Genes Found Essential in Other Mycoplasmas Are Dispensable in *Mycoplasma bovis*

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**Abstract**

Mycoplasmas are regarded to be useful models for studying the minimum genetic complement required for independent survival of an organism. *Mycoplasma bovis* is a globally distributed pathogen causing pneumonia, mastitis, arthritis, otitis media and reproductive tract disease, and genome sequences of three strains, the type strain PG45 and two strains isolated in China, have been published. In this study, several Tn4001 based transposon constructs were generated and used to create a *M. bovis* PG45 insertional mutant library. Direct genome sequencing of 319 independent insertions detected disruptions in 129 genes in *M. bovis*, 48 of which had homologues in *Mycoplasma mycoides* subspecies *mycoides* SC and 99 of which had homologues in *Mycoplasma agalactiae*. Sixteen genes found to be essential in previous studies on other mycoplasma species were found to be dispensable. Five of these genes have previously been predicted to be part of the core set of 153 essential genes in mycoplasmas. Thus this study has extended the list of non-essential genes of mycoplasmas from that previously generated by studies in other species.

**Introduction**

Mycoplasmas are a group of obligately parasitic bacteria that evolved from Gram positive organisms by reductive evolution. In the process, they have lost many dispensable genes and are thought to maintain only regulatory systems essential for their survival *in vivo* [1–6].

The mycoplasmas lack a cell wall and have relatively small genomes (580 to 1380 kbp), but can still perform all the functions required for autonomous life [4,5]. Despite their genetic simplicity, many are pathogenic and can persist for very extended periods in their vertebrate hosts. *Mycoplasma bovis*, a significant pathogen of cattle throughout the world, lies in the hominis phylogenetic group, with *M. agalactiae*, *M. fermentans*, *M. synoviae*, *M. pulmonis*, *M. hyopneumoniae*, *M. arthritidis*, *M. hominis*, *M. conjunctivae*, *M. crocodyli*, *M. mobile* and *M. orale* [7,8].

The genomes of three strains of *M. bovis*, the type strain PG45 [9] and two strains isolated in China, Hubei-1 [10] and HB0801 [11], have been determined. There have been very few functional studies on *M. bovis*, and its virulence factors and the mechanisms involved in its pathogenicity are largely unknown. However, it is clear that it uses complex strategies to invade and avoid the immune response of the host [12,13].

Only a few tools are available to genetically manipulate mycoplasmas. Transposons have been used to disrupt genes to study their role in virulence and their immunogenicity, to define the minimum genetic complement required for independent survival of an organism [14–17], and as vectors for xenogenic expression [18,19]. Only Tn916 and Tn4001, isolated from *Enterococcus faecalis* and *Staphylococcus aureus*, respectively, have been shown to function in mycoplasmas. Tn4001 is smaller (4.7 kbp) than Tn916 (18 kbp) and appears to have a better transformation efficiency [20], and plasmid pISM2062, carrying the transposon Tn4001 [21], has been used to introduce this transposon into *M. bovis* [22].

In the study described here, a library of *M. bovis* strain PG45 mutants was created by transformation with Tn4001-based plasmids. The locations of transposon insertions in the genome were identified by genomic sequencing and the catalogue of disruptable genes compared to those generated in other pathogenic mycoplasmas to identify those genes previously thought to be indispensable in mycoplasmas that are dispensable in *M. bovis*.

**Results**

Functionality of transposon constructs for *M. bovis* strain PG45

The series of constructs based on Tn4001 were initially examined for their ability to transform *M. gallisepticum* strain S6, which was considered a model organism for transformation, as it had been transformed successfully in previous studies in our laboratory [23,25,27]. Following success in transforming *M. gallisepticum*, pTn4001complete was used to transform *M. bovis* strain PG45. Subsequently, *M. bovis* was transformed with pTn4001single and then with the minitransposons containing either the gentamicin or tetracycline resistance genes. Individual colonies on selective agar plates were selected and cultured in appropriate selective broth and the cultures examined by PCR to
confirm the presence of the gentamicin or tetracycline resistance genes.

Randomness of transposon integration

The randomness of transposon integration in the genome was confirmed by direct genomic sequencing of the mutant library (Figure 1), which allowed mapping of the transposon integration site for 319 mutants.

Haystack screening for a xer1 insertion

To identify mutants with a disruption in a specific gene target, transposon insertion sites were initially screened using a PCR-based strategy based on the haystack mutagenesis approach. For each of the four targeted loci, a first round of PCR was performed on each pool using one primer specific for the GOI and a second that would bind to the 5' or 3' end of Tn4001. An amplification product was identified in pool 5 using the oligonucleotide primer pair GKxer1 for and IR inverse (Table S1), indicative of a xer1 disruption. The pool contained 29 individual mutants. The second round of PCR was performed on DNA from each the 29 mutants within the pool individually and mutant number 29, which had the xer1 gene disrupted by Tn4001complete, was identified. The PCR yielded an amplification product of around 350 bp, suggesting that the site of insertion of the transposon was expected to be around 350 bp downstream of the start codon of the gene. This was confirmed by cloning the PCR product in pGEM-T and sequencing the insert. Haystack screening did not detect disruptions in p48, oppD or the restriction endonuclease gene, and the absence of these mutations from the library was confirmed by direct genome sequencing.

Non-essential genes in M. bovis

After initial studies using haystack mutagenesis, we used direct sequencing to identify the insertion sites in all the mutants in the library. Of the 319 mutants, 151 were generated using pTn4001single, 125 using pTn4001complete, 40 using pMiniTn4001-gent and 3 using pMiniTn4001-tet. A total of 191 insertions were in annotated ORFs, 38 within predicted intergenic regions, 40 within ICE elements and 50 within transposase genes. Of the 191 insertions in ORFs, 113 were in predicted genes, 56 in genes encoding membrane proteins or lipoproteins and 22 in genes encoding hypothetical proteins. Based upon the criteria for gene disruption, 129 genes had been disrupted, and of these 48 and 99 genes had homologues in M. mycoides subspecies mycoides SC strain PG1 and M. agalactiae strain PG2, respectively (Table 1). There were 21 additional genes that had transposon insertions within the last 15% of the coding sequence and which were therefore not considered to be disrupted, although this may not have been the case if function was located in this region of the protein (Table S2). Several genes were disrupted in multiple mutants. Intergenic regions contain promoters for genes located downstream, so transposon insertions in intergenic regions may have impaired the function of downstream genes or operons, so while these insertion events were not considered gene disruptions, the mutants carrying them (Table S3) may also be important in assessment of gene function. In addition, a total of 90 insertions were observed within integrative conjugative elements (ICE) (Table S4) and transposase genes (Table S5).

A number of notable genes were disrupted, including those annotated as encoding the heat shock proteins ClpB (MBOVPG45_0720) and DnaJ (MBOVPG45_0839), all the genes in the putative nucleotide transporter operon (MBOVPG45_307 to MBOVPG45_311), one gene in the polyamine ABC transporter system operon (MBOVPG45_0135), two genes in the glycerol ABC transporter system operon (MBOVPG45_0746 & MBOVPG45_0749), and in the genes encoding the glycerol kinase (MBOVPG45_0529) and the glycerol uptake facilitator protein (MBOVPG45_0530).

Figure 1. Location of 319 transposon integration sites in the M. bovis genome. The distribution of the transposon insertion sites indicates that insertions were randomly distributed.
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| ORF  | Product                                      | Gene              | Gene locus | Gene size (bp) | Tn insertion site in gene (%) | MSC orthologue* | MAGPG2 orthologue* |
|------|----------------------------------------------|-------------------|------------|----------------|-------------------------------|----------------|-------------------|
| 0008 | Oxidoreductase, FAD/FMN-binding protein      | 5995–4814         | 1182       | 14.5 & 86.2    | Y                             | Y              |                   |
| 0024 | S41B peptidase family lipoprotein            | 23202–21331       | 1872       | 48.2           |                               |                |                   |
| 0025 | Indigoidine synthase A family protein        | 24378–23473       | 906        | 30.6 & 84.5    |                               |                |                   |
| 0028 | Membrane protein                             | 26308–28284       | 1977       | 39.1           |                               |                |                   |
| 0030 | Hypothetical protein                          | 29413–29760       | 348        | 40.5           |                               |                |                   |
| 0031 | Hypothetical protein                          | 29735–31156       | 1233       | 70.6           |                               |                |                   |
| 0038 | Hypothetical protein                          | 49660–39680       | 9980       | 49.3           |                               |                |                   |
| 0049 | Membrane protein                             | 59330–60364       | 1035       | 80.8           |                               |                |                   |
| 0060 | tRNA modification GTPase                     | tmE               | 69942–68605| 1338           | 45.1                          | Y              |                   |
| 0061 | Hypothetical protein                          | 70070–70744       | 675        | 19.3, 20.3 & 74.8 |                               |                |                   |
| 0065 | SOS ribosomal protein L34                    | rpmH              | 74593–74742| 150            | 7.3                           | Y              |                   |
| 0083 | DNA polymerase IV                            | 94778–96025       | 1248       | 44.1           |                               |                |                   |
| 0086 | Lipoprotein                                  | 97862–99055       | 1194       | 33.6           |                               |                |                   |
| 0093 | Membrane protein                             | 104021–102738     | 1284       | 35.0 & 37.1    |                               |                |                   |
| 0096 | RNA methyltransferase, TrmH family           | 105512–106243     | 732        | 78.1           |                               |                |                   |
| 0116 | Oligopeptide ABC transporter ATP-binding protein | 125479–127881 | 2403   | 72.0           |                               |                |                   |
| 0118 | Membrane protein                             | 128948–129439     | 491        | 1.0 & 42.2     |                               |                |                   |
| 0119 | Membrane protein                             | 129526–130743     | 1218       | 80.9           |                               |                |                   |
| 0120 | Lipoprotein                                  | 130781–132721     | 1941       | 28.8           |                               |                |                   |
| 0123 | Membrane protein                             | 135436–135822     | 387        | 36.2           |                               |                |                   |
| 0131 | LemA family protein                          | 142685–143338     | 699        | 80.8           |                               |                |                   |
| 0133 | Oligoendopeptidase F                         | pepF              | 147853–146012| 1853 | 2.7           | Y                             | Y              |                   |
| 0135 | Polyamine ABC transporter permease           | patB              | 149512–150348| 837  | 80.8           | Y                             | Y              |                   |
| 0139 | Membrane protein                             | 153571–155238     | 1667       | 81.0           |                               |                |                   |
| 0140 | Thiamine biosynthesis protein/tRNA modification protein | thl | 155240–156376| 1137 | 34.7           | Y                             | Y              |                   |
| 0153 | Phosphate acetyltransferase                  | pts_I             | 171898–172854| 957  | 60.4           | Y                             | Y              |                   |
| 0157 | Membrane protein                             | 175094–176596     | 1503       | 1.5 & 49.3     |                               |                |                   |
| 0164 | Lipase/esterase LIP3/BchO family             | 185490–184690     | 801        | 48.4           |                               | Y              |                   |
| 0168 | Type II RM system methylase                  | 191805–190104     | 1702       | 45.4           |                               |                |                   |
| 0169 | Type III RM system methylase                 | 193542–191868     | 1674       | 38.7           |                               |                |                   |
| 0170 | Type III RM system methylase                 | 195267–193599     | 1668       | 80.2           |                               |                |                   |
| 0176 | Membrane protein                             | 203450–201144     | 2307       | 0.9, 58.1 & 71.6 |                               |                |                   |
| 0215 | Membrane nuclease A                          | mnuA              | 250107–248878| 1329 | 2.3           | Y                             |                |                   |
| 0216 | Peptidase, M17 family                        | 250218–251579     | 1362       | 38.0           |                               | Y              |                   |
| 0227 | Methionine adenosyltransferase               | metK              | 261973–263121| 1149 | 1.5           | Y                             | Y              |                   |
| ORF | Product | Gene | Gene locus | Gene size (bp) | Tn insertion site in gene (%) | MSC orthologue* | MAGPG2 orthologue* |
|-----|---------|------|------------|----------------|-------------------------------|----------------|-------------------|
| 0232 | Membrane protein | 267520–269451 | 1932 | 53.5 | Y | Y |
| 0234 | Lipoprotein | 270776–270095 | 624 | 45.8 | Y |
| 0237 | Type I RM system methylase | hsdM-1 | 272909–275587 | 2679 | 18.0 & 52.7 | Y |
| 0238 | Type I RM system, S subunit | 275592–276749 | 1158 | 79.5 | Y |
| 0241 | Type I RM system, S subunit | 282015–280936 | 1194 | 17.2 & 58.2 | Y |
| 0242 | Hypothetical protein | 288402–283690 | 4712 | 36.4, 48.9, 60.2, 82.1 & 99.1 | Y |
| 0245 | Hypothetical protein | 291827–293329 | 1503 | 52.8 & 95.0 | Y |
| 0281 | HAD-superfamily hydrolase | 313881–314750 | 869 | 12.9 | Y |
| 0298 | Lipoprotein | 335476–334424 | 1053 | 44.4 | Y |
| 0300 | Deoxyribose-phosphate aldolase | 337090–336422 | 75.5 | Y | Y |
| 0301 | Pyrimidine-nucleoside phosphorylase | pdf | 338395–337100 | 1296 | 2.1 | Y | Y |
| 0307 | Oligosaccharide ABC transporter perease | 34498–343515 | 983 | 39.3 | Y |
| 0309 | Oligosaccharide ABC transporter ATP-binding protein | 347590–345488 | 2013 | 75.8 & 76.6 | Y | Y |
| 0310 | Lipoprotein, nuclease family | 348768–347599 | 1170 | 70.3 | Y |
| 0311 | Membrane lipoprotein P81 | mb-mp81 | 351020–348834 | 2187 | 44.1 | Y |
| 0316 | Membrane protein | 357175–355469 | 1707 | 35.2 | Y |
| 0317 | Deoxyribonuclease IV phage-T4-induced | nbo | 358043–357207 | 837 | 70.5 | Y | Y |
| 0327 | AEC family transporter | 369686–370930 | 1245 | 18.2 | Y | Y |
| 0333 | Kinase family protein | 379206–377755 | 1452 | 12.5 | Y |
| 0349 | Membrane protein | 396678–395692 | 987 | 25.5 | Y | Y |
| 0353 | Lipoprotein | 401201–400053 | 1149 | 64.7 | Y |
| 0366 | Type II restriction enzyme | 414369–413662 | 109 | 70.9 | Y |
| 0370 | Membrane protein | 419556–418309 | 1248 | 65.9 | Y |
| 0372 | Membrane protein | 421848–424058 | 2211 | 29.9 | Y |
| 0375 | Membrane protein | 429203–431452 | 2250 | 14.0 | Y |
| 0376 | LppD family lipoprotein | 431482–434052 | 2571 | 38.5 & 73.1 | Y |
| 0385 | Lipoprotein | 446177–443793 | 2385 | 43.8 | Y | Y |
| 0390 | Glycosyltransferase | 452829–451825 | 1005 | 24.7 | Y |
| 0402 | Lipoprotein | 465914–463971 | 1944 | 9.2 | Y | Y |
| 0403 | msrA/msrB peptide methionine sulfoxide reductase | 466880–465951 | 930 | 23.8 & 58.2 | Y | Y |
| 0404 | Smr domain-containing protein | 467245–466970 | 276 | 6.9 | Y |
| 0416 | Lipoprotein | 479242–481113 | 1872 | 65.9 | Y |
| ORF | Product | Gene       | Gene locus  | Gene size (bp) | Tn insertion site in gene (%) | MSC orthologue* | MAGPG2 orthologue* |
|-----|---------|------------|-------------|----------------|--------------------------------|----------------|-------------------|
| 0419| Hypothetical protein | 483086-484111 | 82.8 | Y | 5.17 |
| 0421| S41B peptidase family lipoprotein | 487636-485678 | 1959 | Y | 6.2 |
| 0425| Membrane protein | 495066-493078 | 1989 | 46.0, 88.2 & 93.4 |
| 0458| Hypothetical protein | 530597-529914 | 684 | 76.0 |
| 0464| Potassium transporter, Trk family | 535963-533295 | 669 | 26.3 |
| 0466| Membrane protein | 537715-538167 | 453 | 62.9 |
| 0468| Hypothetical protein | 539519-540037 | 519 | 53.2 |
| 0503| Lipoprotein | 580958-578787 | 2170 | 78.9 |
| 0509| Membrane protein | 587125-588648 | 1524 | 31.5 |
| 0519| ABC transporter permease protein | 596244-597584 | 1340 | 37.5 |
| 0520| ABC transporter ATP-binding protein/chromosome segregation protein | 600615-597637 | 2979 | 38.6 |
| 0527| DHH family protein | 606297-607298 | 5002 | 11.3, 20.4 & 64.5 |
| 0529| Glycerol kinase | 609112-610620 | 1509 | 22.4 |
| 0530| Glycerol uptake facilitator protein | 610629-611420 | 792 | 51.9 |
| 0533| Neutral amino acid transporter, L-type amino acid transporter LAT family | 614828-616498 | 1671 | 65.8 |
| 0534| Hypothetical protein | 616549-617004 | 456 | 29.6 |
| 0550| Membrane protein | 633844-633634 | 1791 | 11.5 |
| 0557| S41B peptidase family lipoprotein | 648754-645631 | 1877 | 4.1 |
| 0564| Lipoprotein | 651735-650404 | 2310 | 39.6, 39.6, 46.5 & 92.2 |
| 0565| Lipoprotein | 654047-655885 | 1839 | 43.8 & 47.9 |
| 0568| DAACS family amino acid transporter | 661162-659552 | 1611 | 20.9 |
| 0569| Pyridine nucleotide-disulfide oxidoreductase | 662504-661155 | 1350 | 55.5 |
| 0584| Lipoprotein | 677916-678983 | 1068 | 52.2 |
| 0617| Type I RM system R subunit | 705566-707224 | 1659 | 16.4, 36.1 & 75.3 |
| 0618| Type I RM system M subunit | 707238-708689 | 1452 | 16.3 & 67.9 |
| 0621| Type I RM system S subunit | 712712-711396 | 1272 | 49.3 & 49.6 |
| 0623| Hypothetical protein | 715793-713523 | 2271 | 4.3 & 4.7 |
| 0629| Non-specific serine/threonine protein kinase | 724478-723480 | 999 | 23.3 |
| 0655| Lipoprotein | 746841-747865 | 1024 | 27.6 |
| 0662| Hypothetical protein | 752431-752135 | 297 | 31.3 |
| 0669| CypA family protein | 763380-761983 | 1398 | 80.8 |
| 0673| Membrane protein | 767508-765733 | 1776 | 62.6 |
| ORF | Product | Gene | Gene locus | Gene size (bp) | Tn insertion site in gene (%) | MSC orthologue* | MAGPG2 orthologue* |
|-----|---------|------|------------|----------------|-------------------------------|----------------|-------------------|
| 0685 | Membrane protein | 780789–783107 | 2319 | 11.1 & 57.7 |
| 0690 | Ser/thr protein phosphatase family/S’-nucleotidase, C-terminal domain-containing protein | 793088–791046 | 2043 | 54.9 |
| 0695 | Drug resistance ATPase ABC transporter family, ATP-binding protein | 797866–799479 | 1614 | 17.0 |
| 0719 | S1 RNA binding domain-containing protein | 836712–838871 | 2160 | 18.7 |
| 0720 | ATP-dependent chaperone protein | cipB | 839018–841186 | 2169 | 62.0 |
| 0728 | Phosphoglucomutase/phosphomannomutase domain-containing protein | 848314–851286 | 2973 | 35.4 & 47.1 |
| 0734 | Orotidine 5’-phosphate decarboxylase | pyF | 856735–856082 | 654 | 61.9 |
| 0735 | Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 | 857233–856745 | 489 | 9.8 |
| 0738 | Phosphotriesterase family protein | 860537–859476 | 1062 | 18.6 |
| 0743 | Type III RM system | 865850–867709 | 1859 | 16.5 & 68.9 |
| 0744 | N-6 adenine-specific DNA methylase truncated | 867719–868585 | 867 | 17.4 |
| 0748 | Glycerol ABC transporter permease | gscC2 | 873847–873035 | 813 | 74.2 |
| 0749 | Glycerol ABC transporter permease | gbsB2 | 874811–873837 | 975 | 25.3 |
| 0766 | Site-specific DNA-methyltransferase adenine-specific | 890583–891707 | 1125 | 1.2 & 42.4 |
| 0770 | Ribonuclease HIII | rrnB-2 | 896491–895868 | 624 | 68.8 |
| 0777 | Hypothetical protein | 901078–901689 | 612 | 12.6 |
| 0787 | Membrane protein | 911272–911937 | 666 | 17.0 |
| 0800 | Hypothetical protein | 923232–922786 | 447 | 10.5 |
| 0810 | Variable surface lipoprotein G | vspG | 935674–934715 | 960 | 28.2 |
| 0822 | Site-specific recombinase, phage integrase | xer1 | 948412–949161 | 750 | 45.9 |
| 0825 | Lipoprotein | 951540–952691 | 1152 | 84.5 |
| 0826 | Hypothetical protein | 952881–955298 | 2418 | 22.1 |
| 0831 | Ribosomal large subunit pseudouridine synthase, RluA family | 959814–958960 | 855 | 3.4 |
| 0838 | Hypothetical protein | 965052–964435 | 617 | 6.7 |
| 0839 | Chaperone protein | dnaJ | 966317–965184 | 1134 | 61.0 |
| 0845 | tRNA binding domain-containing protein | 970893–970291 | 603 | 7.1 |
| 0849 | Methyltransferase, HemK family | 973014–972922 | 723 | 50.3 |
| 0855 | SsrA-binding protein | smpB | 978922–979368 | 447 | 31.5 |
| 0858 | Transcriptional regulator | 983085–982126 | 960 | 25.8 |

*M. bovis* strain PG45 has 382 genes homologous to those of *M. mycoides* subspecies mycoides SC strain PG1 (MSC) and 595 homologous to those of *M. agalactiae* strain PG2 (MAGPG2). Y indicates the presence of an orthologous gene. doi:10.1371/journal.pone.0097100.t001
The table below lists genes regarded as essential in previous studies but found to be dispensable in this study.

| ORF | Product                          | Gene | Gene size (bp) | Essential MYPU orthologue \(^1\) | M. genitalium essentiality \(^2\) | Core mycoplasma genes \(^3\) | Gene persistence in mycoplasmas \(^4\) | Essentiality \(^5\) in B. subtilis & E. coli | Gene persistence \(^6\) in B. subtilis & E. coli |
|-----|----------------------------------|------|----------------|----------------------------------|---------------------------------|-------------------------------|--------------------------------------|----------------------------------|--------------------------------------|
| 0060 | tRNA modification GTPase         | trmE | 1338           | 0130 (56)                        | 008 (Y)                        | CEMyc0050                     | 20                                   | N                                | Ec                                   |
| 0065 | SOS ribosomal protein L34        | rpmH | 150            | 1540 (79)                        | 466 (Y)                        |                               | 20                                   | Bs, Ec                           | Bs, Ec                               |
| 0135 | Polyamine ABC transporter permease | podB | 837            | 4260 (49)                        | 043 (Y)                        | CEMyc0750                     | 20                                   |                                   |                                      |
| 0140 | Thiamine biosynthesis protein    | thl  | 1137           | 7180 (52)                        | 372 (Y)                        |                               |                                      |                                   |                                      |
| 0153* | Phosphate acetyltransferase      | pca_1| 957            | 2370 (55)                        | 299 (Y)                        |                               | 18                                   |                                   |                                      |
| 0227 | Methionine adenosyltransferase   | metK | 1149           | 7020 (54)                        | 047 (Y)                        | CEMyc01380                    | 19                                   | Bs, Ec                           | Bs, Ec                               |
| 0300 | Deoxyribose-phosphate aldolase   | deoC | 669            | 3140 (58)                        | 050 (Y)                        | CEMyc0540                     | 20                                   |                                   |                                      |
| 0307 | Oligosaccharide ABC transporter permease | 983 | 0280 (43) | 189 (Y)                          |                               |                               |                                      |                                   |                                      |
| 0309 | Oligosaccharide ABC transporter ATP-binding protein | 2103 | 0260 (41) | 187 (Y)                          |                               |                               |                                      |                                   |                                      |
| 0311 | Membrane lipoprotein P81         | mb-mp81 | 2187 | 0240 (28) | 260 (N)                          |                               |                                      |                                   |                                      |
| 0317 | Deoxyribonuclease IV phage-T4-induced | nfo | 837 | 6210 (61) | 235 (Y)                          | CEMyc01290                    | 20                                   |                                   |                                      |
| 0390 | Glycosyltransferase              |      | 1005           | 7700 (32)                        | 335 (Y)                        |                               |                                      |                                   |                                      |
| 0464 | Potassium transporter, Trk family | ktrA | 669            | 1370 (51)                        | 323 (Y)                        |                               |                                      |                                   |                                      |
| 0520 | Chromosome segregation protein/ABC transporter ATP-binding protein | smc | 2979 | 7140 (53) | 298 (N)                          |                               |                                      |                                   |                                      |
| 0527** | DHH family protein              |      | 1002           | 6920 (49)                        | 190 (Y)                        |                               |                                      |                                   |                                      |
| 0534 | Hypothetical protein             |      | 456            | 6130 (53)                        | NA                             |                               |                                      |                                   |                                      |
| 0629 | Non-specific serine/threonine protein kinase | pknB | 999 | 6850 (41) | 109 (Y)                          |                               |                                      |                                   |                                      |
| 0695 | Drug resistance ATPase family, ATP-binding protein | 1614 | 6900 (71) | NA                             |                               |                                      |                                   |                                      |
| 0728 | Phosphoglucomutase/phosphomannomutase domain-containing protein | 2973 | 4840 (26) | NA                             |                               |                                      |                                   |                                      |
| 0839 | Chaperone protein                | dnaI | 1134           | 7330 (40)                        | 019 (Y)                        | CEMyc01460                    | 12                                   | N                                | Bs, Ec                               |
| 0845 | tRNA binding domain-containing protein | 603 | 4860 (32) | 195 (Y)                          |                               |                               |                                      |                                   |                                      |
| 0849 | Methyltransferase family         | hemK | 723            | 1060 (53)                        | 259 (Y)                        | CEMyc0140                     | 18                                   | N                                | Ec                                   |
| 0855 | SraA-binding protein             | smpB | 447            | 3520 (48)                        | 059 (Y)                        | CEMyc0650                     | 20                                   | N                                | Bs, Ec                               |

1 Essential genes in M. pulmonis, with percentage amino acid sequence identity with M. bovis strain PG45 in parentheses (French et al., 2008).
2 M. pulmonis genes found dispensable in later study (Dybvig et al., 2010).
3 Gene essentiality in transposon mutagenesis studies in M. genitalium (Glass et al., 2006).
4 Y indicates gene essentiality; N indicates gene dispensability; NA indicates orthologous gene not found.
5 Database of predicted essential genes http://tubic.tju.edu.cn/pdeg/ (Lin & Zhang, 2011).
6 Number of mycoplasma species (out of 20) in which gene is conserved (Liu et al., 2012).
7 Essentiality in B. subtilis (Bs) and E. coli (Ec). N indicates conserved.
8 Gene persistence in B. subtilis (Bs) and E. coli (Ec) (Fang et al., 2005).
9 *0323 parologue in M. bovis shares 43% identity with 0153.
10 **0526 parologue in M. bovis shares 48% identity with 0527.

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Fewer essential genes in mycoplasmas than in previous studies

In an early study employing transposon mutagenesis, 310 genes were reported to be essential in *M. pulmonis* [15]. A further study on *M. pulmonis* found an additional 39 of these 310 genes to be dispensable [14], and it has been suggested that there are 153 core essential genes in Mycoplasma species [26]. In the study described here on *M. bovis*, 23 genes considered to be essential in *M. pulmonis* in the initial study [15], 16 of which were still found to be essential in the subsequent study [14], were disrupted (Table 2). Five of these genes, encoding the rRNA modification GTPase TrmE (MBOVPG45_0060), the polyamine ABC transporter permease PotB (MBOVPG45_0135), the methionine adenosyltransferase MetK (MBOVPG45_0227), the chaperone protein DnaJ (MBOVPG45_0839) and the ssaA binding protein SmpB (MBOVPG45_0855), were considered essential in all previous gene essentiality studies in mycoplasmas [14–16,28] and have been predicted to form the core set of 153 essential genes in mycoplasmas [26]. Thus our study has demonstrated that mycoplasmas have fewer core essential genes than predicted previously.

Among the other genes considered essential in earlier studies [14–16,28] that were disrupted in our library were those coding for the 50S ribosomal protein L34 (RpmH, MBOVPG45_0065), the thiamine biosynthesis protein (ThiI, MBOVPG45_0140), oligosaccharide ABC transporter proteins (MBOVPG45_0307 & 0309), a glycosyltransferase (MBOVPG45_0390), the potassium oligosaccharide ABC transporter proteins (MBOVPG45_0307 & 0308), the drug resistance ABC transporter ATP-binding protein (Smc, MBOVPG45_0520), a hypothetical protein (MBOVPG45_0527), which were reported to be essential in earlier studies, were disrupted in the *M. bovis* library, but these genes have paralogues in the *M. bovis* genome and therefore could not be considered to be dispensable based on our study.

**Discussion**

Although the genomes of the type strains of *M. bovis*, *M. agalactiae* and *M. mycoides* subspecies *mycoides* SC, all of which cause disease in ruminants, have been sequenced [7], gene essentiality data are not available for these species. There has been extensive horizontal gene transfer between these species, with many genes in *M. bovis* and *M. agalactiae* probably acquired from the phylogenetically distant *M. mycoides* cluster [8,10] during co-infection of the same host [29]. Therefore, genes found to be non-essential in *M. bovis* are likely to also be non-essential in the other two species. Of the genes disrupted in the *M. bovis* mutant library, 48 had orthologues in *M. mycoides* subspecies *mycoides* SC and 99 had orthologues in *M. agalactiae*. Six of the 23 essential mycoplasma genes that were found to have transposon insertions in our study have essential orthologues in *B. subtilis* [30,31].

In our study, there were insertions in 191 predicted ORFs. In earlier studies in *M. genitalium* 382 genes were found to be indispensable in *M. genitalium* [16], while 310 genes were found to be essential in *M. pulmonis* [15]. A further study in *M. pulmonis* [14] found 39 additional genes to be dispensable. Comparison of the data from our study with that obtained for *M. pulmonis* is of interest as both species have similar genome sizes and lie within the same (hominis) phylogenetic group. We found 23 of the 310 genes found to be essential in the initial study on *M. pulmonis* [15] were disruptable in *M. bovis* (Table 2), with 7 of these 23 among those found to be disruptable in the later study on *M. pulmonis*.

The *M. bovis* genome has 52 ABC transporter genes, in 14 operons, and nine of these transporter gene ORFs, in four operons, were able to be disrupted (Table 3). Acquisition of nutrients by mycoplasmas appears to predominantly involve ABC transporters, and the low level of redundancy in mycoplasmas suggests that they are likely to be required for nutrient acquisition in *vivo*, but clearly some are dispensable in the complex media used for culture in *vitro*.

The dispensability of two genes belonging to the glycerol transport system, along with glycerol kinase and the glycerol uptake facilitator protein, is not surprising considering that there are two predicted glycerol transport systems in *M. bovis* that may be dispensable based on the study.

**Table 3.** Putative ABC transporters of *M. bovis* disrupted by transposon mutagenesis.

| ORF | Product | Gene | Gene locus | Gene size (bp) | Tn insertion site in gene (%) | Operon |
|-----|---------|------|------------|---------------|-------------------------------|--------|
| 0116 | Oligopeptide ABC transporter ATP-binding protein | oppF2 | 125479–127881 | 2403 | 72.0 | 0112–0116 |
| 0135* | Polyamine ABC transporter permease | potB | 149512–150348 | 837 | 80.8 | 0134–0137 |
| 0307* | Oligosaccharide ABC transporter permease | | 344498–345315 | 983 | 39.3 | 0307–0311 |
| 0309* | Oligosaccharide ABC transporter ATP-binding protein | | 347590–345488 | 2103 | 75.8 & 76.6 | 0307–0311 |
| 0519 | ABC transporter permease protein | | 596244–597584 | 2979 | 38.6 | NA |
| 0520* | ABC transporter ATP-binding protein/chromosome segregation protein | smc | 600615–597637 | 2979 | 38.6 | NA |
| 0695* | Drug resistance ABC transporter/ATP-binding protein | | 797866–799479 | 1614 | 17.0 | NA |
| 0748 | Glycerol ABC transporter permease | gtsC2 | 873847–873035 | 813 | 74.2 | 0747–0750 |
| 0749 | Glycerol ABC transporter permease | gtsB2 | 874811–873837 | 975 | 25.3 | 0747–0750 |

*considered essential in earlier mycoplasma studies as described in Table 2; NA: does not appear to be part of any operon.

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complement each other. Earlier studies have reported that the production of hydrogen peroxide by ruminant mycoplasmas involves the glycerol transport system [32,33], and the transport system appears to be dispensable in M. mycoides subspecies mycoides SC, as European strains, which are less virulent compared to their African counterparts, lack both the gtdB and gtc glycerol transport genes [34].

The possibility of gene disruptions in some genes of M. bovis that are essential in other mycoplasmas might be expected, as there are paralogues in M. bovis of the genes encoding phosphate acetyltransferase and the DHH family protein. Similarly, some variations might be expected between different species because of unrecognized redundancy. The serine/threonine protein kinase gene \( pknB \), which was disrupted in the M. bovis library, has been reported to be essential for growth of M. pulmonis and M. genitalium [14–16], however \( pknB \) has been disrupted in M. agalactiae [35].

The genes MBOVPG45_0307 to MBOVPG45_0311, which are part of a putative nucleotide transporter operon [36–38], were disrupted in our study, and transposon insertions in \( mslA \), the MBOVPG45_0311 homologue in M. gallisepticum, have been reported previously [39]. It has been demonstrated recently that \( mslA \) of M. gallisepticum, the MBOVPG45_0311 homologue, binds single and double stranded DNA [40], suggesting that the \( mslA \) may bind and deliver oligonucleotides to the exonuclease, which then processes these oligonucleotides to generate individual nucleotides for transport into the cell via the ABC transporter. The disruption of genes encoding this operon might be tolerated because there are three putative membrane nucleases in the genome of M. bovis strain PG45, MBOVPG45_0089, MBOVPG45_0215 and MBOVPG45_0310.

The dispensability of five genes disrupted in our study, \( tnmE, poIB, metK, dnaJ \) and \( sphB \), which were found to be essential in all previous gene essentiality studies in mycoplasmas, and which were among the predicted set of 153 core mycoplasma genes, could not be explained by predicted redundancy. Although the polynucleotide transporter system is dispensable in B. subtilis, this may result from complementation by another transport system. It is possible that this may also be the case in M. bovis. The chaperone DnaJ has long been considered to be essential for cellular growth. However, as expression of DnaJ increases in response to cellular stress [41], it may be dispensable during the optimal growth conditions used for culture in vitro. There are no obvious explanations for the dispensability of \( rpmH, thiL \) and \( ketA \), nor for the genes encoding the glycosyltransferase and the RNA binding domain-containing protein.

However, it has been pointed out that minimal or core sets of genes are context dependent and it has been suggested that gene persistence is a better indication of the role of specific genes in the long term survival of an organism [42] and that, in defining the minimal requirements for cellular life, it would be more useful to consider those genes that, while not ubiquitous, were conserved in most genomes. Therefore we have assessed which of the genes that we found to be dispensable (Table 2) are found in most mycoplasma genomes [43]. We have also compared the gene dispensability determined in our study with the persistence and essentiality of orthologues in B. subtilis and E. coli [44].

The dispensability of \( rpmH \) is surprising, as it is conserved in all the fully sequenced mycoplasma genomes [43], and not only essential in M. pulmonis and M. genitalium, but also in B. subtilis and E. coli [44]. Similarly \( snc \) is conserved in all the mycoplasma genomes, as well as in B. subtilis. In recent studies, the \( rpmH \) and \( snc \) genes have been reported to be borderline persistent [45], and \( snc \) could be disrupted in M. pulmonis [14] and \( rpmH \) in B. subtilis, although the growth of the mutant was affected [46]. The \( pheB \) and \( thiL \) genes are not highly conserved in the mollicutes, with \( pheB \) absent in M. hyorhinis, M. hypopneumoniae, M. conjunctivae and Acholeplasma laidlawii, and \( thiL \) not found in M. hyorhinis, M. hypopneumoniae, M. conjunctivae or Ureaplasma urealyticum, its absence being correlated with a mutation in tRNA\( K \). The gene \( hmk \), which is predicted to code for a methyltransferase, is absent in M. conjunctivae, U. parvum and B. subtilis, while \( metK \), which codes for methionine adenosyltransferase, is conserved in all Mycoplasma species, B. subtilis and E. coli, but is not annotated in U. urealyticum.

Several potential problems with transposon-generated mutant libraries in mycoplasmas were not seen or were addressed by use of differing techniques in our study. In an earlier study [22] 16–86% of colonies growing on selective agar plates lacked a transposon insertion. In this earlier study, it was assumed that these resulted from acquisition of spontaneous resistance, but attempts to decrease the prevalence of pseudotransformants by increasing the concentration of antibiotic in selective agar failed. The problem was overcome in this earlier study by incubation of M. bovis in selective broth for an extended period after transformation, but this may also result in multiplication of mutants and thus increase the prevalence of replicate clones in the final library. However we did not detect any pseudotransformants following transformation with any of our transposon constructs.

Reproducible transposition, resulting in multiple insertions in the genome, have been a problem in some studies. We developed several derivatives of Tn4001, including Tn4001single, which lacked one of the IS256 arms, and minitransposons, with the transposase outside the transposon, with the aim of creating transposons that would be incapable of secondary transposition and that would thus generate mutants that could be expected to be genetically stable [47]. That this was desirable was demonstrated by the relatively high frequency of multiple insertion events we saw in mutants created using Tn4001 (data not shown).

The potential presence of insertional hotspots has also been raised as a concern in the use of transposons to generate mutant libraries. The randomness of insertion of Tn4001 and its derivatives was confirmed by genomic sequencing of 319 individual mutants, which demonstrated that insertion events were distributed throughout the genome (Figure 1).

Targeted gene knockout remains a challenge in mycoplasmas. Targeted gene disruption in mycoplasmas has occasionally been achieved through homologous recombination, either employing free DNA or replicable \( oriC \) plasmids [25,48,49], but the low rate of recombination has necessitated extensive passage to increase the likelihood of acquiring the desired knockout. In many cases recombination with \( oriC \) plasmids occurs within the \( oriC \) region, or in illegitimate sites, rather than in the desired targets, and if it does occur within the targeted gene it can be difficult to isolate the recombinant clone [50]. Transposon mutagenesis has been the genetic tool most commonly used to manipulate mycoplasmas because of its much greater efficiency, but there have been only limited attempts to identify mutants in libraries with specific phenotypic changes that might be attributable to disruption of specific genes. Mutant libraries have been screened for loss of reactivity with a specific antiserum against \( LppQ \) in M. mycoides subspecies mycoides SC [51], loss of gliding motility in M. pneumoniae [52] or loss of capacity for growth on cell cultures [35,53]. In the absence of a selectable phenotypic trait and to avoid time consuming direct genomic sequencing of all individual mutants, the PCR based haystack mutagenesis approach [3,51] can be used to identify specific gene knockouts. However, the approach may not be suitable for identification of gene disruptions in large coding regions, and particularly if it occurs in middle of coding regions. In our study the haystack mutagenesis approach was used to identify
a serI gene disruption. In earlier haystack mutagenesis studies [51], the transformants were grown in broth as a pool before DNA extraction. This may result in overgrowth of mutants with disruptions in genes not required for optimal growth. Therefore, we picked individual mutants, generated an ordered mutant library, and cultured the mutants to late log phase before creating a series of pools for screening. Instead of using two primer pairs in the Tin4001 region [3,31], a single oligonucleotide primer binding to the IR region of Tin4001 was used, as it could be combined with either a forward or reverse primer flanking the gene of interest to yield a single PCR product in the event of insertion in the desired gene.

Although genome sequences are available for more than 1000 bacterial species, genome-wide essentiality data is available for only 15 species, including three Mycoplasma species, *M. genitalium*, *M. pneumoniae* and *M. pulmonis* [14-17,20]. A set of 153 core essential mycoplasma genes have been predicted [26]. Some genes expected to be essential were identified as disrupted in an early study [20], possibly because mutants were not characterised as clonal cultures, but rather as members of a mixed pool, and some genes that were predicted to be non-essential in this initial study appeared to be essential in later studies [15].

Although the mutant library we have characterised here could not be expected to have included a comprehensive repertoire of mutable genes as the genome was not saturated with insertions, the lack of insertions in several large genes and transport systems suggests the importance of these genes for optimal growth of *M. bovis* in vitro. These include two predicted oligopeptide ABC transporter system operons, a predicted carbohydrate uptake ABC transporter system operon and a predicted cobalt ABC transporter system operon. No gene coding for tRNAs or rRNAs, which are considered essential for cell replication, was disrupted. In addition, some large genes that encode membrane proteins or hypothetical proteins were not disrupted, including MBOVG45_0337 (3419 bp), MBOVG45_0481 (4547 bp) and MBOVG45_0710 (8012 bp), and thus these genes may have a role in optimal growth of *M. bovis* in vitro and may be worthy of further investigation.

One of the largest membrane proteins in *M. bovis*, MBOVG45_0710, which is over 8 kbp in length (2670 amino acids) and has full or partial homologues in *M. agalactiae* MAG6100, *M. fermentans* MFE_02570, *M. crocodyli* MICRO_0279, *M. gyorui* MS53_0226, *M. pulmonis* MYPU_3130, *M. conjunctivae* MCJ_003940, *M. mobile* MMOB4250, *M. hyorhini* MYM_0299 and *M. hyopneumoniae* mhp677 [7], was not disrupted. Homologues of MBOVG45_0710 in *M. fermentans* and *M. mobile* are predicted to possess lipase activity, and the regions between amino acid residues 90 and 395 of MBOVG45_0710 had 31% identity to residues 90 and 395 of MBOVG45_0710 had 31% identity to *M. hyopneumoniae* p65, which has been demonstrated to possess lipase activity [34]. Although the conserved domain is restricted to the amino terminal end of this protein, the large size and lack of disruptions within the gene suggest essentiality of this protein. It may consist of several conserved domains, the functions of which are specific for species related closely to *M. bovis*.

Thus this study has validated the use of haystack mutagenesis to identify mutants with specific genes disrupted in an ordered mutant library, and has characterised the location of more than 300 transposon insertions in the *M. bovis* genome, establishing the dispensability of at least 16 genes previously believed to be essential in mycoplasmas. These data will aid in furthering our understanding of the functions of genes and gene products of mycoplasmas.

**Methods**

**Bacterial strains and culture conditions**

*M. bovis* type strain PG45 (ATCC 25523) was cultured at 37°C in modified Frey’s broth (21 g PPLO, 37 ml yeast extract, 100 ml inactivated swine serum, 4 ml 1.6% phenol red solution, 300 mg penicillin G, 859 ml distilled water, pH adjusted to 7.8) or on mycoplasma agar plates (modified Frey’s broth without phenol red with 1% agar added). For the selection of *M. bovis* transformants, gentamicin (Invitrogen) or tetracycline (Sigma Aldrich) was added to plates at a concentration of 50 μg/ml or 5 μg/ml, respectively.

*Escherichia coli* DH5α cells (Life Technologies) were used for cloning of different transposon constructs and were cultured at 37°C in Luria-Bertani (LB) broth (1% w/v tryptone (Oxoid), 0.5% w/v yeast extract (Oxoid), 0.5% w/v NaCl) with shaking at 200 rpm on an orbital shaker incubator (Ratek) or on LB agar plates (LB broth containing 1% bacteriological agar). Selection of plasmid-transformed *E. coli* DH5α cells was performed on LB agar containing 3-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal) (Sigma) at 40 μg/ml, isopropyl-B-D-thiogalactopyranoside (IPTG) (Sigma) at 50 μg/ml and an appropriate antibiotic. *E. coli* DH5α containing plasmid constructs were grown in LB broth or on LB agar plates containing ampicillin (Amresco) at 100 μg/ml, gentamicin at 20 μg/ml or tetracycline at 4 μg/ml.

**Agarose gel electrophoresis and plasmid extraction**

Polymerase chain reaction (PCR) products and plasmid DNA constructs were analysed using conventional agarose gel electrophoresis in 0.8-2.0% w/v agarose (gels) in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) or 0.5× TPE buffer (1× TPE is 36 mM Tris, 30 mM NaH2PO4, 1 mM EDTA) and stained with ethidium bromide at 0.1 μg/ml. DNA bands were visualised using an ultraviolet transilluminator (Gibco BRL) and imaged using either the Digital Science electrophoresis documentation and analysis system (Kodak) or the Molecular Imager ChemiDoc XRS+ imaging system (Bio-Rad).

PCR products and restriction endonuclease digestion products of plasmids were separated by agarose gel electrophoresis and the DNA in specific bands extracted using the Ultraclean gel spin DNA purification kit (Mo Bio Laboratories) according to the manufacturer’s instructions. The Wizard Plus SV Miniprep DNA purification system (Promega) was used to extract up to 2 μg of plasmid DNA from *E. coli* DH5α cells, whilst for purification of 20 μg or more of plasmid DNA the Qiagen Plasmid Midi kit (Qiagen) was used according to the manufacturer’s guidelines.

**Amplification of PCR products**

The cleavage sites for the restriction endonucleases *Bgl*II and *Nde*I were incorporated into the forward and reverse primers, respectively, used for the amplification of Tin4001 with either a single or both IS236 arms. The same cleavage sites were included in the oligonucleotide primers for the amplification of the gentamicin resistance gene, while *Sal*I and *Kpn*I cleavage sites were included in the forward and reverse primers used for amplification of the transposase (*tnp*) gene (Table S1). PCR reactions were performed in a thermocycler (iCycler, Bio-Rad) with 30 pg of plasmid DNA as template in a 50 μl reaction containing 3 μl of 10× Mg2+ free HiFi buffer, 2 mM MgSO4, 250 nM of each primer, 200 μM of each deoxynucleotidetriphosphate (dNTP) and 2.5 U of Platinum HiFi Taq DNA polymerase (Invitrogen).
FRT site, the reporter gene (operator region contained an inverted repeat (IR) (39 bp, 5'-taggaacttc-3') included the gataaagtccgtataattgtgtaaaagtaaaaaggccat-3'), the bovis tuf transposase gene (the signal (s) and an pMiniTn transposable element (IR, black bar). To generate the plasmid (backbone. The FRT site was ligated to a fragment containing an IR (grey bar) was ligated to a fragment containing an inverted repeat (IR, black bar), the promoter (p), the signal sequence(s) and an FRT site (grey bar) was ligated to a fragment containing an FRT site, the reporter gene (phoA) and an IR using the EcoRI and XhoI cleavage sites in a pUC57 backbone. Tn4001 with one or both insertion sequences was amplified and inserted in between the FRT sites of the construct to generate pTn4001 single (a) and pTn4001 complete (b), respectively. The construct pMiniTn4001 gent (c) was developed by amplifying and inserting the gentamicin resistance gene (aacA-aphD) between the two FRT sites of the construct, then the transposase gene (tnp) was amplified and inserted outside of the transposable element (IR, black bar). To generate the plasmid pMiniTn4001 tet (d), a fragment containing the IR, the promoter (p), the signal (s) and an FRT site was ligated to a fragment containing an FRT site, the reporter gene (phoA) and an IR in the pUC57 plasmid backbone. FRT sites have a unique XbaI cleavage site, so ligation of the fragments produced a construct with a single FRT site. The tnp gene was amplified and ligated into the plasmid outside the transposing element, then the tetM resistance gene with its own promoter and terminator was ligated within the construct.

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Development of novel reporter construct

To create a novel transposon from which the antibiotic resistance marker could be excised following transposon insertion in, and disruption of, a specific gene, operator and gene region fragments were designed and then synthesized commercially and cloned in the EoRV site of pUC57 (GenScript Corporation). The operator region contained an inverted repeat (IR) (39 bp, 5’-gataaagtccgtataattgtgtaaaagtaaaaaggccat-3’) together with the M. bovis tuf promoter (252 bp tuf promoter region located between bases 474270 and 474521 of NCBI Reference Sequence NC_014760.1), a vp signal sequence (84 bp, gene ID 10014768, predicted protein sequence MKKSKFLLLGVSASLISFVAAKGET) and the FRT sequence (34 bp Fbp recognition target, 5’-gaagttcctattctctagagtaaaagtaaaaaggccat-3’), an M. bovis codon optimised alkaline phosphatase reporter gene (phoA) [23] and the IR (39 bp, 5’-atggcctttttacttttataacataggaacttc-3’). The operator and gene segments were digested separately with EcoRI and XhoI and the operator segment ligated to the gene segment in the pUC57 backbone so that the FRT sequences were oriented as direct repeats. The nucleotide sequence of this novel construct, and relevant restriction endonuclease cleavage sites, are shown in Figure S1.

Construction of plasmids carrying transposons

Different Tn4001-based transposon constructs coding for gentamicin or tetracycline resistance and containing a single IS256 arm or both IS256 arms, and minitransposons, were generated (Figure 2). Tn4001 containing either a single or both IS256 arms (Figure S2), including the region coding for the gentamicin resistance gene (aacA-aphD), were amplified from PstI [24] using the primer pairs ISSIS256 for/SSISgent rev and ISSIS256 for/SSISSISgent rev, respectively (Table S1). Each PCR product was ligated to pGEM-T (Promega) and its DNA sequence confirmed by DNA sequencing using ABI PRISM Big Dye 3.1 Terminator chemistry (Life Technologies). Sequencing revealed that use of primer ISSIS256 for had resulted in amplification of the complete Tn4001, resulting in inclusion of the EcoRI cleavage site at the 5’ and 3’ ends. Therefore, pGEM-T plasmids containing either a single IS256 arm or the complete Tn4001 were digested with BglII and NotI or BglII alone, respectively, and ligated between the FRT sites of constructs digested with the same enzymes to generate the pTn4001 single and pTn4001 complete constructs. To facilitate insertion of the complete Tn4001 the construct was incubated with 150 units of bacterial alkaline phosphatase (BAP, Invitrogen) at 65°C for 1 h to prevent plasmid recircularisation.

To overcome potential problems associated with subsequent transposition and multiple insertions, Tn4001-based minitransposons containing the genes coding for either gentamicin or tetracycline resistance were developed. For construction of pMiniTn4001 gent (Figure 2), the complete gentamicin resistance gene, with its promoter and terminator sequences, was amplified by PCR from the pTn4001 single plasmid construct using the Gmgene for/Gmgene rev primer pair (Table S1), which contained engineered restriction endonuclease cleavage sites. The gentamicin resistance gene was cloned in pGEM-T, released by digestion with BglII and NotI, and then ligated between the two FRT sites in the novel construct, which had been digested using the same pair of endonucleases. The tnp gene was then amplified from the pTn4001 single plasmid using the primer pair Tnp for/Tnp rev, ligated into pGEM-T, excised with SacI and KpnI and then ligated into plasmid that had been cleaved with SacI and KpnI in a site external to the transposing element.

Another minitransposon, pMiniTn4001 tet (Figure 2), which had a single FRT site and encoded the tetracycline resistance gene (tetM), was also generated. In this construct, the M. bovis operator region was replaced by the tuf promoter and Tn4001 signal sequence of M. gallisepticum strain S6 [23]. As the FRT sequences contain a single XbaI cleavage site, ligation of the operator and gene segments after digestion with SacI and XbaI produced a single FRT site (Figure S3) in the construct, with pUC57 as the backbone. The tnp gene was then ligated outside of the transposing element in a site exposed by digestion with SacI and KpnI. Finally, the tetM gene with its own promoter and terminator was released from pMlori [25] by digestion with SpeI and ligated into the SpeI site in the plasmid containing the tnp gene at the SacI-KpnI site.
Transformation of *M. bovis* and creation of mutant libraries

Approximately 5 µg of each plasmid construct was used for transformation. The method used was based upon that described by Chopra-Dewasthaly et al. (2005), with some modifications. Briefly, 8 dilutions of a *M. bovis* culture were made in mycoplasma broth (1:5, 1:11.25, 1:12.2, 1:13.3, 1:1.5, 1:17.5, 1:21.65 and 1:30), and these incubated at 37°C for 16 h (late exponential phase). The cultures were pooled and cells were harvested by centrifugation at 16,000 g for 5 min at room temperature (RT) in a bench-top centrifuge. The cells were washed twice in 250 µl ice-cold HEPS–sucrose buffer (8 mM HEPES, 272 mM sucrose, pH 7.4). The cell pellet was then resuspended in 100 µl HEPS–sucrose buffer containing 5 µg plasmid DNA and transferred to a pre-chilled electroporation cuvette (0.2 cm, Bio-Rad). The mixture was kept on ice for 30 min and then pulsed (2.5 kV, 100 µF) using a Gene Pulser (Bio-Rad). The cells were immediately resuspended in 1 ml cold mycoplasma broth (4°C), placed on ice for a further 15 min and then incubated at 37°C for 2 h. The transformed culture was then plated onto a selective mycoplasma plate containing 50 µg gentamicin/ml or 5 µg tetracycline/ml. The plates were allowed to dry, then incubated in the dark in an airtight canister at 37°C until the colour of the medium changed. Individual colonies were picked using a Pasteur pipette, inoculated into 500 µl broth containing an appropriate selective antibiotic, and incubated at 37°C for 24 h. The transformed culture was then transferred to a selective mycoplasma plate containing 50 µg gentamicin/ml or 5 µg tetracycline/ml. The plates were allowed to dry, then incubated in the dark in an airtight canister at 37°C. The transformed cultures were examined for colonies after five days. Individual colonies were identified by direct genome sequencing.

**PCR-based detection of the selectable marker in cloned transformants**

To confirm the presence of the transposable element in the genome of the mutants, a screening PCR was performed that targeted the antibiotic resistance determinant. To verify the presence of either antibiotic resistance gene, cells from 100 µl of culture were pelleted by centrifugation at 16,000 g for 5 min at RT, the supernatant discarded and the cell pellet resuspended in 25 µl of distilled water. The resuspended cells were incubated at 100°C for 5 min and used as template for PCR. The PCR assays used 2 µl of DNA template in a 25 µl reaction mixture containing 1.25 U of Gotaq DNA polymerase (Promega) in 1× buffer supplied by the manufacturer, 200 µM of each dNTP, 1.25 mM MgCl₂ and 250 nM of each oligonucleotide primer for amplification of the gentamicin (Gm for/Gm rev) or tetracycline (LAtetM for/LBatetM rev) resistance genes (Table S1).

**PCR-based screening for specific gene knockouts**

The ‘haystack mutagenesis’ approach [3] was employed to screen the library of transposon mutants for insertions in four targeted genes. To limit the number of PCR reactions, 168 individual transposon-generated mutants were cultured in 1 ml of mycoplasma broth and arranged in seven pools containing 20 to 50 mutants. The genomic DNA was extracted from these pools using the High Pure DNA purification kit (Roche). The insertion of the transposable element in the genome could have occurred in either orientation (Figure 3), so the screening PCR was performed using a pair of primers that included the IR inverse oligonucleotide, which was specific for the transposon but could bind at either end of it, and either a forward or reverse oligonucleotide specific for the gene of interest (GOI) (Table S1) to identify a pool containing the desired GOI-transposon junction. Subsequently, a similar PCR using DNA prepared by boiling a cell pellet suspended in distilled water was performed on all the individual mutants in the positive pool to identify the mutant of interest. The relative position of the transposon insertion within the GOI was estimated from the size of the PCR fragment. To confirm the location of the transposon within the specific gene, the PCR product generated was cloned into pGEM-T and its DNA sequence determined. The location of the transposon in the *xer1* gene was further confirmed by direct genome sequencing.

**Determination of transposon insertion sites in the genome**

After selection from the initial agar plate each mutant was passaged a further two times in selective mycoplasma broth at 37°C to amplify the culture up to a volume of 8–10 ml. The cells were harvested by centrifugation at 11,000 g for 20 min at 4°C, and the supernatant discarded. The cell pellet was resuspended in 500 µl of distilled water. The resuspended cells were incubated at 100°C for 5 min, cooled on ice, and either a forward or reverse oligonucleotide specific for the gene of interest (GOI) (Table S1) was added to each sample.

**Figure 3. PCR-based screening approach to identify transposon insertions in gene targets**. The insertion of the transposable element in a particular gene can occur in two possible orientations. PCR reaction using a primer pair, one based on the 39-bp IR sequence (uppercase) of the transposon and another one being either the forward (in this figure) or reverse primer flanking the gene of interest (GOI) would generate a single PCR product in the event of gene disruption. The relative position of the transposon insertion within the GOI is estimated based on the size of the PCR fragment including the region of binding of forward or reverse primer and primer based on IR region of transposon.

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washed twice in phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), and finally resuspended in 200 μl PBS. Genomic DNA extraction was performed using the High Pure PCR kit (Roche) according to the manufacturer’s protocol, except that the initial lysozyme treatment was omitted and the DNA was eluted in 50 μl of elution buffer. DNA sequencing was performed directly on genomic DNA extracted from transposon mutants. The oligonucleotide sequencing primers tuf inverse and T7 universal (Table S1), which bind within the transposon at distances of 42–67 bp and 59–78 bp, respectively, from its insertion site, were used to sequence across the transposon-genomic DNA junction. Each 20 μl reaction contained 2–3 μg of purified genomic DNA, 30 μM of the primer, 4 μl of Big Dye terminator (BDT) v3.1 enzyme mixture and 4 μl of 5× BDT dilution buffer. The sequencing products were purified and their sequence determined. The resultant DNA sequence was then used to identify the location of each transposon in the M. bovis PG45 genome [9] using BLAST (National Centre for Biotechnology Information, NCBI www.ncbi.nlm.nih.gov). The insertion sites were mapped onto the M. bovis PG45 genome using Geneious Pro 5.1.6 (Biomatters Ltd).

Critera for gene inactivation

To address the question of which M. bovis genes were dispensable for growth in laboratory media, a gene was considered to be disrupted if the transposon insertion was located after the first three codons and within the first 85% of the protein coding sequence. Global transposon disruption studies [14–16] have identified a repertoire of putative essential genes, and a recent study has predicted a set of 153 essential genes for all Mycoplasma species [26]. The dispensable genes in our M. bovis library were compared with the genes defined as essential in these previous studies.

Supporting Information

Figure S1 Nucleotide sequence of novel transposon constructs. Relevant restriction endonuclease cleavage sites used to generate the construct are indicated above the sequence. The inverted repeat (IR) regions that act as transposable elements are marked, as well as the tuf promoter (p), the Vsp signal sequence (s), two directly oriented FRT sites and the phad gene. (TIF)

Figure S2 Nucleotide sequence of Tn4001 (Ptag7) and deduced amino acid sequences of tuf and aacA-aphD. Relevant primer binding sites are marked above the sequence, while start and stop codons of tuf and aacA-aphD are indicated below the sequence. (TIF)

Figure S3 Nucleotide sequence of M. gallisepticum based transposon construct and predicted phad translation. Relevant restriction endonuclease cleavage sites are indicated above the sequence. The transposable element between the inverted repeats (IR) contains the tuf promoter (p), the vbaA1 signal sequence(s), a single FRT site and phad. The predicted translation of phad from the tuf promoter, fused to the vbaA1 signal sequence, following expected excision of the resistance marker is shown. The region outside the IRs contains the multicloning sites of the plasmid into which the region was ligated. (TIF)

Table S1 Primers used for PCR in this study and their products. (DOCX)

Table S2 Transposon insertions in M. bovis strain PG45 considered unlikely to disrupt function. (DOCX)

Table S3 Transposon insertions within intergenic regions in M. bovis strain PG45. (DOCX)

Table S4 Transposon insertions within integrative conjugative elements (ICEs) in M. bovis strain PG45. (DOCX)

Table S5 Transposon insertions within transposase genes in M. bovis strain PG45. (DOCX)

Author Contributions

Conceived and designed the experiments: SS GFB PFM. Performed the experiments: SS. Analyzed the data: SS PFM GFB. Wrote the paper: SS PFM GFB.

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