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Isolation and Characterization of a Temperate Bacteriophage Specific for *Rhodopseudomonas spheroides*

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The isolation of a temperate phage specific for the photosynthetic bacterium *Rhodopseudomonas spheroides* is reported. This phage, Rϕ-1, establishes a state of lysogeny and can be induced from the prophage state by exposure to mitomycin C or UV irradiation. Mutants of Rϕ-1 which grow on a standard laboratory strain (2.4.1) of *Rhodopseudomonas spheroides* were isolated. Although the original Rϕ-1 isolated was chloroform sensitive, the mutant which plates on strain 2.4.1 is chloroform resistant. Rϕ-1 does not grow on closely related bacteria, such as *Rhodopseudomonas palustris* or *Rhodopseudomonas capsulata*. Rϕ-1 mutants forms plaques with the same efficiency whether the plates are incubated under aerobic conditions in the dark or under anaerobic conditions in the light (phototropic conditions).

Bacteria capable of utilizing light energy, the so-called photosynthetic bacteria, have proven to be exceptionally useful in the study of photosynthesis (4, 6). In particular, mutants of these bacteria have been used in analyzing specific components of the photosynthetic apparatus (5, 8). One factor which limits the usefulness of these studies has been the lack of knowledge about a genetic transfer system which would permit ordering of these mutant markers. Marrs (9) has recently reported the discovery of a genetic transfer system for the photosynthetic bacterium *Rhodopseudomonas capsulata*. However, it may be difficult to map relatively large genetic regions using this transfer system, since the particle involved is extremely small and is presumably only capable of transferring small amounts of genetic information (Marrs, personal communication).

In the quest for a more useful genetic transfer system, we set about to isolate a transducing phage for the photosynthetic bacterium *Rhodopseudomonas spheroides*. *R. spheroides*, a member of the nonsulfur purple bacteria (*Athiorhodaceae*), has the capability of growing both aerobically in the dark and anaerobically in the light (12). Since this organism can grow nonphotosynthetically, mutations affecting the photosynthetic apparatus are only conditionally lethal.

Since temperate phage have proven to be the most effective transducers (11), we devised a technique for isolating temperate phage whose natural hosts are members of the *Athiorhodaceae*. We report here this method of isolation and the preliminary characterization of a temperate phage which is specific for the photosynthetic bacterium *Rhodopseudomonas spheroides*. The isolation of a virulent phage specific for *R. spheroides* has recently been reported (1).

MATERIALS AND METHODS

Bacterial strains. Strain 2.4.1 of *R. spheroides* was obtained from E. Gray. Strain 05 is a streptomycin-resistant derivative of 2.4.1, obtained by mutagenesis with *N'*-methyl-*N'*nitronitrosoguanidine (100 μg/ml in broth). Strain 014 is an uncharacterized strain, thought to be a rhodopseudomonad, which was isolated from a pond near Ann Arbor, Michigan. Strain 032 is a derivative of 2.4.1 which was lysogenized with Rϕ-1. Strains of *R. palustris* and *R. capsulata* were supplied by R. Uffen; *Rhodospirillum rubrum* was supplied by P. Datta.

Media. Tryptone broth: 1% tryptone, 0.5% NaCl, 2% maltose, $MgSO_4$ (10^{-2} M), and 1.0 μg thiamine hydrochloride per ml. The agars used were: (i) tryptone agar (1% tryptone, 0.25% NaCl, 1.0 μg thiamine hydrochloride per ml, and 1.1% agar); (ii) tryptone top agar (1% tryptone, 0.5% NaCl, and 0.7% agar); and (iii) LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, $CaCl_2$ (5×10^{-2} M), and 1.1% agar). Tris-(hydroxymethyl)aminomethane - magnesium - gelatin buffer, containing $MgSO_4$ (0.01 M), tris(hydroxymethyl)aminomethane hydrochloride (0.01 M) (pH 7.4), and 0.01% gelatin.

Phage plating. Standard phage overlay techniques were used (2).

Enrichment techniques. Members of the *Athiorhodaceae* were selected by the method of van Niel (13). Positive enrichments were further purified by streaking on tryptone plates. Anaerobic conditions were maintained in a chamber with an atmosphere of

85% nitrogen, 5% carbon dioxide, and 10% hydrogen (3). Illumination was provided by both flourescent and incandescent lamps. Enrichment and purification of various rhodopseudomonads were carried out under anaerobic conditions; isolation and characterization of bacteriophage were done aerobically.

Preparation of lysates. High-titer lysates of Rφ-1 were prepared by the confluent lysis technique. To a 0.5-ml sample of an overnight culture of sensitive bacteria, 10^7 phage were added and allowed to adsorb for 30 min. Then 3 ml of tryptone broth and 2.5 ml of tryptone top agar were added, and the mixture was poured onto an LB agar plate. These plates were incubated overnight at 32 C. The soft agar portion was collected and sedimented by centrifugation. The supernatant fraction was decanted and usually contained between 10^9 to 10^{10} phage/ml.

RESULTS

Isolation of phage Rφ-1. The strategy we employed in looking for temperate phage specific for rhodopseudomonads was to screen for bacterial lysogens. To obtain a great number of bacteria to use in the screening process, we isolated bacteria from the natural environment of *Athiorhodaceae*. Mud and water samples were collected from ponds and ditches in the area of Ann Arbor, Michigan. Portions of these samples were subjected to enrichment procedures known to yield bacteria of the *Athiorhodaceae* (see above). Bacteria isolated in this manner were purified, and a number of cloned stocks were obtained.

These stocks were tested for resident prophage using mitomycin C, a known inducer of some prophages (10). The ability of each bacterial isolate to grow in the presence of 10 μg of mitomycin C per ml was tested. Although growth of all stocks was significantly inhibited, there was no sign of lysis. The supernatant that was obtained after sedimentation of one of these cultures lysed a number of strains of presumptive rhodopseudomonads, which were obtained from natural environments. One of the latter plating strains (014) was then used for further tests.

Plaque formation. Using standard overlay methods, tryptone top agar, and either tryptone or LB agar plates, Rφ-1 forms turbid plaques on a lawn of the sensitive host strain 014 temperatures from 30 to 40 C. In addition, Rφ-1 plaques with equal efficiency whether the plates are incubated under aerobic conditions in the dark or under anaerobic conditions in the light (phototropic conditions). Clear plaque mutants of Rφ-1 have been obtained and are currently being analyzed.

Host range of Rφ-1. Although Rφ-1 is unable to form plaques on a standard laboratory strain of *R. spheroides* (2.4.1), mutant derivatives of the phage were isolated at a frequency of $\sim 10^{-7}$ which formed plaques on strain 2.4.1. These mutants do not form plaques on the originally sensitive host (strain 014). One significant difference was observed between the original Rφ-1 and the mutant which plates on 2.4.1; the former is chloroform sensitive, while the latter is chloroform resistant.

To further test Rφ-1's host range, we determined whether the strain would form plaques on a number of other members of the *Athiorhodaceae*. In these experiments Rφ-1 did not form plaques on *Rhodopseudomonas capsulata*, *R. palustris*, or *Rhodospirillum rubrum*. As yet, no variants of Rφ-1 have been isolated which form plaques on these hosts.

Formation of lysogens carrying Rφ-1. Since Rφ-1 was isolated by a process of induction, it was assumed to be a temperate phage. This assumption was confirmed by showing that stable lysogens could be formed after infection with Rφ-1. Lysogens could easily be obtained by picking centers of turbid plaques and streaking the plaque samples on tryptone-B_1 plates to obtain single colonies. A quick screening procedure was developed to test for lysogens. Colonies of putative lysogens were picked with a sterile toothpick and stabbed onto a prepoured lawn of sensitive bacteria. The plate was then exposed to UV irradiation (2,700 ergs/cm^2) and incubated at 32 C for 48 h. A zone of lysis developed around the site of a lysogenic colony, but no zone of lysis was observable around the site of a nonlysogenic colony.

Growth of Rφ-1. The growth of Rφ-1 in strain 05, a streptomycin-resistant derivative of 2.4.1, was observed after infection and induction. Growth after infection was studied with a standard, single-step growth experiment, and growth after induction was monitored with a modification of that technique so as to limit the assay to free phage. As shown in Fig. 1 and 2, there is little difference in the growth pattern of Rφ-1 whether it begins its life cycle by infection or induction. The period required for maximum burst in each case is about 240 min, and the burst size is ~10 phage per infected bacterium.

Electron microscopy of Rφ-1. Isolates of Rφ-1 purified by CsCl centrifugation were prepared, and electron micrographs were obtained. As shown in Fig. 3, Rφ-1 appeared to have a polyhedral head with an extremely long tail. No tail fibers were observable in any of our preparations.

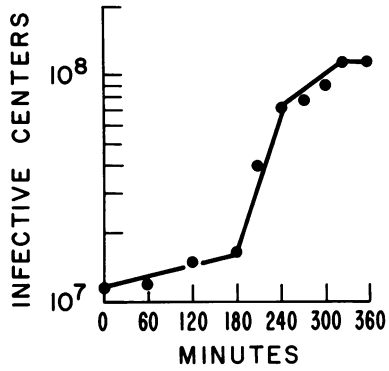

FIG. 1. *Growth of Rφ-1 after infection of a sensitive host. Strain 05 was grown at 31 C in tryptone broth. Approximately 10^7 phage were added to cells in the late logarithmic state of growth and allowed to adsorb for 30 min. Infected cultures were washed with tris-(hydroxymethyl)aminomethane - magnesium - gelatin buffer and resuspended in tryptone broth. Then dilutions were serially made into tryptone broth. These diluted cultures were incubated at 31 C. Samples were removed at the indicated times and plated on a sensitive lawn. Infective centers represented both free phage and infected cells.*

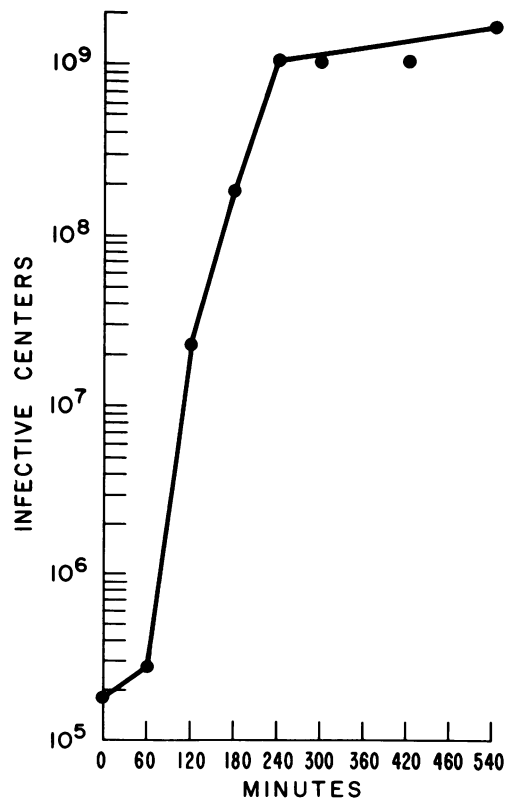

FIG. 2. *Growth of Rφ-1 after induction of a lysogen. Strain 032 was grown in tryptone broth at 31 C, and cells in the late phage of logarithmic growth were diluted into tris(hydroxymethyl)aminomethane-magnesium-gelatin buffer to yield a concentration of 1 × 10^8 cells/ml. Cells in buffer were exposed to UV irradiation (1,800 ergs/cm²) and then diluted into tryptone broth and incubated at 31 C. At the indicated times, samples were removed and plated on streptomycin plates (100 μg/ml) with a Str^r host (05). Since the lysogen is Str^s,* only extracellular phage was assayed, and infective centers indicated free phage.

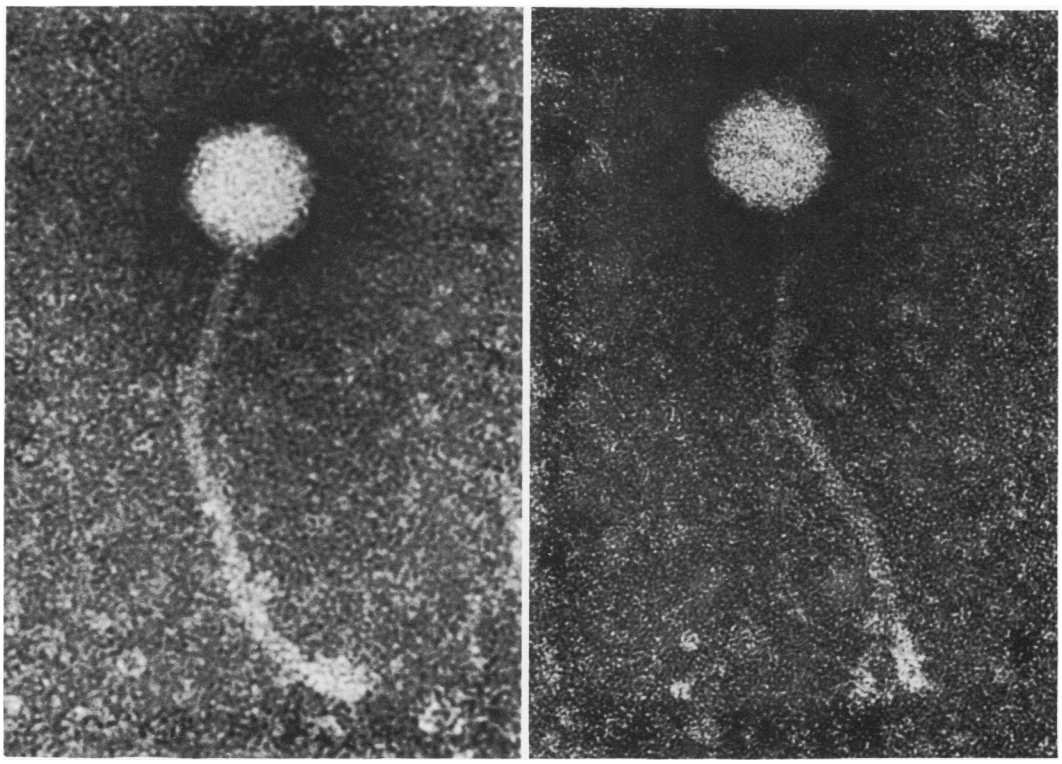

FIG. 3. *Electron micrographs of Rφ-1. Samples were negatively stained with sodium phosphotungstate (pH 6.8) by the drop method (7) on carbon-coated grids. Preparations were viewed in a Philips EM300 electron microscope at 80 kv with an anticontamination device in operation. Magnifications (×254,000) were calibrated using a diffraction-grating replica (54,864 lines/mm; E.F. Fullam Inc., Schenectady, New York).*

DISCUSSION

We designed a technique for isolating temperate phage specific for photosynthetic bacteria. The isolation of Rφ-1 indicates that this technique can be successfully employed. Since our ultimate goal is the isolation of a phage which will transduce the rhodopseudomonads, a number of experiments were performed to determine whether Rφ-1 is, in fact, a transducing phage. We have not been able to demonstrate transduction of any of a number of genetic markers, including amino acid auxotrophic and antibiotic-resistant markers. However, the search for proper transducing conditions is continuing.

Preliminary experiments demonstrated that Rφ-1 is a DNA phage, and a succeeding report will present studies characterizing the structure of the nucleic acid of Rφ-1.

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