A NOVEL METHOD FOR ISOLATION OF CONDITIONAL-LETHAL MUTANTS OF MYCOPLASMA AND MYCOPLASMAVIRUSES

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SUMMARY

A technique is presented that is useful for selecting conditional-lethal mutants of mycoplasma cells and viruses. The method is based on growing mycoplasma on Millipore filters. Mutants can be isolated directly from filters seeded with mycoplasma. The filters can be transferred from condition to condition, acting as its own "master" and "replica" template. Virus mutants from the non-lytic Mycoplasma Group L1 and L2 viruses can also be picked from filters seeded with infected cells. This method is analogous to classical "replica plating" which is not a practical technique for mycoplasmas.

INTRODUCTION

Genetical studies of mycoplasma, the smallest free-living cells, have been largely unsuccessful (cf. reviews 10, 14, 15, 16). Two major problems in this nascent research area have been the limited types of available mutants (16) and the unsuccessful adaption of classical bacterial genetic methods for use with mycoplasmas (6). The discovery of viruses infecting mycoplasma (3, 7) and the observation that mycoplasmas could be transfected (8) suggest mechanisms for genetic exchange (i.e. transformation, transduction) is possible for mycoplasmas. This report notes a novel technique that allows for isolation of conditional lethal mutants of mycoplasma cells and mycoplasmaviruses.

MATERIALS AND METHODS

Medium, Cells, and Viruses

Tryptose broth or tryptose agar (9) supplemented with 10% heat inactivated calf serum (Grand Island Biological Co., Grand Island, N.Y.) was used for this study. The cell used was Acholeplasma laidlawii JA1, previously described by Liss and Maniloff (7). Mycoplasmaviruses from Group L1, MVL51, Group L2, MVL2, and Group L3, MVL3 were used. These viruses have been previously described (7, 4).

Mutagenesis

JA1 was treated with the mutagen N-methyl-N'-nitro-N-Nitrosoguanidine (NG) using the protocol of Miller (13). Exponentially growing JA1 were washed and resuspended in 0.1M citrate buffer at pH 5.5. NG is added to a final concentration of 50 µg/ml. Cell concentration, measured as colony forming units (CFU) is about 10^9 per ml. The cell-NG mixture was incubated at 37°C for 30 min. This resulted in about 50% cell death and optimal mutagenesis (Liss, unpublished results). Cells were then collected on Millipore filters (0.22 µ), washed twice with citrate buffer and resuspended in tryptose broth.

Mycoplasmaviruses were mutagenized by irradiation with ultra-violet light as described by Liss and Maniloff (7). Treated viruses were added to non-mutagenized JA1 which have been seeded onto Millipore filters as noted in the next section.

Mutant Selection

Selection of conditional lethal mutants of mycoplasma viruses and cells was based on the ability of mycoplasmas to grow on Millipore filters (2). Temperature sensitivity was used as the conditional growth parameter. The permissive temperature was 30°C. Non-permissive temperatures of 37°C and 22°C were used to find mutants.

For selection of cell mutants, mutagenized JA1 was diluted to about 10^5 CFU/ml. About 5 ml of cells are seeded onto a 47 mm GSWP Millipore filter (Millipore Filter Corp., Bedford, Mass.) by suction. The filter is then incubated overnight (14 to 16 hrs) at the permissive temperature. The filter is then sterilely transferred to a tryptose broth plate containing 0.1% Chlorazol Black E (Eastman Organic Chemicals, Rochester, N.Y.). This dye is incorporated into growing mycoplasma colonies (1).

The plate is then incubated at the non-permissive temperature. Temperature mutants are those colonies that have not taken up the black dye. Mutants are picked from the filter for retesting at the appropriate temperatures.

Virus mutants were selected as follows. A filter is seeded as noted above. With the suction off, virus is added at a multiplicity of infection of 0.5 to 0.1. The suction is turned on after 15 min at 37°C, and the cells washed with sterile, pre-warmed broth--to remove unadsorbed viruses. This filter is then placed onto a pre-formed lawn of JA1, as previously described (7) and incubated for 2 hrs at the permissive temperature. The filter is then removed and placed on a pre-formed JA1 lawn and incubated for 2 hrs at the non-permissive temperature. The filter is then removed and stored at 4°C. The filter-infected lawns are incubated for 48 hrs at their respective temperatures. Plaques found at the permissive temperature are compared to plaques on the lawns incubated at the non-permissive temperature. The orientation of plaques on both plates is identical due to the filter-based nature of the infection.

Appropriate virus mutants are recovered directly from the "master" filter.

RESULTS AND DISCUSSION

As seen in Table I, the technique noted above was useful in identifying mutant mycoplasma cells and viruses. As expected, mutagenesis increased the frequency of mutation in both cells and viruses (Table I). The identification

TABLE I

FILTER-REPLICA SELECTION OF MUTANTS

	Permissive Temperature (°C)	Non-permissive Temperature (°C)	Frequency of Mutants
Untreated JA1	30	38	10^{-7}
Mutagenized JA1	30	38	10^{-4}
Untreated JA1	30	22	10^{-8}
Mutagenized JA1	30	22	10^{-6}
Untreated MVL51	30	38	10^{-10}
Mutagenized MVL51	30	38	10^{-7}
Untreated MVL51	30	22	10^{-9}
Mutagenized MVL51	30	22	10^{-7}
Untreated MVL2	30	38	10^{-9}
Mutagenized MVL2	30	38	10^{-7}
Untreated MVL2	30	22	10^{-9}
Mutagenized MVL2	30	22	10^{-7}
Untreated MVL3	30	38	$> 10^{-11}$*
Mutagenized MVL3	30	38	$> 10^{-11}$*
Untreated MVL3	30	22	$> 10^{-11}$*
Mutagenized MVL3	30	22	$> 10^{-11}$*

The relative frequencies of mutant isolation of mycoplasma cells and viruses are noted. The frequency value is determined by dividing the number of CFU or PFU at the permissive temperature into the number of CFU or PFU at the non-permissive temperature.

*Using this method, this value was not consistent (cf. Discussion).

of the exact cause for the conditional-lethal mutants is currently being worked on in this laboratory.

Clearly, this report presents a technique analogous to the replica plating technique of Lederberg (5). The Lederberg technique does not work with mycoplasma colonies which are usually imbedded in the agar matrix (Liss, un-

published data). The growth of mycoplasma on filters is useful as noted here as well as for pulse labelling mycoplasma. For this, any radioactive precursor can be added to agar medium and the seeded filter placed onto this medium for various times. One advantage of this method is the ability of re-using the label-containing agar mediums. These plates are not contaminated by the seeded-filter, nor is the radioactive label depleted readily (Liss, unpublished data).

The greatest, perhaps, use of the filter replica is for virus mutant selection. Mycoplasmaviruses have a low spontaneous mutation rate (Table I). Mutagenesis does not greatly increase this rate. The replica-nature of this technique allows for rapid screening of viruses as compared to the alternate technique of resuspended plaques in buffer and spotting these samples under various conditions.

The inability of this technique to produce useful data with MVL3 may be due to the fact that this is a lytic mycoplasmavirus (Liss, MS in preparation) while MVL51 and MVL2 are released from growing cells (6, Liss, unpublished results). The fact that infected cells do not lyse for Group L1 or L2 mycoplasmaviruses make them most useful for filter-replicating under a variety of conditions.

The characterization of the isolated virus mutants will be presented in another publication (MS, in preparation). The development of this and other techniques analogous to classical bacterial methods but appropriate for mycoplasma is the start of the elucidation of the genetics of these very small organisms.

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