gly), GGG>AGA (Gly124Arg), and TAT>TGT (Ile244Val) substitutions, 2 others (9.2.2 and 9.2.6) presented a different mutation in one of the targeted codons, GAT>AGT (Asp85Ser). This suggested the coexistence of two populations in the initially selected 9.2 mutant. Consequently, subclones 9.2.2 and 9.2.6 with an unwanted additional mutation were not analyzed further.

Phenotypic analysis of the subclones was performed as described above, using two subclones from each parental mutant (Table 1). After evaluating the temperature sensitivity phenotype of the subclones by evaluating their growth at two temperatures (Fig. S5), data from the phenotypic analysis of the subclones that showed the most pronounced phenotype (43.7.3E, 43.7.4B, 9.2.1A, and 9.2.3B) were confirmed using qPCR assays (Fig. 3C to F). At 32°C, the cultures of the four subclones reached the stationary phase at T_{72}, showing a 24-h delay compared to the control strain 30.1.1 (Fig. 3C and E). This delay significantly increased at 40°C, a temperature at which almost all subclones reached their optimum growth beyond 96 h of incubation, in contrast to 24 h for the control strain 30.1.1. Only subclone 43.7.3E reached its stationary phase after 72 h of incubation (Fig. 3F). As observed for growth curves, the subclones derived from mutant 9.2 (9.2.1A and 9.2.3B) behaved differently from the parental mutant, as they both grew slow, especially at 40°C (Fig. 3D). In fact, their phenotype became almost identical to that of subclones 43.7.3E and 43.7.4B, which contain the same mutations in the *obg* gene (Asp85Gly [GAT>GGT], Gly124Arg [GGG>AGA], and Ile244Val [TAT>TGT]) plus a mutation in *nadE* (Gln7Arg [CAA>CGA]). This discrepancy, most likely caused by the presence of a mixed population in mutant 9.2, suggested that the mutation GAT>AGT (Asp85Ser) detected in some 9.2 subclones probably does not confer a TS^+ phenotype.

The culture assays (Fig. S5) together with the qPCR measurements confirmed that subclones 43.7.3E, 43.7.4B, 9.2.1A, and 9.2.3B exhibit a marked TS^+ phenotype.

Whole-genome sequencing of subclones derived from the 30.1 control and from the 7.1, 9.2, and 43.7 mutants indicated a number of mutations other than those in the *obg* gene, but none of these correlated with the TS^+ phenotype observed (Table S2).

Evaluation of the virulence of the 9.2.3B *obg* subclone in an experimental animal model. Among the four *M. mycoides* subsp. *capri obg* subclones (43.7.3E, 43.7.4B, 9.2.1A, and 9.2.3B) that were characterized as described above, subclone 9.2.3B, which carried 3 mutations in the *obg* gene and showed the strongest TS^+ phenotype, was selected for subsequent *in vivo* testing of attenuation using a caprine infection model.

Two groups of eight male goats, housed in separated rooms, were infected transtracheally with either *M. mycoides* subsp. *capri* YCpMmyc1.1 (22, 23) (control group) or *M. mycoides* subsp. *capri obg* subclone 9.2.3B (*obg* group). After two injections, one at 0 days postinfection (dpi), with 10^8 CFU, and the other at 7 dpi, with 10^9 CFU, animal health status was monitored daily by measuring key parameters, such as body weight, heartbeat, breathing frequency, and body temperature (Fig. 4). The following endpoint criteria for animal euthanasia were applied: a body temperature higher than 40.5°C for more than 3 consecutive days, the appearance of clinical signs indicating moderate to severe pain or distress, weight loss of more than 10% within 7 days, and a breathing frequency higher than 50 breaths · min^{-1} for more than 3 days. In the control group, 5 goats out of 8 met the endpoint criteria before the envisaged end of the trial (i.e., 35 dpi) and were euthanized in the course of the experiment, whereas only 1 goat (animal CM135) out of 8 met the same criteria in the *obg* group (Fig. 4A) (24). *M. mycoides* subsp. *capri* was cultured from 3 out of 8 animals that were infected with the control strain YCpMmyc1.1. The time points of *M. mycoides* subsp. *capri* isolation coincided with severe pyrexia. Bacterial titers ranged between 100 and 1,000 CFU · ml^{-1} (24). Animals that did not develop the disease had body temperatures ranging

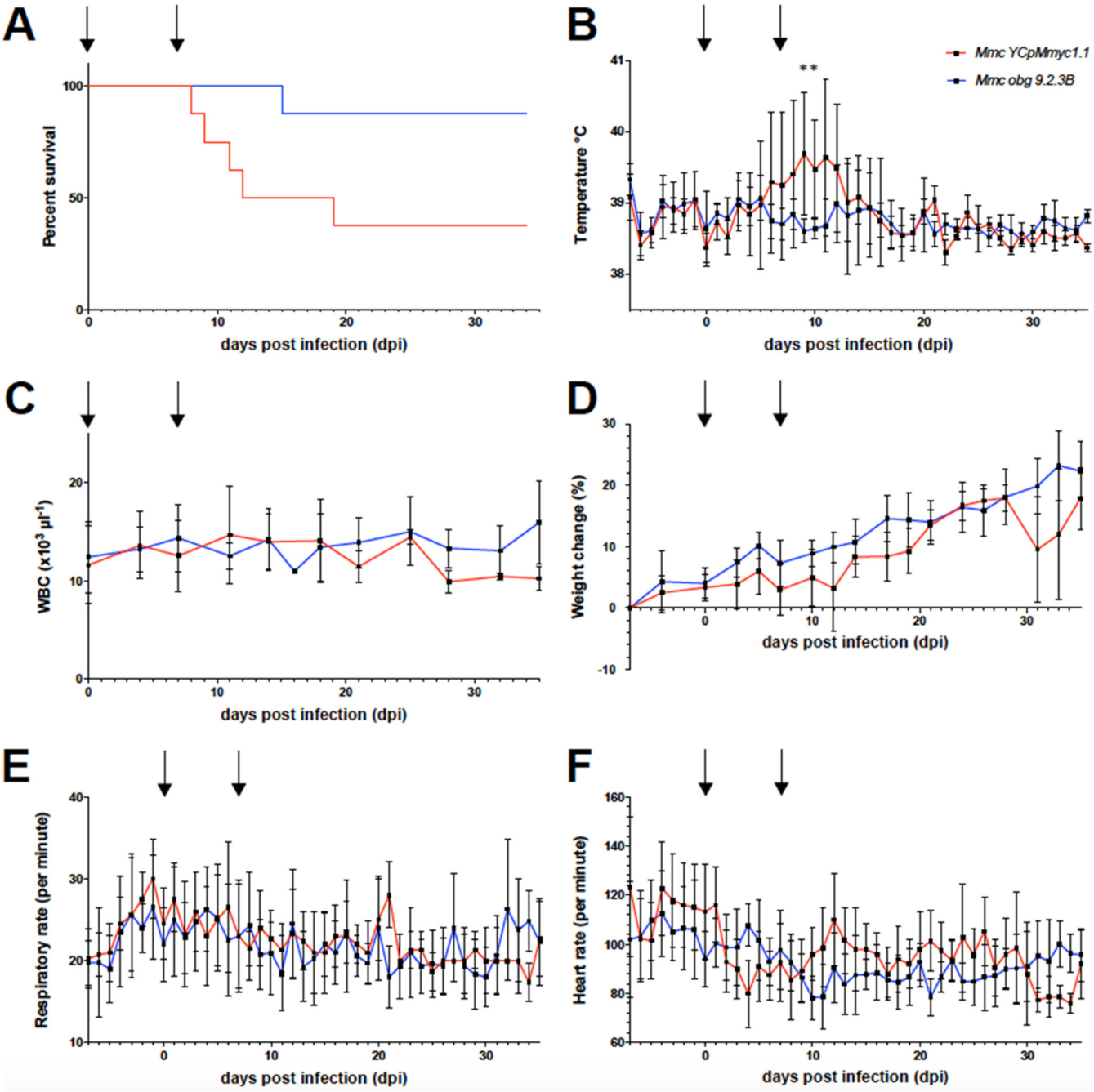

FIG 4 Survival rates and clinical parameters measured during *in vivo* testing of *M. mycoides* subsp. *capri obg* 9.2.3B (*obg* group) and *M. mycoides* subsp. *capri* YcpMmyc1.1 (control group). (A) The difference in survival rates (Kaplan-Meier curve) is significant according to both the Mantel-Cox test ($P = 0.0351$) and the Gehan-Breslow-Wilcoxon test ($P = 0.0325$). There were 8 goats per group. (B) Rectal temperature measured daily, shown as an average per group. The difference in average temperatures between the groups was significant on days 9 and 10 postinfection (day 9, $P = 0.0006$; day 10, $P = 0.0053$) using the Mann-Whitney test, coinciding with the peak of severity of disease in the group that received the *M. mycoides* subsp. *capri* YcpMmyc1.1 strain. (C) The white blood cell (WBC) count (measured twice weekly) was relatively stable throughout the trial, and no major difference between the groups was observed at any time point. (D) Both groups gained weight, measured three times weekly, continuously during the trial. (E) Respiratory rate, measured daily, remained within the normal range throughout the study. (F) Heart rate was measured daily and remained within the normal range for both groups. Arrows indicate days of infection (day 0, 10^8 CFU per animal; day 7, 10^9 CFU per animal). All graphs show the average measured parameters per group, with error bars showing SD.

between 38°C and 39.5°C, which represents the physiological range for goats (Fig. 4B). Within the group infected with *M. mycoides* subsp. *capri* YCpMmyc1.1, those animals showing signs of disease developed fever (rectal temperature of >39.5°C) between days 3 and 10 after the first injection (Fig. 4B), and animal CM135 from the *obg* group

showed fever starting at 13 dpi (data not shown). In most of the animals showing fever, body temperature reached 41°C in less than 3 days. In the *obg* group, the heart rate and breathing rate remained within the normal ranges for all animals, including animal CM135, throughout the study (Fig. 4E and F). We cultivated *M. mycoides* subsp. *capri* from the blood of the animals of the *obg* group, and only blood from the animal (CM135) that succumbed to disease gave a positive culture indicating bacteremia, with a titer of 10^3 CCU · ml^{-1}. Cultivation of *M. mycoides* subsp. *capri* from synovial fluid, urine, and pleural fluid of the same animal did not yield any positive cultures.

Necropsy of animals from the control group revealed severe and extensive necrosis, edema, and inflammation of the skin, subcutis, skeletal muscle of the neck, and the trachea around the site of inoculation in five animals. Histologically, there was extensive coagulation necrosis of these tissues surrounding the trachea in the vicinity of the inoculation site and a marked infiltration of neutrophilic granulocytes (Fig. S6a). These areas were surrounded by a rim of infiltration with mainly macrophages and lymphocytes. Regional lymph nodes showed purulent to necrotizing lymphadenitis, and there were multifocal neutrophilic infiltrates in the spleen. In one animal, there were multifocal, randomly distributed small areas of necrosis in neutrophilic infiltrates in the liver. These lesions were suggestive of septicemic spread of *M. mycoides* subsp. *capri* from the site of inoculation. Three animals of this group (animals CM045, CM047, and CM191) did not show macroscopic or histological lesions. Animal CM135 in the *obg* group had lesions similar to those in the control group (Fig. S6b). Overall, the data clearly demonstrated that even though one animal in the *obg* group developed disease and had to be euthanized, the virulence of *M. mycoides* subsp. *capri obg* subclone 9.2.3B was attenuated compared to its parental strain.

Genotypic analysis of the revertant iCM135. In order to find out the reason for the loss of one animal in the *obg* group (Fig. 4A), the mycoplasma strain isolated from blood samples of the euthanized animal (iCM135 strain) was subjected to further analyses. Whole-genome sequencing showed that iCM135 carried a single reversion in one of the target mutations within the *obg* gene. Indeed, compared to the original TS^+ *M. mycoides* subsp. *capri obg* subclone 9.2.3B, the GAT>GGT (Asp85Gly) triplets reverted back to GAT (Gly85Asp) in the iCM135 strain. The temperature sensitivity of iCM135 was analyzed *in vitro* using culture assays at 32°C or 40°C along with the *M. mycoides* subsp. *capri* control subclone 30.1.1 (intact *obg*). The experiment clearly showed that the iCM135 strain lost its temperature sensitivity and recovered a non-temperature-sensitive (TS^-) phenotype that is similar to that of the parental strain (Fig. S7) and that the loss of the TS^+ phenotype is probably due to the reversion observed at position 85 (Table S2). It should be stressed that a similar reversion of phenotype associated with reversion at position 85 was also observed *in vitro* during culture of subclone 9.2.1C at 40°C (Fig. S7).

Experimental challenge following infection with the 9.2.3B *obg* subclone. Since seven of eight goats infected with the 9.2.3B *obg* subclone survived after 35 dpi, we evaluated whether infection in these animals had triggered an immune response that would provide a certain degree of protection against an experimental challenge. The animals were challenged transtracheally with the *M. mycoides* subsp. *capri* GM12 strain (25). It should be noted that this strain is different from the *M. mycoides* subsp. *capri* YCpMmyc1.1 strain used as the control in the above-described experiment because it has not been submitted to a round of yeast cloning-genome transplantation followed by triple filter cloning. Therefore, the *M. mycoides* subsp. *capri* GM12 strain, at a low passage number, is known for its high pathogenicity, causing septicemia and death when administered at a high dose (27). At 0 days postchallenge (dpc), the seven animals were infected transtracheally with 10^9 CFU of the *M. mycoides* subsp. *capri* GM12 strain, and animal health status was monitored daily for 28 days by measuring parameters such as body weight, heartbeat, breathing frequency, and body temperature (Fig. 5). Between 4 dpc and 10 dpc, 6 out of the 7 goats met the endpoint criteria and were euthanized, with only 1 animal (CM118) surviving until the end of the

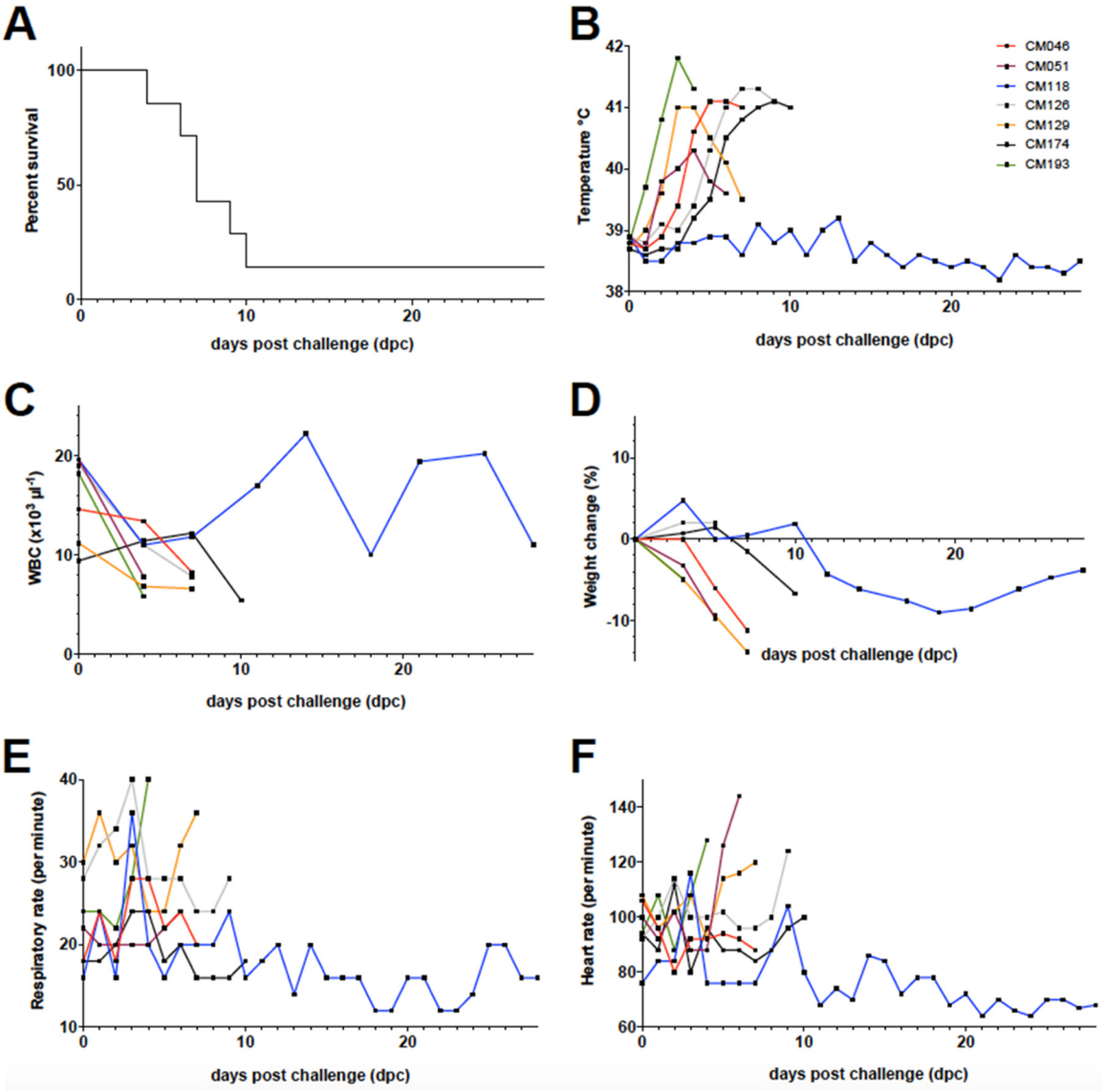

FIG 5 Experimental challenge of surviving goats following infection with the 9.2.3B *obg* subclone. Shown are survival rates and clinical parameters measured during *in vivo* challenge with wild-type *M. mycoides* subsp. *capri* GM12 (10^9 CFU per animal) of the 7 animals that survived the initial infection with *M. mycoides* subsp. *capri obg* 9.2.3B. Due to the small number of animals and the heterogeneity of data, individual curves for clinical parameters are shown in panels B to F. (A) Six out of the seven animals were euthanized due to severity of disease between days 4 and 10 postchallenge. (B) All but one of the seven animals had fever (defined as a rectal temperature of >39.5°C), with peak temperatures ranging from 40.3°C to 41.8°C. One animal did not show an elevated temperature (animal CM118). (C) The white blood cell count dropped initially in 5 of the 7 animals but remained within the normal range throughout the study. (D) Weight loss was seen during severe disease in 5 animals. Goat CM118 lost weight after the other animals in the group had been euthanized but started to recover at around 20 days postchallenge. (E) Breathing frequency was within the normal range throughout the challenge experiment. (F) At the peak of severity of disease, the individual heart rates of animals CM193, CM051, CM129, and CM126 increased. The average heart rate remained within the normal range throughout the trial.

experiment (Fig. 5A). As expected with this highly pathogenic and septicemic strain of *M. mycoides* subsp. *capri*, infection resulted in a peak body temperature reaching ≥41°C for 5 out of the 6 animals that were euthanized. The *M. mycoides* subsp. *capri* challenge strain GM12 was isolated from the blood of 6 of the 7 animals. The bacterial titers ranged from 10^4 to 10^{10} CFU · ml^{-1}. The only animal for which the cultures remained negative was animal CM118, which survived until the end of the experiment, at 30 days postchallenge. Other clinical parameters were also affected although not uniformly among these animals (Fig. 5C to F).

Pathological lesions in 6 out of 7 of the *M. mycoides* subsp. *capri* GM12-inoculated animals were similar to those of the above-described control group, with massive

necrosis and mainly neutrophilic inflammation in the soft tissues and the trachea of the neck extending from the inoculation site (Fig. S6c). All animals showed histopathological signs of septicemia, characterized as multifocal acute necrosis and neutrophilic infiltration in the spleen. In addition, three animals developed mild, purulent bronchopneumonia. Animal CM118 did not show any macroscopic or histological lesions.

DISCUSSION

Using synthetic biology approaches, the essential gene *obg* encoding a P-loop GTPase was mutated on the *M. mycoides* subsp. *capri* genome, cloned in yeast, and subsequently transplanted into *Mycoplasma capricolum* recipient cells to obtain a set of *M. mycoides* subsp. *capri* mutants carrying various mutations. The targeted modifications of *obg* reproduced mutations that are known to be associated with a temperature-sensitive phenotype in various bacteria, including in a commercial live vaccine of *M. synoviae*.

Among the single *obg* mutations that were previously associated with a TS^+ phenotype in *E. coli*, *B. subtilis*, and *M. synoviae*, the only one that showed a significant TS^+ phenotype in *M. mycoides* subsp. *capri* is Gly80Glu (mutant 7.1) (Fig. 2). This mutation has been described in detail in *Caulobacter crescentus* and was also characterized by a marked TS^+ phenotype associated with alterations in the cell cycle (21). In contrast, the single mutation described in the TS^+ vaccine strain of *M. synoviae* (Gly124Arg) did not, by itself, produce a TS^+ phenotype in *M. mycoides* subsp. *capri*. Recently, complementation of the Gly124Arg substitution in *M. synoviae* with a wild-type copy of the *obg* gene did not result in a TS^- phenotype (10), but some of the growth parameters, including growth rate and cell viability, were at least partially restored; it should be stressed that in this case, complemented clones expressed both wild-type Obg and the protein with the Gly124Arg substitution. In the *obg* mutants with more than one mutation, the complex interplay among them is probably due to structure constraints. For example, the reversion at position 85 (Gly85Asp) was associated with the loss of the TS^+ phenotype in the 9.2.3B mutant, although the single mutation Asp85Asn (mutant 11.1) did not produce a significant TS^+ phenotype in *M. mycoides* subsp. *capri*. As the replacement at this position has been done using two different amino acids (Gly and Asn), we cannot exclude the possibility that one change is more drastic than the other one regarding the growth of *M. mycoides* subsp. *capri* at a nonpermissive temperature.

The set of mutants that was obtained in this study finally resulted in a range of TS^+ phenotypes. The mutant that was the most severely affected in its growth at 40°C was selected because the multiple mutations (at least the Asp85Gly and the Gly124Arg mutations) were considered to confer a higher level of safety against possible genetic reversion. These two mutations did not prove to be sufficient, since a single reversion at position 85 (Gly85Asp) was associated with the loss of the TS^+ phenotype both *in vivo* (mutant iCM135) and *in vitro* (subclone A9.2.1C_{40}). This indicates that a structural change in the protein that leads to the TS^+ phenotype occurs only when both of the two mutations (Gly85Asp and Gly124Arg) are present, possibly together with the third mutation (Ile224Val). Therefore, the reversion in one of them was enough to lose the TS^+ phenotype in *M. mycoides* subsp. *capri*. Obviously, the reversion of these mutations is a problem if one envisages obtaining safe vaccine strains with this approach. In fact, this reversion to the TS^- phenotype is already known for the vaccine strain of *M. synoviae* that was produced by chemical mutagenesis, but this attenuation is most likely due to other mutations in other genes, such as the ones encoding the oligopeptide permease (Opp) system (6, 10, 26).

A novel caprine challenge model has been established for *M. mycoides* subsp. *capri* GM12 (24, 27), which we basically followed, with slight modifications, as reported recently (24). This challenge model proved to be robust and reproducible (24, 27). Another caprine challenge model using an atypical *M. mycoides* subsp. *capri* strain was reported elsewhere (28), but as the number of infected animals was small and the outcome differed between groups markedly, we opted not to follow this challenge

model. The model of infection performed here is a significant advancement, as there is a general lack of animal models for studying mycoplasma pathogenicity, which is mostly due to the high host specificity of mycoplasmas. However, this animal model has its own advantages as well as limits. Its main advantage is that it allows evaluation of the attenuation of mutants when derived from a strongly pathogenic strain such as *M. mycoides* subsp. *capri* GM12. Within a few days, the survival curves (Fig. 4) together with the data on bacteremia provide the main tools for evaluation of the outcome of infection. The weight of each clinical parameter, including body temperatures, is more difficult to evaluate because we used outbred goats that are genetically heterogeneous. In addition, as shown in this study, the high dose of infection with mutants provides the possibility of detecting pathogenic revertants. However, the limits of this animal model also need to be recognized. The transtracheal application of the 9.2.3B *obg* subclone was selected for comparison of virulence with the parental strain YCpM-myc1.1. This route was not meant to be used as prospective immunization route. However, we opted to challenge the 7 goats that survived the first phase of the experiment (35 dpi) because we expected the induction of an immune response, and it provides a comparison with similar results obtained with a GM12 *glf* mutant defective in capsule biosynthesis (24). Whereas 3 of the 8 animals infected with the *glf* mutant survived, only 1 of the 7 animals infected in this study with the *obg* mutant survived. This difference is not significant. It is difficult to discuss much more about this difference without a full analysis of the immune response in individual animals; our preliminary attempts using enzyme-linked immunosorbent assays (ELISAs) and immunoblotting did not provide meaningful results because of the high background of antibodies cross-reacting with *M. mycoides* subsp. *capri* antigens in the goats. This is likely due to the exposure of these animals to a number of parasites before being selected for this trial. Finally, it should also be stressed that very little is known about the immune determinants that would provide protection from challenge with highly virulent *M. mycoides* subsp. *capri*.

Until now, the tools from synthetic biology applied to *Mycoplasma* species have been mostly limited to building a minimal cell (20). However, we have shown that these tools are also useful for the elucidation of gene functions (29) and for the deciphering of host-pathogen interactions (30). The versatility of these approaches that combine in-yeast genome engineering and back-transplantation into a recipient cell is further demonstrated in the present study by showing our ability to generate targeted point mutations within the essential *obg* gene. Such an achievement was previously impossible using the genetic tools available for any *Mycoplasma* species. Since it is now possible to use synthetic biology tools for most of the ruminant-pathogenic *Mycoplasma* species that are closely related to *M. mycoides* subsp. *capri* (31), new strategies of vaccine design based on precise and multiple targets using genome engineering have become fully realistic. Within this global aim, the TS^+ phenotype resulting from *obg* mutations described here is of interest if combined with (i) other mutations that hamper completely the possibility of reversion to a virulent phenotype and (ii) other deletion(s), such as deletion of the nonessential *glpO* gene known to be involved in the production of H_2O_2 (32). Owing to this new animal model, we now have the possibility of evaluating the attenuation of virulence of different mutants resulting from genome engineering approaches and the immune response providing protection against an experimental challenge.

MATERIALS AND METHODS

Bacterial and yeast strains and culture conditions. Competent *E. coli* cells (ElectroMAX DH10B; Invitrogen) [F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ− *rpsL nupG*] served as the host for cloning experiments and plasmid propagation. *E. coli* cells transformed with plasmids were grown at 37°C in Luria-Bertani (LB) broth or agar medium supplemented with 100 $\mu g \cdot ml^{-1}$ of ampicillin.

Wild-type *Mycoplasma mycoides* subsp. *capri* strain GM12 (25) and other *M. mycoides* subsp. *capri* derivatives were cultured in SP5 medium as described previously (31). Tetracycline was added to the medium when needed at 5 $\mu g \cdot ml^{-1}$. For genome transplantation experiments, restriction-free *Mycoplasma capricolum* subsp. *capricolum* (McapΔRE) recipient cells (22) were grown at 30°C in superoptimal

broth (SOB) supplemented with 17% (vol/vol) fetal bovine serum, 1% (wt/vol) glucose, 0.002% (wt/vol) phenol red, and penicillin at 0.5 μg · ml^{-1} (SOB^{+} medium) (33).

Saccharomyces cerevisiae strain VL6-48N (*MAT*α *trp1-*Δ*1 ura3-*Δ*1 ade2-101 his3-*Δ*200 lys2 met14 cir*^{o}) (34) was cultured at 30°C in YPDA (yeast extract peptone dextrose adenine) medium (Clontech) according to a standard protocol (35). Yeast transformed with mycoplasma genomes was grown in minimal SD (synthetic defined) base medium (Clontech) complemented with histidine dropout supplement (Clontech) (SD-His medium).

***M. mycoides* subsp. *capri* genome engineering in yeast.** The genome of the modified *M. mycoides* subsp. *capri* clone YCpMmyc1.1 (strain GM12) was previously cloned into *S. cerevisiae* strain VL6-48N (22). Targeted nucleotides corresponding to the amino acids at position 80, 85, or 124 of the *M. mycoides* subsp. *capri* Obg protein (Fig. 1A; see also Fig. S1 in the supplemental material) were mutated in the YCpMmyc1.1 genome cloned into yeast by using the TREC-IN (tandem repeat endonuclease cleavage-insertion) method as previously described (36). The experimental procedure used to obtain these engineered genomes is shown in Fig. 1B and detailed in the legend of Fig. S2 in the supplemental material (primers used during the procedure are listed in Table S1). At the end of the process, YCpMmyc1.1-*obg* genomes were obtained and contained mutations at either position 239/240, 253/255, or 369/371 of the *obg* gene, which corresponds to position 80, 85, or 124 of the Obg protein, respectively. To check this, total DNA was extracted from all selected yeast recombinants, and the DNA was analyzed by simplex PCR (Table S1), multiplex PCR, and pulsed-field gel electrophoresis (PFGE) as described previously (29) (Fig. S3). The presence of the mutations was confirmed by sequencing the 1,601-bp *obg* PCR fragment obtained using the primers obgF1 and obgR2 (Fig. S2). For multiplex PCR, a set of 11 different pairs of primers was used as previously described (22) (Table S1).

The TREC-IN method was also used to build a genome equivalent to the mutant 43.7 genome but with a repaired *nadE* sequence. Using the primer pair obgF1/obgR3 (Table S1) and mutant 43.7 genomic DNA, we amplified the *obg* gene with changes at positions 254, 369, 371, and 732 plus a 113-bp segment located upstream of the *obg* gene. This amplicon was then used as a template for PCR with primers cassette B-forward-F1 and cassette D-reverse-R1 to create a minicassette, named minicassette BC, with 60-bp overlaps with minicassette A at its 5′ end and 60-bp overlaps with the sequence downstream of 5′ Kan (positions 1 to 515) of the YCpMmyc1.1-Δ*obg*::*URA3* genome, at its 3′ end. Minicassettes BC and A were finally cotransformed into VL6-48N yeast clone 34 harboring the YCpMmyc1.1-Δ*obg*::*URA3* genome (step 2 of the TREC-IN method). The next steps of the TREC-IN method as well as all genotypic tests were performed as described above.

Back-transplantation of the engineered YCPMmyc1.1-*obgmod* genomes into mycoplasma recipient cells to produce the *M. mycoides* subsp. *capri obg* mutants. In-yeast-engineered *M. mycoides* subsp. *capri* genomes were isolated in agarose plugs and transplanted back into restriction-free *M. capricolum* subsp. *capricolum* (McapΔRE) recipient cells as previously described (22, 37). The resulting transplants were selected on SP5 medium plates supplemented with 5 μg · ml^{-1} of tetracycline. After 3 successive passages in liquid medium, transplants were analyzed by PCR using *M. mycoides* subsp. *capri*-specific primers (Table S1), multiplex PCR (Table S1), and PFGE, as previously described (22). The *obg* gene was systematically amplified using the primer pair Obg-F1/ObgR1 (Fig. S2B) and sequenced in order to verify the presence of the desired mutations.

Mutants showing temperature-sensitive phenotypes were submitted to three successive rounds of filter cloning using 0.22-μm sterilizing-grade filters (38). Subclones were once more assessed for their temperature-sensitive properties, and their whole genomes were sequenced using Illumina next-generation sequencers (see the supplemental material). Mutant and subclone names and main characteristics are summarized in Table 1.

Assessment of temperature sensitivity by serial dilution and qPCR. Mutants and subclones were grown at 32°C in SP5 medium until the pH reached 6.4. The temperature sensitivity of the samples was then analyzed by serial dilutions (10^{-1} to 10^{-9}) of the cell cultures in SP5 medium, followed by an incubation period of 8 days at a permissive temperature (32°C) or a nonpermissive temperature (40°C). Culture color change was monitored daily and compared to that of a culture of WT *M. mycoides* subsp. *capri* strain GM12 grown under the same conditions. The cell concentration of each initial culture (pH 6.4) was determined by plating dilutions on SP5 plates. Just before putting the samples into the incubator (T_0) and then every 24 h during 6 days (T_{24} to T_{144}), a 100-μl aliquot was taken from the "10^{-8} dilution tube" (for the mutants) and from the "10^{-5} dilution tube" (for the subclones) for qPCR assays. These qPCR assays were performed using a protocol described previously (31). Briefly, qPCR measurements were conducted in 96-well plates using the SsoFast EvaGreen supermix (Bio-Rad) on the LightCycler 480 real-time PCR system (Roche) according to the manufacturers' instructions. Standard curves used to quantify the numbers of *M. mycoides* subsp. *capri* genomes were run independently for each assay by using purified genomic DNA (Wizard genomic DNA purification kit; Promega), with samples containing between 10^2 and 10^8 genomes. Measurements were done in triplicates for all samples. Data analyses were performed using GraphPad Prism statistical software version 7. All qPCR data were previously log transformed to meet model assumptions, and the Benjamini-Hochberg method (39) was used to adjust the false discovery rate (FDR) associated with the test. Analyses were done using an unpaired (independent) *t* test to compare the concentrations (means) of the different mutants with those of the *M. mycoides* subsp. *capri* controls (30.1 or 30.1.1) at each time point.

Animal studies. The animal experiments were performed under strict ethical rules (see "Ethics statement," below). Briefly, 16 male crossbred goats (*Capra hircus*), 1 to 2 years of age, were randomly selected from the International Livestock Research Institute (ILRI) ranch in Kapiti (see the supplemental material for details). Five days before experimental infection, all animals were transferred to the animal

biosafety level 2 (ABSL2) unit and split randomly into two groups of eight animals (positive-control and *obg* groups), and each group was housed in a separate room. On day 0 postinfection (p.i.) and day 7 p.i., goats of the positive-control group and the *obg* group were infected transtracheally (the injection site was 5 to 10 cm distal from the larynx) with *M. mycoides* subsp. *capri* YCpMmyc1.1 and *M. mycoides* subsp. *capri* YCpMmyc1.1-*obg*, respectively. On day 0 p.i., each animal was infected with a 1-ml liquid culture containing 10^8 CFU, followed by a flush with 5 ml of phosphate-buffered saline (PBS); on day 7 p.i., this procedure was repeated, except that each animal was infected with a liquid culture containing 10^9 CFU. The animals were allowed to move freely within the ABSL2 unit and received hay and water *ad libitum* as well as pellets in the morning. Heart rate, breathing frequency, rectal temperature, and oxygen blood saturation were measured daily in the morning hours. Animals showing either a body temperature of >40.5°C for >3 consecutive days, moderate to severe pain or distress, weight loss of >10% within 7 days, or a breathing frequency of >50 breaths/min for >3 days were euthanized. More information regarding the group infected with YCpMmyc1.1 were described previously by Jores et al. (24).

Ethics statement. All protocols of this study were designed and performed in strict accordance with Kenyan legislation for animal experimentation and were approved by the institutional animal care and use committee (IACUC) (reference no. IACUC-RC2016-04, signed by Roger Pelle). Since 1993, the ILRI has complied voluntarily with the United Kingdom's Animals (Scientific Procedures) Act 1986 (http://www.homeoffice.gov.uk/science-research/animal-research/), which contains guidelines and codes of practice for the housing and care of animals used in scientific procedures. The study reported here was carried out in strict accordance with the recommendations in the standard operating procedures of the ILRI IACUC (ILRI IACUC ref no. 2016.06) and with adequate consideration of the 3 R's (replacement of animal with nonanimal techniques, reduction in the number of animals used, and refinement of techniques and procedures that reduce pain and distress). As this project also received financial support from the NSF (NSF proposal IOS-1110151 entitled BREAD: Toward Development of a Vaccine for Contagious Bovine Pleuropneumonia [CBPP]) awarded to the J. Craig Venter Institute (JCVI), the protocols of this study were also approved by the JCVI's own IACUC. The project was approved initially by the IACUC on 17 August 2011, reapproved on 6 October 2014 (no. 011-14), and signed by Mark Adams, JCVI Scientific Director.

Pathomorphological and histological analyses. 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, stained routinely with hematoxylin and eosin (H&E), and evaluated by a board-certified pathologist.

Data availability. We declare that the data supporting the findings of this study are available within the paper and its supplemental material. The sequences from this study are available from the NCBI under SRA accession no. PRJNA510711.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00030-19.

TEXT S1, DOC file, 0.04 MB.
FIG S1, PDF file, 0.6 MB.
FIG S2, PDF file, 0.4 MB.
FIG S3, PDF file, 0.3 MB.
FIG S4, PDF file, 0.3 MB.
FIG S5, PDF file, 0.5 MB.
FIG S6, PDF file, 0.6 MB.
FIG S7, PDF file, 0.5 MB.
TABLE S1, XLSX file, 0.01 MB.
TABLE S2, XLSX file, 0.03 MB.

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We declare no competing interests.

A.B., J.J., and C.L. conceived and designed the research. Y.V.T., F.L., B.B., H.P., and C.L. performed laboratory experiments and analyzed the *in vitro* data. J.J., E.S., A.L., H.P., and

F.S. performed *in vivo* experiments and analyzed the *in vivo* data. P.S.-P. performed genome sequencing and subsequent analyses. A.B., F.L., P.S.-P., Y.V.T., J.J., S.V., and C.L. wrote the paper and contributed to the design of the figures and tables. All authors approved the final version of the paper.

REFERENCES

1. Bébéar CM, Kempf I. 2005. Antimicrobial therapy and antimicrobial resistance, p 535–568. *In* Blanchard A, Browning G (ed), Mycoplasmas: molecular biology pathogenicity and strategies for control. Horizon Bioscience, Norfolk, United Kingdom.
2. Nicholas RA, Ayling RD, McAuliffe L. 2009. Vaccines for Mycoplasma diseases in animals and man. J Comp Pathol 140:85–96. https://doi.org/10.1016/j.jcpa.2008.08.004.
3. Jores J, Mariner JC, Naessens J. 2013. Development of an improved vaccine for contagious bovine pleuropneumonia: an African perspective on challenges and proposed actions. Vet Res 44:122. https://doi.org/10.1186/1297-9716-44-122.
4. Shahid MA, Ghorashi SA, Agnew-Crumpton R, Markham PF, Marenda MS, Noormohammadi AH. 2013. Combination of differential growth at two different temperatures with a quantitative real-time polymerase chain reaction to determine temperature-sensitive phenotype of Mycoplasma synoviae. Avian Pathol 42:185–191. https://doi.org/10.1080/03079457.2013.779363.
5. White MD, Bosio CM, Duplantis BN, Nano FE. 2011. Human body temperature and new approaches to constructing temperature-sensitive bacterial vaccines. Cell Mol Life Sci 68:3019–3031. https://doi.org/10.1007/s00018-011-0734-2.
6. Shahid MA, Markham PF, Markham JF, Marenda MS, Noormohammadi AH. 2013. Mutations in GTP binding protein Obg of Mycoplasma synoviae vaccine strain MS-H: implications in temperature-sensitivity phenotype. PLoS One 8:e73954. https://doi.org/10.1371/journal.pone.0073954.
7. Kint CI, Verstraeten N, Wens I, Liebens VR, Hofkens J, Versees W, Fauvart M, Michiels J. 2012. The Escherichia coli GTPase ObgE modulates hydroxyl radical levels in response to DNA replication fork arrest. FEBS J 279:3692–3704. https://doi.org/10.1111/j.1742-4658.2012.08731.x.
8. Kobayashi G, Moriya S, Wada C. 2001. Deficiency of essential GTP-binding protein ObgE in Escherichia coli inhibits chromosome partition. Mol Microbiol 41:1037–1051.
9. Feng B, Mandava CS, Guo Q, Wang J, Cao W, Li N, Zhang Y, Zhang Y, Wang Z, Wu J, Sanyal S, Lei J, Gao N. 2014. Structural and functional insights into the mode of action of a universally conserved Obg GTPase. PLoS Biol 12:e1001866. https://doi.org/10.1371/journal.pbio.1001866.
10. Shahid MA, Marenda MS, Markham PF, Noormohammadi AH. 2018. Complementation of the Mycoplasma synoviae MS-H vaccine strain with wild-type obg influencing its growth characteristics. PLoS One 13:e0194528. https://doi.org/10.1371/journal.pone.0194528.
11. Kok J, Trach KA, Hoch JA. 1994. Effects on Bacillus subtilis of a conditional lethal mutation in the essential GTP-binding protein Obg. J Bacteriol 176:7155–7160. https://doi.org/10.1128/jb.176.23.7155-7160.1994.
12. Buglino J, Shen V, Hakimian P, Lima CD. 2002. Structural and biochemical analysis of the Obg GTP binding protein. Structure 10:1581–1592. https://doi.org/10.1016/S0969-2126(02)00882-1.
13. Kukimoto-Niino M, Murayama K, Inoue M, Terada T, Tame JR, Kuramitsu S, Shirouzu M, Yokoyama S. 2004. Crystal structure of the GTP-binding protein Obg from Thermus thermophilus HB8. J Mol Biol 337:761–770. https://doi.org/10.1016/j.jmb.2004.01.047.
14. Sato A, Kobayashi G, Hayashi H, Yoshida H, Wada A, Maeda M, Hiraga S, Takeyasu K, Wada C. 2005. The GTP binding protein Obg homolog ObgE is involved in ribosome maturation. Genes Cells 10:393–408. https://doi.org/10.1111/j.1365-2443.2005.00851.x.
15. Bonventre JA, Zielke RA, Korotkov KV, Sikora AE. 2016. Targeting an essential GTPase Obg for the development of broad-spectrum antibiotics. PLoS One 11:e0148222. https://doi.org/10.1371/journal.pone.0148222.
16. Kint C, Verstraeten N, Hofkens J, Fauvart M, Michiels J. 2014. Bacterial Obg proteins: GTPases at the nexus of protein and DNA synthesis. Crit Rev Microbiol 40:207–224. https://doi.org/10.3109/1040841X.2013.776510.
17. French CT, Lao P, Loraine AE, Matthews BT, Yu H, Dybvig K. 2008. Large-scale transposon mutagenesis of Mycoplasma pulmonis. Mol Microbiol 69:67–76. https://doi.org/10.1111/j.1365-2958.2008.06262.x.
18. Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA, III, Smith HO, Venter JC. 2006. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A 103:425–430. https://doi.org/10.1073/pnas.0510013103.
19. Lluch-Senar M, Delgado J, Chen WH, Llorens-Rico V, O'Reilly FJ, Wodke JA, Unal EB, Yus E, Martinez S, Nichols RJ, Ferrar T, Vivancos A, Schmeisky A, Stulke J, van Noort V, Gavin AC, Bork P, Serrano L. 2015. Defining a minimal cell: essentiality of small ORFs and ncRNAs in a genome-reduced bacterium. Mol Syst Biol 11:780. https://doi.org/10.15252/msb.20145558.
20. Hutchison CA, III, Chuang RY, Noskov VN, Assad-Garcia N, Deerinck TJ, Ellisman MH, Gill J, Kannan K, Karas BJ, Ma L, Pelletier JF, Qi ZQ, Richter RA, Strychalski EA, Sun L, Suzuki Y, Tsvetanova B, Wise KS, Smith HO, Glass JI, Merryman C, Gibson DG, Venter JC. 2016. Design and synthesis of a minimal bacterial genome. Science 351:aad6253. https://doi.org/10.1126/science.aad6253.
21. Datta K, Skidmore JM, Pu K, Maddock JR. 2004. The Caulobacter crescentus GTPase CgtAC is required for progression through the cell cycle and for maintaining 50S ribosomal subunit levels. Mol Microbiol 54:1379–1392. https://doi.org/10.1111/j.1365-2958.2004.04354.x.
22. Lartigue C, Vashee S, Algire MA, Chuang RY, Benders GA, Ma L, Noskov VN, Denisova EA, Gibson DG, Assad-Garcia N, Alperovich N, Thomas DW, Merryman C, Hutchison CA, III, Smith HO, Venter JC, Glass JI. 2009. Creating bacterial strains from genomes that have been cloned and engineered in yeast. Science 325:1693–1696. https://doi.org/10.1126/science.1173759.
23. Benders GA, Noskov VN, Denisova EA, Lartigue C, Gibson DG, Assad-Garcia N, Chuang RY, Carrera W, Moodie M, Algire MA, Phan Q, Alperovich N, Vashee S, Merryman C, Venter JC, Smith HO, Glass JI, Hutchison CA, III. 2010. Cloning whole bacterial genomes in yeast. Nucleic Acids Res 38:2558–2569. https://doi.org/10.1093/nar/gkq119.
24. Jores J, Schieck E, Liljander A, Sacchini F, Posthaus H, Lartigue C, Blanchard A, Labroussaa F, Vashee S. 2019. In vivo role of capsular polysaccharide in Mycoplasma mycoides. J Infect Dis 219:1559–1563. https://doi.org/10.1093/infdis/jiy713.
25. DaMassa AJ, Brooks DL, Adler HE. 1983. Caprine mycoplasmosis: widespread infection in goats with Mycoplasma mycoides subsp mycoides (large-colony type). Am J Vet Res 44:322–325.
26. Zhu L, Shahid MA, Markham J, Browning GF, Noormohammadi AH, Marenda MS. 2018. Genome analysis of Mycoplasma synoviae strain MS-H, the most common M. synoviae strain with a worldwide distribution. BMC Genomics 19:117. https://doi.org/10.1186/s12864-018-4501-8.
27. Jores J, Ma L, Ssajjakambwe P, Schieck E, Liljander AM, Chandran S, Stoffel MH, Cippa VL, Arfi Y, Asad-Garcia N, Falquet L, Sirand-Pugnet P, Blanchard A, Lartigue C, Posthaus H, Labroussaa F, Vashee S. 2019. Removal of a subset of non-essential genes fully attenuates a highly virulent Mycoplasma strain. Front Microbiol 10:664. https://doi.org/10.3389/fmicb.2019.00664.
28. D'Angelo AR, Di Provvido A, Di Francesco G, Sacchini F, De Caro C, Nicholas RA, Scacchia M. 2010. Experimental infection of goats with an unusual strain of Mycoplasma mycoides subsp. capri isolated in Jordan: comparison of different diagnostic methods. Vet Ital 46:189–207.
29. Lartigue C, Lebaudy A, Blanchard A, El Yacoubi B, Rose S, Grosjean H, Douthwaite S. 2014. The flavoprotein Mcap0476 (RlmFO) catalyzes m5U1939 modification in Mycoplasma capricolum 23S rRNA. Nucleic Acids Res 42:8073–8082. https://doi.org/10.1093/nar/gku518.
30. Schieck E, Lartigue C, Frey J, Vozza N, Hegermann J, Miller RA, Valguarnera E, Muriuki C, Meens J, Nene V, Naessens J, Weber J, Lowary TL, Vashee S, Feldman MF, Jores J. 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. https://doi.org/10.1111/mmi.13213.
31. Labroussaa F, Lebaudy A, Baby V, Gourgues G, Matteau D, Vashee S, Sirand-Pugnet P, Rodrigue S, Lartigue C. 2016. Impact of donor-recipient

phylogenetic distance on bacterial genome transplantation. Nucleic Acids Res 44:8501–8511. https://doi.org/10.1093/nar/gkw688.
32. Pilo P, Vilei EM, Peterhans E, Bonvin-Klotz L, Stoffel MH, Dobbelaere D, Frey J. 2005. A metabolic enzyme as a primary virulence factor of Mycoplasma mycoides subsp. mycoides small colony. J Bacteriol 187:6824–6831. https://doi.org/10.1128/JB.187.19.6824-6831.2005.
33. Hanahan D. 1983. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166:557–580. https://doi.org/10.1016/S0022-2836(83)80284-8.
34. Larionov V, Kouprina N, Solomon G, Barrett JC, Resnick MA. 1997. Direct isolation of human BRCA2 gene by transformation-associated recombination in yeast. Proc Natl Acad Sci U S A 94:7384–7387. https://doi.org/10.1073/pnas.94.14.7384.
35. Treco DA, Winston F. 2008. Growth and manipulation of yeast. Curr Protoc Mol Biol Chapter 13:Unit 13.2. https://doi.org/10.1002/0471142727.mb1302s82.
36. Chandran S, Noskov VN, Segall-Shapiro TH, Ma L, Whiteis C, Lartigue C, Jores J, Vashee S, Chuang RY. 2014. TREC-IN: gene knock-in genetic tool for genomes cloned in yeast. BMC Genomics 15:1180. https://doi.org/10.1186/1471-2164-15-1180.
37. Lartigue C, Glass JI, Alperovich N, Pieper R, Parmar PP, Hutchison CA, III, Smith HO, Venter JC. 2007. Genome transplantation in bacteria: changing one species to another. Science 317:632–638. https://doi.org/10.1126/science.1144622.
38. Brown DR, Whitcomb RF, Bradbury JM. 2007. Revised minimal standards for description of new species of the class Mollicutes (division Tenericutes). Int J Syst Evol Microbiol 57:2703–2719. https://doi.org/10.1099/ijs.0.64722-0.
39. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.
40. Gietz D, St Jean A, Woods RA, Schiestl RH. 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425. https://doi.org/10.1093/nar/20.6.1425.
41. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, III, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343–345. https://doi.org/10.1038/nmeth.1318.
42. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Eberhard C, Grüning B, Guerler A, Hillman-Jackson J, Von Kuster G, Rasche E, Soranzo N, Turaga N, Taylor J, Nekrutenko A, Goecks J. 2016. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res 44:W3–W10. https://doi.org/10.1093/nar/gkw343.