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Transformation of *Mycoplasma gallisepticum* with Tn*916*, Tn*4001*, and Integrative Plasmid Vectors

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***Mycoplasma gallisepticum* causes respiratory disease in avian species, but little is known about its mechanism(s) of pathogenesis. These studies were undertaken in order to develop genetic systems for analysis of potential virulence factors. *M. gallisepticum* was transformed with plasmids containing one of the gram-positive transposons Tn*916* or Tn*4001*, which inserted randomly into the mycoplasmal chromosome. Plasmids containing cloned chromosomal DNA were also constructed and tested for integration into regions of DNA homology derived either from chromosomal fragments or from the gentamicin resistance marker from Tn*4001*. These studies demonstrate that *M. gallisepticum* is amenable to transformation with both transposons and integrative vectors.**

Mycoplasma gallisepticum causes avian mycoplasmosis (22). The mortality due to *M. gallisepticum* infection is not high, but the chronic nature of the disease results in immunosuppression and increased sensitivity of poultry to environmental factors and associated diseases. Further economic loss can be attributed to retarded growth, downgrading of carcasses, and contamination (22). Although *M. gallisepticum* has been isolated for more than 30 years and numerous vaccines and control programs aimed towards eradicating the disease have had some success, infections of poultry continue to be a world wide problem, especially in developing countries. Vaccine failure has been attributed partially to our lack of understanding of the molecular basis for pathogenesis, due in part to the inability to genetically manipulate *M. gallisepticum* and, until recently, all other mycoplasmal species.

Pioneering work by Dybvig and Cassell (6) and Mahairas and Minion (14) has provided some genetic tools for mutagenesis and cloning in the members of the class *Mollicutes*. Transformation of several mycoplasmal species has been reported (3–5, 11, 13–15, 20), but not with the avian pathogen *M. gallisepticum*. The goal of these studies was to demonstrate the ability to transform *M. gallisepticum* with transposons and integrative plasmid vectors.

Two gram-positive transposons, Tn*4001* and Tn*916*, have been used successfully to transform mycoplasmas (5, 6, 8, 11, 14, 18, 21). The Tn*916*-encoded tetracycline resistance marker *tetM* is one of the few determinants shown to function in both gram-positive and gram-negative bacteria (19) and in mycoplasmas (15).

A cloning system for mycoplasmas involving the integration of plasmids into the chromosome through homologous recombination has been described (13, 15). Recent studies have shown that the original reports were incorrect in that the species used was not *Mycoplasma pulmonis* but rather *Acholeplasma oculi* (14, 15). The integrative plasmid cloning system in the genus *Acholeplasma* was clearly established, but its usefulness in the genus *Mycoplasma* was in question. Reported here are the results from studies showing the introduction of integrative plasmid vectors into *M. gallisepticum* as well as the random insertion of Tn*916* and Tn*4001*.

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were maintained in Luria broth (1). *M. gallisepticum* ATCC 19610 was grown in 2.5% PPLO broth as described before (14). *M. gallisepticum* R was grown in Frey's medium (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% horse serum (GIBCO/BRL), 5% porcine serum (GIBCO/BRL), 2% yeast extract, 5 ml of dextrose (50% stock in water), 5 ml of Cefobid (cefoperazone; 50% stock in 50% ethanol, stored at −20°C), and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; 1 M stock in water at pH 8.0). Species verification was accomplished by PCR as described before (17), by regular examination for hemagglutination activity and colony morphology (10), and by comparing chromosomal DNA restriction patterns with the type strain, ATCC 19610. The specificity of the PCR primers was confirmed by the lack of reaction products when DNAs from *E. coli*, *Serpulina hyodysenteriae*, *A. oculi*, *Mycoplasma bovis*, *Mycoplasma bovoculi*, *Mycoplasma capricolum*, *Mycoplasma dispar*, *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma pneumoniae*, *Mycoplasma pulmonis*, and *Mycoplasma hyosynoviae* were amplified under standard conditions. All *M. gallisepticum* strains gave the expected 730-bp product (data not shown). The parent strain R was serotyped by J. G. Tully (Frederick Cancer Research Facility, National Institute for Allergy and Infectious Diseases, Frederick, Md.) to confirm its identity. Antibiotic solutions were used at the following final concentrations: for *E. coli*, 100 μg of ampicillin per ml and 12.5 μg of tetracycline per ml; for mycoplasmas, 25 μg of gentamicin per ml of agar or PPLO broth and 2 μg of tetracycline per ml of agar or 10 to 15 μg of tetracycline per ml of PPLO broth.

E. coli plasmid DNA was isolated either by the method of Birnboim (2) and further purified by CsCl-ethidium bromide density gradient centrifugation (1) or by Qiagen column chromatography (Qiagen Inc., Chatsworth, Calif.). Mycoplasmal chromosomal DNA for cloning or Southern blotting was prepared in low-melting-point agarose and restricted as previously described (14). DNA fragments were excised and purified with glass milk (GeneClean II; Bio 101, Inc., La Jolla, Calif.). DNA-DNA hybridization was performed by the method of Southern (1) with DNA probes labeled with

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
M. gallisepticum		
19610	Wild type	American Type Culture Collection
R	Wild type	F. Goff
Ω204	19610(Tn*4001*) Gm^r	This study
Ω207	19610(pISM2531) Tc^r	This study
Ω600	19610(Tn*916*) Tc^r	This study
E. coli DH5α	*supE44 ΔlacU169* (φ 80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO/BRL
Plasmids		
pISM1001	13.5 kb, Ap^r Gm^r, pKS with *Eco*RI fragment B of pSK31 (Tn*4001*)	14
pISM1002	7.5 kb, Ap^r Tc^r, *tetM* from pAM120	15
pAM120	21.4 kb, Ap^r Tc^r	9
pSP64	3.0 kb, Ap^r	Promega
pISM1003	5.45 kb, Ap^r Gm^r	15
pISM1025	7.9 kb, Ap^r Tc^r, *tetM* in pSP64	This study
pISM2531	pISM1025 with 3.8-kb *Eco*RI fragment of chromosomal DNA	This study
pISM2532	pISM1025 with 5.0-kb *Eco*RI fragment of chromosomal DNA	This study
pISM2533	pISM1025 with 3.5-kb *Eco*RI fragment of chromosomal DNA	This study
pISM2534	pISM1025 with 3.7-kb *Eco*RI fragment of chromosomal DNA	This study
pISM2535	pISM1025 with 1.7-kb and 7.9-kb *Eco*RI fragments of chromosomal DNA	This study
pISM2536	pISM1025 with 3.6-kb and 7.9-kb *Eco*RI fragments of chromosomal DNA	This study
pISM2537	pISM1025 with 3.6-kb and 7.9-kb *Eco*RI fragments of chromosomal DNA	This study
pISM2538	pISM1025 with 1.5-kb and 1.6-kb *Eco*RI fragments of chromosomal DNA	This study
pISM2539	pISM1025 with 3.1-kb *Eco*RI fragment of chromosomal DNA	This study
pISM2540	pISM1025 with 1.7-kb *Eco*RI fragment of chromosomal DNA	This study
pISM1003.2531	pISM1003 with the *Eco*RI fragment from pISM2531	This study
pISM1003.2533	pISM1003 with the *Eco*RI fragment from pISM2533	This study
pISM1003.2534	pISM1003 with the *Eco*RI fragment from pISM2534	This study

[^{32}P]dCTP (14) or digoxigenin-11-dUTP. The digoxigenin-labeled probe was detected by chemiluminescence with the Genius system (Boehringer Mannheim, Indianapolis, Ind.).

E. coli was transformed by electroporation. *M. gallisepticum* was transformed with polyethylene glycol (14) or by electroporation (8). Transformation frequency was defined as the number of antibiotic-resistant colonies divided by the total CFU plated on selective medium. Insertion of transposons or integrative plasmids was confirmed by DNA-DNA hybridization with the 2.5-kb *Hin*dIII fragment containing the gentamicin marker as a Tn*4001*-specific probe or an IS*256*-specific PCR product generated by using the primer GCCTCGAG GATAAAGTCCGTATAATTGTGTAA in a standard amplification reaction with pISM2061 (12). The product is 1.3 kb in size and is specific for the IS*256* element of Tn*4001* (data not shown).

Two plasmids used in this study have been described previously, pISM1001, containing Tn*4001* (14), and pAM120, containing Tn*916* (7). When introduced into *M. gallisepticum*, both plasmids gave rise to antibiotic-resistant transformants at 2×10^{-5} transformants per CFU for Tn*916* and 1×10^{-6} transformants per CFU for Tn*4001*. When examined by DNA-DNA hybridization, Tn*4001* appeared to insert randomly (Fig. 1), as did Tn*916* (data not shown). When the DNA from *M. gallisepticum* transformants was probed with pKS, the parent plasmid of pISM1001, no plasmid sequences were identified, indicating that the transposon integrated into the chromosome through a transposition event and not by insertion of the plasmid (data not shown). This also showed that the plasmid was not replicating autonomously in the cell.

In earlier studies, addition of yeast tRNA to transformation mixtures was important to transformation of *A. oculi* with purified DNA (15). It had no effect on transformation frequencies with *M. gallisepticum* with pISM1001 (data not shown), but DNA concentrations were important. One microgram of pISM1001 DNA gave 2.9×10^{-7} transformants per CFU, 5 μg of DNA gave 1.1×10^{-6} transformants per CFU, and 10 μg of DNA gave 1.3×10^{-6} transformants per CFU (means of three experiments). Both *M. gallisepticum* strains were transformed at similar frequencies (data not shown).

To investigate the feasibility of using integrative vectors in *M. gallisepticum*, plasmids pISM1003 (15) and pISM1025 (Fig. 2) were used to clone random *Eco*RI chromosomal DNA fragments from *M. gallisepticum*. Plasmid pISM1025 was constructed by subcloning a 4.8-kb *Hin*cII fragment from

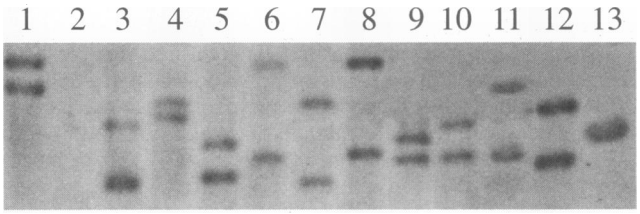

FIG. 1. *M. gallisepticum* Tn*4001* transformant chromosomal DNAs probed with an IS*256*-specific probe. Eleven independent Tn*4001*-transformed *M. gallisepticum* clones were analyzed by published protocols (15). The genomic DNA was digested with *Dra*I, and the fragments were separated by electrophoresis on a 1% agarose gel. The DNA fragments were blotted to a Biodyne Plus membrane (Pall Biosupport, Glen Cove, N.Y.) and probed with random-primed IS*256*-specific PCR product labeled with digoxigenin-11-dUTP. Lane 1, *Dra*I-digested pISM2061 containing Tn*4001*; lane 2, nontransformed *M. gallisepticum Dra*I-digested genomic DNA; lanes 3 to 13, independent Tn*4001*-transformed *M. gallisepticum Dra*I-digested chromosomal DNAs. The image of the autoradiogram was captured with a Cohu model 4900 high-performance CCD camera (Cohu, Inc., San Diego, Calif.), and the image was cropped in Adobe Photoshop on a Macintosh IIci computer. The lettering was added using Aldus Freehand.

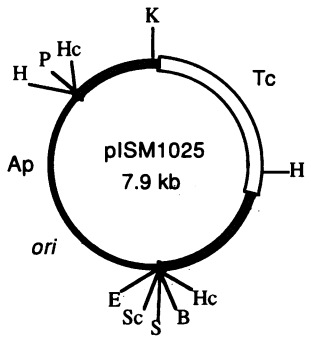

FIG. 2. Restriction map of plasmid pISM1025. The plasmid was constructed as described in the text. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; S, *Sma*I; Ap, ampicillin resistance marker; *ori*, origin of replication; Tc, tetracycline resistance marker (*tetM*) from Tn*916*.

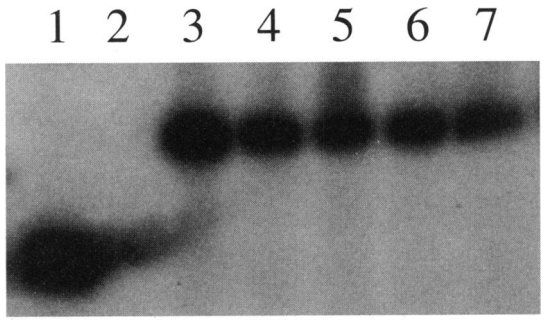

FIG. 3. Analysis of *M. gallisepticum* integrative plasmid transformants by DNA-DNA hybridization. *M. gallisepticum* pISM2531 transformant chromosomal DNAs were digested with *Pst*I, and nitrocellulose blots were prepared. Shown is a blot probed with a random-primed, [^{32}P]dCTP-labeled *Hin*dIII fragment from Tn*4001* containing the gentamicin resistance marker. Lane 1, linear pISM2531 digested with *Pst*I; lane 2, wild-type chromosomal DNA; lanes 3 to 7, independent pISM2531 transformant chromosomal DNAs. The autoradiogram was digitized and the figure was prepared as described in the legend to Fig. 1.

pISM1002 containing the *tetM* marker from Tn*916* into the *Hin*cII site of pSP64 (Promega). One hundred and seven pISM1025 derivatives containing *M. gallisepticum* chromosomal DNA fragments were identified, and 10 of these were chosen randomly for further study (Table 1). When introduced into *M. gallisepticum*, only 3 of the original 10 plasmids resulted in transformants in spite of repeated attempts. Even with these plasmids, the transformation frequency was low (Table 2). In attempts to increase the frequency of transformation, *M. gallisepticum* chromosomal DNA fragments from three pSP64 derivatives were subcloned into a pKS-derived vector (pISM 1003). Fragments which, in a pSP64 vector, transformed *M. gallisepticum* at low frequency or at an undetectable frequency were chosen. Table 2 shows the results of these experiments. All of the plasmids derived from pKS gave higher transformation frequencies in *M. gallisepticum* than did those derived from pSP64. No transformants were ever observed when either of the parent plasmids, pISM1025 (a pSP64 derivative) and pISM1003 (a pKS derivative), were used. This indicated that a region of homology was needed for integration. Further evidence for its site-specific integration is shown in Fig. 3. All transformants from a single transformation mixture (in this case, pISM2531) yielded the same size fragment by DNA hybridization, but the control plasmid was a different size, giving further evidence that the plasmid was not replicating autonomously in the cell.

To assess the stability of transposons and integrative plasmids in *M. gallisepticum* during growth in the laboratory, three recombinant strains, designated Ω204 (Tn*4001*), Ω207 (pISM 2531), and Ω600 (Tn*916*) (Table 1), were passaged in nonselective broth for 100 generations. At the 30th- and 100th-generation passages, the numbers of CFU on selective and nonselective agar were determined and compared. Approximately 20 individual colonies from each strain at the 30th and 100th generations were picked for DNA analysis by DNA-DNA hybridization. Chromosomal DNAs were also prepared from each culture after every 10 generations. The DNA-DNA hybridization patterns of *Eco*RI-digested chromosomal DNAs were compared between individual colonies at the different passages to measure the stability of the transposon or integrative plasmid insert. There was no difference in the number of CFU on the selective and nonselective plates between the 30th and 100th passages with any *M. gallisepticum* strain except for Ω204 (data not shown). This strain had slightly fewer CFU on selective plates than on nonselective plates at the 30th passage but slightly higher numbers at the 100th passage. When chromosomal DNAs from individual colonies from both passages were analyzed by DNA-DNA hybridization, there was no apparent movement of Tn*4001* (data not shown). Additional bands were sometimes observed (data not shown), however, indicating that there is some instability of Tn*4001* in *M. gallisepticum* by replicative transposition of either the intact transposon or one of the IS*256* elements. This did not occur in either the Tn*916* or the integrative plasmid transformants (data not shown).

TABLE 2. Transformation frequencies of integrative vectors in *M. gallisepticum* 19610

Plasmid	Transformation frequency[a]
pISM2535	$6.4 \times 10^{-10} \pm 3.2 \times 10^{-10}$
pISM2540	$3.6 \times 10^{-10} \pm 2.1 \times 10^{-10}$
pISM2531	$1.3 \times 10^{-10} \pm 6.4 \times 10^{-11}$
pISM1003.2531	$5.0 \times 10^{-7} \pm 3.5 \times 10^{-7}$
pISM2533	$<1 \times 10^{-10}$
pISM1003.2533	$3.0 \times 10^{-7} \pm 2.4 \times 10^{-7}$
pISM2534	$<1 \times 10^{-10}$
pISM1003.2534	$3.0 \times 10^{-7} \pm 4.8 \times 10^{-7}$

[a] Number of transformants per CFU. Data represent the mean of at least three experiments.

These studies demonstrate that Tn*916*, Tn*4001*, and integrative plasmid vectors can be used in *M. gallisepticum*. Both transposons integrated in a seemingly random fashion and were stably maintained in the absence of selection. Replicative transposition of Tn*4001* appeared to occur at low frequency, however. Since earlier studies were performed with an *Acholeplasma* species rather than *M. pulmonis*, as reported (14, 15), it was important to confirm not only the use of Tn*4001* (14) but also the ability to integrate plasmids into a *Mycoplasma* chromosome. Attempts to integrate gentamicin-resistant vectors into *M. pulmonis* have failed, but vectors carrying the *tetM* determinant from Tn*916* have integrated into *M. pulmonis* at a low frequency (16).

Interestingly, the parent plasmid background had a significant effect on transformation frequencies; pKS derivatives transformed at a significantly higher frequency than did pSP64 derivatives (Table 2). The failure of 7 of 10 cloned fragments

to yield transformants in the pSP64 background may simply imply that the transformation frequency was below detectable limits. All of the transformation frequencies in the pSP64 background were low ($\leq 1 \times 10^{-10}$ transformants per CFU). When two of the seven nontransforming fragments were transferred at random to the pKS background, the transformation frequency rose to more normal levels for mycoplasmas ($\approx 10^{-7}$ transformants per CFU) (Table 2). The reason for this is unknown, but it suggests that a restriction system which can recognize and cleave pSP64 may be present in *M. gallisepticum*.

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