Genetic Exchange of Transposon and Integrative Plasmid Markers in *Mycoplasma pulmonis*

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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 3.3×10^{-4} to 6.4×10^{-8} transconjugants per CFU. Examination of chromosomal DNA from transconjugants by hybridization revealed that the transposons or integrated plasmids were in the same chromosomal locations as in 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 before 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*; 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 conjugationlike mechanism involving specific trypsin-sensitive membrane components.

Members of the class *Mollicutes* are procaryotes characterized by the lack of a cell wall and a small genome (800 kilobases) that is thought to be the minimum genetic information necessary for autonomous existence (18). This limited genomic potential has resulted in organisms devoid of several biosynthetic pathways, including those involving nucleic acid precursors and membrane phospholipids (18). Mycoplasmas therefore grow only in complex media, and this property has prevented the use of metabolic markers for genetic studies. Antibiotic resistance markers are rare in members of the class *Mollicutes*, and only recently have heavy-metal-resistant mutants been used in genetic studies (1). As a result, 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 commensal organisms or pathogens. Mycoplasmas produce significant diseases in humans and in animal populations, and they 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 *Mycoplasma hominis*. Roberts et al. (20) first reported the occurrence of *tetM* sequences in clinical isolates of *M. hominis*, and Roberts and Kenny subsequently demonstrated exchange of Tn916 from *Streptococcus faecalis* to *M. hominis* (19). The basis for this type of exchange was not addressed, but it presumably occurred through conjugal properties associated with Tn916 (6-8, 22). If mycoplasmas can participate in genetic exchange with streptococci or other bacteria in the natural environment, as is 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 (1, 11), who described the exchange of UV-induced chromosomal mutations in *Spiroplasma citri*. To examine this phenomenon in *Acholeplasma laidlawii* and the sterol-requiring mycoplasmas, genetically marked strains of *A. laidlawii*, *Mycoplasma pulmonis*, and *Mycoplasma gallisepticum* were constructed by using Tn4001 (12-14), Tn916 (15), and integrative plasmids derived from their cloned antibiotic resistance genes (15). Genetic exchange was followed by hybridization studies of chromosomal DNA by 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 effects of various treatments on genetic exchange were also examined.

MATERIALS AND METHODS

Bacteria and culture media. The mycoplasma strains used are described in Table 1. *Escherichia coli* was maintained as previously described (16). Mycoplasmas were grown in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 10% immunoglobulin-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 μ g of Cefobid (Pfizer, Inc., New York, N.Y.) per ml. PPLO agar also contained 1% Noble agar (Difco). Mating mixtures were plated on PPLO agar containing 15 μ g of gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) and 10 μ g of tetracycline (Sigma) per ml, and the plates were 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 (15). Restriction enzymes were purchased from Bethesda Research Lab-

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TABLE 1. Mycoplasmal strains

Strain	Relevant genotype and properties	Source or reference
<i>M. pulmonis</i>		
UAB5782		G. H. Cassell
UABCT		M. K. Davidson
66		G. H. Cassell
JB		G. H. Cassell
ISM1499	UAB6510 high-passage strain	15
ISM1501	Ω (ISM1499::pISM1005)1005 Tc ^r	15
ISM1503	Ω (ISM1499::pISM1007)1007 Gm ^r	15
ISM1506	Ω (ISM1499::Tn4001)5 Gm ^r	This study
ISM1507	Ω (ISM1499::Tn916)101 Tc ^r	This study
ISM3001	Virulent low-passage rat isolate	10
<i>M. gallisepticum</i>		
PG31	Prototype strain	ATCC 19610
ISM1575	Ω (19610::Tn916)750 Tc ^r	This study
ISM1576	Ω (19610::Tn916)751 Tc ^r	This study
ISM1577	Ω (19610::Tn4001)700 Gm ^r	This study
ISM1578	Ω (19610::Tn4001)701 Gm ^r	This study
<i>A. laidlawii</i> B		
ATCC 14192		R. Ross
ISM1585	Ω (14192::Tn916)550 Tc ^r	This study
ISM1586	Ω (14192::Tn916)551 Tc ^r	This study
ISM1587	Ω (14192::Tn4001)500 Gm ^r	This study
ISM1588	Ω (14192::Tn4001)501 Gm ^r	This study

oratories, Inc. (Gaithersburg, Md.) or Promega Corp. (Madison, Wis.) and used as instructed by the manufacturers. 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 previously (15). Integrative vectors consisted of a pMB1 *E. coli* replicon, one of the resistance markers from either Tn4001 or Tn916, and a 3- to 5-kilobase fragment of chromosomal DNA from the recipient mycoplasma (15). Integration of plasmid and transposon markers was confirmed by hybridization. Matings were performed between isogenic and nonisogenic strains of *M. pulmonis* by 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* by using derivatives marked with transposable elements. Matings were generally performed by directly plating 50 μ l of each parent (1×10^9 to 5×10^9 CFU/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 the cells at 37°C at various time intervals before plating them on selective media. For *M. gallisepticum* and *A. laidlawii*, some matings were performed by mixing strains and centrifuging them for 2 min at $12,000 \times g$ before plating. The uptake of DNA from the surrounding environment was tested by incubating *M. pulmonis* strains in the presence of 10 μ g of purified chromosomal DNA from a transposon-containing strain or 10 μ g of pISM1005 plasmid DNA (15). Before plating on selective media, mixtures of mating pairs were treated with various concentrations of PEG (10 to 40%) or 100 mM calcium chloride. Resistant colonies were cloned

by passing cultures through 0.2- μ m-pore-size filters as described previously (25), and chromosomal DNAs were analyzed by blot hybridization (16). Mating frequencies were determined by dividing the number of doubly resistant organisms by the total number of CFU of both parents per mating mixture or, in some instances, by dividing the number of doubly resistant organisms by the number of CFU 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 for the presence of bacteriophages. DNase (Sigma) was added to cell suspensions (250 μ g per reaction) and spread on agar surfaces (250 μ g per plate). To examine temperature effects, donor cells were treated at 56°C for 0, 15, and 30 min before their use in matings. To examine trypsin sensitivity, cell suspensions (10^9 CFU/ml) in phosphate-buffered saline were treated with trypsin (1 mg/ml) at 37°C, and cells were removed at the indicated time points and used directly in mating experiments. The effect of UV exposure 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 were removed at various time intervals. Viability was determined in all treated samples by plating serial dilutions on nonselective medium, and mating efficiencies were 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 supernatants by passage through three 0.1- μ m-pore-size filters (MicronSep mixed cellulose esters; Fisher Scientific Co., Pittsburgh, Pa.) and testing for viable organisms by culture on PPLO agar. To control for nonspecific adherence of bacteriophages to the filters, λ and P1L4 lysates were filtered and decreases in titer were determined (4, 16). *M. pulmonis* ISM1499 was examined for viruses by examining negative-stained (3) pellets of culture supernatants ($200,000 \times g$, 4 h) by transmission electron microscopy and by plaque-forming ability on mycoplasmal lawns (9).

DNA-DNA hybridizations. Chromosomal DNAs from transconjugants were prepared as previously reported (15). Restriction digests consisted of 4 to 5 μ l of agarose containing chromosomal DNA previously melted at 70°C and equilibrated to 37°C, 2 μ l of $10\times$ enzyme buffer, and 3 to 5 U of restriction enzyme in a total volume of 20 μ l. Samples were digested for 1 h, 5 μ l of electrophoresis sample buffer was added, and 10- μ l samples were loaded onto 0.55% agarose gels in Tris-borate buffer (16). DNA fragments were then transferred to GeneScreen (Dupont, NEN Research Products, Boston, Mass.) by capillary transfer. DNA probes were labeled with [³²P]dCTP by the Multiprime labeling system (Amersham Corp., Arlington Heights, Ill.).

RESULTS

Integrating plasmids and strain constructions. *M. pulmonis* ISM1501 and ISM1503 that harbor integrated plasmids have been described elsewhere (15). In addition, *M. pulmonis*, *M. gallisepticum*, and *A. laidlawii* strains carrying the transposons Tn4001 and Tn916 were constructed (Table 1).

Mating in *M. pulmonis*. The frequency of genetic exchange between *M. pulmonis* ISM1499-derived isogenic strains varied from 3.3×10^{-4} to 6.4×10^{-8} transconjugants per CFU,

FIG. 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 CFU of UV-exposed parent and log mating frequency versus time of exposure. Mating frequencies were calculated as number of doubly resistant colonies per total CFU plated (exposed plus unexposed parent). Symbols: ○, log CFU; ●, log mating frequency. (A) Mating between exposed ISM1501 (Tc^r plasmid integrant) $\times$ ISM1506 ($Tn4001$ insert); (B) mating between exposed ISM1507 ($Tn916$ insert) $\times$ ISM1506 ($Tn4001$ insert); (C) mating between exposed ISM1506 ($Tn4001$ insert) $\times$ ISM1501 (Tc^r plasmid integrant; upper curve) or ISM1507 ($Tn916$ insert; lower curve); (D) mating between exposed ISM1503 (Gm^r plasmid integrant) $\times$ ISM1501 (Tc^r plasmid integrant).

depending on the specific mating pair (data not shown). Experiments were performed eight times with different lots of media. Exchange frequencies between pairs were highly reproducible, with standard errors usually less than 20%. Mating occurred between strains containing both integrated plasmids and transposon inserts. In all cases, $Tn4001$ -containing strains mated at higher frequencies than did pISM1003 plasmid integrants (data not shown), and in some matings, differences were significant ($P < 0.05$). Spontaneous mutants arose at a frequency of less than 5×10^{-9} transconjugants per CFU. *M. pulmonis* ISM3001, JB, 66, and UAB5782 failed to mate either with isogenic strains or with permissive ISM1499-derived strains even in the presence of PEG (data not shown). $Tn916$ derivatives of *M. pulmonis* UABCT mated readily with Gm^r 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 to 40%), 100 mM calcium chloride, or 20 mM EDTA to mating mixtures had no effect on mating frequencies (data not shown). In addition, extended incubation of mating mixtures in broth before plating did not increase mating frequency.

The effect of UV exposure on mating frequencies is shown in Fig. 1. One parent of a mating mixture was exposed to UV light for various amounts of time, and the CFU and mating frequencies were determined. Shown are representative samples from experiments in which each type of parent, transposon insertion or plasmid integrant, Tc^r or Gm^r marker, was irradiated. Mating frequencies are expressed as the number of transconjugants per total CFU plated. Exposure of all strains except those harboring $Tn4001$ to UV light resulted in a decrease in transfer frequency paralleling the decrease in viability (Fig. 1A, B, and D). This was not true

FIG. 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 $56^\circ C$ for 0, 15, or 30 min. Mating frequencies are expressed as number of doubly resistant colonies per total CFU plated. (A) ISM1501 heat treated; (B) ISM1506 heat treated. Symbols: ○, log CFU; ●, log mating frequency.

for the $Tn4001$ -containing strains, in which mating frequency was not decreased during an 8- to 10-log loss in viability (Fig. 1C).

Figure 2 shows the effects of heat inactivation of one parent in a mating mixture. After exposure of mycoplasmal suspensions for 15 min at $56^\circ C$, viability was typically reduced 3 to 4 logs. When expressed as transconjugants per total CFU plated, frequency of transfer paralleled the decrease in viability even with strains containing $Tn4001$ inserts. This was in contrast to the results with UV inactivation (Fig. 1C). In Fig. 3, frequencies are expressed as transconjugants per viable treated parent only and plotted versus log CFU of treated parent. To measure the effect of viable cell density on mating frequency, one parent was diluted to reduce the number of CFU plated (Fig. 3A). Under these conditions, at least 10^7 cells of each parent were needed to obtain transconjugants, and mating frequency was not affected by the viable cell density of the treated (diluted) parent. In contrast, mating frequencies increased 1 to 2 logs after heat treatment (Fig. 3B) and as much as 5 logs after UV inactivation (Fig. 3C).

Trypsin treatment of cell suspensions reduced mating

FIG. 3. Effect of dilutions, heat, and UV inactivation on mating frequency. Mating strains were ISM1501 and ISM1506. Mating frequencies are expressed as number of doubly resistant colonies per CFU of treated cells. For comparison, data are plotted as log mating frequency versus log CFU. (A) ISM1501 (○) and ISM1506 (●) diluted; (B) ISM1501 (○) and ISM1506 (●) heat treated; (C) ISM1501 (○) and ISM1506 (●) UV inactivated.

FIG. 4. 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. Mating frequencies are expressed as number of doubly resistant colonies per total CFU plated. Symbols: ○, log CFU of treated parent; ●, log mating frequency. (A) ISM1501 trypsin treated; (B) ISM1506 trypsin treated.

frequency (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 CFU plated or per CFU of the treated parent plated did not significantly alter the frequency of mating. This result was in contrast to findings for heat treatment or UV inactivation (compare Fig. 1 and 2 with Fig. 3).

To examine the possibility that a virus was responsible for gene transfer, bacteriologically sterile filtrates were prepared and tested in standard mating mixtures from culture supernatants of two ISM1499 derivatives giving the highest genetic exchange frequencies, ISM1501 and ISM1506. No doubly resistant transductants were obtained in any of the crosses examined (data not shown). When concentrated culture supernatants 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* (9).

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. In some instances, 35% PEG was added to the mating mixtures to determine whether a membrane barrier could be overcome with a fusogenic agent. The interspecies matings were performed with strains containing transposons to determine whether sequential transposition of either Tn916 (27) or Tn4001 could overcome differences in DNA homology. 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).

DNA-DNA hybridization. The nature of the genetic event that occurred during mating was examined by DNA-DNA hybridization (Fig. 5). The cross ISM1501 × ISM1503 represented matings between strains harboring integrative plasmids, and the cross ISM1506 × ISM1507 represented matings between strains harboring transposons (Table 1). At least 30 to 40 isolated colonies from each mating mixture were examined separately with the *tetM* (Fig. 5A)- and Tn4001 (Fig. 5B)-specific probes to confirm the location of the resistance markers. Plasmid pISM1002 (15) was used as a *tetM*-specific probe; because of an internal *Hind*III restriction endonuclease site in the *tetM* sequences, two bands occurred upon hybridization analysis of chromosomal DNAs from tetracycline-resistant strains containing either an integrative plasmid or Tn916. Plasmid pSK31 (12), a staphylococcal plasmid containing Tn4001, served as the Tn4001-specific probe, since it shared no homology with the integrative vectors other than the gentamicin resistance marker. When reacted with *Eco*RI-digested DNAs from Gm^r Tc^r transconjugants, pSK31 recognized only a single band containing Tn4001 or a plasmid derivative (Fig. 5B). Transconjugants contained resistance markers in the same chromosomal locations as the parents whether matings occurred

FIG. 5. DNA-DNA hybridization studies of *M. pulmonis* transconjugants. Chromosomal DNAs were obtained from doubly resistant colonies arising from two mating mixtures (ISM1501 × ISM1503 and ISM1506 × ISM1507) as described in the text. Restriction enzyme digests of these DNAs (*Hind*III [A] and *Eco*RI [B]) were resolved in agarose gels and transferred to nylon membranes. The membranes were probed with ³²P-labeled, *tetM*-specific pISM1002 (A) and Tn4001-specific pSK31 (B). (A) Lanes: 1, ISM1499 (negative control); 2, ISM1501 (parent); 3, ISM1507 (parent); 4 to 6, transconjugants derived from ISM1501 × ISM1503 mating; 7 to 9, transconjugants derived from ISM1506 × ISM1507 mating. (B) Lanes: 1, ISM1503 (parent); 2, ISM1506 (parent); 3, ISM1499; 4, ISM1501; 5 to 7, transconjugants derived from ISM1501 × ISM1503 mating; 8 to 10, transconjugants derived from ISM1506 × ISM1507 mating.

between strains harboring integrative plasmids or transposons.

To test the possibility that diploidy resulting from membrane fusion may have been responsible for the double resistance (21), 20 transconjugants from two independent crosses were filter cloned twice on nonselective medium, and the resultant colonies were tested for the Gm^r Tc^r phenotype. In every case, the total number of CFU on nonselective medium equaled the number of doubly resistant CFU, 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 have arisen from a spontaneous mutation of either parent.

DISCUSSION

Genetic exchange in *S. citri* has been previously described (1, 11). The results presented here differ from those of previous studies, since exchange in *M. pulmonis* was not affected by PEG or incubation of the parents together in suspension before plating. 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 may be 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 (23), and (iii) transfer did not appear to be due to a virus, but rather cell-cell contact appeared to be required.

In contrast to previous studies (1, 11), *M. pulmonis* transconjugants arose at frequencies ranging from 10^{-4} to 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. For instance, variability in genetic exchange frequency among *M. pulmonis* strains could have been due to differences in the chromosomal locations of the genetic markers, since some strains had significantly higher frequencies than others when mated with the same series of strains (data not shown). This result could be explained by recombinational hot spots in the chromosome (26) or a directional effect associated with genetic exchange (i.e., an Hfr-like element). In addition, our studies showed a lack of enhancement of mating frequency by PEG or progressively longer 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* ATCC 19610 and *A. laidlawii* ATCC 14192 did not participate in mating with either isogenic or nonisogenic strains (data not shown); therefore, genetic exchange was not a general feature of mycoplasmas. This block in mating could not be overcome with PEG (data not shown) (1) even within isogenic strains, which suggested that mating involved more than simple membrane fusion. 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 shown by their acceptance of integrative plasmids. Restriction-modification incompatibilities could be ruled out in these matings, since some isogenic strains failed to undergo exchange. Rather, genetic exchange appeared to involve at least one trypsin-sensitive factor in *M. pulmonis* ISM1499. Other factors might be involved at the membrane or chromosomal levels.

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. In most cases, there was a decrease in frequency when it was expressed as transconjugants per total CFU plated. This trend did not occur with Tn4001-containing strains, in which UV inactivation seemed to have no effect on mating frequency. This result clearly indicated that nonviable cells could participate in genetic exchange. To determine whether viable cell density affected mating frequency, one parent in a mating mixture was diluted to reduce cell numbers, and frequencies were expressed as a function of CFU of diluted parent plated (Fig. 3A). No change in frequency was observed, but at least 10^7 cells were needed for detection of transconjugants (data not shown). This was clearly not the case when cell populations were UV inactivated (Fig. 1) or heat treated (Fig. 2), because transconjugants arose when fewer than 10^7 viable cells were plated. In addition, Tn4001-containing strains could exchange markers in the total absence of viability (Fig. 1C). Therefore, both viable and nonviable cells participated in genetic exchange.

To prevent masking of treatment effects by the higher numbers of viable untreated cells, data were expressed as mating frequency versus CFU of viable treated parent and compared (Fig. 3). When cells were UV inactivated (Fig. 3C), frequency increased 4 to 5 logs, showing that UV inactivation had little effect on mating activity even though viability was significantly reduced (Fig. 1). Heat treatment gave an intermediate effect on mating frequency (Fig. 3B), showing that heat destroyed mating activity in both the viable and nonviable cell populations.

Mating frequencies decreased as a function of trypsin treatment despite the marginal effect on cell viability (Fig. 4). Thus, genetic exchange in *M. pulmonis* apparently involves a trypsin-sensitive membrane protein(s) that may be inactivated or denatured by heat and is not regenerated by viable cells when placed under selective conditions during agar mating.

Molecular probes to the resistance markers ruled out unstable heterozygosity, although we were not able to completely rule out the maintenance of two independent actively transcribed chromosomes (17, 24). An inactive chromosome can sometimes be maintained in a limited fashion (21), but if two chromosomes were present, they failed to segregate upon passage under nonselective conditions (data not shown). Two additional lines of evidence argue against diploidy. First, variation in mating frequencies between different mating pairs argues against protoplastlike fusion and heterozygous diploidy; frequency would have been a function of fusion, and it would not have varied between different pairs. Second, diploidy does not explain the unusual results of the experiments involving UV inactivation of Tn4001-containing strains. There is the possibility that two mechanisms may be operative in genetic exchange, one in which a unidirectional mating involving sequential transposition of Tn4001 is occurring and a second in which mating may be bidirectional and involve membrane fusion and the movement of chromosomal segments (1). It should be noted that Tn4001 has not been reported to possess conjugal properties. It is more likely that mating is independent of the marker or treatment involved. The unusual results with strains harboring Tn4001 may be explained by sequential transposition of Tn4001 due to UV-induced damage to DNA

and induction of DNA repair mechanisms. Studies are under way to examine this possibility.

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 (5), is common in mycoplasmas requiring filtration of cell suspensions for the isolation of clones (25). In addition, genetic exchange appeared to be progressive; i.e., these events appeared to occur over time, and doubly resistant colonies continued to arise 3 to 7 days after plating. This was best illustrated by a marked variation in colony size after 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 result was interpreted as a continual recombination between chromosomes interrupted at different chromosomal locations resulting in various phenotypes (10).

We propose that mycoplasmal genetic exchange involves a conjugationlike mechanism that 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 (2, 6). 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* sp., 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.

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