Genetics and Molecular Research

A replicating plasmid-based vector for *GFP* expression in *Mycoplasma hyopneumoniae*

H.Z.A. Ishag[1,2], M.J. Liu[1], R.S. Yang[1], Q.Y. Xiong[1], Z.X. Feng[1] and G.Q. Shao[1]

[1]Key Laboratory of Veterinary Biological Engineering and Technology, Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Ministry of Agriculture, National Research Center for Engineering and Technology of Veterinary Bio-Products, Nanjing, China
[2]College of Veterinary Sciences, Nyala University, Nyala, Sudan

Corresponding author: G.Q. Shao
E-mail: gqshaojaas@163.com

Genet. Mol. Res. 15 (2): gmr.15027832
Received December 16, 2015
Accepted January 18, 2016
Published April 27, 2016
DOI http://dx.doi.org/10.4238/gmr.15027832

ABSTRACT. *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) causes porcine enzootic pneumonia (PEP) that significantly affects the pig industry worldwide. Despite the availability of the whole genome sequence, studies on the pathogenesis of this organism have been limited due to the lack of a genetic manipulation system. Therefore, the aim of the current study was to generate a general *GFP* reporter vector based on a replicating plasmid. Here, we describe the feasibility of *GFP* reporter expression in *M. hyopneumoniae* (strain 168L) controlled by the *p97* gene promoter of this mycoplasma. An expression plasmid (pMD18-TOgfp) containing the *p97* gene promoter, and origin of replication (*oriC*) of *M. hyopneumoniae*, tetracycline resistant marker (*tetM*), and *GFP* was constructed and used to transform competent *M. hyopneumoniae* cells. We observed green fluorescence in *M. hyopneumoniae* transformants under fluorescence microscopy, which indicates that there was expression of the *GFP* reporter that was driven by the *p97* gene promoter. Additionally, an electroporation method for *M. hyopneumoniae* with an

efficiency of approximately 1 x 10^{-6} transformants/μg plasmid DNA was optimized and is described herein. In conclusion, our data demonstrate the susceptibility of *M. hyopneumoniae* to genetic manipulation whereby foreign genes are expressed. This work may encourage the development of genetic tools to manipulate the genome of *M. hyopneumoniae* for functional genomic analyses.

Key words: *Mycoplasma hyopneumoniae*; oriC plasmid; *GFP*

INTRODUCTION

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes porcine enzootic pneumonia (PEP) that affects the pig industry worldwide. Although the mortality rate of this disease is low, the incidence has remained high. Once an outbreak is established, the control of the disease is difficult, resulting in significant economic losses (DeBey et al., 1992; DeBey and Ross, 1994). *M. hyopneumoniae* infection is often complicated by secondary bacterial and viral infections such as those of *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis*, Porcine reproductive and respiratory syndrome virus (PRRSV), and Porcine circovirus 2 (PCV-2). Currently, control of *M. hyopneumoniae* induced PEP mainly depends on antibiotics and vaccines. Although the current vaccine can protect against *M. hyopneumoniae* infection, methods to control the disease are still lacking. Therefore, the development of more efficient therapeutic approaches is urgently needed.

Recently, the whole genome sequence of *M. hyopneumoniae* strain 168L was determined (Liu et al., 2011). More recently, Maglennon et al. (2013a; 2013b) established methods to transform *M. hyopneumoniae* strain 232 with oriC-plasmids, and suggested the use of transposon mutagenesis to establish libraries of random mutations in this strain. Therefore, evidence supporting the use of *M. hyopneumoniae* as a host for heterologous gene expression has become a prerequisite for genetic studies regarding the pathogenicity of this organism, as the complementation of mutants requires the cloning and expression of exogenous genes.

Expression of reporter systems such as the *lacZ* gene and green fluorescent protein (GFP) in mycoplasmas can help to understand the nature of gene expression in these bacteria. However, in all reported cases, the fluorescent fusion proteins were constructed in standard cloning vectors, and subsequently transferred to mycoplasma transposon delivery vectors (Balish et al., 2003; Kenri et al., 2004; Zimmerman and Herrmann, 2005). Therefore, it would be helpful to have a general *GFP* reporter plasmid that was developed based on a replicating plasmid.

In the current study, an expression plasmid pMD18-TOgfp was constructed, in which the *p97* gene promoter was used to express reporter *GFP* in *M. hyopneumoniae* strain 168L. The method of electro-transformation of *M. hyopneumoniae* was also optimized. The motivation for this work was that obtaining an expression vector for *M. hyopneumoniae* based on replicating plasmids may help to develop genetic tools to engineer the *M. hyopneumoniae* genome.

MATERIAL AND METHODS

Bacterial strains and growth conditions

M. hyopneumoniae strain 168L (GenBank accession No. 507382422) was grown

at 37°C in KM2 cell-free liquid medium (a modified Friis medium) containing 20% (v/v) swine serum as previously described (Bai et al., 2013). For growth on solid medium, a final concentration of 0.7% w/v purified agar was added to the KM2 medium, and plates were incubated at 37°C until colonies were visible. Colonies were visualized by light microscopy. For the selection of transformed *M. hyopneumoniae*, tetracycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.02 μg/mL. The *E. coli* competent cells, XL-10 (Vazyme, Nanjing, China) served as the host for cloning procedures and plasmid amplification, and were grown in Luria-Bertani (LB) broth containing 100 μg/mL ampicillin at 37°C with overnight shaking at 180 rpm.

Vector construction

The primers for the target genes listed in Table 1 were designed based on the sequences available in the National Center for Biotechnology Information (NCBI) GenBank database (*M. hyopneumoniae* strain 168L ID 507382422; tetM ID AGI19285.1; and *GFP* ID AGN96197.1). The origin of replication (*oriC*) of *M. hyopneumoniae* was predicted based on previously described methods (Cordova et al., 2002; Maglennon et al., 2013b), as was the *p97* gene promoter (Maglennon et al., 2013b). Touchdown PCR was performed with the Ex Taq Polymerase Kit (TaKaRa, Otsu, Japan) to obtain the target genes. The oriC and *p97* gene promoter were PCR amplified from *M. hyopneumoniae* 168L genomic DNA, while *GFP* and *tetM* were PCR amplified from the pSE-1 plasmid (provided by Prof. Xiao Shaobo of Huazhong Agricultural University, Wuhan, China).

Table 1. List of primers used to amplify elements of the pMD18-TOgfp vector.

Fragment	Enzyme*	Oligonucleotides sequence (5'-3')	Product (bp)
oriC-MHP	*Eco*RI	F: CCGGT**GAATTCG**GTTAATTATTGCTTGAAATTC	2167
	*Xho*I	R: CGAA**CTCGAG**CCAACATCATAAATTTC	
p97 gene promoter	*Sac*I	F:CCTG**GAGCTC**TAAAAAAATATAGAAGAAAATTTCCTAGTAAAGTTC	620
	*BamH*I	R: CGGA**GGATCC**TAAAATTTGGAGTTAATAGAACCAAGATCC	
tetM (no promoter)	*BamH*I	F:CCG**GGATCC**ATGAAAATTATTAATATTGGAGTTTTAGCTC	1920
	*Xba*I	R:CCG**TCTAGA**TTATTTTATTGAACATATATCGTACTTTATCTATCCG	
p97 gene promoter spliced *GFP*	*Sal*I	F:TATATAT**GTCGAC**TAAAAAAATATAGAAGAAAATTTCCTAGTAAAGTTC	620
		R:CGCCCTTGCTCACCATATTTTAAACCTCAATTATCTTGGTTCTAG	
GFP spliced *p97* gene promoter		F: TAAAAAAATATAGAAGAAAATTTCCTAGTAAAGTTCATGGTGAGCAAGGGCG	750
	*Pst*I	R:CGGA**CTGCAG**TTACTTGTACAGCTCGTCCATGC	

*Restriction enzyme sites are in bold and underlined. MHP = *M. hyopneumoniae*. F = forward, R = reverse.

The pMD18-T vector (TaKaRa) was used to construct the expression vector of *M. hyopneumoniae,* and the cloning sites for insertion of the target fragments into this vector were as follows: the *oriC* at *Eco*RI/*Xho*I sites, the *p97* gene promoter at *Sac*I/*BamH*I sites, *tetM* at *BamH*I/*Xba*I sites, and the *p97* gene promoter spliced with *GFP* at *Sal*I/*Pst*I sites. The resultant plasmid was designated as pMD18-TOgfp. Every cloned fragment was verified by restriction enzyme digestion and DNA sequencing analysis.

Electroporation of *M. hyopneumoniae*

Electroporation of *M. hyopneumoniae* was done according to previously published methods with minor modifications (Maglennon et al., 2013b). Briefly, approximately 40 mL cultured *M. hyopneumoniae* were centrifuged at 12,000 rpm for 20 min at 4°C, and the

pellet washed three times with electroporation buffer (272 mM Sucrose, 200 mM HEPES, pH 7.2) supplemented with 1 mM EDTA. The pellet was incubated on ice for 5 min, and then resuspended in 100 μL electroporation buffer. This preparation of competent *M. hyopneumoniae* cells was either transformed immediately or stored at -80°C.

Plasmid DNA (10-15 μg) was added to 100 μL competent cells and transferred to a chilled 0.2 cm electroporation cuvette (Bio-Rad, Hercules, CA, USA). The mixture was incubated on ice for 20 min. The cells were electroporated with the ECM 630 Electroporation System (Harvard Apparatus BTX, Holliston, MA, USA) at 2.5 KV, 125 Ω, and 25 μF. Immediately after electroporation, 900 μL chilled KM2 medium was added. To this mixture, an additional 10 μg pMD18-TOgfp plasmid was inoculated. The resulting suspension was incubated for 20 min on ice, and then 24 h at 37°C. Subsequently, 0.02 μg/mL tetracycline hydrochloride (minimum inhibitory concentration) was added, and the cells were incubated at 37°C for 5-10 days. The selected transformants were also grown in KM2-Agar for at least for 2 weeks.

PCR-based detection of *GFP* in selected transformants

M. hyopneumoniae DNA was extracted from 2 mL cultures using a genomic DNA purification kit (TIANGEN, Beijing, China). PCR amplification of the *GFP* gene was performed to detect the presence of the pMD18-TOgfp plasmid in the selected KM2 transformants using *GFP* specific primers. The resultant product was 750 bp.

Detection of *GFP* fluorescence

The *M. hyopneumoniae* transformants grown in KM2 containing 0.02 μg/mL tetracycline hydrochloride were then plated on KM2-Agar and cultured until the growth of visible colonies. The *GFP* fluorescence of single *M. hyopneumoniae* clones was observed under a fluorescent microscope (Nikon, Eclipse E600, Tokyo, Japan).

RESULTS

Vector construction

In the current study, the fragments (*GFP*, *tetM*, *oriC*, and *p97* gene promoter) required to construct the heterologous gene expression vector for *M. hyopneumoniae* were PCR amplified (Figure 1A), and cloned into the pMD18-T vector to yield the desired pMD18-TOgfp vector (Figure 1B).

Electroporation of *M. hyopneumoniae*

The constructed expression plasmid isolated from *E. coli* could be introduced into *M. hyopneumoniae* competent cells by electroporation. The electric field, electric resistance, and amount of DNA required for the transformation were then optimized. Accordingly, 1 x 10^{-6} transformants·CFU^{-1}·μg^{-1} plasmid DNA were obtained with an electroporation system under optimal conditions (2.5 KV/cm, 100 Ω, 25 μF). Adding up to 10 μg plasmid DNA to cells immediately after electroporation improved the transformation efficiency where the colonies appeared within 10-14 days of incubation.

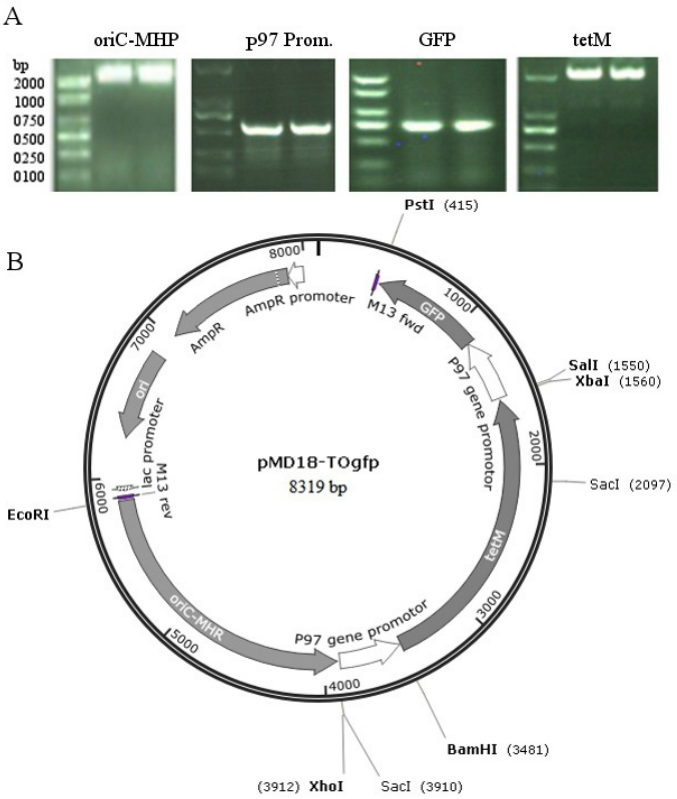

Figure 1. Construction of pMD18-TOgfp. **A.** PCR amplified fragments of the *oriC* of MHP (2167 bp), *p97* gene promoter (620 bp), *GFP* (750 bp), and tetracycline resistance marker *tetM* (1920 bp). **B.** Map of the constructed pMD18-TOgfp vector. Prom = promoter and MHP = *M. hyopneumoniae*.

PCR-based detection of *GFP* in selected transformants

Total DNA was extracted from pooled *M. hyopneumoniae* transformants grown in KM2 medium containing 0.02 µg/mL tetracycline hydrochloride, and the presence of the pMD18-TOgfp plasmid was subsequently analyzed by PCR detection of *GFP* (750 bp). Sequence analyses of the transformants further showed that the amplified products contained *GFP* DNA (Figure 2), which correlated with *GFP* expression observed under fluorescence microscopy. *GFP* was not detected in DNA from untransformed bacteria.

Detection of *GFP* fluorescence

The promoter of the *p97* gene of *M. hyopneumoniae* 168L was used to express the reporter genes including *GFP*. Cells were transformed with the pMD18-TOgfp plasmid, grown in KM2 containing 0.02 µg/mL tetracycline hydrochloride, and plated in KM2-Agar. The cells were then cultured at 37°C for 14 days. The presence of *GFP* was observed under fluorescence microscopy in the transformed colonies (Figure 3), but not in controls, indicating that *GFP* was expressed and functional in *M. hyopneumoniae*.

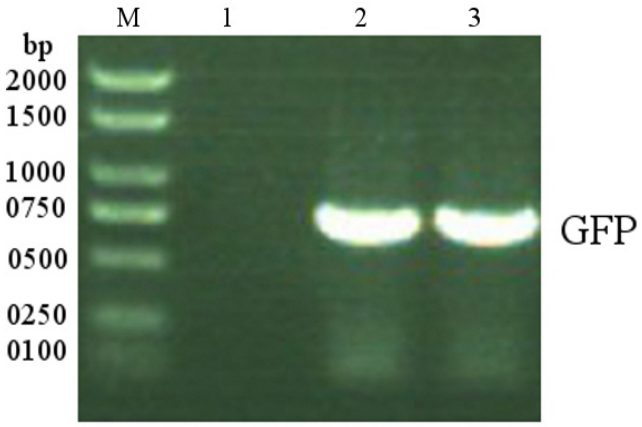

Figure 2. Detection of the pMD18-TOgfp plasmid in *Mycoplasma hyopneumoniae* transformants. *M. hyopneumoniae* competent cells were transformed with 15 µg pMD18-TOgfp, and an additional 10 µg plasmid was added immediately after transformation. The cells were then cultured in KM2 medium or KM2-Agar containing 0.02 µg/mL tetracycline hydrochloride. DNA was extracted from selected cultures. The presence of the plasmid was detected by PCR amplification of *GFP* resulting in a 750 bp product (*lanes 2* and *3*). Untransformed cells served as the control (*lane 1*). *Lane M* = marker.

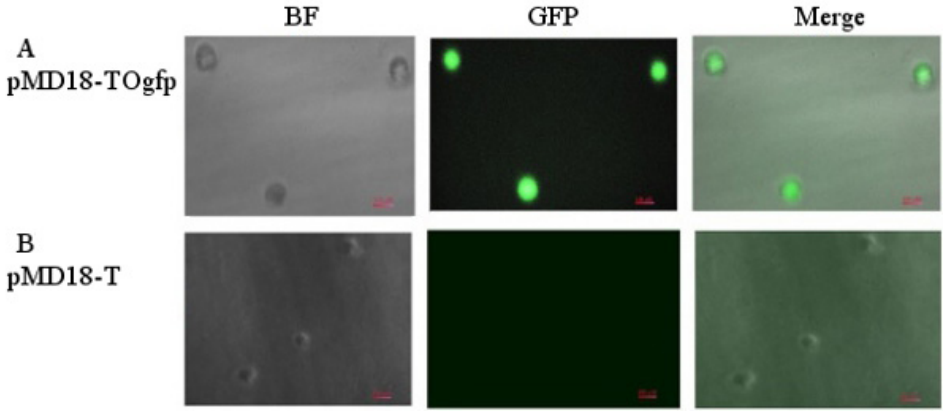

Figure 3. Detection of *GFP* fluorescence. Competent *Mycoplasma hyopneumoniae* cells were transformed with 15 µg of pMD18-TOgfp, and an additional 10 µg plasmid was added immediately after transformation. The cells were then incubated at 37ºC for 4 h, and then selected with 0.02 µg/mL tetracycline hydrochloride for 5-10 days. Subsequently, the cultures were grown on KM2-Agar for at least 14 days. **A.** The expression of *GFP* after transformation with the pMD18-TOgfp vector was observed under fluorescence microscopy. **B.** Control cultures were transformed with the pMD18-T empty vector. Scale bar = 100 µm.

DISCUSSION

M. hyopneumoniae is one of the most important species of mycoplasmas. *M. hyopneumoniae*-induced PEP negatively affects the pig industry worldwide. Although the mortality rate of the disease is low, the incidence has remained high. Once an outbreak is established, the control of the disease is difficult, and thus can result in significant economic losses (DeBey et al., 1992; DeBey and Ross, 1994).

The lack of development of tools for *M. hyopneumoniae* 168L genetic manipulation has been a major obstacle in studies into the pathogenesis of this organism for several years. The slow growth of this pathogen in liquid medium, difficultly of growth in solid medium, and its resistance to accept foreign genes by standard transformation methods have further complicated the development of genetic tools for functional genomics studies of *M. hyopneumoniae* 168L. With the exception of two reports by the British authors describing a replicative plasmid and transposon tool for *M. hyopneumoniae* strain 232 (Maglennon et al., 2013a; Maglennon et al., 2013b), little progress has been made in this area. Therefore, evidence supporting the use of *M. hyopneumoniae* as a host for heterologous gene expression is important as the complementation of bacterial mutants requires the expression of exogenous genes.

Recently, the entire genome of *M. hyopneumoniae* strain 168L was sequenced (Liu et al., 2011), which has provided a large amount of useful data. Herein, we aimed to investigate the expression of the *GFP* reporter in *M. hyopneumoniae* transformed with a vector based on replicative plasmids. The origin of replication has been used to develop oriC-plasmids in some mycoplasmas (Cordova et al., 2002; Chopra-Dewasthaly et al., 2005; Janis et al., 2005; Lee et al., 2008)., which in turn has been used to inactivate targeted genes (Cordova et al., 2002; Lee et al., 2008). The origin of replication in mycoplasma is generally located around the dnaA gene (Cordova et al., 2002). Following this, the *oriC* of *M. hyopneumoniae* was previously identified. Therefore, for the first time, we cloned this *oriC* into the pMD18-T vector along with a tetracycline resistance marker for selection and *GFP* for the first time to monitor heterologous gene expression. We used the *M. hyopneumoniae p97* gene promoter to heterologously express the *GFP* reporter gene in *M. hyopneumoniae*, and optimized methods for electro-transformation these mycoplasma. With this construct, *GFP* fluorescence was obtained, indicating that the expression of heterologous genes in *M. hyopneumoniae* is feasible.

In conclusion, we generated a general *GFP* reporter plasmid for *M. hyopneumoniae* based on a replicating plasmid that may be used as a tool to engineer the genome of *M. hyopneumoniae,* and thus can be used to better understand its pathogenesis.

Conflicts of interest

The authors declare no conflicts of interests.

ACKNOWLEDGMENTS

Research supported by the Postdoctoral Fund of Jiangsu Academy of Agricultural Science, China (#6511318) and the National Natural Sciences Foundation of China (#31370208 and #31550110211). H.Z.A. Ishag designed and carried out experiments, and drafted the manuscript. M.J. Liu assisted in the experimental design. Q.Y. Xiong, Z.X. Feng, R.S. Yang, and G.Q. Shao critically revised and approved the final manuscript.

REFERENCES

Bai F, Ni B, Liu M, Feng Z, et al. (2013). Mycoplasma hyopneumoniae-derived lipid-associated membrane proteins induce apoptosis in porcine alveolar macrophage via increasing nitric oxide production, oxidative stress, and caspase-3 activation. *Vet. Immunol. Immunopathol.* 155: 155-161. http://dx.doi.org/10.1016/j.vetimm.2013.07.004

Balish MF, Santurri RT, Ricci AM, Lee KK, et al. (2003). Localization of Mycoplasma pneumoniae cytadherence-associated protein HMW2 by fusion with green fluorescent protein: implications for attachment organelle structure.

Mol. Microbiol. 47: 49-60. http://dx.doi.org/10.1046/j.1365-2958.2003.03282.x

Chopra-Dewasthaly R, Marenda M, Rosengarten R, Jechlinger W, et al. (2005). Construction of the first shuttle vectors for gene cloning and homologous recombination in Mycoplasma agalactiae. *FEMS Microbiol. Lett.* 253: 89-94. http://dx.doi.org/10.1016/j.femsle.2005.09.021

Cordova CM, Lartigue C, Sirand-Pugnet P, Renaudin J, et al. (2002). Identification of the origin of replication of the Mycoplasma pulmonis chromosome and its use in oriC replicative plasmids. *J. Bacteriol.* 184: 5426-5435. http://dx.doi.org/10.1128/JB.184.19.5426-5435.2002

DeBey MC and Ross RF (1994). Ciliostasis and loss of cilia induced by Mycoplasma hyopneumoniae in porcine tracheal organ cultures. *Infect. Immun.* 62: 5312-5318.

DeBey MC, Jacobson CD and Ross RF (1992). Histochemical and morphologic changes of porcine airway epithelial cells in response to infection with Mycoplasma hyopneumoniae. *Am. J. Vet. Res.* 53: 1705-1710.

Janis C, Lartigue C, Frey J, Wróblewski H, et al. (2005). Versatile use of oriC plasmids for functional genomics of Mycoplasma capricolum subsp. capricolum. *Appl. Environ. Microbiol.* 71: 2888-2893. http://dx.doi.org/10.1128/AEM.71.6.2888-2893.2005

Kenri T, Seto S, Horino A, Sasaki Y, et al. (2004). Use of fluorescent-protein tagging to determine the subcellular localization of mycoplasma pneumoniae proteins encoded by the cytadherence regulatory locus. *J. Bacteriol.* 186: 6944-6955. http://dx.doi.org/10.1128/JB.186.20.6944-6955.2004

Lee SW, Browning GF and Markham PF (2008). Development of a replicable oriC plasmid for Mycoplasma gallisepticum and Mycoplasma imitans, and gene disruption through homologous recombination in M. gallisepticum. *Microbiology* 154: 2571-2580. http://dx.doi.org/10.1099/mic.0.2008/019208-0

Liu W, Feng Z, Fang L, Zhou Z, et al. (2011). Complete genome sequence of Mycoplasma hyopneumoniae strain 168. *J. Bacteriol.* 193: 1016-1017. http://dx.doi.org/10.1128/JB.01305-10

Maglennon GA, Cook BS, Deeney AS, Bossé JT, et al.; BRaDP1T consortium (2013a). Transposon mutagenesis in Mycoplasma hyopneumoniae using a novel mariner-based system for generating random mutations. *Vet. Res.* 44: 124. http://dx.doi.org/10.1186/1297-9716-44-124

Maglennon GA, Cook BS, Matthews D, Deeney AS, et al.; BRaDP1T consortium (2013b). Development of a self-replicating plasmid system for Mycoplasma hyopneumoniae. *Vet. Res.* 44: 63. http://dx.doi.org/10.1186/1297-9716-44-63

Zimmerman CU and Herrmann R (2005). Synthesis of a small, cysteine-rich, 29 amino acids long peptide in Mycoplasma pneumoniae. *FEMS Microbiol. Lett.* 253: 315-321. http://dx.doi.org/10.1016/j.femsle.2005.09.054