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 Rlow 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 (pGTLon) and M. imitans (pMiI) replicated in both species, but Mycoplasma pneumoniae could not support replication of pGTLon. 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 pMiI 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.

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, increased egg production and increased medication costs (Ley & Yoder, 1997). 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; Hedreylea 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
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’-TTWTTMHAAMA-3’ identical for each DnaA box, and the region between dnaN and soj contains a higher than average content of adenine 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 MIsojrev-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 Rlow (GenBank accession no. NC_004829).

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence* (5’–3’)</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW4 for/SW5 rev</td>
<td>GAGCTTTTGGCATGCAAAAAATTC/</td>
<td>Long oriC region (LoriC; 1.96 kb)</td>
</tr>
<tr>
<td>SW6 for/SW7 rev</td>
<td>TTGTTAATTCATGGATGATCAAA</td>
<td>Short oriC region (SoriC; 0.67 kb)</td>
</tr>
<tr>
<td>SW4 for/SW7 rev</td>
<td>CCTCAGTCGACTAGTTAAG</td>
<td>Whole oriC region (WoriC; 3.16 kb)</td>
</tr>
<tr>
<td>SW4 for/SW9 rev</td>
<td>/CTGATGCGTTAGCATAGTGG</td>
<td>Partial LoriC 1 (1.06 kb)</td>
</tr>
<tr>
<td>SW15 for/SW16 rev</td>
<td>CGCTTTAATTTGTAACAAATA/</td>
<td>Partial LoriC 2 (565 bp)</td>
</tr>
<tr>
<td>SW4 for/P Lori-rev-2</td>
<td>GCTGTTATCAAGAATAATTAAAGC/</td>
<td>Partial LoriC 3 (707 bp)</td>
</tr>
<tr>
<td>Start DnaN /P Lori-rev-2</td>
<td>GATAAACTCTGCTGAAACTC/</td>
<td>Partial LoriC 4 (633 bp)</td>
</tr>
<tr>
<td>SW4 for/P Lori-rev-4</td>
<td>/ACAATATTCCGATAAAAAATCAC/</td>
<td>Partial LoriC 5 (510 bp)</td>
</tr>
<tr>
<td>SW4 for/P Lori-rev-5</td>
<td>/TTTACAAACAAATTTGTTAC/</td>
<td>Partial LoriC 6 (459 bp)</td>
</tr>
<tr>
<td>SW4 for/P Lori-rev-6</td>
<td>/ACAGTTTTGTGAAAAGTGA/</td>
<td>Partial LoriC 7 (180 bp)</td>
</tr>
<tr>
<td>SW4 for/pP LoriC8 rev</td>
<td>/GAGTTATCTCTGATTTATC/</td>
<td>Partial LoriC 8 (96 bp)</td>
</tr>
<tr>
<td>pP LoriC6 for/P Lori-rev-6</td>
<td>CCATGATAATTTGTGATAAAATC/</td>
<td>Partial LoriC 9 (119 bp)</td>
</tr>
<tr>
<td>Tetfor/Tetrev</td>
<td>GAAAGATCTGGAGTAGTTGGAAG/</td>
<td>Tetracycline resistance gene (2.36 kb)</td>
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<tr>
<td>AmpR for/AmpR rev</td>
<td>CCAATGTTCAATTTACGAGG/</td>
<td>Ampicillin resistance gene (862 bp)</td>
</tr>
<tr>
<td>SW6 for/dnaA rev</td>
<td>/AAGCATGTCTTAAAGCTCAAATTTGAA/</td>
<td>dnaA gene of <em>M. imitans</em> (2.1 kb)</td>
</tr>
<tr>
<td>SW15 for/Misoj rev-1</td>
<td>/TTTTCTCAATTTTATTTACTC/</td>
<td>partial oriC region and soj gene of <em>M. imitans</em> (2 kb)</td>
</tr>
<tr>
<td>MIdnaN for/Misoj rev-2</td>
<td>CAGAAGAAAGCTCTGGTACAAAAC/</td>
<td>oriC region of <em>M. imitans</em> (2.3 kb)</td>
</tr>
<tr>
<td>P3.03-F-SphI/P3.03-R-Ncol</td>
<td>CTTTAGACTCGTCGAGATGTCG/</td>
<td>Internal fragment of the vlhA3.03 gene (1 kb)</td>
</tr>
<tr>
<td>P3.03-F-PstI/P3.03-R-SalI</td>
<td>CTTTAGACTCGTCGAGATGTCG/</td>
<td>Internal fragment of the vlhA3.03 gene (1 kb)</td>
</tr>
<tr>
<td>SW13 for/vlhA Leader rev</td>
<td>CGACTCATAATAGGGCCGAA/</td>
<td>Integration site of pMIoriC/ΔA3.03</td>
</tr>
</tbody>
</table>

*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 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 R68av, 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 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 Ncol cleavage sites, and the short oriC region was inserted between the PsfI and SalI sites (Fig. 1a). The resulting constructs were designated pGTLori and pGTSoiri. 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 Mlssojrev-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.

**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 Ncol sites located in the multicloning site of pGEM-T, and the short oriC was inserted between the PsfI 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, Ncol; P, PsfI; S, SalI; Se, SpeI.

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 μF 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 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 NsI (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 [³²P]ATP using a random-primed DNA labelling kit (Roche). Unincorporated nucleotides were removed by passage through a BioSpin 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 pGDLori using the AmpRfor and AmpRrev primer pair, and the partial oriC region of M. imitans was amplified using the SW15for and Misolv-rev 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 Rlow using the P3.03-F-PstI and P3.03-R-SalI primer pair or the P3.03-F-SphI and P3.03-R-Ncol 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 pLoriC7, which contained 180 bp of the M. gallisepticum oriC region, and into the SphI and Ncol 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/Δ3.03 was confirmed by DNA sequencing of the PCR product that was obtained using the primer pair SW13 for and vlhALeader 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 pGT Lori plasmid contained a 1.96 kb region upstream of the soj gene, which included three DnaA boxes and four AT-rich clusters; the pGT Sori plasmid contained a 0.64 kb DNA region downstream from the soj gene, which included two DnaA boxes but no AT-rich cluster; and pGT Dori 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 pGT Wori 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
pGTSori, could be detected in cultures of tetracycline-resistant transformants; the frequency of transformation was $\sim6 \times 10^{-7}$ 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 $\mu$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 (oriC1 to oriC9) (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 *Nsi*I; the fragments were separated by gel electrophoresis, Southern-blotted and hybridized to the appropriate radiolabelled probe. Following *Nsi*I 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 kb 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 $\mu$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 *Nsi*I 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 case, we passaged both parental *M. gallisepticum* and a transformant
containing pPLoriC7 in broth containing tetracycline at 0.4 or 1 μg ml⁻¹. Parental M. gallisepticum could only grow in broth containing 0.4 μg 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 μg tetracycline ml⁻¹, suggesting that 0.4 μg tetracycline ml⁻¹ was an inadequate concentration to select for transformants. In the transformant grown in 1 μg 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 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 μg 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 pMIori replicated in both M. gallisepticum strain S6 and M. imitans strain 4229. The stability of this oriC plasmid was investigated following passage of
Targeted inactivation of a gene by homologous recombination using oriC plasmids

pPLOriC7/ΔA3.03-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/ΔA3.03 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/ΔA3.03 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/ΔA3.03 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 μg 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/ΔA3.03, 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 vlhALeader 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
that pMIori/A3.03 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-Dewasthan, 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, 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. 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 pPLoriCl, which contained larger sections of the oriC regions, did not appear to support replication of the plasmids. In contrast to *M. gallisepticum* strain S6, we found that only the AT-rich regions downstream from the soj gene were required for replication of the plasmid. This suggests that the DnaA boxes of *M. 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 pPLoriCl, which contained larger sections of the oriC

![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.](image-url)
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 (pMP05) 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 pMLori was found in all passed 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 pMLori 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 pMLori/ΔA3.03, 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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