1989

Development of molecular genetic approaches to study mycoplasma pathogenesis

Gregory G. Mahairas
Iowa State University

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Development of molecular genetic approaches to study mycoplasma pathogenesis

Mahairas, Gregory G., Ph.D.
Iowa State University, 1989
Development of molecular genetic approaches to study mycoplasma pathogenesis

by

Gregory G. Mahairas

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University
Ames, Iowa
1989
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<tr>
<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ApT</td>
<td>ampicillin resistant</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>G + C</td>
<td>quanine Plus cytosine</td>
</tr>
<tr>
<td>GG</td>
<td>gamma globulin</td>
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<tr>
<td>Gm</td>
<td>gentamicin</td>
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<tr>
<td>GmT</td>
<td>gentamicin resistant</td>
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<tr>
<td>GTP</td>
<td>quanine triphosphate</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>KCl</td>
<td>potassium chloride</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>L</td>
<td>liter</td>
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<tr>
<td>LGT</td>
<td>low gelling temperature</td>
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M     Molar
MCS  multiple cloning site
MgCl₂ magnesium chloride
µg   microgram
min  minute
ml   milliliter
MLV  Mycoplasma Virus
mM   millimolar
MNNG N-methyl-N'-nitro-N-nitrosoguanidine
NaCl sodium chloride
nm   nanometer
nmol nanomolar
PBS  phosphate buffered saline
PEG  polyethylene glycol
pmol picomolar
PPLO pleuropneumonia like organism
psi  pounds per square inch
RNA ribonucleic acid
SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPV  Spiroplasma virus
ssDNA single stranded deoxyribonucleic acid
SVTS2 Spiroplasma virus single stranded
TAE  Tris [hydroxymethyl aminomethane] - acetate - ethylenediamine tetraacetic acid
       buffer
Tc   tetracycline
<table>
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<th>Abbr.</th>
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<tr>
<td>TeRx</td>
<td>tetracycline resistant</td>
</tr>
<tr>
<td>TE</td>
<td>Tris - EDTA buffer</td>
</tr>
<tr>
<td>TNE</td>
<td>Tris [hydroxymethyl aminomethane] - sodium chloride - ethylenediamine tetraacetic acid buffer</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris [hydroxymethyl aminomethane]</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
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EXPLANATION OF DISSERTATION FORMAT

This Dissertation has been prepared in the alternate Dissertation format, and contains three separate manuscripts. Each individual manuscript is complete in itself and includes an Abstract, Introduction, Materials and Methods, Results, Discussion and Literature Cited. The first manuscript describes the introduction and random insertion of Tn4001 into \textit{M. pulmonis} and has been published (Mahairas and Minion, 1989a). The second manuscript which has also been published (Mahairas and Minion, 1989b) deals with the transformation of \textit{M. pulmonis} using integrative plasmids, and describes the optimal conditions for the introduction of exogenous DNA into this mycoplasma. The third manuscript discusses the genetic exchange of transposon and integrated plasmid markers in \textit{M. pulmonis} and has been submitted for publication in the Journal of Bacteriology. Cao Jian is listed as a secondary author on the third manuscript an contributed by providing marked strains of \textit{M. gallisepticum} and \textit{A. laidlawii}. An appendix describing the isolation and purification of \textit{M. pulmonis} DNA-dependent RNA polymerase is also included and follows the discussion and summary portions of this work. In addition, a current review of the literature relevant to this thesis is included as well as a General Discussion and Summary. Literature citations contained within the Literature Review and Appendix are listed in Additional Literature Cited.
LITERATURE REVIEW

Phylogeny

The division Tenericutes, class *Mollicutes* consists of the families *Acholeplasmataceae* (comprised of 1 genus with 10 established species), *Spiroplasmataceae* (comprised of 1 genus with 4 established species), and *Mycoplasmataceae* (comprised of 2 genera, *Mycoplasma* and *Ureaplasma*, with over 70 established species). The *Mollicutes*, a diverse group of organisms characterized by the absence of a cell wall, are thought to have arisen through degenerative evolution of the low G + C portion of the Gram positive phylogenetic tree (Woese, 1985). The closest eubacterial ancestors to the *Mollicutes* based on 5S and 16S rRNA comparisons are *Clostridium innocuum* and *C. ramosum*. Successive genome reductions supposedly gave rise to the non-sterol requiring *Acholeplasma*, the sterol-requiring *Spiroplasma* (*Spiroplasma* supposedly arose from the *Acholeplasma* without a genome reduction) and finally the sterol-requiring *Mycoplasma* and *Ureaplasma* (Rogers et al., 1985; Woese, 1985).

Additional evidence for the phylogenetic relationships within the *Mollicutes* was provided by studies of the DNA polymerases and RNA polymerases. Studies of the DNA polymerase activities of different members of the *Mollicutes* have found that *M. mycoides* and *U. urealyticum* possessed a single enzyme in contrast with *A. laidlawii* and *S. citri* which possessed three different DNA polymerases (Maurel et al., 1989). An immunological relationship existed between the *M. mycoides* and *U. urealyticum* enzymes, but this relationship did not extend to the *Acholeplasma* or *Spiroplasma* enzymes. The activity of the *M. mycoides* and *U. urealyticum* enzymes were similar, both having 3'-5' exonuclease activity.

Rifampin sensitivity, a general property of eubacterial DNA-dependent RNA polymerases (Sippel and Hartman, 1968), also provided supportive evidence of the
phylogenetic relationships. Rifampin acts on the \( \beta \) subunit of the RNA polymerase holoenzyme to inhibit the initiation step of transcription (Rabussay and Zillig, 1969). Gadeau et al. (1986) show that the growth of five representative \textit{Mollicutes} was insensitive to rifampin in contrast to \textit{E. coli} which was fully inhibited. Additional evidence was provided by \textit{in vitro} experiments that showed that \textit{S. apis} and \textit{S. melliferim} enzymes were rifampin resistant even though their subunit structure strongly resembled that of \textit{E. coli} (Gadeau et al., 1986). Interestingly, the RNA polymerase of \textit{A. laidlawii} showed a marked sensitivity to rifampin as compared with the \textit{Spiroplasma} enzymes but there was a greater degree of resistance than with the \textit{E. coli} enzyme (Gadeau et al., 1986). Therefore it is believed that the sensitivity to rifampin was lost during the splitting of the \textit{Acholeplasma} branch of the phylogenetic tree.

The Mycoplasma Genome

The genomes of organisms in the class \textit{Mollicutes} are characterized by their low G + C ratio and small size (750-1500 kilobases). \textit{Acholeplasma} have the largest and most complex genomes of the class with the size of the genome estimated at 1500 kilobases (kb). The \textit{Spiroplasma} genome arose from the \textit{Acholeplasma} branch without a genome reduction and consequently the size of these genomes is also approximately 1500 kb. The \textit{Mycoplasma} and \textit{Ureaplasma} arose from the \textit{Spiroplasmas} through a genomic reduction to yield two genera with genomes on the order of 750-1000 kb. This is the smallest genomic content known in free living organisms.

The coding capacity of these small genomes is about 600-750 genes assuming an average protein of 30,000 daltons encoded by 1.0 kb of DNA. However, two dimensional gel electrophoresis of radiolabeled proteins from \textit{M. capricolum} identified only about 350 proteins as compared to \textit{E. coli} with 1,100 proteins indicating that the coding capacity of mycoplasma genomes may be less than predicted (Muto, 1987). The \textit{M. capricolum} genome was estimated to contain about 40 tRNA genes as well as three rRNA species, 16S, 23S and 5S (Sawada et
al., 1981), but Andachi et al. (1989) sequenced the complete set of tRNAs and found only 29
tRNA species. The *M. capricolum* genome also contained two sets of rRNA genes compared
to the ten sets of *Bacillus subtilis* (Widom et al., 1988).

All species of the *Mollicutes* have a relatively low G + C content although a large
variation exists (Razin and Freundt, 1984). The G + C content of all species of *Ureaplasm* was
reported to be about 28% while that of most organisms in the genus *Mycoplasma* was
between 24-30% (Razin and Freundt, 1984). It should be noted that the G + C content of *M.
gallisepticum* was 32% while that of *M. pneumoniae* was about 38% (Razin and Freundt,
1984). These organisms are the prototypes of a small group of higher G + C mycoplasmas
(Razin and Freundt, 1984). Determinations of G + C content have been made by thermal
renaturation kinetics in the past but are now analyzed by High Performance
Liquid Chromatography (HPLC) with the added advantage of being able to identify modified
bases (Razin and Razin, 1980).

Methylated bases in the *Mollicutes* were first recognized by Razin and Razin (1980)
using HPLC. The DNAs of *Acholeplasma* and several *Mycoplasma* spp. contained m^6Ade,
and *M. hyorhinis* contained m^5Cyt (Razin and Razin, 1980 ). Four of five *Spiroplasma* strains
have as their sole methylated base m^5Cyt (Nur et al., 1985). Chan and Ross (1984) were able
to differentiate *M. hyopneumoniae* from *M. flocculare* by digestion of DNA using the
restriction endonucleases *MboI* and *DpnI*. *MboI* digests both methylated and unmethylated
DNA while *DpnI* digests only methylated DNA. Methylation patterns of DNA serve as
recognition factors for the cellular machinery functioning in DNA replication (Sugimoto et al.,
1979) and restriction (Meselson and Yaun, 1968). Only two restriction systems have been
identified in the *Mollicutes*, the *ScN1* system of *S. citri* and the *MfeI* system of *M. fermentans*
(Halden et al., 1989). *ScN1* is an isoschizomer of *HhaI* that recognizes the sequence 5'-
GCGC-3' (Stephens, 1982) cleaving between the first G and C generating a two base 5'
overhang. *Mfe1* is the only known isoschizomer of *EcoR1* and represents only the second restriction enzyme isolated from the *Mollicutes*.

**Metabolism**

Mycoplasmas require complex media for growth. Defined media for *M. capricolum* and *M. mycoides* require about 20 amino acids, purine and pyrimidine bases, five to six vitamins, cholesterol and fatty acids to support growth (Rodwell, 1983). These complex nutritional requirements are suggestive that many genes for enzymes in metabolic pathways are absent. Muto (1987) postulated that a complex membrane system exists for the transportation of these macromolecular precursors. Mycoplasmas generate energy either by the fermentation of glucose, utilization of arginine or the hydrolysis of urea. Enzymes from the Embden-Meyerhof and pentose pathways were present in all species examined but enzymes of the tricarboxylic acid cycle were absent (Cocks et al., 1985).

A system to generate energy from ATP is also required for autonomous growth. Schiefer and Schummer (1982) examined several mycoplasmas and found that energy is generated by a membrane bound proton-translocating adenosine triphosphatase that hydrolyzes ATP formed in glycolysis leading to proton extrusion. Additionally, Zilberstein et al. (1986) showed that the F1F0 ATPase of several mycoplasmas were immunologically crossreactive with that of *E. coli* in the active site region but differed in that the mycoplasma enzymes had an altered structure and were covalently anchored to the membrane. About 270 genes in the *E. coli* genome encode components of the protein and nucleic acid synthesizing machinery, including RNA and DNA polymerases, ribosomal proteins, tRNAs and additional transcription and translation factors (Muto, 1987). Thus it is thought that in the mycoplasmas, a major portion of the coding regions is devoted to these essential functions as well as the generation of energy making the mycoplasmas the minimal information coding for a living organism.
Although it appears that organisms in the class *Mollicutes* would have much in common, the DNA homology among species within the class is only about 5% (Christiansen, 1987).

**DNA Repair and Recombination**

The mycoplasmas are exceedingly sensitive to ultraviolet (UV) and gamma radiation (Razin, 1985). Survival curves versus UV exposure typically show a shoulder near the origin followed by logarithmic killing (Aoki et al., 1979; Labarere and Barroso, 1984; Maniloff and Morowitz, 1972). Dark repair and photoreactivation have been demonstrated in *A. laidlawii* (Das et al., 1972). Agents that inhibit excision repair such as acriflavin abolish dark repair in UV-irradiated *A. laidlawii* (Ghosh et al., 1978). It should be noted that Aoki et al. (1979) were unable to detect a dark repair system in *M. buccale* and Ghosh et al. (1977) failed to observe both photoreactivation and dark repair in *M. gallisepticum*. This last point raises questions about how an organism that appears to lack these critical repair systems is able to maintain fidelity in the replication of its genome. It has been suggested that due to the lack of DNA repair systems that *M. gallisepticum* may accumulate base changes more frequently; this supports the theory of rapid evolution in the *Mollicutes* (Woese et al., 1980; Woese et al., 1985).

Alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) have been used to produce temperature sensitive mutants of *M. gallisepticum* and *M. pneumoniae* (Lam et al., 1981; Steinberg et al., 1969), hemadsorption-negative mutants of *M. pneumoniae* (Hansen et al., 1981), and lysis resistant mutants of *A. laidlawii* (Dahl et al., 1979). The frequency of induced mutations in the *Mollicutes* using MNNG were on the same order of magnitude as in other procaryotes (Razin, 1985).

**Transfection, Transformation and Recombination**

Transfection in the *Mollicutes* was first demonstrated by Liss and Maniloff (1972) who infected lawns of *A. laidlawii* with DNA isolated from two viral strains of the MLV1 virus.
group. The uptake of DNA in this system was shown to be growth phase dependent and consisted of simple coincubation of the cells with DNA. Transfection frequencies were low and the method was not useful with all viral DNA (Liss and Maniloff, 1972; Sladek and Maniloff, 1983). A transfection system to infect *A. laidlawii* with MLV5 DNA using polyethylene glycol was developed by Sladek and Maniloff (1983). The frequency of transfection was $1 \times 10^{-4}$ transfectants per CFU using both single stranded and double stranded DNA forms of the virus (Sladek and Maniloff, 1985). Sladek and Maniloff (1983) were unable to transfect *A. laidlawii* using MLV3 viral DNA and suggested that the large size of this molecule may have precluded its entry into the cell. The polyethylene glycol method has also been used to transfect *Spiroplasma* strains with SPV4 DNA (Renaudin et al., 1984).

Transformation of *M. salivarium* and *M. hominis* using chromosomal DNA from a tetracycline resistant clinical isolate of *M. hominis* was reported by Furness and Cerone (1979). This method consisted of the incubation of mycoplasma cells in cold calcium chloride prior to adding DNA and subsequent heat shock and is based on a procedure for transforming *Salmonella typhimurium* (Lederberg and Cohen, 1974). Transformation frequencies were $1 \times 10^{-7}$ per CFU and uptake of DNA was abolished by the addition of DNase and competing nucleic acids (Furness and Cerone, 1979).

Tn916 was introduced into *A. laidlawii*, *M. pulmonis* (Dybvig and Cassell, 1987) and *M. hyorhinis* (Dybvig and Alderete, 1988) by polyethylene glycol-mediated transformation. DNA hybridization studies confirmed the presence of Tn916 and demonstrated random insertion of the transposon as well as the presence of cointegrates between pAM120 and the mycoplasma chromosome (Dybvig and Alderete, 1988). These protocols consisted of suspending mycoplasma cells in phosphate buffer followed by the addition of polyethylene glycol. The frequency of introduction of Tn916 ranged from $1 \times 10^{-6}$ PER CFU for *M. pulmonis* to $1 \times 10^{-8}$ per CFU in *M. hyorhinis*. Dybvig (1989) reported the transformation of
A. laidlawii by the polyethylene glycol method using the streptococcal plasmids pVA868 and pVA920 which specified tetracycline and erythromycin resistance, respectively. In A. laidlawii, large portions of these plasmids were deleted and the deleted derivatives were not stably maintained (Dybvig, 1989).

McCammon et al. (1989) used electroporation to transfect S. citri with SVTS2 viral DNA. The frequency of transfection was $1 \times 10^{-4}$ transfectants per CFU and the authors noted that these frequencies were comparable to using the polyethylene glycol method of Sladek and Maniloff (1985). Lorenz et al. (1988) also used electroporation to transfect Acholeplasma laidlawii. Interestingly, McCammon et al. (1989) observed differences in transfection frequencies when cloned viral DNA was purified from different E. coli cloning hosts. These differences in transfection were found to be due to different patterns of adenine methylation found among some E. coli strains (McCammon et al., 1989).

The Expression of Cloned Genes and Codon Usage

Thus far only a few mycoplasma genes encoding characterized proteins are known. These genes were identified by extensive analysis of clones in mini- or Maxi-cells, screening for homologous sequences from other bacteria and the detection of mycoplasmal antigens in E. coli. Mouches et al. (1985) reported the cloning and expression in E. coli of the spiralin major membrane protein of S. citri. In E. coli the spiralin protein was produced and transcription was initiated from its native promoter. Full or nearly full sized proteins of M. hyopneumoniae were occasionally detected in E. coli when fragments of chromosomal DNA were cloned into λgt11, a bacteriophage expression system, but this was not observed when those fragments were subcloned into the plasmid vector pBR322 (Klinkert et al., 1985). Kawauchi et al. (1984) reported the cloning and expression of ribosomal proteins of M. capricolum from their native promoters. Thus it appears that the transcription/translation apparatus of E. coli is capable of expressing some mycoplasmal genes.
Clearly not all mycoplasmal genes express in *E. coli* and a major obstacle in using traditional cloning hosts is the alternate codon usage in the *Mollicutes*. Unconventional codon usage has been reported for *M. mycoides* (Guindy et al., 1989), *M. capricolum* (Yamao et al., 1988) and *M. pneumoniae* (Schaper et al., 1987; Inamine et al., 1988); most notable is the use by mycoplasmas of the universal stop codon UGA as a tryptophan (Yamao et al., 1988). It should be noted that some of the genes previously expressed in *E. coli* such as spiralin do not contain tryptophan (J. Bove, University of Bordeaux, Bordeaux, France. personal communication). The sequences of the complete set of tRNA species in *M. capricolum* have been reported (Andachi et al., 1989). According to Andachi et al. (1989) tRNA anticodons in *M. capricolum* have several unique features: (1) Only one tRNA species exists for alanine, glycine, leucine, proline, serine and valine, and these exist as 4-codon boxes; (2) $tRNA^{thr}$ has anticodons UGU and AGU with the U residue unmodified; (3) Arginine has only one anticodon, ICG; and (4) UGA is read as a tryptophan. Thus it appears that unusual codon usage is a general feature of mycoplasmas.

**Transposons Tn916 and Tn4001**

First reported in *Enterococcus faecalis* (Franke and Clewell, 1981), Tn916 is a 16.4 kb transposon that encodes tetracycline resistance. Tn916 transposes at a frequency of $1 \times 10^{-5}$ to $1 \times 10^{-6}$ per CFU and is the prototype of a unique class of transposons that have conjugational properties. This allows their transfer from a nonconjugative plasmid or chromosomal location to a plasmidless recipient at a frequency of $1 \times 10^{-8}$ per CFU (Franke and Clewell, 1981; Gawron-Burke and Clewell, 1982a & 1982b). The results of restriction endonuclease analyses of plasmids that have lost Tn916 are identical to those of the plasmids prior to the acquisition of the transposon. Additionally, Nida and Cleary (1983) report the restoration of streptolysin S activity with the concomitant loss of tetracycline resistance in *Streptococcus pyogenes* carrying a streptolysin S gene that had been insertionally inactivated by Tn916. Taken together these
data indicate that Tn916 undergoes precise excision (Gawron-Burke and Clewell, 1984). Tn916 has been introduced into *A. laidlawii* and *M. pulmonis* by polyethylene glycol-induced transformation with pAM120 (Dybvig and Cassell, 1987) and found to transpose to diverse chromosomal sites (Dybvig and Cassell, 1987). Also Tn916 was found to conjugally transpose from both plasmid and chromosomal locations in *E. faecalis* to diverse chromosomal locations in *M. hominis* (Roberts and Kenny, 1987). This last observation may explain the presence of naturally occurring tetM (Tn916) sequences in *M. hominis* and *U. urealyticum* (Roberts et al., 1985).

**Tn4001** is a 4.7 kb transposon that confers resistance to the aminoglycoside antibiotics gentamicin, tobramycin, and kanamycin, and was originally isolated in an outbreak of multiply resistant *Staphylococcus aureus* in Australia (Lyon et al., 1984). Lyon et al. (1984) used heteroduplex analysis to show that Tn4001 consists of a 2.0 kb unique central region between inverted repeats of 1.35 kb (IS256), a configuration suggestive of a composite class I transposon (Kleckner, 1981). Lyon et al. (1984) also demonstrated the transposition of Tn4001 in a RecA- mutant of *S. aureus*. The 2.0 kb central region has been shown to encode a 56.9 kDa protein that has both aminoglycoside 2' phosphotransferase and aminoglycoside 6' acetyltransferase activities in separate domains and transcription is initiated from a promoter within IS256R (Lyon and Skurray, 1987; Byrne et al., 1989). Additionally, Mahairas et al. (1989) demonstrated that Tn4001 was capable of occupying numerous and diverse chromosomal sites in *S. aureus*. The nucleotide sequence of Tn4001 has been reported (Byrne et al., 1989; Rouch et al., 1987).

**Genetic Exchange in the Mollicutes**

Labarere and Barroso (1984) reported exchange between stains of *S. citri* using UV-induced markers for resistance to xylitol and arsenate, and arsenate and vanadium oxide (Barroso and Labarere, 1988). Doubly resistant mutants arose at frequencies of $1 \times 10^{-4}$ when
parent strains were mixed. This exchange of genetic markers was not affected by the addition of DNase or RNase but was enhanced by the addition of polyethylene glycol or prolonged coincubation of the parents (Barroso and Labarere, 1988). The possibility that a virus was responsible for genetic exchange was ruled out by U-tube experiments and by the absence of enhancement of exchange by the addition of divalent cations. Exchange occurred following mixing of parent strains, incubation at 32°C for one hour, and subsequent plating on media containing both toxic substances (Barroso and Labarere, 1988). Colonies of cells resistant to both arsenate and xylitol arose at frequencies of $5 \times 10^{-5}$ per CFU (Labarere and Barroso, 1984). The addition of polyethylene glycol, DNase or RNase had no effect on the frequency of transfer, and transfer was thought to involve direct contact possibly through the fusion of cell membranes. Gourlay et al. (1984) reported the recovery of doubly resistant colonies of \textit{A. laidlawii} by the coincubation of neomycin resistant and chloramphenicol resistant cells in the presence of polyethylene glycol. Colonies arose at a frequency of $5 \times 10^{-6}$ per CFU on doubly selective media. Additionally, Lam and Lin (1984) suggested that recombination events following membrane fusion occurred with \textit{M. gallisepticum}.

\section*{Genetic Approaches to Studying Mycoplasma Diseases}

The first mycoplasma was isolated from cattle with contagious bovine pleuropneumonia (Nocard and Roux, 1898). It has also been recognized that many members of the \textit{Mollicutes} are parasites living in plants and animals including arthropods and vertebrates. Of the approximately ninety recognized species in the class \textit{Mollicutes}, over one-third are pathogens of man, birds, insects and plants. Freundt (1958) classified mycoplasmas virulent for man and animals into three categories 1) those associated with respiratory tract infections, i. e., \textit{M. pulmonis} and \textit{M. pneumoniae}; 2) those primarily infecting the urogenital tract, i. e., \textit{M. hominis} and \textit{Ureaplasma} spp.; and 3) those that have an affinity for tissues comprising the joints, i. e. \textit{M. arthritidis}. Mycoplasmas that cause respiratory tract diseases and are often
considered pathogens can be found in healthy hosts (Cassell et al., 1985). Because of this opportunism and disease complexity, little is known of the mechanisms by which mycoplasmas cause respiratory disease. Additionally, incubation periods are quite long and the isolation of the insulting mycoplasma is often difficult (Chanock et al., 1961).

Animal mycoplasmas most often associate with epithelial surfaces which are constantly being shed. As a result some pathogenic mycoplasmas have evolved a specialized attachment organelle (Gabridge et al., 1985). The specialized attachment proteins of *M. pneumonias* and *M. gallisepticum* have been studied extensively and the major cytadhesin gene of *M. pneumonias*, P1, has been cloned and sequenced (Su et al., 1988; Inamine et al., 1988). The pathogenic mechanisms of mycoplasmas remain a paradox because with one exception, no extracellular products or toxins have been identified (Gabridge et al., 1985). Instead, mycoplasmas appear to induce metabolic and morphological changes in host cells through subtle chemical processes (Gabridge et al., 1985). The possibility that pathogenicity may be linked to surface enzymes, particularly proteases, has led to the study of proteolytic activities of mycoplasmas. Freundt (1958) described the liquefaction of gelatin by cultures of *M. mycoides* and *A. laidlawii*. Additionally, the liquefaction of heat denatured or clotted serum has been reported by *M. arthritidis* (Aluotto et al., 1970), *M. bovirhinis* (Aluotto et al., 1970), *M. capricolum* and *M. mycoides* (Tully, 1974). In *M. pulmonis*, additional surface protein-associated activities have been identified including an exonuclease (Minion and Goguen, 1985), a hemagglutinin (Minion et al., 1984), and a hemolysin (Minion and Goguen, 1985; Minion, 1987; Jarvill and Minion, 1989).

The goals of this study were to develop molecular genetic approaches and tools that could be applied to the study of the pathogenic attributes of mycoplasmas. The specific objectives were twofold. The first objective was to develop methods to create discrete mutations in mycoplasmal chromosomal genes. This would allow an initial assessment of a
particular attribute as a potential virulence factor. The second objective was to develop additional genetic techniques to allow genetic complementation and recombinant DNA manipulations in mycoplasmas once mutants to specific activities were identified. This would provide the conclusive evidence of virulence linkage to a particular mycoplasmal activity or characteristic.
SECTION I. RANDOM INSERTION OF THE GENTAMICIN RESISTANCE TRANSPOSON TN4001 IN MYCOPLASMA PULMONIS
Random Insertion of the Gentamicin Resistance Transposon Tn4001 in
*Mycoplasma pulmonis*

by

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ABSTRACT

The staphylococcal transposon Tn4001 was introduced into *Mycoplasma pulmonis* using an *Escherichia coli* derived vector by polyethylene glycol-mediated transformation. Using a reaction mixture containing 10 μg plasmid DNA, 10 μg yeast tRNA, and 34-35% polyethylene glycol per 1 x 10^8 cells, Tn4001 could be introduced into *M. pulmonis* at a frequency of 5 x 10^-5 per colony forming unit. DNA-DNA hybridization studies illustrated that Tn4001 could occupy a diversity of insertion sites in the *M. pulmonis* chromosome. These data indicated that Tn4001 is a potentially useful tool for the introduction of mutations and for genetic studies in *M. pulmonis*. 
INTRODUCTION

Transposable genetic elements are valuable resources for studying microbial pathogenesis at the molecular level through mutational analysis of potential virulence determinants (Salch and Shaw, 1988), studies of genetic control elements in vivo (Casadaban and Cohen, 1979), gene mapping (Kleckner, 1981), and DNA sequencing (Nag et al., 1988). Until recently it has not been possible to use mobile genetic elements to study the Mollicutes. Consequently progress on studies of the organization and regulation of mycoplasma genes, and the identification and characterization of virulence determinants has been slow.

Since no naturally occurring transposons have been identified in the Mollicutes, they must be obtained from other bacterial sources. Progress in this area has been hindered by the lack of selectable markers and functional mobile elements such as insertion sequences. Recently, Dybvig and Cassell (1987) introduced the Enterococcus faecalis transposon Tn916 into Mycoplasma pulmonis and Acholeplasma laidlawii by PEG-mediated transformation. Roberts et al. (1985) reported the natural occurrence of tetM sequences in Mycoplasma hominis as well as the conjugal transfer of Tn916 from E. faecalis to M. hominis (Roberts and Kenny, 1987). Although the observation of transpositional activity of Tn916 in mycoplasmas is a welcome tool in mycoplasma genetics, its large size and complexity (Senghas et al., 1988) has made it a less useful genetic tool than Tn917 (Weaver and Clewell, 1987) or Tn5 (Krebs and Reznikoff, 1988; Nag et al., 1988).

In order to develop additional tools for the study of mycoplasmal genetics, we examined the properties of Tn4001 when transformed into M. pulmonis. Tn4001 is a small (4.7 kb) composite class I transposable element originally isolated from Staphylococcus aureus (Lyon et al., 1984). It encodes a bifunctional peptide specifying both kanamycin and gentamicin resistance in separate domains which is flanked by a 1.3 kb insertion element,
IS256 (Lyon and Skurray, 1987). The compact size and phenotypic characteristics of Tn4001 would allow more precise analysis of gene composition and function in mycoplasmas than is now possible with Tn916.
MATERIALS AND METHODS

*E. coli* strain DH5α (φ80dlacZΔM15 endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1
F- Δ[lacZYA-argF]U169) was used throughout this study. *M. pulmonis* UAB6510 (Minion
et al., 1984) was grown and maintained in PPLO broth (Difco Laboratories, Detroit, Mich.)
supplemented with 10% agamma horse serum (GIBCO Laboratories, Grand Island, N.Y.),
2.5% fresh yeast extract (Flow laboratories, Inc., McLean, Va.), 0.5% glucose and 25 µg/ml
Cefobid (Pfizer, Inc., New York, N.Y.). *M. pulmonis* transformants were selected on PPLO
medium containing 12 g/L Noble agar (Difco) and gentamicin sulfate (10 µg/ml). *E. coli*
and *S. aureus* were grown to saturation density in tryptose (32 g/L tryptose - 20 g/L yeast extract
- 5 g/L NaCl) or GL (3 g/L yeast extract - 3 g/L casamino acids - 5.9 g/L NaCl - 3.3 ml/L 60%
sodium lactate - and 1 ml/L glycerol) broth, respectively.

Plasmid DNAs were prepared by the method of Birnboim and Doly (1979) except that
*S. aureus* SK2205 cells were treated with lysostaphin (1 µg/ml, Sigma Chemical Co., St.
Louis, Mo.) instead of lysozyme. Plasmids were purified on cesium chloride - ethidium
bromide density gradients (Radloff et al., 1967). All DNA manipulations were as described
(Maniatis et al., 1982). *E. coli* DH5α transformants were selected on Luria agar containing
100 µg/ml of ampicillin and the recombinant plasmids screened by the method of Kado and

Tn4001 was introduced into *M. pulmonis* by a modification of the PEG-mediated
transformation system of Sladek and Maniloff (1985). Cells from an overnight culture of *M.
pulmonis* were harvested by centrifugation (8000 x g, 15 min), washed once in PBS (pH
7.4), and resuspended in one half the original volume of ice cold 100 mM CaCl2. After
incubation on ice for 30 min, 250 µl of cells were mixed with 10 µg yeast tRNA (Bethesda
Research Laboratories, Gaithersburg, Md.) and 10 µg of pISM1001, and the volume adjusted
to 300 μl with 100 mM CaCl₂. The addition of yeast tRNA was required for transformation when purified plasmid DNA was used in the transformation mixtures. Two ml of 40% PEG 8000 (Sigma) made up in 0.1 M Tris (Sigma) - 0.5 M sucrose buffer (pH 6.5) was then added. The mixture was incubated for 2 min at room temperature, diluted with 10 ml of PBS, inverted several times, and centrifuged at room temperature (8000 x g, 15 min). The resulting pellet was resuspended in 1 ml of growth media and incubated at 37°C for 1 h prior to plating on gentamicin containing PPLO agar.

Individual mycoplasma colonies were picked from selective agar medium using Pasteur pipettes, dispensed into broth containing 10 μg/ml gentamicin, and grown to mid to late log phase. Cultures were harvested by centrifugation, and DNAs were isolated by the following procedure. Mycoplasma pellets from 1.5 ml of fully grown culture were resuspended in 50μl of TNE (0.01 M Tris - 0.15 M NaCl - 0.001 M EDTA, pH 8.0) containing 100 μg Proteinase K (Sigma) in a microfuge tube. The tube was equilibrated to 37°C and then 50 μl of 2% low temperature melting agarose (Seaplaque, FMC Corp., Rockland, Ma.) in TNE (equilibrated to 48°C) was added and the contents thoroughly mixed. The tubes were placed on ice to harden the agarose, the plug was transferred to a 2 ml screw cap microfuge tube (Sardstadt, Princeton, N.J.) and 100 μl of 2% Tween 20 (Sigma) in TE (0.01 M Tris - 0.001 M EDTA, pH 8.0) was added. Following an overnight proteolytic digestion at 37°C, the plug was dialyzed against 1 ml volumes of TE (4°C) changed every hour for four hours. Restriction enzyme digests consisted of 5 μl DNA and 2 U of EcoR1 (BRL) in a total reaction volume of 20 μl. Fragments were resolved on a 0.55% agarose gel in Tris acetate buffer (Maniatis et al., 1982), transferred to GeneScreen (New England Nuclear Research products, Boston, Mass) by the method of Southern (Southern, 1982) and probed with pISM1003 or pBR322 labeled with ³²P-dCTP using multiprime labeling (Amersham Corp., Arlington Heights, Ill). Labeling efficiencies normally were about 1 x 10⁹
cpm per µg DNA. Hybridization was performed according to Maniatis et al. (1982). After drying, blots were subjected to autoradiography on Kodak X-Omat AR film.
RESULTS

Initial attempts to introduce Tn4001 into M. pulmonis using pSK31 plasmid DNA were unsuccessful. Since this may have been due to the large size of pSK31 (37.3 kb), we cloned the Tn4001-containing fragment from pSK31 onto pKS, a pMB1-derived E. coli replicon to produce pISM1001 (Figure 1). This was accomplished by excising the intact transposon from the S. aureus plasmid pSK31 (Lyon et al., 1984) by EcoR1 digestion followed by ligation into the EcoR1 site of pKS (Stratagene Cloning Systems). A resulting ampicillin resistant (Ap^) 13.45 kb plasmid, pISM1001, contained Tn4001 on a 10.5 kb EcoR1 fragment. Gentamicin resistance was not expressed in E. coli DH5α, but was in M. pulmonis. Plasmid pISM1001 was used as the vehicle to introduce Tn4001 into M. pulmonis. Additionally, the internal 2.5 kb HindIII fragment of Tn4001 which contains the antibiotic resistance determinant was subcloned from pISM1001 into the HindIII site of pKS. The resulting plasmid, pISM1003, was used as a Tn4001 specific probe for Southern blot analysis of mycoplasmal transpositional mutants. Using this construction we were able to introduce Tn4001 into M. pulmonis at a frequency of 5 x 10^-5 per colony forming unit (CFU). No spontaneous gentamicin resistant mutants were observed on control plates (<1 x 10^-9 per CFU).

Figure 2 shows an autoradiogram of a Southern hybridization with a Tn4001-specific probe of EcoR1 digested M. pulmonis chromosomal DNAs derived from gentamicin resistant (Gm^) colonies. Over 40 independent insertions were examined and transposon inserts seemed to occur randomly throughout the chromosome. More than one insertion occurred at a low frequency (Figure 2, lane 19), and occasionally a low molecular weight band smaller than the intact Tn4001 element was observed (Figure 2, lane 24). This band probably represents a copy of IS256 which occasionally transposes at a low frequency independent of the intact...
element. Lanes containing mycoplasma chromosomal DNAs did not react with a radiolabeled plasmid-specific probe (data not shown) indicating that the intact plasmid was not integrated into the chromosome and therefore Tn4001 was transpositionally active in *M. pulmonis*. The stability of Tn4001 insertions in *M. pulmonis* was determined by passing strains more than 10 times under nonselective conditions with subsequent examination of the ratio of the number of CFUs on nonselective media to the number of CFUs on selective media, and by examining Southern hybridization profiles of isolated colonies. In each case, the numbers of CFUs on nonselective and selective media were equal, and the Southern hybridization patterns were identical to the parent. Tn4001 containing *M. pulmonis* strains were able to tolerate concentrations of gentamicin greater than 50 μg/ml.
DISCUSSION

The introduction of Tn4001 into *M. pulmonis* represents the second transposable element of Gram positive origin which expresses both phenotypic (Gm<sup>+</sup>) and transpositional functions in mycoplasmas (Dybvig and Cassell, 1987). Since mycoplasmas are believed to be closely related to the Gram positive eubacteria phylogenetically (Maniloff, 1983), other transposons which are functionally active in Gram positive bacteria may also function in mycoplasmas. Difficulties may arise from the expression and usefulness of other antibiotic resistance markers. In studies in this laboratory, kanamycin and erythromycin have not proven useful for genetic studies because of a high level of spontaneous mutants in *M. pulmonis* (>5 X 10<sup>-5</sup> per CFU). Both tetracycline and gentamicin, however, proved to be excellent antibiotics with frequencies of spontaneous mutants arising at fewer than 1 x 10<sup>-9</sup> per CFU. Additionally, selective concentrations of gentamicin could range from 5-15 μg/ml without significantly effecting the frequency of transformation, and Tn4001 containing strains grew more rapidly in the presence of selection than those which contained Tn916.

The availability of a second transposable element for use with mycoplasmas will provide an additional tool to study the genetic structure and regulation of these organisms. As a mutagen Tn4001 will enhance the search for specific mutants by providing a second selectable marker which complements the tetracycline resistance of Tn916. It will also be valuable as a genetic marker for strain constructions such as vaccine strains for which molecular probes will be available. This laboratory is presently developing derivatives of Tn4001, i.e. fusion vectors (Casadaban and Cohen, 1979; Weaver and Clewell, 1987; Manoil and Beckwith, 1985), for future studies in mycoplasma gene structure and regulation.
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LITERATURE CITED


FIGURE 1. Plasmid constructions. [Plasmid pSK31 carrying Tn4001 was digested with EcoRI and ligated to EcoRI digested, dephosphorylated pKS. A resulting plasmid, pISM1001, carried Tn4001 on a 10.5 kb insert. To construct pISM1003, a 2.5 kb HindIII fragment from pISM1001 was ligated to pKS treated with HindIII and calf intestinal phosphatase. The resulting 5.45 kb plasmid contained the aminoglycoside resistance determinant from Tn4001. Stipled bar indicates resistance determinants in pKS and pISM1001; open bar indicates multiple cloning site (MCS); black bar indicates the IS256 elements of Tn4001. Restriction enzymes are designated as follows: E = EcoRI, H = HindIII.]
FIGURE 2. Southern hybridization of Tn4001 containing *M. pulmonis* chromosomal DNAs probed with pISM1003. [Lane 1, wildtype *M. pulmonis* UAB6510; lane 2, undigested pISM1003; lane 3, undigested pISM1001. The multiple bands in lanes 2 and 3 represent the monomeric, dimeric, and open circular forms of the undigested plasmids. The remaining lanes contain EcoR1 digested chromosomal DNAs from gentamicin resistant transformants of *M. pulmonis* UAB6510. Linear size markers are shown on the left in kilobases.]
SECTION II. TRANSFORMATION OF MYCOPLASMA PULMONIS:
DEMONSTRATION OF HOMOLOGOUS RECOMBINATION,
INTRODUCTION OF CLONED GENES AND THE PRELIMINARY
DESCRIPTION OF AN INTEGRATING SHUTTLE SYSTEM
Transformation of *Mycoplasma pulmonis*: Demonstration of Homologous Recombination, Introduction of Cloned Genes and the Preliminary Description of an Integrating Shuttle System

by

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ABSTRACT

The transposons Tn916 and Tn4001, and a series of integrating plasmids derived from their antibiotic resistance genes, were used to examine polyethylene glycol-mediated transformation of *Mycoplasma pulmonis*. Under optimal conditions, Tn916 and Tn4001 could be introduced into *M. pulmonis* at frequencies of $1 \times 10^{-6}$ and $5 \times 10^{-5}$ per CFU respectively. Integrating plasmids were constructed with the cloned antibiotic resistance determinants of Tn916 and Tn4001, a pMB1-derived plasmid replicon, and mycoplasmal chromosomal DNA, and used to examine recombinational events following transformation into *M. pulmonis*. Under optimal conditions, chromosomal integrations could be recovered at a frequency of $1 \times 10^{-4}$ to $1 \times 10^{-6}$ per CFU depending on the size and nature of the chromosomal insert, and the parental plasmid. Integrated plasmids were stable in the absence of selection and could be rescued in *E. coli* along with adjacent mycoplasma DNA. These studies provide the first direct evidence of a recombination system in the Mollicutes, and describe the first *E. coli - M. pulmonis* shuttle vectors.
INTRODUCTION

The Mollicutes are a diverse class of cell wall-less bacteria which have the smallest genomes of free living organisms, approximately 750 - 1250 kilobase pairs (kb). Little is known about the genetics of these organisms despite their importance as mammalian and plant pathogens (Razin, 1985). Conjugation, transformation, transduction, and recombination, which have been the cornerstone of genetic studies in other bacterial systems have been studied very little in mycoplasmas (Razin, 1985). Viruses are restricted almost exclusively to the more complex genera within the Class, Acholeplasma and Spiroplasma (Razin, 1985). One exception is the recent report of an unusual double stranded RNA virus isolated from Mycoplasma pulmonis (Dybvig and Cassell, 1987b). None of these viruses have been reported to have transducing capabilities.

No conjugal system has been described in mycoplasmas, although genetic exchange has been reported in Spiroplasma citri (Barroso and Labarere, 1988, Labarere and Barroso, 1984) and Acholeplasma (Gourlay et al. 1984), the latter in the presence of polyethylene glycol (PEG). Exchange is thought to occur through a membrane fusion event, and recombination has been implied as an important element (Barroso and Labarere, 1988, Labarere and Barroso, 1984, Razin, 1985), but has never been studied directly. A major hindrance in this area has been the scarcity of genetic markers in the Mollicutes and the relative lack of defined media for growth of these organisms.

The recent introduction of Gram positive transposons into mycoplasmas, offers the potential for examination of the genetic features of these organisms. This introduction by transformation with plasmid replicons containing Tn916 (Dybvig and Cassell, 1987a) and Tn4001 (Mahairas and F. C. Minion, 1989) or by conjugal transfer of Tn916 from
*Streptococcus faecalis* (Roberts and Kenny, 1987) demonstrate that transpositional events occur, and that at least two Gram positive resistance genes can be expressed in mycoplasmas.

As a first step toward expanding genetic capabilities in mycoplasmas, we have cloned the antibiotic resistance genes of Tn4001 and Tn916 in *E. coli*, and constructed plasmids capable of integrating into the *M. pulmonis* chromosome. Using these plasmids and the transposons from which they were derived, we have examined some parameters for the efficient introduction of DNA by polyethylene glycol (PEG) mediated transformation of *M. pulmonis*, and describe a shuttle strategy between *M. pulmonis* and *E. coli*. In addition, we show that homologous recombination occurs at high frequency in the *Mollicutes*, the first such report.
MATERIALS AND METHODS

Bacterial Strains and Culture Media

The strains of bacteria, their relevant genotypes, origins, and plasmids used in this study are listed in Table 1. Strains of E. coli were maintained as previously described (Maniatis et al., 1982). M. pulmonis was grown in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% GG-free horse serum (GIBCO Laboratories, Grand Island, N.Y.), 2.5% fresh yeast extract (Flow laboratories, Inc., McLean, Va.), 0.5% glucose, 2.5 μg of Cefobid (Pfizer, Inc., New York, N.Y.) per ml, and when required, 1% Noble agar (Difco). Strain ISM1499 was derived by passing UAB6510 (Minion et al., 1984) more than 100 times in broth media. Selective levels of antibiotics for M. pulmonis were 2 μg of tetracycline per ml and 10 μg of gentamicin per ml, and resistant cultures were maintained in PPLO medium containing either antibiotic at 10 μg per ml. For E. coli, 20 μg of tetracycline per ml and 100 μg of ampicillin per ml in L agar were used to select and maintain resistant strains. All antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Reagents and Buffers

Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Promega Corp. (Madison, Wis.), and used according to manufacturer's directions. All solutions were prepared in tissue culture grade water and autoclaved before use. Solution compositions were: PEG, 40% (wt/vol) PEG-8000 (Sigma) in 0.01 M Tris - 0.5 M sucrose, pH 6.5; PBS, 10 mM Na2HPO4 - 0.14 M NaCl, pH 7.3; TE, 10 mM Tris - 1 mM EDTA, pH 8.0; TNE, 10 mM Tris - 1 mM EDTA - 140 mM NaCl, pH 8.0; and TAE buffer, 40 mM Tris acetate - 1 mM EDTA.
Transformation

Transformation of *E. coli* was performed as previously described (Hanahan, 1983). The optimal conditions for the transformation of *M. pulmonis* were as follows. Cells from a late log phase 12 - 18 h culture of ISM1499 were harvested by centrifugation (10,000 x g, 10 min), washed once with PBS, resuspended in an original volume of cold 0.1 M CaCl₂, and incubated at 0°C for 30 min. Ten μg of yeast tRNA, 250 μl of cell suspension (approximately 1 x 10⁸ colony forming units), and 10 μg DNA in TE were mixed with PEG to give a final concentration of 35 - 36% PEG. Following a 1 min incubation, the transformation mixture was diluted in 15 ml PBS, the cells collected by centrifugation (10,000 x g, 15 min, room temperature), resuspended in 1 ml of warm (37°C) PPLO broth and incubated for 1 h at 37°C without selection. Transformants were selected on PPLO agar plates and colonies appeared after incubation for 72 - 96 h at 37°C. Transformation frequency was defined as the number of resistant organisms recovered divided by the total number of organisms in each reaction mixture.

Isolation of Plasmid and Chromosomal DNA

Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (1979), and purified by CsCl-ethidium bromide density gradient centrifugation (Maniatis et al., 1982). Mycoplasma chromosomal DNAs for Southern hybridizations were prepared as previously described (Mahairas and Minion, 1989). *M. pulmonis* chromosomal DNAs for the retrieval of integrated plasmids were prepared as follows. Cells from 150 ml of late log/stationary phase culture were lysed in 5 ml of TNE buffer by the addition of 1% Tween 20 (Sigma). After the addition of 100 μg of Proteinase K (Sigma), the preparation was incubated at 37°C for 12 h, extracted with phenol once, and the chromosomal DNA further purified by CsCl - ethidium bromide density gradient centrifugation.
Subcloning In LGT Agarose

The subcloning of the 5.0 kb EcoRI fragment of pISM1004 into the EcoRI site of pISM1003 was carried out in Seaplaque LGT agarose (FMC Bioproducts, Rockland, Maine) according to the following procedure. Five μg of pISM1004 plasmid DNA was restricted with EcoRI and subjected to electrophoresis in LGT agarose in 0.8% TAE buffer containing 0.5 μg of ethidium bromide per ml. The EcoRI fragment B was excised from the gel, diluted with one third vol of sterile water, and melted at 70°C. Ligation mixtures contained 500 ng of EcoRI-digested, alkaline phosphatase treated pISM1003, and varying amounts of gel to 50% of the total ligation mixture. After overnight ligation at 4°C, the gel was melted at 70°C, cooled to 37°C, and then aliquots used to transform competent E. coli DH5α cells.

DNA-DNA Hybridizations

M. pulmonis chromosomal DNAs were digested with the appropriate restriction endonuclease and electrophoresed in a 0.55% agarose gel using TAE buffer. DNA fragments were transferred to Genescreen (New England Nuclear Research Products, Boston, Mass.) by the method of Southern (Southern, 1975) and hybridized as previously described (Maniatis et al., 1982). DNA probes were made using the Multiprime labeling system (Amersham Corp., Arlington Heights, Ill.) incorporating 32P dCTP (ICN Biochemicals, Inc., Irvine, Cal.). The specific activity of the labeled probe was typically 1 x 10^9 cpm/μg DNA.
RESULTS

Construction of Integrating Plasmids

Plasmid pISM1002 was constructed by digesting pAM120 with HincII, ligating, transforming into E. coli DH5α, and selecting for tetracycline resistance. One clone containing a 7.5 kb plasmid specifying only TcR, and consisting of a 2.7 kb fragment containing the pMB1-derived origin of replication, and a 4.8 kb HincII fragment containing the tetracycline resistance gene of Tn916 was designated pISM1002. Plasmid pISM1002 conferred selectable tetracycline resistance in both M. pulmonis and E. coli, and had unique restriction sites for EcoRI, HindIII, KpnI, PstI, and PvuII (Fig. 1). The construction of plasmid pISM1003 which contained the gentamicin resistance marker of Tn4001 has been described previously (Mahairas and Minion, submitted). It specified ApR in E. coli and GmR in M. pulmonis with unique restriction sites for EcoRI, NolI, KpnI, BamHI, EcoRV, Clal, PstI, and SmaI.

Restriction maps of both pISM1002 and pISM1003 are shown in Fig. 1. Integrating plasmids were selected from an M. pulmonis chromosomal DNA library generated by cloning EcoRI digested chromosomal DNA into the unique EcoRI sites of both pISM1002 and pISM1003. The plasmids constructed and used in this study are described in Table 1. Plasmid pISM1008 was constructed by excising the EcoRI fragment B from plasmid pISM1005, and cloning it into the EcoRI site of plasmid pISM1003.

Transformation of M. pulmonis

The transformation frequencies of the plasmids used in this study are shown in Table 2. An examination of the parameters of transformation was done by varying the concentrations of the reaction components described above. In contrast to resuspending the organisms in PBS as described previously (Dybvig and Cassell, 1987a), incubation of cells in 0.1 M CaCl₂ or 0.1 M MgCl₂ for 30 min at 0°C prior to transformation resulted in a four- to five-fold increase in
the number of transformants (data not shown). The results of the determination of optimal yeast tRNA, plasmid DNA, and PEG concentrations are given in Fig. 2. From these data, the optimal conditions for the transformation of *M. pulmonis* using these vectors were determined to be 10 μg yeast tRNA, 10 μg purified plasmid DNA, and a final PEG concentration of 35 - 36 percent. The stability of the plasmid integrants was verified by passing ISM1004 and ISM1005 in broth in the absence of selection 10 - 20 times followed by determination of colony forming units on selective and nonselective media. Colony forming units were always equal (data not shown) indicating that plasmid integrations derived from pISM1002 and pISM1003 were stably maintained in *M. pulmonis* in the absence of selective pressure.

In order to examine the transforming properties of linear DNA, chromosomal DNA from ISM1500 and ISM1501, and plasmids pISM1004 and pISM1005 linearized with *SalI* and *BamHI*, respectively, were used to transform ISM1499. Both ISM1500 and ISM1501 contain plasmid integrations from pISM1004 and pISM1005 (Table 2). Restriction enzyme analysis of pISM1004 and pISM1005 revealed internal restriction endonuclease sites within the cloned chromosomal inserts. The *SalI* site in pISM1004 was 1.2 kb from one end of the cloned 3.0 kb chromosomal fragment, and the *BamHI* site in pISM1005 was 2.3 kb from one end of the 5.0 kb cloned chromosomal fragment. Both chromosomal and linearized plasmid DNA failed to transform *M. pulmonis* (transformation frequency < 1 x 10^{-8} per CFU).

To determine whether parental plasmid sequences could account for differences in transformation frequencies (Table 2), plasmid pISM1008 was constructed carrying the same 5.0 kb *EcoRI* fragment of *M. pulmonis* chromosome as pISM1005. The pISM1002-derived vector (pISM1005) transformed at a 40-fold higher frequency than did its pISM1003-derived counterpart (Table 2). Therefore, parental plasmid sequences appeared to effect recombinational frequencies.
DNA Hybridization Studies

A Southern analysis of transformed *M. pulmonis* chromosomal DNAs probed with their respective parental plasmids, pISM1002 or pISM1003, is shown in Fig. 3. Panel A represents transformants arising from pISM1002 - derived plasmids and panel B, pISM1003 - derived plasmids. Because of an internal *HindIII* site in pISM1002 (Fig. 1), digestion of pISM1002 - derived transformant chromosomal DNAs with *HindIII* resulted in the appearance of two bands, each containing a portion of pISM1002 (panel A). Digestion of pISM1003 - derived transformant chromosomal DNAs with *XbaI* resulted in a single major fragment that shows an increase in molecular weight due to adjoining chromosomal DNA sequences (panel B). Another light, almost nondetectable band was often seen. This band represented 36 base pairs within the multiple cloning site between *ihtXbaI* and *EcoRI* restriction sites which was attached to adjoining chromosomal DNA (Fig. 3). Without exception, every transformant examined from each independent transformation mixture had the same Southern banding pattern depending upon the chromosomal insert (panel A, lanes 4 - 5 and 6 - 7; panel B, lanes 3 - 5, 6 - 8, 9 and 10).

Retrieval of Integrated Plasmids

To test if integrated plasmids could be retrieved in *E. coli*, chromosomal DNAs from plasmid integrants were digested with *EcoRI*, diluted, ligated, and transformed into *E. coli* DH5α. Plasmid DNAs were obtained from these transformants and analyzed by restriction digests. Intact plasmid pISM1002 could be recovered from the chromosome of ISM1004 and ISM1005 at a frequency of 5 x 10² *Tc*⁺ transformants per μg chromosomal DNA. Plasmid pISM1003 was recovered from its chromosomal location in ISM1008 at a frequency of 5 x 10³ *Ap*⁺ transformants per μg chromosomal DNA. Chromosomal DNA adjacent to integrated plasmid pISM1002 could be recovered by digestion with *BamHI* or *SalI* at detectable frequencies which varied depending upon the size of the mycoplasmal chromosomal insert. In
similar fashion, chromosomal DNAs adjacent to plasmid pISM1003 have been directionally rescued by digestion with *PstI* and *XbaI*. 
DISCUSSION

The introduction of Tn4001 (Mahairas and Minion, 1989) and Tn916 (Dybvig and Cassell, 1987a) into M. pulmonis by PEG-mediated transformation has been described previously. Transformation frequencies reported in these studies reflect both the efficiency of DNA uptake and transposition. By constructing integrating vectors containing cloned antibiotic resistance genes from both transposons, we removed the constraints of transposition and were able to examine the properties of mycoplasmal transformation more directly. These studies demonstrate the first introduction of cloned genes into the Mollicutes, and give the first physical evidence of homologous recombination. Additionally, in the absence of a mycoplasmal cloning vector, the integration vectors described in this study can function as an E. coli - M. pulmonis shuttle system.

In order to examine transformation in detail, a protocol was established using purified components with the addition of yeast tRNA. Nonpurified DNA preparations that contain significant amounts of cellular RNA transform at frequencies comparable to those obtained using purified components, leading us to believe that tRNA enhances transformation either by inhibiting cell surface nucleases (Minion and Goguen, 1985) or by acting as a carrier, aiding DNA passage across the membrane.

In contrast to earlier studies ( Furness and Cerone, 1979), linear DNA failed to transform M. pulmonis. Furness and Cerone demonstrated that chromosomal DNA from a tetracycline resistant strain of M. hominis could transform M. hominis and M. salivarium (Furness and Cerone, 1979). This may have resulted from a sequential transposition event (Yost et al., 1988) of Tn916, however. In our studies, both chromosomal and linear plasmid DNA failed to transform M. pulmonis indicating that supercoiled or closed circular DNA was required for PEG-mediated transformation. This may have been due to an increased
susceptibility of linear DNA to nucleases (Minion and Goguen, 1985) or an inability of linear DNA to transverse the membrane.

The introduction of the cloned tetracycline and gentamicin resistance determinants of Tn916 and Tn4001 into M. pulmonis in association with mycoplasma chromosomal DNA represents the first clear evidence of homologous recombination in the Mollicutes. Southern blot analysis confirmed the presence of integrated plasmid sequences that were directed to their chromosomal locations by the original cloned mycoplasma DNA sequence. Although implied in previous studies (Barroso and Labarere, 1988, Labarere and Barroso, 1984, Razin, 1985), homologous recombination had not been demonstrated conclusively in these organisms. A recent report of genetic exchange in S. citri (Barroso and Labarere, 1988) gave strong evidence for recombination, but the authors were unable to examine the recombinational event directly because of the lack of molecular probes for the UV light-induced selectable markers. In contrast, the introduction of cloned antibiotic resistance genes for which we have molecular probes clearly demonstrates homologous recombination.

A comparison of transposition frequencies of Tn916 (1 x 10^{-6} per CFU) with frequencies of recombination in pISM1002-derived plasmids (1 x 10^{-4} to 3.25 x 10^{-5} per CFU) seemed to indicate that recombination occurred at a higher frequency than did transposition assuming that the rate of DNA uptake remained the same. Larger mycoplasma DNA fragments generally mediated vector integration at higher frequencies relative to smaller fragments as shown in Table 2, but the minimum length of homology necessary for integration was not determined. In addition, a comparison of transformation frequencies of pISM1005 and pISM1008 suggested that plasmid sequences could interfere with integration as was the case with the pISM1003-derived plasmids. Alternatively, the lower frequency of transposition of Tn916 relative to Tn4001 may have reflected the differences in size of their respective plasmids (21.4 kb vs 13.45 kb), since there appeared to be an upper limit on plasmid size in
transformation by PEG (Table 2). Both pISM1009 (22.95 kb) and pISM1010 (21 kb) failed to transform *M. pulmonis*. Transfection studies of L1 (Liss and Maniloff, 1973), L2 (Sladek and Maniloff, 1983), and L3 viral DNAs with *Acholeplasma laidlawii* demonstrated an upper limit for PEG transformation as well. Both L1 (4.54 kb ssDNA) and L2 (11.8 kb dsDNA) could transfect *A. laidlawii* using PEG. L3 DNA (39.3 kb dsDNA) could only be introduced by electroporation (Lorenz et al., 1988).

The ability to introduce foreign DNA at predefined locations in *M. pulmonis* serves as the basis for an *E. coli - M. pulmonis* shuttle system. Specific gene mutations could be created in *E. coli* and then introduced into *M. pulmonis* in order to study the effect of the mutations in vivo. *M. pulmonis* could also serve as a cloning host for genes from other mycoplasmas since *E. coli* is a poor host for the expression of mycoplasma genes because of differences in regulatory elements (Tashke and Herrmann, 1988) and codon usage (Yamao et al., 1985). The applicability of these vectors to other mycoplasmas is currently under investigation.
ACKNOWLEDGMENTS

We are grateful to P. A. Pattee, R. F. Ross, and R. Rosenbusch for reviewing this manuscript. This work was supported by Public Health Services grant AI24428 from the National Institutes of Health and by the Biotechnology Grants Program at Iowa State University.
LITERATURE CITED


Table 1. Bacterial strains and plasmids

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Table 2. Transformation frequencies of circular recombinant plasmids in *Mycoplasma pulmonis*

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* a Parent indicates the parental plasmid used to construct each derivative.
* pAM120 contained intact Tn916 and pISM1001 contained intact Tn4001.
* b Size of the mycoplasma chromosomal insert in kb. Inserts in pISM1005 and pISM1008 are identical. pISM1009 contained three chromosomal inserts of 8.0, 5.0, and 4.5 kb.
* c Transformation frequency: Number of transformants/Total number of organisms.
FIGURE 1. Restriction maps of pISM1002 and pISM1003. [The approximate positions of genes and restriction sites are indicated. The white areas in pISM1003 indicate the multiple cloning site of pKS. The black areas indicate positions of the insertion element of Tn4001, IS256. Details of the construction of pISM1003 are given in Mahairas and Minion (1989). The resistance markers for selection in M. pulmonis are indicated by shaded areas. All restriction sites for a particular enzyme are shown. B, BamHI; E, EcoRI; H, HindIII; Hc, HincII; K, KpnI; P, PstI; Pv, PvuII; S, SalI; p X, XbaI.]
FIGURE 2. The determination of optimal conditions for the transformation of *M. pulmonis* using purified components and integrating plasmids. Transformation reaction mixtures contained $1 \times 10^8$ cells in 0.1 M CaCl$_2$. Panel A, reaction mixtures contained 10 μg yeast tRNA, 36% PEG and varying amounts of pISM1004 (closed) and pISM1005 (open) plasmid DNA; Panel B, reaction mixtures contained 10 μg pISM1005 plasmid DNA, 10 μg yeast tRNA, and varying concentrations of PEG; Panel C, reaction mixtures contained 5 μg pISM1005 plasmid DNA, 36% PEG, and varying amounts of yeast tRNA. Five μg pISM1005 was used in the yeast tRNA experiments (Panel C) to enhance sensitivity of the assay. Data represent the mean of 4 experiments (panel A) and 2 experiments (panels B and C).
FIGURE 3. Southern blot analysis of plasmid integrations in *M. pulmonis*. [Mycoplasmal chromosomal DNAs were digested with HindIII (A) or XbaI (B) and separated in 0.55% agarose gels. DNA fragments were transferred to GeneScreen and probe with radiolabeled pISM1002 (A) or pISM1003 (B). Chromosomal DNAs with integrated plasmids () were as follows. The strain numbers, i.e., ISM1501-1, indicate individual transformants from a single transformation reaction mixture as described in Table 1. Panel A: lanes 1 and 8 contained kilobase ladder size standards (Bethesda Research Laboratories). The probe reacted with the 517 bp fragment of pBR322. Lane 2, linearized pISM1002 plasmid DNA (7.5 kb, positive control); lane 3, ISM1499 chromosomal DNA (negative control); lane 4, ISM1500-1 (pISM1004); Lane 5, ISM1500-2 (pISM1004); Lane 6, ISM1501-1 (pISM1005); Lane 7, ISM1501-2 (pISM1005). Panel B: lane 1, linearized pISM1003 plasmid DNA (5.45 kb, positive control); lane 2, ISM1499 chromosomal DNA (negative control); lane 3, ISM1502-1 (pISM1006); lane 4, ISM1502-2 (pISM1005); lane 5, ISM1502-3 (pISM1006); lane 6, ISM1503-1 (pISM1007); lane 7, ISM1503-2 (pISM1007); lane 8, ISM1503-3 (pISM1007); lane 9, ISM1504-1 (pISM1008); lane 10, ISM1504-2 (pISM1008).]
SECTION III. GENETIC EXCHANGE OF TRANSPOSON AND INTEGRATIVE PLASMID MARKERS IN *MYCOPLASMA PULMONIS*
Genetic Exchange of Transposon and Integrative Plasmid Markers in
Mycoplasma pulmonis

by

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ABSTRACT

Matings of genetically marked derivatives of *Mycoplasma pulmonis* resulted in the exchange of chromosomal DNA and the appearance of doubly marked transconjugants. Transposons Tn916 and Tn4001, and a series of integrative plasmids derived from their cloned antibiotic resistance genes, were used to construct antibiotic resistant mycoplasmal derivatives to examine this phenomenon at the molecular level. Genetic exchange occurred on agar surfaces at frequencies ranging from $3.3 \times 10^{-4}$ to $6.4 \times 10^{-8}$ transconjugants per colony forming unit. Examination of chromosomal DNA from transconjugants by hybridization revealed that the transposons or integrated plasmids were in the same chromosomal locations as the parental strains indicating that exchange involved the transfer of chromosomal DNA and homologous recombination. Transfer was not affected by DNase, polyethylene glycol, EDTA or calcium chloride, but was affected by treatment of either parent with trypsin. Mixing of mating strains prior to plating had no effect on mating frequencies, but mating did occur in liquid media. The ability to exchange chromosomal markers was limited to selected strains of *M. pulmonis*, and mating did not occur with *Acholeplasma laidlawii* or *M. gallisepticum*. Heat and UV inactivation studies revealed that nonviable cells could act as donors in matings. The evidence presented supports a conjugation-like mechanism involving specific trypsin-sensitive membrane components.
INTRODUCTION

The Mollicutes are prokaryotes characterized by the lack of a cell wall and a small genome (i.e., 800 kilobases) that is thought to be the minimum genetic information necessary for autonomous existence (Razin, 1985). This limited genomic potential has resulted in organisms devoid of several biosynthetic pathways including those involving nucleic acid precursors and membrane phospholipids (Razin, 1985). Mycoplasmas, therefore, grow only in complex media and this has prevented the use of metabolic markers for genetic studies. Antibiotic resistance markers are rare in the Mollicutes and only recently have heavy metal resistant mutants been used in genetic studies (Barroso and Labarere, 1988). Because of this, little is known about the chromosomal genetics of mycoplasmas.

Many mycoplasmal species are mucosal pathogens and are often found in environments inhabited by other bacterial commensals or pathogens. Mycoplasmas produce significant diseases in humans and in animal populations, and are generally difficult to detect and eradicate. The ability of mycoplasmas to exchange genetic markers, in particular antibiotic resistance markers, has recently gained interest because of the increasing prevalence of tetracycline resistant isolates of *M. hominis*. Roberts et al. (1985) first reported the occurrence of *tetM* sequences in clinical isolates of *M. hominis*, and subsequently demonstrated exchange of Tn916 from *Streptococcus faecalis* to *M. hominis* (Roberts and Kenny, 1987). The basis for this type of exchange was not addressed, but presumably occurred through conjugal properties associated with Tn916 (Fitzgerald and Gasson, 1988; Cawron-Burke and Clewell, 1982; Senghas et al., 1988). If mycoplasmas can participate in genetic exchange with streptococci or other bacteria in the natural environment as suggested by these studies, then control of mycoplasmal infections may become significantly more complex as antibiotic resistances are disseminated.
Genetic exchange between spiroplasma strains was recognized by Labarere and Barroso (Barroso and Labarere, 1988; Labarere and Barroso, 1984) who described the exchange of UV-induced chromosomal mutations in *Spiroplasma citri*. To examine this phenomenon in *Acholeplasma laidlawii* and the sterol-requiring *Mycoplasma*, genetically marked strains of *A. laidlawii*, *M. pulmonis* and *M. gallisepticum* were constructed using Tn*4001* (Lyon et al., 1984; Lyon and Skurray, 1987; Mahairas and Minion, 1989a), Tn*916* (Franke and Clewell, 1981) and integrative plasmids derived from their cloned antibiotic resistance genes (Mahairas and Minion, 1989b). During matings genetic exchange was followed by hybridization studies of chromosomal DNA using marker-specific probes. Exchange occurred in *M. pulmonis* in the absence of fusogenic agents on solid media indicating that genetic transfer may occur naturally in *M. pulmonis* cultures allowing for the free exchange of genetic information. The effect of various treatments on genetic exchange was also examined.
MATERIALS AND METHODS

Bacteria and Culture Media

The strains of mycoplasmas used in this study, their relevant genotypes and origins are listed in Table 1. *Escherichia coli* was maintained as previously described (Maniatis et al., 1982). Mycoplasmas were grown in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% gamma globulin-free horse serum (GIBCO Laboratories, Grand Island, N.Y.), 2.5% fresh yeast extract (Flow laboratories, Inc., McLean, Va.), 0.5% glucose, and 2.5 μg per ml Cefobid (Pfizer, Inc., New York, N.Y.). PPLO agar also contained 1% Noble agar (Difco). Mating mixtures were plated on PPLO agar containing 15 μg per ml gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) and 10 μg per ml tetracycline (Sigma) and incubated for 3 to 5 days at 37°C.

Chromosomal DNA, Reagents and Buffers

Mycoplasma chromosomal DNAs for restriction endonuclease digestions and hybridization analyses were prepared in low melting temperature agarose as described previously (Mahairas and Minion, 1989b). Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Promega Corp. (Madison, Wis.) and used according to the manufacturer's directions. Polyethylene glycol (PEG) solution consisted of 40% (wt/vol) PEG-8000 (Sigma) in 0.01 M Tris - 0.5 M sucrose, pH 6.5. Phosphate buffered saline was 0.01 M sodium phosphate - 0.14 M sodium chloride, pH 7.5.

Transformation and Mating

Tn4001, Tn916 and the recombinant integrative plasmids were introduced into *M. pulmonis*, *M. gallisepticum*, and *A. laidlawii* by PEG-mediated transformation as described (Mahairas and Minion, 1989b). Matings were performed between isogenic and nonisogenic strains of *M. pulmonis* using derivatives containing both integrated plasmids and transposons.
Additional matings were performed between isogenic strains of *M. gallisepticum* and *A. laidlawii*, and between *M. pulmonis* and *M. gallisepticum* using derivatives marked with transposable elements. Matings were generally performed by directly plating 50 μl of each parent (1–5 x 10⁹ CFUs per ml) onto selective media or in some experiments by mixing 0.1 ml of a late log phase, overnight culture of each parent and incubating at 37°C at various time intervals prior to plating on selective media. For *M. gallisepticum* and *A. laidlawii*, some matings were performed by mixing strains and centrifuging for 2 min at 12,000 x g prior to plating. The uptake of DNA from the surrounding environment was tested by incubating *M. pulmonis* strains in the presence of 10 μg of purified chromosomal DNA from a transposon containing strain or 10 μg of pISM1005 plasmid DNA (Mahairas and Minion, 1989b). Mixtures of mating pairs were treated with varying concentrations of PEG (10-40%) or 100 mM calcium chloride prior to plating on selective media. Resistant colonies were filtered cloned as described (Tully, 1983) and chromosomal DNAs analyzed by blot hybridization (Maniatis et al., 1982). Mating frequencies were determined by dividing the number of doubly resistant organisms by the total number of CFUs of both parents per mating mixture or in some instances by dividing the number of doubly resistant organisms by the number of CFUs of the treated parent only. Data were analyzed by the analysis of variance.

The mechanism of genetic exchange was examined by testing for DNase, temperature, trypsin and UV light sensitivities, and the presence of bacteriophages. DNase (Sigma) was added to cell suspensions (250 μg per reaction) and spread on agar surfaces (250 μg per plate). To examine temperature effects, donor cells were treated at 56°C for 0, 15 and 30 min prior to their use in matings. To examine trypsin sensitivity, cell suspensions (1 x 10⁹ CFUs per ml) in PBS were treated with trypsin (1 mg per ml) at 37°C, and cells were removed at the indicated time points and used directly in mating experiments. The effect of UV radiation on genetic exchange was determined as follows. The parent to be irradiated was washed, resuspended in
an original volume of phosphate buffered saline and placed as a thin film in a sterile petri plate 30 cm below a UV germicidal lamp. Cultures were agitated and samples removed at various time intervals. Viability was determined in all treated samples by plating serial dilutions on nonselective medium and mating efficiencies measured by plating identical volumes of treated cells and a corresponding untreated recipient on doubly selective medium.

To examine for potential transducing bacteriophages, bacteriologically sterile culture filtrates were prepared from culture supernates by passing through three 0.1 μm filters (Micron Separations, Inc., MicronSep mixed cellulose esters, Fisher Scientific Co., Pittsburgh, Penn.) and testing for viable organisms by culture on PPLO. To control for nonspecific adherence of bacteriophage to the filters, λ and P1L4 lysates were filtered and decreases in titer determined (Curtiss, 1981; Maniatis et al., 1982). *M. pulmonis* strain ISM1499 was examined for viruses by examining pellets of culture supernatants (200,000 x g, 4 h) by transmission electron microscopy using negative staining (Cole, 1983) and by plaque forming ability on mycoplasmal lawns (Gourlay, 1983).

**DNA - DNA Hybridizations**

Chromosomal DNAs from transconjugants were prepared as previously reported (Mahairas and Minion, 1989a). Restriction digests consisted of 4 to 5 μl agarose containing chromosomal DNA previously melted at 70°C and equilibrated to 37°C, 2 μl 10X enzyme buffer, and 3 to 5 U units of restriction enzyme in a total volume of 20 μl. Samples were digested for 1 h, 5 μl of electrophoresis sample buffer was added, and 10 μl samples loaded onto 0.55% agarose gels in Tris-borate buffer (Maniatis et al., 1982). DNA fragments were then transferred to GeneScreen (Du Pont NEN Research Products, Boston, Mass.) by capillary transfer. DNA probes were labeled with 32P dCTP using the Multiprime Labeling System (Amersham Corp., Arlington Heights, Ill.).
RESULTS

Integrating Plasmids and Strain Constructions

*M. pulmonis* strains ISM1501 and ISM1503 harboring integrated plasmids have been described (Mahairas and Minion, 1989b). In addition, *M. pulmonis*, *M. gallisepticum*, and *A. laidlawii* strains carrying the transposons Tn4001 and Tn916 were also constructed (Table 1).

Mating in *M. pulmonis*

The frequency of genetic exchange between *M. pulmonis* ISM1499-derived isogenic strains varied from $3.3 \times 10^{-4}$ to $6.4 \times 10^{-8}$ transconjugant per CFU depending upon the specific mating pair (data not shown). Exchange frequencies between pairs were highly reproducible with standard errors usual less than 20% in eight replicates with different lots of media. Mating occurred between strains containing both integrated plasmids and transposon inserts. In all cases, Tn4001-containing strains mated at higher frequencies than pISM1003 plasmid integrants (data not shown) and in some matings, differences were significant ($p<0.05$). Spontaneous mutants arose at less than $5 \times 10^{-9}$ transconjugants per CFU. *M. pulmonis* strains ISM3001, JB, 66 and UAB5782 failed to mate either with isogenic strains or with permissive ISM1499-derived strains. Tn916 derivatives of *M. pulmonis* strain UABCT mated readily with Gm$^+$ parents of ISM1499.

Mating frequencies of ISM1499-derived strains were not affected by the addition of DNase to either cell suspensions or agar plates. The addition of PEG (10-40%), 100 mM calcium chloride, or 20 mM EDTA to mating mixtures had no effect on mating frequencies (data not shown). In addition, extended coincubation of mating mixtures in broth prior to plating did not increase mating frequency.

The effect of UV light on mating frequencies is shown in Figure 1. One parent of a mating mixture was exposed to UV light for varying amounts of time and the CFUs and mating
frequencies determined. Shown are representative samples from experiments exposing each
type of parent, transposon insertion or plasmid integrant, Tc\textsuperscript{T} or Gm\textsuperscript{T} marker. Mating
frequencies are expressed as the number of transconjugants per total CFUs plated. Exposure
to U V light of all strains except those harboring Tn\textit{4001} resulted in a decrease in transfer
frequency paralleling the decrease in viability (Panels A, B and D). This was not true for the
Tn\textit{4001} containing strains even though viability was lost (Panel C).

Figure 2 shows the effects of heat inactivation of one parent in a mating mixture.
Viability was typically reduced 3 to 4 logs after exposure for 15 min at 56°C. When expressed
as transconjugants per total CFU plated, frequency of transfer paralleled the decrease in
viability even with strains containing Tn\textit{4001} inserts which was not true for UV-inactivated
strains (Fig. 1C). By expressing frequencies as transconjugants per viable treated parent and
plotting frequency versus log CFUs of treated parent, it is possible to examine the effects of
viability and treatment on mating frequency (Fig. 3). The control for Fig. 3 is Panel A where
cells of one parent were diluted and plated. Under these conditions, at least 1 x 10\textsuperscript{7} CFUs of
one parent were needed to obtain transconjugants, and thus only a narrow range of dilutions
was applicable. In these experiments, mating frequency was constant indicating that the
frequency was not affected by total numbers of treated (diluted) cells. Upon heat treatment,
however, frequency increased 1 to 2 logs (Panel B), and mating frequencies with UV
inactivated cells increased as much 5 logs (Panel C). In addition, it was possible to obtain
transconjugants when fewer than 1 x 10\textsuperscript{7} viable cells of one parent were plated (data not
shown).

Trypsin treatment of cell suspensions reduced mating frequency as shown in Fig. 4. Trypsin
treatment had little effect on cell viability, but mating frequency showed a marked
decrease. Since viability was marginally affected, expression of mating frequencies as the
number of resistant colonies per total CFUs plated, or CFUs of the treated parent plated, did
not significantly alter the frequency of mating. This was in contrast to heat treatment or UV inactivation (compare Fig. 1 with Figs. 2 and 3).

In order to examine the possibility that a virus might be responsible for gene transfer, bacteriologically sterile filtrates of culture supernates of two ISM1499 derivatives giving the highest genetic exchange frequencies, ISM1501 and ISM1506, were prepared and tested in standard mating mixtures. No doubly resistant transductants were obtained in any of the crosses examined (data not shown). When concentrated culture supernates or filtrates were examined by transmission electron microscopy, no bacteriophage or bacteriophage-like particles were observed. Also, no virus plaques were observed when culture supernatants were spotted on lawns of *M. pulmonis* or *A. laidlawii* (Gourlay, 1983).

**Mating in *M. gallisepticum* and *A. laidlawii***

Several different transposon containing strains of *M. gallisepticum* and *A. laidlawii* were constructed and tested in genetic exchange experiments. Matings performed between all isogenic strains of *M. gallisepticum* and *A. laidlawii* failed to result in doubly-resistant colonies, as did matings between *M. pulmonis* (ISM1499) and *M. gallisepticum* (data not shown). The interspecies matings were performed with strains containing transposons to determine if sequential transposition of either Tn916 (Yost et al., 1988) or Tn4001 could overcome differences in DNA homology.

**DNA-DNA Hybridization**

The nature of the genetic event which occurred during mating between strains harboring integrated plasmids was examined by DNA-DNA hybridization. Figure 5 shows restriction endonuclease digested chromosomal DNAs from transconjugants from two representative matings (ISM1501 X ISM1503 and ISM1506 X ISM1507) probed separately with the *tetM*- (Panel A) and Tn4001-specific probes (Panel B). Plasmid pISM1002 was used as a *tetM*-specific probe and it reacted with both *tetM* sequences and plasmid sequences (Panel
A). Because of an internal HindIII restriction endonuclease site in the integrated plasmid sequences, two bands occur upon hybridization analysis of chromosomal DNAs. Plasmid pSK31 (Lyon et al., 1984) is a staphylococcal plasmid containing Tn4001 and does not cross-react with integrated vector sequences derived from pBR322. When labeled pSK31 was reacted with EcoRI-digested DNAs from Gm\(^\text{T}\), Tc\(^\text{T}\) transconjugants, only a single band was recognized that was in the same position as the corresponding parent (Panel B). Thirty to forty isolated colonies from each mating mixture were examined and all contained both resistance markers in the same chromosomal locations as the parents. When transconjugants derived from matings between Tn916 containing strains and either Tn4001 or Gm\(^\text{T}\) integrated plasmid containing strains were examined, similar results were obtained (data not shown).

In order to test the possibility that diploidy resulting from membrane fusion may be responsible for the double resistance (Sanchez-Rivas et al., 1988), twenty transconjugants from two independent crosses were filter-cloned twice on nonselective media and the resultant colonies tested for the Gm\(^\text{T}\), Tc\(^\text{T}\) phenotype. In every case, the total number of CFUs on nonselective media equalled the number of doubly resistant CFUs indicating stability of the markers. The presence of both genetic markers was further confirmed by hybridization analysis (data not shown) eliminating the possibility that the doubly resistant isolates might be due to a spontaneous mutation of either parent.
DISCUSSION

Genetic exchange in *Spiroplasma citri* has been previously described (Barroso and Labarere, 1988; Labarere and Barroso, 1984). The results presented here differ from previous studies since exchange in *M. pulmonis* was not affected by PEG or incubation of the parents together in suspension prior to plating. The use of transposon and integrative plasmid markers at different chromosomal locations allowed a genetic examination of the mechanisms operative in genetic exchange because of the availability of molecular probes. In agreement with previous studies, our results showed that (i) genetic markers in the transconjugants were stable in the absence of selection suggesting that the markers were being maintained through a homologous recombination event between segments of chromosomal DNA; (ii) transfer occurred in a DNase insensitive manner discrediting transformation as a possible transfer mechanism (Stewart and Carlson, 1986); and (iii) transfer did not appear to be due to a virus but rather cell-cell contact appeared to be required. Molecular probes to the resistance markers gave evidence for a homologous recombination event although we were not able to completely rule out the maintenance of two independent actively transcribed chromosomes (Punita et al., 1989; Suwanto and Kaplan, 1989). An inactive chromosome can sometimes be maintained in a limited fashion (Sanchez-Rivas et al., 1988), but if two chromosomes were present, they failed to segregate upon passage under nonselective conditions (data not given).

In contrast to previous studies (Barroso and Labarere, 1988; Labarere and Barroso, 1984), *M. pulmonis* transconjugants arose at frequencies ranging from $1 \times 10^{-4}$ to $1 \times 10^{-8}$ transconjugants per CFU. The frequencies between specific mating pairs were reproducible indicating that mating frequency was affected by factors independent of the mechanism of DNA transfer. Variability in genetic exchange frequency among *M. pulmonis* strains could have been due to differences in the chromosomal locations of the genetic markers. Some strains had
significantly higher frequencies than others when mated with the same series of strains (data not shown). This could be explained by recombinational hotspots in the chromosome (Vagner and Ehrlich, 1988) or a directional effect associated with genetic exchange (i.e., a Hfr-like element). In addition, our studies showed a lack of enhancement of mating frequency by PEG or progressively longer co-incubations of the parents in suspension which could indicate that mating was occurring at maximum frequency on agar surfaces.

Not all species of mycoplasmas or strains of *M. pulmonis* were capable of genetic exchange. Derivatives of *M. gallisepticum* ATCC19610 and *A. laidlawii* ATCC14192 did not participate in mating with either isogenic or nonisogenic strains (data not shown), and, therefore, genetic exchange was not a general feature of mycoplasmas. Recombinational proficiency did not appear to be a limiting factor in these matings since all strains and species of mycoplasmas examined were recombinationally proficient, as seen with their acceptance of integrative plasmids. Restriction/modification incompatibilities could be ruled out in these matings since isogenic strains failed to undergo exchange. Rather, genetic exchange appeared to involve at least one trypsin-sensitive specific factor in *M. pulmonis* ISM1499, and other factors might be involved at the membrane or chromosomal levels.

In order to gain a better understanding of the mechanism of genetic exchange, matings were performed with cells treated to alter surface properties (trypsin treatment) or reduce cell viability (heat and UV light treatments). Figure 1 shows the effects of UV irradiation on mating frequency when frequency was expressed as transconjugants per total CFU plated. In most cases there was a decrease in frequency, but this trend did not occur with Tn4001-containing strains where viability seemed to have no effect on mating frequency. This indicated that nonviable cells could participate in genetic exchange. The decrease in frequency resulted from expressing frequencies as a function of total CFUs or viable cells plated. Expression of frequencies as a function of CFUs of treated parent plated resulted in increased
mating frequencies (Fig. 3) because as the viability of the treated cell population decreased logarithmically, appearance of transconjugants resulted from the participation of nonviable cells. Dilution of one parent had no effect on mating frequency as long as sufficient numbers of cells were plated (Fig. 3A). When cells were UV-inactivated (Fig. 3C), frequency increased 4 to 5 logs supporting the idea that nonviable cells could participate in genetic exchange. In addition, Tn4001 containing strains could exchange markers in the absence of viability (Fig. 1C). Heat treatment gave an intermediate effect on mating frequency (Fig. 3B) showing that as heat destroyed mating activity in the nonviable cell population, mating frequency became more dependent on the remaining viable cells in the treated population. When one parent was treated with trypsin, mating frequencies decreased despite the marginal effect on cell viability (Fig. 4). Thus, genetic exchange in M. pulmonis apparently involves directly or indirectly a trypsin-sensitive membrane protein(s) which may be inactivated or denatured by heat and is not regenerated by viable cells when placed under selective conditions during agar mating.

Comparisons can be drawn between genetic exchange in M. pulmonis and conjugation or protoplast fusion in Gram positive bacteria. Transconjugants appear through cell-cell contact of donor and recipient strains on agar surfaces. Clumping, a pheromone-induced response during streptococcal mating (Dunny et al., 1978), is common in mycoplasmas requiring filtration of cell suspensions for the isolation of clones (Tully, 1983). In addition, genetic exchange appeared to be progressive, i.e., these events appeared to occur over time and doubly resistant colonies continued to arise from three to seven days after plating. This was best illustrated by a marked variation in colony size following the initial mating although once isolated, these variants were indistinguishable either genetically or phenotypically. This was similar to what was observed in Bacillus protoplast fusion where single colonies harvested from nonselective regeneration media gave rise to multiple phenotypes. This was interpreted as
a continual recombination between chromosomes interrupted at different chromosomal locations resulting in various phenotypes (Hopwood, 1981).

We propose that mycoplasmal genetic exchange involves a conjugation-like mechanism which proceeds by cell aggregation at the agar surface or in suspension followed by localized membrane fusion or the movement of DNA through protein channels between cells (Clewell, 1985; Fitzgerald and Gasson, 1988). A chromosomal Hfr-like genetic element might be involved since no plasmids have been identified in *M. pulmonis*. Mating might be limited to cells harboring this element which would explain why some *M. pulmonis* strains and mycoplasmal species fail to participate in genetic exchange. Even though these cell wall-less bacteria may be expected to undergo events similar to protoplast fusion in *Bacillus*, several lines of evidence illustrate the uniqueness of mating in mycoplasmas. These studies seem to indicate that discrete mycoplasmal genes or gene products may be involved in the acquisition of genetic diversity or the dissemination of selectively advantageous markers in *M. pulmonis*. Once additional mycoplasmal genetic markers are identified, studies can be designed to examine the mechanism of genetic exchange in greater detail.
ACKNOWLEDGMENTS

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LITERATURE CITED


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FIGURE 1. Effect of UV inactivation on mating frequency. [One parent in a mating mixture was exposed to UV light for the indicated time, and viability and mating frequencies were determined as described in Materials and Methods. Data are presented as Log CFUs of UV exposed parent and Log mating frequency versus time of exposure. Mating frequencies were calculated as number of doubly resistant colonies per total CFUs plated (exposed plus unexposed parent). Log CFUs (○); Log Mating Frequency (●). A: Mating between exposed ISM1501 (Tc<sup>R</sup> plasmid integrant) X ISM1506 (Tn<sup>4001</sup> insert); B: Mating between exposed ISM1507 (Tn<sup>916</sup> insert) X ISM1506 (Tn<sup>4001</sup> insert); C: Mating between exposed ISM1506 (Tn<sup>4001</sup> insert) X ISM1501 (Tc<sup>R</sup> plasmid integrant, upper curve) or ISM1507 (Tn<sup>916</sup> insert, lower curve); D: Mating between exposed ISM1503 (Gm<sup>R</sup> plasmid integrant) X ISM1501 (Tc<sup>R</sup> plasmid integrant).]
FIGURE 2. Effect of heat treatment on mating frequency. [Matings were performed between ISM1501 (Tc^R plasmid integrant) and ISM1506 (Tn4001 insert) after treatment of one parent at 56°C for 0, 15 or 30 min. Mating frequencies were expressed as number of doubly resistant colonies per total CFUs plated. A: ISM1501 heat-treated; B: ISM1506 heat-treated. Log CFUs (O); Log Mating Frequency (●).]
FIGURE 3. Effect of dilutions, heat and UV inactivation on mating frequency. [Mating strains were ISM1501 and ISM1506. Mating frequencies were expressed as number of doubly resistant colonies per CFU treated cells. For comparison, data were plotted Log Mating Frequency versus Log CFUs. A: ISM1501 diluted (○), ISM1506 diluted (●); B: ISM1501 heat-treated (○), ISM1506 heat-treated (●); C: ISM1501 UV-inactivated (○), ISM1506 UV-inactivated (●).]
Effect of trypsin on mating frequency. [Matings were performed between ISM1501 and ISM1506. Trypsin digestions were performed as described in Materials and Methods. Samples were removed at the indicated time intervals and used directly in agar matings. Log CFUs (○); Log Mating Frequency (●). A: ISM1501 trypsin-treated; B: ISM1506 trypsin-treated.]
FIGURE 5. DNA-DNA hybridization studies of *M. pulmonis* transconjugants. [Chromosomal DNAs were obtained from doubly resistant colonies arising from two mating mixtures (ISM1501 X ISM1503 and ISM1506 X ISM1507) as described in the text. Restriction enzyme digests of these DNAs (*Hind*II, A; *Eco*RI, B) were resolved in agarose gels and transferred to nylon membranes. The membranes were probed with $^{32}$P-labeled, *tetM*-specific pISM1002 (A) and Tn4001-specific pSK31 (B). Panel A: lanes: 1, ISM1499 (negative control); 2, ISM1501 (parent); 3, ISM1507 (parent); 4 - 6, transconjugants derived from ISM1501 X ISM1503 mating; 7 - 9, transconjugants derived from ISM1506 X ISM1507 mating. Panel B: lanes: 1, ISM1503 (parent); 2, ISM1506 (parent); 3, ISM1499; 4, ISM1501; 5 - 7, transconjugants derived from ISM1501 X ISM1503 mating; 8 - 10, transconjugants derived from ISM1506 X ISM1507 mating.]
GENERAL SUMMARY AND DISCUSSION

Although mycoplasmas have been studied extensively for their membrane properties and pathogenic potential, very little progress has been made in understanding the genetics of these organisms. This work entailed the development of methods and tools that lend themselves to genetic applications and the study of virulence properties of mycoplasmas.

In order to expand genetic capabilities in the mycoplasmas, a series of studies was begun to introduce genetic markers into mycoplasmas. Initially transposon Tn916 was introduced into *M. pulmonis* using the transformation method of Dybvig and Cassell (1987) and although this method yielded transformants, success was highly variable and frequencies of transformation were low (approximately $1 \times 10^{-8}$). Tn4001 was also introduced into *M. pulmonis* and transformants could be obtained by selecting for gentamicin resistance (Mahairas and Minion, 1989a). Hybridization studies confirmed the presence of both transposons and showed that insertion of Tn916 and Tn4001 into the *M. pulmonis* chromosome was essentially random (Mahairas and Minion, 1989a). The introduction of transposons Tn916 and Tn4001 into mycoplasmas represent powerful tools for the production of defined mutants (Kleckner et al., 1977; Kleckner, 1981) to study the virulence properties of these organisms. Challenge studies can be used to assess the contributions of potential virulence determinants using defined mutations in genes of interest generated by transposon mutagenesis. Unlike chemical and UV-induced mutations, transposon insertions are polar mutations and often result in the disruption of gene or operon functions. In addition, inserts giving the appropriate phenotypes can be mapped and the gene subcloned and further studied. The resistance genes of Tn916 and Tn4001 are the first genetic markers amenable for use as tools to study the genetics of the Mollicutes.
The antibiotic resistance genes of Tn916 and Tn4001 were cloned, and integrative plasmids constructed that carried a resistance gene and a segment of *M. pulmonis* chromosome and introduced into *M. pulmonis* by transformation. Using these integrative plasmids, we studied and transformation of *M. pulmonis* in the absence of the constraints of transposition and optimized the transformation protocol. Integrated plasmids could be introduced into *M. pulmonis* at frequencies between $1 \times 10^{-4}$ to $1 \times 10^{-8}$ transformants per CFU (Mahairas and Minion, 1989b).

The transformation protocol developed for *M. pulmonis* appears to be broadly applicable to other mycoplasmas and using this protocol Tn916 and Tn4001 have been introduced into *M. gallisepticum, M. capricolum, A. laidlawii* and several strains of *M. pulmonis*. These transposons appear to insert randomly in these strains as indicated by DNA-hybridization except that Tn4001 seems to insert site specifically in *M. capricolum* (data not shown). Additionally, integrated plasmids bearing tetracycline and aminoglycoside resistances have been introduced into *M. gallisepticum* and *M. capricolum*. Synthetic media have been described for *M. capricolum* (Rodwell, 1983) which when coupled with these recently developed genetic tools, allow the dissection of the metabolic capabilities possessed by mycoplasmas. Additionally, metabolic studies will provide additional genetic markers that will further advance genetic capabilities in these organisms.

The traditional cloning and analysis of mycoplasma genes have been carried out in *E. coli* (Razin, 1985). However, many mycoplasma genes do not express or express very poorly due to differences in codon (Yamao et al., 1985; Andachi et al., 1989) and promoter usage (Taschke and Herrmann, 1988). To circumvent these problems and to expand our genetic capabilities, methods were developed to carry out recombinant DNA manipulations in *M. pulmonis, M. gallisepticum* and *M. capricolum*. These three species were chosen for development because *M. pulmonis* is genetically the most developed mycoplasma and is
particularly amenable to genetic manipulations resulting in the technological carryover to other species, and *M. gallisepticum* and *M. capricolum* represent different groups within the genus. *M. gallisepticum* represents those species such as *M. pneumoniae* and *M. genitalium* which have higher G + C contents and *M. capricolum* is considered the prototype species of the group that has been studied most extensively. The cloning system utilizes integrated plasmids in the mycoplasma chromosome as regions of homology with cloning vehicles containing foreign DNA which insert by homologous recombination. These recombinant cloning vehicles are constructed in *E. coli* and moved via polyethylene glycol-mediated transformation into a suitable mycoplasma recipient.

Recombinant plasmids carrying fragments of *M. gallisepticum* chromosomal DNA were introduced individually into *M. pulmonis* by transformation. Approximately 70 independent *M. pulmonis::M. gallisepticum* recombinants were produced and screened with anti-*M. gallisepticum* sera absorbed against *M. pulmonis*. None of the recombinants expressed a detectable cloned *M. gallisepticum* gene product. A likely explanation for this was that only a few *M. gallisepticum* gene products were recognized by the hyperimmune sera and only 70 recombinants had been screened. Also, there may be genetic differences between these species which prevent proper expression the cloned genes. These could include codon usage and promoter recognition differences. The production of this gene library was cumbersome due to the need to introduce recombinant plasmids one at a time because of relatively low transformation frequencies and the need for high concentrations of plasmid DNA (Mahairas and Minion, 1989b). The best application of this method is the introduction, into the host mycoplasma of a single recombinant molecule that is already known to carry or express a gene of interest.

From this perspective recombinant plasmids were constructed that carried the structural gene of the P1 protein of *M. pneumoniae* (Su et al., 1987) and a *Brucella abortus* cell surface
protein (Mayfield et al., 1988). Each of these plasmids was transformed into *M. pulmonis* and the presence of the cloned sequences confirmed by hybridization. The *B. abortus* sequence was expressed from its native promoter in *E. coli*, but was found not to express in *M. pulmonis* when probed with monospecific polyclonal antisera (Mayfield et al., 1988). The P1 structural gene also did not express in *M. pulmonis* which could be explained by its separation from its regulatory sequences (Inamine et al., 1988). The P1 operon was reconstructed from subcloned fragments and introduced into *M. pulmonis*, but the P1 gene not expressed as determined by western blotting using anti-P1 monoclonal antibody. Again, differences in codon and promoter usage may account for the inability to detect recombinant P1 protein in *M. pulmonis*. It should be noted that a significant difference in the G + C content exists between *M. pneumoniae* (38%) and *M. pulmonis* (26%). Additionally the possibility exists that one or more of the P1 constructs supplied to us are either from an cryptic or defective P1 (Su et al., 1988) determinant or lack an essential regulatory element. Future studies to determine if P1 mRNA is produced may shed light on whether regulatory sequences of the operon allow transcription to proceed. Also, the recombinant P1 operon should be transformed into *M. gallisepticum* which is closer in G + C content (36%) to *M. pneumoniae* than *M. pulmonis*.

The integrated plasmid system can also be used as a chromosome mapping tool. The integrative plasmid vectors contain several rare restriction endonuclease cleavage sites such as *Sma*I and *Nol*I within the multiple cloning site. These same sites are present infrequently in the chromosome; *Sma*I cuts the *M. pulmonis* chromosome at five sites (Minion and Tigges, Iowa State University, Ames, IA. unpublished data). *Sma*I digestion of the chromosomal DNA of a plasmid integrant would result in four of the five original bands and two new bands as subfragments of the fifth original band. Since the *Sma*I site is located near the plasmid-chromosome junction, the plasmid encoded sequence should be associated with only one fragment. By partial digestion with *Sma*I and using intact plasmid as a probe, the insert and
every other Smal site can be mapped in a single blot. Additionally, since the digestion of the chromosome of a plasmid integrant results in one of the fragments containing a selectable marker and an origin of replication compatible with *E. coli*, the cloning of sequences adjacent to the site of integration can be accomplished in a single step.

Additional features of the mycoplasma cloning system include 1) the ability to do complementation and allelic exchange studies in the *Mollicutes*, and 2) the rescue of cloned sequences for further analysis in *E. coli*, i.e., deletion analysis, *in vitro* mutagenesis or sequencing analysis. The ability to introduce a specific DNA sequence in a predetermined location on the host chromosome allows the complementation of specific mutations. Furthermore, mutant genes can be created *in vitro* by interrupting the cloned sequences with either *tetM* or the aminoglycoside resistance determinant of Tn4001 resulting in an interposon (Black and Falkow, 1987; Stibitz et al., 1986). Introduction of this mutant gene is accomplished by selecting for the phenotypic marker while scoring for the desired mutant phenotype. Resistant colonies containing the interposon that have phenotypically lost the marker of interest should represent double crossover events generated by allelic exchange.

An important step in improving the efficiency of this cloning system would be to increase the frequency of which a cloning vector can be introduced. Vagner and Ehrlich (1987) studied recombination around the chromosome of *Bacillus subtilis* and found that the frequency of the introduction of a plasmid marker into a homologous sequence present at different locations on the chromosome varied with respect to the location of the recombination site. In the context of developing a cloning system that is dependent on recombination at a chromosomal (integrated plasmid) site, it would be advantageous to identify a plasmid that is integrated in a region where recombination occurs at the highest frequency. To carry out this study in *M. pulmonis* a small library (10-20) of transposon insertions (either Tn4001 or Tn916) can be generated and the chromosomal location of the inserts determined by Field
Inversion Gel Electrophoresis. An integrating plasmid that bears both tetM and the cloned aminoglycoside resistance gene is then used to transform strains carrying the transposon insertions. The transposon acts as a portable region of homology to the integrating plasmid and selection for the counterselectable marker should result in site specific recombination within the transposon sequences. Strains that carry the integrated plasmid sequences in areas where recombinational frequencies are highest represent candidates as recipient strains for cloning experiments. Using this approach it may be possible to make significant improvements in the ability to introduce recombinant DNA in M. pulmonis.

An interesting application of the cloning system developed for M. pulmonis is the potential as a vaccine delivery vehicle. Presently there is no efficient method of delivering antigens to the bronchial associated lymphoid tissues (BALT). Davis et al. (1982) have shown that a pronounced hyperplasia of the BALT is accompanied by respiratory infections of M. pulmonis. Genetically engineered strains of M. pulmonis that express and present antigen on their surface could serve as a method of controlling and studying immunological properties of the BALT.

The construction of genetically marked strains of mycoplasma has allowed the detailed examination of genetic exchange in the Mollicutes. Barroso and Labarere (1988) first described genetic exchange using UV-induced mutations in S. citri but they were unable to follow the chromosomal events during the exchange because of the nature of their markers. Using tetracycline and gentamicin markers we examined genetic exchange in M. pulmonis. The mixing of genetically marked strains of M. pulmonis on the agar surface resulted in the appearance of doubly resistant transconjugants at frequencies between 1 x 10^-4 and 1 x 10^-8 transconjugants per CFU. Transconjugants from these mating had integrated plasmid and transposon markers in the same chromosomal locations as the parents. The M. pulmonis mating system demonstrated a marked insensitivity to DNase ruling out natural transformation.
as a means of transfer. Also there was no enhancement of genetic exchange by the addition of polyethylene glycol as was previously reported by Barroso and Labarere (1988), and marker transfer was not effected by the addition of EDTA or calcium chloride. Viral involvement was ruled out as a mechanism of marker transfer due to the inability of filtered lysates to transfer resistance, the inability to visualize viral particles both in cell culture supernatants and on the surface of cells by electron microscopy and the lack of effects due to EDTA or calcium.

Genetic exchange experiments conducted over a range of temperatures were inconclusive except that the frequency of marker transfer did not vary over the growth range of *M. pulmonis*. Additionally, the frequency of genetic exchange was proportional to the number of viable cells of each parent when cells were killed by heat (55°C) indicating that following heat treatment only viable cells could participate in marker transfer. Further studies using UV-inactivated cultures showed that nonviable cells could transfer their resistance markers effectively. Tn4001-containing strains were particularly effective and participated in genetic exchange despite extensive UV-killing. Experiments in which one or both parents were treated with trypsin to remove surface proteins implicated surface components that play a role in genetic exchange.

To examine if genetic exchange could occur in infected animals, attempts were made to establish recombinant *M. pulmonis* infections in the respiratory tracts of germ-free mice. Several attempts to colonize the respiratory tracts of these mice failed, and infection could not be established even following treatment of the mice with cyclophosphamide, an agent that kills rapidly dividing cells such as those of the immune system (Singer et al., 1972). *M. pulmonis* has been shown to lose the ability to colonize respiratory systems of mice (Davidson et al., 1988), and since all engineered strains of *M. pulmonis* used in these studies are derived from the high passage strain ISM1499, it is possible that factors required for colonization have become cryptic or were lost. Davidson et al. (1988) assessed the virulence potential of several
strains of *M. pulmonis* and showed that of the commonly studied strains of *M. pulmonis*, the UAB-CT strain was most virulent. Transposon and integrated plasmid markers introduced into a low passage CT strain marker should allow the colonization of mice with subsequent infection and an examination of genetic exchange during infection.

In conclusion, it is clear that the advent of genetic capabilities such as the use of transposons and shuttle vectors for gene cloning will facilitate the detailed study of the basic life functions and pathogenic potential of the mycoplasmas. The development of methods to analyze and detect mutants in virulence-associated activities and metabolic deficiencies remains the next major obstacle to the broad application of this genetic system. In addition studies of genetic exchange in *M. pulmonis* may give a new perspective on the mechanisms operative in Gram positive conjugation.
ADDITIONAL LITERATURE CITED


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APPENDIX ISOLATION AND PURIFICATION OF THE DNA-DEPENDENT RNA POLYMERASE OF *MYCOPLASMA PULMONIS*
INTRODUCTION

The cloning of mycoplasma genes in *E. coli* has shown that mycoplasma gene regulatory elements function poorly in *E. coli*, and that truncated proteins often result, possibly due to aberrant codon usage in mycoplasmas (Minion, unpublished observations; Yamao et al., 1985). Previous reports have shown that some mycoplasma genes are capable of expression in *E. coli* (Taylor et al., 1983), but specific details concerning the structure of the regulatory elements has not been obtained. In addition, our results with screening mycoplasmal chromosomal gene banks constructed in *E. coli* lambda phage has shown that most mycoplasma genes are not expressed from their native regulatory elements. This indicates that the *E. coli* transcriptional and translational machinery cannot utilize mycoplasma gene regulatory sequences. We have chosen to examine this problem at the transcriptional level by purifying *Mycoplasma pulmonis* RNA polymerase and comparing its activities with *E. coli* RNA polymerase in vitro.
MATERIALS AND METHODS

Mycoplasma Culture Conditions and Buffers

M. pulmonis (UAB 6510) was grown in standard mycoplasma medium containing 10% agamma horse serum, 2.5% fresh yeast extract, 1% glucose, and 2.5 \( \mu \)g/ml Cefobid. Organisms were grown statically at 37°C to mid log phase.

Buffer A consisted of 10 mM Tris - 10 mM MgCl\(_2\) - 1 mM EDTA - 50 mM KCl - 10% glycerol (pH 8.0). Buffer B consisted of 10 mM Tris - 2 mM MgCl\(_2\) - 1 mM EDTA - 50 mM KCl - 20% glycerol (pH 8.0). Cell lysis buffer consisted of Buffer A plus 1 mM each phenyl methyl sulfonyl fluoride and Aprotinin.

Preparation of Cell-Free Extract

Cells from 10 liters of mycoplasma broth were collected by centrifugation and washed with Buffer A and resuspended in 25 ml of the same buffer (4°C). Sodium deoxycholate was added to 0.5% and the cells incubated on ice for 15 min. The suspension was then passed through a French press twice at 8,000 psi to reduce viscosity, and centrifuged at 30,000 \( \times \) g for 10 min to remove cell debris. The resulting clear amber supernatant was designated as the cell free extract.

Affinity Chromatography

A Heparin Agarose (BioRad) column (30 ml bed volume) was poured and equilibrated with Buffer A followed by application of the cell free extract. The column was washed extensively with Buffer A until the optical density (280 nm) reached baseline. The bound proteins were eluted with a 100 ml 50 mM - 1 M linear gradient of KCl in Buffer A. Column eluent was monitored by optical density (280 nm) and protein containing fractions assayed for RNA polymerase activity as described below. Active fractions were pooled, dialyzed against 100 volumes of Buffer B, and applied to a single-stranded DNA agarose column (15 ml bed
volume) prepared by the method of Schaller et al. (1972) and equilibrated with Buffer B. The pooled RNA polymerase containing fractions were washed through the column with Buffer B until the optical density (280 nm) reached baseline, and then eluted with a 50 ml 50 mM - 1 M linear KCl gradient in Buffer B. Fractions were assayed for RNA polymerase activity and those active fractions pooled, dialyzed against buffer A containing 50% glycerol for storage at -70°C.

In Vitro Transcription Assay Conditions

For the quantitation of polymerase activity during purification, the 100 μl assay mixture contained 100 mM Tris (pH8.0) - 10 mM MgCl2 - 0.1 mM EDTA - 1.6 mM spermidine - 0.5 mM dithiotreitol - 0.4 mM each ATP, GTP, CTP, and [3H]-UTP (2.5 x 10^5 cpm/nmol) or [32P]-UTP (250 cpm/pmol), and 100 μg/ml DNA template (plasmid or chromosomal) (Davison et al., 1979). RNA synthesis was initiated by the addition of 5 μl of the column fraction followed by incubation at 37°C for 15 min. The reaction was terminated by the addition of ice cold 10% trichloroacetic acid and 50 mM sodium pyrophosphate. Acid insoluble material was collected on glass fiber filters and washed with 4 vol of 5% trichloroacetic acid, 25 mM sodium pyrophosphate followed with 2 washes with ethanol. Radioactivity was measured by liquid scintillation. To examine rifampin susceptibility, 5 μg/ml of rifampin was added to the reaction mixture prior to the incubation at 37°C. Optimal KCl concentrations was determined by adding the appropriate concentrations of a 1 M KCl solution.
RESULTS

RNA Polymerase Purification

The chromatographic behavior of *M. pulmonis* cell-free extract on Heparin agarose is shown in Figure 1. RNA polymerase activity eluted with a linear salt gradient has a double peak between 0.5 M and 1 M KCl. Active fractions were pooled and dialyzed against Buffer B and applied to a ssDNA agarose column. The elution profile of the pooled fractions from the Heparin agarose column on the ssDNA agarose column is shown in Figure 2. Polymerase activity eluted with a linear salt gradient as a single peak at ionic strength of 0.2 M KCl. Active fractions were analyzed by SDS-PAGE and contained subunit proteins of similar molecular weights as those of *E. coli* RNA polymerase (data not shown).

Optimization of RNA Polymerase In Vitro Assay Conditions

To study RNA polymerase assay conditions, KCl concentration was varied and different DNA templates (covalently closed circular vs. linear) were examined. Figure 3 demonstrates the effect of KCl concentration on *M. pulmonis* RNA polymerase activity. The optimum concentration was 100 mM KCl. This compares with the 150 mM KCl concentration optimum for *E. coli* RNA polymerase (New England BioLabs, Inc.). DNA concentration was examined at 10 and 100 µg/ml and 100 µg/ml was found to be optimal. Covalently closed circular DNA consistently gave a 2-fold increase in label incorporation over linear (chromosomal) DNA. Studies with Rifampin showed a typical inhibition of eubacterial RNA polymerase (*E. coli*), but did not affect the mycoplasma enzyme (Fig. 4).
CONCLUSIONS

1. The conditions for chromatographic purification of \textit{M. pulmonis} RNA polymerase was established using Heparin Agarose and ssDNA agarose affinity chromatography.

2. Optimal conditions for in vitro transcription reactions using \textit{M. pulmonis} RNA polymerase were also established and included 100 mM KCl with 100 μg/ml DNA template.

3. \textit{M. pulmonis} RNA polymerase was found to be insensitive to Rifampin, a characteristic of all mycoplasma RNA polymerases. This is in contrast to common eubacteria which are inhibited by Rifampin.

4. SDS-PAGE analysis of chromatographic fractions revealed two subunits similar in apparent molecular weight to the β and β' \textit{E. coli} RNA polymerase subunits (155,000 kDa and 145,000 kDa, respectively).
FIGURE 1. Chromatography of *M. pulmonis* cell free extract on Heparin Agarose. [Cell free extract from *M. pulmonis* was loaded onto a Heparin Agarose column and eluted with increasing KCl concentrations. RNA Polymerase activity eluted in two peaks at an ionic strength between 0.5 and 1 M KCl.]
FIGURE 2. Chromatography of pooled fractions from a Heparin Agarose column on a ssDNA Agarose column. [Fractions containing RNA polymerase activity from a Heparin Agarose column were pooled, dialyzed against Buffer B, and chromatographed on a ssDNA Agarose column as described in Materials and Methods. RNA Polymerase activity eluted as a single peak at an ionic strength between 0.2 and 1M KCl concentrations.]