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Construction of a DNA vector system for *Mycoplasma gallinarum*

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Abstract

Diseases caused by mycoplasmas bring significant problems for livestock and poultry production with major economic and social consequences worldwide. However, the genetic studies of these bacteria are limited by the paucity of a replicable vector system. Mycoplasma gallinarum, an opportunistic pathogen that has a wide range of hosts, is not an exception. This study describes the development of the first vectors for this species using the origin of replication of the chromosome (oriC) region from closely related Mycoplasma gallisepticum. Additionally, this report provides the first evidence of the successful genetic transformation of M. gallinarum. Three heterologous oriC plasmids were constructed, two of which were functional. The replication of the third vector was not supported, probably because of the mutation upstream the second DnaA box. Passaging in vitro caused the reduction in growth rate and colony size of most of the transformants. The oriC plasmids described may therefore become valuable tools to study M. gallinarum through inactivation or expression of selected genes.

Keywords: *Mycoplasma gallinarum*, *Mycoplasma gallisepticum*, *oriC* vectors.

Introduction

Mycoplasmas are considered as major animal pathogens worldwide, causing infections of the respiratory and urogenital tracts, mammary glands, joints or eyes of various poultry and livestock species [1]. Since they are responsible for significant economic losses due to increased medication costs and reduced feed conversion efficiency, a lot of effort is put into unraveling the pathogenicity of these bacteria. To date, a large pool of data has been obtained from sequenced mollicute genomes, however, genetic studies have been limited by the lack of genetic tools. In order to study gene function, transposons Tn*916* and Tn*4001* [2-5] have mostly been used. However, random integration of the transposon into the chromosome may complicate analysis of gene expression and does not allow specific targeting of a gene of interest. The only plasmids that are known to replicate in *Mycoplasma* species are cryptic plasmids in *Mycoplasma mycoides* subsp. *mycoides* and *Mycoplasma capricolum* [6, 7]. In contrast, replicative plasmids have been developed by using the origin of replication of the chromosome (*oriC*) and selectable antibiotic resistance markers. Such plasmids are able to replicate in their respective hosts and can be used to inactivate target genes by homologous recombination, support heterologous gene expression and to functionally complement mutants [8-11]. As previous studies have shown, *oriC* plasmids containing larger *oriC* fragments tend to integrate into the *oriC* region of the genomic DNA by homologous recombination. Shorter *oriC* fragments are being used to make plasmids more stable and suitable for targeted homologous recombination [8, 11, 12]. *OriC* plasmids are

usually host specific, but functionality of heterologous plasmids in related mollicute species has also been demonstrated [9].

The aim of this study was to develop plasmid vectors capable of replicating in *M. gallinarum*. This species is considered to be a commensal for a broad range of hosts, including poultry, cattle, pigs and sheep [13, 14]. Some researchers have linked *M. gallinarum* with respiratory diseases in poultry, where it serves as a cofactor in combination with infectious bronchitis virus or vaccines for infectious bronchitis virus, and Newcastle disease virus [15]. Moreover, *M. gallinarum* provides a model to study host adaptation, as this mycoplasma is found in different animal species [16]. Thus, the development of gene transfer methods and genetic tools for this bacterium has become a prerequisite. Since *M. gallinarum* genome has not been sequenced yet, the nucleotide sequence of its *oriC* region is still unknown. However, *oriC* plasmids for a close relative, *M. gallisepticum*, were constructed recently [11]. In this study, the *oriC* region from *M. gallisepticum* was used to develop heterologous plasmids that replicate in *M. gallinarum*.

Materials and Methods

Bacterial strains and culture conditions

Mycoplasma strain *M. gallisepticum* S6 and *M. gallinarum* field isolate [17] were used in this study. Mycoplasmas were grown at 37 °C in modified Frey media supplemented with 500 U/ml of penicillin. For growth in solid media, mycoplasmas were incubated at 37 °C under anaerobic conditions. Transformants were selected on Frey agar plates containing 5 μg/ml of tetracycline. *Escherichia coli* DH5α [φ80dlacZΔM15Δ(*lacZYargF*) U169 *deoR recA1 endA1 hsdR17* (r^-_k m^+_k) sup E44 *thr1 gyr* A96 *relA1*] (HANAHAN & al.) served as the host for cloning procedures and plasmid amplification. *E. coli* cells were grown in nutrient broth (NB) and nutrient agar (NA) at 37 °C. *E. coli* transformants were grown in NA media supplemented with 50 μg/ml of ampicillin and 5 μg/ml of tetracycline.

Cloning procedures

Three fragments of the *oriC* region of *M. gallisepticum* were amplified by PCR (Fig. 1). Specific primer pairs SW4for and SW9rev, SW4for and PLori-rev-4, and SW4for and PLori-rev-5 were used [11]. PCR assays were conducted using 10 ng *M. gallisepticum* R_{low} genomic DNA as template in 25 μl reaction mixtures, with 1 U Platinum *Taq* High Fidelity DNA polymerase (INVITROGEN) in 1× buffer supplied by the manufacturer, 200 μM dNTPs, 2 mM MgSO4 and 12.5 μM of each primer. The amplification was performed in an Eppendorf Mastercycler Personal thermocycler 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 chain termination at 68° C for 7 min. PCR products were separated by gel electrophoresis and extracted from gel slices using the Silica Bead DNA Gel Extraction Kit (FERMENTAS) following the manufacturer's instructions. All three *oriC* fragments were cloned separately into pTZ57R/T vector (FERMENTAS). The tetracycline-resistance gene (*tetA*) from the plasposon p34S-Tc [18] was then cloned into *KpnI* restriction site of the vectors, resulting in the plasmids pG3-Tc, pG5-Tc and pG6-Tc (Fig. 1). Prior transformation, the purified plasmids were checked by DNA sequencing.

Transformation of *M. gallinarum*

Cells of mycoplasma in late log phase were transformed by electroporation as described previously [19]. For each transformation, 5 ml cultures of mycoplasma were used. After harvesting by centrifugation at 12000 g for 5 min at room temperature, the cells were washed in 5 ml ice-cold HEPES-sucrose buffer (8 mM HEPES, 272 mM sucrose, pH 7.4). The cell pallet was then resuspended in 100 μl HEPES-sucrose buffer containing ~100 ng of

plasmid DNA. The mixture was transferred to a cuvette with a 0.2 cm electrode gap and pulsed. Gene Pulser (BIO-RAD) was set at 2.5 kV, 100 Ω and 25 μF. After electroporation the cells were resuspended in 1 ml cold broth and incubated firstly at room temperature for 10 min and then at 37 °C for 2 hours. Transformants were selected on Frey solid media containing 5 μg/ml of tetracycline. The plates were kept at 37 °C and examined for colony development after seven days of incubation. Tetracycline-resistant colonies were subcultured in 1 ml of broth containing 5 μg/ml of tetracycline. Transformants were passaged by inoculating 100 μl of culture into 900 μl of broth containing 5 μg/ml of tetracycline. A minimum of 10 independent transformations were made for each of the *oriC* plasmids.

PCR-based detection of *tetA* in selected transformants

M. gallinarum DNA was extracted from 1 ml cultures using the Wizard genomic DNA purification kit (PROMEGA). PCR assays were conducted using 1 μl of mycoplasma DNA as template in 20 μl reaction mixtures with 1 U of Maxima Hot Start *Taq* DNA polymerase (FERMENTAS) in 1× Hot Start PCR buffer supplied by the manufacturer and 1 μM of each primer, TET-for (5'-TCTAACAATGCGCTCATCATCGTCATC-3') and TET-rev (5'-TTCCGAATACCGCAAGCG ACAG-3'). The amplification was performed in an Eppendorf Mastercycler Personal thermocycler under the following conditions: 95° C for 4 min, followed by 25 cycles of 95° C for 30 s, 53° C for 1 min, and 72° C for 1 min, with a final chain termination at 72° C for 10 min. This PCR resulted in 740 bp *tetA*-product in the presence of free or integrated *oriC* plasmids.

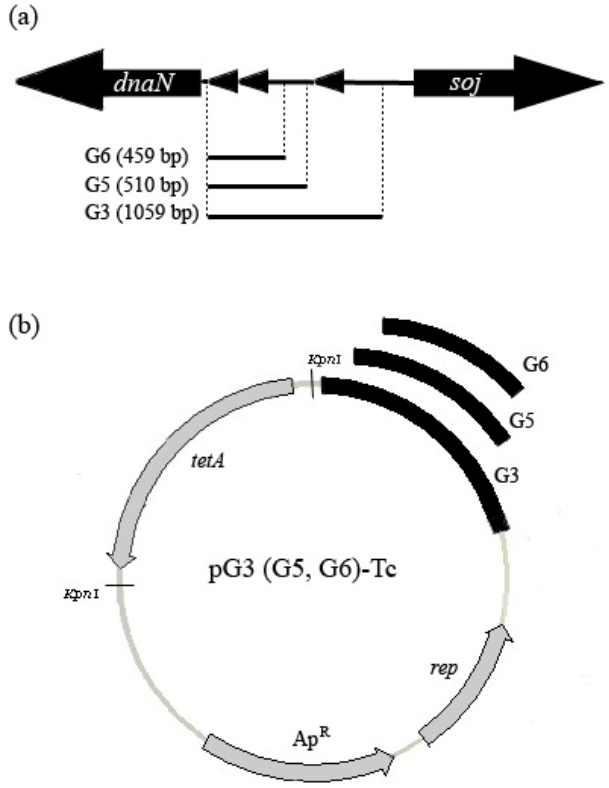

Figure 1. Construction of *M. gallisepticum oriC* plasmids. (a) Schematic representation of *oriC* regions amplified by PCR. The solid lines indicate PCR products, black triangles indicate DnaA boxes. (b) Plasmid constructs containing different *oriC* regions. The backbone of the plasmid was pTZ57R/T, containing the

ampicillin-resistance gene (Ap^R) and the replicon responsible for the replication of the plasmid in *E. coli* (*rep*). The tetracycline-resistance gene (*tetA*) was cloned into the *KpnI* restriction site afterwards.

Results and discussions

Cloning of the *oriC* region of *M. gallisepticum* and construction of replication vectors

OriC plasmids have been previously developed for *M. gallisepticum* [11]. We decided to investigate whether *oriC* region of *M. gallispeticum* could be used to maintain replication of vectors in *M. gallinarum*. Although *oriC* plasmids are usually host specific, compatibility between closely related species is not uncommon, and it has been previously shown between mycoides cluster species, *M. mycoides* subsp. *mycoides* SC, *M. mycoides* subsp. *mycoides* LC, and *M. capricolum*, as well as between *M. gallisepticum* and *M. imitans* [9 , 11]. Moreover, one of the very few examples of *oriC* plasmids that are compatible between different bacterial genera has also been shown in mollicutes, when *M. capricolum* was successfully transformed by *Spiroplasma citri oriC* plasmids [9].

Thus, we constructed plasmids, which include the *tetA* selection marker and the *oriC* region of *M. gallisepticum*. Three plasmids containing different extents of the *oriC* were constructed. The pG3-Tc plasmid contained a 1059 bp fragment consisting of 3 DnaA boxes. Both, the pG5-Tc and the pG6-Tc plasmids, contained shorter fragments with two DnaA boxes, which were 510 bp and 459 bp in length, respectively. The sequences of G3, G5 and G6 fragments from *M. gallispeticum* used in this study were aligned with respective sequences of *M. gallisepticum* available in the GenBank [2] (Fig 2). There were nine mismatches in the longest *oriC* fragment of 1114 bp.

Transformation of *M. gallinarum* with heterologous *oriC* plasmids

Constructed *oriC* plasmids were introduced into *M. gallinarum* by electroporation. Using the pG3-Tc and the pG6-Tc plasmids, tetracycline-resistant colonies appeared within 7-14 days of incubation. During the indicated time period, no spontaneous tetracycline-resistant colonies were noticed for untransformed controls. Transformation efficiency was of 6×10^{-7} transformants/c.f.u./μg plasmid DNA. PCR analysis confirmed that the presence of *tetA* determinant correlated with the tetracycline resistance. These results indicated that *M. gallisepticum oriC* plasmids could be functional in *M. gallinarum*. However, no tetracycline-resistant colonies of *M. gallinarum* transformed with the pG5-Tc plasmid were detected. As seen from the sequence data of the pG5-Tc plasmid, it had a mutation upstream the second DnaA box where A-T pair of nucleotides had been changed into G-C (Fig. 2). Although the location of the mutation was neither within the DnaA box nor within AT-rich region, A-T pair in that position appeared to be important for the functionality of the plasmid.

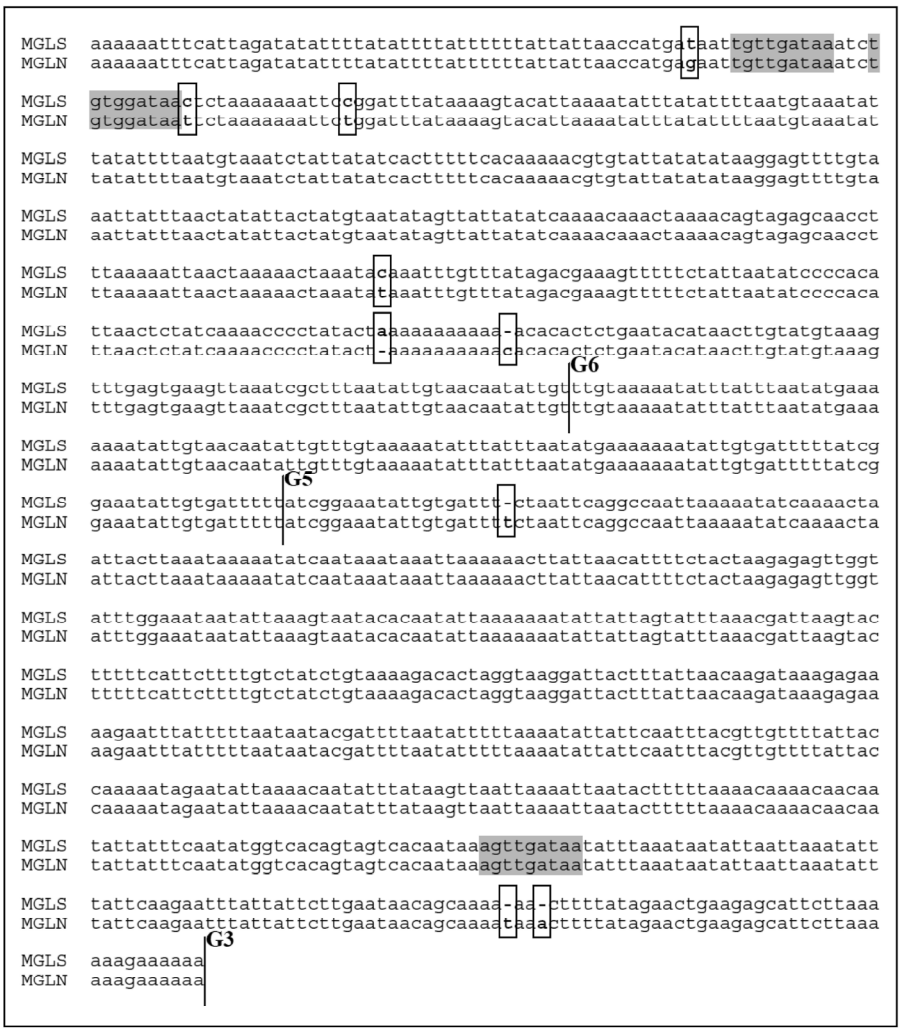

Figure 2. Alignment of the sequences of fragments G3, G5 and G6 from *M. gallisepticum* (MGLS) [2] and *M. gallisepticum* used in this study (MGLN). Putative DnaA boxes are colored in grey, mismatches are indicated with frames and the position of the mutation in the pG5-Tc plasmid is noted by an asterisk.

Stability of *oriC* plasmids in *M. gallinarum*

Distinct transformants of *M. gallinarum* were subjected to 20 serial passages in selective Frey media. Only 3 out of 50 selected transformants kept replicating until the last passage, meanwhile 34 lost viability before the 5th passage. All of the subcultured transformants were plated on solid selective media after each passage. We noticed that some of them, instead of forming usual colonies of *M. gallinarum*, which are approximately 400 μm in diameter, began forming very small colonies of 25 μm in diameter (Fig. 3). The size of the colonies did not change after transformants were plated on a solid media without tetracycline. However, in the case of some transformants, small colonies reverted into usual ones after several passages. This change of colony morphology correlated with the loss of viability as transformants used to stop growing several passages after their colonies morphology had changed.

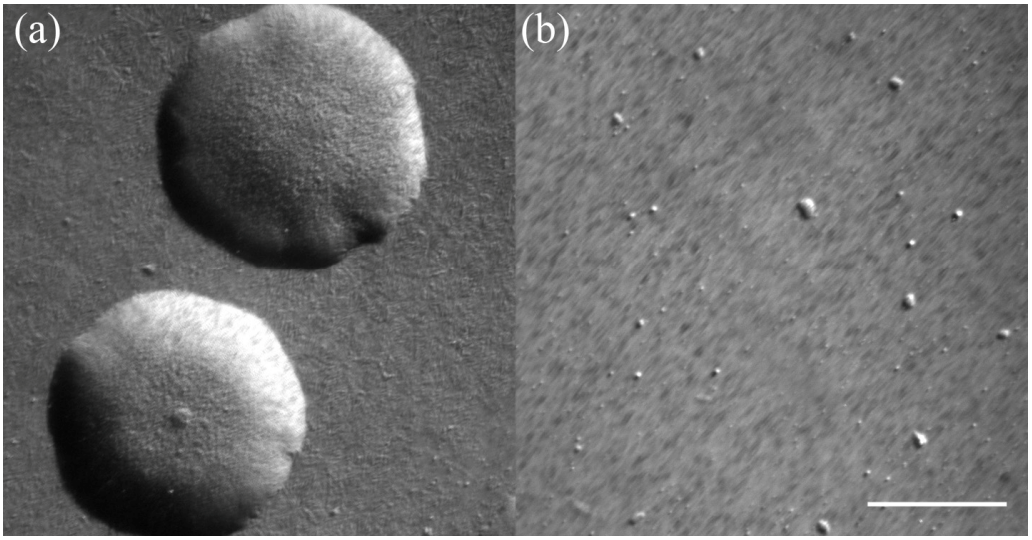

Figure 4. Normal colonies formed by untransformed *M. gallinarum* and most of the transformants (a) and small colonies formed by some of the transformants (b). Scale bar, 200 μm.

A reduction in growth rate and the size of colonies, which are almost 20 times smaller than normal ones, might indicate vector integration into the *oriC* region of the chromosome through homologous recombination. Recombination of heterologous *oriC* plasmids has already been shown for *M. capricolum* [9]. If such event could also be confirmed for *M. gallinarum*, this would show that gene targeting strategies using heterologous *oriC* plasmids may be applied for this bacterium.

We have shown that *M. gallinarum* is amenable for genetic transformation, which proves functional genomics for this bacterium to be technically feasible. Using the sequence of *oriC* region, not only heterologous, but also homologous *oriC* plasmids could be developed, enabling genetic studies of *M. gallinarum*. Because of a general lack of knowledge regarding the mycoplasmal host adaptation mechanisms, such plasmids would be of particular interest, since *M. gallinarum*, which has a large host range, provides an excellent model to study these phenomena.

Conclusions

In this study, we have constructed *M. gallisepticum oriC* plasmids that are functional in heterologous host *M. gallinarum*. The *oriC* fragment containing two DnaA boxes has been shown to be sufficient to drive replication. We are also the first to successfully transform *M. gallinarum*. The findings from our research may be applied in further studies of mycoplasma genetics.

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