

Characterization of the Cytoplasmic Ribosomes and Ribosomal RNA from Mycelium of Various Species of *Phytophthora*

J. V. Leary, J. R. Roheim and G. A. Zentmyer

Assistant Professor, Graduate Student, and Chairman, respectively, Department of Plant Pathology, University of California, Riverside 92502. Present address of J. R. Roheim is Department of Plant Pathology, University of Nebraska, Lincoln.

Supported by California Avocado Advisory Board Grant No. 20649.

Accepted for publication 22 March 1973.

ABSTRACT

Ribosomes from the vegetative mycelium of several species of *Phytophthora* were characterized by sucrose density-gradient centrifugation. The monosomes had a Svedberg sedimentation coefficient (S-value) of approximately 80, with the large and small subunits being 60S and 40S, respectively. The ribosomal RNA, isolated in the total cellular RNA, sedimented to a position in the gradient

corresponding to RNA's of known 28S and 18S values. These data indicate that the ribosomes of *Phytophthora* spp. are typical of eukaryotes and that the susceptibility of these species to certain antibacterial antibiotics is not due to prokaryotic 70S ribosomes.

Phytopathology 63:1148-1151.

The genus *Phytophthora* has long been of great interest to plant pathologists because it includes many serious plant pathogens (6). In addition, the genus offers material uniquely suited to the study of morphogenesis because the life cycle includes several distinct morphological stages; i.e., mycelium, sporangia, free-swimming zoospores without cell walls, encysted (walled) zoospores, chlamydospores, and the oospores which result from sexual reproduction (2, 13, 17).

Important to the investigation of the molecular events in fungal morphogenesis is an understanding of the components of the protein synthesizing apparatus (7, 9, 10, 14, 15). This type of study on *Phytophthora* is of additional importance because this genus and other oomycetes differ from most other fungi and eukaryotes in their susceptibility to antibiotics known to inhibit protein synthesis by 70S ribosomes (8, 11, 12, 16).

Therefore, we began this project by isolating and characterizing the ribosomes from the vegetative mycelium of various *Phytophthora* spp. in order to first define the organism as a typical eukaryote, and secondly, to provide a basis for further studies on the molecular events involved in morphogenesis.

MATERIALS AND METHODS.—*Phytophthora* species and strains.—The species and the particular strains used were all obtained from the collections in this department. They included the homothallic species *Phytophthora citricola*, *P. megasperma*, and *P. vignae* and the heterothallic species *P. capsici*, *P. cinnamomi*, *P. drechsleri*, and *P. palmivora*.

Culture techniques.—All cultures were maintained on plates of Erwin's synthetic (ES) agar medium (5). Five-day-old cultures, incubated at 25 C, were macerated in a Waring Blendor in 25 ml sterile H₂O for 15 sec. Five ml were pipetted into 300-ml flasks containing 25 ml ES liquid medium and incubated at 25 C for 4 hr. These cultures were combined, macerated for 15 sec in a Waring Blendor, 10 ml inoculated into 500-ml Erlenmeyer flasks containing 75 ml ES medium, and incubated on a reciprocal shaker at room temperature.

The mycelia were harvested by filtration through cheesecloth over ice. The mycelial mats were washed with 2,000 ml chilled distilled water, collected, and immediately lyophilized.

Radio-labeled mycelia were obtained by growing the mycelium under the above conditions in the presence of 1-10 μ Ci of ³H-uridine (specific activity 10.2 mCi/mg).

Preparation of crude extract.—Lyophilized mycelium (100-250 mg dry wt) was ground with glass beads in a mortar and pestle until complete breakage of the mycelium was attained. The ground mycelium was resuspended in 5 mM Tris-HCl - 5 mM MgCl₂ (Tris - Mg²⁺), pH 7.4 buffer and centrifuged for 15 min at 15,000 g. One to two ml of the supernatant fraction was layered over linear 5-20% sucrose gradients and centrifuged at 51,000 g (avg) for 3.5 hr at 5 C. The gradients were then pumped through an ISCO density gradient fractionator at the speed and A₂₅₄ maximum previously determined. One-ml fractions were collected directly into scintillation vials after passage through the fractionator and

radioactivity assayed in 10 ml of Bray's scintillation fluid (4).

Preparation and characterization of purified ribosomes.—The crude extract was prepared as described above, layered over 3 ml 2 M sucrose in Tris Mg^{+2} buffer, and centrifuged at 49,000 rpm for 2–2.5 hr in the 50 Ti rotor of the Spinco Model L2-65B ultracentrifuge. The ribosomal pellet was resuspended in Tris- Mg^{+2} buffer. The resuspended ribosomes were layered over 5–20% linear sucrose gradients, centrifuged, and analyzed as described above.

Characterization of ribosome subunits.—Subunits were prepared by resuspending purified ribosomes in 3 mM Tris-HCl - 3 mM EDTA (pH 7.5) at 5 C and incubated at room temp for 1 hr. Two ml of the suspension were layered over linear 15–30% gradients made of sucrose in Tris-EDTA buffer, centrifuged 18 hr at 51,000 g (avg) (4 C) and analyzed as previously described. Radioactivity was determined for 1.0-ml fractions.

Characterization of ribosomal RNA.—RNA was obtained by phenol-sodium dodecyl sulfate (SDS) extraction of 100–250 mg dry mycelium according to the method of Brakke & Van Pelt (3). The mycelium was ground in a Virtis homogenizer containing phenol, RNA extraction buffer, SDS, and bentonite. The RNA was precipitated with absolute ethanol three times. The final pellet was resuspended in 3 ml Tris-EDTA buffer. One to two ml of the resuspended RNA was layered over a 28 ml 5–20% linear sucrose gradient, centrifuged for 20 hr at 51,000 g (avg) (5 C), and analyzed with the ISCO density-gradient fractionator.

***Neurospora crassa* strains and culture techniques.**—The wild-type strains of *Neurospora crassa* used were the St. Lawrence strains 74A and 77a. The complete and minimal media were essentially those of Beadle & Tatum (1).

One-day-old cultures on minimal agar medium were macerated in 25 ml sterile distilled water for 30 sec in a Waring Blender. Five-ml portions were inoculated in 25 ml liquid medium, and incubated in still culture for 48 hr at 25 C. These cultures were subsequently macerated as described above, 15 ml inoculated into 100 ml liquid

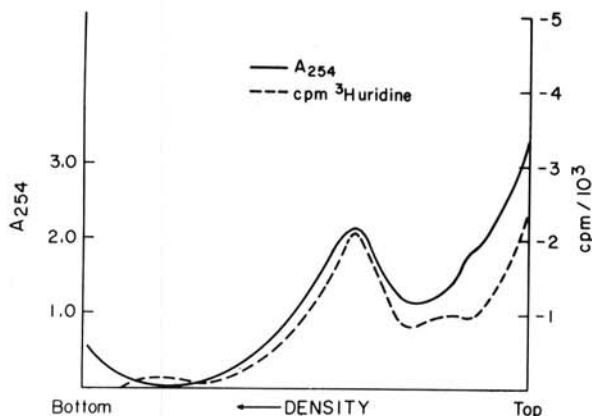


Fig. 1. Sucrose density-gradient profile (5–20%) of crude mycelial extracts from *Phytophthora megasperma* (---) and *Neurospora crassa* (—).

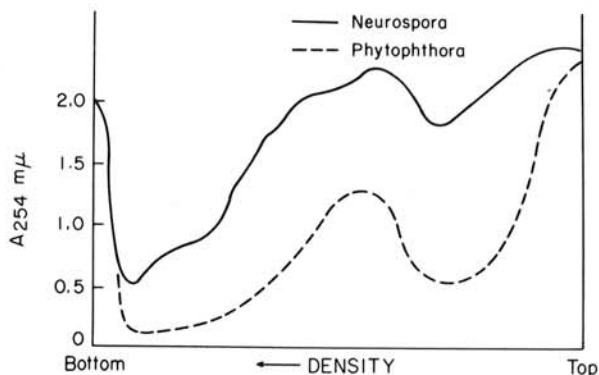


Fig. 2. Sucrose density-gradient profile (5–20%) of a crude mycelial extract from *Phytophthora palmivora* grown in the presence of 1 μ Ci 3 H-uridine (— = A_{254} ; --- = radioactivity).

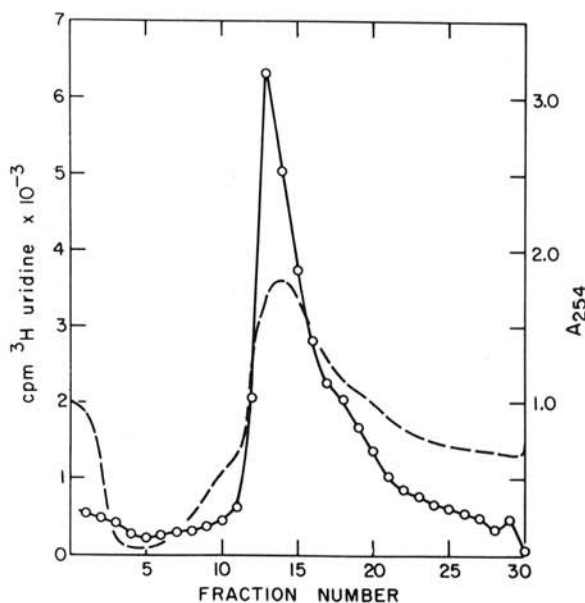


Fig. 3. Sucrose density-gradient profile (5–20%) of purified ribosomes isolated from 3 H-uridine-labeled *Phytophthora palmivora* mycelium and unlabeled *Neurospora crassa* mycelium (—O— = A_{254} ; --- = radioactivity).

medium, and incubated at 25 C on a reciprocal shaker. Mycelia were harvested by filtration, washed extensively with chilled water, and lyophilized. The techniques used for the preparation of crude extracts, purified ribosomes, ribosomal subunits, and the sucrose density-gradient centrifugation were those described above.

RESULTS AND DISCUSSION.—As a first step in the characterization of the *Phytophthora* ribosomes, crude extracts of *Phytophthora* mycelia were subjected to sucrose density-gradient centrifugation (Fig. 1). By comparison to the profile of crude extract of *Neurospora crassa* mycelium run in a separate gradient in each experiment, the predominant peak appears to consist of

80S monosomes. Material sedimenting faster than the monosomes, and presumed to be polysomes, is also present. When the crude extracts of ^3H -uridine labeled *Phytophthora* mycelium were analyzed by sucrose density-gradient centrifugation, the radioactivity and absorbancy profiles correspond exactly (Fig. 2). Additional confirmation of the similarity of *Phytophthora* and *N. crassa* ribosomes was obtained when ^3H -uridine labeled, purified *Phytophthora* ribosomes and unlabeled, purified *N. crassa* ribosomes were mixed and co-sedimented (Fig. 3). The exact correspondence between radioactivity and absorbancy could only result from the ribosomes being of similar S values.

Since the above data indicate that *Phytophthora* ribosomes can be characterized as typical of eukaryotes in that the monosomes have an S-value of 80, it was possible that the unique sensitivity to antibacterial antibiotics could result from properties inherent in one of the ribosomal subunits. If the sedimentation coefficients of the subunits were atypical of eukaryotic ribosomal subunits, this would be significant. However, this is not the case as the data in Fig. 4 demonstrate. The subunits obtained by EDTA treatment of purified *Phytophthora* ribosomes sediment to positions in the gradient identical to those of *N. crassa* ribosomal subunits. The large and small subunits of *Phytophthora* ribosomes can be considered 60S and 40S, respectively.

The total cellular RNA prepared by phenol-SDS extraction was characterized by sucrose density-gradient centrifugation (Fig. 5). When the absorbancy profile and radioactivity peaks were compared to *N. crassa* RNA, run in separate gradients, the two predominant species were determined to be 28S and 18S ribosomal RNA.

In conclusion, our studies demonstrate that the

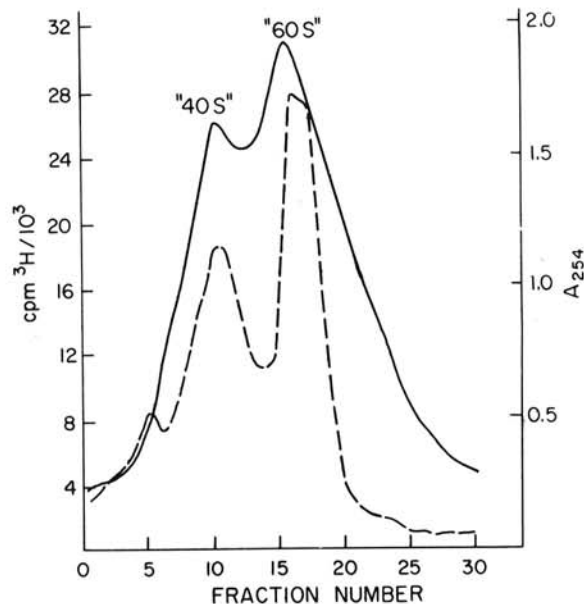


Fig. 4. Sucrose density-gradient profile (15-30%) of ribosomal subunits prepared from ^3H -uridine labeled *Phytophthora cinnamomi* mycelial ribosomes and unlabeled *Neurospora crassa* mycelial ribosomes (— = A_{254} ; --- = radioactivity).

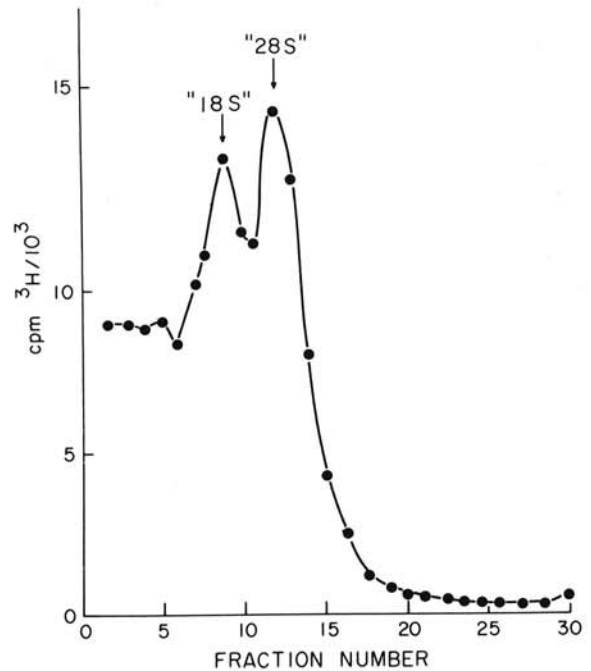


Fig. 5. Sucrose density-gradient profile (5-20%) of RNA isolated from 72-hr-old mycelium of *Phytophthora palmivora* grown in the presence of ^3H -uridine. The S values were assigned by comparison with *Neurospora crassa* RNA centrifuged in separate gradients.

cytoplasmic ribosomes in the genus *Phytophthora* are typically 80S. The ribosomal subunits and subunit RNA's are typical of such ribosomes. These findings do not explain the unusual sensitivity of members of this genus to antibacterial antibiotics such as chloramphenicol. We know, however, that this property is not due to the cytoplasmic ribosomes being the 70S prokaryotic type.

The development of methods for radioactively labeling, isolating, and purifying the ribosomes from *Phytophthora* spp. should permit the characterization of the ribosomes from the various morphological stages such as the zoospores, chlamydozoospores, and oospores and allow the study of RNA and protein biosynthesis and turnover during morphogenesis.

LITERATURE CITED

1. BEADLE, G. W., & E. L. TATUM. 1945. *Neurospora*. II: Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* 32:678-686.
2. BLACKWELL, E. M., & GRACE M. WATERHOUSE. 1930. Spores and spore germination in the genus *Phytophthora*. *Trans. Brit. Mycol. Soc.* 15:294-310.
3. BRASSE, M. K., & NANCY VAN PELT. 1969. Influence of bentonite, magnesium, and polyamines on degradation and aggregation on tobacco mosaic virus. *Virology* 39:516-533.
4. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
5. ERWIN, D. C., & H. KATZNELSON. 1961. Studies on the nutrition of *Phytophthora cryptogea*. *Can. J. Microbiol.* 7:15-25.

6. ERWIN, D. C., G. A. ZENTMYER, J. GALINDO, & J. S. NIEDERHAUSER. 1963. Variation in the genus *Phytophthora*. *Ann. Rev. Phytopathol.* 1:375-396.
7. GOTTLIEB, D. 1966. Biosynthetic processes in germinating spores. p. 217-233. *In* M. F. Madelin [ed.]. *The fungus spore*. Butterworths, London.
8. GOTTLIEB, D., & P. D. SHAW. 1967. *Antibiotics*. Vol. I. Mechanism of Action. Springer, New York. 785 pp.
9. LEARY, J. V., A. J. MORRIS, & A. H. ELLINGBOE. 1969. Isolation of functional ribosomes and polysomes from lyophilized fungi. *Biochim. Biophys. Acta* 182:113-120.
10. OBRIG, T. G., J. CERNA, & D. GOTTLIEB. 1969. Characteristics of in vitro protein synthesis systems from *Rhizoctonia solani* and *Sclerotium bataticola*. *Phytopathology* 59:187-192.
11. SISLER, H. D. 1969. Effect of fungicides on protein and nucleic acid synthesis. *Ann. Rev. Phytopathol.* 7:311-330.
12. TSAO, P. H. 1970. Selective media for isolation of pathogenic fungi. *Ann. Rev. Phytopathol.* 8:157-186.
13. TUCHER, C. M. 1931. Taxonomy of the genus *Phytophthora* de Bary. *Missouri Univ. Agric. Exp. Stn. Res. Bull.* 153. 208 p.
14. VAN ETTEN, J. L., & R. M. BRAMBL. 1968. Protein synthesis during fungal spore germination. II. Amino-acyl-soluble ribonucleic acid synthetase activities during germination of *Botryodiplodia theobromae* spores. *J. Bacteriol.* 96:1042-1048.
15. VAN ETTEN, J. L., R. K. KOSHI, & M. M. E.-ALEMY. 1969. Protein synthesis during fungal spore germination. IV. Transfer ribonucleic acid from germinated and ungerminated spores. *J. Bacteriol.* 100:1182-1186.
16. VASQUEZ, D. 1966. Mode of action of chloramphenicol and related antibiotics. p. 169-191. *In* B. A. Newton & P. E. Reynolds [ed.]. *Biochemical studies of antimicrobial drugs*. Cambridge: Cambridge Univ. Press, London.
17. WATERHOUSE, GRACE M. 1970. The genus *Phytophthora* de Bary. *Commonwealth Mycol. Inst., Mycol. Pap. No.* 122. 59 p.