Unmarked insertional mutagenesis in the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* SC: characterization of a *lppQ* mutant

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Mycoplasma mycoides subspecies *mycoides* small colony (SC) is the aetiological agent of contagious bovine pleuropneumonia (CBPP), a respiratory disease causing important losses in cattle production. The publication of the genome sequence of *M. mycoides* subsp. *mycoides* SC should facilitate the identification of putative virulence factors. However, real progress in the study of molecular mechanisms of pathogenicity also requires efficient molecular tools for gene inactivation. In the present study, we have developed a transposon-based approach for the random mutagenesis of *M. mycoides* subsp. *mycoides* SC. A PCR-based screening assay enabled the characterization of several mutants with knockouts of genes potentially involved in pathogenicity. The initial transposon was further improved by combining it with the transposon $\gamma\delta$ TnpR/res recombination system to allow the production of unmarked mutations. Using this approach, we isolated a mutant free of antibiotic-resistance genes, in which the gene encoding the main lipoprotein LppQ was disrupted. The mutant was found to express only residual amounts of the truncated N-terminal end of LppQ. This approach opens the way to study virulence factors and pathogen–host interactions of *M. mycoides* subsp. *mycoides* SC and to develop new, genetically defined vaccine strains.

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INTRODUCTION

Mycoplasma mycoides subspecies *mycoides* biotype small colony (SC) is the causal agent of contagious bovine pleuropneumonia (CBPP), a respiratory disease of cattle that is widespread in most of sub-Saharan Africa and causes important economic losses in the cattle industry. Although the disease was eradicated from the USA, Canada and most of Europe in the 19th century (for review, see Provost *et al.*, 1987), several outbreaks occurred in southern European countries during the 1980s and 1990s, and molecular typing studies have demonstrated that the isolated strains were from a lineage distinct from the African ones (Bischof *et al.*, 2006; Cheng *et al.*, 1995; Lorenzon *et al.*, 2003; Vilei *et al.*, 2000).

Although CBPP was controlled in Europe by the systematic slaughter of infected herds, such a strategy is difficult to

propose in many African countries because of the negative effects on short-term economics and population health (Boonstra *et al.*, 2001). Consequently, the main method recommended to control the spread of CBPP is vaccination with live-attenuated vaccine strains. Several vaccine strains have been produced since the 1990s by serial culture of the organism in liquid medium or in embryonated eggs (Thiaucourt *et al.*, 2003, 2004). The first vaccine strains, V5 and KH₃J, conferred only a low level of protection upon the vaccinated animals and were replaced by derivatives of the T1 strain. The T1/44 strain was obtained after 44 passages in embryonated eggs, and subsequent passages resulted in the streptomycin-resistant variant T1sr. Although both vaccine strains confer a certain level of protection, frequent inoculations are required to obtain satisfactory results. The mutations leading to the attenuation are unknown for all of these strains. The precise characterization of the mutation would improve the biological safety of live vaccine strains significantly, and is required for licensing of recombinant live bacterial vaccine strains in most countries (Frey, 2007). Marked

Abbreviations: CBPP, contagious bovine pleuropneumonia; IR, inverted repeat.

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post-vaccine reactions have been observed with the T1/44 strain, suggesting that it may have retained significant virulence, which has been confirmed by the report of CBPP cases following T1/44 vaccine inoculation via the endo-bronchial route (Mbulu *et al.*, 2004).

New genetically defined vaccine strains should be developed to confer long-term protection, but they should be exempt from any residual pathogenicity or risk of reversion towards virulence. Only a few molecular determinants of *M. mycoides* subsp. *mycoides* SC have been proposed as virulence factors. Arguably the best-characterized factor is the glycerol catabolism system, which includes an ATP-binding cassette (ABC) transporter (GtsABC) and a membrane-bound glycerol-phosphate oxidase (GlpO) that results in the production of hydrogen peroxide (Vilei & Frey, 2001). High amounts of H₂O₂ are released by virulent African strains, but not by less-virulent European strains, which has been directly associated with cytotoxic effects (Pilo *et al.*, 2005). However, this is not the only virulence attribute of mycoplasma, because vaccine strains, including T1/44, also show high cytotoxicity and high H₂O₂ release (Bischof *et al.*, 2008). As in most mycoplasma species, the *M. mycoides* subsp. *mycoides* SC genome encodes various lipoproteins that are suspected to play an important role in the interaction with the host (Westberg *et al.*, 2004). Some of these lipoproteins have been characterized, including LppB (Vilei & Frey, 2001) and LppQ, which has been described as a major antigen recognized during the immune response (Abdo *et al.*, 2000; Bonvin-Klotz *et al.*, 2008). A specific ELISA based on the detection of LppQ-antibodies has been developed to identify infected or vaccinated animals (Bruderer *et al.*, 2002). However, the specific role of the LppB and LppQ lipoproteins in pathogenicity is unknown, due to the lack of genetic tools available to generate mutants. Ideally, such mutants would be devoid of antibiotic-resistance markers, allowing experimental field trials for the evaluation of virulence.

The first step in the development of efficient genetic tools was the construction of *oriC* plasmids for *M. mycoides* subsp. *mycoides* SC, *M. mycoides* subsp. *mycoides* large colony (LC) and *Mycoplasma capricolum* subsp. *capricolum* (Lartigue *et al.*, 2003). These plasmids harbour the replicative origin of the chromosome and have been shown to replicate in mycoplasma cells. The use of these *oriC* plasmids for efficient gene targeting in *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* LC has been demonstrated (Janis *et al.*, 2005). Taking into account the phylogenetic proximity of the SC and LC biotypes, our first attempts to inactivate genes in *M. mycoides* subsp. *mycoides* SC were based on the same strategy. However, no recombination events were detected, despite several attempts and the choice of various target genes, including *lppQ*, *glpO* and the *gtsABC* operon (unpublished results). Similar difficulties have hampered the isolation of *Mycoplasma pulmonis* mutants using the same strategy (Cordova *et al.*, 2002).

Consequently, another strategy based on the use of a modified transposon was chosen. We took advantage of the development of a Tn4001 derivative (pMT85) used in *Mycoplasma pneumoniae* (Zimmerman & Herrmann, 2005). In the present work, pMT85 was first evaluated as a molecular tool for efficient gene mutagenesis in *M. mycoides* subsp. *mycoides* SC. In a second step, we further modified pMT85 to be able to eliminate the antibiotic-resistance gene after selection of transformants. Using this new strategy, a *lppQ* mutant of *M. mycoides* subsp. *mycoides* SC strain T1/44 was isolated and characterized.

METHODS

Bacterial strains and culture conditions. The vaccine strain T1/44 (CIRAD-EMVT/PANVAC; Batch 002) of *M. mycoides* subsp. *mycoides* SC was grown at 37 °C in modified Hayflick medium (Freund, 1983) without thallium acetate and supplemented with BBL IsoVitalax Enrichment (Becton Dickinson). For cloning procedures and propagation of plasmids, the *Escherichia coli* DH10B strain (Stratagene) was used; gentamicin (100 µg ml⁻¹) or tetracycline (5 µg ml⁻¹) was added when required. For selection of mycoplasma transformants, gentamicin and tetracycline were used at 300 and 20 µg ml⁻¹, respectively.

Plasmid constructions. Plasmid pMT85 is a plasmid harbouring a transposon derived from Tn4001 (Zimmerman & Herrmann, 2005). This plasmid contains a *colE1* replicative origin for propagation in *E. coli* and the *aacA-aphD* gene that confers resistance to gentamicin, tobramycin and kanamycin (Fig. 1). This selection marker is located

Fig. 1. Schematic outline of the PCR-based screening procedure for transposon insertions. Thick black arrows indicate the IRs of the transposon. *colE1*, replication origin in *E. coli*; *gent^R*, gentamicin-resistance marker encoded by the *aacA-aphD* gene. The transposase-encoding gene (TnpA) is located outside the transposed region of the pMT85 plasmid. The locations of primers used for the PCR-based screening are shown by thin arrows. The forward and reverse primers are specific to the gene of interest; the MT85.1 and MT85.2 primers are complementary to transposon sequences. Two orientations of the transposed elements are possible, as indicated by (1) and (2).

between the two inverted repeats (IRs) that define the extremities of the transposed fragment. In contrast, the transposase-encoding gene (*tnpA*) is located outside the transposed fragment.

The *res* sequences of the $\gamma\delta$ transposon were retrieved from the pSD6 plasmid (Duret *et al.*, 2005). The first *res* sequence was PCR-amplified with the *res7/res8* primers (Table 1) and cloned in the pMT85 plasmid after digestion with *NheI* and *Acc65I*. The second *res* sequence was PCR-amplified with the *res5/res6* primers (Table 1) and cloned in the intermediate plasmid after digestion with *SpeI* and *BamHI*. The resulting plasmid was named pMT85/2res.

The *oriC* plasmid pMYSO1 is a plasmid that replicates in *M. mycoides* subsp. *mycoides* SC cells and confers tetracycline resistance due to the *tet(M)* gene (Lartigue *et al.*, 2003). For the complementation of the *lppQ* mutant, the wild-type *lppQ* gene and surrounding sequences were PCR-amplified using the primers LppQ7 and LppQ8 (Table 1) and cloned at the *SphI* site of the pMYSO1 plasmid to generate pCJ13. The sequence of the cloned *lppQ* gene was verified by DNA sequencing.

The $\gamma\delta$ resolvase gene (*tnpR*) was PCR-amplified from the pSD25 plasmid (Duret *et al.*, 2005) using the PSresR and PSresL primers (Table 1) and cloned into the *SphI* site of the pMYSO1 plasmid; the resulting plasmid was named pCJ15. The sequence of the resolvase gene was verified by DNA sequencing.

Construction of transposon insertion libraries. PEG-mediated transformation of *M. mycoides* subsp. *mycoides* SC was performed as described elsewhere (King & Dybvig, 1994), using 30 μ g pMT85 (or pMT85/2res). Transformed cells were resuspended in 500 μ l pre-warmed Hayflick medium and incubated for 3 h at 37 °C. A 2 ml volume of medium supplemented with gentamicin (300 μ g ml⁻¹) was then added and serial dilutions were performed in selective medium to determine the transformation efficiency. For the pMT85-based library, the volume of the culture was increased to 10 ml with selective medium after 1–3 days, and further incubated for 1–3 days. One millilitre was kept as a stock at -80 °C and the remaining 9 ml was used for DNA extraction. For the pMT85/2res-based library, the

2.5 ml culture was incubated for 17–24 h at 37 °C and then plated onto solid medium supplemented with gentamicin (300 μ g ml⁻¹). Isolated colonies were picked after 7–10 days and cells were grown in 1.5 ml gentamicin-containing liquid medium. From each culture, 750 μ l was stored at -80 °C and the remaining 750 μ l was used for DNA extraction.

DNA isolation, PCR screening of the library and Southern blot hybridization. Genomic DNA was prepared from each pool of transformants using the NucleoSpin Tissue DNA purification kit (Macherey–Nagel). Genomic DNAs from the pools of transformants generated by each of the 122 transformations were gathered into 14 superpools for a first round of PCR. A second round was performed for each pool contained in a positive superpool. PCRs (25 μ l) were performed according to the recommendations of the *Taq* DNA polymerase supplier (Promega).

For Southern blot hybridization, 2 μ g genomic DNA or 30 ng plasmid DNA was digested with *EcoRI*, and hybridization was performed in the presence of DIG-labelled DNA probes (20 ng ml⁻¹). Detection of hybridized probes was achieved using anti-DIG antibodies coupled to alkaline phosphatase, and the fluorescent substrate CDP-star (Roche Molecular Biochemicals).

Protein analysis, and Western and colony blotting. Mycoplasma cells were collected from exponential-phase cultures by centrifugation (8000 g, 10 min, 4 °C). Whole-cell antigens of strains T1/44, T1/*lppQ*⁻RSP1 and Afadé were prepared by mixing the concentrated cells in PBS with one volume SDS sample buffer [0.06 M Tris/HCl, pH 6.8, 2 % SDS, 10 % glycerol (v/v), 2 % β -mercaptoethanol, 0.025 % Bromophenol Blue], and boiling for 5 min. The antigens were separated by SDS-PAGE using 12 or 10 % polyacrylamide gels and then blotted onto a nitrocellulose membrane with a pore size of 0.2 μ m (Bio-Rad). The membrane was then blocked with blocking buffer (1 % milk powder, 0.1 M Tris/HCl, pH 8.0, 1 M NaCl, 0.5 % Tween 20, 0.1 % merthiolate) for 1 h at room temperature, and incubated with the sera in blocking buffer (dilutions of 1:500 and 1:1000 for mouse sera and rabbit sera, respectively) for 1 h at room

Table 1. Primers used in this study

Primer	Sequence 5'–3'	<i>T_m</i> (°C)
Primers used for library screening and junction sequencing		
MT85.1	ACAGTAATTGCGGGTGGATC	60
MT85.2	TTACCGCCTTTGAGTGAGCT	60
GlpO6'	GCTCTTGCACTTGTGTTC	60
GlpO7	ACAGATTGCCACTGCTCATTT	60
GtsA3	ATGGATCCGCATGCTAGGACCTTCTGGGAGTGGA	62
GtsB3	GCTCAGCTGCCGACGCTCTAATAAGGCTGCTGCAAATAAAAT	58
GtsB1	GATCGCATGCAAGATTGACCAAATGAAGC	58
GtsC4	ATCTGCAGGCATGCCATAGCACCAGCTGCTTGAA	58
LppQ1	GATCGCATGCAAAAATGAGTGACAAAACAAGAT	60
LppQ2	ATATGCATGCTGTGTCAACCAAAGGCATC	58
Primers used for the construction of pCJ13, pCJ15 and pMT85/2res		
LppQ7	ATATGCATGCTGGTGTGCTACTGAAATTTGT	60
LppQ8	ATATGCATGCCCTGTTCCTCTTGCCCATCAT	60
PSresR	ATATGCATGCATTAAGCAATTTATTTGGAAAATC	56
PSresL	ATATGCATGCTTAGTTGCTTTTCATTATTACTT	56
res5	ATATACTAGTGGATCTCATAAAAATGTATCCTA	60
res6	ATATGGATCCGCAACCGTCCGAAATATTATAA	60
res7	ATATGCTAGCGGATCTCATAAAAATGTATCCTA	60
res8	ATATGGTACCGCAACCGTCCGAAATATTATAA	60

temperature and washed with deionized H₂O. The membranes were then incubated for 1 h at room temperature with alkaline phosphatase-labelled conjugates, which were diluted in blocking buffer. The conjugates used were polyclonal antibody anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories) diluted 1:2000 and polyclonal antibody anti-rabbit IgG (H+L) (Kirkegaard & Perry Laboratories) diluted 1:2000. The membranes were then washed with deionized H₂O. The colour reaction was initiated by adding 0.3 mg ml⁻¹ nitro blue tetrazolium (NBT) (Roche Diagnostics), followed by 0.15 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics) in alkaline buffer (100 mM Tris/HCl, pH 9.5, 5 mM MgCl₂), and was stopped with deionized H₂O.

For immunodetection of LppQ at the surface of mycoplasma cells, colonies were lifted from solid medium onto nitrocellulose membranes. After 5 min air drying, membranes were incubated for 1 h at room temperature in blocking buffer (25 mM Tris/HCl pH 8.0, 125 mM NaCl, 0.1 % Tween 20, 5 % defatted dry milk). Anti-LppQ-N' serum (Abdo *et al.*, 2000) (diluted 1:500) was then added for 1 h incubation at room temperature; the subsequent steps of the immunoblotting procedure were as described above.

Immunization of mice. Seven BALB/c mice, two males and five females, were immunized subcutaneously with 5×10^8 live cells of *M. mycoides* subsp. *mycoides* SC. The two male mice and two of the female mice were immunized with strain T1/44 and three female mice were immunized with strain T1/lppQ-RSP1. After 18 days, the immunizations were repeated, and after another 18 days, blood was collected from the animals. Blood samples were centrifuged at 1000 g for 5 min at 4 °C to recover the serum.

RESULTS

Construction of a library of *M. mycoides* subsp. *mycoides* SC mutants using pMT85

The plasmid pMT85 used in this study harbours a transposon derived from Tn4001 which confers gentamicin resistance (Zimmerman & Herrmann, 2005). We first determined the MIC for gentamicin of the T1/44 strain of *M. mycoides* subsp. *mycoides* SC. This MIC ranged from 15.6 to 32.2 µg ml⁻¹, and a concentration of 300 µg ml⁻¹ was chosen for the selection of transformants. The yield of each transformation of *M. mycoides* subsp. *mycoides* SC with pMT85 was found to vary for unknown reasons, generating between 10 and 1000 mutants. A set of 122 transformation experiments was conducted to generate the transposon library. From each experiment, the transformants were amplified in liquid medium as a pool, before DNA extraction. The total number of mutants in the library was estimated to be in the range of 40 000–400 000. Considering that 1016 genes were identified in the genome of the type strain PG1 of *M. mycoides* subsp. *mycoides* SC (Westberg *et al.*, 2004), each non-essential gene should be targeted several times by the transposon in this library of mutants, assuming random insertions of the transposon.

PCR-based detection of mutants of interest within the transposon insertion library

To evaluate the diversity of mutants within the library, transposon insertion sites were screened using a PCR-based

method. Three loci with genes potentially involved in the pathogenicity of *M. mycoides* subsp. *mycoides* SC were searched: the *gtsABC* operon, the *glpO* gene and the *lppQ* gene. For the first two targets, there is experimental evidence showing that the genes are not essential. Indeed, the *gtsABC* operon is partly deleted and therefore not functional in European strains of *M. mycoides* subsp. *mycoides* SC (Vilei & Frey, 2001), and a glycerol-phosphate oxidase-deficient strain has been described (Wadher *et al.*, 1990).

The PCR-based strategy for screening mutants of interest is described in Fig. 1. Briefly, two primers flanking the locus of interest were used in combination with two other primers directed against specific sequences of the transposon, MT85.1 and MT85.2 (Table 1). Accordingly, a PCR product should be generated with two of the four possible pairs of primers whenever a transposon is inserted between the positions of the target-specific oligonucleotides.

To limit the number of PCR reactions, genomic DNAs from the pools of transformants generated by each of the 122 transformations were gathered into 14 superpools. For each of the three targeted loci, a first round of PCR was performed on each superpool. Amplification products compatible with the locus size were observed for each target in at least one superpool. For example, in the search for *lppQ* mutants, a fragment of about 1300 bp was amplified from the DNA superpool SP1 in the reaction mix containing the LppQ2/MT85.2 pair of primers (Fig. 2a). Taking into account the length of the *lppQ* gene (1338 bp) and the nucleotide position of the primers, this amplification suggested that the transposon had integrated in the chromosome in the reverse orientation, at about 200 bp downstream from the start codon. An amplification of the other junction should also have been observed using the LppQ1/MT85.1 pair of primers. However, in this particular case, further analysis indicated that the transposon had integrated into the chromosome exactly within the sequence corresponding to the LppQ1 primer. Consequently, because of LppQ1 mispairing, no amplification product was observed. Another primer, LppQ7, located upstream from LppQ1 and used with MT85.1, enabled PCR amplification of the junction (data not shown).

For each target, after identifying positive superpools, a second round of PCR was performed to determine the pool in which the mutant of interest could be found. In every case, PCR products were observed for at least one pool. For example, the superpool SP1 resulted from the mixing of genomic DNAs extracted from 10 pools of transformants and was PCR-positive for a *lppQ* mutant. During the second round of PCR, a fragment of about 1300 bp was amplified from pool P1.4, as in the superpool SP1 (Fig. 2b). Another pool, P1.5, was also found to be positive during this screening, with a PCR product of about 1500 bp (Fig. 2b), which suggested that the transposon was inserted within *lppQ* at a site different from that of the mutant in pool P1.4.

PCR products obtained from pools of transposon mutants were sequenced to identify the sites of insertion and to

Fig. 2. PCR-based screening for transposon insertions within the *lppQ* gene. (a) First round of PCR screening for *lppQ* mutants within superpool SP1 using different combinations of PCR primers (see Table 1) as indicated above the lanes. T, untransformed T1/44 control; P, superpool SP1; M, DNA Smart Ladder (Eurogentec). (b) Second-round PCR using primers LppQ2 and MT85.2 for the identification of the pools containing *lppQ* mutants. Pool P1.4 gave the strongest signal, of the same size as that detected in superpool SP1.

predict the lengths of the truncated proteins (Table 2). Sequencing of the amplification products confirmed integrations of the transposon in each target locus. Two

insertions were detected in the *gtsABC* locus, one in *gtsB* and one in *gtsC*. Three different mutants were characterized with transposon insertions within the *glpO* gene. Predicted GlpO proteins were 383, 37 and 79 aa in length, instead of 392 aa for the wild-type protein. Finally, two insertions were identified within the *lppQ* locus. The first one, detected in the P1.4 pool, was located 209 bp downstream of the ATG, leading to a truncated putative protein of 70 aa in length, instead of 445 aa for the wild-type protein. The second insertion, identified in the P1.5 pool, was found 205 bp upstream of the ATG, in a non-coding sequence (Table 2).

Our results show that pMT85 can be used for efficient mutagenesis of *M. mycoides* subsp. *mycoides* SC and that insertion events in genes of interest can be detected by a PCR-based procedure.

Isolation and characterization of a *lppQ* mutant

Different methods can be used to isolate specific mutants from a pool identified as positive for a specific targeted gene. Taking into account the relatively low complexity of the pools generated by transformation of *M. mycoides* subsp. *mycoides* SC (about 10–1000 transformants), this could be achieved by a PCR-screening assay. Alternatively, mutants can be screened using antibodies directed against the encoded protein. Such a strategy was used for the isolation of a *lppQ* mutant by negative immunoscreening. After filtration through a 0.45 µm pore-size filter to eliminate cell aggregates, transformants from pool P1.4 were plated on solid medium supplemented with gentamicin. A colony-blot assay was then performed using anti-LppQ-N antibodies. Colonies that were revealed by a non-specific Ponceau red staining but not by the anti-LppQ-N antibodies were picked and grown in selective liquid medium. After a second round of filtration and negative immunodetection, a single mutant, named T1*lppQ*[−]MT1, was obtained.

Genomic DNA was extracted from the T1*lppQ*[−]MT1 mutant and the transposon integration site was verified

Table 2. Transposon integration sites within specific target genes from *M. mycoides* subsp. *mycoides* SC

Gene	Gene length (bp)	Wild-type protein length (aa)	Screening primers	Positive superpool	Positive pool	Insertion site*	Predicted length of truncated protein (aa)
<i>glpK</i>	1518	505	GlpO6', GlpO7	SP11	P11.11	+29	10
<i>glpO</i>	1179	392	GlpO6, GlpO7	SP10	P10.9	+1148	383
			GlpO6', GlpO7	SP10	P10.11	+109	37
			GlpO6', GlpO7	SP11	P11.9	+217	79
<i>gtsB</i>	1029	342	GtsA3, GtsB3	SP3	P3.10	+505	175
<i>gtsC</i>	810	269	GtsB1, GtsC4	SP2	P2.1	+588	200
<i>lppQ</i>	1338	445	LppQ1, LppQ2	SP1	P1.4	+209	70
			LppQ1, LppQ2	SP1	P1.5	−285	Untruncated

*From the ATG start codon.

by DNA–DNA hybridization (Fig. 3). Total DNA was *Eco*RI-digested and the restriction fragments were separated by gel electrophoresis. After transfer onto nylon membranes, the DNA was hybridized with probes complementary to inserted parts of the plasmid (*co*/E1) or *lppQ* sequences. A unique *Eco*RI fragment of about 5500 bp was detected by both probes. This length was in agreement with the theoretical genomic organization inferred from the transposon integration site identified by sequencing. Moreover, only one band was detected using the *co*/E1 probe, indicating that a single copy of the transposon had integrated the chromosome (Fig. 3). It should be noted that the genome of the T1/44 strain contains a single copy of the *lppQ* gene (Bischof *et al.*, 2006), in contrast to that of the PG1 type strain, which contains two copies (Westberg *et al.*, 2004). To evaluate its

Fig. 3. Location of the transposon within the *lppQ* gene of the T1/*lppQ*[−]MT1 mutant. (a) Schematic integration of the transposon within the *lppQ* gene. Grey bars indicate the location of the probes *co*/E1 and *lppQ* used in Southern blots. E, *Eco*RI restriction site. Elements are not drawn to scale. (b, c) Total genomic DNA from the T1/*lppQ*[−]MT1 mutant was *Eco*RI-digested and fragments were separated by gel electrophoresis, transferred to nylon membranes and hybridized with a *co*/E1 (b) or a *lppQ* probe (c). P, pMT85 control; T, T1/44; M, T1/*lppQ*[−]MT1 mutant.

stability, the T1/*lppQ*[−]MT1 mutant was cultured in the absence of gentamicin for 15 passages, and the *lppQ* locus was checked again by DNA hybridization. The same DNA pattern was observed, showing that the integrated transposon was maintained at the same locus (data not shown).

In order to confirm that the LppQ antigen was not expressed by the T1/*lppQ*[−]MT1 mutant, total proteins extracted from both wild-type and mutant cells were separated by SDS-PAGE (Fig. 4). After transfer onto a nitrocellulose membrane, immunodetection of the LppQ protein was performed using anti-LppQ-N antibodies. Whereas the LppQ protein was detected in the T1/44 control extract as a band corresponding to a protein of about 48 kDa, it was not found in the extract from the mutant strain, which confirmed that the transposon integration prevents LppQ expression. It is still possible that a 70 aa truncated LppQ protein was produced that could not be detected under the experimental conditions used. The T1/*lppQ*[−]MT1 mutant was then transformed with the pCJ13 plasmid, which is a derivative of the *oriC*-based plasmid pMYSO1 and carries the *lppQ* gene under the control of its own promoter. This transformation complemented the *lppQ* mutation, as the production of the LppQ protein was restored (Fig. 4b).

Improvement of the pMT85 plasmid to generate unmarked mutations

With the goal of being able to delete the antibiotic-resistance marker from the mycoplasma genome to obtain

Fig. 4. Lack of LppQ expression in the T1/*lppQ*[−]MT1 mutant. (a) Total proteins were extracted using SDS from untransformed and mutant cells and separated by 12% PAGE. T, T1/44 (60 µg); M, T1/*lppQ*[−]MT1 mutant (60 µg); MC, T1/*lppQ*[−]MT1 mutant complemented with wild-type *lppQ* gene (120 µg). Proteins were stained with Coomassie Blue. Molecular mass markers LMW were used (Amersham Biosciences). (b) Immunodetection of LppQ after transfer onto a nitrocellulose membrane. A polyclonal serum directed against the N-terminal part of LppQ was used to detect the lipoprotein (dilution 1 : 500). The two panels correspond to three independent strips that were parts of a single experiment and that were grouped for the purpose of this figure.

unmarked mutations, the pMT85 plasmid was modified by cloning *res* sequences within the element surrounded by the IR sequences, yielding the pMT85/2*res* plasmid (Fig. 5). The *res* sequences are recognized specifically by the transposon $\gamma\delta$ resolvase, which is encoded by the *tnpR* gene carried by the pMYO1-derived pCJ15 plasmid.

The pMT85/2*res* plasmid was used to create a new library of *M. mycoides* subsp. *mycoides* SC insertion mutants using conditions similar to those described above. However, instead of keeping the mutants as a pool in liquid medium, after 17–24 h culture with antibiotic, mycoplasmas were plated on selective medium containing gentamicin. A total of 984 clones were collected from a set of eight transformations, and were amplified in 1.5 ml cultures.

Isolation and characterization of an unmarked *lppQ* mutant

The new library of *lppQ* mutants was screened by PCR, using the same strategy as described in Fig. 1, which resulted in the selection of the clone T2A108. The sequencing of PCR products from this clone indicated that the transposed element from pMT85/2*res* had inserted between nucleotides 989 and 990 of the *lppQ* gene. Clone T2A108 was transformed with the pCJ15 plasmid in order to remove the *aacA-aphD* marker. After a single passage of the transformed clone in the presence of tetracycline, the antibiotic was removed to facilitate the loss of the pCJ15

plasmid, and a new clone, named T1*lppQ*[−]RSP1, was obtained after plating on a medium without antibiotic. We verified that the clone T1*lppQ*[−]RSP1 had the same sensitivity to tetracycline and gentamicin as the original T1/44 strain, which indicated that it had lost the *aacA-aphD* gene and the *tet*(M) gene carried by the pCJ15 plasmid. However, further sequencing analysis showed that the T1*lppQ*[−]RSP1 clone had retained the *tnpA* gene, which indicated that the initial transposition had not occurred exactly as expected (Fig. 6). Indeed, analysis of the junction between the 5' end of the *lppQ* gene and the inserted element showed that an IR-like sequence was used during transposition. This sequence is only partially homologous to the genuine IR, with 17 conserved positions out of 26. This clone, which showed the required phenotype, was selected for further characterization.

Mice immunized with live *M. mycoides* subsp. *mycoides* SC strain T1/44 or T1*lppQ*[−]RSP1 showed no side-effects from immunization with either strain. Sera collected from these animals were used to detect mycoplasma antigens obtained from *M. mycoides* subsp. *mycoides* SC strains T1/44, T1*lppQ*[−]RSP1 and the virulent strain Afadé. The immunoblot results were compared with that of an identical immunoblot incubated with monospecific anti-LppQ-N antibodies (Fig. 6). Sera from mice infected with the vaccine strain T1/44 of *M. mycoides* subsp. *mycoides* SC

Fig. 5. Schematic outline of the two-step procedure to obtain unmarked mutants. In the first step the transposon integrates into the chromosome. After selection of a mutant of interest, resolution of most of the transposed sequence is induced by the $\gamma\delta$ resolvase that is encoded by a gene carried by the *oriC* plasmid pCJ15. Thick black arrows indicate the IRs of the transposon harboured by the plasmid pMT85/2*res*. Open arrows indicate the resolvase target sequences (*res*). *co/E1*, replication origin in *E. coli*; *gent^R*, gentamicin-resistance marker. The transposase-encoding gene is located outside the transposed region of the plasmid.

Fig. 6. Molecular characterization of the mutant T1*lppQ*[−]RSP1. (a) Genomic structure of the disrupted *lppQ* gene in the T1*lppQ*[−]RSP1 mutant. The position of the transposon within the nucleotide sequence of *lppQ* is indicated. *tnpA*, transposase gene; black arrows, transposon IRs; open arrow, remaining *res* sequence. (b) Immunodetection of LppQ in T1/44, T1*lppQ*[−]RSP1 and Afadé. Total proteins were extracted from mycoplasma cells of T1/44 (lanes a), T1*lppQ*[−]RSP1 (lanes b) and Afadé (lanes c) and separated by 10% PAGE. The blot in panel 1 was reacted with anti-T1/44 antibodies. The blot in panel 2 was reacted with serum from a mouse that was immunized with T1*lppQ*[−]RSP1. The blot in panel 3 was reacted with anti-LppQ-N antibodies. M, prestained molecular mass markers. The black arrows indicate the 48 kDa LppQ antigen.

showed a strong reaction to the 48 kDa protein LppQ in whole-cell preparations of strains T1/44 and Afadé, whereas no reaction to this antigen could be detected with a whole-cell preparation of strain T1lppQ⁻RSP1 (Fig. 6a). When sera from mice immunized with the T1lppQ⁻RSP1 clone were tested against the same bacterial preparations from the three strains, there was no reaction to LppQ (Fig. 6b). Anti-LppQ-N antibodies showed that the 48 kDa protein LppQ was present in strains T1/44 and Afadé. However, they reacted only weakly to a 37 kDa antigen from T1lppQ⁻RSP1 (Fig. 6c, lane b), which suggests that there was production of minor amounts of the residual N-terminal part of LppQ in strain T1lppQ⁻RSP1. The size of the detected antigen was consistent with the calculated mass of the truncated LppQ protein (38.36 kDa) in T1lppQ⁻RSP1. Interestingly, sera from all three mice infected with T1lppQ⁻RSP1 did not react to a protein of ~85 kDa present in all three strains, T1/44, T1lppQ⁻RSP1 and Afadé, while serum from all four mice immunized with T1/44 reacted predominantly to this 85 kDa protein band in all strains. This 85/80 kDa antigen of *M. mycoides* subsp. *mycoides* SC is known as a major antigen of experimentally or naturally infected cattle (Abdo *et al.*, 2000). The lack of antigenicity of the 85 kDa protein in strain T1lppQ⁻RSP1 could be due to a number of factors, such as the reduced immunogenicity of the cell antigens because the major LppQ lipoprotein is not produced. Another possibility is that there is altered production or membrane exposure of the 85 kDa protein, for reasons that are currently unknown.

DISCUSSION

Two transposons and various derivatives have already been used for gene inactivation in mollicutes. Transposon Tn916, originating from *Enterococcus faecalis* and carrying the *tet*(M) gene that confers resistance to tetracycline, has been used with success in *Acholeplasma laidlawii*, *M. pulmonis*, *Mycoplasma hyorhinis* (Dybvig & Cassell, 1987; Dybvig & Alderete, 1988), *M. mycoides* subsp. *mycoides* LC (King & Dybvig, 1991) and *Mycoplasma gallisepticum* (Whetzel *et al.*, 2003). However, the instability of the integrated transposon has been identified as a potential drawback (Dybvig & Alderete, 1988). A second transposon, Tn4001, initially isolated from *Staphylococcus aureus*, has been used to create mutants in *M. pneumoniae*, *Mycoplasma genitalium* (Hedreyda *et al.*, 1993; Reddy *et al.*, 1996), *M. gallisepticum* (Mudahi-Orenstein *et al.*, 2003) and *Spiroplasma citri* (Foissac *et al.*, 1997). This transposon carries the gene *aacA-aphD*, which confers resistance to gentamicin, kanamycin and tobramycin. Because gentamicin resistance could not be used as a selective marker in *M. pulmonis* and *M. hyorhinis*, derivatives carrying the *tet*(M) gene (Tn4001T) or the *cat* gene (Tn4001C) were developed for use in these two species (Dybvig *et al.*, 2000). Another derivative of Tn4001 with the transposase gene cloned outside the transposable region of the harbouring plasmid

pMT85 was recently used for haystack mutagenesis in *M. pneumoniae* (Halbedel & Stulke, 2007; Zimmerman & Herrmann, 2005). In the present study, we extended this work by modifying this Tn4001 derivative to create unmarked mutations in the genome of *M. mycoides* subsp. *mycoides* SC, a major bovine pathogen.

In this study, we have shown that the mutant T1lppQ⁻MT1, obtained by using the pMT85 plasposon, is stable. Furthermore, we also have shown that the *lppQ* mutation can be complemented by using derivatives of the pMYSO1 *oriC* plasmid (Lartigue *et al.*, 2003). The combination of a transposon that confers resistance to gentamicin and a replicative plasmid that harbours a marker for tetracycline resistance constitutes an efficient, and as yet unreported, panel of molecular tools for the identification of the genetic determinants of the pathogenicity of *M. mycoides* subsp. *mycoides* SC.

Three particular loci of the *M. mycoides* subsp. *mycoides* SC genome have been chosen as targets for this study because of their proposed involvement in mycoplasma pathogenicity (Pilo *et al.*, 2007). Disruption of the genes *lppQ*, *glpO*, *gtsB* and *gtsC* indicates that these genes are not essential, at least for growth in rich medium. For both *glpO* and *gtsABC*, this conclusion is in agreement with previous studies reporting *M. mycoides* subsp. *mycoides* SC strains in which these genes were inactivated or missing (Vilei & Frey, 2001; Wadher *et al.*, 1990). Our study indicates that the gene encoding the major LppQ antigen is not essential, as we were able to isolate and characterize two independent mutants, T1lppQ⁻MT1 and T1lppQ⁻RSP1. These mutants are of interest because LppQ is hypothesized to play a role in producing adverse reactions in vaccinated animals. Indeed, a recent study has shown that cattle immunized with purified recombinant LppQ, using different adjuvant methods, were significantly more susceptible to challenge with *M. mycoides* subsp. *mycoides* SC than cattle that were not vaccinated with LppQ (Nicholas *et al.*, 2004). LppQ may therefore contribute to the immunopathology induced by *M. mycoides* subsp. *mycoides* SC, including the strong post-vaccine reactions observed after immunization of animals with the T1/44 vaccine strains. The *lppQ* mutants derived in this study from strain T1/44 provide a unique opportunity to test this hypothesis. The unmarked T1lppQ⁻RSP1 mutant is particularly suited for such studies because of the removal of the antibiotic-resistance marker, which may be regarded as a potential environmental hazard if left in a genetically modified vaccine. The development of a vaccine strain that does not produce LppQ, such as the T1lppQ⁻RSP1 mutant, follows the modern vaccinology concept of DIVA (differentiating infected from vaccinated animals); hence, existing serological assays could be employed to differentiate between infected and vaccinated animals (Bruderer *et al.*, 2002; Le Goff & Thiaucourt, 1998).

The development of improved *M. mycoides* subsp. *mycoides* SC vaccine strains remains an important goal for the

control of CBPP in countries where the disease is endemic. The inactivation of specific genes is now a practicable strategy for attenuating virulent strains, or existing vaccine strains for which side-effects indicative of residual virulence have been reported. One of the main challenges of vaccine strain development is to eliminate virulence while maintaining protective immunogenicity. This last constraint implies that gene-inactivation strategies should minimize the number of passages from the initial strain to the isolated mutant. The genetic tools that we have adapted for *M. mycoides* subsp. *mycoides* SC enable the isolation of specific mutants in fewer than 10 passages, including steps for the removal of the antibiotic-resistance marker.

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