Development of a replicable *oriC* plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*

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The genome of *Mycoplasma gallisepticum* strain R_{low} has been sequenced completely, but subsequent genetic studies have been limited by the lack of a replicable vector system. In this study, replicable plasmids were constructed for *M. gallisepticum* and *Mycoplasma imitans* using the *oriC* region upstream from the *soj* gene. The *oriC* plasmids of *M. gallisepticum* (pGTLori) and *M. imitans* (pMLori) replicated in both species, but *Mycoplasma pneumoniae* could not support replication of pGTLori. A 180 bp section of the *oriC* region of *M. gallisepticum* was found to be the minimal region required for plasmid replication in *M. gallisepticum* strain S6, the shortest *oriC* region defined for mycoplasmas. Targeted gene disruption of *vlhA1.1* of *M. gallisepticum* S6 was attempted using these *oriC* plasmids. Constructs made in pPLoriC7 integrated into the *M. gallisepticum* genomic *oriC* region, not into the targeted gene, whereas those made in pMLori disrupted the *vlhA1.2* gene, which has 97% DNA sequence identity with the *vlhA1.1* gene.

During *in vitro* passages, antimicrobial selection pressure did not influence the rate of chromosomal integration. These *oriC* plasmids will thus be useful for genetic studies, including inactivation or expression of selected genes, in *M. gallisepticum* and *M. imitans*, and will lead to a better understanding of their molecular biology. They are, to our knowledge, the first replicable plasmids developed for the *Pneumoniae* phylogenetic group of mycoplasmas.

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INTRODUCTION

Mycoplasma gallisepticum is an important pathogen of poultry, causing chronic respiratory disease in chickens and infectious sinusitis in turkeys. It is responsible for considerable economic losses in poultry production worldwide due to downgrading of carcasses, reduced feed conversion efficiency, decreased egg production and increased medication costs (Ley & Yoder, 1997). Recently the genome of *M. gallisepticum* was sequenced completely (Papazisi *et al.*, 2003), allowing prediction of the function of specific genes. However, genetic studies of *M. gallisepticum* have been limited by the lack of genetic tools for its manipulation, with most studies using transposons Tn916 (Dybvig & Cassell, 1987; Dybvig & Alderete, 1988; Whetzel *et al.*, 2003) and Tn4001 (Bearson *et al.*, 2003; Hedreyda *et al.*, 1993; Hudson *et al.*, 2006; Mahairas & Minion, 1989; Mudahi-Orenstein *et al.*, 2003; Papazisi *et al.*, 2002; Whetzel *et al.*, 2003; Winner *et al.*, 2003) or suicide vectors (Markham *et al.*, 2003) to study gene function. However, the random insertion of the transposon in the genome of

the organism does not allow specific targeting of a gene of interest, and random integration can confound analyses of gene expression (Dybvig & Cassell, 1987; Mahairas & Minion, 1989). There has been some success in development of vectors for mollicutes using homologous origins of replication (*oriC*) and selectable antibiotic resistance markers. These plasmids are able to replicate extrachromosomally and have been used to inactivate target genes by homologous recombination (Cordova *et al.*, 2002; Duret *et al.*, 1999; Janis *et al.*, 2005; Lartigue *et al.*, 2002). In many cases *oriC* plasmids containing larger *oriC* regions have been found to integrate into the *oriC* region in the genomic DNA by homologous recombination following *in vitro* passage. Plasmids containing a shorter *oriC* region are more stable and have been used to generate targeted homologous recombination (Cordova *et al.*, 2002; Lartigue *et al.*, 2002; Renaudin *et al.*, 1995). Most of the *oriC* plasmids of mollicutes show host specificity, but plasmids containing the *oriC* of *Mycoplasma mycoides* subsp. *mycoides* LC and SC replicate in the closely related species *Mycoplasma capricolum* subsp. *capricolum* (Lartigue *et al.*, 2003). *Mycoplasma imitans*, which has been isolated from ducks and geese, is phylogenetically closely related to *M. gallisepticum*. DNA–DNA hybridization studies show

The GenBank/EMBL/DDBJ accession number for the *oriC* region sequence of *M. imitans* is EF028085.

that *M. gallisepticum* and *M. imitans* share 40–46 % genetic identity (Bradbury *et al.*, 1993), whilst PFGE and random amplified polymorphic DNA studies suggest a DNA sequence identity of 53–60 % (Bradbury *et al.*, 1993; Marois *et al.*, 2001). Papazisi *et al.* (2003) reported that the predicted *oriC* region of *M. gallisepticum* was located between the *dnaN* and *dnaA* genes. The predicted *oriC* region contains five DnaA box sequences, with the consensus sequence 5'-TTWTMHAMA-3' identical for each DnaA box, and the region between *dnaN* and *soj* contains a higher than average content of adenosine and thymidine (80 %). However, the DNA sequence of the *M. imitans* *oriC* region has not been determined. The aims of this study were to determine the DNA sequence of the *M. imitans* *oriC* region and to produce vectors containing different regions of the *oriC* of *M. gallisepticum* and *M. imitans* to assess their replication and stability during passage. We also attempted to inactivate a target gene in *M. gallisepticum* using these *oriC* plasmids. We chose to knockout the expressed *VlhA1.1* gene of *M. gallisepticum* as the genome sequence is available and the target gene is not essential for the survival of the organism (Glew *et al.*, 2000).

METHODS

Bacterial strains and growth media. *M. gallisepticum* strain S6 (Markham *et al.*, 1992) and *M. imitans* strain 4229 (Bradbury *et al.*, 1993) were grown in modified Frey's medium to late exponential phase at 37 °C (Whithear, 1993). *Mycoplasma pneumoniae* strain FH (ATCC No. 15531) (donated by V. Peters, Department of Virology, Royal Children's Hospital, Melbourne) was grown in modified Hayflick's medium in tissue-culture flasks. For the growth and selection of antibiotic-resistant mycoplasma transformants, tetracycline was added to a concentration of 4 µg ml⁻¹ in broth or agar medium (Markham *et al.*, 2003). *Escherichia coli* strain DH5α was used as the host for gene cloning and was grown in Luria–Bertani (LB) broth at 37 °C and subjected to standard molecular biological techniques (Sambrook & Russell, 2001).

Amplification and DNA sequencing of the *oriC* region of *M. imitans* strain 4229. Part of the *oriC* region and the *dnaA* gene of *M. imitans* was amplified using the primer pairs SW15for and MIsjrev-1, and SW6for and dnaArev, respectively (Table 1), and cloned into pGEM-T for DNA sequencing. The remainder of the *oriC* region was sequenced directly from genomic DNA. The DNA sequence was aligned with the homologous region from the complete genome of *M. gallisepticum* strain R_{low} (GenBank accession no. NC_004829).

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequence* (5'–3')	PCR product
SW4 for/SW5 rev	GAGCTTTTGCATGCAAAAAATTC/ TTGTTAATCCATGGAATGATCATAA	Long <i>oriC</i> region (LoriC; 1.96 kb)
SW6 for/SW7 rev	CCTCAGCTGCAGTAGGTTATG/ GAACTGAAATGTCGACTAAAAATC	Short <i>oriC</i> region (SoriC; 0.67 kb)
SW4 for/SW7 rev		Whole <i>oriC</i> region (WoriC; 3.16 kb)
SW4 for/SW9 rev	/CTGATGCCGTTAGCATTAGG	Partial LoriC 1 (1.06 kb)
SW15 for/SW16 rev	CGCTTTAATATTGTAACAATA/ GCTGTTATTCAAGAATAATAAATTC	Partial LoriC 2 (565 bp)
SW4 for/PLori-rev-2	/CAGATAGACAAAAGAATGAAAAAG	Partial LoriC 3 (707 bp)
Start DnaN f/PLori-rev-2	GATAAATCTGTGGATAACTC/	Partial LoriC 4 (633 bp)
SW4 for/PLori-rev-4	/CACAATATTCCGATAAAAAATCAC	Partial LoriC 5 (510 bp)
SW4 for/PLori-rev-5	/TTTTACAAACAATATTGTTAC	Partial LoriC 6 (459 bp)
SW4 for/PLori-rev-6	/ACACGTTTTGTGAAAAAGTGA	Partial LoriC 7 (180 bp)
SW4 for/pPLoriC8rev	/GAGTTATCCTCTGATTTATC	Partial LoriC 8 (96 bp)
pPLoriC9for/PLori-rev-6	CCATGATAATTGTTGATAAATC/	Partial LoriC 9 (119 bp)
Tetfor/Tetrev	GAAAAGATCTGGAGTAATTGGAAG/ ACTAGTCCATATTTATATAACAACCT	Tetracycline resistance gene (2.36 kb)
AmpR for/AmpR rev	CCAATGCTTAATCAGTGAGG/ GTATGAGTATTCAACATTTCCG	Ampicillin resistance gene (862 bp)
SW6 for/dnaA rev	/AAGCATGTCTAAAGTCTAATTAGAA	<i>dnaA</i> gene of <i>M. imitans</i> (2.1 kb)
SW15 for/MIsj rev-1	/TTTTCCATTTTGTATTCTC	partial <i>oriC</i> region and <i>soj</i> gene of <i>M. imitans</i> (2 kb)
MIdnaN for/MIsj rev-2	CAGAAGAGAAGTTGCTTGAAAAC/ CTAGTGTGCTCTTCAAAACAC	<i>oriC</i> region of <i>M. imitans</i> (2.3 kb)
P3.03-F-SphI/P3.03-R-NcoI	CTTAGCTGCATGCGCAGATTCG/ TTTTCCATGGGTTTTGCTTGG	Internal fragment of the <i>vlhA3.03</i> gene (1 kb)
P3.03-F-PstI/P3.03-R-SalI	CTTAGCTCCATCTGCAGATTCG/ CATTAGGTTTTGCGTCGACGTTTAATG	Internal fragment of the <i>vlhA3.03</i> gene (1 kb)
SW13 for/vlhALeader rev	CGACTCACTATAGGGCGAA/ CAAATGAACCAATACCTAATAA	Integration site of pMioriC/Δ3.03

*Underlined bases are restriction endonuclease cleavage sites.

Construction of *oriC* plasmids. Several regions of the predicted *oriC* region of *M. gallisepticum* were amplified by PCR (Fig. 1a). The long *oriC* region, the short *oriC* region, and the whole *oriC* region were amplified with the specific primer pairs SW4for and SW5rev, SW6for and SW7rev, and SW4for and SW7rev, respectively (Table 1). Each PCR contained 5 µl 10× reaction buffer (Invitrogen), 10 µM each deoxynucleoside triphosphate, 2.5 mM MgSO₄, 12.5 µM each primer, 1.25 U Platinum *Taq* High Fidelity DNA polymerase (Invitrogen), 10 ng genomic DNA of *M. gallisepticum* strain R_{low} as template, and water to a final volume of 50 µl. PCRs were performed in a thermocycler (iCycler, Bio-Rad) under the following conditions: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 2 min, and 68 °C for 4 min, with a final extension at 68 °C for 7 min. PCR products were separated by agarose gel electrophoresis and extracted from gel slices using the QIAEX II kit (Qiagen) according to

Fig. 1. Construction of *M. gallisepticum* and *M. imitans* *oriC* plasmids. (a) *M. gallisepticum* genomic *oriC* region and plasmid constructs containing different *oriC* regions. The plasmid backbone was pGEM-T (Promega) containing the tetracycline-resistance gene (*tetM*). The long *oriC* region was inserted between the *SphI* and *NcoI* sites located in the multicloning site of pGEM-T, and the short *oriC* was inserted between the *PstI* and *SalI* sites. The whole *oriC* region was amplified by PCR and cloned into pGEM-T, and the *tetM* gene was then cloned into the *SpeI* site. (b) *M. imitans* genomic *oriC* region and plasmid construct containing the *oriC* region. The *oriC* region was amplified and cloned into pGEM-T and then the *tetM* gene was cloned into the *SpeI* site. The black triangles indicate the locations of the DnaA box consensus sequences and shaded rectangles indicate the locations of AT-rich clusters. Sp, *SphI*; N, *NcoI*; P, *PstI*; S, *SalI*; Se, *SpeI*.

the manufacturer's instructions. The long and short *oriC* regions were cloned separately into pGEM-T (Promega), into which the tetracycline-resistance gene had already been inserted (*tetM*/pGEM-T) (Markham *et al.*, 2003). The long *oriC* region was inserted between the *SphI* and *NcoI* cleavage sites, and the short *oriC* region was inserted between the *PstI* and *SalI* sites (Fig. 1a). The resulting constructs were designated pGTLori and pGTSori. A further construct was produced that contained both the regions upstream and downstream from the *soj* gene, and was named pGTDori. The whole *oriC* region was amplified similarly and cloned into pGEM-T, and the *tetM* gene was then cloned into the *SpeI* site of the vector, resulting in pGTWori (Fig. 1a). The *oriC* region of *M. imitans* was amplified from the genome of *M. imitans* strain 4229 using the MIdnaNfor and MIsjrev-2 primer pair (Table 1), introduced into the cloning site of pGEM-T, and the *tetM* gene was then ligated into the *SpeI* cleavage site of the vector (Fig. 1b). To determine the minimal *oriC* region for plasmid replication in *M. gallisepticum*, different regions upstream of the *soj* gene were amplified by PCR with specific primer pairs (Table 1). To generate the *oriC* plasmids containing the PCR products shown in Fig. 2, each PCR product was cloned separately into pGEM-T, and the *tetM* gene was then ligated into the *SpeI* site of the vector.

Transformation of *M. gallisepticum* strain S6, *M. imitans* strain 4229 and *M. pneumoniae* strain FH. *M. gallisepticum* strain S6, *M. imitans* strain 4229 and *M. pneumoniae* strain FH were transformed by electroporation, as described by Hedreyda *et al.* (1993). Briefly, 5 ml cultures of mycoplasmas were grown to late exponential phase and harvested by centrifugation at 12 000 g for 5 min in a bench-top centrifuge at room temperature. The cells were washed twice in 250 µl ice-cold HEPES-sucrose buffer (8 mM HEPES, 272 mM sucrose, pH 7.4). The cell pellet was then resuspended in 100 µl HEPES-sucrose buffer containing ~10 µg plasmid DNA and transferred to a Gene Pulser cuvette with a 0.2 cm electrode gap (Bio-Rad), and the mixture was pulsed using a Gene Pulser (Bio-Rad) set at 2.5 kV, 100 Ω and 25 µF. The cells were resuspended in 1 ml broth and incubated at 37 °C until the medium showed a colour change, after which 500 µl culture was plated onto agar medium containing 4 µg tetracycline ml⁻¹. The plates were incubated at 37 °C for 7–10 days in the dark in an airtight jar for *M. gallisepticum* and *M. imitans*, and incubated for 21 days for *M. pneumoniae*. Individual tetracycline-resistant colonies

Fig. 2. Schematic representation of reduced *oriC* regions amplified by PCR and cloned into pGEM-T. The solid lines indicate the PCR products which generated plasmids that could be detected following transformation of *M. gallisepticum*, while the dashed lines indicate those that generated plasmids that could not be detected in *M. gallisepticum*. Black triangles indicate DnaA boxes and shaded rectangles indicate AT-rich clusters.

were selected and subcultured in 1 ml broth containing 4 µg tetracycline ml⁻¹ and incubated until the medium changed colour. To confirm the presence of the plasmid, *tetM* was amplified by PCR with the Tetfor and Tetrev primer pair (Table 1). Transformants were passaged by inoculating 1 ml late-exponential-phase culture into 19 ml broth containing 4 µg tetracycline ml⁻¹.

Southern blot analysis. *M. gallisepticum* strain S6 and *M. imitans* strain 4229 genomic DNA, and *oriC* plasmid DNA were digested to completion with the restriction endonuclease *NsiI* (New England Biolabs). The fragments were separated in a 0.7 % agarose gel and blotted onto Hybond-N⁺ membranes (Amersham). The DNA was fixed to the membrane by exposure to UV light for 5 min and the blot was prehybridized in a buffer containing 7 % SDS, 1 % BSA, 1 mM EDTA and 0.25 M Na₂HPO₄ (pH 7.2) for 2 h at 58 °C (Church & Gilbert, 1984). A probe containing 50 ng of a 1.96 kb PCR product produced with the primer pair SW4for and SW5rev was radiolabelled with [γ -³²P]ATP using a random-primed DNA labelling kit (Roche). Unincorporated nucleotides were removed by passage through a Bio-Spin 30 chromatography column (Bio-Rad), following the manufacturer's instructions. The radiolabelled probe was denatured by incubation at 100 °C for 10 min and then added to the hybridization buffer and incubated with the membrane at 58 °C overnight. The next day the membrane was washed three times in 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 0.1 % SDS at 58 °C for 20 min each. The membrane was autoradiographed at room temperature overnight on BioMax film (Kodak). The same method was used to produce probes to detect the ampicillin-resistance gene (*ampR*) and the *oriC* region of *M. imitans*. The *ampR* gene was amplified from pGEM-T using the AmpRfor and AmpRrev primer pair, and the partial *oriC* region of *M. imitans* was amplified using the SW15for and Misojrev-1 primer pair (Table 1), together with the products radiolabelled as described above. The hybridization and washing conditions were the same as those used for the *oriC* probe.

Targeted gene inactivation by homologous recombination using *oriC* plasmids. To construct an *oriC* plasmid that could integrate into a target gene by homologous recombination, an internal fragment of the *vlhA3.03* gene (which also has 96 % DNA identity with *vlhA1.1* and *vlhA1.2* of *M. gallisepticum* strain S6), including regions that diverged significantly from most other *vlhA* genes, was amplified from the genome of *M. gallisepticum* strain R_{low} using the P3.03-F-*PstI* and P3.03-R-*SalI* primer pair or the P3.03-F-*SphI* and P3.03-R-*NcoI* primer pair (Table 1). The PCR products were digested with the appropriate restriction endonucleases and cloned separately into the *PstI* and *SalI* restriction sites in pPLoriC7, which contained 180 bp of the *M. gallisepticum oriC* region, and into the

SphI and *NcoI* restriction sites in the *M. imitans oriC* plasmid pMIori (Fig. 3). Approximately 10 µg of these plasmids was introduced into *M. gallisepticum* strain S6 by electroporation, as described above. Seven days after electroporation, individual tetracycline-resistant colonies were selected from the agar plate and incubated in 500 µl medium containing 4 µg tetracycline ml⁻¹ until a colour change was observed in the medium. The cultures were then screened for the presence of the *tetM* gene by PCR. To promote homologous recombination, each transformant was passaged 10 times in medium containing tetracycline, and then an additional five times without tetracycline. Transformants were spread on agar containing tetracycline and individual tetracycline-resistant colonies were selected 7 days after plating. Insertion of the construct into *vlhA3.03* was assessed by Southern blotting. The genomic DNA from transformants and plasmid DNA were digested with *PstI* (New England Biolabs) and Southern-transferred. The membrane was hybridized to radiolabelled *ampR* and the amplified fragment of *vlhA3.03*, and binding of the probes was detected as described above. The integration of pMIori/ Δ 3.03 was confirmed by DNA sequencing of the PCR product that was obtained using the primer pair SW13 for and *vlhA*leader rev (Table 1).

RESULTS

Functional analysis of *M. gallisepticum oriC* plasmids for *M. gallisepticum*, *M. imitans* and *M. pneumoniae*

Several *oriC* plasmids containing different extents of the putative *oriC* region of *M. gallisepticum* were produced and investigated for their ability to replicate in *M. gallisepticum* strain S6. The pGTLori plasmid contained a 1.96 kb region upstream of the *soj* gene, which included three DnaA boxes and four AT-rich clusters; the pGTSori plasmid contained a 0.64 kb DNA region downstream from the *soj* gene, which included two DnaA boxes but no AT-rich cluster; and pGTDori contained both the 1.96 kb region upstream and the 0.64 kb region downstream from the *soj* gene, which was replaced in the plasmid by the *tetM* gene. The pGTWori plasmid contained the 3.16 kb putative *oriC* region, including the *soj* gene (Fig. 1a). All plasmid constructs were introduced into *M. gallisepticum* strain S6 by electroporation and all, with the exception of

Fig. 3. Schematic representation of alignment of the *vlhA* multigene family and plasmid constructs containing an internal part of the *vlhA3.03* gene. Black rectangles indicate regions of conserved DNA, grey rectangles indicate regions of variable DNA sequence and white rectangles indicate regions of highly variable DNA sequence; results were obtained through DNA sequence alignment of the *vlhA* multigene family. The amplified internal part of the *vlhA3.03* gene was cloned separately into the *PstI* and *SalI* restriction sites in pPLoriC7 and into the *SphI* and *NcoI* restriction sites in the *M. imitans oriC* plasmid pMIori.

pGTSori, could be detected in cultures of tetracycline-resistant transformants; the frequency of transformation was $\sim 6 \times 10^{-7}$ transformants per c.f.u. These results suggested that the AT-rich regions were important for plasmid replication, and in addition that the *dnaA* and *soj* genes were not essential for the replication of the *oriC* plasmid. In order to determine the replicability of the *oriC* plasmid of *M. gallisepticum* in *M. imitans* strain 4229 and *M. pneumoniae* strain FH, plasmid pGTLori, harbouring a 1.96 kb region from *oriC* of *M. gallisepticum*, was introduced into *M. imitans* and *M. pneumoniae* by electroporation. Seven days after transformation, several tetracycline-resistant *M. imitans* colonies were observed on mycoplasma agar plates containing 4 μ g tetracycline ml^{-1} , but no tetracycline-resistant colonies of *M. pneumoniae* were detected up to 21 days of incubation, in spite of repeated attempts to introduce pGTLori into *M. pneumoniae* strain FH.

The minimal functional *oriC* region in *M. gallisepticum*

Previous studies have reported that *oriC* plasmids containing larger *oriC* regions can easily integrate into the *oriC* region of genomic DNA through homologous recombination after *in vitro* passage (Cordova *et al.*, 2002; Lartigue *et al.*, 2002; Renaudin *et al.*, 1995). In order to reduce the likelihood of this, a number of plasmids containing different lengths of the *oriC* region were tested for replication in *M. gallisepticum*. We amplified the partial *oriC* regions (*oriC*1 to *oriC*9) (Fig. 2) using specific primer pairs (Table 1) and cloned the products into *tetM*/pGEM-T, and these plasmids were then used to transform *M. gallisepticum* strain S6. Following transformation, the constructs pPLoriC1, pPLoriC3, pPLoriC5, pPLoriC6 and pPLoriC7, but not pPLoriC2, pPLoriC4, pPLoriC8 or pPLoriC9, were able to replicate in *M. gallisepticum* (Fig. 2). These results suggested that the minimal *oriC* region that was functional in *M. gallisepticum* strain S6 was around 180 bp and included two DnaA boxes and two AT-rich regions.

Determination of the DNA sequence of the *oriC* region in, and development of an *oriC* plasmid from, *M. imitans*

Part of the *oriC* region and the *dnaA* gene of *M. imitans* were amplified using the corresponding *M. gallisepticum* primer pairs. PCR products and the remainder of the *oriC* region were sequenced. The 2.17 kb region upstream from the *soj* gene of *M. imitans* contained six DnaA boxes, which had a 9 nt consensus sequence (5'-TTWTMHAMA-3'), and was 90% A+T (Fig. 1b). Alignment of the *oriC* regions of *M. imitans* and *M. gallisepticum* strain R revealed 56% DNA sequence identity, whilst their *dnaA* genes had 85% DNA sequence identity. The pMIori plasmid contained the 2.3 kb region upstream from the *soj* gene (Fig. 1b). The pMIori plasmid was introduced into *M.*

imitans strain 4229 and *M. gallisepticum* strain S6 by electroporation and was found to replicate in both species.

Stability of *M. gallisepticum* *oriC* plasmids in *M. gallisepticum* and *M. imitans*

We investigated the stability of *oriC* plasmids in transformants by Southern blot analysis after repeated passage. DNA was extracted from cells of transformants after passaging and, together with the genomic DNA of the untransformed organism and the *oriC* plasmid purified from *E. coli*, was digested with *NsiI*; the fragments were separated by gel electrophoresis, Southern-blotted and hybridized to the appropriate radiolabelled probe. Following *NsiI* digestion, the *M. gallisepticum* *oriC* probe is predicted to bind to an extrachromosomal plasmid with a predicted size of 7.0 kb (Fig. 4a). The 1.96 kbp *oriC* probe bound a 3.9 kb fragment in *M. gallisepticum* strain S6 (Fig. 4b, lane 1) and to a 7 kb fragment in pGTLori transformants (Fig. 4b, lane 2); a similar-sized fragment was also detected after five passages of the transformants (Fig. 4b, lane 3). The detection of 4.8 and 6 kb fragments by the probe at the 10th passage (Fig. 4b, lane 4) indicated integration of the plasmid into the genome in the *oriC* region. In transformants obtained with pPLoriC1, which contained a 1.06 kb *oriC* region, the *oriC* probe hybridized to the 4.5 kb fragment predicted for an extrachromosomal plasmid (Fig. 4b, lanes 5 and 6). The detection of 3.4 and 4.8 kb fragments at the 10th passage indicated that the plasmid had integrated into the genome (Fig. 4b, lane 7). To determine the stability of pPLoriC7, the replicative plasmid containing the shortest section of the *oriC* region, we performed Southern blot analysis on two transformants passaged under antibiotic selection pressure (4 μ g tetracycline ml^{-1}) after 5, 10 and 15 passages. Due to the low sensitivity of the *oriC* probe for the 180 nt *oriC* region, a longer probe that detects the *ampR* gene, which would indicate the location of the *oriC* plasmid, was used. It was expected that the probe would not hybridize to DNA from untransformed *M. gallisepticum* but would hybridize with a 3.5 kb DNA fragment after *NsiI* digestion of the DNA extracted from transformants containing extrachromosomal plasmid, whilst hybridization to a 4.0 kb fragment would suggest that the plasmid had integrated into the chromosome (Fig. 5a). The probe did not hybridize to untransformed *M. gallisepticum* (Fig. 5b, lane 1), but bound to a 3.5 kb fragment derived from the *oriC* plasmid, and to a similar-sized band in both transformants at the fifth passage (Fig. 5b, lanes 2, 3 and 6). At the 10th passage a 4.0 kb fragment was detected in all clones, but extrachromosomal plasmid was also detected (Fig. 5b, lanes 4 and 7). By the 15th passage, extrachromosomal plasmid could not be detected and the plasmid appeared to have integrated into the chromosome of each transformant (Fig. 5b, lanes 5 and 8). One possible reason for the integration of pPLoriC7 was the selection pressure exerted by the antibiotic. To investigate if this was the cause, we passaged both parental *M. gallisepticum* and a transformant

Fig. 4. Stability of *oriC* plasmids in *M. gallisepticum*. (a) Schematic diagram showing the *NsiI* (N) cleavage sites in the extrachromosomal plasmid and the predicted integrated forms of pGTLori and pPLoriC1 in the *M. gallisepticum* chromosome. The predicted sizes of fragments generated by *NsiI* cleavage that are expected to hybridize with the radiolabelled *oriC* probe are shown in bold type. The unshaded arrow indicates the *tetM* gene. (b) Southern blot analysis of *NsiI*-digested DNA. Lanes: 1, untransformed *M. gallisepticum*; 2, pGTLori; 3 and 4, *M. gallisepticum* transformed with pGTLori after 5 and 10 passages, respectively; 5, pPLoriC1; 6 and 7, *M. gallisepticum* transformed with pPLoriC1 after 5 and 10 passages, respectively.

containing pPLoriC7 in broth containing tetracycline at 0.4 or 1 $\mu\text{g ml}^{-1}$. Parental *M. gallisepticum* could only grow in broth containing 0.4 $\mu\text{g tetracycline ml}^{-1}$. Neither extrachromosomal nor integrated forms of the plasmid could be detected in the transformant after five passages in media containing 0.4 $\mu\text{g tetracycline ml}^{-1}$, suggesting that 0.4 $\mu\text{g tetracycline ml}^{-1}$ was an inadequate concentration to select for transformants. In the transformant grown in 1 $\mu\text{g tetracycline ml}^{-1}$, pPLoriC7 had completely integrated into the genome by the 15th passage. There appeared to be no difference in the rate of integration of

Fig. 5. Stability of the plasmid pLoriC7, containing a 180 bp *oriC* region, in *M. gallisepticum*. (a) Schematic representation of the integration of pLoriC7 into the *M. gallisepticum* genome. The predicted sizes of fragments generated by *NsiI* cleavage that would hybridize with the radiolabelled *ampR* probe are shown in bold type. The unshaded arrow indicates the *tetM* gene. (b) Southern blot analysis of *NsiI*-digested DNA. Lanes: 1, untransformed *M. gallisepticum* strain S6; 2, pLoriC7; 3–8, fifth, 10th and 15th passages of *M. gallisepticum* clones 1 (lanes 3–5) and 2 (lanes 6–8) transformed with pLoriC7.

the plasmid into the chromosome in transformants cultured in low concentrations of tetracycline, suggesting that antimicrobial selection pressure did not influence the rate of chromosomal integration. The stability of pGTLori was tested in two transformants of *M. imitans* strain 4229, which were selected on solid media containing 4 $\mu\text{g tetracycline ml}^{-1}$ and then passaged 15 times in broth with tetracycline. Southern blot analysis was performed at the fifth, 10th and 15th passages of each transformant using the radiolabelled *M. gallisepticum oriC* probe. The *oriC* probe did not hybridize with the *oriC* region of untransformed *M. imitans* (results not shown). At all passage levels examined, a 7 kb DNA band, indicative of the extrachromosomal form of pGTLori, was detectable in both transformants (results not shown).

Stability of *M. imitans oriC* plasmids in *M. gallisepticum* and *M. imitans*

The *oriC* plasmid pMlori replicated in both *M. gallisepticum* strain S6 and *M. imitans* strain 4229. The stability of this *oriC* plasmid was investigated following passage of

transformants. *Nsi*I-digested DNA was hybridized with the radiolabelled 2 kb *M. imitans* *oriC* probe, which contained part of the *oriC* region and the *soj* gene. A 3.5 kb band indicative of the genomic *oriC* region of *M. imitans* was detected in untransformed *M. imitans* and all passaged transformants (Fig. 6a, lanes 2–8). At the 15th passage the 7.4 kb extrachromosomal form of plasmid was still detectable in both transformants (Fig. 6a, lanes 5 and 8). In *M. gallisepticum*, the *oriC* probe, which contained part of the *M. imitans* *soj* gene, hybridized to a 3.9 kb band containing the *M. gallisepticum* *soj* gene (Fig. 6b, lane 2). A 7.4 kb band, corresponding to the extrachromosomal plasmid, was detectable until the 15th passage in transformant C1 (Fig. 6b, lane 5). However, the plasmid had integrated into the genome by the 10th passage, although not into the *oriC* region (Fig. 6b, lanes 4, 5, 7 and 8).

Targeted inactivation of a gene by homologous recombination using *oriC* plasmids

pPLoriC7/ Δ 3.03-transformed *M. gallisepticum* S6 produced several tetracycline-resistant colonies after 7 days of incubation. To investigate the integration site of the plasmid, Southern blot analysis was performed using DNA extracted from 16 pPLoriC7/ Δ 3.03 transformants that had been passaged 15 times. Identical membranes were

hybridized to the radiolabelled *ampR* gene or the internal fragment of the *vlhA3.03* gene. The *ampR* probe bound to a 6.5 kb band in the digest of the plasmid pPLoriC7/ Δ 3.03 and in most passaged transformants to a 10.5 kb band, corresponding to the predicted size of the fragment containing the chromosomal *oriC* region of *M. gallisepticum* S6 after the plasmid had integrated into this region. However, no band indicative of targeted homologous recombination was detected (results not shown).

Tetracycline-resistant colonies of the pMlori/ Δ 3.03 transformants of *M. gallisepticum* S6 were passaged 15 times and the integration site of the plasmid was investigated by hybridizing Southern blots with radiolabelled *ampR* gene and *vlhA3.03* probes. Several bands were detected in all passaged transformants, indicating that the plasmid had integrated at least twice into the *M. gallisepticum* genome. The *vlhA3.03* probe detected bands of a different size in one of the transformants. This transformant was passaged an additional five times in media that did not contain tetracycline, to allow the transformant to cure itself of the extrachromosomal plasmid, and then inoculated onto mycoplasma agar containing 4 μ g tetracycline ml⁻¹. Two single tetracycline-resistant colonies were selected for Southern blot analysis. The *ampR* probe detected an 8.77 kb band in the digest of pMlori/ Δ 3.03, while an ~15 kb band was detected in these transformants (Fig. 7 lanes P, 2 and 3). The gene that was predicted to have been interrupted was amplified using the primer pair SW13 for and *vlhA*Leader rev, generating a PCR product of ~2 kb. DNA sequencing of the PCR product and searches of the databases determined that the amplicon was identical to part of the *vlhA1.2* gene (previously *pMGA1.2*) of *M. gallisepticum* S6 and the section of the *vlhA3.03* gene that was contained within the plasmid construct. The product also contained part of the pGEM-T vector. This suggested

Fig. 6. Stability of *oriC* plasmid pMlori in *M. gallisepticum* and *M. imitans*. (a) Southern blot analysis with *Nsi*I-digested DNA hybridized with *ampR* radiolabelled probe. Lanes: 1, pMlori; 2, untransformed *M. imitans* strain 4229; 3–8, fifth, 10th and 15th passages of *M. imitans* clones 1 (lanes 3–5) and 2 (lanes 6–8) of pMlori transformants. (b) Southern blot analysis with *Nsi*I-digested DNA. Lanes: 1, pMlori; 2, untransformed *M. gallisepticum* strain S6; 3–8, 5th, 10th and 15th passages of clones 1 (lanes 3–5) and 2 (lanes 6–8) of *M. gallisepticum* transformed with pMlori.

Fig. 7. Integration of pMlori/ Δ 3.03 into the *M. gallisepticum* strain S6 chromosome. Southern blot analysis of *Pst*I-digested DNA. The blot was hybridized to radiolabelled *ampR* DNA. Lanes: P, pMlori/ Δ 3.03; 1, untransformed *M. gallisepticum* strain S6; 2 and 3, 20th passage of *M. gallisepticum* pMlori/ Δ 3.03 transformants 1 and 2, respectively.

that pMIori/Δ3.03 had integrated into the *vlhA1.2* gene rather than the *vlhA1.1* gene.

DISCUSSION

In this study, we produced functional *oriC* plasmids containing the putative *oriC* regions of *M. gallisepticum* and *M. imitans*. In earlier studies, *oriC* plasmids have been constructed for *Spiroplasma citri*, *M. mycoides* subspecies *mycoides* LC and SC, *M. capricolum* subsp. *capricolum*, *Mycoplasma agalactiae* and *Mycoplasma pulmonis* (Chopra-Dewasthaly *et al.*, 2005; Lartigue *et al.*, 2003). Apart from *M. pulmonis*, which belongs to the Hominis phylogenetic group, all these species belong to the Spiroplasma phylogenetic group, and all have a conserved gene order within the *oriC* region (Fig. 8), which contains the *dnaA* gene. The functional *oriC* plasmids for these species require regions upstream and downstream from the *dnaA* gene, with the exception of the smallest *oriC* plasmid for *S. citri*, which contains only a 163 bp region downstream from the *dnaA* gene (Cordova *et al.*, 2002; Lartigue *et al.*, 2002, 2003). The gene order in the *oriC* regions of *M. gallisepticum* and *M. imitans* is similar to those of *M. pneumoniae* and *Mycoplasma genitalium* (Fig. 8). These mycoplasmas belong to the Pneumoniae phylogenetic group and the putative *oriC* regions surround the *soj* gene but lie upstream of the *dnaA* gene (Cordova *et al.*, 2002; Papazisi *et al.*, 2003). We produced four *oriC* plasmid constructs that contained the *soj* gene and regions upstream and downstream of the *soj* gene in *M. gallisepticum*. Only the plasmids containing the region upstream from the *soj* gene could be detected in *M. gallisepticum*, indicating that only the AT-rich sequences found in this region were essential for plasmid replication, even though the region downstream from the *soj* gene included two DnaA boxes. This suggests that, at least in *M. gallisepticum*, the *soj* gene is not required for replication of an *oriC* plasmid. The *M. gallisepticum* *oriC* plasmid, pGTLori, and the *M. imitans* *oriC* plasmid pMIori were able to replicate in both species. Alignment of the *oriC* region of *M. imitans* with that of *M. gallisepticum* showed that the sequences were very different, but that the surrounding genes had high levels of DNA sequence identity. Both species contained similar DnaA box consensus sequences. Therefore, *M. gallisepticum* and *M. imitans* might be expected to support replication of heterologous *oriC* plasmids. In contrast to *M. imitans*, *M. pneumoniae* did not appear to support replication of the *M. gallisepticum* *oriC* plasmid pGTLori. Though both species have a conserved gene order in the *oriC* region (Fig. 8), the DNA sequence of their *oriC* regions appears to be poorly conserved, and alignment of the peptide sequence of the DnaA proteins of *M. gallisepticum* and *M. pneumoniae* revealed only 23 % peptide sequence identity. Several AT-rich clusters were identified in the putative *oriC* region of *M. pneumoniae*, but consensus DnaA box nonamers were not found. This suggests that the DnaA boxes of *M.*

Fig. 8. Gene order surrounding the chromosomal *oriC* regions of mollicutes and the structure of their putative *oriC* regions. Triangles indicate the locations of the DnaA box consensus sequences and shaded rectangles indicate the locations of AT-rich regions.

pneumoniae may have a more relaxed consensus sequence. The minimal *oriC* region of *M. gallisepticum* that was found to be functional in *M. gallisepticum* strain S6 was 180 bp in size. This region was upstream from the *soj* gene and included two DnaA boxes and two AT-rich regions of 31 and 22 bases (Fig. 2). The size of the minimal *oriC* region in *M. gallisepticum* was similar to that required in the functional *oriC* vector for *S. citri*, which was a 163 bp region downstream from the *dnaA* gene containing three DnaA boxes and two AT-rich regions (Lartigue *et al.*, 2002). For *M. gallisepticum* strain S6, we found that only two DnaA boxes were necessary for plasmid replication, and this is the smallest *oriC* region capable of supporting plasmid replication in mycoplasmas. Both pGTLori and pPLoriC1, which contained larger sections of the *oriC*

region, integrated readily into the chromosome during passage, as has been seen with the *oriC* plasmids pBOT1 in *S. citri* and pMPO1 in *M. pulmonis* (Cordova *et al.*, 2002; Renaudin *et al.*, 1995). We produced a smaller *oriC* plasmid in an attempt to generate a vector that would not integrate readily into the genome. While pPLoriC7 did not integrate into the chromosome as rapidly as pGTLori and pPLoriC1, it had integrated completely into the chromosome by the 15th passage. In *M. pulmonis*, the minimal *oriC* regions necessary for plasmid replication are 262 bp upstream and 327 bp downstream from the *dnaA* gene. This *oriC* plasmid (pMPO5) remains extrachromosomal for at least 15 passages (Cordova *et al.*, 2002). The *oriC* plasmid pGTLori could replicate in *M. imitans* strain 4229 and appeared to be more stable in *M. imitans* than in *M. gallisepticum*. In Southern blot analysis, free plasmid was detectable until the 15th passage, with only limited integration into the chromosome. This suggested that the *oriC* plasmid of *M. imitans* might be more stable in *M. gallisepticum* than the homologous *oriC* plasmid. However, while some plasmid remained extrachromosomal until the 15th passage, a portion had integrated into the genome at sites outside the *oriC* region by the 10th passage. Interestingly, an extrachromosomal form of pMIori was found in all passaged transformants of *M. imitans*. These results suggest that the homologous recombination system in *M. gallisepticum* has a higher efficiency than that of *M. imitans*.

Targeted gene inactivation was attempted in *M. gallisepticum* using the pPLoriC7 and pMIori plasmids. In a previous study, only five of 16 transformants containing the smallest *S. citri* *oriC* plasmid (containing 163 bp of the *oriC* region) had integration of the plasmid into the chromosomal *oriC* region after 15 passages, and this construct was successfully used to inactivate *scm1* (Lartigue *et al.*, 2002). However, pPLoriC7, containing the 180 bp *M. gallisepticum* *oriC* region, integrated readily into the chromosomal *oriC* region by the 15th passage, and attempts to inactivate *vlhA3.03* using pPLoriC7 were unsuccessful, with the plasmid integrating into the chromosomal *oriC* region in all transformants rather than into the target gene region, even though the *vlhA3.03* fragment in the plasmid was 986 bp in size.

Of the 16 *M. gallisepticum* transformants containing pMIori/ Δ 3.03, only one showed evidence of an interruption of a *vlhA* gene in Southern blot analysis. The inactivated gene was identified as *vlhA1.2*. At the DNA level, *vlhA1.2* and *vlhA1.1* are 98 % identical, so this result is not unexpected. Targeted gene inactivation has, to our knowledge, only been achieved once before in *M. gallisepticum*, but use of the *M. imitans* *oriC* plasmid could improve the efficiency of recombination. Thus, in this study, we constructed several *oriC* plasmids that could replicate successfully in *M. gallisepticum* and *M. imitans*. This is the first report, to our knowledge, of *oriC* plasmids for members of the Pneumoniae phylogenetic group. In other mollicutes, *oriC* plasmids have been used to inactivate genes or to express exogenous genes (Cordova *et al.*, 2002; Duret *et al.*, 1999;

Janis *et al.*, 2005; Lartigue *et al.*, 2002), so these *oriC* plasmids are likely to be useful tools for genetic research in *M. gallisepticum* and *M. imitans*.

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