

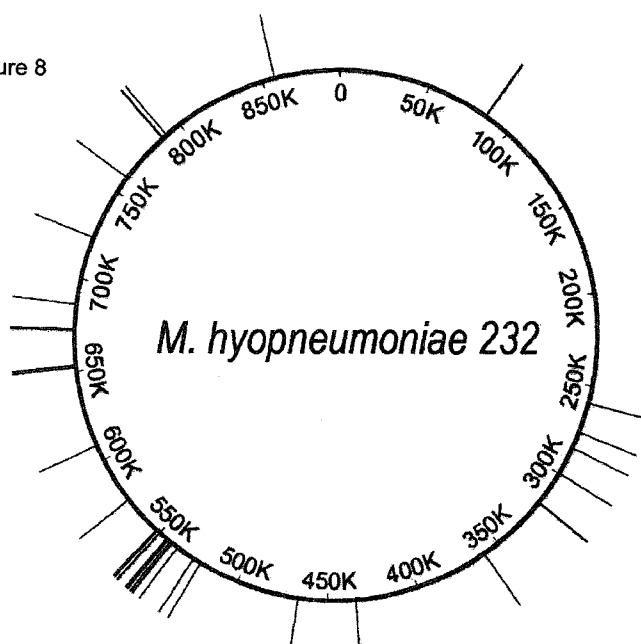


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[Continued on next page]

(54) Title: METHODS

Figure 8



(57) Abstract: A plasmid for transforming *Mycoplasma hyopneumoniae* comprising an *OriC* region comprising both AT-rich regions from a predicted *OriC* region of *M. hyopneumoniae* 232, or comprising a variant thereof that retains the ability to act as an *OriC* region in *M. hyopneumoniae* and has at least 90% sequence identity with a sequence comprising both AT-rich regions from a predicted *OriC* region of *M. hyopneumoniae* 232. A *M. hyopneumoniae* cell transformed with a plasmid of the invention may be useful in a vaccine composition. A nucleic acid construct comprising a nucleic acid sequence encoding a transposase enzyme and a promoter sequence, wherein the promoter sequence is active in *M. hyopneumoniae*; optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiral-in* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*; optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* *ldh*, *P97*, *secD*, *Tuf*, *rpoB*, *P146*, ATP transporter ATP binding protein, Asparagine-tRNA synthetase or Translation elongation factor gene. The transposase enzyme may be a Mariner family transposase, optionally Himar1 transposase or Himar1 C9 mutant transposase.



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Methods

The present invention relates to genetic manipulation of *Mycoplasma hyopneumoniae*.

5 *Mycoplasmas* are a genus of bacteria that are characterised by their lack of a cell wall and a small genome size, and are considered to be the smallest free-living self-replicating organisms. As such, they are of considerable interest in synthetic biology (Gibson et al.). They are responsible for a number of important diseases in humans and animals. *Mycoplasma hyopneumoniae* is the cause of enzootic pneumonia, a chronic
10 swine respiratory disease of global economic importance to pig producers [1]. Partial control of *M. hyopneumoniae* is achieved by a combination of management practices, antimicrobials and immunisation with commercially available vaccines [1]. Although current vaccines improve performance parameters and reduce the severity of lung lesions, there is a need for more effective vaccines, particularly in response to concerns
15 regarding the current overuse of antimicrobials in production animals. Progress in understanding the molecular basis of *M. hyopneumoniae* pathogenesis is essential to improved vaccine development, but has been severely hampered by the lack of tools to genetically manipulate the organism [2]. Advances have been made with other mycoplasmas using transposon mutagenesis to generate random insertion mutants that
20 can be screened for the expression of virulence factors using *in vivo* or *in vitro* model systems [3-9]. A few mycoplasmas have also been transformed with artificial plasmids that are capable of self-replication [10-14]. These plasmids contain the origin of replication (*oriC*) of the *Mycoplasma* along with an antimicrobial resistance gene. Following the transformation of mycoplasmas, transformants expressing the resistance
25 gene are selected for by addition of antimicrobial. *oriC* plasmids have proven valuable in demonstrating the amenability of *Mycoplasmas* to genetic manipulation and in some cases they have been useful in promoting the generation of mutations in target genes by homologous recombination [11, 12]. The ability to reproducibly transform *M. hyopneumoniae* has been a long anticipated, but until now, unachieved, development in
30 the study of this important organism.

Respiratory disease is a major problem facing swine producers and *Mycoplasma hyopneumoniae*, the cause of enzootic pneumonia (EP), is a swine-specific mycoplasma of global prevalence that is one of the leading causes of disease (Maes et al., 2008;
35 Thacker and Minion, 2012). EP is characterised by a chronic non-productive cough that is most evident during the growing and fattening stages of production, although all ages of animal may be affected (Simionatto et al., 2013). Mortality rates are usually low, but

morbidity may be high with associated economic losses due to increased medication costs, lower growth rates and lower feed conversion efficiencies (Thacker and Minion, 2012). The pathogenesis of EP involves entry of *M. hyopneumoniae* into the respiratory tract by inhalation, largely from nose-to-nose contact with other pigs (Thacker and Minion, 2012), and colonisation of the ciliated epithelial cells of the trachea, bronchi and bronchioles (Blanchard et al., 1992; Zielinski and Ross, 1993). Adherence of the organism to the epithelium causes ciliostasis and loss of cilia, thereby preventing effective clearance of debris, pathogens and mucus from the airways (DeBey and Ross, 1994). In addition, it is likely that *M. hyopneumoniae* causes direct damage to epithelial cells by production of cytotoxic metabolites such as hydrogen peroxide (Razin et al., 1998). Typically, a chronic inflammatory process ensues that is characterised histologically by hyperplasia of lymphoid follicles surrounding bronchioles and infiltration of mononuclear cells into peribronchiolar tissue and alveolar septae (Livingston et al., 1972; Rodriguez et al., 2004). The chronic nature of disease may result from modulation of the host immune response by *M. hyopneumoniae* (Rodriguez et al., 2004; Thacker and Minion, 2012) and possibly by variable expression of bacterial surface antigens, enabling the organism to evade effective clearance (Bogema et al., 2012). *M. hyopneumoniae* infection can be associated with and exacerbated by co-infection with other pathogens involved in the Porcine Respiratory Disease Complex (PRDC) such as Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Circovirus type 2 (Thacker and Minion, 2012). Additionally, concurrent infection may occur with upper respiratory tract bacteria such as *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Haemophilus parasuis* and *Pasteurella multocida* (Thacker and Minion, 2012). The impact of *M. hyopneumoniae* on pig herds is currently limited by a combination of management practices, anti-microbial medications and vaccination (Maes et al., 2008). Commercial vaccines are in widespread use in the pig industry where they are reported to lessen the economic effects of the disease by reducing clinical signs and lung lesions and by improving performance parameters (Maes et al., 1998; Maes et al., 1999; Maes et al., 2008). However, current vaccines are not completely effective and do not prevent colonisation of the respiratory tract with *M. hyopneumoniae* or eliminate infection from the herd (Thacker et al., 2000).

Central to the design of more effective vaccines is an understanding of the pathogenesis of EP. Despite the availability of whole genome sequences of several *M. hyopneumoniae* strains and the small genome size, the functions of most of the protein coding sequences are still unclear. The chronic nature of *M. hyopneumoniae* infection implies a complicated relationship between the pathogen and the host that is poorly

understood. The small genome size of *M. hyopneumoniae* offers an appealing opportunity to understand the molecular basis of disease formation, and to unravel the interplay between the host and the pathogen. Traditionally the exploration of gene function in a bacterium is investigated through the generation of random insertional mutants by transposon mutagenesis. Typically for other Mycoplasmas, the Tn4001 transposon derived from *Staphylococcus aureus* (Lyon et al., 1984) has been utilised (Cao et al., 1994; Chopra-Dewasthaly et al., 2005; Dybvig et al., 2000; Hedreyda et al., 1993; Pour-El et al., 2002). As reviewed by Halbedel and Stülke (Halbedel and Stulke, 2007), a mini Tn4001 transposon is used containing an antimicrobial resistance cassette flanked by inverted repeat sequences. The mini transposon and transposase enzyme are delivered into the organism by transformation and expression of the resistance cassette enables selection of mutated organisms. Insertions in essential genes result in lethality but many transformants have mutations in genes that are not necessary for growth *in vitro*, but may encode for particular pathogenicity determinants that are necessary for growth, survival, invasion or disease in the animal. Such mutants could be exploited in a number of different assays, including *in vivo* screening of mutants using the powerful technique of signature tagged mutagenesis (Mazurkiewicz et al., 2006), for example. Through the screening of large numbers of random mutants *in vivo*, mutants that are attenuated *in vivo* can be identified. Such mutants may encode for virulence factors and may be useful in the identification of live attenuated vaccine candidates, or in other vaccine approaches.

We describe the first successful transformation of *M. hyopneumoniae* strain 232 using an artificial self-replicating *oriC* plasmid system (see also Maglennon et al., 2013). We provide a set of useful transformation conditions, antimicrobial selection cassettes and promoter sequences for *M. hyopneumoniae*. We have developed a transposon mutagenesis system for *M. hyopneumoniae*. The system may make use of a Mariner family transposon, for example the Himar1 transposon belonging to the Mariner family of transposons. Insertion occurs at any TA dinucleotide (Lampe et al., 1996) offering an excellent potential for complete coverage of the AT-rich genome of *M. hyopneumoniae*. We describe the development of this system and its use in generating a large pool of several thousand *M. hyopneumoniae* mutants. This is a major advancement in the study of this important swine pathogen and potentially other Mycoplasmas.

We have generated an *oriC* based system for *M. hyopneumoniae* and have demonstrated susceptibility of the organism to transformation and demonstrated suitable

transformation conditions. The system may be used in, for example, generating non-virulent live vaccine candidates, which are considered to provide protection and to be distinguishable from infection (for example using current diagnostic techniques).

- 5 A first aspect of the invention provides a plasmid for transforming *Mycoplasma hyopneumoniae* comprising an *oriC* region comprising both AT-rich regions from the predicted *oriC* region of *M. hyopneumoniae* strain 232 [2], or comprising a variant thereof that retains the ability to act as an *oriC* region in *M. hyopneumoniae* and has at least 90% sequence identity, optionally at least 95% or 98% sequence identity with a
 10 sequence comprising both AT-rich regions from the predicted *oriC* region of *M. hyopneumoniae* 232.

Thus, the *oriC* region of the plasmid may comprise both AT-rich regions as identified in Figure 1A. These AT-rich regions are considered to flank the *DnaA* gene. The upstream
 15 AT-rich sequence is 208 bp in size and is considered to have the sequence
 TTTTCTCCTTTTTTTTTGCTTAAATTTAAGTTTTAGAAGTTATTTTAAGGTATTTTAAA
 AAAATATTGTTAAAATTTAAACCAAATCAACTTTATTAAATGTGCTAAATAAAGTTG
 ATAAAATGTTTGCAAAAACATTTTTGTTGTTTTAAACAAAACAAATTGATTTAAAAATT
 ATACTACAAAATTAAAGGAAAATTTATAAA. The down stream AT-rich sequence is 144
 20 bp in size and is considered to have the sequence
 AAATATTAAACAGCTTTTTGGGCAAAAAGTACTATAAATTAAAGTGGTTTTTTATCTTT
 TCTACATTTCTACACCCTTATTATTTTTATTATTAAACTAAATAAAAATTAATACTAAATA
 TAATATAATTATTAAAGGTAAAAAT. Thus the *oriC* region of the plasmid may comprise
 a sequence having at least 90, 95 or 98% sequence identity with the upstream AT-rich
 25 sequence indicated above, and/or having at least 90, 95 or 98% of the length of any
 such sequence (with any deletions preferably being at the ends of the sequence). The
oriC region of the plasmid may comprise a sequence having at least 90, 95 or 98%
 sequence identity with the downstream AT-rich sequence indicated above, and/or having
 at least 90, 95 or 98% of the length of any such sequence (with any deletions preferably
 30 being at the ends of the sequence). The *oriC* region of the plasmid may comprise a
 sequence of about 700bp comprising both AT-rich regions as identified in Figure 1A.
 The *oriC* region of the plasmid may comprise the *DnaA* gene from *M. hyopneumoniae* or
 a sequence having at least 70, 80, 90, 95 or 98% sequence identity with the *DnaA* gene.
 The inclusion of the *DnaA* gene sequence within the *oriC* region is considered to
 35 enhance the transforming efficiency of the plasmid.

"Percent (%) sequence identity" will be well known to those skilled in the art, and is defined as the percentage of nucleic acid residues in a candidate sequence that are identical with the nucleic acid residues of the sequence of interest, after aligning the sequences and introducing gaps, if necessary to achieve maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Methods for performing sequence alignment and determining sequence identity are known in the art, may be performed without undue experimentation, and calculations of % identity values may be obtained for example, using available computer programs such as WU-BLAST-2 (Altschul *et al.*, Methods in Enzymology 266:460-480 (1996). One may optionally perform the alignment using set default parameters in the computer software program (Blast search, MacVector and Vector NTI). Many other systems may be used instead. Typically DNASTar (Lasergene) may be used, though the other programs indicated are equally suitable.

The *oriC* region of the plasmid may comprise or consist of the following sequence, considered to be 2121bp in length and to comprise the DnaA gene and both of the AT rich regions

```

TTGTTAATTATTGCTTGAAATTCCCAATTTTTTTTAATACTACGAATTTTTTGCAAACT
ATCTCAACAAAAATTATTTATCAGAAACAGTTAGTCTTTTCCGACCTTTTGCGCGGCG
TGCAGCAAGAATTTTTCGCCCGTCGGCTGTTGACATTCGCGCGCGGAATCCGTGTG
TTTTTAAATGTTTTCTTTATTTGGTTGATAAGTTCGCTTCATTTTTTCTCCTTTTTTTT
TGCTTAAATTTAAGTTTTAGAAGTTATTTAAGGTATTTTAAAAAATATTGTTAAAT
TAAACCAAAATCAACTTTATTAATGTGCTAAATAAAGTTGATAAAATGTTTGCAAAA
ACATTTTTGTTGTTTTAAACAAAACAAATTGATTTAAAAATTATACTACAAAATTAAAG
GAAAATTTATAAAATGCAACAAATAAAAATAATTTAAAGGTTAGAACACAGCAAATT
AGGCAACAAATTGAAAATTTATTAATGATCGAATGTTGTATAACAACCTTTTTTAGCA
CAATTTATGTACTCAACGAGACAGAACTGAAATTATTATAGATTTTACAGACTTAAT
CGCGAAACAGGAAGTGATTTACGCTGAGTTGATACGGTTGAAAAAGCTATTAAAAA
TCTTGAAATTTCAAAAATCCTAACTTTTAACAATACAAATAATTATACCATTAATTCAA
AAGAAAGCCAAAACTTTTCCATAAAAAATAAATATTGCAGCTTTAATTAACAATGTT
TTAAACAAATTTACCTTTAGAAATTTTATAAAATCAAGTTATAATTTCCAAATTTTTAGT
ATTTATGACGCAATAGTCGCAAATTCAAGACTAAATTACTACCAATTTTTATTTTACG
GACCATCAGGAATTGGAAAAACGCATTTTATTAATGCGATTGGAAATTTACTTGTAGA
AAAACAGAAGAAAGTTTTCTACATTAACGACTATAAATTTATCAGTTGCGTTTCTTCC
TGGATGCAAAATGGTCAAAATGAAAAAATTAGTGAATTTTTTAACTGATTGTCTCAAG
TTGACGCTTTTCTTTTTGATGATATCCAAGGTTTGGCTAACAAACAACAACTTCAAT
TGTTGCACTTGAAATTTTAAATAGATTTATCGAAGAGGATAAAACAGTGATAATAACA

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TCTGATAAATCGCCTTCTTTACTTGGTGGATTTGAAGAAAGATTTATAACGCGATTTA
 GTTCAGGGTTGCACATTAAATTAACAAGCCGAAAAAGAAGACTTTTTGCGGATTT
 TTAAGCATAAATTAGTTGAAGAAAAATTAGAAAAACATATTTGAACAAATGATGCTTTT
 GAATTTTTGTCAAAGCATTTTCGAAATTCGATTCGTGAGCTTGAAGGTGCGCTAAAA
 5 TCAATTGTTTTTATATCCAAACAAATAAAAAATAAATTTGAGGATGAAATTTATTTTGA
 TAAGAAAAAATGTTTGAAATTTTTGTTGAAAAATATGAAATCGAACAAACAATCACC
 CCTGATTTAATCATTGAGGTTGTCTCAAAATATTATGGCGTCTCAATTTTAGATATAA
 AAAGTGAAAAAAGAGGCCAAAAATATTGTGCATGCCCGCGATATTGCAATCTGATTAA
 TAAAAATATTCTGGATTTAACTCATAATAGCGTAGGAACTTTTTTTAAACAACAGAAG
 10 ACATTCAACAATAATTTCTACCCTTAAAAAAATTGATACTTTAAACAAAGCAACAATA
 ATGAACTTGAAATTGCCCTTAACCATATTTATAACAATTAACTGAAGTTTTAAACA
 GCGAAAATAAAAAATATTAACAGCTTTTTGGGCAAAAAGTACTATAAATTAAAGTGGT
 TTTTATCTTTTCTACATTTCTACACCCTTATTATTTTTATTATTAATAAACTAAATAAAAA
 TTAATAAATATAATATAATTATTAAGGTAAAAATATGAAATTTACAATTGAAAAAAA
 15 AATTATTGAATGACAAATTGAAAGAATGCAATCAGCAATTCTTAATAATGTAAATTCA
 CCATTAAGTTCATTTTTTTTAAACTTACAAGAAGCGGATTATTTATAATTTCAACAAA
 TAGCGAACTTTCCTATAAAG. The *oriC* region of the plasmid may comprise or consist

of a variant thereof that retains the ability to act as an *oriC* region in *M. hyopneumoniae* and has at least 90, 95 or 98% sequence identity with said sequence. It is considered
 20 that other sequences considered (see the examples below) not to act as an *oriC* region have a lower % sequence identity than indicated above.

The ability of a variant sequence to act as an *oriC* region may be assessed by, for example, replacing the sequence known to act as an *oriC* region in *M. hyopneumoniae*
 25 with the variant sequence and determining its ability to act as an *oriC* region, for example by assessing whether transformed colonies are obtained on transforming *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) with the plasmid containing the variant *oriC* region, for example under the same conditions as transformed colonies are obtained with a plasmid containing the sequence known to act as an *oriC* region.
 30 Preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the number of transformed colonies obtained with the plasmid containing the known *oriC* region are obtained with the plasmid containing the variant *oriC* region. Transformed colonies may be identified using a marker such as tetracycline resistance (for example as described in the Examples), as will be well known to those skilled in the art.

35 The plasmid may further comprise a marker gene. The marker gene may comprise a promoter derived from *M. hyopneumoniae* or the *spiralin* gene promoter of *Spiroplasma*

citri[15], or the promoter from the *tetM* (tetracycline resistance) gene, for example from *Staphylococcus aureus*. The promoter may be the P97 gene (1B) or *ldh* gene (1C) promoter, for example as shown in Figure 1 or indicated below; or a variant thereof that is capable of acting as a promoter in *M. hyopneumoniae*, for example *M.*

5 *hyopneumoniae* 232 and has at least 90% sequence identity with the relevant promoter sequence, for example as shown in Figure 1 or indicated below. The promoter may be a promoter comprising the motif TATAAT up to 200bp upstream of the native gene start codon. The promoter may be a promoter comprising the motif TAAAAT up to 200bp upstream of the native gene start codon.

10

It is considered that promoter regions typically (but not always) have the motif TATAAT or TAAAAT within approximately 200 bp upstream of the start codon. This has been described in more detail by Weber Sde et al 2012 in DNA Research, for example.

15 Suitable marker sequences will be well known to those skilled in the art. As noted above, the *tetM* resistance gene (conferring resistance to tetracycline) may be used, as may the *pac* (puromycin-N-acetyl-transferase) sequence (conferring resistance to puromycin). We have shown that both of these genes can be expressed in the *oriC* system to levels sufficient to confer resistance to respective antimicrobials in transformed

20 organisms. The *pac* gene has, for example, been utilised in the pMiniHimar1BSC1 transposon mutagenesis system described in Example 6. The *teM* gene is present in the pMHC9-1 transposon construct described in Example 3.

Examples of additional suitable antimicrobial resistance cassettes include the *cat*

25 (chloramphenicol acetyltransferase) sequence (conferring resistance to chloramphenicol) and the *aacA-aphD* determinant (conferring resistance to aminoglycosides such as gentamicin). Both of these resistance genes have been shown to be functional in conferring resistance to respective antimicrobials in other *Mycoplasma spp.*

30

The P97 gene promoter sequence is considered to be:

ACGGGGATTATAAACAGAACTCCAAAATTGACTATGCTTTTATTAAAAAAAACAG
AAAGAACTCTCACTTTTACCTATTTTTTTTAGGATTATTTTTGGATTTTTACTTTTAATT
35 ATTAGTCTTCCTTTTTTAATTCAAATTAATAAAAAAATATAGAAGAAAATTCCTAGT
AAAGTTCTAAAATTATTAACAGCATTAAAACTGCTGTTTTTTAATAATCCTA
ATTTAATATTGGATTATTTATTAAATTGTTCAAATTTTTTATACTAAATAAATTATA

AAAGAAGACTGATTAGAAATTTAGAACTATTCAAATCTTCAAAAAAGTGGCCTAAAA
 ACAATGATAAAAAAATTTCCAACTTTTTTTGTTGCAAAAAAAAAAAAAAAAAAGTATAAT
 TTTAATTGTACAAGTTAAATAAATTTTCACTTATCTTTTTTTATTTTGCAAACTTTTA
 AAAAAATTAGATATCTAAATTATATTATATGATTGAGAAAATGAAAAATTTATTTCTTA
 5 GAACCAAGATAATTGAGGTTTAAATATGAGTAAAAAATCAAAAACATTTAAATTGG
 TTTGACTGCCGGAATTGTTGG

The lactate dehydrogenase gene promoter sequence is considered to be:

10

ATAGAATTTTGCAATTAAAGCAAGCATCATTGTCATTAAGAAGGAAAGAGTAATGAAA
 ACATAAATTACTTTTTTTTTTAAACTTAACTTGAATAGAAATCTAAAATATTGATTAGAT
 CTAAAAATATTTGTCCTGACTTGTAACAAAGTCAACCTCTTCTACTTATCTAATATTGT
 TGTATAATATTAGATAATAATTTGTAAAATTTTAAATTTTTACTAAATTAAAAAGCGG
 15 AAAACCACTTTTAAAAAAATAAAATTAATATCTAAAAGTGAAATTATAACATAAATTAT
 CAAAAAGTTACTGTTTTTTATTTTTAAGGAAAAAATTTATGATAATTGGCTTTATCGGG
 ATAACAAATACGAGTATAAATTTAATTTCTAACCTAATTTTGACAAGAAATGAGGTGG
 AAT

20

The *tetM* (tetracycline resistance) gene promoter sequence from *Staphylococcus aureus* is considered to be or comprise the following sequence:

CTAGTCATGTGATTCTAAAGTATCCGGAGAATATCTGTATGCTTTGTATGCCTATGGT
 25 TATGCATAAAAATCCCAGTGATAAGAGTATTTATCACTGGGATTTTTATGCCCTTTTG
 GGCTTTTGA

We have verified that the lactate dehydrogenase gene and P97 ciliary adhesin gene
 30 promoter sequences from *M. hyopneumoniae* are functional in driving the expression of
tetM in *M. hyopneumoniae*. See, for example, Example 1. We have also shown that the
 spiralin gene (from *Spiroplasma citri*) promoter sequence is active in driving *tetM*
 expression and *pac* expression in *M. hyopneumoniae* using the same system.
 Additionally, we have confirmed that the native promoter sequence of *tetM* (as derived
 35 from *S. aureus*, for example) is active in driving *tetM* expression in *M. hyopneumoniae*.
 In Example 3, we show that the P97 promoter sequence is a suitable promoter for
 controlling expression of the Himar1 transposase enzyme.

We consider that other predicted *M. hyopneumoniae* promoter sequences, for example promoter sequences that are constitutively active (or variants thereof), could alternatively be used to drive expression of a marker gene or other gene, for example a transposase gene, for example the Himar1 transposase gene, as discussed further below. Indicated below are seven further potential promoter sequences from the *M. hyopneumoniae* strain 232 genome. These were selected based on the following criteria: 1) known function of the open-reading frame; 2) identification of a TATA binding site; 3) arrangement of genes such that there is a high likelihood that a promoter must be within the selected sequence. The sequences are shown 5'-3' and the co-ordinates of the sequence within the *M. hyopneumoniae* strain 232 genome are shown. As noted above, the promoter sequence may be, for example, a promoter sequence as shown below or a variant thereof that is capable of acting as a promoter in *M. hyopneumoniae*, for example *M. hyopneumoniae* 232 and has at least 90% sequence identity with the relevant promoter sequence, for example as shown in Figure 1 or indicated below.

secD (167635-167934)

GCAAAAAAGTACCTAATTTTCATGCTTTTCCTTTCTCGAGTTTACAATTATATCATATT
TTAATAATATGAATAAGTATTTTTATGGTTTTTTTGACGAAAAGGTTAGAAGAAAGCTA
ATTAACAAAAATATTATCAAATTTTTCAAATTTTCAAGCTTTTCTTATAAATTTTTTAA
TATAAATTAGTTAGAATTTTAATTAGTGAAAAAAATTATGAAATCCGAAAAACTTATTA
AAAAATTCCTTCCATATTTCTTTAAAGTTTGACCATTTTTTGGATGTTTTTAAAAAATA
ATTG

25

Tuf (elongation factor) (682581-682880)

TCCTGTCGTTTTAACTGCCATATTTTTTCCTTTGCTTATCATTTATTATATCGTA
ATTATATTATATTATTGTTTTAATAAAACCTTGCAAAAAACCGCTGAATAGCCTTG
CAACTATTTCTATCCTTTTTTTGCAGGTGCAATTCTAAAAAAATAAACATTTTCAAA
AATAATTTTTAAATGATTCTTTTTATAATTATACCAAATTTGAAAAATTATTTTAAGA
ATTTTTTTAACAAAAAAATAATTTAGAAATTTTACTTAAAAACCGCGGCTTTTAACTG
ATTT

35 rpoB (801604-801903)

TTTTAATTTATATATGTGATTCATTTTTCCCTTCTTACCATTTTTTCAATAAGTTTTTAA
ATTATATTAGAAACGTAAAAAAATAAAATGTTTTTATAATTTAATCCAAAAAAATTAA

GAAAAATTTTATTGGGATATTTTTTATAGAAAAATAAAAAATAAACAAAAACGGTGT
TTAAACACCTTTTTGAATTTTAATTAAGAACTAATTTTTCTAAGAAAAATTAGTCAA
TCGAAACTTCTGCTCCCGCTGCGACTAATTTTGCTTTATATTCTTCAGCTTCTTCAGG
TTTT

5

P146 (864811-865110)

TATCCTTTCGAAGATATAATATTTTTACCTCGCCGCCTTAGCAGCAGCAGCTGCTGC
CGGAGCACTAGAAGAAGATCCTGAATTTTGGCTTTCATGTTTGGCATTTCATCGC
TTGCCTACTTGTTTGCTGATTTGAGGCCTCTGCTCCTTGATAATCATCTTTTATAAGT
10 CTAACCTTGAAGAAAAATGCTGACTGGGGAACCTTGGCCTTGGGAACCTTGTTTCTTTT
TGTTCCCTTTTCTTTTCTTTTCTTTCTCTTTTCTTTTGTTCCTGCTGCTGTTCTTCT
TTTCTAACCAC

15 ATP transporter ATP binding protein (207459-207758)

GATCTAATTGATTTAATTCGTGAACCTTCAACAAGAATAAGAAGTTAAAAACAAGTG
GAACTGGCAAGAGTAAAGCTAAAATTTTTCTAAATTTTTTTTCAACATTTTGATTTTT
AATTAAAAAGAGCGATATTTTTTAACTTCACTTTCTTTTTTGATAAATTAATAAGAG
ACAATAAATAAATGGAAAAAGATGAAAATTTAACTAGTTATTTTTCAATTTTATTGTA
20 TAATATTATACCATAAAAAATTAGAATTAAGGAAAATAAAATGCTAGAAGTTAGAGA
ATTATCG

Asparagine-tRNA synthetase (493214-593513)

AATTCGTTAATAGTTTGGAACATATTTTCACCCCATATTATTATAAAAACTCTTTTAT
25 TATATCATAAAATTTAGGTTTTTTTACTATAATTAAAATAAAAAATAAGTTAAAATTAAAA
CATTTTCTATCAAAAATACAAGAATTATAAAATTTTCTAAATTTTTTAAATTTAATCTTA
TGTTCTGTAATAAATTTGTTATTTCTAATAAAAAAATTTAAATTTTTAGAAAATTAACT
TTTTTTGTTGTAAATAATTTTTTTATCCTTTTTATAGCCTTAACATAGATAACTAAAAA
A

30

Translation elongation factor (507412-507711)

TCGAAATTCATTAACATTAATCATCCCGCCTCCACTTGACAATAAACAAAGTAACTAT
AAATTATAGCATAAAAAAAAAAAAAAAAAAATCTAAAATGAAATTATTGAATATTTTTA
AAAACAAGCTTTTAATTCAGAAAAGGATAACATAAAATTTTAAATATTATTAATAAATT
35 GTGATATAATTTATAAGGTTAAAGTTTCTTTGAAACTTTTGGTTTAAGTTTTGAGTTTT
GATTTACTAAATTTGAATTGTAAATGTAAATAAGTAAGCAAAAACCTACGTCGACCTT
TTAAGTG

The ability of a variant sequence to act as promoter in *M. hyopneumoniae* may be assessed by, for example, replacing the sequence known to act as a promoter in *M. hyopneumoniae* with the variant sequence and determining its ability to act as a promoter, for example by assessing whether marked colonies are obtained on transforming *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) with the plasmid containing the variant promoter controlling a marker (for example tetracycline resistance), for example under the same conditions as marked colonies are obtained with a plasmid containing the sequence known to act as an promoter controlling the same marker. Preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the number of marked colonies obtained with the plasmid containing the known promoter are obtained with the plasmid containing the variant promoter.

The method may similarly be performed using a transposon-based system as described herein, though it may most conveniently be performed using the plasmid based system as indicated above.

The plasmid may, for example, comprise an epitope-tagged sequence under the control of a promoter sequence wherein the promoter sequence is active in *M. hyopneumoniae*, (or putative promoter sequence, as appropriate), where the epitope is optionally GPF, optionally hemagglutinin tag, his6 tag or other epitope which will be well known to those skilled in the art.

The plasmid may further (compared with any of the previous embodiments) comprise a sequence that can undergo recombination with the homologous DNA in the bacterial chromosome, thus causing disruption of the homologous gene at a specific site (site-directed mutagenesis). Thus, the plasmid may further comprise a further sequence that has at least 80, 90, 95 or 98% identity over a sequence length of at least 100, more typically at least 300, 400 or 500 nucleotides, for example between 300, 400 or 500 and 3000, 2000 or 1500 nucleotides, for example between 500 and 1500 nucleotides, with a sequence of *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) chromosomal nucleic acid.

The site-directed mutagenesis may be, for example, the addition of an in-frame epitope tag sequence for expression of an endogenously expressed native tagged protein.

A further aspect of the invention provides a method of transforming *M. hyopneumoniae*, the method comprising the step of exposing a *M. hyopneumoniae* cell to a plasmid according to the first aspect of the invention. The method may further comprise the step of performing electroporation on the cell in the presence of the plasmid according to the first aspect of the invention. The method may further comprise the step of culturing the exposed (and optionally electroporated) cell under conditions where transformed cells have a selective growth advantage or otherwise can be identified.

A further aspect of the invention provides an *M. hyopneumoniae* cell transformed with a plasmid according to the first aspect of the invention. The plasmid may remain as a separate plasmid within the cell or may be integrated or partially integrated into the cell's genomic DNA.

A further aspect of the invention provides a vaccine composition comprising a transformed *M. hyopneumoniae* cell of the invention. The *oriC* based plasmid is considered to be able to, for example, introduce DNA into *M. hyopneumoniae* that could then undergo recombination with the homologous DNA in the bacterial chromosome, thus causing disruption of the homologous gene at a specific site (site-directed mutagenesis). See, for example, Examples 2 and 5. Such a mutant could be useful as, for example, a live, attenuated vaccine for protection of pigs against porcine enzootic pneumonia. The mutated/disrupted gene may be, for example, the gene for P46, P97 or P146 adhesion proteins, involved in cell adhesion. Other suitable genes may be identified making use of the transformation techniques of the present invention, which may be used, for example, in identifying sequences involved in virulence of this pathogen. For example, suitable genes may be identified through formation of and analysis of mutant cells, for example as described further below, for example in Examples 2 to 5. The transformed *M. hyopneumoniae* of the invention, for example in a vaccine composition of the invention, may comprise a gene, for example the gene for P46, P97 or P146 adhesion proteins, or a gene identified as involved in virulence of *M. hyopneumoniae*, («virulence factor») that has undergone site-directed mutagenesis as a consequence of transformation with a plasmid of the invention. The transformed *M. hyopneumoniae* of the invention, for example in a vaccine composition of the invention may be a live-attenuated cell e.g. a cell that has reduced ability to replicate and is not capable of causing disease in a normal (not immunocompromised) animal.

35

The skilled person would readily be able to determine that a cell is a *M. hyopneumoniae* cell, for example a *M. hyopneumoniae* 232 cell. For example *M. hyopneumoniae* can be recognised making use of PCR-based techniques [16-18]. *M. hyopneumoniae* 232 can be recognised making use of whole genome DNA sequencing techniques [2]. Examples of techniques for identifying *M. hyopneumoniae* are also indicated in the Examples, for example Example 6.

A further aspect of the invention provides a plasmid, transformed *M. hyopneumoniae* cell or vaccine composition of the invention for use in medicine. A further aspect of the invention provides a plasmid, transformed *M. hyopneumoniae* cell or vaccine composition of the invention for use in aiding in treating or preventing infection by *M. hyopneumoniae* or disease caused by infection by *M. hyopneumoniae*. A further aspect of the invention provides a method for aiding in treating or preventing infection by *M. hyopneumoniae* or disease caused by infection by *M. hyopneumoniae*, the method comprising administering to a subject a transformed *M. hyopneumoniae* cell (typically a live, attenuated *M. hyopneumoniae* organism) or vaccine composition (typically comprising a live, attenuated *M. hyopneumoniae* organism) of the invention. Diseases and conditions linked to *M. hyopneumoniae* will be well known to those skilled in the art and some examples are indicated above.

Typically, the vaccine would be used on the farm. It would typically be given by the respiratory route (intranasal or as a spray or aerosol) to piglets or to sows before farrowing. It is also possible that the vaccine could be given by a non-respiratory route such as intramuscular or subcutaneous injection.

The vaccine may be formulated or administered alongside other agents intended as vaccines (for example as part of a combination vaccine), as will be well known to those skilled in the art. The vaccine may be formulated or administered with other pharmaceutically acceptable components, which may be considered to be adjuvant components, as will also be well known to those skilled in the art.

A further aspect of the invention comprises a method for identifying a sequence capable of acting as an *oriC* region in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence to act as an *oriC* region with the ability of a sequence comprising both AT-rich regions from the predicted *oriC* region of *M. hyopneumoniae* 232 (for example as set out above) to act as an *oriC* region in *M. hyopneumoniae*. A sequence may be identified as capable of acting as an *oriC* region if

transformed colonies are obtained on transforming *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) with the plasmid containing the candidate *oriC* region, for example under the same conditions as transformed colonies are obtained with a corresponding plasmid containing the sequence known to act as an *oriC* region. Preferably at least
5 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the number of transformed colonies obtained with the plasmid containing the known *oriC* region are obtained with the plasmid containing the candidate *oriC* region.

A further aspect of the invention comprises a method for identifying a sequence capable
10 of acting as a promoter in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence to act as a promoter with the ability of the *spiralin* gene promoter of *Spiroplasma citri*, promoter of the *M. hyopneumoniae* P97 gene or *M. hyopneumoniae* *ldh* gene, (or other promoter sequence active as a promoter in *M. hyopneumoniae*, for example a sequence indicated above as active as a promoter
15 in *M. hyopneumoniae*) to act as a promoter in *M. hyopneumoniae*. A sequence may be identified as capable of acting as a promoter if marked colonies are obtained on transforming *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) with the plasmid (typically a plasmid comprising an *oriC* region as set out above) containing the candidate promoter controlling a marker (for example tetracycline resistance), for example under
20 the same conditions as marked colonies are obtained with a corresponding plasmid containing the sequence known to act as an promoter controlling the same marker (for example a promoter and/or marker indicated above). Preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the number of marked colonies obtained with the plasmid containing the known promoter are obtained with the plasmid containing
25 the candidate promoter.

A further aspect of the invention comprises a method for identifying a sequence capable of acting as a selection marker in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence (for example with at least 90% sequence
30 identity to a sequence known to confer resistance to a compound (challenge compound) in another organism), under the control of a promoter known to work in *M. hyopneumoniae*, to confer resistance to a challenge compound, with the ability of a construct not containing the candidate sequence to confer resistance to the challenge compound. A sequence may be identified as capable of acting as a marker if marked
35 colonies are obtained on titrations of the challenge compound upon transforming *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) with the plasmid (typically a plasmid comprising an *oriC* region as set out above) containing the candidate marker

under the control of a promoter (for example the *spiralin* gene promoter of *Spiroplasma citri*), for example under the same conditions as no marked colonies are obtained with a corresponding plasmid lacking the candidate marker sequence; or under similar conditions (which may be the same conditions, or may be conditions adapted for a different marker, for example using a different antibiotic) in which marked colonies are obtained with a known marker sequence. Preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the number of marked colonies obtained with the plasmid containing a known marker sequence are obtained with the plasmid containing the candidate marker sequence.

A further aspect of the invention provides a nucleic acid construct comprising a nucleic acid sequence encoding a transposase enzyme and a promoter sequence, wherein the promoter sequence is active in *M. hyopneumoniae*;

optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*;

optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* *ldh*, *P97*, *secD*, *Tuf*, *rpoB*, *P146*, *ATP transporter* *ATP binding protein*, *Asparagine-tRNA synthetase* or *Translation elongation factor* gene. Such promoter sequences are indicated above. As also indicated above, the promoter sequence may be a variant thereof that is capable of acting as a promoter in *M. hyopneumoniae*, for example *M. hyopneumoniae* 232, and has at least 90% sequence identity with the relevant promoter sequence, for example as shown in Figure 1 or indicated above.

Typically the transposase-encoding sequence is under the control of the promoter sequence, as will be well known to those skilled in the art.

The term "transposase" and examples of transposases will also be well known to those skilled in the art. See, for example, discussion in Example 6, section 1.5.2 and references cited therein. DNA transposons (class II transposons) consist of a transposase gene and terminal inverted repeats (TIRs). They have been grouped into families based on their transposase sequence and inverted repeat regions (Munoz-Lopez and Garcia-Perez, 2010). The families include Superfamily Tc1/*mariner*, PIF/*Harbinger*, hAT, Mutator, Merlin, Transib, P, *piggyBac* and CACTA. Active transposases of the TC1/*mariner* family include *Mos1* and *Himar1* (Lampe et al (1999)). Others indicated in Munoz-Lopez and Garcia_Perez, 2010 include Tc1, Tc3, *Minos*,

Famar1, *Osmar5*, *Fot1*, *Impala*, *ISY100*, *Mboumar-9*, *Sleeping Beauty*, *Frog Prince* and *Hsmar1*. See also, for example, PLoS One. 2013 Sep 11;8(9):e73227. doi: 10.1371/journal.pone.0073227. Functional characterization of the human mariner transposon hsmar2. Gil E, Bosch A, Lampe D, Lizcano JM, Perales JC, Danos O, Chillon M., which reports a further active Tc1/*mariner* family transposase.

Typically the transposase enzyme is a Mariner family transposase, optionally Himar1 transposase or Himar1 C9 mutant transposase. Such a transposase is considered to be particularly useful in relation to *Mycoplasma*, for example *M. hyopneumoniae*, in view of its AT-rich genome and the insertion site of Tc1/*mariner* transposases, for example Himar 1 transposase or the C9 mutant, being at a TA dinucleotide. The C9 transposase is a hyperactive mutant of Himar1 with two amino acid substitutions, Q131R and E137K, that are considered to result in an increase in activity relative to wild type. See also, for example US6,368,830. Other mutations of the Himar1 transposase or Himar1 C9 mutant transposase may also be possible whilst retaining appropriate transposase activity. Thus, it will be appreciated that variants are also included, for example with at least 90% amino acid sequence identity with the amino acid sequence of Himar1 or Himar1 C9 mutant and typically at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the mutant-forming activity of Himar1 or Himar1 C9 mutant, for example as judged by comparison of the number of independent transformed colonies (for example of *M. hyopneumoniae*) recovered with the Himar1 or Himar 1 C9 mutant sequence and with the mutated Himar1 or Himar 1 C9 sequence, for example as set out in the Examples.

The coding sequence of the Himar1 gene is;

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ATGGAAAAAAGGAATTCGTGTTTTGATAAAATACTGTTTTCTGAAGGGAAAAATA
CAGTGAAGCAAAACTTGGCTTGATAATGAGTTTCCGGACTCTGCCCCAGGGAAA
TCAACAATAATTGATTGGTATGCAAAATTCAAGCGTGGTGAAATGAGCACGGAGGAC
GGTGAACGCAGTGGACGCCCGAAAGAGGTGGTTACCGACGAAACATCAAAAAAAT
CCACAAAATGATTTTGAATGACCGTAAAATGAAGTTGATCGAGATAGCAGAGGCCTT
AAAGATATCAAAGGAACGTGTTGGTCATATCATTCATCAATATTTGGATATGCGGAA
GCTCTGTGCAAAATGGGTGCCGCGCAGCTCACATTTGACCAAAAACAACACGTG
TTGATGATTCTGAGCGGTGTTTGCAGCTGTAACTCGTAATACACCCGAGTTTTTCC
GTCGATATGTGACAATGGATGAAACATGGCTCCATCACTACACTCCTGAGTCCAATC

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GACAGTCGGCTGAGTGGACAGCGACCGGTGAACCGTCTCCGAAGCGTGGAAGAC
 TCAAAAGTCCGCTGGCAAAGTAATGGCCTCTGTTTTTTGGGATGCGCATGGAATAAT
 TTTTATCGATTATCTTGAGAAGGGAAAAACCATCAACAGTGACTATTATATGGCGTTA
 TTGGAGCGTTTGAAGGTGCGAAATCGCGGCAAACGGCCCCACATGAAGAAGAAAAA
 5 AGTGTTGTTCCACCAAGACAACGCACCGTGCCACAAGTCATTGAGAACGATGGCAA
 AAATTCATGAATTGGGCTTCGAATTGCTTCCCCACCCGCCGTATTCTCCAGATCTGG
 CCCCCAGCGACTTTTTCTTGTTCTCAGACCTCAAAGGATGCTCGCAGGGAAAAAAT
 TTGGCTGCAATGAAGAGGTGATCGCCGAAACTGAGGCCTATTTTGAGGCAAAACCG
 AAGGAGTACTACCAAATGGTATCAAAAAATTGGAAGGTCGTTATAATCGTTGTATC
 10 GCTCTTGAAGGGAACATATGTTGAATAA

The amino acid sequence of the Himar1 transposase is considered to be;

15 MEKKEFRVLIKYCFLKGKNTVEAKTWLDNEFPDSAPGKSTIIDWYAKFKRGEMSTEDGE
 RSGRPKEVVTDENIKKIHKMILNDRKMKLIEIAEALKISKERVGHIIHQYLDMRKLCAKWW
 PRELTFDQKQQRVDDSERCLQLLTRNTPEFFRRYVTMDETWLHHYTPESNRQSAEWT
 ATGEPSPKRGKTQKSAGKVMASVFWDAHGIIFIDYLEKGKTINSDYIMALLERLKVEIAA
 KRPHMKKKKVLHFHQDNAPCHKSLRTMAKIHGELGFELLPHPPYSPDLAPSDFFLFSDLKR
 20 MLAGKKFGCNEEVIAETEAYFEAKPKEYYQNGIKKLEGRYNRCIALEGNYVE

The coding sequence of the Himar1 C9 mutant gene is considered to be;

25 ATGGAAAAAAGGAATTTCTGTGTTTTGATAAAATACTGTTTTCTGAAGGGAAAAAATA
 CAGTGGAAGCAAAAACCTTGGCTTGATAATGAGTTTCCGGACTCTGCCCCAGGGAAA
 TCAACAATAATTGATTGGTATGCAAAATTCAAGCGTGGTGAAATGAGCACGGAGGAC
 GGTGAACGCAGTGGACGCCCCGAAAGAGGTGGTTACCGACGAAAACATCAAAAAAAT
 CCACAAAATGATTTTGAATGACCGTAAAATGAAGTTGATCGAGATAGCAGAGGCCTT
 30 AAAGATATCAAAGGAACGTGTTGGTCATATCATTCATCAATATTTGGATATGCGGAA
 GCTCTGTGCGAAATGGGTGCCGCGCGAGCTCACATTTGACCAAAAACAACGACGTG
 TTGATGATTCTAAGCGGTGTTTGCAGCTGTAACTCGTAATACACCCGAGTTTTTCC
 GTCGATATGTGACAATGGATGAAACATGGCTCCATCACTACACTCCTGAGTCCAATC
 GACAGTCGGCTGAGTGGACAGCGACCGGTGAACCGTCTCCGAAGCGTGGAAGAC
 35 TCAAAAGTCCGCTGGCAAAGTAATGGCCTCTGTTTTTTGGGATGCGCATGGAATAAT
 TTTTATCGATTATCTTGAGAAGGGAAAAACCATCAACAGTGACTATTATATGGCGTTA
 TTGGAGCGTTTGAAGGTGCGAAATCGCGGCAAACGGCCCCACATGAAGAAGAAAAA

AGTGTGTTCCACCAAGACAACGCACCGTGCCACAAGTCATTGAGAACGATGGCAA
 AAATTCATGAATTGGGCTTCGAATTGCTTCCCCACCCGCCGTATTCTCCAGATCTGG
 CCCCCAGCGACTTTTTCTTGTCTCAGACATCAAAAGGATGCTCGCAGGGAAAAAAT
 TTGGCTGCAATGAAGAGGTGATCGCCGAACTGAGGCCTATTTTGAGGCAAAACCG
 5 AAGGAGTACTACCAAATGGTATCAAAAAATTGGAAGGTCGTTATAATCGTTGTATC
 GCTCTTGAAGGGAACCTATGTTGAATAA

10 The amino acid sequence of the Himar1 C9 mutant transposase is considered to be;

MEKKEFRVLIKYCFLKGKNTVEAKTWLDNEFPDSAPGKSTIIDWYAKFKRGEMSTEDGE
 RSGRPKEVVTDENIKKIHKMILNDRKMKLIEIAEALKISKERVGHIIHQYLDMRKLCAKWW
 PRELTFDQKQRRVDDSKRCLQLLTRNTPEFFRRYVTMDETWLHHYTPESNRQSAEWT
 15 ATGEPSPKRGKTQKSAGKVMASVFWDAHGIIFIDYLEKGKTINSDYIMALLERLKVEIAA
 KRPHMKKKKVLHFHQDNAPCHKSLRTMAKIHGELGFELLPHPPYSPDLAPSDFFLFSDIKR
 MLAGKKFGCNEEVIAETEAYFEAKPKEYYQNGIKKLEGRYNRCIALEGNYVE

Sites of the two amino acid substitutions are underlined.

20

As noted in Munoz-Lopez and Garcia-Perez, 2010, transposase proteins from different
 Tc1/*mariner* elements are not very similar in sequence, but they have two characteristic
 domains: an amino-terminal region containing the helix-turn-helix (HTH) motif necessary
 25 for recognition and binding of TIRs, and a carboxy-terminal domain harbouring the
 catalytic motif formed by three amino acids DDD (*mariner* family) or DDE (Tc1-like
 family). The first and second D residues are typically separated by 92 amino acids,
 whereas the separation of the second and third residues is variable, between 31 and 39
 amino acids in the different families forming the superfamily Tc1/*mariner*. Other features
 30 include a nuclear localisation signal (NLS) and a WVPHEL linker motif, which may
 participate in the interaction between transposase monomers. Typically the transposase
 will have one or more or all such features.

It is considered that the Hsmar1 transposase (reported in Claeys et al 2010, Nuc Acids
 35 Res and also a Mariner family transposase) may also be suitable.

It is considered that the Tn4001 transposase (which is a member of the Mutator family) may not be functional in *M. hyopneumoniae*. Thus, the transposase may be a transposase other than a Mutator family transposase; or other than the Tn4001 transposase. The sequence of the Tn4001 transposase may be found at <http://www.uniprot.org/uniprot/P19775>, for example. It is considered that the transposase Tn916 is functional in *Mycoplasma* species. However the large size and tendency to insert into hotspots in the genome make Tn916 less preferred in relation to the current invention, as it may be difficult to achieve even coverage or saturation of the genome with mutant. Thus the transposase may be a transposase other than the Tn916 transposase.

The nucleic acid construct (which may typically be circular but which may be, for example, linear) may further comprise other transposon/mini transposon components, for example may comprise (two) inverted repeats and optionally a marker gene, optionally comprising a promoter sequence active in *M. hyopneumoniae*, optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*, as discussed above; optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* *ldh*, *P97*, *secD*, *Tuf*, *rpoB*, *P146*, ATP transporter ATP binding protein, Asparagine-tRNA synthetase or Translation elongation factor gene (as discussed above, including variants).

Alternatively or in addition, a further nucleic acid construct may be used alongside the nucleic acid construct comprising a sequence encoding a transposase. The further nucleic acid construct (transposon delivery vector) may comprise transposon/mini transposon components, for example may comprise (two) inverted repeats and optionally a marker gene, optionally comprising a promoter sequence active in *M. hyopneumoniae*, optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*; optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* *ldh*, *P97*, *secD*, *Tuf*, *rpoB*, *P146*, ATP transporter ATP binding protein, Asparagine-tRNA

synthetase or Translation elongation factor gene (as discussed above, including variants).

In either case, the inverted repeats of a transposon are recognised by the transposase.

5 Thus, typically the inverted repeats are chosen for compatibility with the transposase, as well known to those skilled in the art. Tc1/*mariner* terminal inverted repeats (TIRs) may typically be between 17 and 1100bp in length, for example between 19 and 50bp in length. Requirements for TIRs are discussed further in, for example, Choi, KH & Kim, KJ (2009) J Microbiol Biotechnol Mar 19(3) 217-28 "Applications of
10 transposon-based gene delivery systems in bacteria"; Nagy, Z & Chandler, M (2004) Res Microbiol Jun 155(5); 387-98 "Regulation of transposition in bacteria"; Lampe et al (1996) EMBO Journal vol 15 no 19 p5470-5479 "A purified transposase is sufficient to mediate transposition in vitro". See also other references concerning transposon mutagenesis in other organisms cited in the Introduction section above.

15

The sequence considered to be the one unit of the repeat sequence may be or comprise;

TAACAGGTTGGCTGATAAGTCCCCGGTCT

Both repeats of the inverted repeat may have this 29bp sequence, which is based on the
20 sequence described by Lampe et al (1996).

The repeat sequence may alternatively be

ACAGGTTGGCTGATAAGTCCCCGGTCT

Or

ACAGGTTGGCTGATAAGTCCCCGGTCTGACA, for example.

25

It is considered that some variation may be possible, for example no more than 5, 4, 3, 2, 1 or zero nucleotide changes from any of the sequences indicated. It is preferred that the sequences are exact inverted repeats, but this may not be necessary,

30 In any case, the marker gene may be a selectable marker gene, optionally an antibiotic resistance gene, optionally a gene conferring resistance to puromycin (optionally puromycin-N-acetyl-transferase), tetracycline (optionally *tetM*), chloramphenicol (optionally chloramphenicol acetyltransferase) or aminoglycosides such as gentamicin (optionally *aacA-aphD*). Marker sequences are also discussed above.

35

Typically the marker gene is located between the inverted repeats, for example such that the marker gene sequence would be expected to be inserted into the chromosomal

nucleic acid by the action of the transposase on the construct comprising the inverted repeats. The inverted repeats typically flank the marker gene in a 5'-3' direction towards the marker gene. The sequence within the construct would therefore read (for example);

5 TAACAGGTTGGCTGATAAGTCCCCGGTCT – promoter – marker gene –
AGACCGGGGACTTATCAGCCAACCTGTTA

10 It is considered possible to deliver the transposon and transposase into the cell on the same or different nucleic acid construct(s), for example DNA (or modified DNA) construct(s). Alternatively, one could use *in vitro* transposition to produce a transpososome. The transposon and genome are “mixed” together *in vitro* along with transposase enzyme and transposition occurs. The mutated genomes are then transferred back into mycoplasmas and the genome is mutated by allelic exchange.

15

The nucleic acid construct typically is a DNA construct, as will be apparent to those skilled in the art. It may alternatively be a synthetic or modified nucleic acid construct, as also well known to those skilled in the art. It is not considered that there are any special requirements for the form of the nucleic acid constructs that may be used for
20 transforming *M. hyopneumoniae* or other *Mycoplasma*.

A further aspect of the invention provides a *M. hyopneumoniae* or other *Mycoplasma* cell transformed with or comprising a construct or constructs of the invention (for example a
25 nucleic acid construct comprising a transposase-encoding sequence under the control of a promoter active in *M. hyopneumoniae*; and the same or a different construct comprising inverted repeats and optionally a marker gene comprising a promoter active in *M. hyopneumoniae*). The cell may comprise the construct or constructs as extrachromosomal constructs, or at least the inverted repeat sequence (and typically
30 marker gene sequence, if present) may be integrated into the chromosomal nucleic acid.

The plasmid constructs typically may be propagated in *E. coli* strain DH5a (although other competent strains would suffice). It is considered that the transposase promoter is active in *E. coli* but this does not appear to interfere with propagation.

35

Methods for transforming *M. hyopneumoniae* cells are indicated above and discussed in the Examples. For example, electroporation may be used. Factors such as growth

phase and cell density may affect the efficiency of transformation, as noted in the Examples. Known techniques for transforming other *Mycoplasma* cells may be used with other *Mycoplasma* cell types; or methods as described herein for *M. hyopneumoniae* may be used, for example electroporation.

5

A further aspect of the invention provides a method for forming a collection of mutant *M. hyopneumoniae* or other *Mycoplasma* cells, the method comprising the steps of exposing a sample or samples of *M. hyopneumoniae* or other *Mycoplasma* cells to multiple copies of a construct encoding a transposase, optionally a construct of the invention comprising a sequence encoding a transposase and a promoter sequence active in *M. hyopneumoniae*; and a construct (separate or otherwise) comprising inverted repeats and optionally a marker gene optionally comprising a promoter sequence active in *M. hyopneumoniae*; and

10

15

optionally culturing the exposed cells under conditions such that transformed cells can be detected.

Typically the construct encoding the transposase is a construct in which transcription of the sequence encoding the transposase is under the control of a promoter sequence active in *M. hyopneumoniae*.

20

It is considered that said construct or constructs lead to the insertion of the sequence in the construct (for example transposon vector construct) between the inverted repeats into the chromosomal nucleic acid. It is expected that the insertion occurs at TA dinucleotides (typically when a Tc1/*mariner* transposase is used, for example a Himar1 or Himar1 C9 mutant transposase). Thus, a collection of cells with typically differently mutated chromosomal sequence can be generated.

25

A further aspect of the invention provides a method for forming a collection of mutant *M. hyopneumoniae* or other *Mycoplasma* cells, the method comprising the steps of

30

exposing a sample or samples of *M. hyopneumoniae* or other *Mycoplasma* nucleic acid (for example genomic nucleic acid, for example genomic nucleic acid intended to be complete) to a transposase, optionally a *Mariner* family transposase, optionally Himar1 or Himar1 C9 mutant; and to a construct comprising inverted repeats and optionally a marker gene optionally comprising a promoter sequence active in *M. hyopneumoniae*; and transforming *M. hyopneumoniae* or other *Mycoplasma* cells with the exposed nucleic acid; optionally incubating under conditions where allelic exchange is expected to occur; and

35

optionally culturing the putative transformed cells under conditions such that transformed cells can be detected.

A further aspect of the invention provides a collection of *M. hyopneumoniae* or other
 5 *Mycoplasma* cells transformed with or comprising a construct or constructs according to the invention encoding a transposase, optionally a construct of the invention comprising a sequence encoding a transposase and a promoter sequence active in *M. hyopneumoniae*; and (in a separate construct or otherwise) comprising inverted repeats and optionally a marker gene optionally comprising a promoter sequence active in *M.*
 10 *hyopneumoniae*;
 or obtained or obtainable by a method according to the preceding methods of the invention; optionally wherein the collection comprises at least 10, 20, 50, 100, 200, 400, 500, 800 or 1000 different transformants. Typically the transformants comprise the genomic insertion at a TA dinucleotide of the inverted repeats and the intervening DNA,
 15 optionally a marker gene, optionally comprising a promoter sequence active in *M. hyopneumoniae* (for example as discussed above),
 optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*, as discussed above;
 20 optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* *ldh*, *P97*, *secD*, *Tuf*, *rpoB*, *P146*, ATP transporter ATP binding protein, Asparagine-tRNA synthetase or Translation elongation factor gene (as discussed above, including variants); the marker gene is optionally an antibiotic resistance gene, optionally a gene
 25 conferring resistance to puromycin (optionally puromycin-N-acetyl-transferase), tetracycline (optionally *tetM*), chloramphenicol (optionally chloramphenicol acetyltransferase) or aminoglycosides such as gentamicin (optionally *aacA-aphD*), optionally any gene conferring resistance to a compound as identified from the invention..

30

It is considered that a pool of around 1000 or more transformants may be suitable for genomic analysis in *M. hyopneumoniae*, though it will be appreciated that a smaller pool,
 35 for example of at least 50 transformants, may also be useful.

A further aspect of the invention provides a method for analysing gene function in *M. hyopneumoniae* or another *Mycoplasma*, the method comprising the step of providing a collection of *M. hyopneumoniae* or other *Mycoplasma* transformants according to the preceding aspect of the invention; and

5 analysing nucleic acid sequences of said transformants, optionally identifying sites of insertion of said mini transposon sequence in said transformants; and optionally identifying a gene in which a mini transposon has inserted as a potential site for mutation in a vaccine composition.

10 The method may further comprise the steps of culturing a sample of the collection of transformants under selective conditions, for example in vitro or in vivo, and identifying transformants, and the location of their insertion, that have a selective advantage or selective disadvantage under those selective conditions, for example using techniques such as Signature Tagged Mutagenesis (STM) or TraDIS, as known to those skilled in
15 the art and discussed further in the Examples.

A further aspect of the invention provides a vaccine composition of the invention comprising a gene that has undergone site-directed mutagenesis as a consequence of transformation with a plasmid according to any one of claims 1 to 6, wherein the gene is
20 a gene that has been identified by a screening method of the invention.

A further aspect of the invention provides a vaccine composition comprising a *M. hyopneumoniae* cell transformed with a transposon system constructs of the invention. The transformed *M. hyopneumoniae* cell may have been identified by a screening
25 method of the invention.

A further aspect of the invention provides a vaccine composition comprising a polypeptide encoded at least in part by a coding portion of a gene (for example at least 21 nucleotides, for example at least 30, 39, 45 or 60 nucleotides of the coding portion of
30 the gene) that has been identified by a screening method according to the invention. Typically the polypeptide is not the wild-type *M. hyopneumoniae* polypeptide. The polypeptide may be mutated or truncated relative to the wild-type *M. hyopneumoniae* polypeptide.

35 A further aspect of the invention provides a plasmid of the invention typically comprising a marker sequence, wherein the plasmid further comprises a nucleic acid sequence with the ability to undergo homologous recombination with the *Mycoplasma hyopneumoniae*

chromosome wherein the nucleic acid sequence with the ability to undergo homologous recombination may comprise a sequence that has at least 80, 90, 95 or 98% identity over a sequence length of at least 100, more typically at least 300, 400 or 500 nucleotides, for example between 300, 400, 500, 600, 700, 800, 900 and 3000, 2000 or 1500 nucleotides, for example between 500 and 1500 nucleotides, for example between 700 and 1500 nucleotides with a sequence of *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) chromosomal nucleic acid. As will be understood by the skilled person, the said sequence with the ability to undergo homologous recombination with the *M. hyopneumoniae* chromosome nucleic acid is not a sequence otherwise required in the plasmid, for example the *OriC* sequence, nor any promoter sequence otherwise required in the plasmid. It may be desirable to use a promoter sequence, for example, in the plasmid that is not naturally present in wild-type *M. hyopneumoniae* in order to avoid or minimise the possibility of unwanted homologous recombination through this sequence.

The plasmid may comprises two nucleic acid sequences with the ability to undergo homologous recombination with the *Mycoplasma hyopneumoniae* chromosome as defined in the preceding aspect of the invention, separated by a marker sequence, optionally a selectable marker gene flanked by recombinase recognition sites, optionally FRT flip recombinase recognition sites or, for example, Cre-LoxP system recognition sites. The FRT recognition sequences are discussed in Example 2 below, for example. A LoxP recognition site for Cre recombinase is considered to be, for example, ATAAGTTCGTATA – NNNTANNN – TATACGAAGTTAT. This is a 34 bp recognition site that flanks the DNA region to be 'excised' The 34 bp sequence consists of 13 bp regions flanked by an 8 bp spacer with variable nucleotide sequence (N).

A further aspect of the invention provides a system comprising a plasmid according to the preceding aspect of the invention and a further plasmid of the invention comprising a marker sequence, wherein the marker sequence in the first said plasmid is different from the marker sequence in the further plasmid, optionally wherein the marker sequence in the first plasmid confers resistance to a different challenge compound to the marker sequence in the further plasmid; and wherein optionally the further plasmid comprises a sequence encoding a recombinase, optionally a flip recombinase, optionally under the control of a promoter active in *M. hyopneumoniae*. Such a system may be useful in generating transformed cells with mutated genomic nucleic acid that does not include a marker sequence, for example does not include an antibiotic resistance gene. Thus,

such a system may be useful in generating *M. hyopneumoniae* cells with one or more gene disruptions, mutations or fusions. Such cells may be useful for research purposes or as vaccine candidates.

- 5 Further information is provided in Example 2 below, for example further information on recombinases and recombination sequences, which will in any case be well known to those skilled in the art.

A further aspect of the invention provides a *M. hyopneumoniae* cell transformed with a plasmid or a system of the preceding two aspects of the invention.

The *M. hyopneumoniae* cell may have genomic nucleic acid which comprises a marker sequence, optionally a selectable marker sequence, as discussed above, flanked by recombinase recognition sites, optionally FRT flip recombinase recognition sites or Cre-LoxP recognition sites; or wherein the genomic nucleic acid of the cell comprises a recombinase recognition site. It is considered that a "scar" is left following recombination, where the scar may be recognised by the presence of the FRT (or other) recognition site – one FRT (or other) site is considered to remain in the chromosome following resolution with flip recombinase (for example).

20

A further aspect of the invention provides a plasmid of the invention wherein the plasmid further comprises a nucleic acid sequence encoding an epitope tag in-frame upstream or downstream of a sequence with the ability to undergo homologous recombination (as discussed above), such that following homologous recombination the epitope tag is expected to be expressed in-frame with the target protein. Such a plasmid may be useful in generating a transformed *Mycoplasma*, for example *M. hyopneumoniae* cell, in which it is expected that the tagged target protein is expressed, which may be useful when investigating gene function, for example.

30

A further aspect of the invention provides a method for expressing an epitope tagged or other fusion protein coding sequences in *M. hyopneumoniae* the method comprising the steps of introducing a nucleotide sequence downstream of a promoter that functions in *M. hyopneumoniae* consisting of a epitope coding sequence (or other fusion sequence) inframe with a protein coding sequence in the plasmid according to the invention.

35

The mention of any apparently published document herein is not to be taken as an acknowledgement that the document constitutes part of common general knowledge.

- 5 Any published document referred to herein is hereby incorporated by reference.

The invention is now described in more detail by reference to the following, non-limiting, Figures and Examples.

10 **Figure Legends**

Figure 1

A 2.1 kbp *oriC* region of *M. hyopneumoniae* 232 was predicted based on the location of
15 putative DnaA boxes (black arrowheads), the location of the *dnaA* gene and the
presence of short AT-rich regions of 144 bp and 208 bp with an AT content of 0.83 and
0.84 respectively (1A). The P97 gene (1B) and *ldh* gene (1C) promoter sequences were
predicted based on proximity to the ATG start codon (shown in bold) and putative TATA
box location (underlined). The *oriC* sequence was cloned into the *NcoI* and *SpeI*
20 restriction sites of pGEM-T to generate plasmid pMHO. The *tetM* gene with the *E.*
faecalis, *spiralin* gene, P97 and *ldh* promoter sequence was cloned into the *PstI* and
SpeI sites to produce plasmids pMHO-1, pMHO-2, pMHO-3 and pMHO-4 respectively
(1D). *M. hyopneumoniae* 232 was transformed with each plasmid and transformants
(pMHO-2 in 1E) grown in Friis medium with tetracycline selection. After 3 passages,
25 plasmid DNA was extracted from two individual clones (C1 and C2) and analysed by
agarose gel electrophoresis along with plasmid (P) DNA control (1F).

Figure 2

30 The predicted *oriC* region of *M. hyopneumoniae* 232 is shown with the location of two
putative DnaA boxes (black arrowheads) lying within AT-rich intergenic regions (grey
boxes). The *oriC* region from plasmid pMHO (*oriC*-i) was reduced in a series of steps to
produce five further plasmids (*poriC*-ii, *poriC*-iii, *poriC*-iv, *poriC*-iii/iv and *poriC*-iv/v). *M.*
35 *hyopneumoniae* 232 was transformed in triplicate with each plasmid and the mean
transformation frequencies (TF) are shown, expressed as the number of transformants
per colony forming unit of mycoplasma transformed.

Figure 3

5 Individual transformants generated with four plasmids containing various *oriC* regions were passaged up to 19 times in Friis medium containing tetracycline (3A). Genomic DNA was extracted from cultures and analysed by Southern hybridization using a probe annealing to the *bla* ampicillin resistance gene. For each plasmid, there was no decline in extrachromosomal DNA over time, and plasmid DNA could still be readily detected
 10 after 19 passages. For plasmid *poriC-iv/v*, three transformants were passaged up to six times without tetracycline before undergoing selection with tetracycline (3B). With each passage without selection, the number of tetracycline-resistant colonies reduced, demonstrating a loss of tetracycline-resistance. Possible integration events were predicted for plasmid pMHO-2 containing *oriC-I* (3C). Three further pMHO-2
 15 transformants were passaged 19 times and total DNA extracts subjected to Southern hybridization with a *tetM* DIG-labeled probe, along with plasmid DNA control (P) and untransformed *M. hyopneumoniae* 232 DNA (C) (1D). Two clones showed the presence of 7.4 kbp plasmid DNA and also a 4.6 kbp fragment consistent with plasmid integration by homologous recombination.

20

Figure 4

Plasmid pTn4001-RVC1 was constructed by replacing the *pac* gene of pMiniTn4001PsPuro with *tetM* from plasmid pSRT2 (1A). Plasmid pMiniHimar1BSC1 contains a mini transposon incorporating *pac* conferring resistance to puromycin and the
 25 wild-type Himar1 transposase downstream of the Tn4001 native promoter sequence (1B) (Cook, 2013). In the pMHW-2 plasmid, the *pac* in pMiniHimar1BSC1 has been replaced with *tetM* from pSRT2 (1C). In plasmid pMHW-1, the promoter sequence of the *M. hyopneumoniae* strain 232 P97 gene has been placed upstream of the wild-type Himar1 gene (1D). In pMHC9-1, the wild-type Himar1 gene has been replaced with the
 30 hyperactive C9 mutant version of the gene (1E) (Lampe et al., 1999).

Figure 5

M. hyopneumoniae strain 232 was transformed with plasmid pMHC9-1 and within 14 days, tetracycline resistant colonies formed on Friis agar. No colonies were observed on
 35 'no DNA' control transformations. Individual colonies exhibited a similar appearance to non-transformed *M. hyopneumoniae* colonies.

Figure 6

M. hyopneumoniae strain 232 was transformed with pMHC9-1. PCR was performed on extracted total DNA from 15 individual transformants (lanes 1-15) for identification of the tetM gene (6A) and the pGEM-T plasmid backbone (6B). A further 11 transformants underwent a further two rounds of 'colony purification'. Total DNA was extracted and linker PCR performed using two primer pairs at either end of the transposon (left and right) (6C). The same 11 transformants were passaged 15 times in Friis medium without selection, and all subsequently retained their resistance to tetracycline. Linker PCR was repeated using the same two primer pairs (left and right) (62D). Control samples for PCR reactions included: 'no template' control (NT); untransformed *M. hyopneumoniae* strain 232 (NC); plasmid DNA positive control (PC).

Figure 7

Total DNA was extracted from 11 individual transformants of *M. hyopneumoniae* 232 generated using plasmid pMHC9-1, and subjected to Southern analysis using a probe specific for tetM. Single bands were generated for 10 of the mutants suggesting single transposon insertion sites. Two separate bands were present for mutant 1. No band was present for the untransformed *M. hyopneumoniae* strain 232 negative control and an intense band was present for the pMHC9-1 positive control as expected.

Figure 8

Transposon insertion sites were determined by linker PCR and sequencing of PCR products for 44 individual mutants. The location of these insertion sites in the *M. hyopneumoniae* strain 232 genome is depicted by lines emanating from the circular genome (drawn to scale). Thicker lines indicate the presence of more than one insertion at a given location.

Figure 9

Plasmid pOriCP146 was generated by cloning a 974 bp fragment of the *M. hyopneumoniae* 232 ORF into the *Mlu*I and *Pst*I restriction enzyme cut sites of pOriC-iv/v. The 974 bp fragment was located a short distance from the promoter region (Pr) and start codon (ATG) of the P146 gene (9A). The plasmid was designed such that a single cross over homologous recombination event would result in integration of the entire plasmid into the chromosome of the organism and causing disruption of the P146 open reading frame.

Figure 10

Following transformation of *M. hyopneumoniae* 232 with plasmids pOriCP146 and pOriC-iv/v, randomly selected tetracycline-resistant colonies were isolated and passaged 19 times in Friis medium containing tetracycline. After 19 passages, extrachromosomal plasmid DNA could not be detected for pOriCP146 transformed organisms (A) but was readily detected for pOriC-iv/v transformed cells (lacking the 974 bp region of homology with the P146 gene) (B). Plasmid DNA extracted from *E. coli* is shown (P) along with plasmid DNA extracts from untransformed *M. hyopneumoniae* 232 (232). A PCR strategy was used to detect integrated plasmid DNA in transformed mycoplasmas, but not extrachromosomal plasmid DNA. Primer sets A and B were designed to amplify DNA at either end of the plasmid/chromosome integration site.(C). Following 19 passages of with tetracycline and then a further 6 passages without tetracycline (to promote further loss of extrachromosomal plasmid DNA), PCR was performed using primer sets A (D) and B (E) on total DNA extracts from mycoplasma cultures transformed with pOriCP146. For all four selected tetracycline resistant clones, it was possible to amplify DNA at either end of the targeted integration site (C1-C4) but not in untransformed *M. hyopneumoniae* (232), free miniprep plasmid DNA (P) or no template control (NTC).

Figure 11

Plasmid pOriC379 was designed to allow targeted gene disruption in the ORF of mhp379 following plasmid integration by homologous recombination (A). Following successful transformation of *M. hyopneumoniae* 232 with pOriC379, extrachromosomal plasmid DNA could be detected in five clones following 19 serial passages in tetracycline, similar to plasmid DNA derived from *E. coli* (P) but not in untransformed *M. hyopneumoniae* 232 (232) (B). At the same time, evidence of plasmid integration into mycoplasma chromosomal DNA was detected in all five clones by PCR (primer location shown by red arrows in (A)), but not in untransformed *M. hyopneumoniae* 232 (232) (C). Similarly, no PCR product was generated with extrachromosomal plasmid DNA derived from *E. coli* (P) or in no template control (NTC). An additional six passages were performed without tetracycline, but it was still possible at this time to detect extrachromosomal plasmid DNA in all five clones (D).

Figure 12

Plasmid pOriCpepF was designed to promote targeted integration within the pepF open reading frame following a homologous recombination event (A). Following 19 passages

of six randomly selected pOriCpepF transformed clones, extrachromosomal plasmid DNA was readily detected in clone 2 and a faint plasmid band was also detected in clone 1 (B). No extrachromosomal plasmid DNA was detected in clones 3-6 or in untransformed mycoplasma cells (232). Similar findings were apparent following an additional six passages without tetracycline selection pressure (C). At this time, it was also possible to detect (by PCR) DNA spanning the integration site within the mycoplasma genome using two primer sets (A) for all six clones but not for untransformed *M. hyopneumoniae* (232), *E. coli* derived plasmid DNA (P) or no template control (NTC) (D and E).

Figure 13

Photographic demonstration of the phenotypic change due to loss of P146. The loss of protein band is easier to discern in enhanced image (Figures 13b and c).

Example 1: Development of a self-replicating plasmid system for *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae is a prevalent swine respiratory pathogen that is a major cause of economic loss to pig producers. Current vaccines only offer partial control and there is a need for improved preventative strategies. A major barrier to advances in understanding the pathogenesis of *M. hyopneumoniae* and in developing new vaccines is the lack of tools to genetically manipulate the organism. We describe the development and optimisation of the first successful plasmid tools for the genetic manipulation of *M. hyopneumoniae*. This represents a major step forward in the study of this important swine pathogen.

The *OriC* of *M. hyopneumoniae* strain 232 [2] was predicted based on homology with other *Mycoplasma* species for which a functional *OriC* has been identified [11, 12, 14]. Typically, the *OriC* encompasses short AT-rich regions flanking the *dnaA* gene and containing DnaA boxes. A search of the *M. hyopneumoniae* 232 genome [2] revealed single DnaA boxes (each with two mismatches with the *E. coli* consensus sequence 5'-TTATCCACA-3') upstream and downstream of the *dnaA* gene within AT-rich regions (figure 1A). Plasmid pMHO (figure 1D) was generated by cloning a ~2.1 kbp sequence (indicated above and in the claims) into pGEM-T (Promega Ltd, UK), encompassing the *dnaA* gene and the flanking AT-rich regions. The *tetM* gene (conferring tetracycline

resistance) was chosen as a selection marker given successful use with other *Mycoplasma* species [10, 12]. The *tetM* gene and promoter from *Enterococcus faecalis* were amplified from plasmid pIVT-1 [5] and cloned into pMHO to produce pMHO-1. *M. hyopneumoniae* 232 [2] was grown in Friis medium (see Supplementary Files) at 37 °C in a static incubator in airtight culture tubes until late logarithmic phase (~ 10⁸ CFU/ml), as determined by an acid colour change in the phenol red pH indicator. Cells were collected by centrifugation (9000 xg at 4°C for 10 minutes) and washed three times in electroporation buffer (272 mM sucrose, 8 mM HEPES, pH 7.4). After the final wash, 100 µl cells (~ 2 mL of mycoplasma culture) were incubated on ice with 10 µg plasmid DNA for 30 minutes. Electroporation (2.5 kV, 100 Ω and 25 µF) was performed in a 0.2 cm cuvette using a Gene Pulser system (Bio-Rad Ltd, UK) and 900 µL chilled Friis medium immediately added. Cells were incubated for 3 hours at 37 °C and then plated onto Friis agar containing 2 µg/mL tetracycline hydrochloride (Sigma-Aldrich Ltd, UK). Triplicate repeats were performed for all transformations, and after 14 days incubation at 37 °C in 5% CO₂, no growth was observed for pMHO-1 or 'no DNA' controls. We considered that the *tetM* promoter sequence may not be active in *M. hyopneumoniae* and/or that the tetracycline concentration may be too high to be overcome by transformed organisms. The *tetM* gene of pMHO-1 was replaced with the *tetM* gene from plasmid pSRT2 [11] to produce pMHO-2 (figure 1D). This utilises the *spiralin* gene promoter of *Spiroplasma citri* [15] that has been shown to be active in several *Mycoplasma* species [11, 12]. Additionally, the predicted promoter regions of the *M. hyopneumoniae* 232 P97 ciliary adhesin gene [19] (619bp) and *lactate dehydrogenase (ldh)* gene (421bp) were placed upstream of the *tetM* gene to produce plasmids pMHO-3 and pMHO-4 respectively (figure 1D). Following the methods of Hannan *et al*, the MIC for tetracycline was found to be 0.03 µg/mL in liquid medium and 0.015 µg/mL in solid medium [20]. *M. hyopneumoniae* 232 was transformed with each plasmid and plated onto Friis agar containing a low concentration of tetracycline (0.2 µg/mL). After 14 days incubation, tetracycline resistant colonies were present for all four *oriC* plasmids but not for the 'no DNA' control, demonstrating activity of the four promoter sequences. Morphologically, colonies were identical to untransformed *M. hyopneumoniae* colonies, but colony growth lagged approximately 3 days behind (figure 1E). The highest number of transformants was generated using pMHO-2 (2.5x10⁻⁴/CFU) with fewer for pMHO-3 (5.8x10⁻⁵/CFU) and pMHO-1 (1.7x10⁻⁵/CFU) and least with pMHO-4 (8.3x10⁻⁶/CFU). Individual colonies were picked from agar and grown in Friis medium containing 0.5 µg/mL tetracycline, after which they were sub-cultured every 2-3 days in 1 µg/mL tetracycline. After three serial passages, plasmid DNA was extracted from 10ml culture using a PureLink Plasmid Mini

kit (Invitrogen Ltd, UK) and separated by electrophoresis on 1% agarose. Plasmid DNA was readily identified for each transformant whereas no plasmid DNA was seen with untransformed *M. hyopneumoniae* 232 (figure 1F). However, less plasmid DNA was detected for pMHO-3 and pMHO-4 transformants. Given that both of these plasmids contain promoter sequences 400 - 600bp in size that are identical to *M. hyopneumoniae* genomic DNA, we suspected that plasmid integration had occurred by homologous recombination.

Initially, we selected electroporation conditions of 2.5 kV, 100 Ω and 25 μ F as described by Hedreyda *et al* [7]. We varied the electroporation voltage from a maximum possible of 2.5 kV down to 0 kV in 500 V increments and used pMHO-2 to transform *M. hyopneumoniae* 232. Electroporation voltage was found to have a profound effect on transformation frequency; decreasing voltage to 2kV resulted in a 10-fold drop in transformation frequency, and below 2kV no transformants were obtained (data not shown). Similarly, decreasing plasmid DNA was associated with a drop in transformation frequency; a drop from 10 μ g down to 1 μ g resulted in a 10-fold drop in transformants (data not shown). Thus the transformation efficiency appears to be very poor for *M. hyopneumoniae* requiring large amounts of plasmid DNA, as is the case for other *Mycoplasmas* but in contrast to the very low quantities of DNA required to transform other bacteria.

We suspected that the large 2.1 kbp *oriC* in our plasmids would favour integration by homologous recombination. We therefore determined whether the predicted *oriC* regions of four phylogenetically closely related *Mycoplasma* species would function in *M. hyopneumoniae*, possibly reducing the likelihood of recombination. The genomes of *M. hyorhinis* [21], *M. pulmonis* [22], *M. conjunctivae* [23] and *M. ovipneumoniae* [24] exhibited a similar arrangement of genes in the *oriC* region to *M. hyopneumoniae*, with AT-rich regions and DnaA boxes in the intergenic regions flanking the *dnaA* gene. The PCR-amplified *oriC* regions were used to replace the *M. hyopneumoniae* 232 *oriC* in plasmid pMHO-2. *M. hyopneumoniae* 232 cells were electroporated in triplicate with each of the plasmids in two separate experiments and plated out onto Friis agar containing 0.2 μ g/mL tetracycline. As expected, tetracycline resistant colonies were obtained with the pMHO-2 plasmid within 10 days. However, after 14 days, no colonies were visible in any culture transformed with plasmids containing the *oriC* regions of the other *Mycoplasma* species.

As an alternative strategy to reduce the incidence of homologous recombination, we attempted to reduce the size of *oriC* used in our plasmids. To define the minimum functional *oriC* in *M. hyopneumoniae*, initially the *oriC* of pMHO-2 (*oriC*-i) was reduced by 300bp by removing excess DNA lying upstream and downstream of the AT-rich intergenic regions (p*OriC*-ii). With both AT-rich regions and the *dnaA* gene still present, as expected, there was no reduction in transformation efficiency (figure 2). To determine if the AT-rich intergenic regions would function as an *oriC* independently, we produced plasmids p*OriC*-iii and p*OriC*-iv containing the upstream and downstream intergenic regions respectively. p*OriC*-iii contained the AT-rich region upstream of the *dnaA* gene, and also extended downstream into the *dnaA* gene to include three putative DnaA boxes. Transformation of *M. hyopneumoniae* 232 failed to produce any transformants, showing that each AT-rich region could not function as an *oriC* independently. However, by placing both intergenic regions adjacent to each other in plasmid p*OriC*-iii/iv, transformants were produced, but the transformation efficiency was approximately 10-fold lower than when the entire *dnaA* gene was present (figure 2). Finally, we determined whether or not the entire *dnaA* gene sequence could be removed leaving the AT-rich intergenic regions alone (p*OriC*-iv/v). We concluded that the minimum *oriC* necessary for functionality in self-replicating plasmids was approximately 700bp long and included both AT-rich intergenic regions only, but with a transformation efficiency approximately 100-fold lower than that achieved when the *dnaA* gene sequence was included (figure 2). To determine how robust the resistance to tetracycline of p*OriC*-iv/v transformants was, we determined the MIC of three individual colonies in triplicate. After initial growth in 1 µg/mL the MIC for tetracycline of all three clones in Friis broth was 32 µg/ml compared to 0.03 µg/mL for untransformed *M. hyopneumoniae* 232.

To determine the stability of our plasmids, individual transformants were picked into Friis medium containing 0.5 µg/mL tetracycline and passaged 19 times. At passages 3, 8 and 19, total DNA was extracted using a Qiagen DNA Blood & Tissue kit (Qiagen Ltd, UK) and 1 µg were digested to completion with HindIII to linearise the plasmid DNA. DNA was separated by electrophoresis on 0.8% agarose and Southern hybridisation performed with a DIG-labelled probed specific for the *bla* resistance using standard methods [25]. For each plasmid, we could readily detect extrachromosomal DNA at every passage, but not for the untransformed *M. hyopneumoniae* 232 control (figure 3A). Additionally, we did not observe a reduction in plasmid DNA quantity that may be suggestive of plasmid integration into the *M. hyopneumoniae* genome by homologous recombination. We next determined the stability of our minimal *oriC* plasmid, p*OriC*-iv/v,

over serial passages without selection. Three individual transformants were passaged five times in Friis medium with 0.5 µg/mL tetracycline followed by six passages without tetracycline. At each of the six passages without selection, cultures were plated (in triplicate) onto Friis agar with and without 0.2 µg/mL tetracycline. After 14 days growth, colony counts were performed. We observed a rapid reduction in tetracycline resistant colonies following serial passage in medium containing no tetracycline, showing that selection pressure is necessary to maintain resistance, and suggesting that the transformants can readily be 'cured' of plasmid DNA (figure 3B). We anticipate that this stable plasmid may be useful in generating targeted gene disruptions of *M. hyopneumoniae*. We therefore examined three clones of pMHO-2 (containing *oriC-i*) transformed *M. hyopneumoniae* at passage 19. We predicted that integration of plasmid pMHO-2 into the *M. hyopneumoniae* genome would generate a 4.6 kbp HindIII digestion fragment that would be detected by a DIG-labeled probe annealing to the *tetM* gene. By separating a larger amount (2.5 µg) of HindIII-digested total DNA and using a DIG-labelled *tetM* gene probe, we were able to visualise apparent a 7.4 kbp band consistent with plasmid DNA in three transformants and a 4.6 kbp band consistent with integrated plasmid DNA in two transformants. This would suggest that recombinase systems active in *M. hyopneumoniae* are capable of allowing homologous recombination to occur, and therefore our minimum *oriC* plasmid may be useful in targeting homologous recombination to target genes (Example 4).

Finally, we found that many of our UK field isolates exhibited greater resistance to tetracycline than *M. hyopneumoniae* 232, making it unachievable to select for transformed colonies on Friis agar containing 0.2 µg/ml tetracycline (data not shown). To circumvent this problem, we replaced the *tetM* gene of pMHO-2 with the PCR-amplified *puromycin N-acetyltransferase* (PAC) gene from pMiniTn4001PsPuro [26] to generate plasmid pMHO_{puro}. This PAC gene also contains the *spiralin* gene promoter of *S. citri* and confers resistance to puromycin, an aminonucleoside that has no veterinary clinical use. Transformation of *M. hyopneumoniae* 232 with pMHO_{puro} resulted in a transformation frequency of 1.6×10^{-4} /CFU compared to 4.6×10^{-5} for plasmid pMHO-2 (containing the *tetM* gene). Thus puromycin may be a suitable selection marker for *M. hyopneumoniae* isolates that are resistant to tetracycline.

Discussion

The inability to genetically manipulate *M. hyopneumoniae* has been a major obstacle to progress in understanding the pathogenesis of the organism. Using simple artificial self-

replicating plasmids containing the *oriC* of *M. hyopneumoniae* and an antimicrobial resistance gene, we have shown for the first time that the organism is susceptible to transformation. We found that the transformation conditions optimized by Hedreyda *et al* and used widely in other *Mycoplasmas* are suitable for *M. hyopneumoniae* [7]. However, other important factors include titration of the tetracycline concentration to a level that can readily be overcome by the *tetM* resistance protein and use of a large excess of plasmid DNA. Additionally, we found that the choice of promoter affected the expression of the *tetM* gene, and as is the case with other *Mycoplasmas*, the *spiralin* gene promoter of *Spiroplasma citri* is a suitable choice for *M. hyopneumoniae*. Our *oriC* system allowed us to assess the function of putative *M. hyopneumoniae* promoter sequences, and confirmed the identity of two promoter sequences that were active in controlling *tetM* expression. The *P97* promoter sequence was recently predicted and we have verified that this is functional in our plasmid system [19]. Using the criteria identified by Weber Sde *et al* [19], we also predicted the location of the *ldh* promoter, and again demonstrated its activity in our plasmid system. These *M. hyopneumoniae* specific promoters will be valuable in the generation of further tools for the genetic manipulation of the organism. Using our system, we verified that the origin of replication of *M. hyopneumoniae* lies in the region of the *dnaA* gene and includes two short AT-rich regions. It has been suspected that the *oriC* of *M. hyopneumoniae* lies in this region, but unusually within the AT-rich regions there are only two DnaA boxes that resemble the *E. coli* consensus sequence, whereas for other *Mycoplasma* species, there are typically multiple DnaA boxes [2]. Additional DnaA boxes are present within the *dnaA* gene, but these are not required for a functional *oriC*. We hypothesized that the *oriC* regions of *Mycoplasmas* showing a high degree of homology to *M. hyopneumoniae* would function in *M. hyopneumoniae*, and enable stable plasmid maintenance without integration by homologous recombination. However, it appears that *oriCs* are very specific to their host species and none of the *oriC* regions from four closely related *Mycoplasma* species functioned in *M. hyopneumoniae*.

The minimum *M. hyopneumoniae* sequence that functioned in an *oriC* plasmid was ~700bp in length and included both AT-rich regions. This plasmid was maintained extrachromosomally over at least 19 passages with selection pressure, equivalent to over 8 weeks post-transformation. However, when selection pressure was removed, tetracycline resistance was rapidly lost within 5 passages. Having demonstrated that plasmids carrying a high degree of homology with *M. hyopneumoniae* can undergo integration by homologous recombination, it is likely that this plasmid will be useful in generating targeted disruptions in genes of interest. By incorporating sequences

encoding mycoplasma genes of interest, it is likely that we will be able to promote targeted homologous recombination and disruption of these genes, as has been shown with other *Mycoplasmas* [11, 12].

- 5 In summary, we have shown that *M. hyopneumoniae* is indeed susceptible to genetic manipulation by transformation with self-replicating plasmids. In doing so, we have developed a system that we consider is useful in genetic studies of *M. hyopneumoniae*. Additionally, we consider that our findings will allow development of a transposon-mediated system to generate random mutants of *M. hyopneumoniae*, which will take us
10 a step closer to unraveling the pathogenic mechanisms of this organism.

Cloning details

PCR was performed using the Phusion High Fidelity kit (NEB Ltd, UK) according to the manufacturers instructions. Oligonucleotide sequences used in PCR reactions are
15 shown in table 1. Molecular cloning was performed using standard methods as described by Sambrook and Russell [25]. For cloning, *E. Coli* strain DH5 α was grown in Luria-Bertani medium with 100 μ g/ml ampicillin.

Table 1: Oligonucleotide sequences

20

PCR product	Size (bp)	Restriction site	Oligonucleotide sequence (5' - 3')
<i>M. hyopneumoniae</i> strain 232	2130	<i>NcoI</i>	F - AGGCCATGGTTGTTAATTATTGCTTGAAATTC
<i>oriC- i</i>		<i>SpeI</i>	R - AGTACTAGTAACTTTATAGGAAAGTTTCG
<i>M. hyopneumoniae</i> strain 232	1823	<i>NcoI</i>	F - GATGCCATGGTTGGTTGATAAGTTTCGCTTC
<i>oriC- ii</i>		<i>SpeI</i>	R - GTAGACTAGTCATTCTTTCAATTTGTCATTC
<i>M. hyopneumoniae</i> strain 232	859	<i>NcoI</i>	F - GATGCCATGGTTGGTTGATAAGTTTCGCTTC
<i>oriC- iii</i>		<i>SpeI</i>	R - GCTCACTAGTGACAATCAGTTTAAAAATTCAC
<i>M. hyopneumoniae</i> strain 232	859	<i>AatII</i>	F - GCAGGACGCTCTTGGTTGATAAGTTTCGCTCC
<i>oriC- iii</i>		<i>NcoI</i>	R - GACGCCATGGGACAATCAGTTTAAAAATTCAC
<i>M. hyopneumoniae</i> strain 232	422	<i>NcoI</i>	F - GCATCCATGGATATTGTGCATGCCCGCGGATAT
<i>oriC- iv</i>		<i>SpeI</i>	R - GTAGACTAGTCATTCTTTCAATTTGTCATTC
<i>M. hyopneumoniae</i> strain 232	280	<i>AatII</i>	F - GCAGGACGCTCTTGGTTGATAAGTTTCGCTCC
<i>oriC- v</i>		<i>NcoI</i>	R - CGTACCATGGCCTAATTTGCTGTGTTCTAA
<i>tetM</i> + spiralin promoter from plasmid pSRT2	2320	<i>PstI</i>	F - TAACTGCAGCAAAAGCTTGCATGCCTGCA
		<i>SpeI</i>	R - GTCCTAGTCAGTTCAGATCTTTATATAAC
<i>tetM</i> from plasmid pIVT	2109	<i>SpeI</i>	F - ATCTACTAGTCATGTGATTCTAAAGTATCC
		<i>PstI</i>	R - GATCTGCAGCTTATTTAACAAGAAACC
<i>tetM</i> (without promoter) from plasmid pSRT2	1963	<i>BamHI/PstI</i>	F - GAACCTGCAGGATCCATGGAGGAAAATCACATGA
		<i>SalI</i>	R - CCCGGTCCGACTTATATAACAACCTTAAATTAC
<i>M. hyopneumoniae</i> strain 232	619	<i>BamHI</i>	F - GAATGGATCCCCAACAATTCGGCAGTC
P97 promoter		<i>SpeI</i>	R - GCTAACTAGTACGGGGATTAAAACAGAAAC

<i>M. hyopneumoniae</i> strain 232	421	<i>SpeI</i>	F - GGCTAA <u>CTAGT</u> ATAGAATTTTGAATTAAG
<i>ldh</i> promoter		<i>Bam</i> HI	R - CTTAGGATCCATTTATACTCGTATTTGTTAT
<i>M. hyorhinis</i> <i>OriC</i>	1479	<i>NcoI</i>	F - GACTCCATGGTCGGAATCGAGATTCCTAATT
		<i>SpeI</i>	R - GCCTA <u>CTAGT</u> CTACACCTTCGATTTCTCTAA
<i>M. ovipneumoniae</i> <i>oriC</i>			
<i>M. pulmonis</i> <i>oriC</i>	1909	<i>NcoI</i>	F - ATTAGCCATGGCACTCTGGTCAGCGCTAGATC
		<i>NheI</i>	R - TCGCGCTAGCCTAACTTGAAAATAAGCTCC
<i>M. conjunctivae</i>	2307	<i>NcoI</i>	F - GATA <u>CCATGGT</u> ATGCCTTTGGTCTAGGATG
<i>oriC</i>		<i>XbaI</i>	R - CGATGTCTAGACCCCTGAACAAATATACCA
<i>bla</i> gene (DIG-labelled probe)	860	N/A	F - CCAATGCTTAATCAGTGAGG
			R - GTATGAGTATTCAACATTTCCG
<i>tetM</i> (DIG-labelled probe)	406	N/A	F - GTGGACAAAGGTACAACGAG
			R - CGGTAAAGTTCGTCACACAC

Oligonucleotide sequences used in this study are shown along with the size of the PCR product generated and the template DNA used. Restriction enzyme sites (where applicable) that were added to the 5' end of the oligonucleotide are underlined. Forward (F) and reverse (R) oligonucleotides are shown.

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Example 2: Methods for generating targeted mutants with a small scar site

Example 1 describes the generation of an *oriC* based plasmid system for *M. hyopneumoniae*. One of the potential applications of this system is utility in producing targeted gene disruptions in *M. hyopneumoniae* by promoting homologous recombination at target sites. In the simplest form, an approximately 1000 bp region of a selected target gene can be incorporated into the *oriC* plasmid. By persisting in the mycoplasma over serial passages under tetracycline selection, the plasmid can be used to drive integration of the entire plasmid into the target site by homologous recombination, therefore disrupting an open reading frame. This has been accomplished as detailed in Example 4b.

However, this leaves a large 'scar' ie a large amount of foreign plasmid DNA including a resistance cassette in the genome. An alternative strategy would be to achieve a double crossover whereby a resistance cassette is flanked by two approximately 1000 bp fragments of *M. hyopneumoniae*. A double crossover event would result in disruption of a target sequence by the resistance cassette. The resistance cassette itself would be flanked by short FRT flip recombinase recognition sites. Mutants carrying the resistance cassette, for example *tetM*, can be selected for by tetracycline. These mutants can then be transformed with another *oriC* plasmid carrying an alternative selection marker eg *pac* (puromycin) and a flip recombinase gene. Mutant *M. hyopneumoniae* carrying the flip recombinase plasmid can be selected for using puromycin (for example). Expression of the recombinase will lead to 'excision' of the *tetM* marker (for example) at the FRT recognition sites and the resulting *M. hyopneumoniae* mutant will retain a disrupted ORF but with only a very small scar.

The 34bp minimal FRT site sequence has the sequence

5'GAAGTTCCTATTC~~tctagaaa~~GtATAGGAACTTC3'

for which flippase (Flp) binds to both 13-bp 5'-GAAGTTCCTATTC-3' arms flanking the 8 bp spacer, i.e. the site-specific recombination (region of crossover) in reverse orientation. FRT-mediated cleavage occurs just ahead from the asymmetric 8bp core region (5'~~tctagaaa~~3') on the top strand and behind this sequence on the bottom strand. Several variant FRT sites exist, but recombination can usually occur only between two *identical* FRTs but generally not among *non-identical* ("heterospecific") FRTs.

Many available constructs include an additional arm sequences (5'-GAAGTTCCTATTC-3') one base pair away from the upstream element and in the same orientation:

5'GAAGTTCCTATTCCGAAGTTCCTATTCTctagaaaGtATAGGAACTTC3'

This segment is dispensable for excision but essential for integration, including Recombinase-mediated cassette exchange. See, for example Zhu XD, Sadowski PD (1995). "Cleavage-dependent Ligation by the FLP Recombinase". *Journal of Biological Chemistry* 270 (39): 23044–54. doi:10.1074/jbc.270.39.23044. PMID 7559444; Schlake T, Bode J (1994). "Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci". *Biochemistry* 33 (43): 12746–12751. doi:10.1021/bi00209a003. PMID 7947678; Turan, S.; Kuehle, J.; Schambach, A.; Baum, C.; Bode, J. (2010). "Multiplexing RMCE: Versatile Extensions of the FLP-Recombinase-Mediated Cassette-Exchange Technology". *J. Mol. Biol.* 402 (1): 52–69. doi:10.1016/j.jmb.2010.07.015. PMID 20650281; Turan, S., Bode, J. (2011). "Site-specific recombinases: from tag-and-target- to tag-and-exchange-based genomic modifications". *FASEB J.* 25: 4088–4107. doi:10.1096/fj.11-186940. PMID 21891781.

Example 3. A novel Himar1 transposon mutagenesis system for generating random *Mycoplasma* (for example *hyopneumoniae*) gene mutants

Mycoplasma hyopneumoniae is the cause of enzootic pneumonia in pigs, a chronic respiratory disease associated with significant economic losses to swine producers worldwide. The molecular pathogenesis of infection is poorly understood due to the lack of genetic tools to allow manipulation of the organism and more generally for the *Mycoplasma* genus. The objective of this study was to develop a system for generating random transposon insertion mutants in *M. hyopneumoniae* that could prove a powerful tool in enabling the pathogenesis of infection to be unraveled. A novel delivery vector was constructed containing a hyperactive C9 mutant of the Himar1 transposase along with a mini transposon containing the tetracycline resistance cassette, *tetM*. *M. hyopneumoniae* strain 232 was electroporated with the construct and *tetM*-expressing transformants selected on agar containing tetracycline. Individual transformants contained single transposon insertions that were stable upon serial passages in broth medium. The insertion sites of 44 individual transformants were determined and confirmed disruption of several *M. hyopneumoniae* genes. A large pool of over 10,000

mutants was generated that should allow saturation of the *M. hyopneumoniae* strain 232 genome. This is the first time that transposon mutagenesis has been demonstrated in this important pathogen and could be generally applied for other Mycoplasma species that are intractable to genetic manipulation. The ability to generate random Mutant libraries is a powerful tool in the further study of the pathogenesis of this important swine pathogen.

Materials & methods

Plasmid construction

PCR was performed using a Phusion High Fidelity kit (NEB Ltd, Hitchin, UK) according to the manufacturer's instructions. Sequences of oligonucleotides used in the study are shown in table 2. To construct pTn4001-RVC1 (figure 4A), the 2293 base pair (bp) tetM gene of the plasmid pSRT2 (Cordova et al., 2002) was amplified by PCR (primers TetM1 and TetM2) and cloned into the XbaI and PstI restriction sites of the pMiniTn4001PsPuro plasmid (Algire et al., 2009). Mariner transposon plasmids were constructed from the plasmid pMiniHimar1BSC1 (figure 4B) (Cook, 2013). To construct pMHWT-2, firstly the 2293 bp tetM gene in pSRT2 (Cordova et al., 2002) was amplified by PCR (primers TetM3 and TetM4) and used to replace the puromycin N-acetyl-transferase (pac) gene of pMiniHimar1BSC1 using the MluI and SbfI restriction sites (figure 4C). pMiniHimar1BSC1 contains the Himar1 transposase gene downstream of the native promoter sequence from the Tn4001 transposase. To produce plasmid pMHWT-1, the 1050 bp Himar1 transposase gene minus the Tn4001 promoter sequence was PCR-amplified from pMiniHimar1BSC1 (primers Tpn1 and Tpn2), introducing an XhoI cut site at the 3' end and NdeI and NcoI restriction sites at the 5' end. This PCR product was cloned into NdeI and XhoI sites of pMHWT-2, replacing the Himar1 gene and Tn4001 promoter sequence, and introducing a new NcoI cloning site. Next, the 619 bp P97 ciliary adhesin gene promoter sequence was amplified from *M. hyopneumoniae* strain 232 (Minion et al., 2004) genomic DNA with NcoI and NdeI restriction sites added (primers P97-1 and P97-2). The P97 promoter sequence was then cloned into the NcoI and NdeI restriction sites upstream of the Himar1 transposase gene, placing Himar1 under its control, and completing plasmid pMHWT-1. To generate plasmid pMHC9-1, the 1050 bp hyperactive C9 mutant of Himar1 (Lampe et al., 1999) was PCR amplified from plasmid pCAM45 (May et al., 2004), adding XhoI and NcoI restriction sites (primers Tpn1 and Tpn2). This PCR product was used to replace the wild-type Himar1 transposase gene of pMHWT-1, generating plasmid pMHC9-1 such that the C9 mutant Himar1 is under control of the P97 promoter sequence.

Bacterial culture

M. hyopneumoniae strain 232 (Minion et al., 2004) was grown in Friis broth medium at 37 °C in a static incubator (Kobisch and Friis, 1996). For growth on solid medium, Friis medium was solidified by addition of 0.8% w/v purified agar (Oxoid Ltd, Basingstoke, UK) and 0.01% w/v DEAE-dextran (Sigma-Aldrich Ltd, Gillingham, UK) and incubated at 37 °C with 5% CO₂. For the selection of transformants, tetracycline hydrochloride (Sigma-Aldrich Ltd, Gillingham, UK) was added to Friis broth medium and Friis agar medium at final concentrations of 0.5 µg/mL and 0.2 µg/mL respectively. Molecular cloning was performed using *Escherichia coli* strain DH5α, grown in Luria-Bertani medium according to standard methods (Sambrook and Russell, 2001). Transformants were selected by the addition of 100 µg/mL ampicillin (Sigma-Aldrich Ltd, Gillingham, UK) to the medium. Additionally, for the selection of transformants containing the *tetM* gene, tetracycline hydrochloride was added to a final concentration of 5 µg/mL.

Transformation of mycoplasmas

M. hyopneumoniae strain 232 was grown to mid-late logarithmic phase in Friis broth as determined by an acid colour change in the phenol red pH indicator. Mycoplasmas were harvested by centrifugation of culture at 9000 xg for 10 minutes at 4 °C and washed three times in electroporation buffer (272 mM sucrose, 8 mM HEPES, pH 7.4). 100 µl cells (corresponding to 3 mL culture) were incubated on ice with approximately 10 µg plasmid DNA for 30 minutes. Electroporation was performed in a 0.2 cm cuvette (Bio-Rad Ltd, Hemel Hempstead, UK) at 2.5 kV, 100 Ω, 25 µF and 900 µL ice-cold Friis medium were immediately added. After 15 minutes incubation on ice, cells were transferred to a 1.5 mL tube and incubated at 37 °C for 3 hours. Culture was then plated onto Friis agar containing tetracycline and incubated at 37 °C in 5% CO₂ for up to 18 days. Individual tetracycline-resistant transformants were picked using a sterile pipette tip into Friis broth medium containing tetracycline and grown for 5-7 days until evidence of growth as determined by the pH indicator.

Determination of transposon insertion sites

Transposon insertion site determination was based on the method described by Chaudhuri et al (Chaudhuri et al., 2009). Genomic DNA was extracted from *M. hyopneumoniae* strain 232 culture using a phenol-chloroform method (Bashiruddin, 1998). 2.5 µg DNA were digested overnight at 37 °C with 10 units of AluI, which cuts the 892 kbp genome of *M. hyopneumoniae* strain 232 a total of 2239 times and generates blunt ends. AluI also cuts within the *tetM*-containing transposon sequence six times. The digested DNA was purified using a MinElute PCR Purification kit (Qiagen Ltd,

Manchester, UK). Oligonucleotide linkers were attached to the digested DNA fragments. To generate the linker, 10 μ M oligonucleotides Linker-A and Linker-B (table 1) were heated together in a boiling water bath for 3 minutes in annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0) and then allowed to cool for 1 hour at room temperature. 50 ng of Alul digested DNA were blunt-ligated to 4 μ L annealed oligonucleotides Linker-A and Linker-B in a 10 μ L reaction using a Quick Ligation kit (NEB Ltd, Hitchin, UK) for 1 hour at room temperature. The ligated DNA was purified using a MinElute PCR Purification kit (Qiagen Ltd, Manchester, UK). PCR was performed using HotStarTaq (Qiagen Ltd, Manchester, UK) according to the manufacturer's instructions. Thermocycler conditions were as follows: 95°C for 15 minutes; 33 cycles of 94 °C for 45 s, 55 °C for 60 s, 72 °C for 120 s; 72 °C for 10 minutes. PCR products were visualised by electrophoresis in 1.6% agarose. Transposon insertion sites were determined by the direct sequencing of PCR products.

Southern analysis

Southern blotting was performed based on previously described methods (Maglennon et al., 2013; Example 1). Total DNA was extracted from 20 mL mycoplasma broth culture using a phenol-chloroform method (Bashiruddin, 1998; Sambrook and Russell, 2001) and 2.5 μ g were digested to completion with HindIII. DNA was separated by electrophoresis on 0.9% agarose, blotted onto Hybond-N+ membrane (GE Healthcare Ltd, Little Chalfont, UK) and then fixed to the membrane by exposure to UV light. A digoxigenin (DIG)-labeled probe specific for the tetM gene was generated from PCR-amplified DNA (primers TetMF and TetMR, Table 2) using a DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science Ltd, Burgess Hill, UK). This kit was also used to perform pre-hybridisation and hybridisation in accordance with the manufacturer's instructions. The membrane was autoradiographed at room temperature using CL-XPosure Film (Fisher Scientific Ltd, Loughborough, UK).

Results

Construction of transposon delivery vectors

Plasmid pTn4001-RVC1 (figure 4A) was constructed containing the Tn4001 transposase and a mini Tn4001 transposon consisting of the tetM gene under control of the spiralin gene promoter sequence of *Spiroplasma citri* bounded by inverted repeats. We previously optimised a set of transformation conditions for *M. hyopneumoniae* using an oriCoriC-based self-replicating plasmid system, and showed that tetM under control of

the spiralin gene promoter region was successfully expressed in *M. hyopneumoniae* strain 232 allowing the selection of transformants on Friis agar plates containing 0.2 µg/ml tetracycline (Maglennon et al., 2013; Example 1). However, despite three separate attempts at transforming *M. hyopneumoniae* strain 232 with pTn4001-RVC
5 using our optimised conditions and our *oriC* plasmid pMHO-2 (Maglennon et al., 2013; Example 1) as a positive control for transformation, we obtained no transformants. This suggested that the Tn4001 transposase was not functional in *M. hyopneumoniae*.

A Himar1 transposon delivery vector (pMiniHimar1BSC1) has been prepared that
10 successfully generated transposon insertion mutants in the *M. gallisepticum* R_(low) strain and very low numbers of transposon insertion mutants in *M. hyopneumoniae* strain 232 (Cook PhD thesis prepared 2013 but not in the public domain [and example 3]). pMiniHimar1BSC1 consists of the Himar1 transposase under control of the promoter sequence from Tn4001 and a transposon carrying the *pac* gene conferring resistance to
15 puromycin (figure 4B). Low numbers of mutants (less than 10 per transformation) were generated in *M. hyopneumoniae* using this vector, but transformation frequencies were very low and protracted recovery times (several days) were required after transformation prior to selection of transformants on solid medium. Thus, the system is functional, but higher transformation frequencies would be desirable. We attempted to transform *M.*
20 *hyopneumoniae* with pMiniHimar1BSC1 and using a self-replicating *oriC* plasmid (pMHOpuro) as a positive control for transformation (Maglennon et al., 2013; Example 1). Approximately 10⁶ CFU were transformed with pMiniHimar1BSC1 and with pMHOpuro. Plasmid pMiniHimar1BSC1 failed to generate any puromycin resistant transformants compared to an average of 1.5 x 10⁻⁴ transformants/CFU for the
25 pMHOpuro positive control. To determine whether *tetM* selection would increase the yield of transformants, *pac* was replaced with *tetM* in plasmid pMHWT-2 (figure 4C). An alternative hypothesis for the paucity of transformants was that the Tn4001 promoter sequence controlling Himar1 transposase expression was poorly active in *M. hyopneumoniae*, even although there had been no need to replace the Tn4001 promoter sequence in other systems in which this transposase had been used. Hence pMHWT-1
30 (figure 4D) was made by replacing the Tn4001 promoter sequence regulating Himar1 with the P97 gene promoter of *M. hyopneumoniae* strain 232 (Maglennon et al., 2013; Example 1). Plasmid pMHC9-1 (figure 4E) was constructed by replacing the wild-type Himar1 transposase with the C9 mutant form which exhibits increased activity in vitro
35 (Lampe et al., 1999). Additionally, as a positive control for transformation, mycoplasmas were transformed with the *oriC*-based plasmid pMHO-2 (containing *tetM*). Approximately 10⁶ CFU were transformed with pMHWT-2, pMHWT-1, pMHC9-1 and pMHO-2 and

selection plates examined after incubation for 14 days. Plasmid pMHWT-2 failed to generate any tetracycline resistant colonies compared to an average of 4.6×10^{-5} transformants/CFU for the pMHO-2 positive control. An average of 123 colonies per transformation were found for pMHC9-1 containing the hyperactive C9 mutant transposase compared to 33 colonies for pMHWT-1 containing the wild-type transposase. Transformation was repeated using pMHWT-1 and pMHC9-1 but with approximately $\times 10^8$ CFU per transformation. Again, more transformants were generated with the hyperactive transposase (33 per transformation) compared to the wild-type transposase (10 per transformation). Transformation of an increased number of cells resulted in a drop in the overall transformation efficiency. However, our experiments suggested that the hyperactive C9 mutant Himar1 transposase could account for a 3-4-fold increase in transformation efficiency compared to the wild-type transposase. Morphologically, tetracycline-resistant mycoplasma colonies grown on Friis agar from transformed cells had a uniform appearance that resembled that of colonies grown from non-transformed cells (figure 5). However, transformants took approximately 3-5 days longer to reach the same colony size as non-transformed cells. Transformed cells formed discrete and well-separated colonies allowing the isolation of individual colonies. Additionally, colonies were relatively non-adherent to the agar surface and could be easily removed by gentle washing with Friis medium. Importantly, in none of the experiments described did we observe colonies in our 'no DNA' control transformations, indicating the absence of pseudo-resistance or spontaneous resistance to tetracycline.

Analysis of transformants

Transformation of *M. hyopneumoniae* strain 232 with plasmid pMHC9-1 consistently generated tetracycline resistant colonies on Friis agar containing tetracycline. To confirm that tetracycline resistance was due to transposition into the host chromosome and not, for example, due to maintenance of extrachromosomal plasmid DNA, 15 individual colonies from pMHC9-1 transformed *M. hyopneumoniae* strain 232 were analysed. Each was transferred into 1 ml Friis broth medium containing 0.5 $\mu\text{g/mL}$ tetracycline. All transformants grew within 5 days, confirming the maintenance of tetracycline resistance upon sub-culturing into liquid medium while no growth was observed for untransformed controls. PCR amplification of tetM from total DNA extracts prepared from 20 ml culture confirmed the presence of tetM in all transformants but not un-transformed *M. hyopneumoniae* strain 232 (figure 6A). In addition, PCR for a region of the pUC19 plasmid backbone failed to amplify DNA for any transformant showing that tetracycline resistance was not due to maintenance of extrachromosomal plasmid DNA,

or due to integration of the entire plasmid, for example by homologous recombination at the P97 promoter sequence site (figure 6B).

Analysis of transposon insertion sites

5 The presence of *tetM* and lack of plasmid backbone suggested that transposition of the transposon into the host chromosome had occurred. To locate the site of transposition in randomly selected transformants, a linker PCR technique was employed. Eleven individual tetracycline resistant colonies were colony-purified on Friis agar and cultured in Friis medium containing 0.5 ug/mL tetracycline. PCR was used to amplify across the
10 site of insertion from the *tetM* to the linker. This was separately performed on DNA extracts using primer pairs to recognise both ends of the transposon. Analysis showed that a single dominant band was amplified for each transformant using one of the oligonucleotide primer pairs, consistent with integration of the transposon into the host chromosome at one site only. Occasionally, larger faint bands were also present, most
15 likely due to the use of low annealing temperatures for PCR amplification, long extension times, a high number of cycles and incomplete digestion of chromosomal DNA by the restriction enzyme *AluI*. For several transformants, no PCR product was obtained with one of the two oligonucleotide primer pairs, but in all cases, where one primer failed, a PCR product was generated with the other primer. Therefore, it was possible to confirm
20 transposon insertion for all of the 11 transformants. Additionally, the stability of transposon insertion sites following multiple serial passages of individual transformants in non-selective Friis medium was determined. Following a total of 15 passages in Friis medium containing no tetracycline all individual transformants retained their resistance to tetracycline upon subsequent culture in medium containing tetracycline. Linker PCR was
25 repeated for each individual transformant, and agarose gel electrophoresis showed that the amplicons generated were identical in size to those obtained prior to serial passaging. Direct DNA sequencing of PCR amplicons was performed and this confirmed that the transposon had inserted into the host cell chromosome between thymine and adenine residues as expected. Where sequencing at both ends of the
30 transposon was possible, the transposon insertion sites were found to match. Southern analysis was performed on total DNA extracted from the same 11 transformants to confirm that only single transposon insertions were present. For 10 of the mutants, a single band was present using a DNA probe specific for *tetM* contained within the transposon (figure 7). For mutant 1, a small intense band was present along with a
35 larger faint band. Only one insertion site was identified for this transformant by linker PCR and this insertion site was confirmed by DNA sequencing. It is possible that two

bands were generated by Southern analysis by a failure to adequately digest the DNA to completion with HindIII.

Insertion sites of transposon mutants

5 Insertion site sequencing of a further 33 individual transformants was performed so that the locations of a total of 44 transformants were mapped to the *M. hyopneumoniae* strain 232 genome (figure 8). Of these 44 transformants, 41 contained unique insertion sites (table 4). Mutants 49, 68 and 161 all contained an insertion at the nucleotide 549968 in the *M. hyopneumoniae* strain 232 genome and mutants 56 and 66 contained identical
10 insertion sites at nucleotide 574428. It was not possible to determine whether these mutants arose from a single transposition event or from multiple events at the same location. Following electroporation, mycoplasmas are given 3 hours to recover and express *tetM* in Friis broth without tetracycline prior to plating onto Friis agar containing tetracycline. It is possible that a single transposition event was able to undergo several
15 rounds of multiplication during this period. In total, transposon insertions were present in 22 open reading frames (ORFs) and 5 non-coding regions (NCRs). For 5 ORFs, there was more than one transformant containing an insertion. The average size of an ORF in the *M. hyopneumoniae* genome is 388 amino acids (aa). All 5 of these ORFs were larger than 388 aa and it is therefore possible that more than one transformant was
20 generated due to the larger size of these ORFs. However, a total of 11 transformants contained a transposon insertion in mhp447. Although this ORF is particularly large at 3970 aa, representing 1.3% of the *M. hyopneumoniae* strain 232 genome, it is likely that this represents a 'hotspot' for transposon insertions. Similarly, 4 transformants contained insertions in the smaller pepF gene (608 aa) and this too may be over-represented.
25 Most of the transposon insertions are in genes of unknown function but that appear to be dispensable for axenic growth, or in NCRs. Of the insertions in genes of known function, three were in the P97 ciliary adhesin and the P95 outer membrane protein. Insertions were found in 3 genes encoding phosphotransferase system subunit proteins specific for fructose and ascorbate and in the molecular chaperone Trigger Factor. Several
30 insertions were also identified in the pepF gene encoding oligoendopeptidase F and the pepP gene encoding a proline aminopeptidase. These are two of a small number of proteases that may be associated with processing of *M. hyopneumoniae* cell surface proteins (Burnett et al., 2006).

Generation of a large pool of *M. hyopneumoniae* mutants

We sought to determine whether our pMHC9-1 transposon delivery vector could be used to produce a large pool of random mutants, sufficient to provide an adequate number of

'hits' in all of the non-essential genes of *M. hyopneumoniae*. A pool of at least 10,000 individual mutants would be expected to give a very high probability of an insertion into every one of the 692 protein coding sequences in the *M. hyopneumoniae* 232 genome determined by Minion et al (Minion et al., 2004). It was calculated that a pool of 10,000 individuals would, on average, contain a mycoplasma cell with an insertion every 89 bp, and that there would be 14.45 insertions per coding sequence. From the Poisson distribution, 10,000 independent insertions would give a 99.99995% probability of an insertion into each one of the 692 coding sequences. 20 individual transformations were performed using plasmid pMHC9-1 with approximately 10^6 CFU per transformation. After 14 days growth at 37°C on agar plates containing tetracycline, colonies were counted and harvested together in one large pool. A total of 11,759 colonies were counted across all 20 plates, representing an average transformation frequency of 3.4×10^{-4} transformants/CFU (standard error 5.3×10^{-5}).

Discussion

The inability to genetically manipulate *M. hyopneumoniae* has stood as a hurdle to advancements in the understanding of the pathogenesis of enzootic pneumonia in pigs. We have reported the successful transformation of *M. hyopneumoniae* using an artificial plasmid system containing the origin of replication of *M. hyopneumoniae* and an antimicrobial resistance cassette, that was capable of self-replicating and maintenance in transformed bacteria (Maglennon et al., 2013; Example 1). We have used this system to optimise a number of conditions for the successful transformation of *M. hyopneumoniae* by electroporation with plasmid DNA. This knowledge has now assisted in the design and construction of a transposon-based system for generating random mutants in *M. hyopneumoniae*.

The Tn4001 transposon is effective in generating insertional mutations in a number of different Mycoplasma species (Chopra-Dewasthaly et al., 2005; French et al., 2008; Hedreyda et al., 1993; Pour-El et al., 2002) but even using our optimised transformation conditions, we were unable to demonstrate transposition in *M. hyopneumoniae*. A Himar1 transposon delivery vector that was shown to be active in *M. gallisepticum* generated very low numbers of transformants in *M. hyopneumoniae* with a very restricted range of unique insertion sites (Cook, thesis; Example 3). By using an *M. hyopneumoniae* strain 232-specific promoter sequence to control the Himar1 expression and (optionally) switching the wild-type Himar1 transposase for a hyperactive mutant form, the frequency of transposition increased significantly. Analysis of transformants confirmed that resistance to tetracycline was due to a transposition event into the

mycoplasma genome and it appeared that transposition occurred at a single site in each individual transformant. The transposon insertions appeared to be stable over serial passages and were maintained even in the absence of antimicrobial selection. These features are desirable when the phenotypes of mutants are studied in vitro, and particularly for in vivo studies of pools of mutants by TraDIS or signature-tagged mutagenesis. Additionally, we did not encounter any "pseudoresistant" colonies in our "no DNA" control transformations, which have been documented in transposon mutagenesis systems described for other mycoplasmas (Chopra-Dewasthaly et al., 2005; Cook, 2013). "Pseudoresistant" colonies can complicate the isolation of true transformants.

In the course of this study, it was evident that there were significant differences in the transformation frequencies obtained using plasmid pMHC9-1. We suspect that differences in transformation frequency can be partially accounted for by variations in the number of cells electroporated and/or the growth phase of the mycoplasma culture used. It appeared that an increase in the number of cells transformed did not necessarily result in an increase in transformation frequency. However, even where similar numbers of cells were electroporated, there could be significant differences in transformation frequencies, implying that other factors could be important. Growth of *M. hyopneumoniae* is assessed and monitored by a simple change in the phenol red pH indicator incorporated into the medium, owing to the fact that turbidity is not generated. Thus pH can serve as a rough guide for growth phase, but the actual number of cells can only be estimated after plating the culture out and allowing growth on agar medium for 5-7 days. Additionally, complex and undefined medium is used to grow *M. hyopneumoniae*, with 20% equine/porcine serum and freshly prepared yeast extract contributing to variations in batches of medium. Thus, it may be necessary to repeat transformations, for example with some variation of these parameters, in order to optimise transformation in a particular setting, should such optimisation be desirable.

Transposon insertion sites were determined for a limited number of individual transformants using a linker PCR technique and direct sequencing of PCR products by Sanger sequencing. From our limited analysis, we found transposon insertions in a number of *M. hyopneumoniae* genes of both known and unknown function, and in non-coding regions. We observed a clustering of transposon insertions in mhp447, a large open-reading frame of unknown function. The Himar1 transposon inserts at 5'-TA-3' dinucleotides and we considered that the large number of insertions may be due to an increase in AT content. However, analysis of this ORF showed that the AT content was

not unusual at 0.681 compared to 0.714 for the entire *M. hyopneumoniae* strain 232 genome. Alternatively, it is possible that there are hotspots for Himar1 insertions in the *M. hyopneumoniae* strain 232 genome, that may be affected by factors such as DNA topology, as is the case with other transposons, including Mariner elements (Claeys
5 Bouuaert and Chalmers, 2013). It is difficult to draw any conclusions from analysis of such a small pool of mutants, but determination of the insertion sites of a large pool of mutants by next generation sequencing would answer the question of whether favoured insertion sites do occur. Using plasmid pMHC9-1, we were able to produce a large pool of mutants. Over 10,000 colonies were generated through a number of transformations
10 that, given an even distribution should be expected to have a high probability of insertions into all of the non-essential genes of *M. hyopneumoniae*. This pool of mutants may prove a valuable tool in future studies of *M. hyopneumoniae* in allowing a list of minimal essential genes to be determined. Such a pool of mutants could also be subjected to an in vivo screen for attenuated mutants using powerful functional genomic
15 techniques such as TraDIS.

Conclusion

A novel transposon delivery constructed has been generated for *M. hyopneumoniae*, allowing for the first time the production of large numbers of random transposon insertion
20 mutants. This system has been used to generate mutants with random transposon insertions in individual *M. hyopneumoniae* protein coding and non-coding sequences. It has also been used to generate a large pool of over 10,000 transposon insertions. These mutants are powerful tools in elucidating the functions of *M. hyopneumoniae* genes, and in turn unraveling the pathogenesis of this important swine pathogen. The
25 system is also considered to be useful for other mycoplasmas.

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Table 2

Sequences of oligonucleotides

Oligonucleotide	Restriction sites	Oligonucleotide sequence (5'-3')
TetM1	PstI	GCCGCTGCAGAATTAAGTTAGTG
TetM2	XbaI	GAAATCTAGATTATATAACAACTTAAATTAC
TetM3	SbfI	CGATCCTGCAGGCAGAATTAAGTTAGTG
TetM4	MluI	CAGTACGCGITTTATATAACAACTTAAATTAC

Tpn1	NdeI/NcoI	GCGCATATGCCATGGAAAAAAGGAATTCGTG
Tpn2	XhoI	CGCCACTCGAGATTATTCAACATAGTTCCTTC
P97-1	NdeI	CGATCATATGACGGGGATTATAAACAGAAAC
P97-2	NcoI	GAATCCATGGCACCAACAATTCCGGCAGTC
TetMF	N/A	GTGGACAAAGGTACAACGAG
TetMR	N/A	CGGTAAAGTTCGTACACAC
Linker-A	N/A	CGACTGGACCTGGA
Linker-B	N/A	GATAAGCAGGGATCGGAACCTCCAGGTCCAGTCG
L-PCR-C	N/A	GATAAGCAGGGATCGGAACC
L-PCR-L	N/A	GATAGGGTTGAGTGTGTTC
L-PCR-R	N/A	TAGTCGGATAGATAAAGTAC

Sequences of oligonucleotides used in the study are shown. For PCR, forward (F) and reverse (R) oligonucleotides are given with a description and size of the amplicon generated. Where appropriate, the restriction enzyme recognition sites added to the 5' end of the oligonucleotides are underlined.

Table 3

Transformation frequencies using Himar1 constructs

Plasmid	Mean transformation frequency (transformants/CFU) [SE]	
	Experiment 1	Experiment 2
pMHWT-2	0	NT
pMHWT-1	2.5×10^{-6} [1.3×10^{-6}]	9.0×10^{-8} [2.4×10^{-8}]
pMHC9-1	9.3×10^{-6} [4.5×10^{-6}]	3.0×10^{-7} [4.6×10^{-8}]

- 10 Mean transformation frequencies (with standard errors) are shown for three separate transformations of *M. hyopneumoniae* strain 232 using plasmid constructs pMHWT-1, pMHWT-2 and pMHC9-1 in two separate experiments. In experiment 1, 10^6 CFU were transformed with each plasmid while in experiment 2, 10^8 CFU were transformed.

Table 4

Insertion sites of *M. hyopneumoniae* strain 232 mutants

Mutant	Transposon Insertion location	ORF disrupted	Gene function
72	84920	Mhp069	Unknown
11	85345	Mhp069	Unknown
40	259306	Tig	Trigger factor
159	275721	NCR	Unknown
50B	284811	Mhp261	Unknown
4	299711	Mhp271	P97 ciliary adhesion
96	318725	P95	Outer membrane protein
165	318913	P95	Outer membrane protein
91	359210	Mhp313	Unknown
9	434381	Mhp366	Unknown
34	466299	ulaA	PTS system ascorbate-specific transporter subunit IIC
37	522533	Mhp443	Unknown
54B	527019	Mhp445	Unknown
156	537514	Mhp446	Unknown
75	540819	Mhp447	Unknown
53B	541339	Mhp447	Unknown
102	542436	Mhp447	Unknown
90	542541	Mhp447	Unknown
1	544071	Mhp447	Unknown
78	544309	Mhp447	Unknown
24	544767	Mhp447	Unknown
5	545264	Mhp447	Unknown
68	549968	Mhp447	Unknown
49	549968	Mhp447	Unknown
161	549968	Mhp447	Unknown
52B	551617	NCR	Unknown
13	552503	NCR	Unknown
56	574428	NCR	Unknown
66	574428	NCR	Unknown
17	608509	Mhp490	PTS system fructose-specific transporter subunit IIABC
16	652232	pepF	oligoendopeptidase F

162	653224	pepF	oligoendopeptidase F
101	653333	pepF	oligoendopeptidase F
92	653430	pepF	oligoendopeptidase F
47	672237	Mhp535	Unknown
170	672318	Mhp536	Unknown
63	672979	Mhp537	Unknown
50	686279	Mhp542	Unknown
19	722926	ulaA	PTS system ascorbate-specific transporter subunit IIC
2	759509	NCR	Regulatory region of glpD
10	788260	Mhp630	Unknown
7	788457	Mhp630	Unknown
12	790862	Mhp631	Unknown
51B	856887	pepP	xaa-pro aminopeptidase

The locations of transposon insertions within the *M. hyopneumoniae* strain 232 genome for 44 individual pMHC9-1 transformants are shown. The open reading frame (ORF) or non-coding region (NCR) containing the insertion is shown and, where known, the function of the protein encoded.

Example 4: In vitro and in vivo screening of *M. hyopneumoniae* mutant pool

Having generated a set of genetic tools for *M. hyopneumoniae*, these techniques can be used to study the pathogenesis of the organism further.

Determination of the minimum core genes required for *in vitro* growth

We have produced a large number of random *M. hyopneumoniae* mutants using the pMHC9-1 transposon system. All of the mutants generated are capable of growth in axenic growth conditions. By performing TraDIS (transposon directed insertion site sequencing: see, for example Langridge G C, Phan M-D, Turner D J et al. (2009) Simultaneous assay of every *Salmonella typhi* gene using one million transposon mutants. Genome Research Available online at: <http://dx.doi.org/10.1101/gr.097097.109>) on the entire pool of mutants, we will know the locations of the transposon inserts for each of the individual mutants. Identification of the insertion sites gives us a list of individual ORFs that are

dispensable for growth in such conditions, and leaves us with a list of 'core genes' that are essential for growth. *M. hyopneumoniae* already has a very small genome, and mycoplasmas themselves are the smallest known organisms capable of self-replication. Thus defining a minimum complement of genes in a bacterium with a minimal genome is of intense interest

In defining the minimum set of genes necessary for growth *in vitro*, it is possible to deduce which genes are not necessary for *in vitro* growth, and that these genes may therefore have a role in causing disease/survival in the animal. These genes may therefore serve as potential vaccine candidates.

Determination of *M. hyopneumoniae* pathogenicity factors

Techniques can be used in identifying genes that are necessary for growth *in vivo* but that are dispensable for growth *in vitro*. For example, a large pool of pMHC9-1 *M. hyopneumoniae* mutants (for example) is screened in pigs. The pool of mutants is grown up in broth culture and the insertion sites of all of the individual mutants determined by TraDIS. The entire pool of mutants is then used to infect several pigs. After 3, 6, and 9 days, individual animals are culled and the lungs harvested. *M. hyopneumoniae* organisms are re-isolated from the lungs by culturing in broth and the transposon insertion sites of all of the residual mutants are determined by TraDIS. By a 'subtraction' of the output pool of mutants from the input pool, mutants are identified that have lost ability to survive in the pig. Such mutants may have transposon insertion sites in genes that represent potential virulence factors for *M. hyopneumoniae*, and these may be useful as candidates for vaccine studies.

We have characterised the transposon insertion sites of a large pool of transposon mutants by next generation sequencing, and have found that there are approximately 33700 independent transposon insertions into the genome of *M. hyopneumoniae* 232. A number of the genes of 232 have no, or very few, insertions indicating that they may be essential genes. Some have a number of insertions less than might be expected indicating that the mutation may render the organism less fit to grow and compete with the others.

We have also shown in a preliminary study that we are able to infect pigs with a large pool of mutants and then recover mutants 3-8 days later from animals at post-mortem.

Example 5

Method for gene targeting

A self-replicating plasmid was produced (pOriC-iv/v) (Example 1) that contains the minimum functional *oriC* of *M. hyopneumoniae* strain 232. The plasmid is stably maintained in transformed *M. hyopneumoniae* 232 cells when selection for *tetM* expression is present using tetracycline, but the cells are rapidly 'cured' of the plasmid when selection is removed. This plasmid was used as part of a strategy to generate targeted gene disruptions in *M. hyopneumoniae* strain 232 by promoting homologous recombination within selected regions of the chromosome. It was anticipated that incorporation of a region of homology of approximately 1000 bp into the plasmid (for example between 700, 800 or 900 and 1100, 1200 or 1300 bp, for example between 800 and 1200 bp) would promote integration of the entire plasmid including *tetM* resistance cassette into the *M. hyopneumoniae* chromosome, with recombinants selected for using tetracycline. Longer regions of homology could be used, but it is considered that it is not necessary to use a length greater than 1000 bp. Using the minimum *oriC* plasmid pOriC-iv/v, we generated plasmid pOriCP146 by inserting a 974 bp homologous region of the P146 gene of *M. hyopneumoniae* strain 232 (figure 9A). Containing the *oriC* of *M. hyopneumoniae* this plasmid is maintained by transformed cells over serial passages in the presence of tetracycline, and was hypothesised to increase the frequency of homologous recombination.

Initially we tried using a simple suicide vector to generate targeted gene disruptions. This consisted of a pGEM-T plasmid vector and *tetM* resistance cassette along with a 974 bp region of the P146 ciliary adhesion gene of *M. hyopneumoniae*. It was anticipated that incorporation of a region of homology of approximately 1000 bp into the plasmid would promote integration of the entire plasmid including *tetM* resistance cassette into the *M. hyopneumoniae* chromosome, with recombinants selected for using tetracycline. Following repeated attempts, no tetracycline resistant organisms were grown, suggesting that successful integration of the plasmid DNA had not occurred. We hypothesised that homologous recombination may be an infrequent event in *M. hyopneumoniae*, and that the frequency may be increased by exposing the cells to plasmid DNA containing the homologous sequence for a prolonged period of time. Using the minimum *oriC* plasmid pOriC-iv/v, we generated plasmid pOriCP146 by inserting the same 974 bp homologous region of the P146 gene of *M. hyopneumoniae* strain 232 (figure 1). Containing the *oriC* (functional origin of replication) of *M. hyopneumoniae* this plasmid should be maintained by transformed cells over serial passages in the presence of tetracycline, affording a wider window for homologous

recombination to occur. The particular region of the P146 gene targeted for homologous recombination was chosen such that integration of plasmid DNA would lead to a disruption in the open reading frame and production of a truncated protein (figure 9A).

Having demonstrated that plasmid integration could be targeted to a selected region within the P146 gene, two further target regions within the *M. hyopneumoniae* 232 genome were selected: the pepF gene encoding the protein oligoendopeptidase F and the open reading frame of mhp379, which encodes a nuclease and is part of an operon involved in nucleotide acquisition.

Materials and Methods

A 974 bp region of the *M. hyopneumoniae* strain 232 P146 ORF was amplified by PCR using primers P146F and P146R. These primers incorporate *MluI* and *PstI* restriction enzyme recognition sites at their 5' ends respectively to facilitate cloning of the fragment into plasmid pOriC-iv/v, generating plasmid pOriCP146. Plasmid pOriCpepF was generated by cloning a 948 bp region of the pepF ORF, produced by PCR using primers pepF-F and pepF-R, into the same *MluI* and *PstI* restriction sites of pOriC-iv/v. Plasmid pOriC379 was generated by cloning a 549 bp fragment of the mhp379 ORF, produced by PCR using primers mhp379-F and mhp379-R, into pOriC-iv/v. *M. hyopneumoniae* strain 232 cells were cultured and prepared for transformation as previously described. Mycoplasmas were electroporated (2.5 kV, 25 μ F, 100 Ω) with 7.5 μ g of plasmid DNA, for example pOriCP146, in a 0.2 cm electroporation cuvette and following recovery for 3 hours in Friis broth medium, were plated onto Friis agar containing 0.2 μ g/ml tetracycline. Plates were incubated for approximately 14 days and then examined for the presence of mycoplasma colonies using a stereoscopic microscope. Colonies were picked using a sterile pipette tip into Friis broth medium containing 0.5 μ g/ml tetracycline and cultured for 3-5 days. In some instances they were then subcultured a further six times with 0.5 μ g/mL tetracycline followed by six times in Friis broth medium without tetracycline. After the final subculture, organisms were plated out onto Friis agar containing 0.2 μ g/mL tetracycline.

At various time points, mycoplasmas were grown in 10 or 20-30 mL Friis broth cultures and pelleted by centrifugation at 9000 xg. Total DNA was extracted using a phenol-

chloroform method (Sambrook & Russell 2001) and plasmid DNA was extracted using a PureLink Mini Plasmid Purification Kit (Invitrogen Ltd, UK). Plasmid DNA was analysed by agarose gel electrophoresis using a 1% w/v gel. Total DNA was analysed by Southern analysis using standard methods (Sambrook & Russell 2001) and as described in Maglennon *et al* 2013. Briefly, approximately 2.5 µg/ml DNA were digested to completion with *Hind*III, separated by electrophoresis on 1% w/v agarose and transferred onto Hybond N+ membrane (GE Healthcare Ltd, UK). Hybridisation was performed using a DIG-labelled DNA probe that anneals to a region of the *tetM* resistance cassette in plasmid pOriCP146. Alternatively, total DNA was analysed for the presence of plasmid integration by using a PCR strategy to amplify regions of DNA spanning the presumed integration sites. PCR was performed using a Qiagen HotStarTaq kit (Qiagen Ltd, UK) according to the manufacturers instructions.

Table 1: Oligonucleotide primer sequences

Primer name	Primer sequence 5'-3'
P146F	CAGC <u>ACGCGT</u> GAACTTGTCTGTGGAGCTAC
P146R	GCAT <u>CTGCAGT</u> CCGATGTCTTTGATAATTC
pepF-R	
pepF-F	
Mhp379-F	
Mhp379-R	
Mhp379-intF	CAAGACCAGCTTTGACAATTTC
Mhp379-intR	ATACTCAAGCTATGCATCCAAC
P146-intF	ATTCGTTATAAAGGTGAAAATC
P146-intR	ATACTCAAGCTATGCATCCAAC
P146-intF2	GTTAGATCAAGCTGAAACACTTG
P146-intR2	TTCAGAACTGGTGCTTCACTGT
pepF-intF	TTGAATCAACATAATGATAGTC
pepF-intR	ATACTCAAGCTATGCATCCAAC
pepF-intF2	GGTTCAGAACTGGTGCTTCAC
pepF-intR2	CTGAAATTCCTGAGAAATACCG

Sequences of oligonucleotide primers are shown with restriction sites highlighted in bold and underlined.

Results

5

Following the transformation of *M. hyopneumoniae* strain 232 cells with plasmid pOriCP146, many tetracycline resistant colonies (>300) were observed after 14 days growth on Friis agar containing tetracycline in three identical triplicate repeats. Plasmid pOriC-iv/v generated similar numbers of colonies but no colonies were observed for the 'no DNA' mock transformations. From the pOriCP146 transformation, three colonies were selected at random and were picked into Friis agar containing 0.5 µg/ml tetracycline. Colonies underwent serial passage as described in the materials and methods section. Analysis of plasmid DNA confirmed the presence of plasmid pOriCP146 following transformation of *M. hyopneumoniae* and isolation of three individual transformants after growth on agar plates (Figure 9B). After a further 6 passages with tetracycline selection pressure maintained, plasmid DNA had reduced considerably and following a further 6 passages without tetracycline (passage 13), no plasmid DNA was present at all. However, all three transformants subsequently demonstrated tetracycline resistance when tetracycline was reintroduced into broth cultures and agar plates. It was also possible to detect the *tetM* resistance cassette and plasmid backbone in transformants by PCR, suggesting that the failure to detect extrachromosomal plasmid DNA may be because it had integrated into the chromosome of the mycoplasma. To confirm this, total DNA extracts from each transformant were subjected to Southern analysis. At passage 1 following isolation of the individual colonies with tetracycline selection, extrachromosomal plasmid DNA was readily detectable corresponding to a 6.9 kbp band (Figures 9C & 9D). At passage 6, there was a reduction in free extrachromosomal DNA and an alternative band was present at 8.7kbp representing plasmid DNA that had integrated into the chromosome of *M. hyopneumoniae* by homologous recombination (Figures 9C & 9D). By passage 13 (after 7 passaged with tetracycline followed by 6 passages without) no extrachromosomal plasmid DNA was detectable and only plasmid DNA intergrated into the *M. hyopneumoniae* chromosome was present (Figure 9D).

Further, individual colonies were picked from agar plates transformed with pOriCP146 and pOriC-iv/v and were grown in Friis broth containing 0.5 µg/ml tetracycline. Plasmid DNA was extracted from approximately 10 mL broth culture and confirmed the presence of plasmid pOriC-iv/v in six randomly selected clones. (Figure 10). However, free

plasmid DNA was not readily detectable in four randomly selected pOriCP146 clones, despite successful growth in the presence of tetracycline. It was suspected that the plasmid DNA containing the tetM resistance cassette was still present, but that it had integrated into the *M. hyopneumoniae* 232 chromosomal DNA. Each clone was

5 passaged a further 19 times in Friis broth containing tetracycline to select for further plasmid integration, followed by a further 6 passages without tetracycline to promote 'loss' of extrachromosomal plasmid DNA. Subsequently, all four clones continued to demonstrate retention of tetracycline resistance when tetracycline selection pressure was re-introduced. Furthermore, it was not possible to detect extrachromosomal DNA in

10 any clone using a simple plasmid extraction technique (data not shown). To show that the retention of tetracycline resistance was due to integration of plasmid DNA into the *M. hyopneumoniae* 232 chromosomal DNA at the P146 gene target site by homologous recombination, a PCR approach was adopted. Using two sets of PCR oligonucleotide primers (primer set A (P146-intF / P146-intR) and set B (P146-intF2 / P146-intR2)), we

15 attempted to amplify DNA spanning the suspected integration site at either end of the integrated plasmid (figure 10). In all four colonies, a PCR product of the correct anticipated size was generated, but no PCR product was generated for control reactions consisting of circular plasmid DNA template and genomic DNA from untransformed *M. hyopneumoniae* 232. Additionally, the PCR products generated were sequenced which

20 confirmed that plasmid DNA had indeed integrated into the chromosomal DNA at the target site.

A similar strategy was used to disrupt mhp379 (figure 11A) and pepF (figure 12A) as was used for P146 with the targeted integration site lying around 100 bp downstream from

25 the start codon such that plasmid integration would only produce a truncated protein. However, mhp379 is only 933 bp long, thus it was not possible to select an approximately 1000 bp region of homology as a target integration site, thus a much shorter approximately 550 bp region was chosen.

30 Following transformation of *M. hyopneumoniae* 232 with plasmids pOriCpepF and pOriC379, many tetracycline resistant colonies were generated on Friis agar containing 0.2 µg/mL tetracycline yet as expected no colonies were generated for 'no DNA' controls. Individual colonies were randomly selected and grown in Friis broth medium containing 0.5 µg/mL tetracycline. Plasmid DNA was extracted from approximately 10 mL culture

35 grown from five or six colonies for each plasmid and analysis showed that plasmid DNA could readily be identified for all clones (data no shown). Mycoplasmas were then subcultured a further 19 times in Friis medium containing 0.5 µg/mL tetracycline and a

further plasmid DNA extract performed. Analysis by agarose gel electrophoresis showed that plasmid pOriC379 could be identified in all five clones, demonstrating that the continued tetracycline resistance was likely to be due, at least in part, to the presence of extrachromosomal plasmid DNA (figure 11B). However, using PCR analysis (primers mhp379-intF and mhp379-intR), it was possible to amplify a region of DNA spanning the anticipated target site of integration (figure 11C). Thus we were able to demonstrate plasmid integration by homologous recombination in at least some of the mycoplasma cells within each culture. A further six passages were performed of each clone in the absence of tetracycline in an attempt to 'cure' the mycoplasmas of extrachromosomal plasmid DNA, and then tetracycline was re-introduced. However, at this time we were still able to readily detect extrachromosomal plasmid DNA (figure 11D). Further attempts were made to rid mycoplasmas of extrachromosomal plasmid DNA by performing further serial passages without tetracycline, followed by re-introducing tetracycline selection pressure and analysing mycoplasmas for the presence of extrachromosomal plasmid DNA versus plasmid integration (data not shown). It was concluded that while it was possible to detect integration events at the select target integration site, integration and ORF disruption was not entirely favourable to growth and survival of such cells, hence the continued presence of plasmid DNA in an extrachromosomal form.

Analysis of plasmid extracts from pOriCpepF transformed clones following 19 passages in Friis broth containing 0.5 µg/mL tetracycline showed that extrachromosomal plasmid DNA could only be readily identified in two clones and could not be easily identified in four of the clones (Figure 12). The continued presence of tetracycline resistance in the four clones in which extrachromosomal plasmid DNA could not be found suggested that tetM was still present but that plasmid integration into the mycoplasma chromosome had occurred. A further six passages were performed without tetracycline and then tetracycline selection was re-introduced. In the same four clones, it was still not possible to detect extrachromosomal plasmid DNA. PCR was performed using primers pepF-intF/pepF-intR (primer set C) and pepF-intF2/pepF-intR2 (primer set D) to amplify DNA spanning the targeted plasmid integration site within the *M. hyopneumoniae* 232 genome (figure 12). It was possible to detect PCR product for all six clones demonstrating that plasmid integration had occurred. Sequencing of the PCR products provided additional confirmation that integration had occurred at the anticipated target site. Thus in four pOriCpepF transformed clones, we were able to demonstrate that the continued presence of tetracycline resistance was associated with an absence of free extrachromosomal plasmid DNA and with integration of plasmid into the host chromosomal at a specific chosen target site within the pepF ORF.

5 Discussion

We have demonstrated targeted gene disruptions in *M. hyopneumoniae*. A self-replicating stably maintained *oriC*-based plasmid was utilised to promote homologous recombination within the chromosomal DNA of *M. hyopneumoniae* 232 cells transformed
10 with the plasmid, at selected target sites. Integration of the plasmid into the host chromosome was confirmed by an absence of free plasmid DNA but with retention of tetracycline resistance associated with *tetM* and by Southern analysis or PCR analysis of total DNA extracted from transformed cells showing amplification of plasmid integration sites. The location of the P146 ORF targeted for homologous recombination is such that
15 we are likely to have achieved disruption of the ORF leading to production of a truncated protein with altered or loss of function. It is possible that this mutant may exhibit an altered phenotype in *in vitro* assays of cellular attachment and/or *in vivo* pathogenesis testing. More importantly, this work illustrates that targeted gene disruptions are achievable in *M. hyopneumoniae*. Additionally, the targeting of exogenous DNA
20 sequences to specific endogenous sequences makes possible the ability to modify coding sequences with epitope tags or other mutations, rearrangements, fusions or deletions. For example incorporating a region of a gene into the plasmid in-frame with the *gfp* coding sequence allows, following homologous recombination, the expression of the *gfp* tagged gene under its native promoter from its native locus.

25

Three specific sites (P146, *mhp379* and *pepF*) were chosen within the *M. hyopneumoniae* strain 232 genome for targeted integration of plasmid DNA by homologous recombination following a single crossover event. We were able to demonstrate plasmid integration within the open reading frames at each target site. For
30 the P146 and *pepF* open reading frames, it was possible to select for cells containing integrated plasmid DNA only by serially passaging cells in the absence of tetracycline selection and then by re-introducing it to select for cells that still retained integrated plasmid DNA. It is likely that in both of these cases, the mutated organisms are unable to generate a full length *pepF* or P146 protein, although it is possible that truncated
35 proteins are produced. In the case of *mhp379*, although we could demonstrate successful disruption of the open reading frame, we were unable to generate pure clones

consisting only of integrated plasmid DNA. The persistence of extrachromosomal plasmid DNA suggests that disruption of this open reading frame may be detrimental to the long term survival of mycoplasmas.

5 The target genes were chosen for a number of reasons. We selected the P146 gene on the basis that it encodes a protein that undergoes proteolytic cleavage and is expressed on the cell surface where it is involved in the interaction with host cells eg adhesion. We therefore considered that it may be an important determinant in the interaction between the host and the pathogen, but that it would not be necessary for growth in vitro, and
10 therefore would be amenable to disruption. The mhp379 ORF was chosen because it encodes a cell surface nuclease and is part of an operon that is involved in nucleotide acquisition (Schmidt et al 2007). The primary reason for choosing this ORF was that if we could disrupt this ORF, then we may be able to detect a phenotypic change in vitro. *M. hyopneumoniae* expresses potent nuclease activity that can readily be detected using
15 a number of simple assays. If we could disrupt this ORF then we would be able to reduce detectable nuclease activity and show a change in phenotype. The pepF ORF was chosen because using the Himar1 transposon system, we found that we were easily able to generate transposon mutations in this gene. It therefore seems that this gene is not essential and is therefore a good candidate for demonstrating gene disruption.

20
Disruption of *M. hyopneumoniae* 232 genomic DNA using this method may be a useful means of studying the function of genes that are not essential for in vitro growth and survival, but that may be associated with virulence/pathogenesis in the host. Although
25 gene disruption is possible, currently using this technique, a large amount of foreign DNA is integrated into the chromosome of the host cell. It is considered that by modification of the technique, double crossover recombination events can be generated that will result in integration of the *tetM* determinant alone.

30
Example 6: Development of genetic tools for functional genomic analysis of *Mycoplasma hyopneumoniae*.

We refer to a thesis completed by Beth Cook in 2013, included in the priority application (when not yet in the public domain), and placed in the public domain on 16 April 2014,
35 when it was made available in the Royal Veterinary College's Learning Resources Centre.

Abstract

Mycoplasma hyopneumoniae is the cause of enzootic pneumonia: a respiratory disease of pigs worldwide. The lack of genetic tools for functional genomic analysis, have hampered efforts to improve the understanding of genes related to pathogenicity within this mycoplasma. Growth of *M.hyopneumoniae* on solid medium was improved following assessment of the purity of the solidifying agar. Incorporation of 2µg/ml kanamycin into solid medium inhibited the growth of *M.hyorhinis* allowing the positive selection of *M.hyopneumoniae*. A transposon delivery system for *M.hyopneumoniae* was required for the generation of mutant genotypes. Conjugative transfer of the *Himar1* transposon pMiniHimarRB1 into *M.hyorhinis* and transformation by electroporation and PEG-treatment with Mini-Tn4001PsPuro were unsuccessful. Therefore a more suitable plasmid, pMini-Himar1BSC1, was constructed and assessed in *M.gallisepticum* resulting in a transformation frequency of 1×10^{-3} /CFU generating a large transposon mutant pool. Southern blot and PCR analysis confirmed insertion of the Mini-Himar1 transposon. An *M.gallisepticum* R(low) infection model was established in chickens with an optimal infectious dose of 107CFU/bird with maximum re-isolation from the trachea and lungs after 7 days post infection. These data will be used to perform an *in vivo* screen of the large *M.gallisepticum* transposon library generated. Transformation of *M.hyopneumoniae* with pMini-Himar1BSC1 by electroporation was successfully achieved following method adaptations. Southern blot and PCR analysis confirmed the generation of six different transposon mutants, suggesting transformation had occurred but was very inefficient. Sequencing of transposon insertion sites identified insertions into 4 different ORF and one intergenic region. This is the first report of the successful introduction of a *Mariner*-based transposon into *Mycoplasma* species. With further improvements, this transposon delivery system will allow functional genomic analysis of this poorly understood and economically important swine pathogen.

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Claims

1. A plasmid for transforming *Mycoplasma hyopneumoniae* comprising an *OriC* region comprising both AT-rich regions from the predicted *OriC* region of *M. hyopneumoniae* 232, or comprising a variant thereof that retains the ability to act as an *OriC* region in *M. hyopneumoniae* and has at least 90% sequence identity with a sequence comprising both AT-rich regions from the predicted *OriC* region of *M. hyopneumoniae* 232.
2. The plasmid according to claim 1 wherein the *OriC* region of the plasmid comprises both AT-rich regions as identified in Figure 1A.
3. The plasmid according to claim 1 or 2 wherein the *OriC* region of the plasmid comprises a sequence of about 700bp comprising both AT-rich regions as identified in Figure 1A.
4. The plasmid according to any one of claims 1 to 3 wherein the *OriC* region of the plasmid comprises the *DnaA* gene from *M. hyopneumoniae* or a sequence having at least 70, 80 or 90% sequence identity with the *DnaA* gene.
5. The plasmid according to any one of claims 1 to 3 wherein the *OriC* region of the plasmid comprises or consists of the sequence
TTGTTAATTATTGCTTGAAATTCCCAATTTTTTTTAATACTACGAATTTTTTGCAAACT
ATCTCAACAAAAATTATTTATCAGAAACAGTTAGTCTTTTCCGACCTTTTGCGCGGCG
TGCAGCAAGAATTTTTCGCCGTCGGCTGTTGACATTCGCGCGCGGAATCCGTGTG
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 5 TCTGATAAATCGCCTTCTTTACTTGGTGGATTGGAAGAAAGATTTATAACGCGATTTA
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 10 TAAGAAAAAATGTTTGAAATTTTTGTTGAAAAATATGAAATCGAACAAACAATCACC
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 15 ATGAACTTGAAATTGCCCTTAACCATATTTATAACAATTAACTGAAGTTTTAAACA
 GCGAAAATAAAAAATATTAAACAGCTTTTTGGGCAAAAAGTACTATAAATTAAAGTGGT
 TTTTATCTTTTCTACATTTCTACACCCTTATTATTTTTATTATTAAACTAAATAAAAA
 TTAATAATATAATATAATTATTAAAGGTAAAAATATGAAATTTACAATTGAAAAAAA
 AATTATTGAATGACAAATTGAAAGAATGCAATCAGCAATTCTTAATAATGTAAATTCA
 20 CCATTAAGTTCATTTTTTTTAAACTTACAAGAAGCGGATTATTTATAATTTCAACAAA
 TAGCGAACTTTCCTATAAAG or a variant thereof that retains the ability to act as an
OriC region in *M. hyopneumoniae* and has at least 90% sequence identity with said
 sequence.

25 6. The plasmid according to any one of claims 1 to 5 wherein the plasmid further
 comprises a marker gene, optionally wherein the marker gene comprises a promoter
 derived from *M. hyopneumoniae* or the *spiralin* gene promoter of *Spiroplasma citri*,
 optionally wherein the promoter derived from *M. hyopneumoniae* is the P97 gene (1B) or
 Idh gene (1C) promoter, for example as shown in Figure 1; or a variant thereof that is
 30 capable of acting as a promoter in *M. hyopneumoniae*, for example *M. hyopneumoniae*
 232 and has at least 90% sequence identity with the relevant promoter sequence, for
 example as shown in Figure 1.

7. A method of transforming *M. hyopneumoniae*, the method comprising the step of
 35 exposing a *M. hyopneumoniae* cell to a plasmid according to any one of claims 1 to 6.

8. The method of claim 7 further comprising the step of performing electroporation on the cell in the presence of the plasmid according to any one of claims 1 to 6; optionally further comprising the step of culturing the exposed (and optionally electroporated) cell under conditions where transformed cells have a selective growth advantage or otherwise can be identified.

9. A *M. hyopneumoniae* cell transformed with a plasmid according to any one of claims 1 to 6.

10. A vaccine composition comprising a transformed *M. hyopneumoniae* cell according to claim 9, optionally comprising a gene, optionally a gene for P46, P97 or P146 adhesion protein, that has undergone site-directed mutagenesis as a consequence of transformation with a plasmid according to any one of claims 1 to 6, optionally wherein the transformed *M. hyopneumoniae* cell is a cell according to claim 29 or claim 30.

15

11. A plasmid, transformed *M. hyopneumoniae* cell or vaccine composition of the preceding claims for use in medicine.

12. A plasmid, transformed *M. hyopneumoniae* cell or vaccine composition of the invention for use in aiding in treating or preventing infection by *M. hyopneumoniae* or disease caused by infection by *M. hyopneumoniae*.

13. A method for aiding in treating or preventing infection by *M. hyopneumoniae* or disease caused by infection by *M. hyopneumoniae*, the method comprising administering to a subject a transformed *M. hyopneumoniae* cell or vaccine composition according to the preceding claims.

14. A method for identifying a sequence capable of acting as an *OriC* region in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence to act as an *OriC* region with the ability of a sequence comprising both AT-rich regions from the predicted *OriC* region of *M. hyopneumoniae* 232 (for example as set out above) to act as an *OriC* region in *M. hyopneumoniae*.

15. A method for identifying a sequence capable of acting as promoter in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence to act as a promoter with the ability of the *spiralin* gene promoter of

Spiroplasma citri, promoter of the *M. hyopneumoniae* P97 gene or *M. hyopneumoniae* Idh gene, to act as a promoter in *M. hyopneumoniae*.

16. A method for identifying a sequence capable of acting as a selection marker in *M. hyopneumoniae* the method comprising the step of comparing the ability of a candidate sequence (optionally with at least 90% sequence identity to a sequence known to confer resistance to a compound (challenge compound) in another organism), under the control of a promoter known to work in *M. hyopneumoniae*, to confer resistance to a challenge compound, with the ability of a construct not containing the candidate sequence to confer resistance to the challenge compound.

17. A nucleic acid construct comprising a nucleic acid sequence encoding a transposase enzyme and a promoter sequence, wherein the promoter sequence is active in *M. hyopneumoniae*;
optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*;
optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* Idh, P97, secD, Tuf, rpoB, P146, ATP transporter ATP binding protein, Asparagine-tRNA synthetase or Translation elongation factor gene.

18. The nucleic acid construct of claim 17 wherein the transposase enzyme is a Mariner family transposase, optionally Himar1 transposase or Himar1 C9 mutant transposase.

19. The nucleic acid construct of claim 17 or 18 or a further nucleic acid construct (transposon delivery vector) wherein the nucleic acid construct further comprises or comprises a mini transposon comprising inverted repeats and optionally comprising a marker gene, optionally comprising a promoter sequence active in *M. hyopneumoniae*,
optionally wherein the promoter sequence comprises a promoter sequence from *M. hyopneumoniae*, or *spiralin* gene promoter of *Spiroplasma citri* or *tetM* promoter sequence from *S. aureus*;
optionally wherein the promoter sequence from *M. hyopneumoniae* is a constitutively active promoter sequence, optionally a promoter sequence from the *M. hyopneumoniae* Idh, P97, secD, Tuf, rpoB, P146, ATP transporter ATP binding protein, Asparagine-tRNA synthetase or Translation elongation factor gene.

20. The nucleic acid construct of claim 19 wherein the marker gene is a selectable marker gene, optionally an antibiotic resistance gene, optionally a gene conferring resistance to puromycin (optionally puromycin-N-acetyl-transferase), tetracycline (optionally *tetM*), chloramphenicol (optionally chloramphenicol acetyltransferase) or
5 aminoglycosides such as gentamicin (optionally *aacA-aphD*)
21. A *M. hyopneumoniae* or other *Mycoplasma* cell transformed with or comprising a construct or constructs according to any one of claims 17 to 18.
- 10 22. A method of forming a collection of mutant *M. hyopneumoniae* or other *Mycoplasma* cells, the method comprising the steps of
exposing a sample of *M. hyopneumoniae* or other *Mycoplasma* cells to multiple copies of a construct according to any one of claims 17 to 20;
optionally culturing the exposed cells under conditions such that transformed cells can
15 be detected.
23. A collection of *M. hyopneumoniae* or other *Mycoplasma* cells transformed with or comprising a construct or constructs according to any one of claims 17 to 20 or obtained or obtainable by a method according to claim 22; optionally wherein the collection
20 comprises at least 10, 20, 50, 100, 200, 400, 500, 800 or 1000 different transformants.
24. A method for analysing gene function in *M. hyopneumoniae* or another *Mycoplasma*, the method comprising the step of providing a collection of *M. hyopneumoniae* or other *Mycoplasma* transformants according to claim 23; and
25 analysing nucleic acid sequences of said transformants, optionally identifying sites of insertion of said mini transposon sequence in said transformants; and
optionally identifying a gene in which a mini transposon has inserted as a potential site for mutation in a vaccine composition.
- 30 25. A vaccine composition according to claim 10 comprising a gene that has undergone site-directed mutagenesis as a consequence of transformation with a plasmid according to any one of claims 1 to 6 or a plasmid according to claim, wherein the gene is a gene that has been identified by a method according to claim 24.
- 35 26. The plasmid according to claim 6 wherein the plasmid further comprises a nucleic acid sequence with the ability to undergo homologous recombination with the

Mycoplasma hyopneumoniae chromosome wherein the nucleic acid sequence with the ability to undergo homologous recombination may comprise a sequence that has at least 80, 90, 95 or 98% identity over a sequence length of at least 100, optionally at least 300, 400 or 500 nucleotides, for example between 300, 400, 500, 600, 700, 800, 900 and 3000, 2000 or 1500 nucleotides, for example between 500 or 700 and 1500 nucleotides, with a sequence of *M. hyopneumoniae* (for example *M. hyopneumoniae* 232) chromosomal nucleic acid.

27. A plasmid according to claim 26 wherein the plasmid comprises two nucleic acid sequences with the ability to undergo homologous recombination with the *Mycoplasma hyopneumoniae* chromosome as defined in claim 26 separated by a marker sequence, optionally a selectable marker gene flanked by recombinase recognition sites, optionally FRT flip recombinase recognition sites or Cre-LoxP recombinase recognition sites.

28. A system comprising a plasmid according to claim 27 and a further plasmid according to claim 6, wherein the marker sequence in the first said plasmid is different from the marker sequence in the further plasmid, optionally wherein the marker sequence in the first plasmid confers resistance to a different challenge compound to the marker sequence in the further plasmid; and wherein optionally the further plasmid comprises a sequence encoding a recombinase, optionally a flip recombinase or Cre recombinase, optionally under the control of a promoter active in *M. hyopneumoniae*.

29. A *M. hyopneumoniae* cell according to claim 9 transformed with a plasmid according to claim 27 or a system according to claim 28.

30. A *M. hyopneumoniae* cell according to claim 9 or 29 wherein the genomic nucleic acid of the cell comprises a marker sequence, optionally a selectable marker sequence, flanked by recombinase recognition sites, optionally FRT flip recombinase recognition sites; or wherein the genomic nucleic acid of the cell comprises a recombinase recognition site.

Figure 1 part 1 of 2

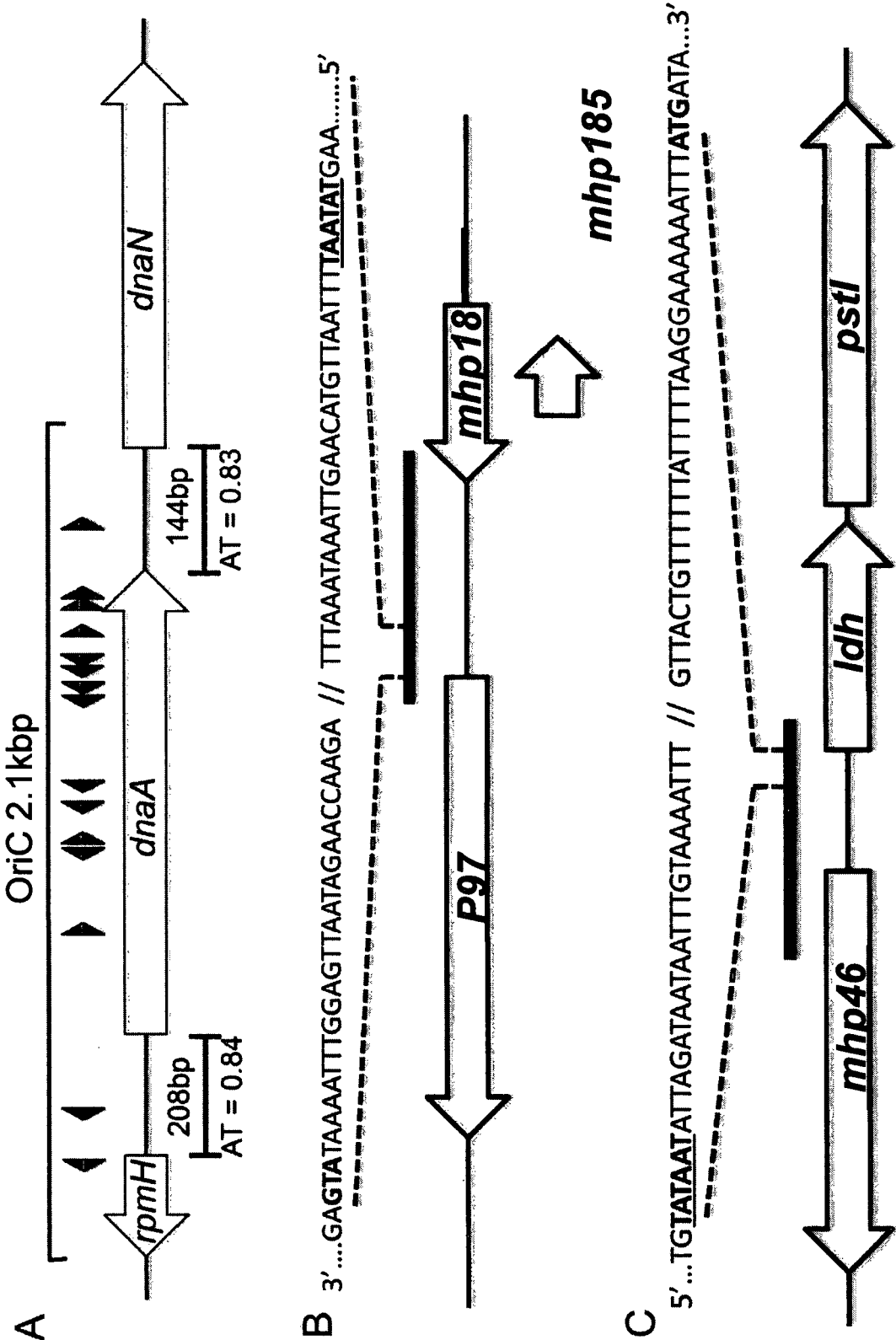


Figure 1 part 2 of 2

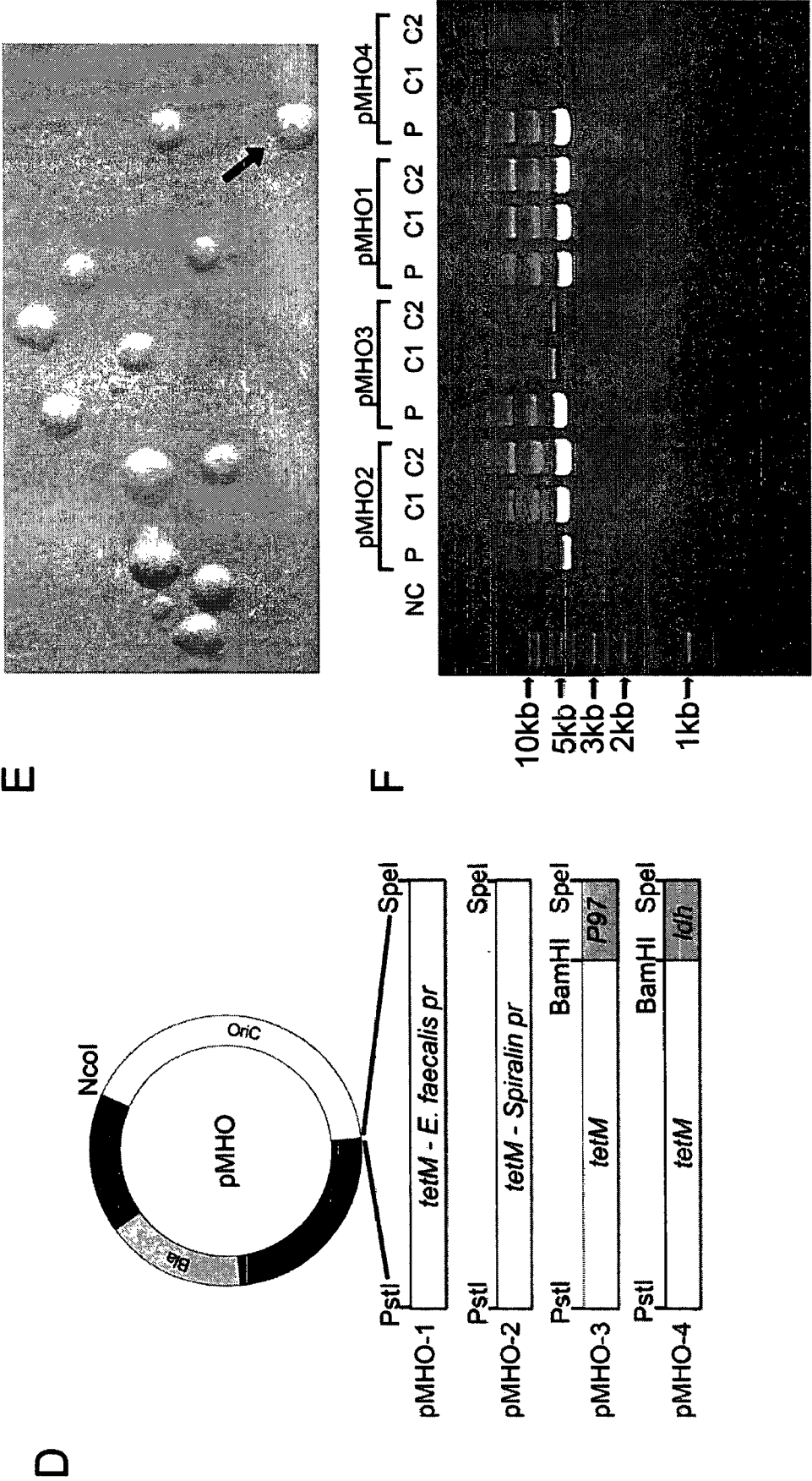


Figure 2

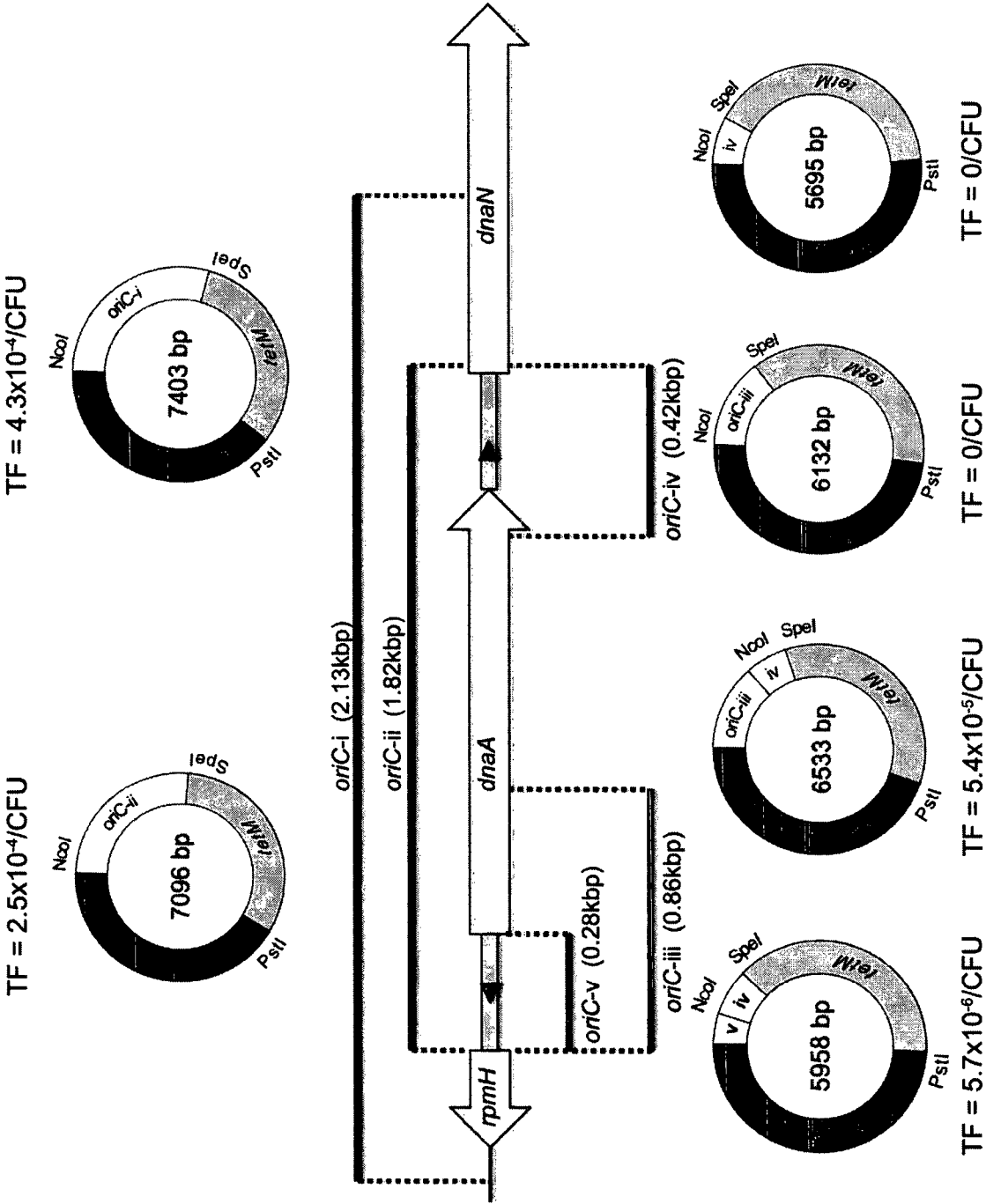


Figure 3 part 1 of 2

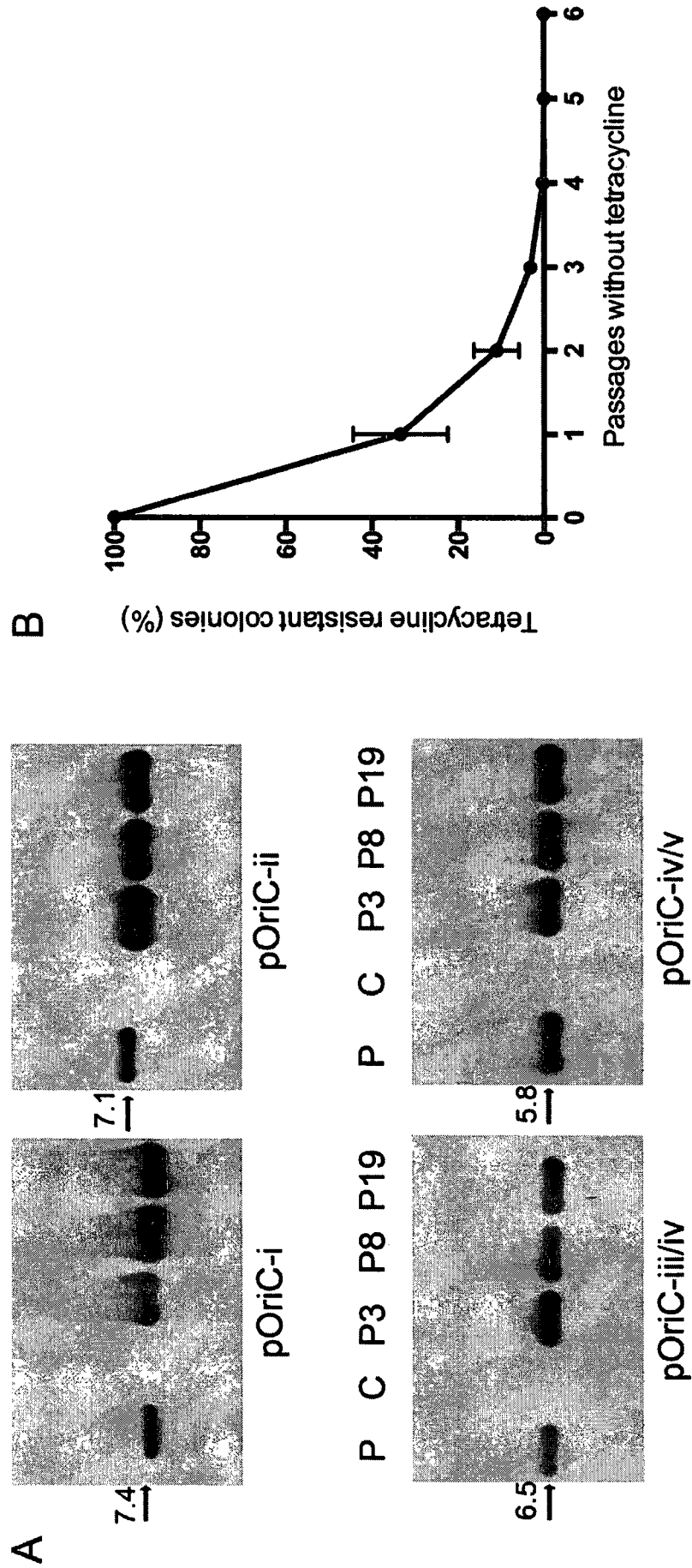
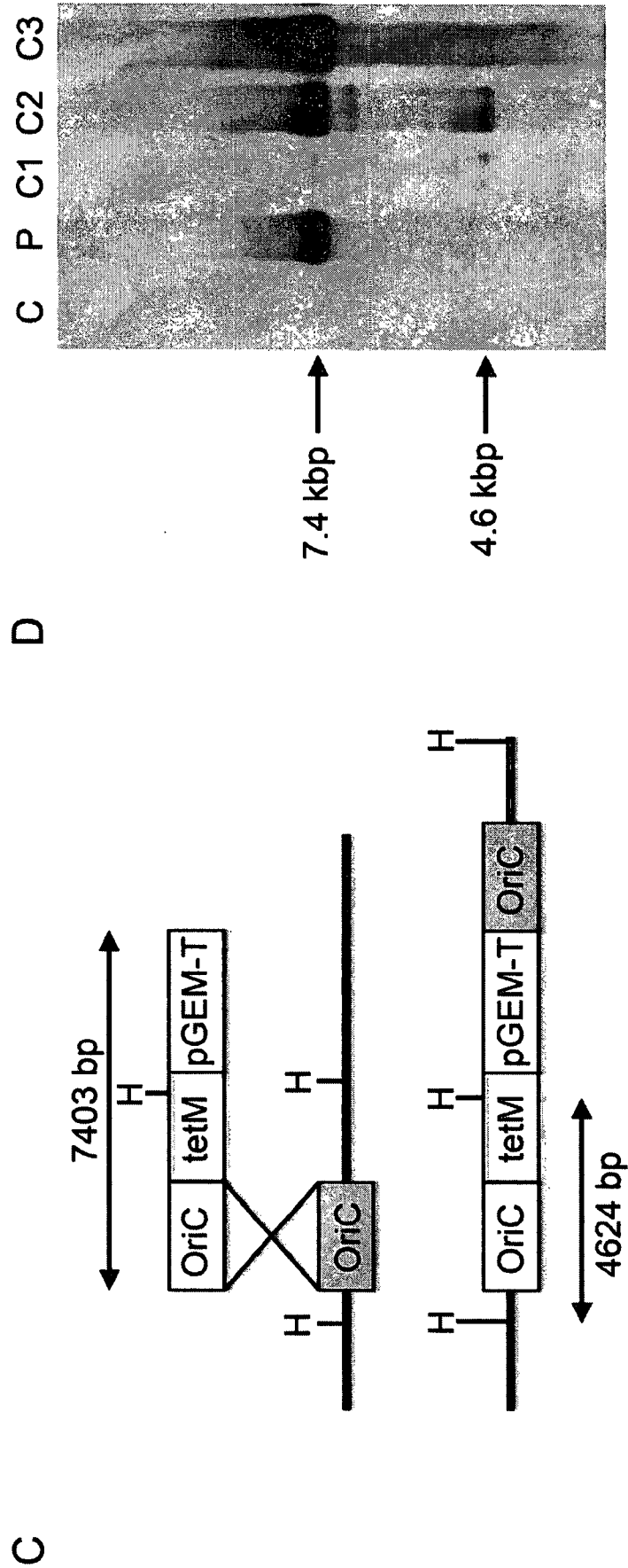
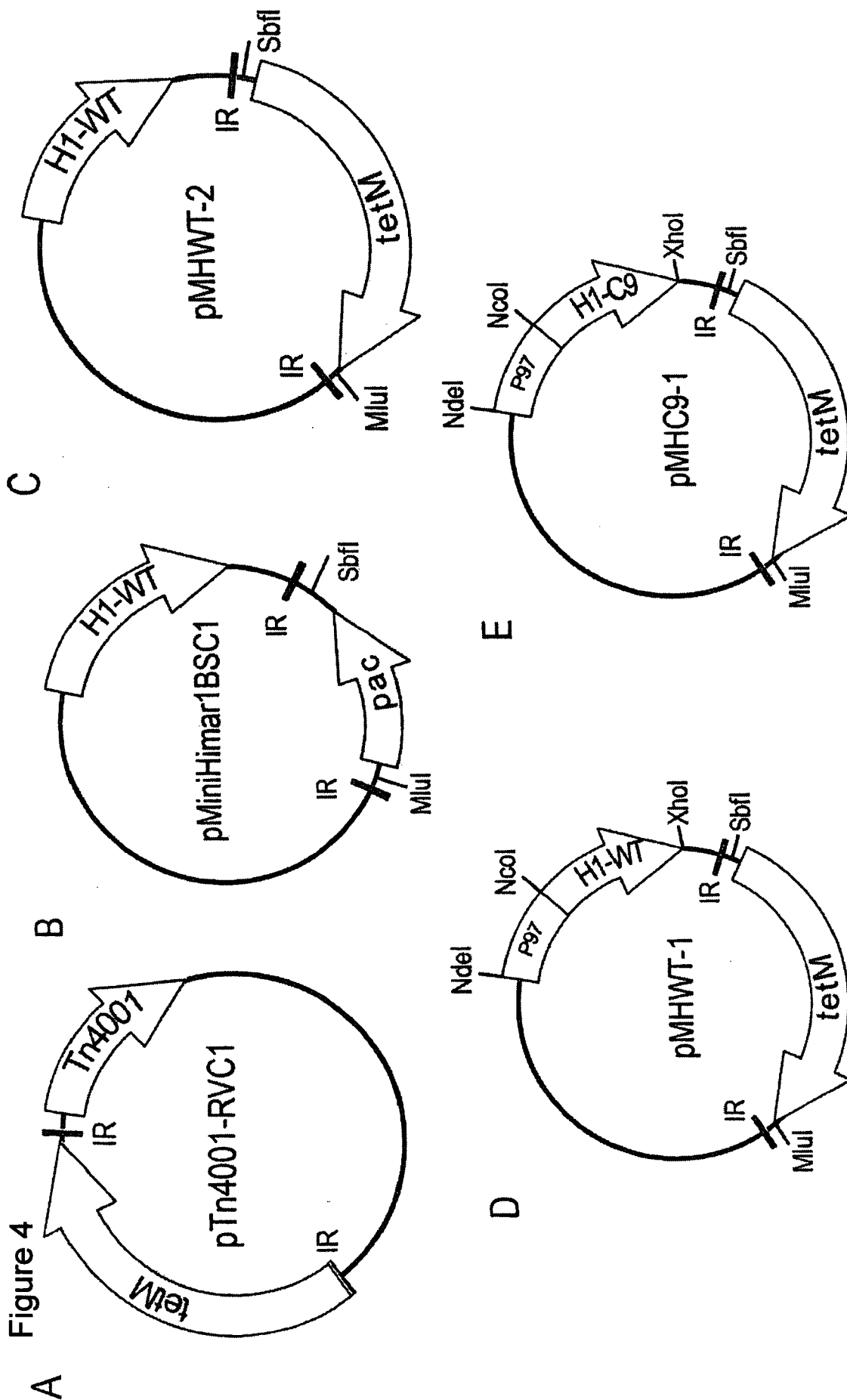


Figure 3 part 2 of 2





B

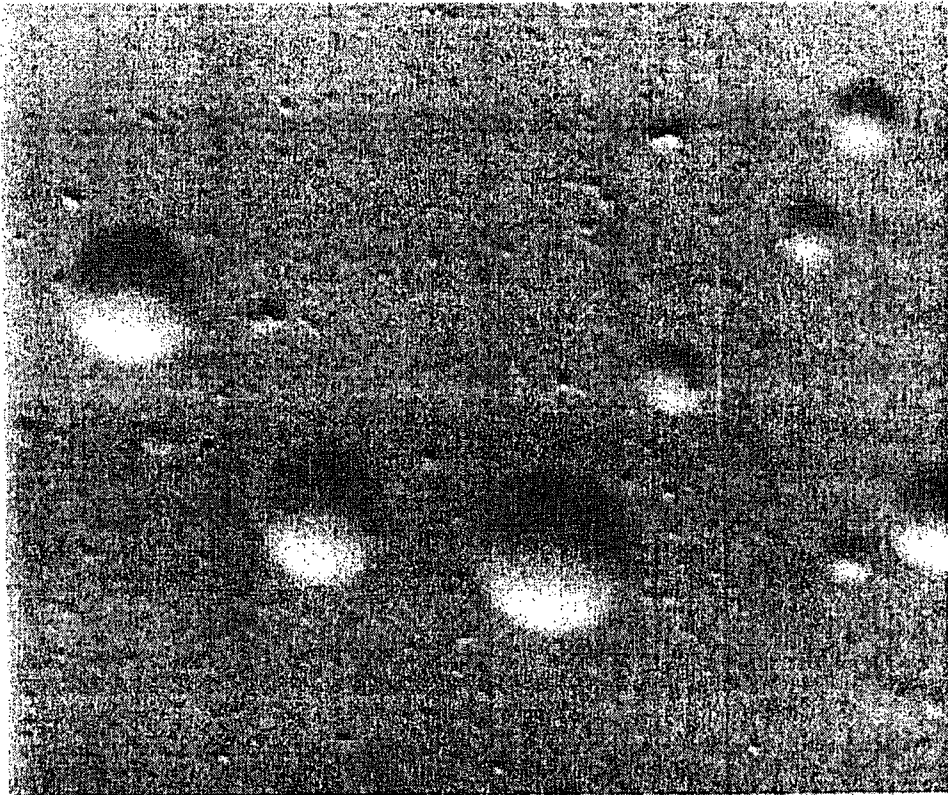


Figure 5

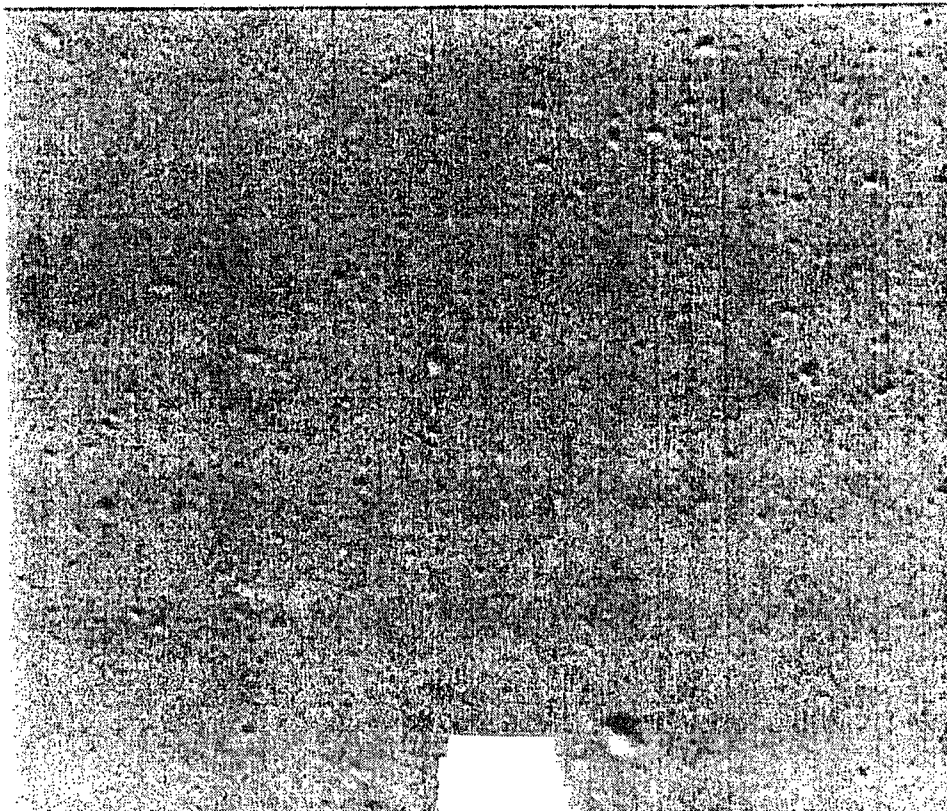


Figure 6 (part 1 of 2)

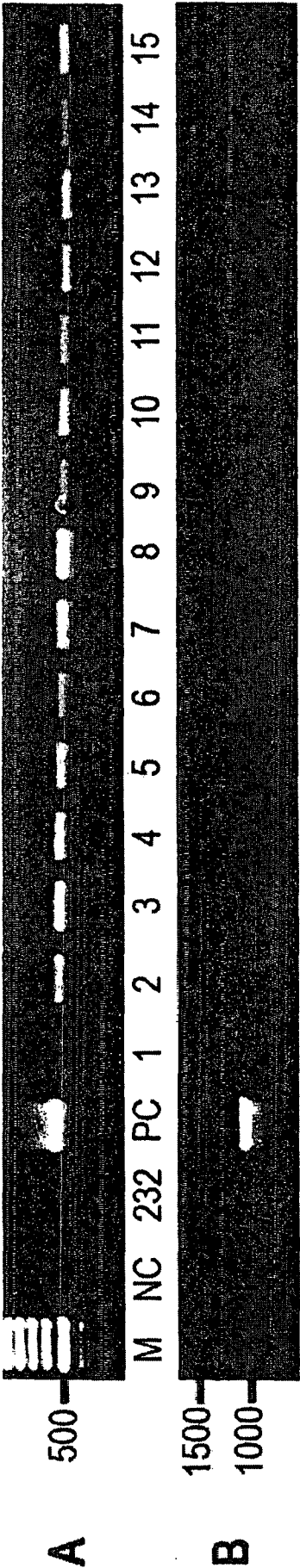
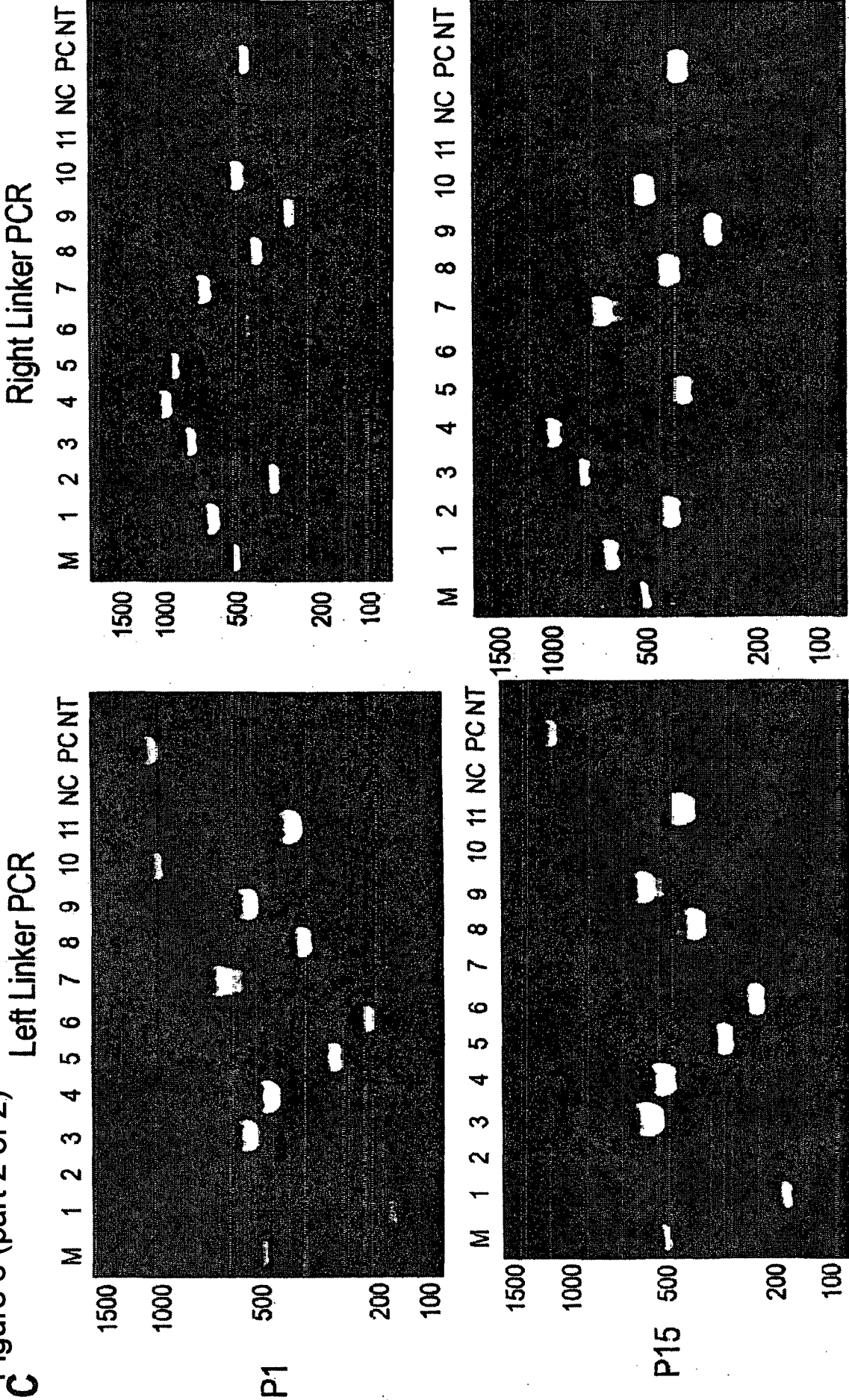
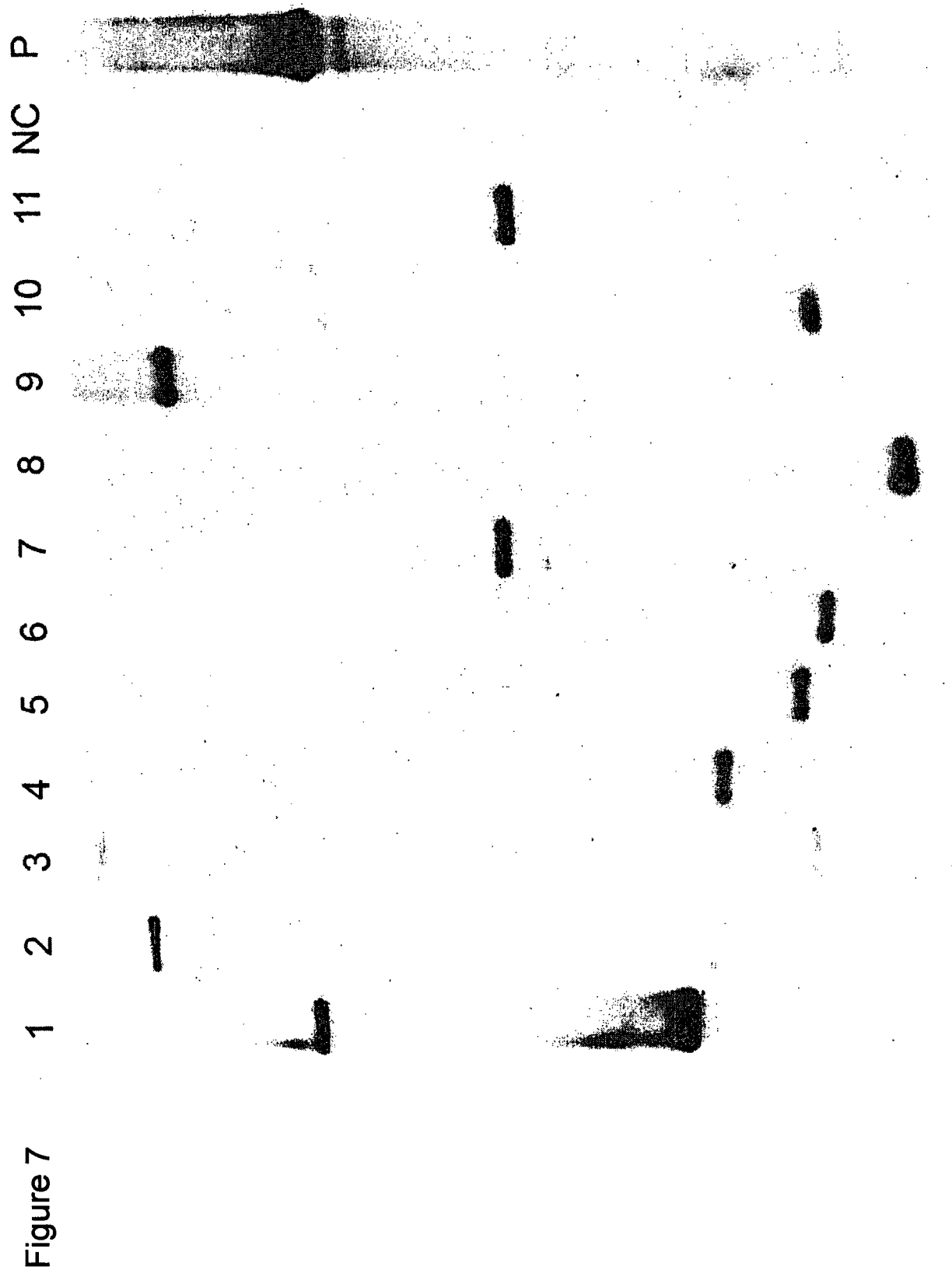


Figure 6 (part 2 of 2)
C





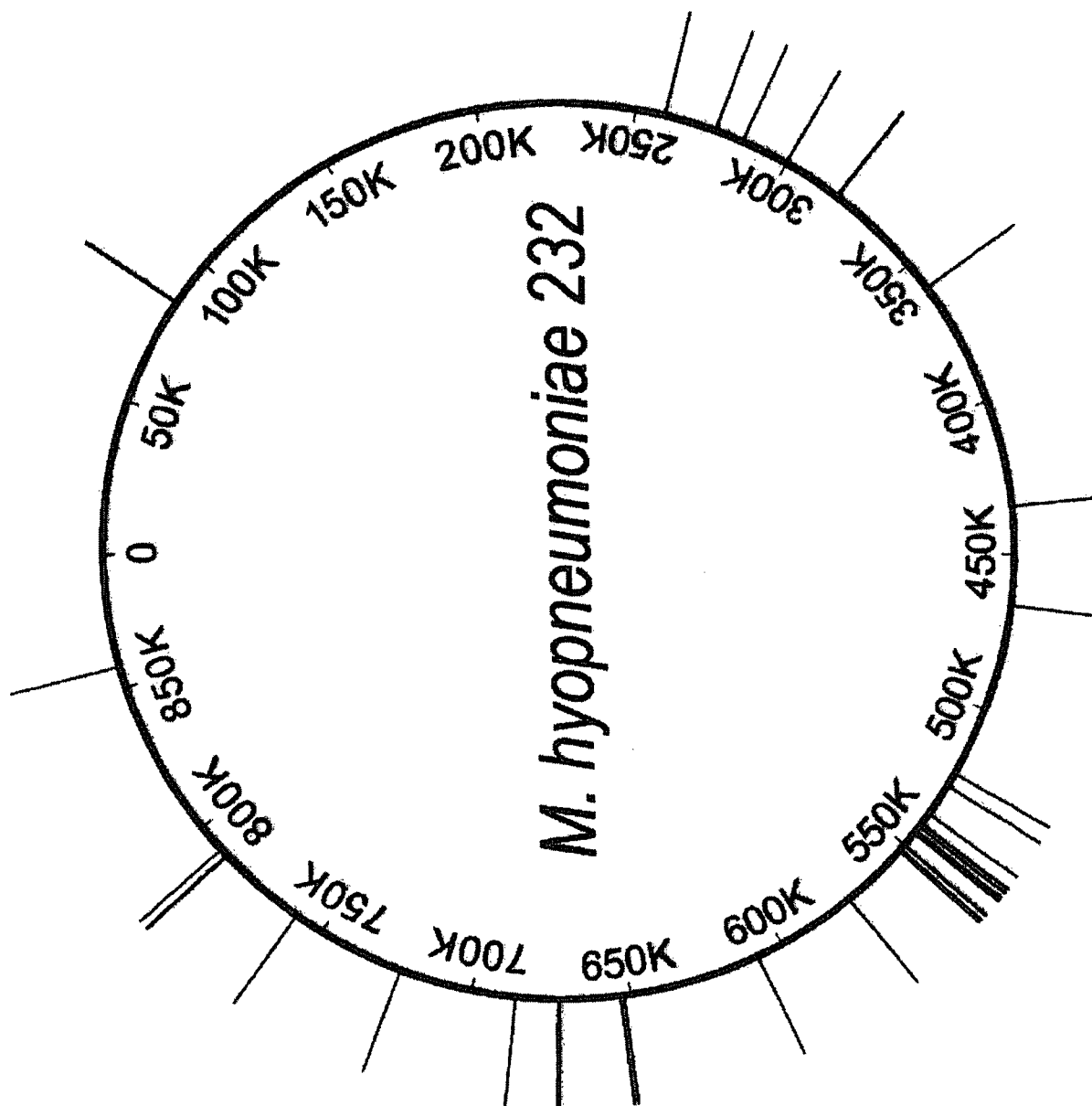


Figure 8

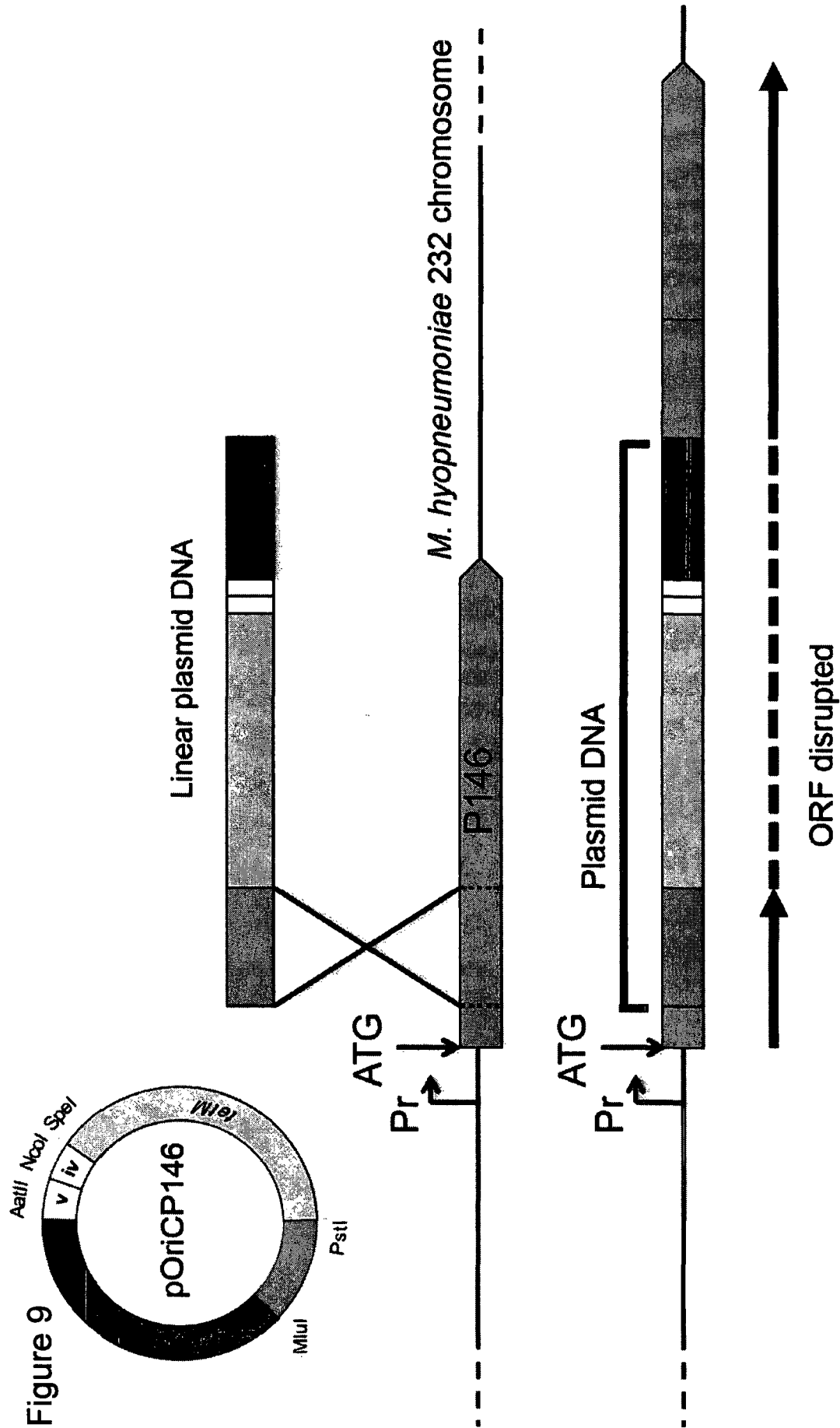


Figure 10

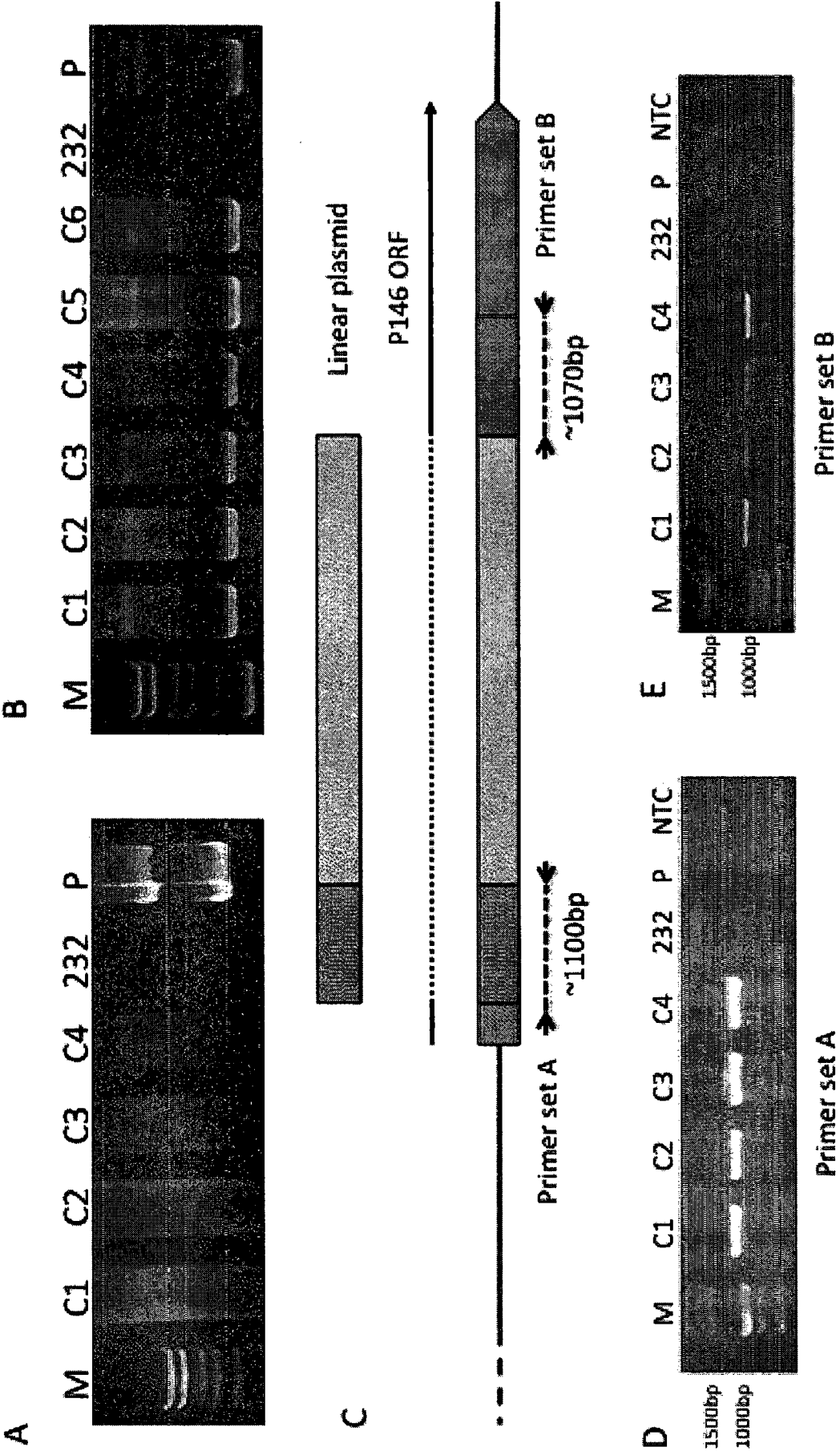


Figure 11 part 1 of 2

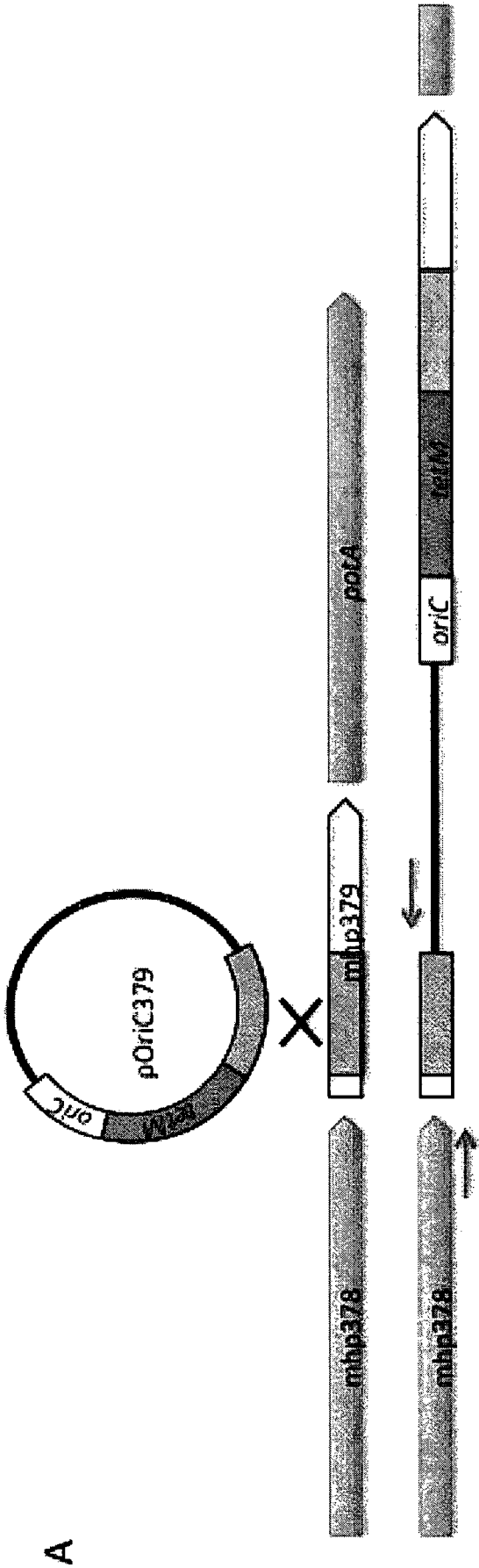


Figure 11 part 2 of 2

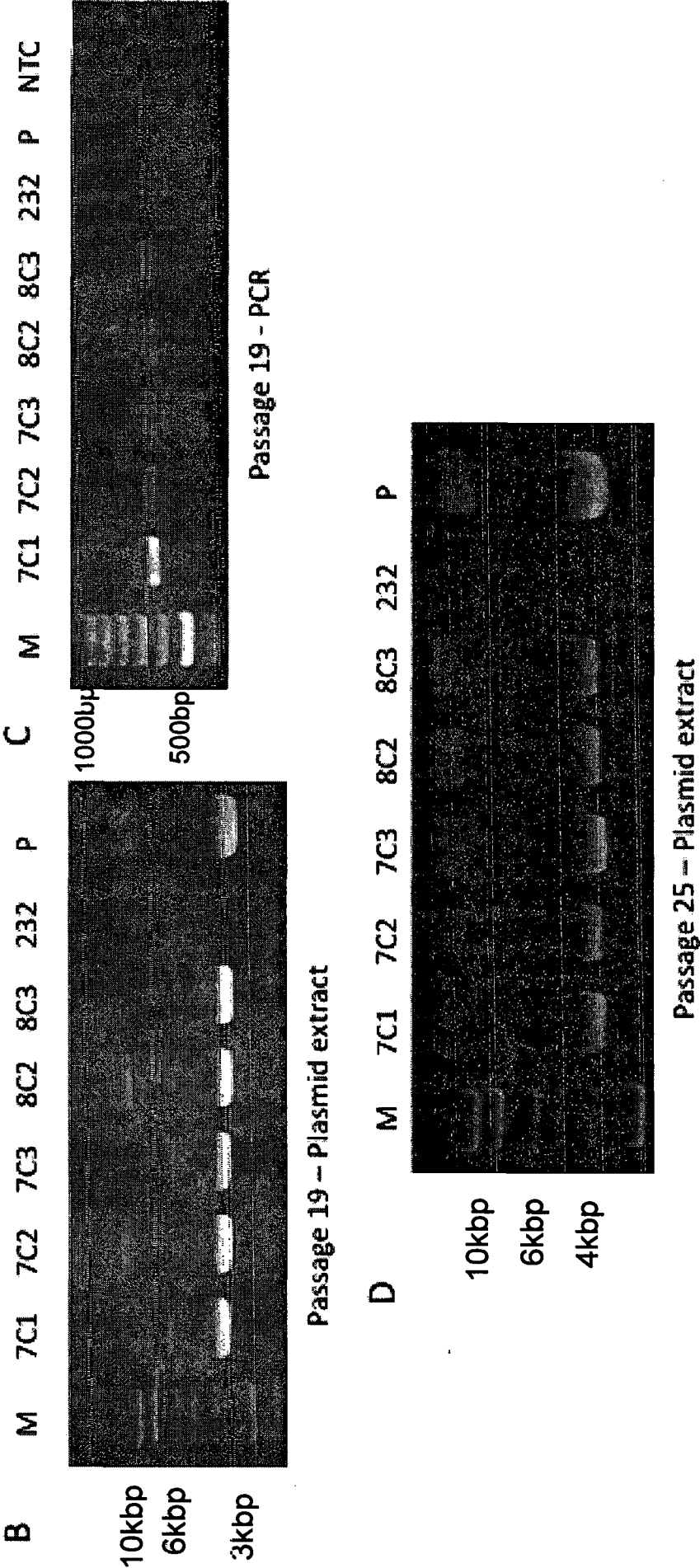


Figure 12 part 1 of 2

A

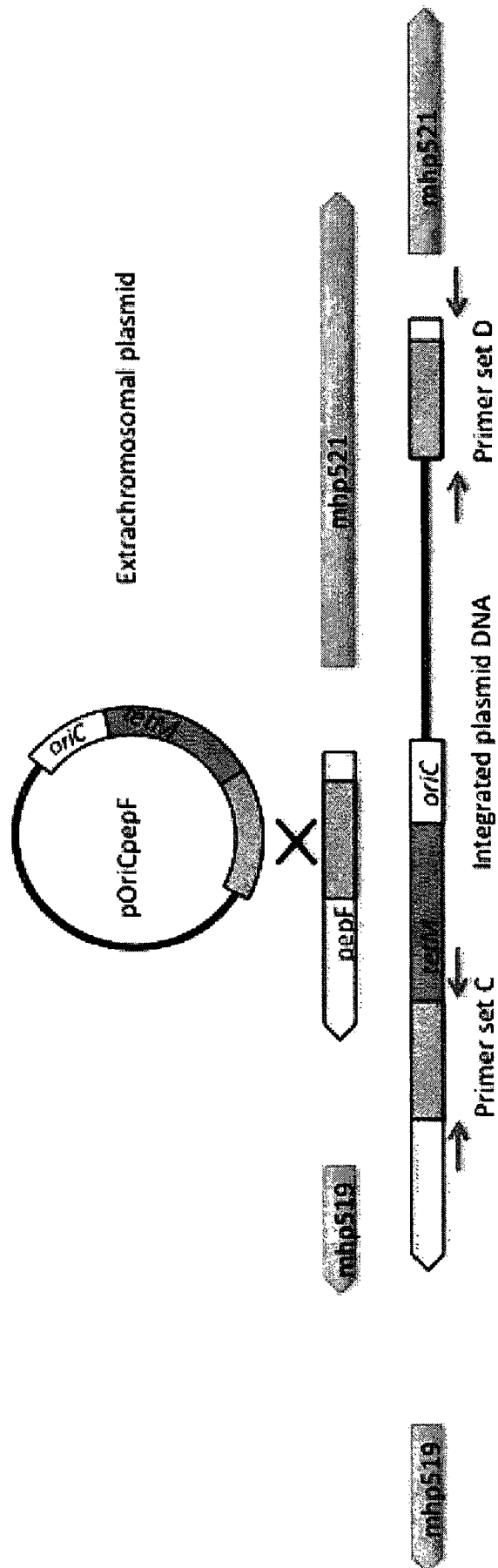


Figure 12 part 2 of 2

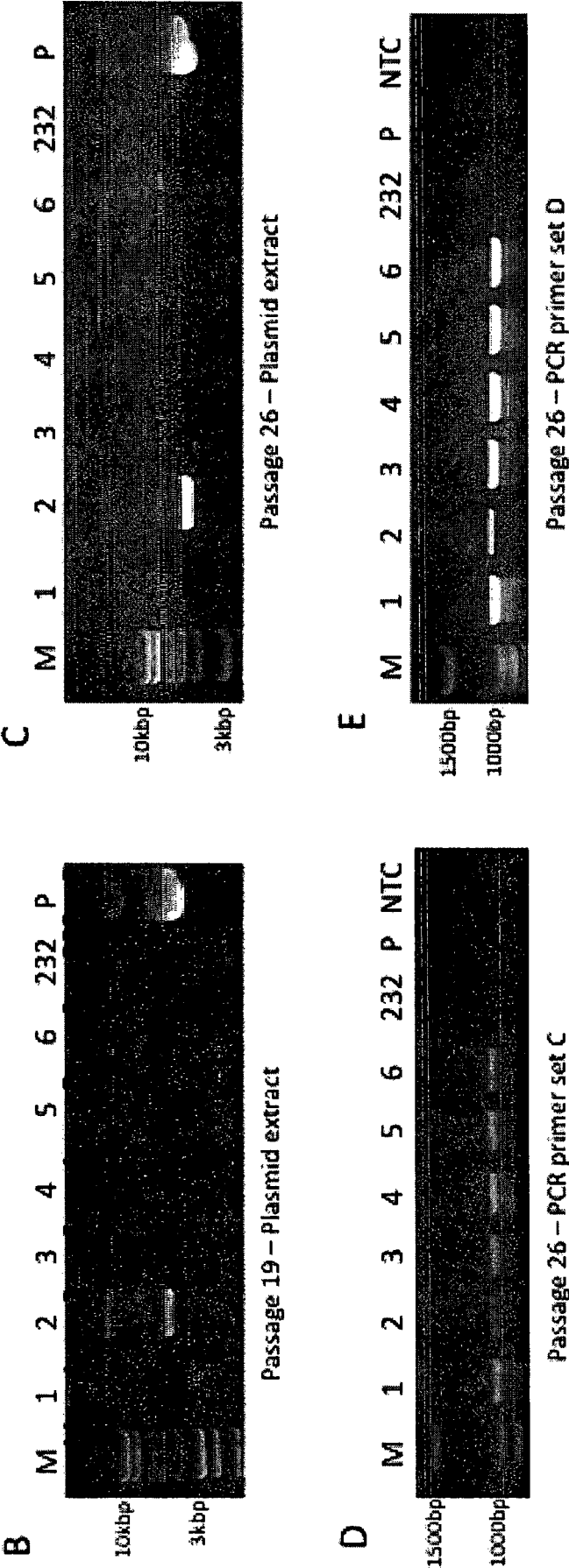


Figure 13 part 1 of 3

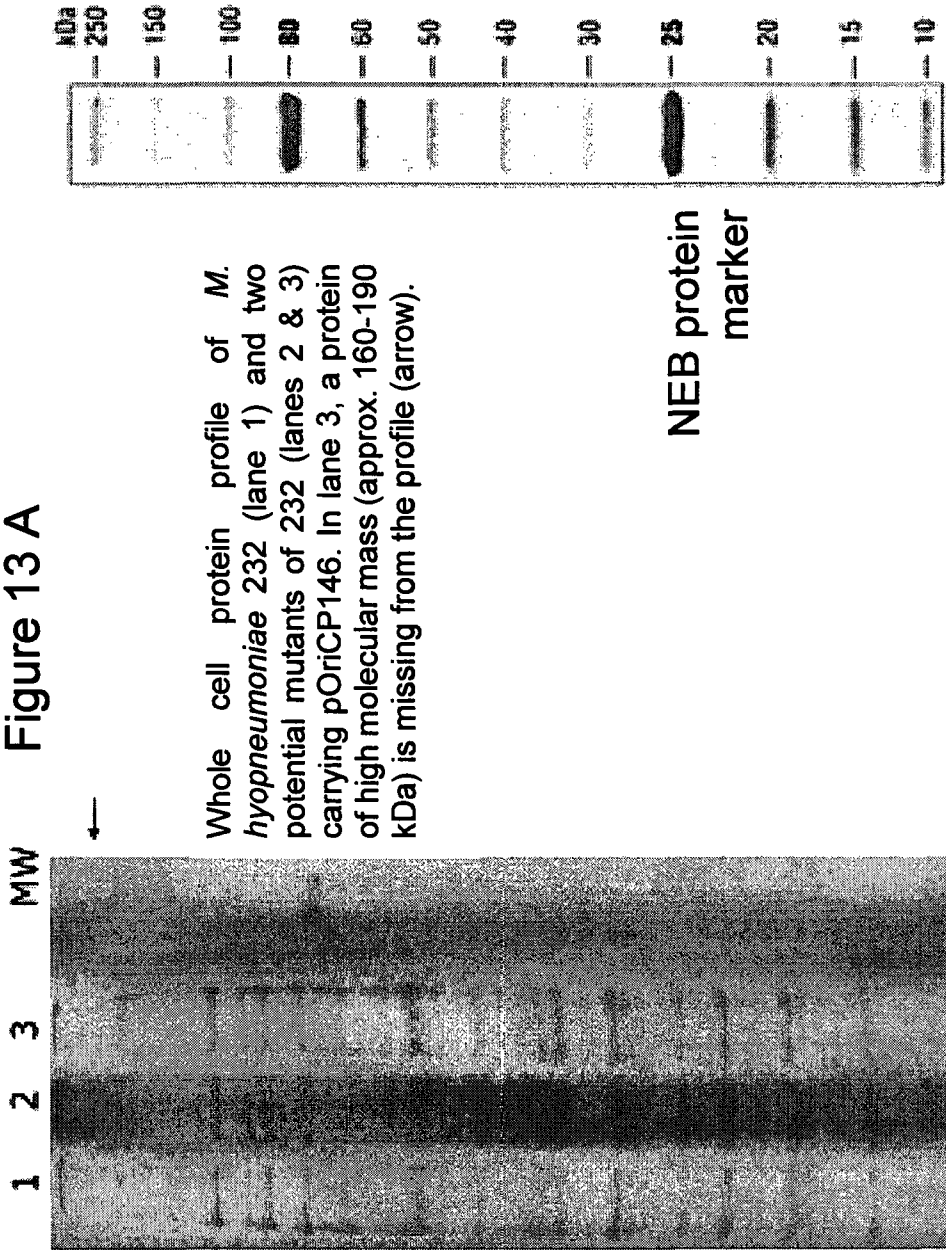


Figure 13 part 2 of 3

Figure 13 B

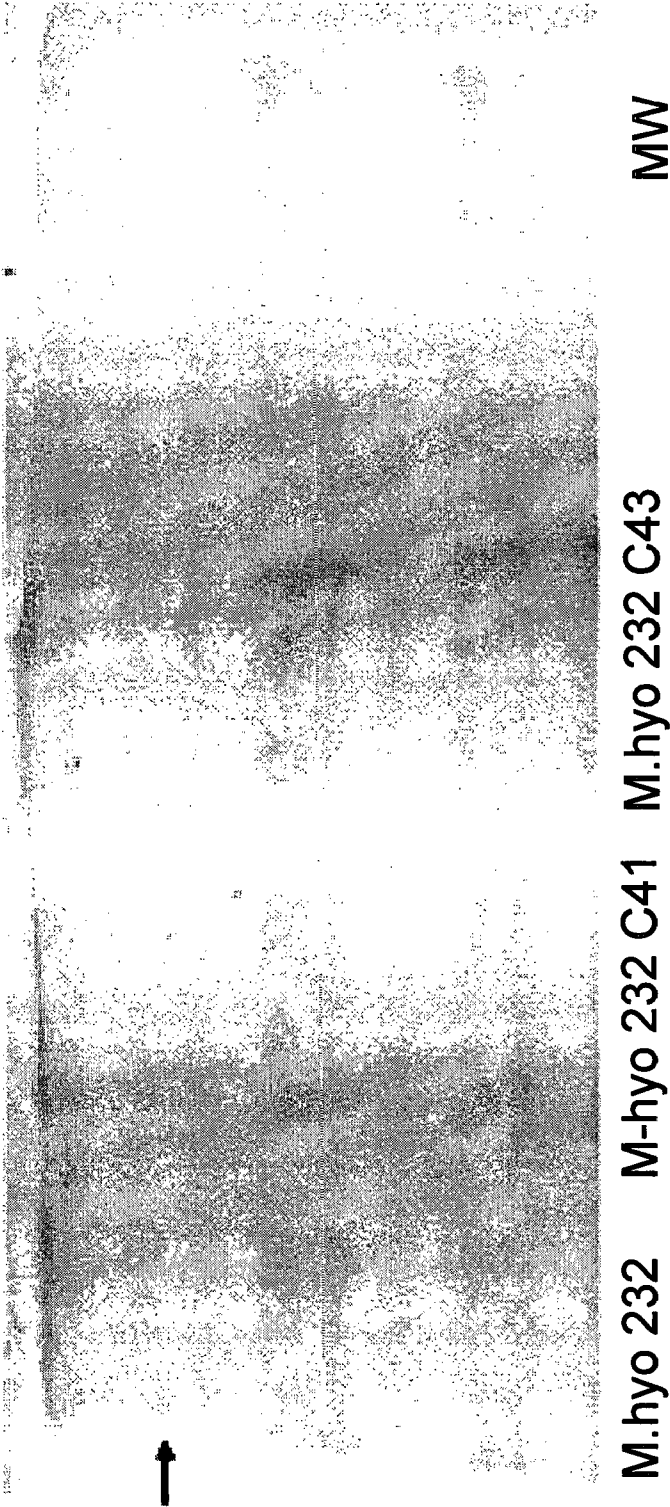


Figure 13 part 3 of 3

Figure 13 C

