# LIPOSOME ENCAPSULATED DSRNA FOR CELL-FREE TRANSMISSION OF HYPOVIRULENCE

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*ABSTRACT.--Unilamellar lipid vesicles prepared by the ether injection method were used to capture dsRNA prepared from Endothia parasitica strain EP-113. The liposomes were fused with protoplasts of an E. parasitica virulent, methionine auxotrophic strain (EP-6). Protoplasts were regenerated on potato dextrose agar containing 0.55 M salt. Cell-free infection of protoplasts was also attempted using free dsRNA and dsRNA containing membrane-bound particles. Presence of the white phenotype was initially used as an indication of cell-free transmission. However, conversion experiments using EP-34 (arg ) indicated otherwise and attempts to extract dsRNA from the white colonies were unsuccessful. The relatively high percentage of white colonies probably resulted from osmotic shock.*

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The evidence for dsRNA involvement as the carrier of genetic information of transmissible hypovirulence (TH) is based entirely upon correlative data (Elliston in press). While this correlative evidence is strong enough to justify research on the biology of the dsRNA, direct evidence of dsRNA involvement by cell-free transmission of the dsRNA is still needed. Development of a successful system for cell-free transmission of dsRNA will make possible studies such as synchronous infection, mixed infections, single segment infections and introduction of the dsRNA into new strains without mixing cytoplasmic genes.

Cell-free transmission of mycoviruses has proven to be very difficult. Relatively few reports exist of successful transmission of mycoviruses (Ghabrial 1980). There are no reports of transmission of dsRNA to fungi without the viral capsid and other associated peptides (Buck 1980). Thus, the task of cell-free transmission of the TH associated dsRNA will not be easy. To increase the likelihood for transmission, the dsRNA will be packaged within membrane vesicles. These vesicles can then be fused with fungal protoplasts releasing the vesicle contents into the protoplasts. Two different types of membrane vesicles for fusion with the fungal protoplast can be used: 1) the naturally occurring membrane-bound particles containing dsRNA that have been isolated from *Endothia parasitica* strain EP-113, and 2) artificially prepared liposomes that contain the dsRNA. While we have not yet successfully transmitted the dsRNA into virulent *E. parasitica* using either of these procedures, we have worked out most of the necessary details. The procedures for obtaining and regenerating protoplasts of virulent *E. parasitica* and the capture of dsRNA in liposomes is described.

## Materials and Methods

Protoplasts of *E. parasitica* strain EP-6 are obtained by incubating approximately 0.3 ml of packed mycelium in the following protoplasting solution: 2.7 ml 5 mM phosphate buffer, pH 5.5 containing 0.55 M NaC1, 45 mg Sigma cellulase, and 0.3 ml of Sigma B-glucuronidase. The mycelium is prepared by inoculating flasks of *E. parasitica* complete medium (Puhalla and Anagnostakis 1971) with conidia and incubating with shaking. Mycelium is harvested by removing the desired amount and washing by centrifugation twice with 20 volumes of 5 mM phosphate buffer, pH 5.5 followed by one wash with 5 mM phosphate buffer with 0.55 M NaCl. The mycelium is incubated with the protoplasting solution at 25 C in a 50 ml Erlenmeyer flask shaking at 150 rpm for 4 to 8 hrs. The protoplasts are separated from mycelial debris by very low speed (250 rpm) centrifugation for 1 minute. The supernatant containing the protoplasts is pelleted by centrifuging at 1000 rpm for 3 minutes. Protoplasts are resuspended in 0.7 M NaCl. Protoplasts were regenerated by plating onto Inolex potato dextrose agar supplemented with 7.5 g/1 Difco malt extract, 2.5 g/1 Difco yeast extract, 0.1 g/1 methionine and 0.55 M NaCl.

Liposomes were prepared using the ether injection method (Ostro et al. 1977). The procedure of Matthews et al. (1979) was used as the primary guide. The liposomes were prepared from an 8:2 molar ratio of phosphatidyl choline and dicetyl-phosphate. The lipids were dissolved in chloroform:petroleum ether (1:10) to a concentration of 4 umole/ml. Five ml of lipid phase were injected into 1 ml aqueous phase at a rate of 0.5 ml/min. The aqueous phase consisted of dsRNA dissolved in 5 mM HEPES buffer, pH 7.4 with 0.15 M NaC1 and 1 mM EDTA. After ether injection, any residual ether was removed by bubbling nitrogen through the liposome suspension.

Preparation of dsRNA and the dsRNA containing membrane-bound particles from E. *parasitica* strain EP-113 were as reported by Dodds (1980a; 1980b).

## Results

Protoplast release and regeneration. The release of protoplasts from the mycelium occurs not by total dissolution of the cell wall but rather by extrusion of the protoplast from weakened portions of the wall. One of the first visible effects of the enzyme is the breaking apart of the mycelial cells at the cross-walls. Later protoplasts are seen extruding from these weakened walls or from hyphal tips. The particular combination of enzymes that we use appears to cause extensive release of protoplasts from cells. Some bursting of protoplasts occurs but shaking in an Erlenmeyer flask rather than a test tube reduces the number of burst protoplasts. Osmotica other than NaC1 can be used. We tried 0.5 M mannitol and found no difference in survival rate. However, regeneration of protoplasts on mannitol resulted in colonies that were large and diffuse as opposed to the desired compact colonies on NaC1 agar.

We tried a number of methods to separate the protoplasts from the mycelial cell ghosts. Because they both pellet upon centrifugation at about the same rate, even through dense solutions, separation is difficult. The mycelial ghosts do pellet faster at very low speed (250 rpm) than do protoplasts.

Using this procedure most of the protoplasts remain suspended. The protoplast suspension is contaminated with 0.1 to 2 percent colony-forming propagules that do not burst when diluted with water.

Yield of protoplasts from the approximate 0.3 ml packed volume of mycelium was about $10^4$ regenerated colonies/ml. Efficiency of regeneration is difficult to judge because of ambiguity concerning which round bodies in the solution are protoplasts and which are vacuoles, etc. Counting all distinct round bodies as being protoplasts our efficiency of regeneration is approximately 5 percent.

Liposome preparation. Liposomes prepared using the ether injection method are large, unilamellar bodies. The liposomes prepared in our lab were sized by passing them through a 1.2 pm Millipore filter, with only 5 percent of the liposome suspension passing through. The amount of aqueous phase captured by the liposome was estimated by adding potassium chromate to the aqueous phase. After separating liposomes from free chromate by gel filtration chromatography, the amount of chromate in the liposomes was photometrically determined after correcting for the light scattering caused by the liposome suspension. We estimate that approximately 5 percent of the aqueous phase is captured in the liposomes.

Fusion experiments. The use of the $met^-$ auxotroph, EP-6, as the recipient strain for cell-free infection assures that there will be no question of the origin of any TH strains that may result from cell-free infection experiments. The EP-113 is used as the source of dsRNA and membrane-bound dsRNA containing particles because of the high yield of both from this strain. We have attempted cell-free infection of protoplasts with free dsRNA, liposome captured dsRNA, and dsRNA containing membrane-bound particles. In attempting cell-free infection we combine equal volumes of protoplasts and the cell-free dsRNA preparation. After 2 hours of shaking at 100 rpm the suspensions are diluted and plated. Conidia from EP-6 were also plated onto the regeneration medium with and without salt as controls. Regenerated colonies are transferred to agar plates without NaC1 and evaluated for colony color after 1 week. It was our assumption that dsRNA containing colonies would assume the white phenotype of EP-113 while those not containing dsRNA would retain the orange phenotype of EP-6 (Puhalla and Anagnostakis 1971; Van Alf en et al. 1978). The results of representative experiments are shown in Table 1. The relatively high percentage of regenerated colonies that were white suggested that we were successful in transmitting dsRNA. The controls however suggested that the white colonies resulted not from dsRNA transmission but rather from osmotic shock. Conversion experiments (Anagnostakis and Day 1979) using an orange strain of the same vegetative compatibility type, EP-34 ($arg^-$), indicated that the white color was not transmissible. Attempts to extract dsRNA from selected white regenerated colonies were also negative. Thus, the white colonies that resulted from our fusion experiments apparently did not contain dsRNA.

## Discussion

The induction of high percentages of white colonies from orange ones by ultraviolet (UV) irradiation has previously been reported (Van Alf en et al. 1978). We can only assume that osmotic shock affects *E. parasitica* in an

Table 1. Percentages of white colonies after plating onto regeneration medium. a/

| Propagules plated | Percentage of white colonies |
|---|---|
| Conidia plated onto regeneration medium without salt | 0 |
| Conidia | 35 |
| Protoplasts | 4 |
| Protoplasts + dsRNA | 23 |
| Protoplasts + dsRNA containing liposomes | 24 |
| Protoplasts + dsRNA containing membrane-bound particles | 11 |

a/ The composition of the regeneration medium is given in the text.

unknown way that results in a phenotype similar to that induced by TH and UV light. The lack of dsRNA in the sampled white colonies indicates that we have not successfully transmitted the dsRNA even though the expected phenotype occurred in high percentages. It is possible that we are losing the enzyme(s) necessary for replication or expression of the dsRNA.

This induction of white colonies by osmotic shock complicates our attempts of cell-free transfer of dsRNA. Our current plan is to test all white colonies for their ability to convert orange ones to white before determining whether they contain dsRNA. We also will increase the numbers of colonies tested. In past experiments we have transferred about 100 colonies per experiment for color development. In the different experiments 2 to 30 white colonies have been tested for ability to convert orange colonies to white with negative results. Obviously large numbers of whites will need to be tested in each experiment. A concerted effort to use naturally occurring membrane-bound particles may also prove useful if we are losing essential enzymes upon transmission.

The cause of variability in percentage of white colonies that are induced from experiment to experiment is not known. We know too little of the mechanism involved in this generation of high percentages of white colonies from virulent ones to speculate about such variability.

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