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Transfection Mediated by *Mycoplasmatales* Viral DNA

(viral DNA isolation/genetic transfer/competence)

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ABSTRACT DNA isolated from *Mycoplasmatales* viruses MVL51 and MVGs51 was infectious when mixed with *Acholeplasma laidlawii* BN1-Nal^R cells. Infectivity was destroyed by deoxyribonuclease but not by ribonuclease, Pronase, or specific antiserum to the virus. Host mycoplasma cells were only competent for transfection during late-log growth phase. The rates of the establishment of DNase insensitivity of viral DNA transfectants were similar to those of bacteriophage systems. The dose-response curve for transfection suggested that an average of six molecules of DNA must interact with a cell in order to produce one infectious center. *Mycoplasmatales* virus DNA exhibited a low efficiency of infection; one infectious center required 4×10^6 virus equivalents of DNA.

Mycoplasmas are a group of small prokaryotic cells (Order, *Mycoplasmatales*) bounded only by a single lipoprotein membrane. They are the smallest free-living cells: different mycoplasma species carry enough genetic information to only code for 600–1000 cistrons per cell (1). Previous attempts to study mycoplasma genomes have suffered from the lack of a system for performance of conjugation, transformation, or tranduction in these cells (2). Folsome (3) has reported that one mycoplasma species, *Acholeplasma laidlawii* A, was capable of binding in DNase-resistant form double- or single-stranded DNA of high molecular weight; however, the existence of transformation could not be demonstrated.

Transfection, the infection of cells with isolated viral DNA, has been reported for several bacterial species: *Escherichia coli* (4, 5), *Streptomyces kanamyceticus* (6), *Proteus* (7), *Bacillus* species (8, 9), and *Staphylococcus aureus* (10). Infectious DNA has also been reported for many animal virus systems: polyoma (11), simian virus-40 (12, 13), and Shope rabbit papilloma virus (14).

This paper reports a transfection system in *A. laidlawii* by DNA from several *Mycoplasmatales* viruses. The lack of cell walls of the mycoplasmas means that this system may be similar to those of animal cells or bacterial protoplasts.

MATERIALS AND METHODS

Cells and Media. The mycoplasma used for virus propagation and as an indicator strain was *A. laidlawii* BN1-Nal^R (Nal^R). It is a nalidixic acid-resistant mutant of a strain, *A. laidlawii* BN1 (15). The isolation of Nal^R and the tryptose broth and agar media used in these studies have been described (16). Cells were assayed as colony-forming units (CFU/ml) (17).

Abbreviations: PFU, CFU, Plaque- or colony-forming units, respectively.

Virus Strains. The two *Mycoplasmatales* viruses used were MVL51, isolated from a spontaneous plaque on a Nal^R lawn, and MVGs51, isolated from a lawn of *Mycoplasma gallisepticum* A5969. Both viruses plaque on Nal^R, but are distinguishable by their different growth rates on Nal^R and their different inactivation rates by antiserum against MVL51. The viruses were assayed as plaque-forming units (PFU) on Nal^R lawns.

Preparation of Viruses. Viruses were propagated as described (16). Lawns were started by plating an 18-hr culture, which had been diluted 1:1 with fresh medium. 6-hr Lawns of Nal^R on tryptose–agar plates were infected with 0.2 ml of a high-titer virus (>10^9 PFU/ml). After incubation at 37° for 20 hr, the plates were flooded with phosphate-buffered saline (pH 7.4) and incubated an additional 18–24 hr. The plate wash was decanted and filtered through a 0.22-μm filter (Nalge, Rochester, N.Y.). The filtrate was centrifuged for 3 hr at 40,000 rpm in a Spinco 42.1 rotor. The resulting virus pellet was suspended in phosphate-buffered saline, plus 0.02% (v/v) Triton X-100 and centrifuged as above. Triton X-100 solubilizes cell membrane debris, but does not affect the virus titer. This second virus pellet was suspended in 0.01 M Tris·HCl buffer (pH 8.1).

Preparation of DNA. Virus DNA was phenol extracted by a modification of the Saito and Miura procedure (18). The virus suspension was mixed with an equal volume of phenol saturated with 0.01 M Tris·HCl, (pH 8.1) and the mixture was shaken for 30 min at 37°. The resulting emulsion was separated into two layers by low-speed centrifugation, and the lower phenol phase was removed with a Pasteur pipette. Phenol treatment was repeated twice. The final aqueous-DNA phase was placed in a dialysis tube, which had been boiled for 20 min in 5% (w/v) sodium bicarbonate to remove any DNase activity associated with the tubing (19). The DNA was dialyzed for 24 hr against 1 M NaCl in 0.01 M phosphate buffer (pH 8.1) and for another 24 hours against 0.15 M NaCl–15 mM Na citrate. To concentrate the DNA, two volumes of cold ethanol was slowly added to the solution; DNA was then collected by centrifugation at 10,000 rpm in a Beckman J-20 rotor for 15 min, dissolved in 0.15 M NaCl–15 mM Na citrate or 15 mM NaCl–1.5 mM Na citrate, and stored at 4° over a drop of chloroform. The ultraviolet absorption ratio (260 nm/280 nm) of the viral DNA prepared this way was 1.7 to 2.0, indicating that relatively pure DNA had been isolated. The DNA concentration was determined by the diphenylamine method (20), with calf-thymus DNA as standard.

TABLE 1. *Effects of various agents on Mycoplasmatales virus and viral DNA titers*

Agent	Infectious material (PFU/ml)			
	MVL51		MVGs51	
	virus ($\times 10^{-5}$)	DNA ($\times 10^{-6}$)	virus ($\times 10^{-5}$)	DNA ($\times 10^{-4}$)
DNase (100 μg/ml)	7.0	0	6.8	0
RNase (100 μg/ml)	7.1	5.2	6.8	4.8
Pronase (500 μg/ml)	7.0	5.8	7.0	5.1
Nonimmune control serum	7.1	6.0	6.9	4.9
Antiserum to MVL51	0	6.1	0	5.0

Enzymatic Treatment of DNA. Beef pancreas DNase 1 (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.01 M Tris·HCl (pH 7.5) containing 0.05 M $MgCl_2$. Beef pancreas RNase and Pronase (Calbiochem, Los Angeles, Calif.) were dissolved in the same buffer, but containing 1 mM EDTA. Concentrations used were 100 μg/ml each of DNase and RNase, and 500 μg/ml of Pronase. Enzymic treatment was at 37° for 30 min.

Assay for Infectious DNA. Viral DNA was added directly to lawns of the indicator Nal^R in most experiments. Where noted, in some experiments the DNA was mixed with Nal^R cells in broth culture, and this DNA-cell mixture was subsequently assayed for infectivity on Nal^R lawns. The lawns, in all cases, were 3- to 5-hr old; the plates were incubated at 37° for 20 hr and examined for plaques.

Preparation of Antiserum. Antiserum to MVL51 was prepared in rabbits. Each animal initially received a purified MLV51 virus pellet resuspended in 2 ml of Eagle's Basal Medium (titer>8×10^{11} PFU/ml) (17) and 2 ml of complete Freund's adjuvant. The injections were repeated 2 weeks later. After another 2 weeks, the virus suspension alone was injected, and this was repeated every other week for 4 weeks. The rabbits were bled before the first injection and every third week afterwards. After cross-adsorption with concentrated suspending medium, the antiserum showed specificity to MVL51 by Ouchterlony double-diffusion assay.

RESULTS

Preliminary observations

Isolated *Mycoplasmatales* virus MVL51 and MVGs51 DNA was tested for infectivity by addition directly to lawns of

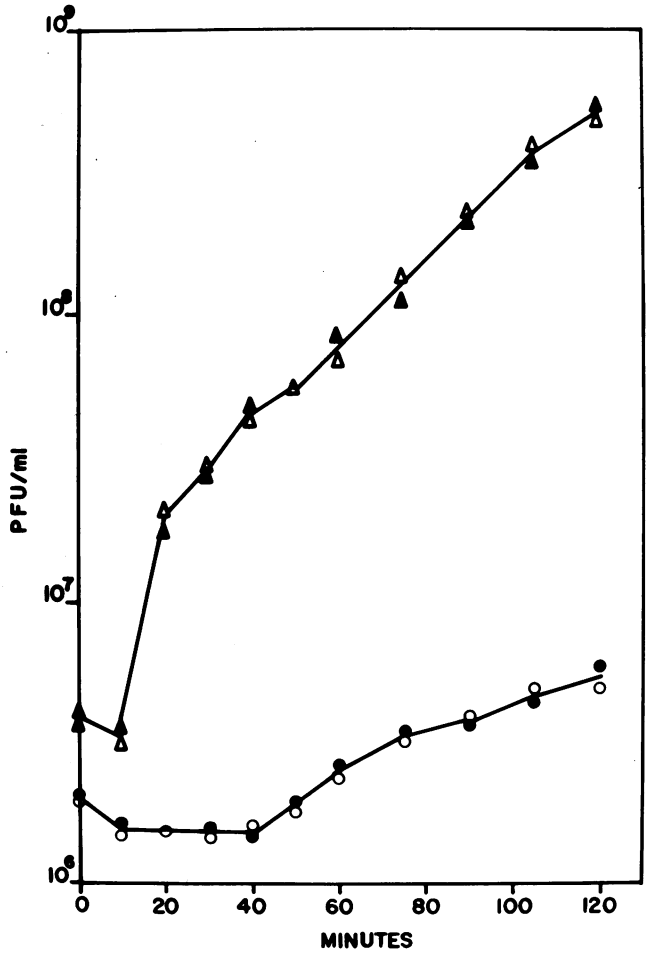

FIG. 1. Growth curves of MVL51 virus (△), MVGs51 virus (○), and of viruses produced by transfection experiments with MVL51 viral DNA (▲) and MVGs51 viral DNA (●). Under these conditions, typical virus yields per infectious center at 120 min are >100 for MVL51 and 3–5 for MVGs51.

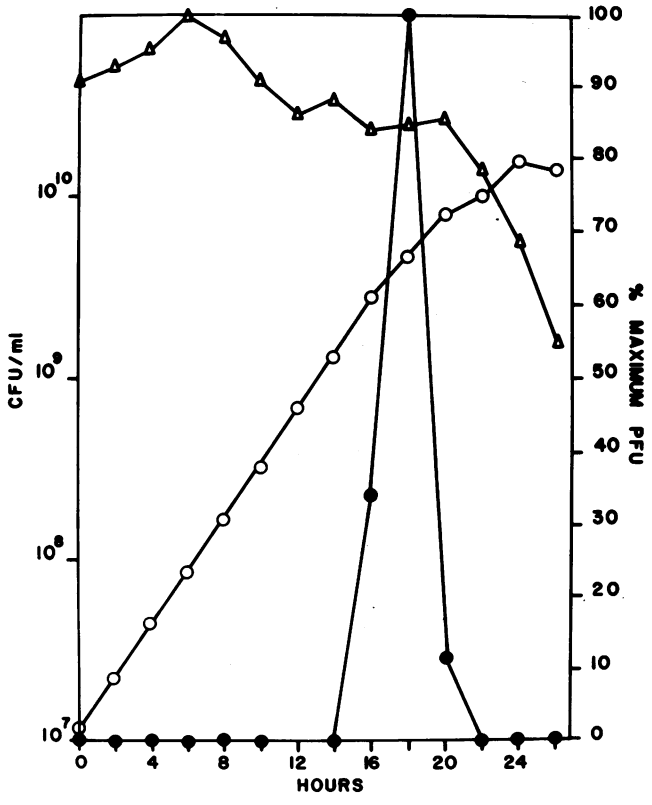

FIG. 2. Efficiency of plaque formation by Nal^R cells of different ages infected with MVL51 virus or viral DNA. The cell titer of the growing culture is shown as CFU/ml (○). Plaque formation at each time by MVL51 virus (△) and MVL51 viral DNA (●) is expressed as the percent of the maximum PFU/ml measured. For MVL51 virus the maximum was the 6-hr value, and for MVL51 viral DNA it was the 18-hr value.

Nal^R. Plaques were observed after these lawns were incubated overnight (20 hr) at 37°. High-titer PFU were washed out of these plaques and shown to be virus particles, as described below.

Effects of enzymes on infectivity of Mycoplasmatales virus DNA

Table 1 reports the infectivity of *Mycoplasmatales* virus DNA after treatment with several agents. It can be seen that the viral DNA lost its infectivity only after treatment with DNase. Intact virus particles were inactivated only by treatment with the antiserum to the virus, confirming the assumption that the infectivity measured in these studies is a DNA-mediated transfection phenomenon.

Characterization of progeny virus of a DNA transfection

As noted above, the host cells in these experiments, *A. laidlawii* BN1-Nal^R, carry a virus, MVL51. In order to determine whether the PFUs observed in the transfection experiments were viruses specified by the viral DNA that was added to the lawns, or whether they represented some type of release of MVL51 as a cellular response to the exogenous DNA, a series of transfection experiments were performed with DNA from a virus (MVGs51) that was isolated from *M. gallisepticum*. This virus had characteristics very distinct from those of MVL51. Fig. 1 shows the viral growth curve for MVGs51 and for the virus produced in a transfection experiment with MVGs51 DNA. Both curves are identical. Preliminary analysis of the inactivation rates by antiserum to MVL51 virus (data not shown) showed that the progeny of the MVGs51 DNA transfection were MVGs51. For comparison, growth curves for viruses from a transfection experiment with MVL51 DNA and MVL51 virus are also shown in Fig. 1. The progeny of a given virus DNA transfection had the same properties as the viral source of that DNA.

Virus infection and transfection through cell growth phases

The ability of mycoplasmas of different ages to sustain a viral infection or to permit transfection (i.e., competence) was investigated. The assay for virus infection involved a constant multiplicity of infection of 0.001. Virus and cell cultures of different ages were mixed for 5 min at 37°, long enough to adsorb >90% of the viruses but too short a time for the release of progeny virus (16). An aliquot was then plated onto Nal^R to assay for PFU. A parallel sample was filtered through a 0.22-μm filter and the filtrate was titered for PFU to determine the unadsorbed virus fraction. All data were corrected for unadsorbed virus. 2 μg/ml of DNA was added to cells of various ages to assay for competence. After allowing sufficient time for adsorption, but not for release of progeny virus (30 min, as determined from experiments similar to those shown in Fig. 3), the reaction was terminated by the addition of DNase. A peak in cellular competence was observed for cells 16- to 20-hr old (late-log growth phase) (Fig. 2). In comparison, cells can successfully maintain a viral infection throughout the first 24 hr of cell growth, with the highest efficiency being in 6-hr-old cells.

Appearance of DNase-resistant transfecting DNA

Rates of the establishment of a DNase-resistant state for the transfecting DNA were studied (Fig. 3). 2 μg/ml of DNA was added to 10^8 18-hr old cultures; at various times, two aliquots were taken. The control was plated immediately (Fig. 3, *curve A*). In the other samples, the reaction was stopped by the addition of 100 μg/ml of DNase and, after a 30-min incubation at 37°, samples were assayed for PFU (Fig. 3, *curve B*). For each time, the percentage of DNase-resistant transfectants was calculated (Fig. 3, *curve C*). As noted by Green (21), when this curve is extrapolated back to the x-axis, the intercept is the time required for the establishment of DNase resistance; data points were fitted to a straight line by least-squares analysis, and the time-axis intercept is 13 min. This falls in the interval between 10 and 15 min, when DNase-resistant PFUs being to appear (Fig. 3). Hence, it takes about 13 min for establishment of the DNase-resistant state in this transfection system.

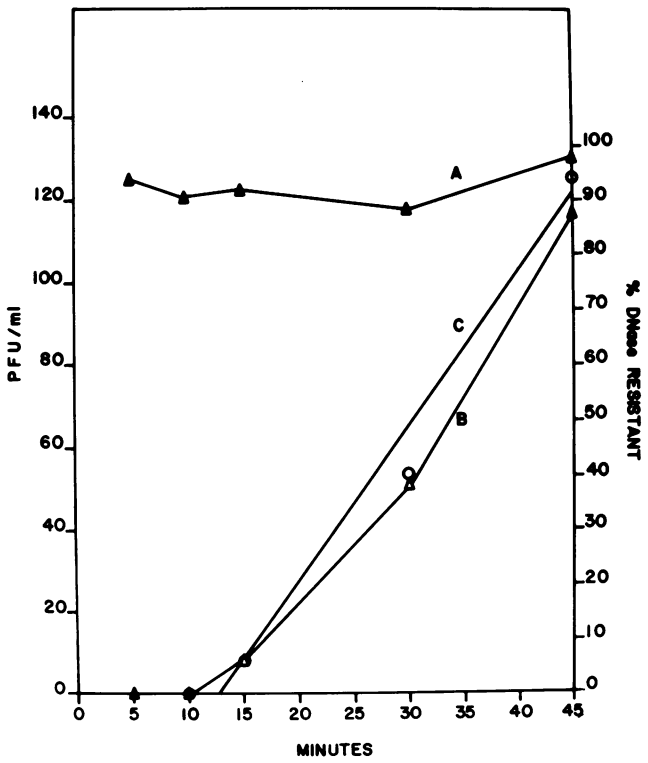

FIG. 3. Appearance of DNase-resistant transfecting DNA. *Curve A* (▲) is the control showing PFU/ml produced as a function of time during a transfection experiment. *Curve B* (△) shows the PFU/ml at each time that are resistant to added DNase. *Curve C* (○) shows the percent of transfecting DNA each time that is DNase resistant.

DNA-concentration dependence for transfection

Various concentrations of DNA, as noted in Fig. 4, were added to 10^8 cells of an 18-hr culture and, after 30 min at 37°, the reaction was stopped with DNase (as described above) and the DNA–cell mixture was assayed for PFU. Note that these data are given as a log–log plot. The slope of the linear part of the curve is about six; hence, the number of PFUs (transfectants) is proportional to about the sixth power of the DNA concentration.

DISCUSSION

The data presented here show that transfection does occur in mycoplasmas. The time course of viral DNA–cell interaction

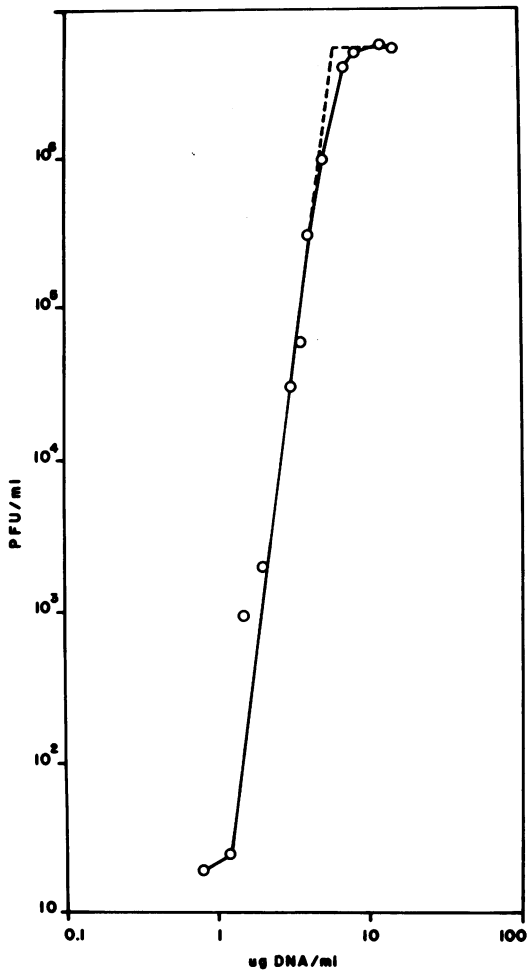

FIG. 4. DNA-concentration dependence for transfection. These are cumulative data from three experiments. The *dotted line* is an extrapolation of the linear increase to the saturation value and is used to calculate the minimum amount of DNA required to produce one infectious center, as described in the text.

and the transition of the viral DNA to a deoxyribonuclease-resistant state resembles that found by other workers (e.g., ref. 21). As proposed for other systems, this type of interaction may represent a two-step uptake of DNA; first, an irreversible attachment of the DNA to the cell during the first 10–15 min, then the entry of the DNA into the cell, with the concomitant loss of DNA sensitivity to DNase.

Analysis of the slope of the dose–response curve (Fig. 4) shows a nonlinear relationship between the number of transfectants and the viral DNA concentration. Similar complex reaction schemes in bacterial transfection systems have been interpreted as indicating either a recombination process involving several viral DNA molecules in the cell (21) that may be required for the formation of the infectious unit, or the variation of some internal experimental parameter (22, 25), such as DNA size or nuclease activity.

In Fig. 4, the dotted line shows the extrapolation of the linear part of the sixth-order curve to the saturation level (the maximum number of transfectants that can be obtained in this experimental system); the intercept is the minimum amount of DNA required to make this number of transfectants. The intercept is 5.5×10^6 PFU/ml at a concentration of 7 μg of DNA/ml, or 1.3×10^{-6} μg of DNA is required to make one infectious center. The molecular weight of the viral genome has been estimated to be about 2×10^6 (Roger Hull and David Garwes, personal communication*). By use of this value in these calculations, we estimate that about 4×10^5 viral DNA equivalents are needed per PFU. This value is of the same order of magnitude as those reported for bacteriophage transfection (21, 22). As for other systems, the low efficiency of transfection may be: (*a*) due to damage to the DNA during isolation or during incubation with the cells, or (*b*) due to a low percentage of competent cells in the culture.

In conclusion, we can examine the various possibilities available for mycoplasma genetic studies. All such studies require stable genetic markers; these have been extremely difficult to isolate in the mycoplasmas (23). The previously reported unsuccessful attempts to demonstrate DNA-mediated transformation in the mycoplasmas (2, 3) may reflect (*a*) the lack of different stable genotypes; (*b*) since it has been shown that transformation frequency is greatly reduced in lysogenic bacteria (24), and that the *Acholeplasmas* used for previous mycoplasma studies are carrying viruses (16), the frequencies for mycoplasma transformation may be so low as to be experimentally difficult to demonstrate; (*c*) competence may be narrowly defined by the medium and/or the culture growth phase; and (*d*) most simply, mechanisms for transformation (and perhaps recombination of cellular DNA) may be absent in the mycoplasmas. However, the transfection data reported here clearly show that mycoplasma cells can take up and express exogeneous DNA. These studies have defined a period of cell competence for the incorporation and phenotypic expression of viral DNA and have suggested that the intracellular recombination of viral DNA may occur. The fact that mycoplasma cells can perform the functions necessary for genetic transfer and expression indicates that *Mycoplasmatales* virus genetics may be readily developed and that DNA-mediated transformation of mycoplasmas may, in principle, be possible.

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