Transformation and homologous recombination in *Mycoplasma gallisepticum*

by

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
Ap^r	ampicillin resistance
bp	base pairs
CFUs	colony forming units
CIAP	calf intestine alkaline phosphatase
dsDNA	double-stranded DNA
ELISA	enzyme-labeled immunosorbant assay
Gm^r	gentamicin resistance
HA	hemagglutination
h	hour
IS	insertion sequence
kb	kilobases
kDa	kilodaltons
min	minute
mol. wt	molecular weight
ORF	open reading frame
ori	origin of replication
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed-field gel electrophoresis
PPLO	pleuropneumonia like organism
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	single-stranded DNA
Tc^r, Tc^s	tetracycline resistant/sensitive
Tn	transposon
Tnp	transposase
ts	temperature sensitive
WT	wild-type

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INTRODUCTION

General Characteristics of Mycoplasmas

Mycoplasmas are the smallest free-living prokaryotes known, and are bounded by a single limiting membrane. There are six genera within the class *Mollicutes*: *Mycoplasma, Ureaplasma, Acholeplasma, Spiroplasma, Anaeroplasma and Thermoplasma.* The genus *Mycoplasma* contains more than 70 species, including a number of human and animal pathogens such as *M. pneumoniae* (human), *M. mycoides* (bovine), *M. hyopneumoniae* (swine), *M. synoviae* (avian), and *M. fermentans* (incognitus strain), a pathogen that has recently been identified in many human AIDS patients (78). Due to the lack of cell walls, mycoplasmas are resistant to penicillin and sensitive to lysis by osmolarity, detergents and specific antibody plus complement. When incubated at 37°C, almost all mycoplasma species can form colonies that have a characteristic "fried egg" appearance on the agar surface. Because of their limited biosynthetic capability, many mycoplasma species need complex media for growth in vitro and most members require sterol and phospholipids as essential components of their membrane.

Antigenic variation

Antigenic variation or phenotypic switching refers to the changes that occur in protein epitopes of an organism. For pathogens, phenotypic switching in general is a means of dealing with the host immune response, it allows adaptation to various environments, and it also expands the host range of the organism. Antigenic variation has been identified in many bacteria (129). Some examples are flagella phase variation in *Salmonella typhimurium* (131), pilin and protein II expression in

Neisseria gonorrhoeae (10, 162), and variable lipoprotein expression in *Mycoplasma hyorhinis* (124, 125, 161). Although in each of these examples the mechanism of antigenic variation is different, it seems that the mechanism is associated with the genomic changes. Recently, antigenic variation has been observed in several mycoplasma species but it has only been characterized at the molecular level for one operon of a swine pathogen, *M. hyorhinis* (115, 153). *M. hyorhinis* undergoes high frequency phase transitions in colony morphology, opacity and in the expression of diverse lipid-modified cell surface protein antigens (variant lipoproteins, Vlp). The Vlps (VlpA, VlpB, and VlpC) are encoded by a cluster of distinct genes within a 4 kilobase (kb) chromosomal segment. Deletion or insertion of the repetitive sequences encoding the Vlp external domain is responsible for Vlp size variation. Phase switching of Vlp is controlled by the length of a polyadenosine tract in a 250 base pair (bp) upstream region of the structural genes. Evidence for antigenic variation in other mycoplasma species is also accumulating. For example, a cytadherence phase-variable protein, HMW3, has been identified in *M. pneumoniae* (104). Heterogeneity of a major surface antigen complex, V1, has been related to colony size variation in *M. pulmonis* (139, 150). A family of variant membrane lipoproteins of *M. fermentans* (132) and some uncharacterized variant proteins from other species such as *M. arthritidis* (149), *M. mycoides* (148), and *M. ovipneumoniae* (142) have also been reported. More recently, chromosomal rearrangements in *M. pulmonis* have been correlated with changes in the susceptibility of cells to virus, an evidence of surface antigen structure change (11).

Virulence factors

There is very limited information about mycoplasma virulence factors, and the molecular basis of pathogenesis of mycoplasmal diseases has not been resolved. Although mycoplasmas lack a cell wall and have a smaller genome, their pathogenicity is probably a multifactoral event. A human pathogen, *M. pneumoniae*, has been studied most extensively and several related virulence "factors" have been identified. In general, these virulence factors include motility, adherence to host cells through a specific adhesin, adaptation to the host environment, secretion of toxic products damaging host cells, evasion of phagocytosis, and the induction of pathological immune responses. It should be pointed out that the cellular and molecular basis for these factors is still not well understood. The well characterized 170 kilodalton (kDa) membrane protein, P1, of *M. pneumoniae* is believed to mediate adherence to respiratory epithelium. In *M. gallisepticum*, similar factors are believed to exist because of its antigenic relatedness to *M. pneumoniae*. For example, there is serological cross-reactivity and genetic homology between the P1 adhesin in *M. pneumoniae* and a similar size protein in *M. gallisepticum*. Additional evidence came from a recent report in which a putative cytadhesin gene in *M. gallisepticum* had been identified by polymerase chain reaction (PCR) using two primers from the conserved region in P1 of *M. pneumoniae* and in MgPa of *M. genitalium* (31). These findings and other studies with *M. genitalium* (27, 28, 100) suggest that a family of P1-like adhesins exists among the pathogenic mycoplasmas (21, 26). However, in a separate report, Markham et al. (93) found no significant homology in N-terminal sequence of 17 amino acids in pMGA, a 67 kDa hemagglutinin of *M. gallisepticum*, with the P1 protein of *M. pneumoniae*.

Chromosomal structure

Our knowledge about the mycoplasma genome is limited compared to our understanding of *E. coli* and other bacterial genomes. Mycoplasmas are considered "the minimal living unit" because they have the smallest genome size of any free living organisms (114), thus limiting their metabolic capabilities. The structure of the mycoplasma chromosome, like that of the *E. coli* genome, consists of a circular double-stranded DNA molecule varying in size from 600 - 2,200 kb (52). Currently, the genomic size of over 50 mycoplasmal species has been determined by pulsed-field gel electrophoresis (PFGE) or DNA hybridization (9, 70, 99, 102, 109, 110, 151, 157, 159), including a few *M. gallisepticum* strains such as strain PG31 (1,050 kb)(109), strain S6 (1,435 kb)(148), strain 5969 (1,340 kb)(7) and *M. gallisepticum* (1,170 kb)(102). The DNA replication site is associated with a bleb structure in the *M. gallisepticum* membrane. Chromosomal rearrangements involving inversion of a genetic segment that causes on/off switching in gene expression have been identified in some mycoplasma species (11, 73).

Physical and genetic maps

Physical and genetic maps of a bacterial genome basically reflect the locations of restriction sites and genetic loci. Despite the fact that mycoplasmas have a smaller genome, physical and genetic maps of only a few species have been completed. Some conserved genes have been cloned including tRNAs (*trn*), rRNAs (*rrn*), ATPase (*uncH, A, GD*), RNA polymerase (*ropC, B*), ribosomal proteins (*rpn*), gyrase (*gyrA/B*), elongation factor-Tu (*tuf*), and deoxyribose phosphate aldolase (*deoC*). Physical and partial genetic maps have been established for *M. genitalium* (25), *M. mobile* (9), *Ureaplasma urealyticum* (22), *M. capricolum* (93, 149) and

Spiroplasma citri (159). Recently, Ladefoged et al. (73) reported the restriction maps of five *M. hominis* strains along with the locations of a number of genetic loci. A physical and partial genetic map of *M. pneumoniae* has also been published with an average resolution of 4.0 kb and a number of cloned genes were located including the P1 operon, tRNA genes, ATPase operon and ribosomal protein genes (70, 151, 153).

Nucleotide composition and codon usage

Mycoplasmas generally contain a low G+C ratio falling within the 25 - 40 mol% range (117). A few mycoplasma species have G+C ratios slightly lower than the theoretical minimum value of 26 mol% G+C thought necessary to code for proteins with a normal amino acid composition (41, 122). The low G+C ratio may suggest that during evolution, a selective advantage for adenine-thymidine base pairs resulted in their preferential accumulation in the genome (101, 137). The G+C mol% value has been used in the classification of mycoplasmas and of prokaryotes in general. A difference in a G+C content larger than 1.5 - 2.0% between two bacteria is considered sufficient to rule out the possibility that they are the same species. Since the G+C content of coding regions in mycoplasmas is much lower than that of corresponding genes in *E. coli* (25% in *M. capricolum* compared to 51% in *E. coli*), it has been predicted that mycoplasmas prefer to use codons having A or U, especially at the third position (105).

An alternative codon usage has been well documented in mycoplasmas. The codon UGA is a universal stop codon in most eubacteria such as *E. coli*, but in the genera *Mycoplasma* and *Spiroplasma*, UGA codes for tryptophan (158). This results in premature truncation of cloned mycoplasma genes in *E. coli*. The codon CGG has

been assigned a termination function in mycoplasmas indirectly because of the absence of a tRNA with a CCG anticodon (2), but $tRNA_{CCG}$ encodes arginine in *E. coli*. To compound the problem, the UGA tryptophan codon is preferentially used in mycoplasmas. This unusual codon usage has been reported in *M. gallisepticum* (55, 56), *M. pneumoniae* (55, 57, 80), *M. genitalium* (55), *M. hyorhinis* (33), *M. arginini* (69), *Spiroplasma citri* (15, 20) and *Spiroplasma* sp strain MQ-1 (119), suggesting that UGA for tryptophan is common in these two genera.

Transformation and transposition

Until recently, the tools for genetic manipulation of mycoplasmas have not been available. Pioneering work by Dybvig (36) and Mahairas and Minion (86) has provided some genetic tools for mutagenesis and cloning in the *Mollicutes*. Since these early reports, the transformation of several mycoplasma species has been reported (14, 34, 35, 64, 84, 86, 87, 133, 134).

Two Gram-positive transposons, Tn*4001* and Tn*916*, have been used successfully to transform mycoplasmas. Tn*4001* (Figure 1) is a typical composite class I transposon originally isolated from plasmids in multiple resistance strains of *Staphylococcus aureus* (48, 83). It is 4.5 kb in length and consists of a 2.0 kb gentamicin resistance gene and two flanking insertion sequence (IS) elements, IS*256*R and IS*256*L. The resistance determinant has been cloned in *E. coli* on a 2.45 kb *Hin*dIII fragment. It encodes a single protein of 59 kDa which specifies both aminoglycoside acetyltransferase and aminoglycoside phosphotransferase activities (126). The mechanism of resistance involves a modification of the aminoglycoside at either amino groups by the aminoglycoside acetyltransferase activity or at hydroxyl groups by the aminoglycoside phosphotransferase activity. This prevents the binding

of the aminoglycoside to the ribosome, its mechanism of action. Sequence analysis of the two IS elements (17) indicated that they are identical and each contains 26 bp imperfect terminal inverted repeats and a single open reading frame (ORF) coding for a 45.6 kDa transposase (Tnp) protein. The detailed mechanism of Tn*4001* transposition is not clear. However, there is some evidence implying that at least one inverted repeat of Tn*4001* can transpose independently of the whole transposon (82, 108). Tn*4001* has been shown to transpose to diverse chromosomal sites in *S. aureus* (85) and mycoplasmas (86) without plasmid integration. A large conjugal transposon, Tn*916,* has also proven functional in mycoplasmas. Tn*916* is a 16.4 kb tetracycline resistant transposon originally identified on the chromosome of *Enterococcus faecalis* DS16 (44). Transposition of Tn*916* requires the excision and integration activities of an integrase encoded by the transposon (135). The

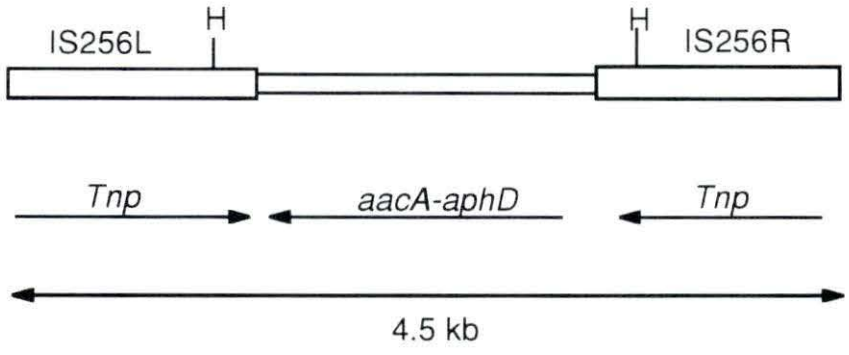


Figure 1. Transposon Tn*4001*. The gentamicin resistance gene, *aacA-aphD* in the middle region, is flanked by two identical insertional sequences IS*256*L (left) and IS*256*R (right) each encoding a transposase (Tnp) enzyme responsible for transposition. The direction of transcription is indicated by the single headed arrows.

tetracycline resistance marker of Tn*916*, *tetM*, has also been cloned and expressed both in *E. coli* and in several mycoplasma species. Tn*916* has been shown to function in *M. pulmonis* (35, 36), *M. hyorhinis* (35), *M. mycoides* subsp. *mycoides* (64, 156), *M. hominis* (121) and *Acholeplasma laidlawii* (36).

DNA repair

DNA repair processes correct DNA damages caused by environmental factors such as radiation and mutagenic chemicals or that occur as a result of inaccurate replication mechanisms. DNA damage can be repaired in any of the following ways: proofreading by DNA polymerase to remove mismatched bases; photoreactivation by DNA photolyase to reverse thymine dimerization; excision repair or dark repair by an endonuclease to remove and replace the damaged sequences; recombinational repair by the intact complementary strand to replace the mutated segment; SOS response by the RecA and LexA proteins to repair lesions in error-prone sites; and repair activity by O^6-methylguanine DNA methyltransferase to remove alkyl groups from the O^6 positions of guanine and thymine. DNA synthesis in *M. gallisepticum* after UV radiation has been studied by Ghosh et al. (47). The survival curves of *M. gallisepticum* are almost exponential, and the absence of a shoulder in the curve suggests that *M. gallisepticum* lacks normal DNA repair functions. DNA synthesis was reduced after UV irradiation, and there was no photoreactivation. Maniloff suggested that *M. gallisepticum* may accumulate base changes far more frequently than other prokaryotes because of a lack of DNA repair mechanisms (90) which may explain the rapid evolution in the *Mollicutes*. There is no experimental support, however, for high mutation rates in *M. gallisepticum*. Surprisingly, *M. gallisepticum* seems to be less heterogeneic than other mycoplasma strains as determined by

slight variation in DNA cleavage patterns (118), antigenic patterns and total cell protein composition (113, 120). Contrary to the above observations, Cao and Minion (unpublished data) in a preliminary study have found that late-log phase *M. gallisepticum* cells following UV radiation show a slight decline in colony forming units (CFUs), similar to that of *Acholeplasma* ISM1499 cells in which efficient repair mechanisms are believed to exist (29, 130).

Homologous recombination

Genetic recombination is a common phenomenon in all living organisms. Most of our knowledge on recombination is derived from studies in *E. coli* where many of the enzymes and molecular events have been documented. *E. coli* has three basic recombination systems: homologous recombination involves repeated DNA segments in the size range of 1 - 10 kb that include IS elements, rRNA genes, and fragments of defective prophages; site-specific recombination is related to phage lambda integration and excision and is highly phage-specific; and illegitimate recombination includes the joining of DNA sequences with little or no homology. All three mechanisms could result in chromosome rearrangements. In recent years, homologous recombination has become a powerful tool in genetic manipulation since recombination between DNA sequences residing in the chromosome and newly introduced DNA sequences could allow the transfer of any cloned gene into the genome of recipient cells, thus constructing mutants with altered phenotypes. Although the detailed mechanism and efficiency of recombination in each organism may be slightly different, the major mechanism of homologous recombination is believed to occur through a Campbell-like model (18). As explained in Figure 2, recombination occurs through the breakage of homologous sequences, a crossover

of single strands to form a Holliday junction, and the migration of that junction prior to resolution of the heteroduplex complex (72). If recombination occurs between two circular molecules such as a plasmid and a bacterial chromosome, a single crossover event will lead to the integration of the plasmid sequence into the chromosome. The site of exchange can occur anywhere within the homologous region. The breaking and reunion process is mediated by the RecA protein and the RecBCD complex in *E. coli* (72, 154).

RecA enzyme

The RecA protein has important functions in homologous recombination, DNA damage repair, gene regulation, and mutagenesis (96). Since the isolation of *E. coli recA* mutants in 1965, *recA* analogs have been identified by interspecies complementation in many Gram-negative and Gram-positive bacteria and even in some eucaryotes (96). As a key enzyme in homologous recombination, RecA protein can promote the pairing and strand exchange between two homologous regions in an ATP-dependent reaction. Despite the well-known recombination systems in *E. coli* as stated above, and *recA* analogs in many other bacteria, very little information is available regarding *recA* gene in the *Mollicutes*. Recently, a putative *recA* gene from *Acholeplasma laidlawii* has been identified in a 3 kb fragment of the chromosome and has been sequenced (38). Sequencing information indicated that it is similar in homology to those from other bacteria, especially from *Bacillus subtilis* (38). In a subsequent report (37), the authors showed that the *recA* gene from *A. laidlawii* and *M. pulmonis* did share homology with *recA* from other

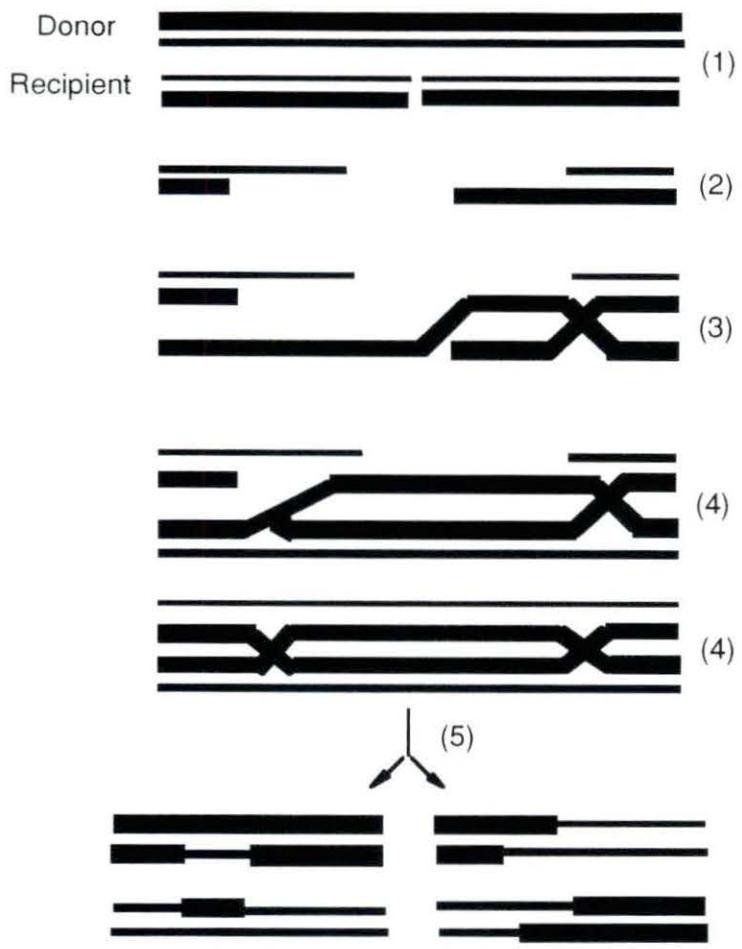


Figure 2. Mechanism of homologous recombination. (1) The two homologous DNA molecules align together and an endonuclease makes a double-stranded break in the recipient molecule. (2) The break is enlarged by an exonuclease to form a gap with single-stranded 3' ends. (3) The 3' end displaces the homologous strand in the donor duplex that forms a D-loop. (4) DNA repair/synthesis functions enlarge the duplex D-loop and extends the two recipient strands. (5) Isomerization and cleavage of the Hollidy junctions give alternative products. (Adopted from Kornberg and Baker, 70).

bacteria including *B. subtilis, Enterococcus faecalis, Streptococcus pyogenes, Streptococcus mutans*, and *E. coli.*

In addition, there are some experimental data that support the existence of RecA-like activities such as homologous recombination in the *Mollicutes.* For example, Mahairas and Minion (87) demonstrated that *Acholeplasma* ISM1499 had a recombination system by introducing homologous DNA fragments into wild-type cells. Introduction of cloned fragments could occur into either chromosomal DNA sites or into previously integrated plasmids in the chromosome (84). Dybvig et al. have found that *recA* mutants of *A. laidlawii* lack DNA repair activity (3). Unfortunately, there is no report on the characterization or isolation of RecA protein in mycoplasmas. Phylogenically, mycoplasmas are closely related to *B. subtilis*, the only Gram-positive bacterium in which *recA* mutants have been identified and sequenced (136). Furthermore, repetitive DNA sequences occur in many mycoplasma species (94) and recombination between these sequences may result in chromosome rearrangements as has been suggested in *M. pulmonis* (11).

Repetitive DNA sequences

Short, interspersed repetitive DNA sequences are present in the genomes of all living organisms (81). These sequences can be classified into two groups. One group includes well characterized sequences such as tRNA and rRNA genes. The second group includes insertion elements, and short, non-coding, randomly-distributed sequences (81). Repetitive sequences play significant roles in homologous recombination, in gene rearrangements, and may affect transcription and translation (107). They may also provide a survival mechanism for adaptation to

different environments by ensuring genomic flexibility, and indirectly, antigenic variation.

Mycoplasmas are a diverse group of organisms morphologically, biochemically and antigenically. Repetitive sequences may be involved in that diversity since they have been identified in several mycoplasma species and spiroplasma species (42, 54, 94, 103, 106, 140, 152). Some of them have been characterized in detail. Two repetitive sequences, RepMp1 and RepMp2, have been detected in *M. pneumoniae* (94). They are present at 8 - 10 copies per genome. RepMp1 is found frequently in association with RepMp2, and both are associated with copies of the P1 adhesin gene. Although it has been postulated that these sequences are related to genetic rearrangements of the P1 gene, no detectable variation of the protein has been observed. In *M. genitalium*, repetitive sequences sharing 85% homology with the intact *M. gallisepticum*Pa operon were found to occupy 3% of the genome (106). Two IS elements, RS-1 and RS-2, have been cloned from two swine pathogens, *M. hyorhinis* and *M. hyopneumoniae* (42). RS-1 is a 1,513 bp segment with inverted repeats and is structurally similar to the IS*3* family in *E. coli.* RS-2, located 2.5 kb upstream of RS-1, is a 1,348 bp segment and may be associated with gene amplification (94). It has also been shown that an IS element of 1,550 bp lying between the VlpA and VlpB of *M. hyorhinis* affects *vlp* gene expression leading to antigenic switching (124, 125, 161) as stated previously. Hu et al. (54) identified the presence of multiple copies of IS-like elements in *M. incognitus.* The size of the element is 1.4 kb, and it contains 29 bp terminal inverted repeats and two actively transcribed ORFs. Subsequent studies indicated that the genes contained in the IS-like element were actively expressed in *M. fermentans* (incognitus strain)(79). In *M. pulmonis*, an IS*3*-related element of 1,285 bp has been found to transpose to a 1.2

kb target sequence (12). DNA hybridization indicated that this IS element is present at 4 - 15 copies per chromosome in almost all strains of *M. pulmonis*.

Mycoplasma gallisepticum

Diseases of poultry

M. gallisepticum is one of the most significant pathogens in avian mycoplasmosis (160). The name was proposed by Edward and Kanarek for the pathogen causing chronic respiratory disease of chickens and infectious sinusitis of turkeys (8). The mortality due to *M. gallisepticum* infection is not high, but the chronic nature of the disease results in increased sensitivity of poultry to environmental factors and associated diseases. Further economic loss can be attributed to retarded growth, downgrading of carcasses and contamination (160). Although *M. gallisepticum* has been isolated for over 30 years and numerous vaccines and control programs aimed to eradicate the disease have met with some success, infections of poultry continue to be a world-wide problem, especially in the developing countries. Vaccine failure has been partially attributed to our lack of understanding of the molecular basis for pathogenesis. This has been due to our inability to genetically manipulate *M. gallisepticum* and until recently, all other mycoplasmal species.

Physical characteristics

M. gallisepticum, like most other mycoplasma species, form typical "fried-egg" colonies on agar surfaces and produce acid from glucose. It can agglutinate red blood cells from a variety of animal species including chicken, turkey, guinea pig and human (46, 88). Hemagglutination is inhibited by sialic acid and its derivatives

leading to the suggestion that sialic acid on the erythrocyte surface provides the binding sites for *M. gallisepticum* (46). In addition, *M. gallisepticum*, along with *M. pneumoniae* (53), *M. genitalium* (24), *M. pirum* (30) and *M. pulmonis* (19), possesses a highly-structured polar body of unknown function protruding from one or both ends of the cell (65, 76, 116, 138, 147). All gliding mycoplasmas, including *M. gallisepticum*, are able to attach to glass (61) and plastic (141). It has been postulated that these blebs or terminal structures are attachment organelles that may govern motility (123) or host-pathogen interactions. In the studies of chicken tracheal organ cultures infected with *M. gallisepticum*, this terminal structure seems to play an important role in *M. gallisepticum* pathogenesis (77). It may also be involved in DNA replication (91, 92, 111, 112). *M. gallisepticum* cells divide by binary fission, but the division is synchronous with DNA replication, unlike most other species of mycoplasmas where cell division and DNA replication are not closely coordinated (71, 112). Isolation of the terminal structures showed that they contained the sites of DNA replication and that the chromosome origin attached to the bleb throughout the cell cycle (92, 111). These structures might function as a primitive mitotic-like structure for chromosomal organization and segregation.

Hemagglutinin

Hemagglutination, as described above, has been studied extensively in *M. gallisepticum* and *M. pneumoniae*. The hemagglutinin is believed to be involved in adherence to epithelial surfaces and pathogenesis. The size of the *M. gallisepticum* hemagglutinin is in dispute, however. A 75 kDa adhesin molecule in *M. gallisepticum* was first characterized by Kahane et al. after extraction with 1% deoxycholate and partial purification by affinity chromatography (62). Bradley et al. have shown that a

69 kDa polypeptide in strain S6 is species-specific and may be related to all or part of the *M. gallisepticum* hemagglutinin (16). Forsyth et al. used immunoelectron microscopy to demonstrate the localization of a 64 kDa lipoprotein (LP-64) hemagglutinin in the base of the *M. gallisepticum* terminal structure. Monospecific polyclonal rabbit antiserum against the LP-64 inhibited hemagglutination of chicken erythrocytes and attachment of *M. gallisepticum* to chicken tracheal cells (43). More recently, Markham et al. reported the characterization of a hemagglutinin from *M. gallisepticum* strain S6 (75). A 67 kDa protein (pMGA) was shown to be located on the cell surface and the monoclonal antibodies against the pMGA can also block hemagglutination and attachment. The authors suggested that there might be a hemagglutinin family of approximately 20 variant genes within a contiguous locus on the *M. gallisepticum* genome. Only one gene seems to be expressed at a time, and genetic recombination may provide for antigenic variation within the gene family. Dohms et al. (31) isolated a putative *M. gallisepticum* cytadhesin gene by PCR using two primers derived from amino acid homology regions of the cytadhesin genes of *M. pneumoniae* and *M. genitalium.* They found that the 583 bp PCR product is unique to *M. gallisepticum* for diagnostic purpose and it encodes a single predicted open reading frame of 193 amino acids. However, its role in hemagglutination has not been investigated.

Hemolysin

Many biological processes, including general membrane toxicity, phospholipase activity and ATPases, have been suggested as the causes of epithelial membrane toxicity. A blood agar plate hemolysis has been identified in *M. gallisepticum*, but its role in pathogenecity has not been established. This hemolysis has been linked to

the production of hydrogen peroxide released in direct proximity to the host cell membrane and consequently leading to oxidative stress, local tissue destruction and membrane disorganization or hemolysis (23, 146). More recently, a trypsin-sensitive, membrane-associated hemolysis activity has been identified in most mycoplasma species including *M. gallisepticum* (98).

Toxins

Mycoplasmas produce few true toxins. Previous investigation indicated that a material produced by *M. gallisepticum* cells primarily affected the central nervous system of turkey poults, resulting in endothelial swelling and increased permeability in the brain (145). This toxicity was dependent on cell viability at a density of 10^8 to 10^9 CFUs per bird, but was not found in culture supernatants. This suggests that the neurotoxin is not a secretory protein and is associated with intact viable cells. Unfortunately, the toxin has never been purified and its role in pathogenicity has not been studied either. Speculation that neurotoxicity was related to sialic acid receptors within the central system has linked the toxic activity with the hemagglutination activity of *M. gallisepticum*.

Antigenic variation in *M. gallisepticum*

Heterogeneity in restriction cleavage patterns and membrane protein profiles among different strains of *M. gallisepticum* is commonly observed. Thomas et al. (143) used laser densitometry of SDS-PAGE gel and polyclonal antisera in an enzyme-linked immunosorbent assay (ELISA) to demonstrate diversity of protein profile among three *M. gallisepticum* strains (F, S6 and A5969). In a subsequent study (144), the authors also noticed a difference in the number, electrophoretic

mobility, and morphology of periodic acid-Schiff positive, lectin-reactive bands of five *M. gallisepticum* strains, indicating a phenotypic diversity of carbohybrate-containing component among five *M. gallisepticum* strains (F, S6, A5969, ATCC 19610, and R). Stantha et al. (128) compared six *M. gallisepticum* strains from different parts of the world. Their observation showed that differences in DNA restriction patterns were significant while the polypeptide patterns less pronounced. However, none of the reports can correlate the variation with the restriction fragment length polymorphism in *M. gallisepticum*.

Vaccination and disease control

Control of *M. gallisepticum* infection in chicken and turkey flocks has met with limited success. A number of vaccines have been developed by numerous investigators, but none of the current vaccines are considered satisfactory (66). Vaccination with a 75 kDa adhesin from R strain resulted in specific antibody, but no protection was reported (5, 6). Subunit vaccines are not pragmatic because of the low yield and the high growth cost of the organism. *M. gallisepticum* bacterins can reduce clinical disease and egg production losses, but cause a local inflammatory reaction and do not prevent colonization (67). The F strain of *M. gallisepticum*, an attenuated live vaccine strain, is recommended primarily for multiple-age commercial layers. It can induce a serological response and persist for a long period, but there is no correlation between the serological response and protection. It retains some pathogenicity, however, and is completely virulent for turkeys. Temperature sensitive (ts) strains have been isolated by exposure of *M. gallisepticum* to a chemical mutagen (NTG, N-methyl-N'-nitro-N-nitrosoguanidine) and selection for a ts phenotype (growth at 34°C but not at 40°C) (51, 63, 74, 155). Following aerosol

administration of the vaccine, the ts strain did not elicit a substantial serum antibody response as measured by the rapid serum agglutination test or by ELISA, and no protection was demonstrated.

Goals

Recently, transposons have been introduced into various mycoplasmal species via transformation (35, 36, 86), but transformation of *M. gallisepticum* has not been reported. Likewise, homologous recombination is a universal phenomenon in all living organisms as stated above, but it has never been examined in *M. gallisepticum*. The goals of the present study are the following: (1) to introduce Tn*4001* and Tn*916* into *M. gallisepticum* and determine the stability of the transposon inserts in vitro, (2) to determine if integrative plasmids can be introduced into *M. gallisepticum* via homologous recombination, and if so, to determine the stability of cloned genes introduced via integrative plasmids and (3) to examine the homologous recombination in *M. gallisepticum* utilizing transposon, chromosomal, and *lacZ* sequences. These studies will serve as the basis for future work for the development of a cloning system in *M. gallisepticum*, the development of live, attenuated mutant strains of *M. gallisepticum* for vaccine purposes, and for the examination of basic genetic mechanisms operative in the *Mollicutes*.

MATERIAL AND METHODS

Bacterial Strains and Culture Media

The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were maintained in Luria broth (4). *M. gallisepticum* ATCC strain 19610 was grown in 2.5% PPLO broth (Difco Laboratories, Detroit, MI) supplemented with 10% gamma globulin-free horse serum (GIBCO/BRL, Gaithersburg, MD), 2.5% fresh yeast extract, 0.5% dextrose, 25 μg of cefobid (cefoperazone sodium, Roerig, Pfizer, NY) per ml and 1% Agar Noble (Difco) when required. *M. gallisepticum* R strain was grown in Frey's media (Becton Dickinson Microbiology Systems, Cockeysville, MD). Completed Frey's broth consisted of the following components: 22.5 g base in 1 liter water plus 50 ml horse serum (donor herd, GIBCO/BRL), 50 ml porcine serum (GIBCO/BRL), 20 ml yeast extract, 5 ml dextrose (50% stock in water), 5 ml cefobid (50% stock in 50% ethanol stored at -20°C) and 10 mM HEPES (1 M stock in water at pH 8.0). All of the supplements were added when broth was cooling to 55°C after autoclaving. Wild-type *M. gallisepticum* was regularly examined for hemagglutination activity, colony morphology as described (59), and its sensitivity to the antibiotics. Antibiotic solutions were prepared and filter-sterilized, aliquoted, frozen at -20°C, and used at the following final concentrations: for *E. coli*, 100 μg of ampicillin (Ap) per ml or 12.5 μg of tetracycline (Tc) per ml; for mycoplasmas, 25 μg of gentamicin (Gm) per ml agar or PPLO broth, 2 μg of Tc per ml agar or 10 - 15 μg of Tc per ml PPLO broth. For mycoplasmas, X-gal, a substrate that yields a deep blue precipitate upon cleavage by ß-galactosidase, was incorporated into PPLO agar at a concentration of 70 μg per ml (50 μg per μl stock in N, N' dimethylformamide stored at -20°C).

Table 1. Strains and plasmids used in this study

Strains and plasmids	Relevant genotype or properties	Source or reference
M. gallisepticum		
19610	wild-type	ATCC
R	wild-type	F. Goll
Ω204	19610 (Tn*4001*) Gm^r	This study
Ω600	19610 (Tn*916*) Tc^r	This study
Ω204.1	Ω204 (pISM2025) Gm^r Tc^r	This study
Ω207	19610 (pISM2541) Gm^r Tc^r	This study
Ω701	19610 (Tn*4001*::*lacZ*) Gm^r, *lacZ* in a 4 kb *Eco*RI chromosomal fragment	This study
Ω709	19610 (Tn*4001*::*lacZ*) Gm^r, *lacZ* in a 7 kb *Eco*RI chromosomal fragment	This study
Ω709.1	A filter-cloned isolate from Ω709	This study
E. coli		
DH5α	*supE44 ΔlacU169*(ø80 *lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO/BRL
GM2163	*hsdR dam dcm supE*	NEB
HB101	*supE44 hsdS20 recA13 ara-14 galK2 lacY1 proA2 rspL20 xyl-5 mtl-1 mcrB mrr*	
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55l lacY1 mcrA*	
TG1	*supE hsdΔ5 thi Δ(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]	Amersham
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F'[*proAB* $^+$*lacI*^{q} *lacZΔM15* Tn*10*]	Stratagene
Plasmids		
pISM1001	13.5 kb, Ap^r Gm^r, pKS with *Eco*RI fragment B of pSK31 (Tn*4001*)	(86)
pISM1001	13.5 kb, Ap^r Gm^r, pKS with *Eco*RI fragment B of pSK31 (Tn*4001*)	(86)

Table 1. (continued)

pAM120	21.4 kb, Ap^r Tc^r	(58)
pISM1002	7.5 kb, Ap^r Tc^r, *tetM* from pAM120	82
pSP64	3.0 kb, Ap^r	Promega
pISM1003	5.45 kb, Ap^r Gm^r	(87)
pISM1025	7.9 kb, Ap^r Tc^r, *tetM* in pSP64	This study
pISM2025	10.35 kb, Gm^r Tc^r, pISM1025 with the 2.45 kb Gm^r marker	This study
pISM2511-2517 series	Tc^r, pISM1002 with fragment of *lacZ* structural gene from 184 to 1,440 bp	This study
pISM2562	12.55 kb, Ap^r Gm^r Tc^r, rescued plasmid from *Eco*RI-digested chromosomal DNA of strain Ω204.1 containing a 1.2 kb fragment	This study
pISM2531	pISM1025 with 3.8 kb chromosomal DNA	This study
pISM2532	pISM1025 with 5.0 kb chromosomal DNA	This study
pISM2533	pISM1025 with 3.5 kb chromosomal DNA	This study
pISM2534	pISM1025 with 3.7 kb chromosomal DNA	This study
pISM2535	pISM1025 with 1.7 & 7.9 kb chromosomal DNA	This study
pISM2536	pISM1025 with 3.6 & 7.9 kb chromosomal DNA	This study
pISM2537	pISM1025 with 3.6 & 7.9 kb chromosomal DNA	This study
pISM2538	pISM1025 with 1.5 & 1.6 kb chromosomal DNA	This study
pISM2539	pISM1025 with 3.1 kb chromosomal DNA	This study
pISM2540	pISM1025 with 1.7 kb chromosomal DNA	This study
pISM2541	pISM2531 with the 2.45 kb Gm marker, Gm^r Tc^r	This study
pISM2542	pISM2533 with the 2.45 kb Gm marker, Gm^r Tc^r	This study
pISM2543	pISM2534 with the 2.45 kb Gm marker, Gm^r Tc^r	This study
pISM2545	pISM1003 with the *Eco*RI fragment from pISM2531	This study
pISM2546	pISM1003 with the *Eco*RI fragment from pISM2533	This study
pISM2547	pISM1003 with the *Eco*RI fragment from pISM2534	This study

Reagents and Buffers

All restriction enzymes and T_4 DNA ligase were purchased from GIBCO/BRL or Promega (Madison, WI) and used according to the manufacturers' directions. Chemicals and other reagents were obtained from Sigma (Sigma Chemical Co., St. Louis, MO) or Fisher (Itaska, IL) except where indicated. All solutions were prepared in tissue culture grade water and autoclaved before use. Solution compositions were the following: 40% (wt/vol) polyethylene glycol (PEG): PEG 8000 in 10 mM Tris-500 mM sucrose (pH 6.5)(autoclaved at 121°C for 15 min); PBS: 10 mM sodium monobasic phosphate-140 mM sodium chloride (pH 7.4), and 10 mM dextrose when needed; TE buffer: 10 mM Tris-1 mM ethylenediamine tetraacetic acid (pH 7.8); TNE buffer: 10 mM Tris HCl-140 mM sodium chloride-1 mM ethylenediamine tetraacetic acid (pH 8.0); TAE buffer: 40 mM Tris acetate-1 mM ethylenediamine tetraacetic acid; and TBE buffer: 89 mM Tris HCl-89 mM boric acid-2 mM ethylenediamine tetraacetic acid. For the last three buffers, 10x or 50x concentrated solutions were made as stocks.

DNA Isolation and Manipulation

E. coli plasmid DNA was isolated either by the method of Birnboim and Doly (13) and further purified by CsCl-ethidium bromide density gradient centrifugation (4) or by Qiagen column chromatography (Qiagen Inc., Chatsworth, CA) according to the manufacturer's directions. Mycoplasma chromosomal DNA for cloning or Southern blotting was prepared in low melting agarose or by phenol extraction as previously described (86). Briefly, three ml of actively growing cells (about 10^9 CFUs per ml, medium pH changes to 6.8 from 7.8) was centrifuged in a microfuge (12,000 x *g* for 4 min), washed once and resuspended in 30 µl of TNE buffer. Proteinase-k

(10 μg per μl stock in water) was added to a final concentration of 1 μg per μl, and then an equal volume (30 μl) of low melting agarose (1.6% in TNE buffer, NuSieve GTG, FMC Bioproducts, Rockland, ME) was added and mixed thoroughly. After 1 - 3 h incubation at 37°C, the agarose was hardened by placing the tube on ice for 5 min, the plug was removed and placed into a 12 x 75 mm plastic tube, and incubated overnight at 37°C in the presence of 0.5 ml 1% Tween-20 in TE buffer. The DNA plug was then dialyzed against cold TE buffer for 4 - 5 h with a fresh buffer change every hour. If the original cell density was lower, final dialysis was done with water to allow for a higher volume of the DNA-agarose in the reaction mixtures and prevent interference of the reaction with ethylenediamine tetraacetic acid. Proteinase-k concentrations were also carefully controlled. The DNA plug could be stored in TE buffer at 4°C for one year or more.

When higher concentrations of DNA were needed, chromosomal DNA was prepared by phenol extraction. In a typical procedure, 500 ml of freshly-grown cells were harvested by centrifugation and washed twice with PBS. The cell pellet was resuspended in 5 ml TNE buffer, and 100 μg of proteinase-k was added. After incubation for 3 - 5 h at 37°C, 1 ml lysis buffer (1% NP-40, 1% Tween-20 and 1% deoxycholate in water) was added and the tube was sharply inverted 1 - 2 times to ensure lysis. One more ml of lysis buffer and 100 μg proteinase-k were added for completion. After incubation overnight at 37°C, the mixture was extracted 3 times or more with equilibrated phenol/chloroform until there was no interface left. The DNA was then precipitated with one fourth volume of 7.5 N ammonium acetate and 2 volumes of ethanol, and the fibrous DNA was spun on a glass rod, vacuum-dried and resuspended in water or TE buffer. DNA concentrations were measured by a fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco, CA).

Restriction digests consisted of 4 - 6 µl of melted DNA-containing agar, 2 µl 10x buffer and restriction enzyme (0.5 - 1 unit) in a total volume of 20 µl. The DNA plug was melted at 70°C and equilibrated at 37°C prior to removing an aliquot for digestion. Restriction buffer and enzyme were prewarmed to 37°C prior to adding the DNA. In some instances, additional enzyme was needed and the reaction tube incubated for an additional 1 h at 37°C. Following digestion, DNA fragments were directly used for cloning, or were separated by electrophoresis on 0.6% agarose gels in TBE or TAE buffer. The digestion mixture was equilibrated to 70°C before adding electrophoresis sample buffer (89) and loading onto the gel. After electrophoresis at 100V for 2 - 3 h, the agarose gels were stained in ethidium bromide solution (0.5 µg per ml in TAE buffer) and visualized under UV light. Specific DNA fragments were either excised and further purified by the glass milk-sodium iodide procedure (GeneClean II, BIO 101, Inc., La Jolla, CA) or transfered onto Nytron nylon membrane. DNA hybridization was performed by the method of Southern (132) using standard stringency conditions (4) in a hybridization chamber (Model 310, Robbins Scientific, Sunnyvale, CA). DNA probes were made by using the Multiprime Labeling System (Amersham Corp., Arlington Heights, IL) incorporating [^{32}P]dCTP (ICN Biochemical, INC. Irvine, CA.) according to the manufacturers' directions.

Transformation Procedure

Transformation of *E. coli* was performed either by the calcium-shock method (50) or by electroporation (32) using an Electro Cell Manipulator 600 (BTX Inc., San Diego, CA). Transformation of *M. gallisepticum* was performed using the PEG procedure as described previously (86) with some modifications. Briefly, *M. gallisepticum* cells were grown to late-log stage in PPLO broth to a density of

approximately 1.5 x 10^9 CFUs per ml, harvested by centrifugation (10,000 x *g* for 10 min), washed once, resuspended in one-half of the original volume of cold 100 mM calcium chloride, and then incubated on ice for 30 - 60 min. A portion of the cell suspension (250 µl) was mixed with 1, 5 or 10 µg of plasmid DNA, 0 or 10 µg yeast tRNA and 2 ml of PEG solution. Plasmid DNA, tRNA and cells were premixed on ice before adding to the PEG solution. Once the cells were in PEG, vigorous shaking was performed to thoroughly mix the suspension. The time of cell contact with the PEG was also minimized. After 1 min of incubation at room temperature, the transformation mixture was diluted with 30 ml of PBS or 10 mM TrisHCl (pH 7.5) buffer, centrifuged at 10,000 x *g* for 10 min, and resuspended in 1.5 ml non-selective PPLO broth. The maximum amount of PBS was used to dilute the PEG and avoid salt precipitation during the procedure. After incubation at 37°C for 30 min, a portion of the cells (10 µl) was taken for measuring CFUs and the rest of the cells were spread onto selective agar plates. The plates were incubated at 37°C for 4 - 6 days. Individual colonies were calculated, picked, and grown in selective PPLO broth for further analysis. Transformation frequency was defined as the number of antibiotic resistant colonies divided by the total CFUs plated on selective media. To obtain total CFUs, 10 µl of cells from each transformation mixture was serially diluted 10-fold to 10^{-8}, and 10 µl from each dilution was plated in duplicate or triplicate. Transformed cells were also stored at -70°C for future plating and isolation of transformants.

Construction of Cloning Vectors

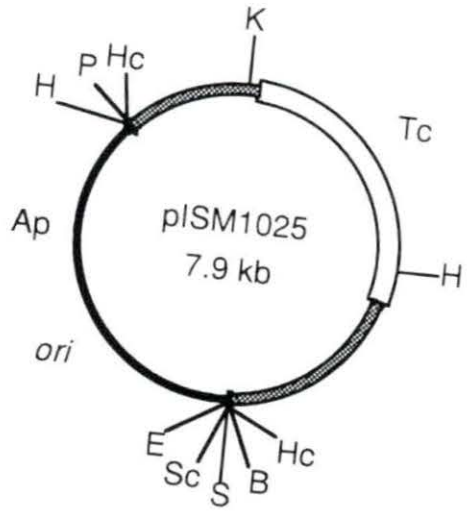
Two plasmids, pISM1001 containing Tn*4001* and pAM120 containing Tn*916*, have been described previously (45, 86). The cloning of the resistance markers from

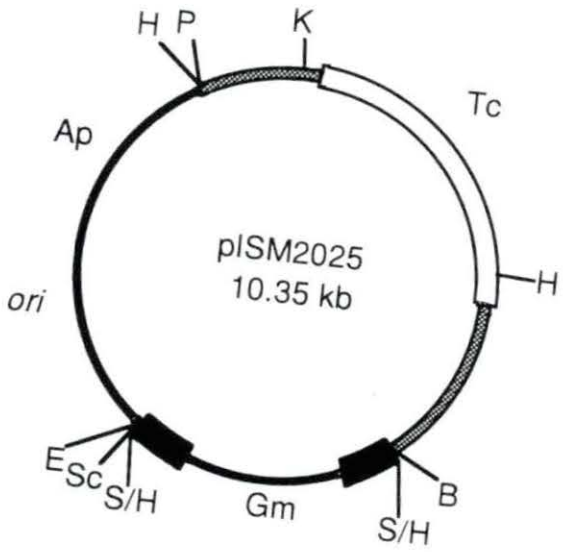
both transposons has also been described (87). These two plasmids were used to transform wild-type *M. gallisepticum* strains 19610 and R.

To construct plasmid pISM1025 for cloning random chromosomal fragments, a *tetM* marker from Tn*916* was inserted into pSP64 (Promega), a 3.0 kb plasmid with an *E. coli* replicon and an ampicillin resistance marker. Briefly, pSP64 was digested with *Hin*cII, and the 3' ends dephosphorylated with calf intestine alkaline phosphatase (CIAP) as described (4). The *tetM* gene in a 4.9 kb fragment was obtained from pISM1002 by *Hin*cII digestion and the reaction products were resolved on a 0.7% agarose gel. The 4.9 kb fragment was isolated and purified by GeneClean. The insert fragment and the dephosphorylated vector were ligated at molar ratios of 1:1, 1:3, and 3:1. Following overnight ligation at 4°C, the ligation mixture was transformed into competent cells of *E. coli* DH5α (49). Controls included undigested vector, digested vector, and digested/dephosphorylated/ligated vector. Ampicillin and tetracycline resistant colonies were screened for recombinant plasmids by the method of Kado (60). The structure of pISM1025 was confirmed by restriction enzyme digestion and agarose gel electrophoresis (Figure 3).

Plasmid pISM2025 (Figure 3), a derivative of pISM1025, was constructed for integration into Tn*4001* inserts in *M. gallisepticum*. The gentamicin resistance marker was obtained from *Hin*dIII-digested pISM1001 in a 2.45 kb fragment. The DNA fragment was first isolated from an agarose gel as described above, and a fill-in reaction was performed with DNA polymerase I (Klenow fragment). The fragment was then ligated into *Sma*I-digested plasmid pISM1025 using blunt end ligation reaction conditions (127). The resulting plasmid contained both gentamicin and tetracycline resistance markers. Both the *Sma*I and the *Hin*dIII sites were lost during the construction.

Figure 3. Restriction maps of plasmids pISM1025 and pISM2025. The plasmids were constructed as described in the Materials and Methods. *Sma*I and *Hin*dIII sites were lost in pISM2025 (S/H). 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.


K
P Hc
H
Tc
Ap
pISM1025
7.9 kb
H
ori
E
Sc
S
B
Hc


H P
K
Tc
Ap
pISM2025
10.35 kb
H
ori
E
Sc
S/H
Gm
B
S/H

Portions of the *E. coli lacZ* sequence were subcloned into pISM1002, a derivative of pBR322, by the following procedure. A 3.0 kb fragment containing intact the *lacZ* structural gene was isolated from *Bam*HI-digested p3-27 (Minion, unpublished data) and purified by GeneClean. This 3.0 kb fragment was further digested by the restriction enzyme *Tsp*509I (New England Biolabs, Beverly, MA) in a total volume of 60 µl containing 1 µg DNA, 6 µl 10x buffer and 2 units of the enzyme. The reaction mixture was incubated at 65°C for 2 h and a small sample was analyzed by agarose gel to monitor the digestion. The residual *Tsp*509I was removed by StrataClean resin (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Ligation (at room temperature for 3 h) was performed in a total volume of 30 µl with 30 ng *Tsp*509I-restricted DNA, 240 ng *Eco*RI-digested pISM1002, T_4 DNA ligase (1 unit) and buffer. The ligase was heat-inactivated at 65°C for 15 min, and 3 units of *Eco*RI were added to digest the ligated vectors. This eliminated background arising from religation of the vector. A 5 µl ligation mixture was used for electroporation of *E. coli* DH5α, and tetracycline resistant colonies were isolated and screened for recombinant plasmids. Since there are five *Tsp*509I restriction sites in the *lacZ* structural gene, attempts were made to cover all the different portions of *lacZ* sequence in a series plasmids (designated pISM2511 - 2517). The resulting plasmids were mapped with *Tsp*509I and other enzymes and probed with the 3.0 kb *lacZ* fragment to confirm the structures. One representative plasmid, pISM2514, containing an internal 1.4 kb fragment of the *lacZ* sequence is shown in Figure 4.

Examination of Homologous Recombination

In order to examine homologous recombination in *M. gallisepticum*, four distinct approaches were used. 1) Tn*4001* sequences were targeted for integration using a plasmid containing both the gentamicin and tetracycline resistance markers. 2) Random fragments of *M. gallisepticum* chromosomal DNA were cloned, and the fragments provided the regions of homology. 3) Single-stranded (ss) plasmid DNA from plasmids containing *M. gallisepticum* chromosomal sequences were also used. 4) The *E. coli lacZ* gene previously inserted into the *M. gallisepticum* chromosome by the introduction of a modified transposon Tn*4001lac* was targeted for integration. Also examined was the probability of a double cross-over event to generate an interposon mutation.

Targeting Tn*4001* sequences for integration

Since preliminary transformation results showed that transposition of Tn*4001* did occur in *M. gallisepticum*, initial experiments were designed to target the transposon sequence for integration. Tn*4001*-containing *M. gallisepticum* strains were constructed by transformation with plasmid pISM1001 and selection for gentamicin resistance. Single insertions of Tn*4001* were confirmed by DNA-DNA hybridization using the gentamicin marker as a sequence-specific probe. One recipient strain, K-43, was transformed again with the integrative plasmid pISM2025 to target the gentamicin resistance marker sequences for integration as shown in Figure 5. Transformants were selected on tetracycline containing agar. The integration of pISM2025 was confirmed by DNA-DNA hybridization using the 4.9 kb *tetM*-containing fragment from pISM1002 and the 2.45 kb Gm-containing fragment from pISM1003 as sequence-specific probes. Transformation frequencies were calculated

as described above. This approach also allows for the rapid isolation of sequences adjacent to the transposon insertion site by digesting the chromosomal DNA of a strain such as K43-2025-1 with restriction enzymes *Eco*RI or *Pst*I (Figure 5), ligation, and transformation of the ligation mixture into *E. coli* selecting for tetracycline resistance. The adjacent chromosomal DNA sequences obtained in the rescued plasmid can be sequenced using plasmid-specific primers (1), or the recombinant plasmid can be used to identify genes by screening a recombinant library and analyzing cross-hybridizing clones.

Targeting *M. gallisepticum*-specific DNA sequences

In order to target *M. gallisepticum*-specific DNA sequences, fragments of chromosomal DNA from wild-type strain 19610 were cloned into plasmid pISM1025. This was accomplished by completely digesting chromosomal DNA with *Eco*RI in a 30 µl volume containing 20 µg DNA, 3 µl 10x buffer, 14 µl water and 20 units enzyme. After a 1 h incubation at 37°C, 10 more units of *Eco*RI were added and incubation continued for another hour. The vector pISM1025 was also digested with *Eco*RI using similar conditions except that 10 µg DNA was digested. For dephosphorylation of the vector, 20 µl of the digestion mixture was diluted with water to 100 µl and 11 µl of 10x buffer and 2 units of CIAP were added. After incubation at 37°C for 20 min, one additional unit of CIAP was added and incubation continued for 10 min. The digested DNAs were diluted with TE to 200 µl and extracted twice with phenol/chloroform and precipitated with ammonium acetate/ethanol. The DNAs were resuspended in 20 µl (for vector DNA) and 40 µl (for chromosomal DNA) of TE buffer. Ligation was performed overnight at 4°C in a 30 µl total volume containing 1 µl of vector DNA, 1, 2, 3 or 4 µl of chromosomal DNA (for different vector /insert

Figure 4. Construction of pISM2514. The *E. coli lacZ* structural gene was obtained on a 3.0 kb *Bam*HI (B) fragment and was digested with *Tsp*5091 (5'-AATT-3'). The resulting fragments (184 - 1,440 bp) were ligated into *Eco*RI-digested pISM1002. Recombinant plasmids containing the partial *lacZ* inserts were obtained by tetracycline selection. Shown is one plasmid pISM2514 which contains an internal 1.4 kb of *lacZ* (hatched region). Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I, P, *Pst*I; Pv, *Pvu*II.

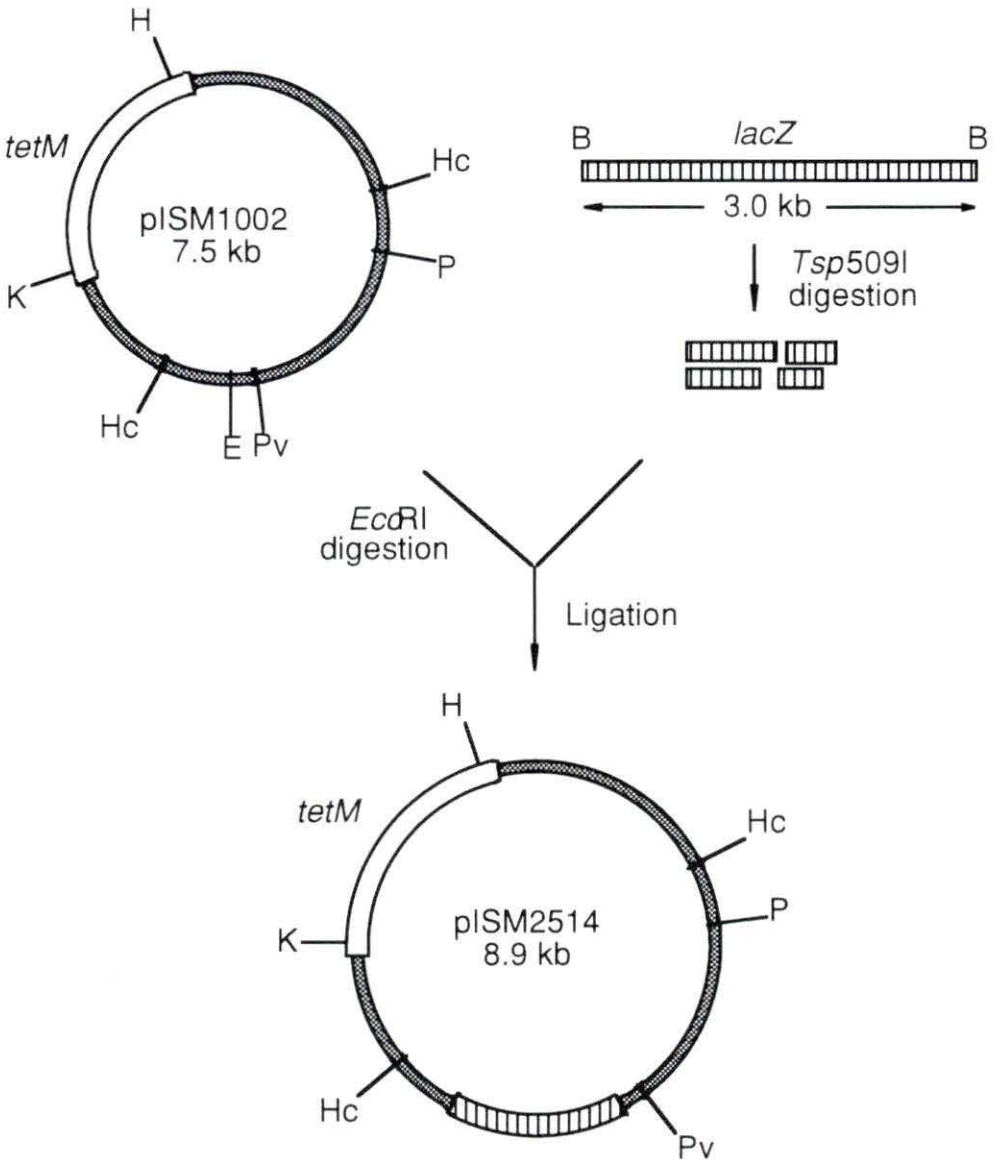
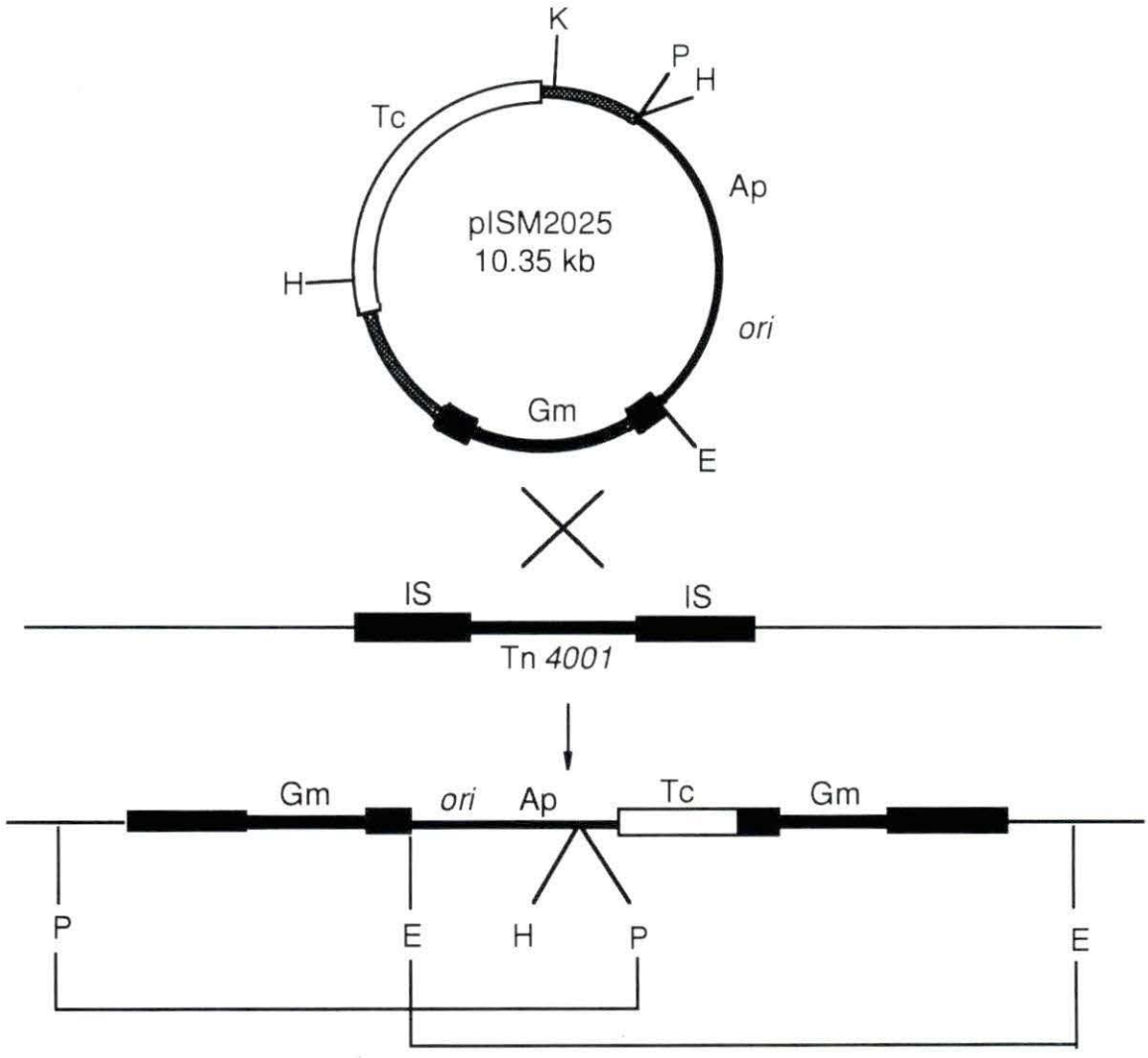

H
tetM
Hc
pISM1002
7.5 kb
P
K
Hc
E Pv
B
lacZ
B
3.0 kb
Tsp509I
digestion
EcoRI
digestion
Ligation
H
tetM
Hc
P
K
pISM2514
8.9 kb
Hc
Pv

Figure 5. Mechanism of homologous recombination based upon a partial transposon sequence and the rescue of adjacent chromosomal sequences. Plasmid pISM2025 carrying the Gm marker and a partial IS*256* element from Tn*4001* integrates into a *M. gallisepticum* strain (Ω204) harboring one copy of native Tn*4001*. The entire plasmid integrates by homologous recombination resulting in a Gm^r Tc^r strain (Ω204.1) as shown. The chromosomal DNA from this strain can be digested with either *Eco*RI (E) or *Pst*I (P) to recover chromosomal the sequences flanking the transposon insertion site. Abbreviations: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; S, *Sma*I; Ap, ampicillin resistance marker; Gm, gentamicin resistance marker; Tc, tetracycline resistance marker; *ori,* origin of replication; IS, IS*256*.


K
P
H
Tc
Ap
pISM2025
10.35 kb
H
ori
Gm
E
IS
IS
Tn 4001
Gm
ori
Ap
Tc
Gm
P
E
H
P
E

ratios), 3 µl 10x ligation buffer, 1 µl ATP (25 mM stock in pH 7.5 Tris·HCl at -20°C) and 0.1 unit of T_4 DNA ligase. The ligation controls included uncut vector, cut vector, cut/ligated vector, and cut/dephosphorylated/ligated vector. The ligation mixtures were used to transform competent *E. coli* DH5α cells by a standard calcium-shock procedure. Tetracycline and ampicillin resistant *E. coli* transformants were screened for inserts by purifying DNA from 2 ml cultures by the method of Birnboim and Doly (13), digesting with restriction enzyme *Eco*RI, and analyzing the digests on 0.7% agarose gels. Ten recombinant plasmids designated pISM2531 - pISM2540 (see Table 1) were randomly chosen and used to transform wild-type *M. gallisepticum* via the standard PEG procedure selecting for tetracycline resistance. Plasmid pISM1025 was used as the control. Integration of each recombitant plasmid into *M. gallisepticum* chromosome was confirmed by DNA-DNA hybridization using the 4.9 kb *tetM*-specific marker as a probe. The mechanism of integration is shown in Figure 6.

Integration of single-stranded DNA into *M. gallisepticum*

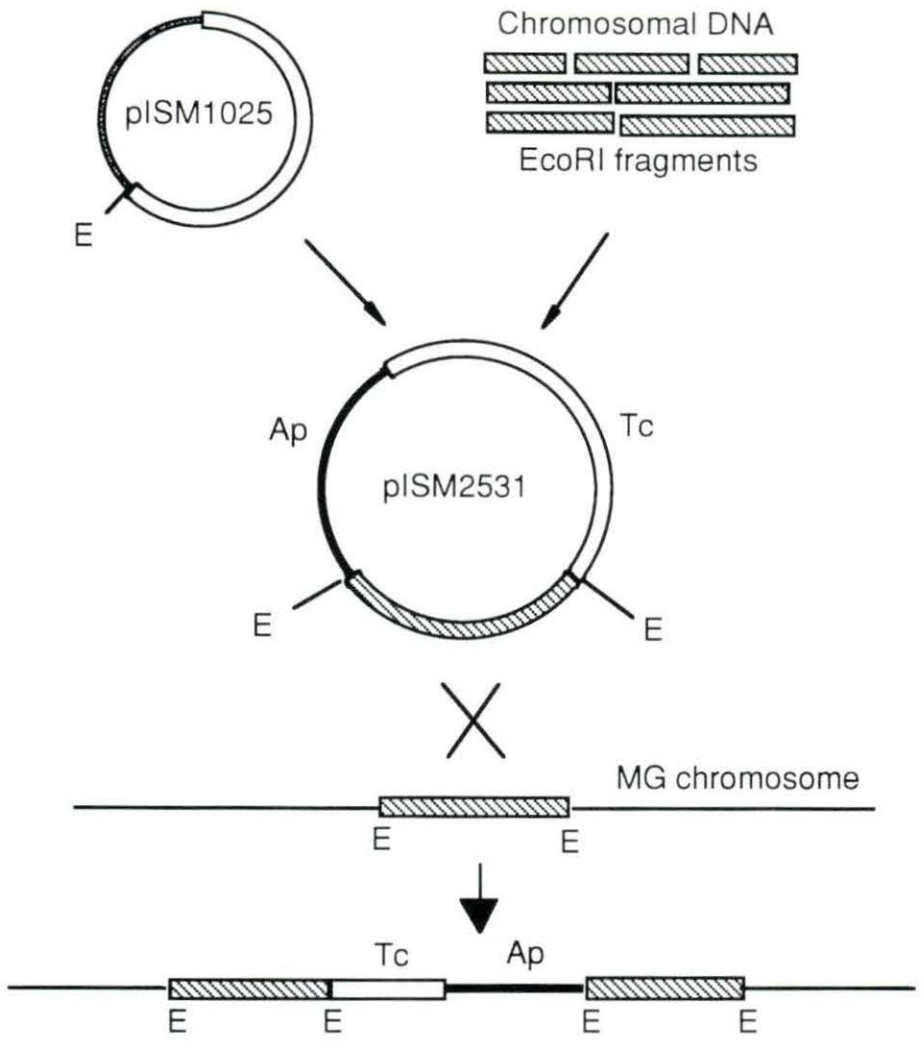
DNA recombination is believed to occur through a single-stranded intermediate and therefore it was of interest to determine if ssDNA could initiate homologous recombination in *M. gallisepticum*. To test this, the *Eco*RI *M. gallisepticum* chromosomal fragments from plasmids pISM2531, pISM2533, and pISM2534 were subcloned into pISM1003, a pKS derivative which contains the f1 origin of replication and the Gm^r marker, resulting in plasmids pISM2545, pISM2546, and pISM2547 (Table 1). The three new plasmids were then transformed into *E. coli* F^+ strain XL1-Blue by electroporation. The f1 helper phage, R204, was added to a fresh culture of 2x TYP broth (per liter: Bacto-tryptone, 16 g; Bacto-yeast extract, 16 g; NaCl, 5 g;

and K_2HPO_4, 2.5 g) containing 10^8 bacteria per ml at a multiplicity of infection of 20:1 (phage to cell). This mixture was incubated for 8 h at 37°C with constant shaking. The *E. coli* cells were removed by centrifuging twice at 10,000 x *g* for 10 min. The supernatant containing the phage was then precipitated by adding one-fourth volume of a 3.5 N ammonium acetate (pH 7.5)/20% PEG solution and extracted with phenol/chloroform (1:1). Finally, ssDNA was precipitated by ammonium acetate/ethanol and resuspended in TE buffer. Single-stranded DNA was confirmed by digestion with either restriction enzymes or nucleases, or by staining with acridine orange (95). Single-stranded DNA was transformed into wild-type *M. gallisepticum* as usual and transformants were selected by gentamicin resistance.

Interposon mutagenesis

In order to develop a system for interposon mutagenesis in *M. gallisepticum* as shown in Figure 7, plasmids containing randomly-cloned chromosomal DNA fragments were isolated and screened for unique restriction enzyme sites. Again, three of the ten recombinant plasmids, pISM2531, pISM2533, and pISM2534, each were found to contain a unique *Hin*dIII site. Since there is a second *Hin*dIII site in the multiple cloning region of pISM1025 (see Figure 5), these three plasmids were linearized by a partial *Hin*dIII digestion in the following way. The reaction mixture consisted of 15 µg plasmid DNA, 15 µl 10x buffer and 15 unit enzyme in a total volume of 150 µl. Digestion was allowed to proceed for 15 min at 37°C, and the reaction was inactivated at 70°C for 10 min. The partially-digested fragments were separated by electrophoresis, and the corresponding linear-sized fragments were isolated, purified by GeneClean, and dephosphorylated as described above. The gentamicin marker isolated from Tn*4001* was ligated into the *Hin*dIII site and the

Figure 6. Homologous recombination based upon random chromosomal sequences. *M. gallisepticum* chromosomal DNA (hatched regions) digested with *Eco*RI was cloned into pISM1025. Recombinant plasmids, e.g. pISM2531, were used to transform wild-type *M. gallisepticum*. Homologous recombination between the plasmid insert and the corresponding chromosomal sequences results in the integration of the plasmid into the chromosome. The target sequence is duplicated during the process, and the Tc^r marker can be used to select for the integration event. Abbreviations: E, *Eco*RI; Ap, ampicillin resistance marker; Tc, tetracycline resistance marker; MG, *M. gallisepticum*.


Chromosomal DNA
EcoRI fragments
pISM1025
E
Ap
Tc
pISM2531
E
E
MG chromosome
E
E
Tc
Ap
E
E
E
E

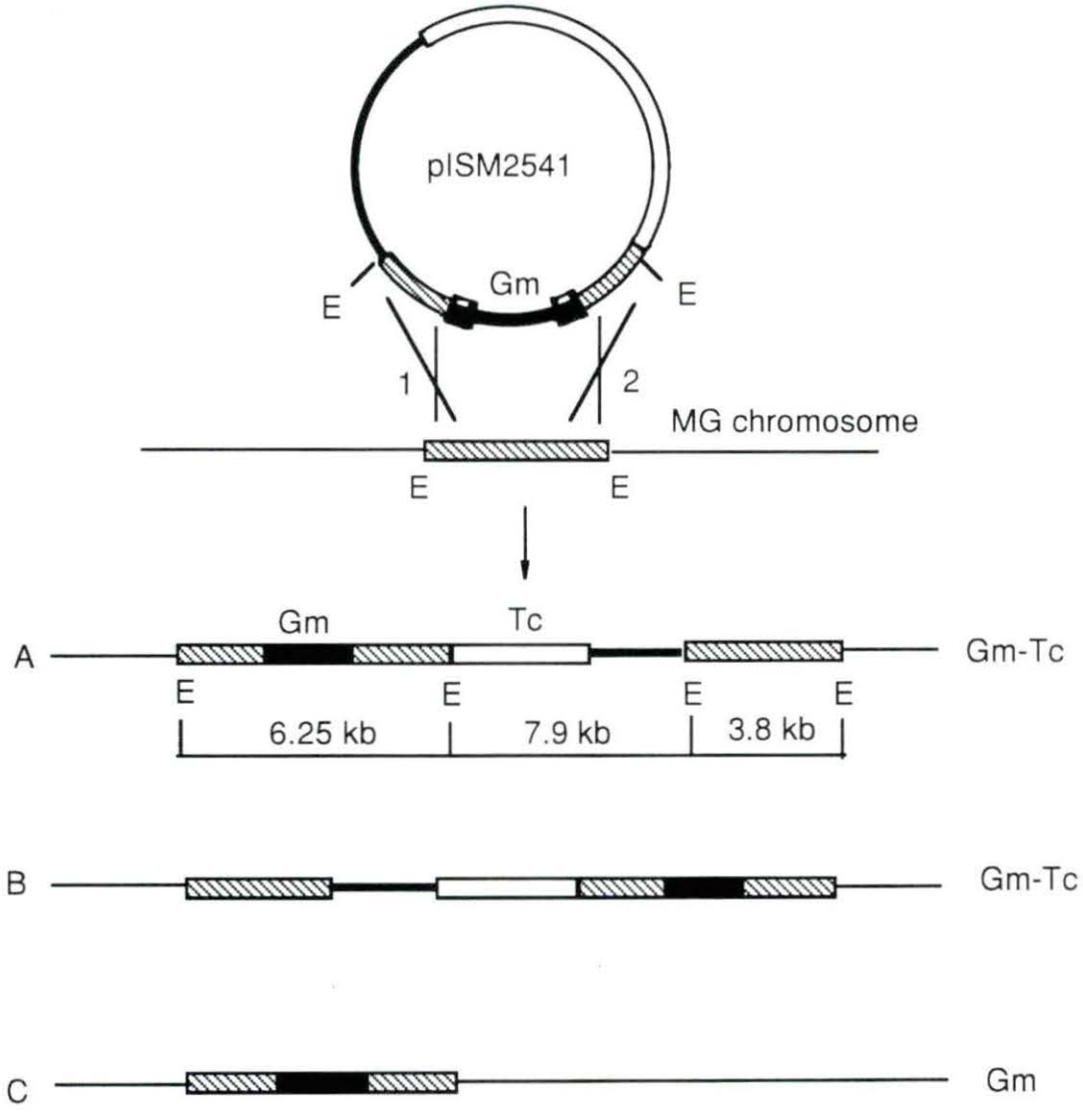
ligation mixture transformed into *E. coli* DH5α. Three recombinant plasmids (interposons), pISM2541, pISM2542and pISM2543, were isolated and their structure confirmed by restriction enzyme digestion.

As shown in Figure 7, when the three plasmids were transformed into *M. gallisepticum*, a single crossover either in the No. 1 or No. 2 location will lead to the integration of the complete plasmid sequence into the chromosome, producing a duplication of the target sequence with a mutant gene and an intact one. Because both gentamicin and tetracycline are functional in mycoplasmas, these transformants should be double resistant. A double crossover event, however, will inactivate the gene and the transformants will be tetracycline sensitive and gentamicin resistant. Double crossover events can be identified by selecting for Gm^r colonies and then scoring for tetracycline sensitivity.

lacZ gene as target sequence

The gene that codes for *E. coli* β-galactosidase, *lacZ,* was first introduced into wild-type *M. gallisepticum* via transposition with Tn*4001lac* (68), and the resulting blue, Gm^r colonies on X-gal agar were isolated and used as recipient strains to transform the series plasmids carrying a portion of *lacZ* gene. The strategy for constructing *lacZ* interposon mutants is shown in Figure 8. Inactivation of *lacZ* by interposon mutagenesis would result in a white (Lac^-) phenotype readily discernible on X-gal containing agar. Fourteen blue (Lac^+) *M. gallisepticum* transformants were obtained and further characterized. To determine the number of *lacZ* copies in the chromosome, chromosomal DNAs were digested with three restriction enzymes, *Hin*dIII, *Kpn*I, and *Xba*I, respectively, and a standard DNA-DNA hybridization was performed using the internal 1.1 kb *Sac*I-*Cla*I fragment of *lacZ* or the 3.0 kb *lacZ*

Figure 7. Interposon mutagenesis. Interposon mutagenesis uses homologous recombination to generate an insert in a structural gene interrupting its expression. The cloned sequence (hatched region) representing a structural gene's coding sequence or an internal fragment is interrupted with foreign DNA (Gm marker) in plasmid pISM2541. During recombination, duplication of the target sequence does not result in an intact gene because only a portion of the gene will be duplicated. During transformation of *M. gallisepticum*, a single crossover event between the pISM2541 and the chromosome can occur at either end of the interrupted sequence (location 1 or 2). This results in a doubly resistant recombinant (Gm^r Tc^r phenotype) as shown in (A) and (B). In rare instances, a double crossover event (C) occurs resulting in a Gm^r Tc^s phenotype. These events can be sorted by selecting for gentamicin transformants and scoring for tetracycline resistance. They can also be sorted by DNA-DNA hybridization using the Gm marker, the Tc marker and the intact cloned fragment as specific probes. An example of this kind of analysis is shown in Figure 11. Abbreviations: E, *Eco*RI; Gm, gentamicin resistance marker; Tc, tetracycline resistance marker; MG, *M. gallisepticum*.

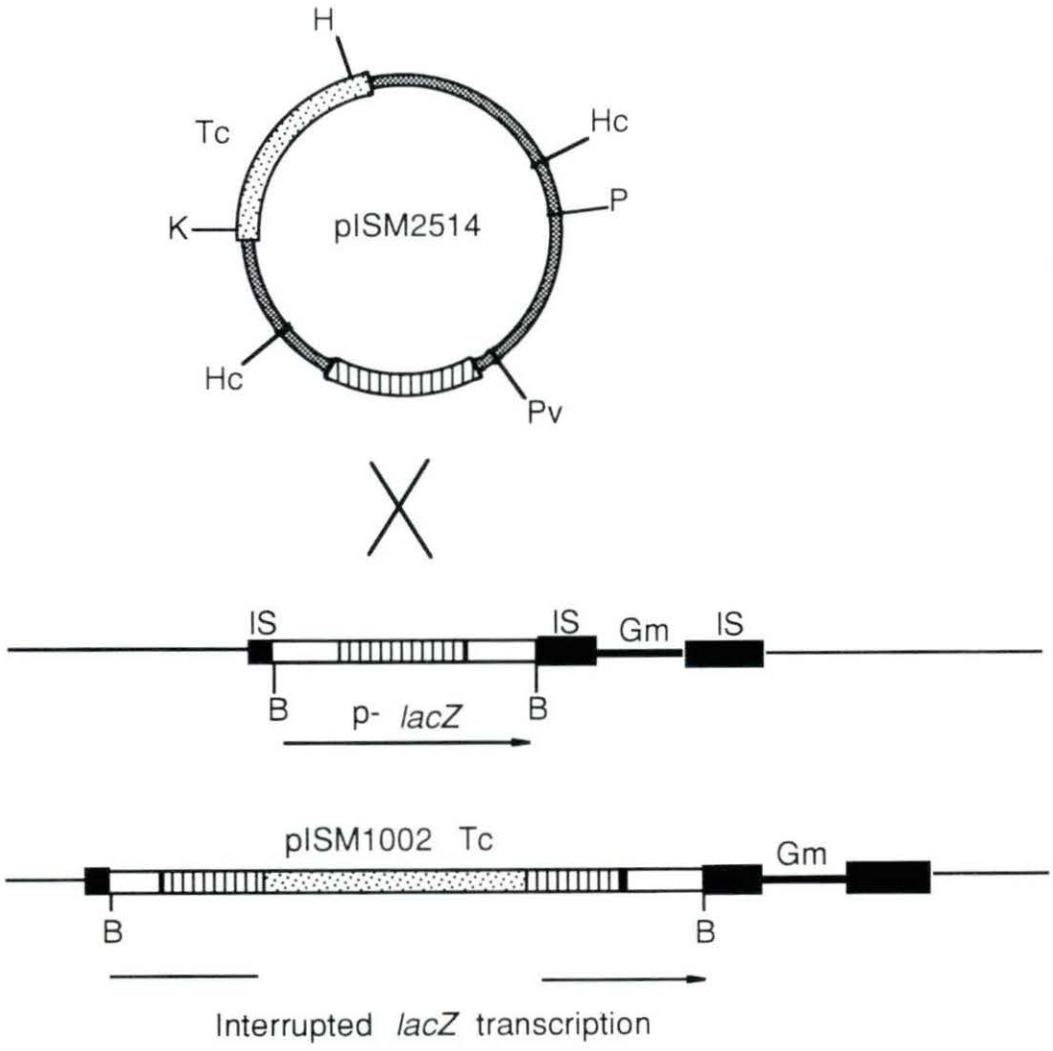

pISM2541
Gm
E
E
1
2
MG chromosome
E
E
Gm
Tc
A
Gm-Tc
E
E
E
E
6.25 kb
7.9 kb
3.8 kb
B
Gm-Tc
C
Gm

fragment as probes to analyze the blots. The stability of the blue phenotype in two strains, Ω709 and Ω701, was also determined by passing them in gentamicin-containing PPLO broth, filter-cloning them using a 0.22 μm filter (Costar Corp, Cambridge, MA) and observing their Lac phenotype on X-gal/gentamicin agar plates. Chromosomal DNA from these white revertants were digested with *Eco*RI and probed with the internal *lacZ* fragment. The hybridization patterns were also compared.

In Vitro Stability of Recombinant Strains

The stability of the *M. gallisepticum* recombinant strains was evaluated in the following way. To assess the in vitro stability of transposons and integrative plasmids in *M. gallisepticum*, four recombinant strains (designated Ω204, Ω204.1, Ω207, and Ω600 (see Table 1) were used. Briefly, each *M. gallisepticum* strain was passed in non-selection broth for about 100 generations (10 μl culture inoculated into 10 ml fresh broth and grown to late-log phase was considered to be 10 generations). At the 30th and 100th generation passages, CFUs were determined on selective and non-selective agar and compared accordingly. Approximately 20 individual colonies from each strain were picked for DNA analysis by DNA-DNA hybridization. Also, chromosomal DNAs were prepared for every 10 generations. DNA was digested with *Eco*RI and separated by electrophoresis as usual. DNA hybridization patterns using specific markers as probes were compared between individual colonies at the different passages to measure the stability of the insert.

Figure 8. Homologous recombination within *lacZ*. Plasmid pISM2514 is transformed into a *M. gallisepticum* strain containing Tn*4001lac* displaying the Lac$^+$ phenotype and homologous recombination occurs in the shared *lac* sequences as shown. This results in integration of the plasmid into the original *lac* sequence, a duplication of the common sequences and the interruption of β-galactosidase expression. The resulting transformant will be Gm^r Tc^r and display a Lac$^-$ (white) phenotype. A shift in the size of the hybridizing bands will be observed for *Bam*HI-digested chromosomal DNA using either the *lacZ* or Tc resistance marker as a probe. Abbreviations: B, *Bam*HI; p-*lacZ*, promoterless *lacZ*.



Interrupted *lacZ* transcription

RESULTS

Insertion of Transposons Tn*4001* and Tn*916* into *M. gallisepticum*

Table 2 shows the results of the transformation studies of *M. gallisepticum* 19610 with pISM1001 as a function of the plasmid DNA and yeast tRNA concentrations. Transformation frequency increased one log as the DNA concentration increased from 1 to 5 μg per reaction. This is expected. However, when the DNA concentration was increased from 5 to 10 μg, the frequency was unaffected. Contrary to studies with other mycoplasmas (36, 87), yeast tRNA had no effect on transformation frequency in *M. gallisepticum*. Consistency in transformation frequencies with pISM1001 could be obtained if cells were grown to late-log phase and the plasmid DNA was CsCl-purified (data not shown). In subsequent transformation studies, pISM1001 was often used as a positive control. Transformation frequencies with pAM120 (Tn*916*) was higher than that for pISM1001(Tn*4001*), 2×10^{-5} versus 1.0×10^{-6} transformants per CFU. In Figure 9, ten randomly chosen Gm^r transformants from strain 19610 and twelve Gm^r transformants from strain R were analyzed by DNA-DNA hybridization for Tn*4001* insertions using the 2.45 kb internal fragment containing the gentamicin resistance marker. In addition, eleven Tc^r transformants from strain R were examined for insertions of Tn*916* using the 4.9 kb *Hin*cII *tetM*-containing fragment. From the distribution of the hybridizing DNA bands, it appeared that the transposons integrated into the *M. gallisepticum* chromosome in a random fashion and that there was a single copy. When the same DNAs were probed with pKS, the parent plasmid of pISM1001, no plasmid sequences were identified indicating that the transposon

Table 2. Effect of DNA concentration and yeast tRNA on transformation of *M. gallisepticum* 19610 with pISM1001

pISM1001 (μg)	Yeast tRNA (μg)	Frequency[a]
1	10	2.9×10^{-7}
5	10	1.1×10^{-6}
10	10	1.3×10^{-6}
1	0	2.2×10^{-7}
5	0	5.8×10^{-6}
10	0	3.9×10^{-6}
10	-	3.4×10^{-8}[b]

[a] Number of transformants per CFU

[b] Frequency with *M. gallisepticum* R strain

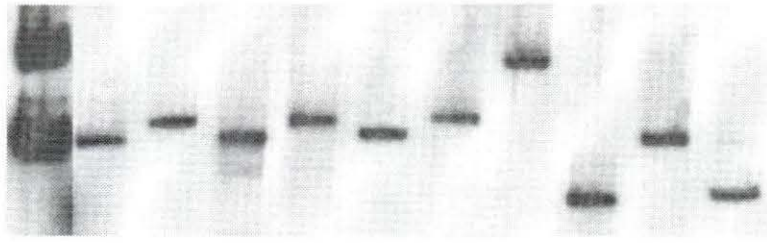
integrated into the chromosome through a transposition event and not by homologous recombination (87) (data not shown).

Rescue of the Sequences Adjacent to Tn*4001* Inserts

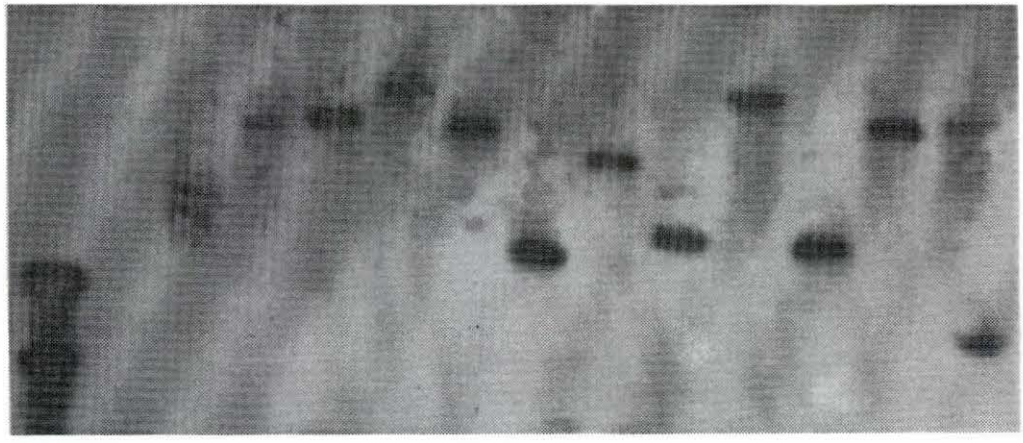
M. gallisepticum strain Ω204 was transformed with pISM2025 at a low frequency (Table 3). The wild-type strain, 19610, failed to transform with pISM2025 (data not shown). One of the resulting Gm^r Tc^r transformants, designated Ω204.1, was chosen for further study. DNA-DNA hybridization studies using Gm- and Tc-specific markers showed the integration of the complete pISM2025 sequence into the recipient strain. Chromosomal DNAs were prepared and plasmids rescued by *Pst*I and *Eco*RI digests as described in the Materials and Methods. Two rescued plasmids from Ω204.1 were designated pISM2526 (map not shown) for *Pst*I digestion (left end) and pISM2562 for *Eco*RI digestion (right end), respectively (see

Figure 9. DNA-DNA hybridization of *M. gallisepticum* transformants. Shown are the results of DNA-DNA hybridization studies with *M. gallisepticum* 19610 transformants. Chromosomal DNAs were digested with *Eco*RI (top and middle panels) or *Hin*dIII (lower panel), blotted and analyzed with the indicated probe. Top panel: Lanes 2 - 11 represent ten independent Tn*4001* transformants. Lane 1 is a positive control (pISM1003). The blot was probed with radiolabeled pISM1003. Middle panel: Lanes 3 - 14 represent *M. gallisepticum* R Tn*4001* transformants. Lane 1 contains pISM1001 (positive control) and lane 2 contains wild type chromosomal DNA (negative control). The blot was hybridized with a [^{32}P]-labeled 2.45 kb fragment from pISM1003 containing the Gm marker of Tn*4001*. Bottom panel: Lanes 3 - 13 represent independent Tn*916* (pAM120) *M. gallisepticum* R transformants. Lane 1 contains pISM1002 (positive control) and lane 2 contains wild type chromosomal DNA (negative control). The blot was analyzed using the 4.9 kb *tetM* containing DNA fragment from pISM1002.

1 2 3 4 5 6 7 8 9 10 11



1 2 3 4 5 6 7 8 9 10 11 12 13 14



1 2 3 4 5 6 7 8 9 10 11 12 13

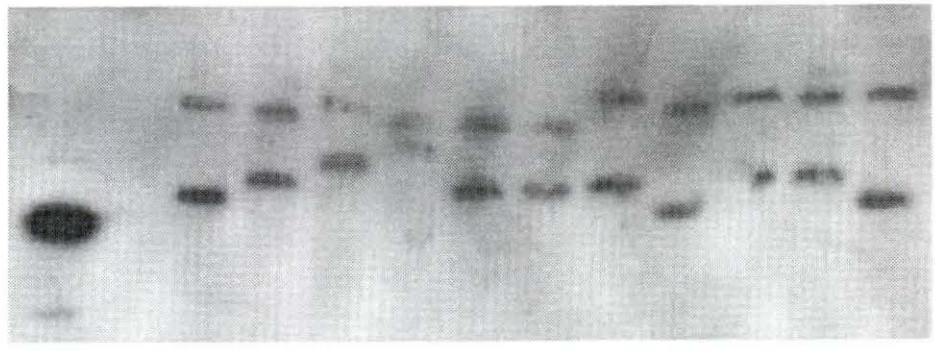


Figure 5). When the two plasmids were radiolabeled with [^{32}P] and used to probe the *Eco*RI-digested wild-type chromosomal DNA, positive bands were observed (data not shown), indicating the recovery of adjacent chromosomal sequences. A preliminary restriction map of pISM2562 is shown in Figure 10. Plasmid pISM2562 contained at least a 700 bp sequence of the chromosomal DNA of strain Ω204.1. The same Southern blotting pattern was observed when only this 700 bp fragment was used as a probe for wild-type chromosomal DNA (data not shown).

Homologous Recombination in *M. gallisepticum*

The construction of a random chromosomal library of *M. gallisepticum* DNA in pISM1025 as described in the Materials and Methods resulted in obtaining 107 recombinant plasmids. Ten plasmids (Table 1) carrying chromosomal DNA ranging in size from 1.7 - 7.9 kb were randomly-chosen to transform back to wild-type *M. gallisepticum*. In order to confirm that homologous sequences existed between these recombinant plasmids and the *M. gallisepticum* chromosome, *Eco*RI fragments from four plasmids (pISM2531, pISM2533, pISM2534 and pISM2540) were isolated from an agarose gel, radiolabeled and each was used to probe wild-type chromosomal DNA digested with *Eco*RI, *Hin*dIII, *Pst*I, or *Xba*I, respectively. The results showed that each plasmid had homologous sequences with the wild-type chromosome (data not shown). When all ten plasmids were used to transform *M. gallisepticum*, only three of them, pISM2531, pISM2535 and pISM2540, produced Tc^r transformants (Table 3). The transformation frequencies with those plasmids were low, but DNA hybridization analysis with the 4.9 kb *tetM* marker and the vector showed the integration of the intact plasmid into the chromosome (data not shown).

Table 3. Transformation frequencies of integrative vectors.

Plasmids	Transformation Frequency[a]
pISM2531	8.0×10^{-11}
pISM2535	9.7×10^{-10}
pISM2540	2.0×10^{-10}
pISM2541	2.0×10^{-10}
pISM2545 (ds)[b]	6.0×10^{-7}
pISM2545 (ss)[c]	1.0×10^{-8}
pISM2546 (ds)	4.2×10^{-7}
pISM2546 (ss)	2.0×10^{-8}
pISM2547 (ds)	8.5×10^{-7}
pISM2547 (ss)	0[d]
pISM2025	3.8×10^{-10}

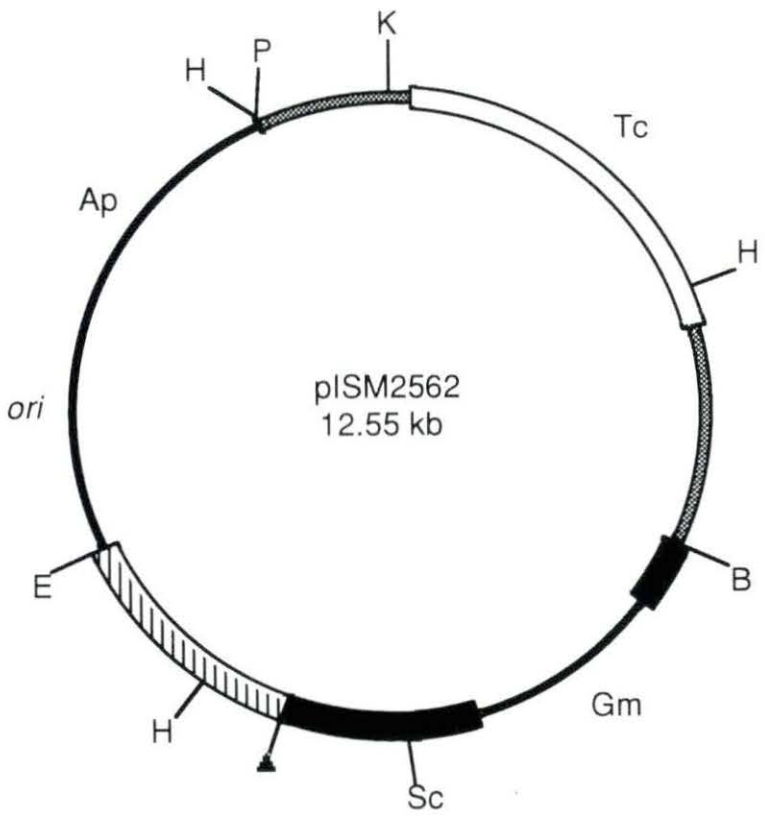
[a] number of transformants per CFU

[b] double-stranded

[c] single-stranded

[d] transformation unsuccessful

Figure 10. Restriction map of the rescued plasmid pISM2562. Plasmid pISM2562 was rescued as described in the Materials and Methods as shown in Figure 5 using *Eco*RI-digested *M. gallisepticum* chromosomal DNA from strain Ω204.1. All of the restriction sites and antibiotic markers in pISM2025 were retained in pISM2562. A 1.2 kb fragment of *M. gallisepticum* chromosomal DNA and an intact IS*256* arm were recovered on the plasmid. Sequence information is needed to resolve the junction site (black triangle) between the IS*256* element and the adjacent chromosomal sequence.


K
H
P
Tc
Ap
H
pISM2562
12.55 kb
ori
E
B
H
Gm
Sc

When the ten plasmids were screened for internal restriction enzyme sites, three plasmids, pISM2531, pISM2533, and pISM2534, contained a single internal *Hin*dIII site in the cloned *Eco*RI fragments to facilitate the insertion of the gentamicin marker. Three correspondent plasmids, pISM2541, pISM2542, and pISM2543, were constructed as described in the Materials and Methods. When these three plasmids were used to transform wild-type *M. gallisepticum* 19610, only pISM2541 produced gentamicin-resistant colonies. Approximately 100 Gm^r colonies were obtained after several transformations with plasmid pISM2541 and studied further. These colonies were first grown in 200 μl non-selective PPLO broth and then subcultured in gentamicin and tetracycline broth media. Seven of the 100 colonies (7%) were found to be tetracycline sensitive. Analysis of several of these strains by DNA-DNA hybridization is shown in Figure 11. All seven gentamicin-resistant colonies (lanes 4-10) and nine tetracycline resistant colonies (lanes 11-19) were examined. Blots from three identical gels were probed with the gentamicin-specific (upper panel), tetracycline-specific (middle panel) or the cloned fragment-specific (lower panel) markers. Despite the loss of tetracycline resistance in the seven colonies, the *tetM* marker was clearly present in all of the pISM2541 transformants indicating only a single crossover event, not a double crossover, had occurred. Also, double bands by the cloned fragment-specific probe indicated that the 3.8 kb target sequence was duplicated, a suggestion that a typical Campbell-like homologous recombination event had occurred.

Because the overall transformation frequency was low in the homologous recombination system, attempts were made to improve the transformation frequency. Plasmid DNAs were amplified in *E. coli* backgrounds other than DH5α because previous studies have shown that DNA amplified in different backgrounds

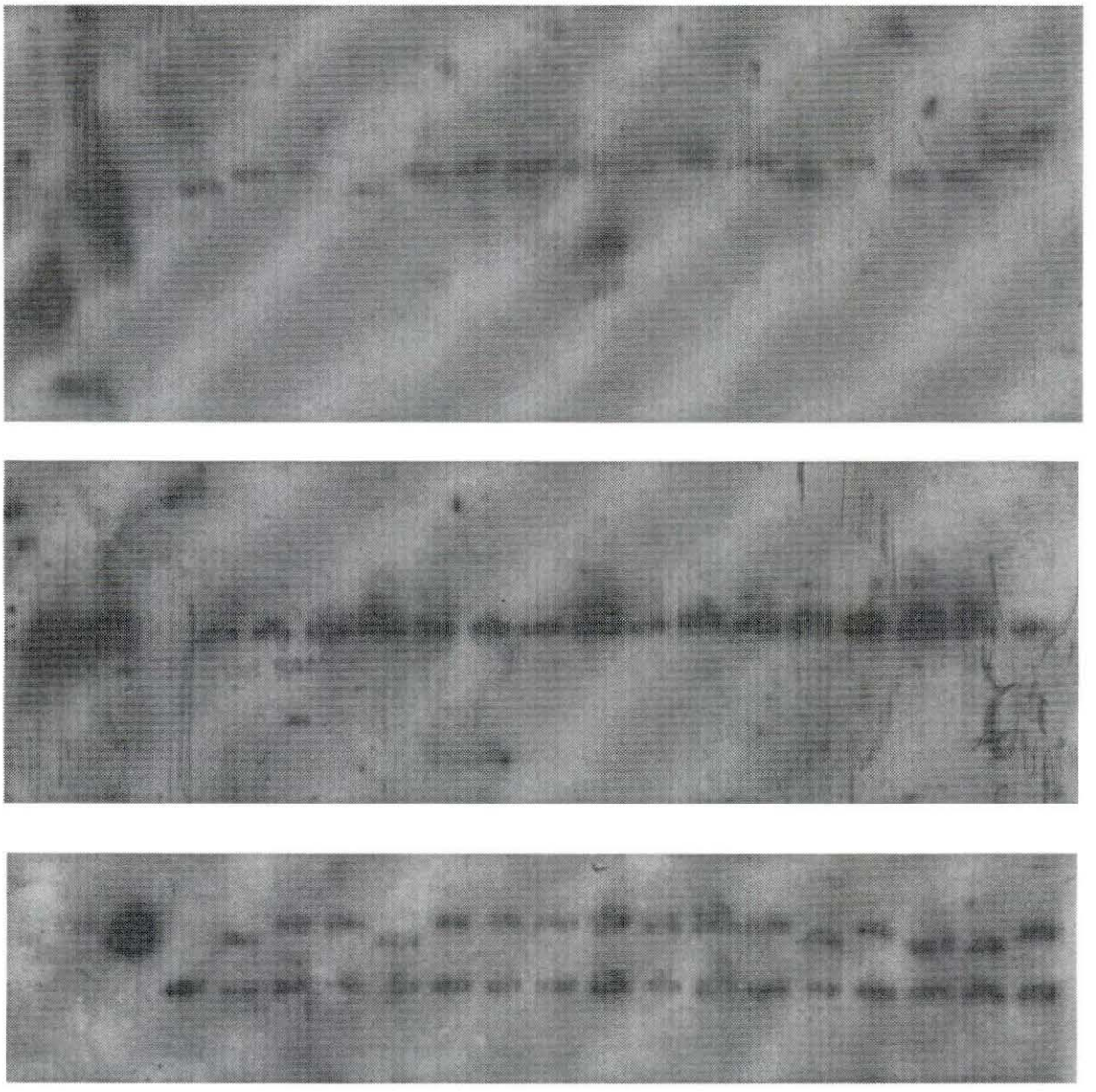
could behave differently due to restriction and modification systems of the host. Plasmid pISM2541 was transformed into *E. coli* strains GM2163, HB101, LE392, and TG1 (Table 1). Plasmid DNAs were then purified from these strains and transformed into wild-type *M. gallisepticum* 19610 using the established procedures. The transformation frequencies of the plasmids from all *E. coli* backgrounds were similar (data not shown). Transformations with *Bam*HI-linearized plasmids pISM2541 and pISM2542 were unsuccessful as well. Also, changes in the growth stage of *M. gallisepticum* cells, $CaCl_2$ treatment, concentration of PEG, quality of the purified DNA (Quigen-purified versus DNA purified by the Birnboim and Doly method), and the antibiotic concentrations in the selective plates were found not to improve transformation frequencies. The vector background was also tested. The three *Eco*RI fragments from pISM2531, pISM2533 and pISM2534 in the pSP64 background were subcloned into pISM1003, a pKS derivative. When these pISM1003 derivatives were used to transform *M. gallisepticum*, the transformation frequency was improved significantly as compared with ssDNA and the pSP64 parent background (Table 3).

Interposon Mutagenesis of *lacZ* Inserts in *M. gallisepticum*

Tn*4001lac* was transformed into *M. gallisepticum* using the PEG protocol and the transformants plated onto gentamicin, Xgal-containing agar as described in the Materials and Methods. Of the fourteen blue strains screened by DNA hybridization, surprisingly, at least eight colonies showed two or more copies of transposon insertion and only four strains clearly gave a single insert of *lacZ* in the chromosome (Figure 12). In addition, instability in *lac* expression resulting in a conversion from blue to white phenotype was observed in all three strains studied. Some of the

Figure 11. DNA-DNA hybridization analysis of putative *M. gallisepticum* interposon mutants. *M. gallisepticum* 19610 was transformed with pISM2541. Gentamicin resistant transformants were scored for tetracycline resistance. The three gels represent DNA-DNA hybridization analysis of sixteen mutants; lanes 4 - 10 were Gm^r Tc^s and lanes 11 - 19 were Gm^r Tc^r. Upper panel: The membrane was hybridized with the 2.45 kb fragment from pISM1003 containing the Gm marker. Lane 1 contained pISM1001 (positive control), lane 2 contained pISM2541 (positive control), and lane 3 contained wild type chromosomal DNA. The 16 samples showed a positive band of 6.3 kb. Middle panel: The membrane was probed with the *tetM* specific marker from pISM1002. Lane 1 contained linear pISM1002 (7.5 kb), lane 2 contained *Eco*RI-digested pISM2541 (a 7.9 kb *Eco*RI band is positive), and lane 3 contained wild type chromosomal DNA. All of the *M. gallisepticum* transformants showed a 7.9 kb positive band. Lower panel: The membrane was probed with the cloned 3.8 kb *Eco*RI *M. gallisepticum* chromosomal DNA fragment from pISM2541. Lane 1 contained pISM1001 (positive control), lane 2 contained pISM2541 (positive control), and lane 3 contained wild type chromosomal DNA. A single 3.8 kb positive band is observed in wild-type chromosomal DNA (lane 3). Two positive bands are shown in each transformant, 3.8 and 6.3 kb in size as predicted (Figure 7). The result indicates a duplication of the target sequence and not a double crossevent.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



revertants were analyzed by DNA-DNA hybridization. As shown in Figure 13, during in vitro passage, some white colonies clearly showed extra copies of *lacZ* in the chromosome. Apparently, intrachromosomal transposition had occured.

When one Lac$^+$ colony from Ω709 was transformed with pISM2514 in an attempt to construct a *lac* interposon mutant, the transformation frequency was about 10^{-8} transformants per CFU. Among the Tc^r colonies obtained, only 5.6% demonstrated the expected white phenotype, a value slightly higher than the spontaneous reversion rate. These results suggested that the integration event was not disrupting *lac* activities as expected. Ten Tc^r colonies were used to study the structure of the Tn*4001lac* insert more fully. *Bam*HI-digested chromosomal DNA were analyzed by DNA-DNA hybridization and the results of this analysis is shown in Figure 14. The chromosomal DNAs were probed with the 3.0 kb *lacZ* fragment (upper panel) and the Tc marker (lower panel). The multiple bands found in the chromosomal DNAs probed with the *lacZ*-specific probe suggested the existence of two copies of Tn*4001lac* in the chromosome.

In Vitro Stability of Transposons and Integrative Plasmids in *M. gallisepticum*

Table 4 shows the results of the in vitro stability studies of Tn*4001*, Tn*916* and integrative plasmids in *M. gallisepticum*. There was no difference in CFUs between the 30th and the 100th passages with any *M. gallisepticum* strain except with K-43 (Tn*4001*). Variation in this strain was inconsistent. At the 30th passage, Ω204 had more colonies on non-selective than on selective media. At the 100th passage, this was reversed. When individual colonies were picked at random at different passages and analyzed by DNA-DNA hybridization, there was no apparent movement of Tn*4001*. Additional bands were sometimes observed (data not shown).

Figure 12. DNA-DNA hybridization analysis of Lac+ *M. gallisepticum* Tn*4001lac* transformants. Chromosomal DNAs from fourteen Lac+ *M. gallisepticum* Tn*4001* transformants were digested with *Pst*I (upper panel), *Xba*I (middle panel) and *Hin*dIII (lower panel) and hybridized with a *lacZ*-specific probe. Lane 15 contained chromosomal DNA from a filter-cloned derivative of strain Ω709shown in lane 14. Most strains showed multiple copies (multiple bands) of *lacZ* except for strains in lanes 1, 4, 11, and 13 which have a single band. An extra band is evident in strain Ω709.1 (lane 15) not found in its parent Ω709 (lane 14).

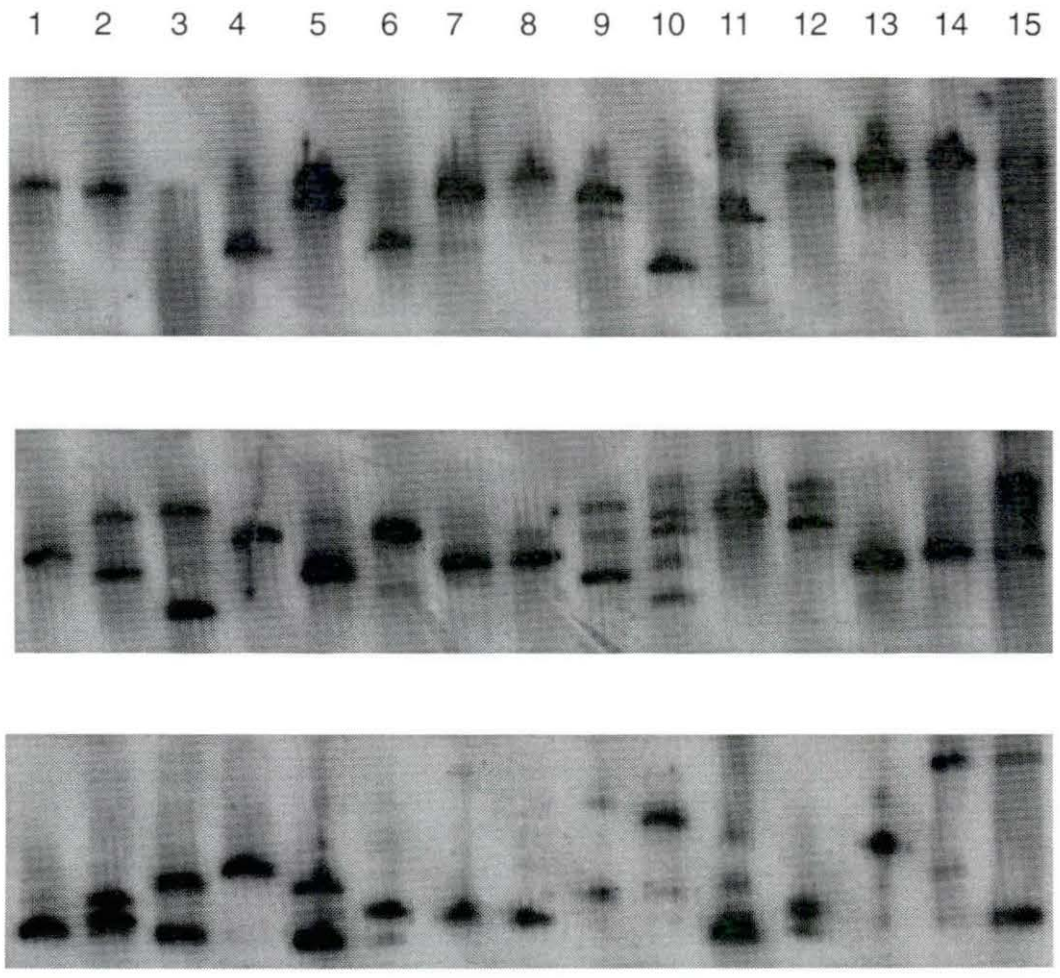
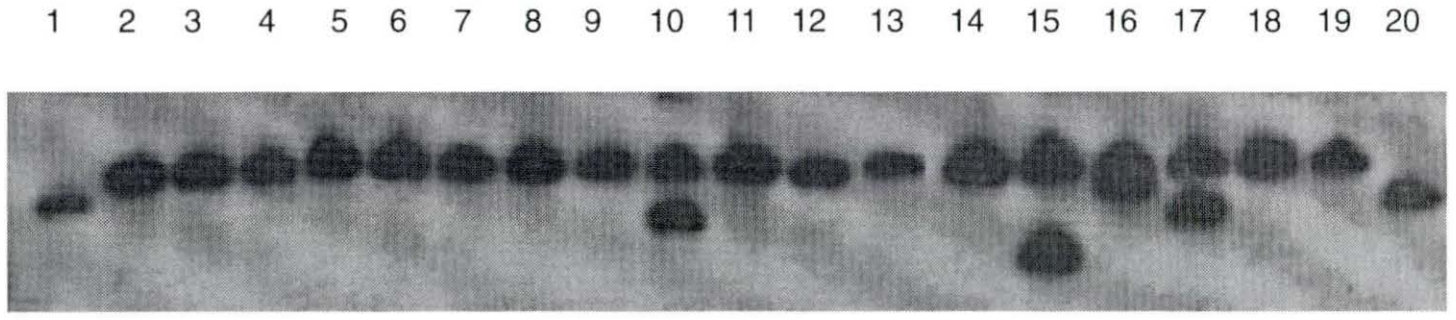

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 13. DNA-DNA hybridization analysis of Lac$^-$ *M. gallisepticum* Ω701 and Ω709 revertants. Cultures of *M. gallisepticum* Lac$^+$ strains Ω701 and Ω709 were filter cloned and plated for single colonies on X-Gal containing media. The upper panel represents the analysis of the Ω701 parent and siblings, and the lower panel represents the analysis of the Ω709 parent and siblings. Both blots were probed a 1.1 kb *SacI*/*ClaI* internal fragment of *lacZ*. In both panels, lane 1 contained the parent chromosomal DNA, and lanes 2 and 3 Lac$^+$ filter-cloned isolates. The remaining lanes contained filtered-cloned Lac$^-$ isolates from the corresponding strain.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

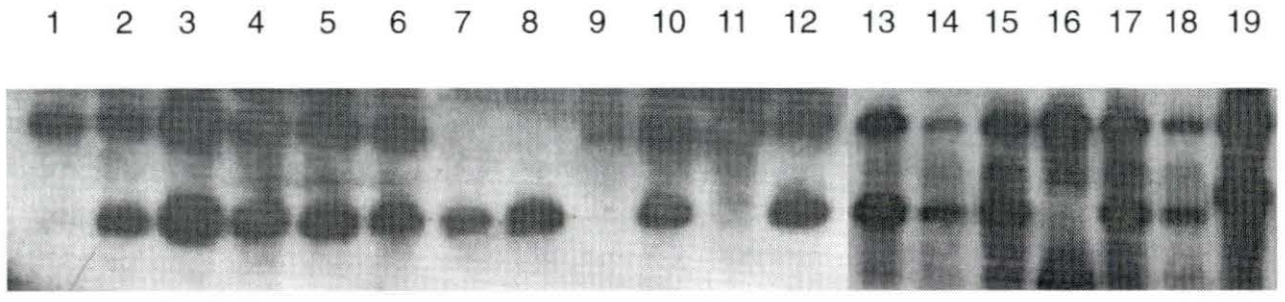

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 14. DNA-DNA hybridization analysis of *M. gallisepticum* strain Ω709.1 pISM2514 transformants. *M. gallisepticum* strain strain Ω709.1, a Lac^+ filter-cloned isolate of strain Ω709, was transformed with pISM2514 in an attempt to construct a Lac^- interposon mutant (Figure 8). Ten Tc^r colonies were analyzed by DNA-DNA hybridization using either a 3 kb DNA fragment of the *lacZ* gene (upper panel) or a 4.9 kb *tetM*-containing DNA fragment (lower panel). Lane 1, pISM1002; lane 2, pISM2514; lane 3, wild-type *M. gallisepticum* DNA; lane 4, Ω709 parent; lane 5, transformant arising from the transformation control mixture with pISM1002; lanes 6-15, pISM2514 transformants.

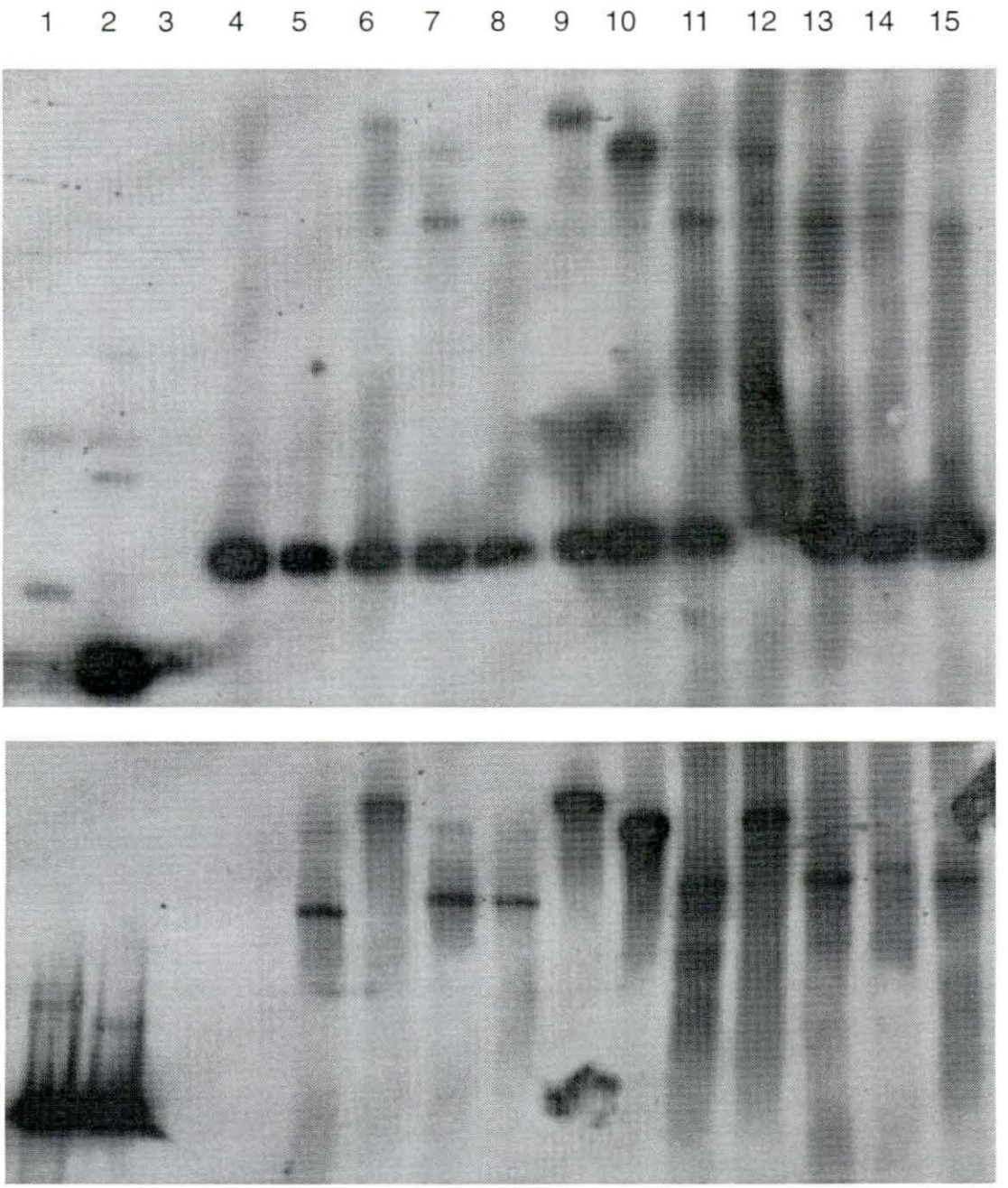

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 4. In vitro stability of recombinant *M. gallisepticum* strains

Strains	non-selection	selection	statistics P[c]
CFUs in 30th passage			
Ω204	382 ± 52	165 ± 35	< 0.05
Ω204.1	33 ± 4	27 ± 6	> 0.05
Ω207	76 ± 13	85 ± 8	> 0.05
Ω600	534 ± 66	529[b]	-
CFUs in 100th passage			
Ω204	287 ± 13	327 ± 9	< 0.05
Ω204.1	110 ± 5	133 ± 21	> 0.05
Ω207	483 ± 63	460 ± 78	> 0.05
Ω600	171 ± 7	191 ± 13	> 0.05

[a] average number of CFUs from three plates

[b] only two plates available: (560 + 497)/2 = 529

[c] values from T table:

P	0.500	0.400	0.200	0.100	0.050	0.025	0.010	0.005	0.001
T	0.741	0.941	1.533	2.132	2.776	3.495	4.604	5.598	8.601

DISCUSSION

Transformation of *M. gallisepticum* was accomplished with Tn*4001*, Tn*916* and with integrative plasmids containing both the gentamicin and tetracycline resistance markers. The control reactions containing no plasmid DNA were generally clean indicating a low background level of spontaneous resistance to the antibiotics. Transformation frequencies were lower than those observed with some other mycoplasmal species (35, 36, 86, 87), especially with the integrative plasmids. Examination of different transformation procedures did not improve the transformation frequencies significantly, and therefore it seems reasonable that the system has been optimized for transformation of *M. gallisepticum* using chemical means (PEG, calcium, etc.). In unpublished transformation studies, electroporation of *M. gallisepticum* with plasmid pISM2065 which also contains Tn*4001* gave a two log higher frequency than did the PEG procedure, 2×10^{-5} as compared to 3.4×10^{-7} transformants per CFU (Tigges and Minion, unpublished observations). Electroporation may provide a means of obtaining more recombinants than PEG, and therefore rare genetic events such as double cross-overs during recombination may be more readily observed. It should be pointed out however, that electroporation may also create chromosomal abberations as a function of the electrical pulse.

Transposition of Tn*4001* and Tn*916* into the *M. gallisepticum* chromosome was a random event, and generally only one copy of the transposon was observed. This is consistent with the results with other mycoplasmas (36, 86). In some instances, there appeared to be an independent transposition of one of the flanking IS*256*

elements as indicated by additional faint bands of low molecular weight in some transformant DNAs analyzed with a Tn*4001*-specific probe. This may suggest an independent movement of IS*256* elements as has been observed in *Staphylococci* (40). Also, this could indicate that in *M. gallisepticum*, the control of Tn*4001* transposition is not as tightly regulated as in other mycoplasmal species. Instability of Tn*4001* has been observed in *Staphylococcus aureus* (P. Pattee, personal communication), but the instability seems to be related to the initial transposition event(s) and not to the loss of the transposon after its stable insertion. Tn*916*, however, did not show evidence of double insertions or instability in *M. gallisepticum*, and therefore may be of more value for future studies of gene analysis in this host. Studies of this type, however, will be seriously hampered by the complexity and size of Tn*916* as compared to the much smaller Tn*4001*.

Both transposons were stable in vitro in *M. gallisepticum* in the absence of selective pressure. This included both the avirulent strain 19610 and the virulent R strain. Neither transposon was lost upon extended cultivation in the absence of selection, and there was also no indication of transposon movement. Therefore, either transposon may be useful for analysis of virulence-related genes in vivo. There may be indications, however, that selective pressures may result in transposon movement. Strains containing Tn*4001lac* did not appear to be as stable as those containing the parent unmodified transposon. This may have been due to the modifications made in the transposon structure or it could have been due to a selective pressure against the expression of β-galactosidase since it is not an essential gene. It should be pointed out that these studies were performed only with Lac+ *M. gallisepticum* strains. It was not possible to further examine this hypothesis

given the limited genetic tools and Tn*4001* derivatives available for use with mycoplasmas.

These studies also clearly indicated that integrative plasmids could be used in *M. gallisepticum*. The reason for the lower frequency of plasmid insertion is not clear. *M. gallisepticum* has been shown to contain membrane nucleases (F. C. Minion, unpublished observations), but the presence of yeast tRNA which serves to inhibit nonspecific nucleases in the transformation mixture had little effect on the transformation frequency. The presence of membrane-associated nucleases may explain the one log decrease in transformation frequency using ssDNA as compared to dsDNA, however (Table 3). Single-stranded DNA is more sensitive to nuclease digestion than is dsDNA; a single nick results in linearization of the plasmid molecule as compared to relaxation with dsDNA. Mycoplasmas have never been transformed with linear DNA (87) (Kapke and Minion, unpublished observations). An inefficient system for homologous recombination may explain the low transformation frequency with integrative plasmids. Previous studies by Ghosh et al. (47) have led to the conclusion that *M. gallisepticum* is inefficient in DNA repair. Obviously, some type of recombination system is present in *M. gallisepticum* to allow for insertion of integrative plasmids, but it may lack some enzymatic activities and the efficiency of other bacterial recombination systems (38, 39, 71, 115). Also, there is indirect evidence of a restriction-modification system in *M. gallisepticum*. Preliminary data have been obtained using a modified Tn*4001* transposon containing rare restriction sites. In some instances, restriction enzyme sites introduced into the chromosome were refractory to enzymatic digestion indicating that methylation or some other modification was occurring at these sites preventing their recognition (Tigges and Minion, unpublished observations). This modification system might interfere with

homologous recombination, but more likely, an associated endonuclease might be recognizing plasmid sequences and cleaving the DNA.

Some chromosomal fragments did not support homologous recombination. This is in contrast to the results with *Acholeplasma* ISM1499 which demonstrated recombination with every cloned chromosomal DNA fragment tested (87, 97). The reasons that *M. gallisepticum* failed to do this are not clear. It is possible that insertion of the plasmid at these sites may have been lethal resulting in the inactivation of an essential gene. In one instance where detailed analysis of the insertion event was monitored, pISM2541(Figure 11), there was a duplication of the homologous sequences, but if the insertion occurred within a gene or an operon, the gene would have been inactivated. This did not appear to be a matter of concern with *Acholeplasma*, however, since all plasmids containing homologous sequences readily integrated into the chromosome (87, 97). There also appeared to be a significant contribution of the plasmid replicon to the transformation frequency. Plasmid pISM2025 (which is a pSP64 derivative) integrated into Tn*4001* inserts at a frequency of 3.8×10^{-10} transformants per CFU, but plasmids derived from pKS gave much higher frequencies of integration (Table 3). The reason for this is not known but it may be related to the conformation of the plasmid, its superhelicity, or whether a large proportion of the plasmid molecules is in a multimeric form. Both parent plasmids are derived from the pUC series of plasmids and would be expected to have similar DNA structure. The alterations done to these plasmids and the large number of passages, however, may have resulted in genetic alterations that could affect transformation under these conditions. Both plasmids were isolated from DH5α to rule out the possibility of host variables.

Attempts were made to construct a *lac* interposon mutant in Tn*4001lac* by identifying double crossover events. These experiments were designed so that a shift in the size of the *lacZ*-containing DNA fragment visulaized by DNA-DNA hybridization would indicate a recombination event as shown in Figure 8. The DNA hybridization patterns using the *lacZ* probe suggested the existence of two copies of Tn*4001lac* in the chromosome. This was an unfortunate choice of parent strains, and prevented the identification of the double crossover because of two inserts of Tn*4001lac* in the chromosome. Unfortunately, this was not apparent in early DNA-DNA hybridization blots when the choice of the Lac+ parent strain was made. The double insertion could have resulted from an intrachromosomal transposition event during in vitro passage in the recipient strain B_1A_{10}-1. Additional support for this explanation is also shown in Figure 12 in which lane 15 (Ω709.1) has an extra faint band that is missing in the parent strain (lane 14, Ω709), and the results shown in Figure 13. It was clear that homologous recombination did occur in one copy of *lacZ* in the chromosome, but the other copy remained intact. It was not possible to determine which copy was actually expressing β-galactosidase and consequently, these experiments failed to demonstrate clearly the feasibility of using interposon mutagenesis to create site-specific mutations in *M. gallisepticum* as originally intended. Studies are in progress to repeat these studies with strains containing single inserts of Tn*4001lac*.

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