Development of oriC-plasmids for use in Mycoplasma hyorhinis

Hassan Z. A. Ishag1,2, Qiyan Xiong3, Maojun Liu3, Zhixin Feng3 & Guoqing Shao3

Mycoplasma hyorhinis (M. hyorhinis) is an opportunistic pig pathogen, belonging to the class Mollicutes. It causes polyserositis, arthritis and cancers in vitro, increasing attention of the researchers. Currently, there is no available genetic tool to manipulate its genome. This study describes a development of oriC-plasmids harboring either large (pGEMT-LoriC) or minimum (pGEMT-MoriC) origin of replication (oriC) of M. hyorhinis along with tetracycline resistance marker. These plasmids were successfully transformed into M. hyorhinis with average transformation frequency of $1.5 \times 10^{-4}$ and $2.0 \times 10^{-6}$ transformants/CFU for pGEMT-LoriC and pGEMT-MoriC respectively, and were integrated at the chromosomal oriC as well as remained freely replicating. We also constructed a Mini-oriC-HT1 targeting plasmid by inclusion of hlyC arms and was used to inactivate hlyC at average frequency of 50%. The efficiency of hlyC inactivation was further improved (by 90%) when Mini-oriC-HT2 that contains E. coli recA was used. In both cases, hemolysin mutant bacteria diminished the ability to lyse mouse RBCs compared to wild-type ($P < 0.001$). OriC-plasmids described in this study may, therefore open the way for functional genomics in M. hyorhinis. Furthermore, this is a first study demonstrated the gene associated with a hemolytic phenotype in mycoplasmas.

Mycoplasma hyorhinis (M. hyorhinis), is an opportunistic pathogen of pigs associating with respiratory tract infections and arthritis1. M. hyorhinis is most often detected as contaminants in cell cultures2. In addition, chronic M. hyorhinis infection has been found to play a role in the development of several types of cancers in vitro3,4, increasing attention of the researchers.

The genome sequence of M. hyorhinis has been completed and published several years ago5. However, studies to manipulate the genome of M. hyorhinis and understand the molecular pathogenesis suffers from the lack of genetic tools available for the pathogen.

For many mycoplasmas including M. hyorhinis, the transposon mutagenesis and its derivatives have been developed for genetic studies6,7. However, random integration of the transposon into the chromosome did not allow the specific gene targeting analysis.

Suicide plasmids have also been used in Mollicutes to inactivate targeted genes via homologous recombination between the gene cloned in the plasmid and its homologues sequence in the host genome. Because of low recombination frequency that may cause by the low transformation efficiency, these vectors were applied only in very few mycoplasmas including Mycoplasma mycoides subsp. capri7, Mycoplasma gallisepticum8, Acholeplasma laidlawii9 and Mycoplasma genitalium10,11. Indeed, in our laboratory, we have developed suicide plasmids and have been successfully used to inactivate hlyC in M. hyorhinis12.

Recently, replicative plasmids have been established for a number of Mollicutes including Spiroplasma citri13, Mycoplasma pulmonis14, Mycoplasma mycoides subsp. capri, M. mycoides subsp. Mycoides small-colony type15, Mycoplasma capricolum subsp. capricolum16,17, Mycoplasma agalactiae17 and Mycoplasma gallisepticum15,18. The typical oriC-plasmid contains an origin of replication (oriC) along with tetracycline resistance marker and sufficient sequence homologous to chromosomal DNA to insert into the chromosome of the organism via homologous recombination. These oriC-plasmids have been used in some studies to disrupt targeted genes, complement mutants15,16,17, and also to express foreign genes16.

More recently, in-Yeast engineering of Mycoplasma mycoides subsp. capri GM12 (Mmc) genome using CRISPR/Cas9 system, has also been established and was used to disrupt mycoplasma glycerol-3-phosphate oxidase-encoding gene (glpO)19. However, cloning and assembly of a synthetic bacterial genome in yeast followed by a back transplantation into recipient bacterial cells is a challenge.

1Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture, National Research Center for Engineering and Technology of Veterinary Bio-products, Nanjing, 210014, China. 2College of Veterinary Science, University of Nyala, Nyala, Sudan. Correspondence and requests for materials should be addressed to Q.X. (email: qiyanxiongnj@163.com)
At present, the oriC-plasmids have not been studied in *M. hyorhinis*. The aim of this study was to construct and evaluate the usefulness of these plasmids as genetic tools in *M. hyorhinis*. Here, the oriC-region of *M. hyorhinis* was characterized and used for the development of oriC-plasmids that replicate in *M. hyorhinis*.

In many cases, the oriC-plasmids harboring large oriC-fragments tend to integrate into the oriC-region of the genomic DNA by homologous recombination between the gene fragment cloned on the plasmid and the corresponding site on the chromosome due to the sequence homology. The stability of the oriC-plasmids can be improved by utilizing a minimum oriC that reduces the possibility of the integration of the plasmid into the host chromosome. Herein, a mini-oriC plasmid that could replicate and maintained stably extra-chromosomally has been developed and evaluated. This study also provides evidence of homologous recombination occurring in *M. hyorhinis*.

The hemolytic activity has been described in other mycoplasmas. Since the HUB-1 genome sequence has a hlyC (NCBI Reference Sequence: WP_014582557.1) related sequence identified by automated prediction, it is hypothesized that this sequence is involved in hemolysis. Therefore, hlyC was chosen as the target gene for these experiments. We initially confirmed that, the supernatant of wild-type *M. hyorhinis* can lyse mouse RBCs (Supplementary Fig. S1). When the mini-oriC plasmid that contained relatively small regions (~500 bp) of homology with chromosomal DNA of hlyC flanking tetM used to transform *M. hyorhinis*, the tetM inserted into the chromosome and disrupted the hlyC. The supernatant of the hemolysin mutant strain diminished the ability to lyse mouse RBCs. Therefore, this manuscript demonstrates that hlyC may be involved in mouse RBC lysis.

RecA plays an important role in DNA recombination and repair. In *M. mycoides* subsp. *capric*, the efficiency of homologous recombination using suicide plasmids was enhanced by the inclusion of a recA (from *E. coli*) into the constructs. Therefore, in this study, we further augmented the occurrence of the homologous recombination at the hlyC site by introducing recA into our targeting oriC-plasmids.

The ability to transform *M. hyorhinis* has been long described. However, the transformation procedure and the oriC-plasmid systems developed in this study, have the potential to be used in targeted gene disruption, and may also in gene complementation and expression studies in this organism and possibly other mycoplasmas.

**Results**

**Vectors construction.** No functional oriC had previously been isolated from *M. hyorhinis*. The large oriC-region (LoriC) of *M. hyorhinis* (1935-bp) which consisted of the complete dnaA and intergenic regions upstream and downstream of the dnaA (NCBI Reference Sequence: WP_020104468.1) were identified (Fig. 1). The genomic regions upstream of the dnaA contained a single putative DnaA box and the AT-rich region is about 85% (Fig. 1). Likewise, the genomic regions downstream of the dnaA contained a single putative DnaA box and the AT-rich region is about 85% (Fig. 1). The minimum oriC region (Moric) of about 876-bp, only contains the regions upstream and downstream the dnaA (Fig. 1).

The PCR products of tetM 1917-bp (without promoter), spiralin gene promoter 313-bp, large oriC (LoriC) 1935-bp, mini-oriC (Moric) 876-bp, recA 1062-bp, hemolysin left arm (LA) 437-bp and hemolysin right arm (RA) 405-bp were prepared (Fig. 2) and separately cloned into pGEM-T vector. To determine whether the LoriC region is able to function as an autonomously replicating sequence, we first constructed pGEMT-LoriC plasmid, harboring LoriC, and later reduced the oriC to a minimum and cloned to generate pGEMT-Moric. To target hlyC, both hemolysin arms were cloned flanking the tetM to yield Mini-oriC-HT1 plasmid. Finally, the efficiency of hemolysin targeting had improved by introducing recA expressed by spiralin gene promoter to yield Mini-oriC-HT2 plasmid. The general strategy used to construct the oriC-plasmids was shown in (Fig. 3).

**Transformation of *M. hyorhinis*.** Following transformation with pGEMT-LoriC and pGEMT-Moric plasmids, tetracycline resistant colonies appeared within 3 to 5 days on KM2-agar plates containing 2.5 µg/ml tetracycline. During the indicated time period, no spontaneous tetracycline-resistant colonies appeared for untransformed controls. The presence of the plasmids in the transformants was confirmed when tetM (part of the plasmid) was detected by tetM specific PCR (with P9 primers, Table 1) using DNA template prepared from...
sub-cultured colonies (Supplementary Fig. S2). The transformation frequency of pGEMT-LoriC plasmid was $1.5 \times 10^{-4}$ transformants per colony-forming unit (CFU) compared to $2.0 \times 10^{-5}$ transformants per CFU for pGEMT-MoriC plasmid.

**Analysis of homologous recombination at the oriC site of M. hyorhinis genome.** The oriC-plasmids containing LoriC fragment, have been shown to integrate into the host chromosome via homologous recombination due to the sequence homology with oriC-region. Here, we evaluated whether our pGEMT-LoriC had integrated into the M. hyorhinis genome, and also investigated the site at which the integration had occurred. Briefly, M. hyorhinis DNA extracted from transformed (at 5th passages) and untransformed control cultures, were subjected to the PCR analysis. Based on M. hyorhinis genomic sequence and pGEMT-LoriC plasmid, we designed integration primers (P10, Table 1) to investigate the predicted integration site of the plasmid at the oriC-region (Fig. 4A). The forward integration primer (P10-F, Table 1) was designed to specifically bind up-stream the LoriC at the M. hyorhinis genome and the reverse integration primer (P10-R, Table 1) was designed to specifically bind the tetM of the pGEMT-LoriC plasmid. Following plasmid integration by single cross-over at the oriC-region of M. hyorhinis, the predicted size of the PCR product was found to be 4037-bp (Fig. 4A). Indeed, the PCR product using integration primers (P10, Table 1) and DNA extracted from pGEMT-LoriC transformants, produced fragments of 4037-bp (Fig. 4B) indicating the integration of pGEMT-LoriC at the predicted oriC region. Analysis of the DNA extracted from pGEMT-LoriC transformants by tetM specific PCR (with P9 primers, Table 1), had detected the tetM and further confirmed the presence of the plasmid (Supplementary Fig. S3). In contrast, if the plasmid replicating in an extrachromosomal form (did not integrate), then the PCR fail to produce a band due to the specificity of the specific integration primers (P10, Table 1) (because the forward integration primer binds specifically to M. hyorhinis genome upstream to LoriC, while reverse integration primer binds specifically to tetM of the pGEMT-LoriC plasmid). We also predicted the size of the PCR product when pGEMT-MoriC integrated at the oriC-site (2122-bp), where we get no PCR product (Supplementary Fig. S4), indicating the absence of plasmid integration at this region by single cross-over.

**Inactivation of hlyC using Mini-oriC-HT1.** The hemolytic activity has been described in other mycoplasmas. However, there is no specific gene in mycoplasmas identified to be associated with the hemolysis. In this study, we first confirmed that, the supernatant collected from M. hyorhinis can lysse mouse RBCs (Supplementary Fig. S1). Since the HUB-1 genome sequence encodes a hlyC related sequence determined by automated prediction, this sequence is hypothesized to be involved in hemolysis. Therefore, we chose to inactivate the hlyC as it’s easy to measure the phenotype of the mutant. In other mycoplasmas, the LoriC fragment drives the plasmid to integrate at the oriC-region of the host due to sequence homology. Therefore, we constructed an oriC-plasmid that contains minimum oriC-region (876-bp) and was named pGEMT-MoriC. Since we aimed to evaluate whether pGEMT-MoriC plasmid could be used for hlyC inactivation. We cloned hemolysin left and right arms into the pGEMT-MoriC plasmid to flank tetM and the spiralin gene promoter and to yield Mini-oriC-HT1 targeting plasmid. The resultant mutant strain expected to confer resistance to tetracycline when added to the medium at the appropriate concentration. The following transformation with Mini-oriC-HT1 plasmid and plating in KM2-agar containing 2.5 μg/ml tetracycline, we observed the growth of tetracycline resistance colonies. Selected colonies sub-cultured in KM2 medium containing 5.0 μg/ml tetracycline and subjected to PCR analysis using hlyC flanking primers (P11, Table 1) located upstream and downstream of the hlyC respectively. The wild-type hlyC produces a predicted PCR product of about 1566-bp with these primers (Fig. 5) indicating the absence of

**Figure 2.** PCR products of fragments required to construct oriC-plasmids for M. hyorhinis. The tetM 1917-bp (A), spiralin gene promoter 313-bp (B), spiralin gene promoter joined with recA to form a single fragment of about 1375-bp (C), large oriC (LoriC) 1935-bp (D), mini-oriC (MoriC) 876-bp (E) and hemolysin left arm (LA) 437-bp and right arm (RA) 405-bp (F) were shown. The gels images were cropped and full-length gels are included in the Supplementary Fig. S8.


tetM insertion at hlyC site. When hlyC is disrupted by inserting tetM along with spiralin gene promoter, the predicted sequence will increase (3072-bp) and could be PCR amplified with hlyC flanking primers (P11, Table 1) (Fig. 5). In our study, we observed a larger PCR product, presumably 3072-bp in size, following insertion of tetM and the spiralin gene promoter into hlyC via homologous recombination (Fig. 6A). Five colonies out of ten (50%) from two repeated experiments were produced with the predicted larger PCR product targeting hlyC when Mini-oriC-HT1 was used. This indicates the success of the specific targeting and inactivation of the hlyC. It also indicates the occurrence of homologous recombination in M. hyorhinis.

Improvement hemolysin targeted inactivation by the inclusion of recA. To improve the frequency of gene targeting, we inserted recA (from E. coli) controlled by the spiralin gene promoter into Mini-oriC-HT1 to yield Mini-oriC-HT2 targeting plasmid. The previous study indicated the usefulness of recA to enhance homologous recombination in M. mycoides subsp. capri. Similar results were observed with our suicide vectors.

Following transformation with Mini-oriC-HT2 plasmid and sub-culture of 5 resistance colonies, we observed the predicted size of PCR product (3072-bp) in all the selected colonies indicating the insertion of tetM at the hemolysin site (Fig. 6B). Similar results were obtained when the experiment repeated. Overall, an about 9/10 (90%) hemolysin targeting efficiency from two repeated experiments was obtained when recA incorporated into the targeting oriC-plasmid (Mini-oriC-HT2). No significant change in the phenotype of the wild-type and mutant colonies was observed (Fig. 6C).

The tetM insertion at the hemolysin site was further investigated with tetM specific PCR (with P9 primers, Table 1), using the 3072-bp PCR product amplified with hlyC flanking primers (P11, Table 1) as a template (Supplementary Fig. S5). The 3072-bp PCR product amplified with hlyC flanking primers was directly sequenced to analyze the junctions and the presence of tetM along with spiralin gene promoter. Analysis of the DNA sequencing results indicates the presence of sequences of hlyC arms, spiralin gene promoter as well as the tetM (Fig. 7), confirming the insertion of tetM along with the spiralin gene promoter at hlyC site.

Further, we predicted the occurrence of single cross-over between the plasmid and any arm of hlyC leading to the integration of the full Mini-oriC-HT1 plasmid that would result in PCR amplification of tetM thus generating positive PCR results. The phenotype of such transformants should have a hemolysin positive phenotype. To investigate this event, we predicted the integration of the Mini-oriC-HT1 plasmid by a single cross-over at the hemolysin right arm site. We designed single cross-over forward and reverse primers (P12, Table 1). The single-cross forward primer (P12-F, Table 1) binds the upstream region of hemolysin right arm (absent if double-cross over had occurred) while the single-cross reverse primer (P12-R, Table 1) binds the tetM present in the Mini-oriC-HT1 plasmid. In our experiment, we failed to obtain a positive PCR product (about 1017-bp) using single-cross primers (Supplementary Fig. S6) indicating that, the insertion of tetM at the hlyC site is due to a double cross-over event.
Scientific Reports  | 7: 10596  | DOI:10.1038/s41598-017-10519-3

**Table 1.** List of primers and their sequences used in this study. Primer sequences are shown along with the cloning site.

| Primer information | Primer sequence (5′-3′) | Cloning site |
|--------------------|-------------------------|--------------|
| P1 (1917-bp): tetM (no promoter) | F: GAAATATAAGAAGCTATGATGAAAAATTATTAATGAGTTTTAGCTCATGTGTTAGC | SpeI PstI |
| P2 (313-bp): (Spiralin gene promoter) | F: GAGCATGAGCTGACTTACCTTAGAAGGAGAATACGTTGTTGAA | SpeI |
| P3 (1935-bp): (LoriC) | F: GTAATGGGGAATTGAGCCCTACTTGGCTCCTGTGCTGAAAGCT | ApaI |
| P4A (563-bp): (MoriC) | F: TCTACCTGTTTCTGCTTGAATATCAGCAGCTCTTGTGAA | ApaI |
| P4B (313-bp): (MoriC) | F: TCGATGAGCTGACTTACCTTAGAAGGAGAATACGTTGTTGAA | SacII |
| P5 (437-bp): (Hemolysin-LA) | F: CCGGATATGCACATGCCAATCCAAAAGAGATCAC | Sall |
| P6 (405-bp): (Hemolysin-RA) | F: ATACCTTCGAGCAGCAATGAGCTTTAGTAAACCTACAG | NcoI |
| P7 (1062-bp): (RecA-splicing-Spiralin gene promoter) | F: GAGAAGGAATAATAAGAAATGCTATGCGAGGAAAACAAACAGAG | -- |
| P8 (313-bp): (Spiralin gene promoter-splicing-recA) | F: TCTACCGCGCCGCAATGTTAGTGAACAGAAGACATGGAAGACCCAG | -- |
| P9 (339-bp): (tetM screening) | F: GCAGTTATGGAAGGAGATACG | -- |
| P10 (4037-bp): (Integration predicted product) | F: CAGCATGAGGAAATTCTGCAATGCGAG | -- |
| P11 (1566-bp for Wild-type and 3072-bp for the mutant): (hlyC flank and sequencing) | F: CATTAGCAAGTTAGAATACAGACACCCC | -- |
| P12 (single crossover) | F: GAGTTAAGTTTTGAGAGTTAGTGCAGT | -- |

**Loss of hemolytic phenotype in the mutants.** In *M. hyorhinis*, disruption of hlyC was expected to diminish its ability to lyse mouse RBCs. The wild-type and the mutant cells grown in K2 medium and the supernatant were used to assay the hemolytic activity. Mouse RBCs were incubated with the supernatant while the control with PBS. The hemolytic activity was measured at 405 nm. We found that, the supernatant collected from the mutant colonies significantly (*P < 0.001*) lacks the ability to lyse mouse RBCs compared to wild-type bacterial supernatants (Fig. 8). Therefore, this is a first study demonstrated the gene associated with a hemolytic phenotype in mycoplasmas.

**Discussion**

In this study, we have described the construction and the use of a series of *M. hyorhinis* oriC-plasmids. Although the transformation of *M. hyorhinis* has been reported earlier with polyethylene glycol (PEG), the usage of the host-vector systems for genetic studies of *M. hyorhinis* has not so far been implemented. Therefore, a host-vector system which can be utilized for *M. hyorhinis* genetic studies is a prerequisite. The electro-transformation conditions optimized in this study are suitable for *M. hyorhinis*. However, some factors such as the use of minimum inhibitory concentration of tetracycline, high voltage and the excess amount of plasmid DNA should be considered.

OriC-plasmids have been successfully used in a number of Mollicutes. Here, the origin of replication region of *M. hyorhinis* genome has been identified and was used to construct *oriC*-plasmids for this bacterium for the first time. The prediction of large *oriC* (LoriC) region of *M. hyorhinis* chromosome has identified two dnaA boxes upstream and downstream the dnaA, and this LoriC was proved to be functional in the *pGEMT*-LoriC plasmid. In the *M. hyorhinis* transformants, the *pGEMT*-LoriC plasmid was found to replicate as a free extra-chromosomally before integrating into the host chromosome during passageing, similarly to the *pBOT1* plasmid in *Spiroplasma citri*. The length of the *oriC* sequences has been shown to influence the recombination efficiency. Furthermore, the presence of a complete dnaA is not essential to driving the replication in *oriC*-plasmids as in *Spiroplasma citri*. Therefore, a plasmid containing a minimum *oriC* fragment was constructed to decrease the frequency of plasmid integration into the *oriC*-region of *M. hyorhinis*. Indeed, free extra-chromosomal *oriC*-plasmids were obtained when the *pGEMT*-MoriC plasmid containing a minimum *oriC*-fragment (876-bp) used, similar to previous observations.
Tetracycline was chosen as the selection marker as it has been used in multiple mycoplasma species and that M. hyorhinis strain HUB-1 stored in our laboratory is tetracycline sensitive. The oriC-based plasmids described here have several useful features that will provide flexibility in future genetic studies. The multiple cloning sites located upstream and downstream of tetM and the spiralin gene promoter make these oriC-plasmids compatible for the subsequent cloning of foreign DNA (such as GFP or other antigens) for many applications in M. hyorhinis. Indeed, this may encourage in vivo imaging following M. hyorhinis infection or even development of recombinantly engineered vaccines when foreign antigens are expressed.

The hemolytic activity has been previously described in some mycoplasmas, however, there has been no previous study showing that a particular gene has hemolytic activity in mycoplasmas. In this study, we investigated the function of hlyC by using our oriC-plasmids, and the data show that, the hlyC is responsible for the hemolytic activity observed in M. hyorhinis. Analysis of the hlyC DNA sequence present in different strains of M. hyorhinis (HUB-1 Accession CP002170 Region: 304679..305920, DBS 1050 Accession CP006849 Region: 438049..439291, GDL-1 Accession CP003231 Region: 438066..439308, MCLD Accession CP002669 Region: 170809..172050, MDBK-IPV Accession CP016817 Region: 437951..439192 and SK76 Accession CP003914.1 Region:305879..307120) by the blast and Clustal V methods, has revealed only one base different (in DBS 1050 and GDL-1 strains) in the hlyC sequence (Supplemental Fig. S7). The conservancy of hlyC sequence in these strains may indicate its potential in the pathogenesis of these strains although of the fact that the strains of M. hyorhinis express differences in virulence. However, the association between the hlyC and virulence of M. hyorhinis remained to be evaluated. Due to the available genome sequences of M. hyorhinis and the importance

Figure 4. Prediction of the possible integration of pGEMT-LoriC plasmid containing large oriC (LoriC) at the oriC-region of M. hyorhinis (A). Following integration at the oriC region, a fragment of about 4037-bp could be detected with PCR using integration primers (P10, Table 1): forward integration primer (P10-F, Table 1) binds specifically to M. hyorhinis genome upstream the LoriC, while reverse integration primer (P10-R, Table 1) binds specifically to tetM of the pGEMT-LoriC plasmid (B) to yield a product of predicted size of 4037 bp. The gel image was cropped and full-length gel is included in the Supplementary Fig. S9.

Figure 5. Prediction of the possible insertion of tetM along with spiralin gene promoter at the hemolysin site using Mini-oriC-HT1 and Mini-oriC-HT2 targeting plasmids. Following insertion at hemolysin site, a fragment of about 3072-bp could be amplified with PCR using hlyC flanking primers. Wild-type hlyC exhibits 1566-bp. LA = left arm, RA = right arm.
of this bacterium in the development of arthritis as well as cancers in vitro, the development of oriC-plasmids for M. hyorhinis, could allow identification of virulence factors, and understand its pathogenesis. In conclusion, our study has developed useful oriC-plasmids for M. hyorhinis and also identified the association between hlyC and the hemolysis phenotype for the first time.

Figure 6. Analysis of hlyC disruption: DNA was extracted from the grown culture of M. hyorhinis transformed with Mini-oriC-HT1 (A) and Mini-oriC-HT2 (B) targeting plasmids along with control untransformed cultures, was subjected to PCR analysis using hlyC flanking primers (P11, Table 1) to investigate the integration of the tetM into M. hyorhinis genome at hlyC site. Wild-type hemolysin exhibits 1566-bp while mutant hemolysin that encodes tetM along with spiralin gene promoter has about 3072-bp. (C) the phenotype of the wild-type and mutant colonies of M. hyorhinis. The gel image was cropped and full-length gel is included in the Supplementary Fig. S10.

Figure 7. DNA sequencing analysis of the large DNA fragment (mutant hlyC, 3072-bp) amplified with hlyC flanking primers (P11, Table 1) from DNA of M. hyorhinis cells transformed with Mini-oriC-HT1 (A) and Mini-oriC-HT2 (B) targeting plasmids. The sequencing was performed in both forward and reverse directions using hlyC flanking primers, P11 (Table 1)
type and negative control. The released hemoglobin was determined by measuring the OD at 405 nm. All reactions were performed in triplicate. Data are presented as Means ± SD (n = 3). P < 0.001 versus control.

Material and Methods

Bacterial strains and cultural conditions. The M. hyorhinis strain HUB-1 (GenBank accession CP002170.1) was kindly provided by Professor Xiao Shaobo (Huazhong Agricultural University, Wuhan, China) and cultured in a modified Friis medium (KM2 medium) containing 20% (v/v) swine serum at 37 °C. The solid medium (KM2-Agar) was prepared by adding 0.7% Agar (Biowest Agarose® G-10; Gene Company Limited, Chi Wan, Hong Kong) to the liquid KM2 medium and was incubated at 37 °C until the growth of the visible colonies. For the growth of mutants, tetracycline hydrochloride (Sigma-Aldrich) was added at 5.0 μg/ml and cultured in a modified Friis medium (KM2 medium) containing 20% (v/v) swine serum at 37 °C. The solid and liquid media respectively.

For the growth of mutants, tetracycline hydrochloride (Sigma-Aldrich) was added at 5.0 μg/ml to the liquid KM2 medium and was incubated at 37 °C until the growth of the visible colonies.

Hemolytic activity of the wild-type and hlyC mutants of M. hyorhinis were collected and incubated with mouse RBCs. PBS was used as a negative control. The released hemoglobin was determined by measuring the OD at 405 nm. All reactions were performed in triplicate. Data are presented as Means ± SD (n = 3). P < 0.001 versus control.

Construction of oriC-plasmids. We used pGEM®-T vector to construct the oriC-plasmids. Images of PCR ethidium bromide-stained agarose gels were acquired and with Gel Doc™ XR Software (Bio-Rad, USA). The tetracycline gene, tetM (ID: AGI19285.1) was PCR amplified (using P1 primers, Table 1) from pSE-1 vector provided by Professor Xiao Shaobo (Huazhong Agricultural University, Wuhan, China) and cloned into pGEM®-T vector at SpeI/PstI restriction enzyme sites by infusion cloning methods to generate pGEMT-tetM plasmid. Since common bacterial cloning vectors such as pGEM®-T vector or genes from *E. coli* cannot express in the *M. hyorhinis* from their own promoter, the spiralin gene promoter (from *Spiroplasma citri*, GI: 2384684, amplified from *S. citri* Plasmid) was cloned upstream of *M. hyorhinis* genomic DNA using P3 primers, Table 1 and cloned at ApaI restriction enzyme site to generate pGEMT-MoriC plasmid.

To amplify a minimum oriC fragment (MoriC), the flanking regions of *dnaA* that contains the AT-rich, were PCR amplified with P4A primers, Table 1 as fragment A (upstream *dnaA*, 563-bp) and with P4B primers, Table 1 as fragment B (downstream *dnaA*, 313-bp). These two fragments were joined by overlapping PCR (using P4A-F and P4B-R primers, Table 1) to form a single fragment of 876-bp as minimum oriC (Fig. 1). This minimum oriC PCR product was cloned into pGEMT-Sp/tetM at ApaI restriction enzyme site to generate the pGEMT-MoriC plasmid as a minimum oriC-plasmid. The design of the fragments A and B were shown in the diagram presented in (Fig. 1), while the primers in (Table 1).

We chose to disrupt *hlyC* (NCBI Sequence: WP_o14582557.1) because we hypothesized that if *hlyC* confers hemolytic activity, it may be easy to measure the hemolytic phenotype. To construct plasmid targets *hlyC*, both hemolysin left and right arms were at least 450-bp to provide adequate homologous sequences to facilitate recombination at the target gene. These arms were PCR amplified and cloned to flank *tetM* as well as the spiralin gene promoter since we aimed to knock-in a functional *tetM* at the hemolysin site. The left arm (LA) of *hlyC* (amplified from *M. hyorhinis* DNA using P5 primers, Table 1) was cloned into pGEMT-MoriC plasmid upstream spiralin gene promoter at SacII restriction enzyme site while right arm (RA) of *hlyC* (amplified from *M. hyorhinis* DNA using P6 primers, Table 1) cloned downstream of *tetM* at SalI restriction enzyme site. The resultant plasmid named mini-oriC targeting plasmid-1 (*Mini-oriC-HT1*) and was used to inactivate *hlyC* by homologous recombination.

The recombinase gene (*recA*) of *E. coli* was found to enhance the homologues recombination in *M. mycoides* subsp. *capri*. Therefore, the *recA* (GI:446885887) was PCR amplified from *E. coli* BL-21 DNA using P7 primers, Table 1. At the same time, the spiralin gene promoter was PCR amplified with P8 primers, Table 1 and joined by overlapping PCR with *recA* (using P8-F and P7-R primers, Table 1) to form a single fragment which was cloned into *Mini-oriC-T1* plasmid at NcoI restriction enzyme site. The resulted plasmid named mini-oriC targeting plasmid-2 (*Mini-oriC-HT2*) and was also evaluated for its functionality to inactivate *hlyC* by homologous recombination. All cloning steps were verified by colony PCR and analysis of DNA sequencing. The diagram of the vectors constructions was shown in (Fig. 3), while the primers used to amplify or join the PCR fragments into each, were listed in (Table 1).
Electroporation of *M. hyorhinis*. *M. hyorhinis* were used at approximately 10E8 CCU. Electroporation of *M. hyorhinis* was carried out by a procedure similar to previously described methods with minor modifications. Brieﬂy, the culture centrifuged at 6,000 rpm for 15 min at 4 °C, and the pellet washed three times with ice-cold electroporation buffer (272 mM Sucrose, 200 mM HEPES, pH 7.2) and resuspended in 100 µl ice-cold electroporation buffer. Aliquot of 0.1 ml of competent cells was mixed with 20 µg plasmid DNA. The mixture was then placed in a pre-chilled sterile electroporation cuvette (2 mm electrode gap, Bio-Rad Laboratories, Hercules, CA) and pulsed immediately with ECM 630 Electroporation System (Harvard Apparatus BTX, Holliston, MA, USA) at 2.5 kV, 125 Ω, and 25 μF. The mixture was immediately diluted with 900 µl of cold KM2 broth, incubated on ice for 5 min, and recovered at 37 °C for 3 hours. The cells were diluted 1:10 and selected on KM2-Agar containing 2.5 µg/ml tetracycline and grown at 37 °C till colonies appeared. Tetracycline-resistant colonies were sub-cultured in 2 ml of KM2-broth containing 5.0 µg/ml tetracycline. Transformants were passaged by inoculating 100 µl of culture into 900 µl of broth containing 5.0 µg/ml of tetracycline.

**Screening of the plasmids by PCR.** Following transformation with pGEMT-LoriC and pGEMT-MoriC plasmids, the cells were diluted and plated on KM-Agar containing 2.5 µg/ml tetracycline. Individual colonies of *M. hyorhinis* were sub-cultured in KM2 medium containing 5.0 µg/ml tetracycline at 37 °C several times to eliminate the residual plasmid from the medium. To detect the presence of the plasmids in the transformants, total DNA was extracted from 2 ml *M. hyorhinis* cultures using a genomic DNA purification kit (TIANGEN, Beijing, China). We performed a PCR amplifying a fragment of the tetM (part of the oriC-plasmid backbone) using universal tetM screening primers (P9, Table 1). This PCR resulted in 339-bp tetM-product in the presence of free or integrated oriC plasmids.

**Analysis of hemolytic phenotype.** A single clone of the mutant and wild-type *M. hyorhinis* was grown in KM2 medium containing 5.0 µg/ml tetracycline and the culture centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were carefully collected and analyzed for the hemolytic activity using fresh mouse RBCs collected from healthy mice. The hemolytic activity was determined as previously described with minor modifications. Briefly, mouse RBCs were incubated with the supernatant collected from the mutant and wild-type *M. hyorhinis* for 2 hours at 37 °C. Control samples were incubated with PBS. The samples were centrifuged at 1,500 × g for 10 min and the released hemoglobin was determined by measuring the OD at 405 nm. All reactions were performed in triplicate.

**Statistical analysis.** Data obtained from three individual experiments were recorded as Mean ± SD and subjected to one-way analysis of variance (ANOVA) using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA). P < 0.05 was considered statistically significant.

**References**

1. Razin, S., YogeV, D. & Naot, Y. Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews* 62, 1094–1156 (1998).
2. Naimiki, K. et al. Persistent exposure to Mycoplasma induces malignant transformation of human prostate cells. *Plos one* 4, e6872, doi:10.1371/journal.pone.0006872 (2009).
3. Yang, H. et al. *Mycoplasma hyorhinis* infection in gastric carcinoma and its effects on the malignant phenotypes of gastric cancer cells. *BMC gastroenterology* 10, 132, doi:10.1186/1471-230X-10-132 (2010).
4. Liu, W. et al. Complete genome sequence of *Mycoplasma hyorhinis* strain HUB-1. *Journal of bacteriology* 192, 5844–5845, doi:10.1128/JB.00946-10 (2010).
5. Dybvig, K. & Alderete, J. Transformation of *Mycoplasma pulmonis* and *Mycoplasma hyorhinis*: transposition of Tn916 and formation of cointegrate structures. *Plasmid* 20, 33–41, doi:10.1016/0147-619X(88)90058-I (1988).
6. Mahairas, G. G. & Minion, F. C. Random insertion of the gentamicin resistance transposon Tn4001 in *Mycoplasma pulmonis*. *Plasmid* 21, 43–47, doi:10.1016/0147-619X(89)90085-1 (1989).
7. Allam, A. B., Reyes, L., Assad-Garcia, N., Glass, J. I. & Brown, M. B. Enhancement of targeted homologous recombination in *Mycoplasma mycoides* subsp. capri by inclusion of heterologous recA. *Applied and environmental microbiology* 76, 6951–6954, doi:10.1128/AEM.00056-10 (2010).
8. Burgos, R., Pich, O. Q., Querol, E. & Pinol, J. Deletion of the *Mycoplasma genitalium* MG_217 gene modifies cell gliding behaviour by altering terminal organelle curvature. *Molecular microbiology* 69, 1029–1040, doi:10.1111/j.1365-2958.2008.06343.x (2009).
9. Dybvig, K. & Woodard, A. Construction of recA mutants of *Acholeplasma laidlawii* by insertional inactivation with a homologous DNA fragment. *Plasmid* 28, 262–266, doi:10.1016/0147-619X(92)90058-I (1992).
10. Dhandayuthapani, S., Blaylock, M. W., Bebear, C. M., Rasmussen, W. G. & Baseman, J. B. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. *Journal of bacteriology* 183, 5645–5650, doi:10.1128/JB.183.10.5645-5650 (2001).
11. Dhandayuthapani, S., Rasmussen, W. G. & Baseman, J. B. Disruption of gene mg218 of *Mycoplasma genitalium* through homologous recombination leads to an adherence-deficient phenotype. *Proceedings of the National Academy of Sciences of the United States of America* 96, 5227–5232, doi:10.1073/pnas.96.9.5227 (1999).
12. Ishag, H. Z., Xiong, Q., Liu, M., Feng, Z. & Shao, G. E. coli recA gene improves gene targeted homologous recombination in *Mycoplasma hyorhinis*. *Journal of microbiological methods* 136, 49–56, doi:10.1016/j.mimet.2004.07.007 (2014).
13. Renaudin, I. et al. Integrative and free Spiroplasma citri oriC plasmids: expression of the Spiroplasma phoeniceum spiralin in *Spiroplasma citri*. *Journal of Bacteriology* 177, 2870–2877, doi:10.1128/JB.177.10.2870-2877 (1995).
14. Cordova, C. M. et al. Identification of the origin of replication of the *Mycoplasma pulmonis* chromosome and its use in oriC replicative plasmids. *Journal of bacteriology* 184, 5426–5435, doi:10.1128/ jb.184.19.5426-5435 (2002).
15. Lartigue, C., Blanchard, A., Renaudin, J., Thiacoort, F. & Sirand-Pugnet, P. Host specificity of mobillicites oriC plasmids: functional analysis of replication origin. *Nucleic acids research* 31, 6610–6618, doi:10.1093/nar/gkg848 (2003).
16. Janis, C. et al. Versatile use of oriC plasmids for functional genomics of *Mycoplasma capricolum* subsp. capricolum. *Applied and environmental microbiology* 71, 2888–2893, doi:10.1128/AEM.71.6.2888-2893 (2005).
17. Chopra-Dewasthaly, R., Marenda, M., Rosengarten, R., Jechlinger, W. & Cittı, C. Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. *FEBS microbiology letters* 253, 89–94, doi:10.1016/j.femsle.2005.09.021 (2005).
18. Lee, S. W., Browning, G. F. & Markham, P. F. Development of a replicable oriC plasmid for Mycoplasma gallisepticum and Mycoplasma imitans, and gene disruption through homologous recombination in M. gallisepticum. Microbiology (Reading, England) 154, 2571–2580, doi:10.1099/mic.0.019208-0 (2008).
19. Maglennon, G. A. et al. Development of a self-replicating plasmid system for Mycoplasma hypopneumoniae. Veterinary research 44, 63, doi:10.1186/1297-9716-44-63 (2013).
20. Kent, B. N., Foecking, M. F. & Calcutt, M. J. Development of a novel plasmid as a shuttle vector for heterologous gene expression in Mycoplasma yeatsii. Journal of microbiological methods 91, 121–127, doi:10.1016/j.mimet.07.018 (2012).
21. Tsarmpopoulos, I. et al. In-yeast Engineering of a Bacterial Genome Using CRISPR/Cas9. ACS synthetic biology 5, 104–109, doi:10.1021/acssynbio.5b00196 (2016).
22. Duret, S., Andre, A. & Renaudin, J. Specific gene targeting in Spiroplasma citri: improved vectors and production of unmarked mutations using site-specific recombination. Microbiology (Reading, England) 151, 2793–2803, doi:10.1099/mic.0.28123-0 (2005).
23. Kannan, T. R. & Baseman, J. B. Hemolytic and hemoxidative activities in Mycoplasma penetrans. Infection and immunity 68, 6419–6422, doi:10.1128/IAI.68.11.6419-6422 (2000).
24. Horii, T., Ogawa, T. & Ogawa, H. Organization of the recA gene of Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 77, 313–317 (1980).
25. Glass, J. I. et al. Essential genes of a minimal bacterium. Proceedings of the National Academy of Sciences of the United States of America 103, 425–430, doi:10.1073/pnas.0510013103 (2006).
26. Duret, S., Danet, J. L., Garnier, M. & Renaudin, J. Gene disruption through homologous recombination in Spiroplasma citri: an scm1-disrupted motility mutant is pathogenic. Journal of Bacteriology 181, 7449–7456 (1999).
27. Ishag, H. Z. et al. GFP as a marker for transient gene transfer and expression in Mycoplasma hyorhinis. SpringerPlus 5, 769, doi:10.1186/s40064-016-2358-3 (2016).
28. Gois, M. & Kuksa, F. Intranasal infection of gnotobiotic piglets with Mycoplasma hyorhinis: differences in virulence of the strains and influence of age on the development of infection. Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B 21, 352–361, doi:10.1111/j.1439-0450.1974.tb00510.x (1974).
29. Xiong, Q. et al. The functions of the variable lipoprotein family of Mycoplasma hyorhinis in adherence to host cells. Veterinary microbiology 186, 82–89, doi:10.1016/j.vetmic.01.017 (2016).
30. Sharma, S., Markham, P. F. & Browning, G. F. Genes found essential in one other mycoplasmas are dispensable in Mycoplasma bovis. PLoS one 9, e97100, doi:10.1371/journal.pone.0097100 (2014).
31. Chu, L. & Holt, S. C. Purification and characterization of a 45 kDa hemolysin from Treponema denticola ATCC 35404. Microbial pathogenesis 16, 197–212, doi:10.1016/mpat.1020 (1994).

Acknowledgements
The work was supported by the postdoctoral fund of Jiangsu Academy of Agricultural Sciences, China (Grant No. 6511318) and National Natural Sciences Foundation of China (Grant No. 31550110211 and No. 31770193).

Author Contributions
H.Z.A.I. designed, carried and drafted the manuscript. Q.Y.X., M.J.L., Z.X.F. and G.Q.S., were critically revised and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-10519-3

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017