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# Viral R Plasmid Rφ6P: Properties of the Penicillinase Plasmid Prophage and the Supercoiled, Circular Encapsidated Genome

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Properties of the viral R plasmid Rφ6P are described. As a temperate bacteriophage, it plaques on the facultative phototroph *Rhodopseudomonas sphaeroides*. Under aerobic conditions the phage had a latent period of 180 min, a burst time of 200 min, and a burst size of 15 to 20 particles per infective center. The encapsidated viral genome occurred as a supercoiled, circular DNA duplex with a mean contour length of 16.5 ± 1.0 μm. Percent guanine plus cytosine, as calculated from thermal denaturation profiles, was 63.5. Mitomycin C-induced loss of the prophage suggested an extrachromosomal location in the host cell. Use of this curing agent enabled the isolation of a plasmid-free strain of *R. sphaeroides*. Biophysical analysis of the plasmid-free strain lysogenized with Rφ6P confirmed that the prophage occurred as a plasmid in the host cell.

Cohen (4) states that plasmids can be viewed as primitive bacteriophages that have not as yet acquired those specialized functions necessary for a complex vegetative cycle or the production of infectious particles capable of existing outside the host cell. There are, however, a number of bacteriophages which do exist as nonintegrating plasmids in lysogenic cells. One such example is the coliphage P1, which possesses a linear genome in viral particles (16). Only after injection into the host cell does this genome circularize to form a plasmid. The ability of P1 to lysogenize its host appears to be independent of *recA*, *-B*, or *-C* functions (37). Circularization of the P1 genome to form a plasmid is carried out by a phage-promoted recombinational system (30).

Unlike λ and P22, whose prophages insert into the host chromosome (2, 9, 13), the plasmid prophage P1 would appear to have greater opportunity to undergo recombination with other plasmids in the same cell. With such recombinational events, the size and location of the inserted DNA determines whether nondefective phages are produced after induction (18, 22, 34).

Gene transposition is an efficient mechanism for inserting extra genes into phage genomes (4). The coliphage P7 is an example of a nondefective, temperate bacteriophage carrying an inserted sequence encoding antibiotic resistance (33). Walker and Walker (35) have suggested that P7 may have evolved from P1 by addition of a β-lactamase transposon. P7 is nondefective because the relatively small transposon is inserted in such a way as to displace the terminally redundant sequences of P1 (36). Nondefective, specialized transduction by phages such as P7 is a rare but highly efficient mechanism for the distribution of antibiotic resistance genes (29). In a previous communication (28) we reported the isolation of the viral R plasmid Rφ6P, whose natural host is the facultative phototroph *Rhodopseudomonas sphaeroides*. Rφ6P has a number of properties in common with P7, possessing a β-lactamase gene and existing as a plasmid in the prophage state. Nevertheless, as the data presented in this report will show, Rφ6P is different from other known plasmids and bacteriophages.

## MATERIALS AND METHODS

**Bacterial and bacteriophage strains.** The bacterial strains used were derivatives of the prototrophic, penicillin-resistant, lysogenic, wild-type strain RS601(Rφ6P). The penicillin-susceptible, nonlysogenic, plasmid-free strain RS630 was obtained from RS601 after two cycles of curing with mitomycin C. The lysogenic strain RS640(Rφ6P) was constructed by reinfecting RS630 with Rφ6P. Streptomycin-resistant strain RS650 *str-1* was a spontaneous mutant selected by plating about $10^9$ colony-forming units of RS630 on peptone-yeast extract (PYE) agar supplemented with 5 μg of streptomycin sulfate per ml.

**Media and cultural conditions.** PYE broth consisted of 3 g of peptone (Difco Laboratories, Detroit, Mich.), 3 g of yeast extract (Difco), and 1,000 ml of distilled water. PYE agar consisted of PYE broth and 1.5% (wt/vol) agar. PYE layer agar consisted of PYE broth, 0.01 M $MgSO_4$, and 0.8% (wt/vol) agar. TMG buffer contained 0.01 M tris(hydroxymethyl)aminomethane, 0.01 M $MgSO_4$, and 0.01% (wt/vol) Difco bacteriological gelatin, adjusted to pH 7.4 before autoclave sterilization.

All cultures were grown at 32°C unless otherwise stated. Liquid cultures were aerated by shaking at 350

rpm in a New Brunswick G24 Environmental Incubator (New Brunswick Scientific Co., New Brunswick, N.J). Penicillin G and streptomycin sulfate were supplied by Glaxo Laboratories, Boronia, Victoria, Australia. Mitomycin C was supplied by Sigma Chemical Co., St. Louis, Mo.

**Bacteriophage titration.** The soft-layer overlay technique of Adams (1) was used for bacteriophage titration. A 0.5-ml sample of an exponential-phase culture (about $5 \times 10^8$ colony-forming units per ml) of the indicator bacterium and 1 ml of the appropriate dilution of the bacteriophage were added to a molten 3-ml PYE layer. This was mixed and immediately poured over the surface of a PYE agar plate, left to set, and then incubated at 32°C for 24 h. Phage titers were determined by counting the resulting plaques.

**Preparation of high-titer lysates of Rφ6P.** Strain RS630 was used as the host bacterium for growth of Rφ6P. Layers from plates showing confluent lysis were suspended in PYE broth with the aid of a Vortex mixer. The resulting suspension was centrifuged at 12,000 × *g* for 10 min, and the supernatant was collected and filter sterilized through 0.45-μm membrane filters. The lysates were stored at 4°C.

**Extraction of viral nucleic acid.** Layers from 25 plates showing confluent lysis were suspended in 100 ml of TMG buffer. Most of the agar and cellular debris was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant was further cleared of cellular material by a 20-min period of centrifugation at 12,000 × *g*. The virus particles were pelleted from the supernatant by centrifugation at 80,000 × *g* for 2 h. The pellet was resuspended in 5 ml of TMG buffer, and the nucleic acid was extracted with an equal volume of water-saturated phenol by gently mixing for 2 min. After centrifugation on a bench centrifuge for 30 min at 2,000 × *g* to break the phenol-water emulsion, the upper, aqueous layer was collected and freed of phenol by manual shaking with five changes of anesthetic-grade ether. Residual ether was removed by bubbling air through the solution.

**Extraction of plasmid DNA.** The general method of Guerry et al. (14) was used to extract plasmid DNA from bacterial cells. Cells from a PYE agar plate were used as the inoculum for 100 ml of PYE broth in a 250-ml Erlenmeyer flask, and the culture was incubated aerobically for 24 h. The cells were harvested by centrifugation at 12,000 × *g* for 10 min, and the pellet was suspended in 2 ml of 25% sucrose in 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0. To this was added 0.4 ml of lysozyme (Calbiochem, San Diego, Calif.), 5 mg/ml in 0.25 M tris(hydroxymethyl)-aminomethane (pH 8.0), and the mixture was held in an ice bath for 5 min; 0.8 ml of 0.25 M ethylenediaminetetraacetic acid was then added, and the mixture was chilled for a further 5 min. A 10% aqueous solution of sodium dodecyl sulfate was added dropwise until lysis occurred, indicated by a slight gelling of the mixture. NaCl (5 M) was added to give a final concentration of 1 M, and the lysate was stored at 4°C overnight. The preparation was then centrifuged for 30 min at 17,000 × *g*, and supernatant was collected and extracted gently with an equal volume of water-saturated phenol, as described for viral nucleic acid. The final solution was dialyzed overnight at 4°C against 0.01 M tris(hydroxymethyl)aminomethane-0.25 mM ethylenediaminetetraacetic acid, pH 7.4.

**Sucrose density gradient centrifugation of plasmid DNA.** Plasmid DNA centrifugation was performed in 5 to 20% neutral sucrose gradients containing 4 μg of ethidium bromide (ICN Pharmaceuticals Inc., Cleveland, Ohio) per ml. DNA samples (0.1 ml) were layered onto 5-ml gradients and centrifuged at 100,000 × *g* for 100 min in an SW 39L rotor of a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The pink fluorescent DNA bands were observed visually under long-wave UV irradiation.

**Preparation of antiserum to Rφ6P.** To prepare antiserum to Rφ6P, 1 ml of a high-titer (about $10^{10}$ plaque-forming units per ml) preparation of the virus in PYE broth was mixed with an equal volume of Freund complete adjuvant (a kind gift of W. J. Halliday) and emulsified by sonic treatment. The sonic extract (0.5 ml) was injected intramuscularly into each hind leg of the a rabbit. This procedure was repeated after 14 days, and the rabbit was bled from the ear after a further 14-day period. The blood was allowed to clot, and the serum was collected and centrifuged to remove any remaining blood cells. The supernatant was filter sterilized through a 0.45-μm membrane filter and stored at −20°C.

**Staining of viral nucleic acid.** Samples of the extracted viral nucleic acid were stained to determine the nature of the molecule by the method of Bradley (3).

**Thermal denaturation of viral nucleic acid.** Viral nucleic acid was precipitated from solution with 2 volumes of ice-cold 95% ethanol and dissolved in 0.2 ml of 0.1× standard saline citrate (15 mM NaCl-1.5 mM sodium citrate) (20) to give an absorbance of between 0.4 and 0.8 at 260 nm. Thermal denaturation was carried out in 1-ml quartz cuvettes in a Gilford 2400 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). DNAs of known percentages of guanine plus cytosine, from *Pseudomonas putida* UQM 493 (63.5 to 63.9%) and *Proteus vulgaris* UQM 734 (37%), were a generous gift of L. I. Sly (Culture Collection, Department of Microbiology, University of Queensland, St. Lucia, Queensland, Australia) and were included in each set of experiments. The percent guanine plus cytosine was obtained from the melting temperature, using equations derived by Mandel and Marmur (20).

**Mitomycin C curing of plasmid DNA.** Exponential-phase cells of RS601 were inoculated into PYE broth supplemented with 1 μg of mitomycin C per ml and incubated in the dark with shaking for 24 h. The survivors were plated onto PYE agar plates and incubated at 32°C. The resulting clones were replica plated onto PYE agar containing 1 μg of penicillin per ml. Penicillin-susceptible clones were again treated with mitomycin C as before, the putative plasmid-free survivors were purified, and their plasmid DNA content was surveyed by extraction and screening by electron microscopy and ethidium bromide density gradient centrifugation as described elsewhere in this report.

**Electron microscopy.** The grids used for electron microscopy were prepared by the method of Kleinschmidt and Zahn (17) as modified by Pemberton (26)

and Pemberton and Clark (27). The spreading solution (1.3 ml) contained 1 ml of 0.5 M ammonium acetate, 0.1 ml of cytochrome *c* (1 mg/ml)-0.5 M ammonium acetate (pH 5.0), and 0.2 ml of a dialyzed sample of DNA. The hypophase was 0.1 M ammonium acetate, pH 5.0. All molecules were photographed with a Phillips 300 electron microscope (N. V. Philips Gloeham-penfabrieken, Eindhoven, The Netherlands), and their lengths were obtained by making enlarged prints and measuring their contours with a map measurer. Usually, 10 molecules having well-defined open contours were used to determine the length of a particular class of plasmids.

Samples to be negatively contrasted were spotted onto copper grids (200 mesh, coated with a Parlodion film supported by a thin layer of carbon deposited by vacuum evaporation), and the excess was removed with filter paper. Staining was carried out by the method of Miller et al. (21), using 1% ammonium molybdate, pH 6.5.

## RESULTS

**Adsorption of Rφ6P.** As previously reported (28), the Rφ6P particle has an isometric head 65 nm in diameter and a long noncontractile tail 12 by 250 nm in length possessing four tail fibers at the base. Electron microscopic examination of phage-infected cells revealed that Rφ6P attaches to the host cell via the distal end of its noncontractile tail.

Phage adsorption can be followed either indirectly, by titrating unadsorbed phage in the phage-bacterium mixture, or directly, using electron microscopic examination of phage-infected cells. Using the indirect method, appreciable adsorption of Rφ6P to susceptible cells was not detected until 20 min after mixing the phage and bacteria (Fig. 1). After this initial lag period (20 min), adsorption proceeded rapidly, so that 80% of phage adsorption occurred in the period between 20 and 45 min after mixing. Viable counts performed on infected and uninfected cultures showed no significant decrease in viability among the phage-infected cells during this period (unpublished data).

Direct electron microscopic examination of the mixture at total multiplicity of infection (plaque-forming units/colony-forming units) of 1 after 60 min revealed that 10% of the cells had adsorbed one or two particles, whereas 1 to 2% of the cells were completely encapsulated by phage particles. Whatever the explanation for this nonrandom adsorption, it results in an effective 10- to 100-fold reduction in the multiplicity of infection for the total culture.

**Single-step growth.** A standard single-step growth experiment (Fig. 2) revealed that Rφ6P has a latent period of 180 min, the burst time being 200 min, with release of 15 to 20 plaque-forming units per infective center. Mural and Friedman (23) obtained similar results with Rφ-1, another temperate bacteriophage of *R. sphaeroides*.

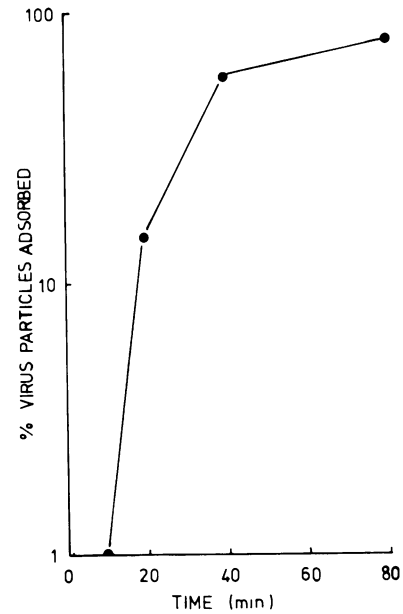


FIG. 1. *Kinetics of adsorption of bacteriophage Rφ6P to strain RS630 of R. sphaeroides. An exponential-phase culture (0.9 ml, about $10^8$ colony-forming units per ml) in PYE broth was infected with 0.1 ml of Rφ6P (about $10^9$ plaque-forming units per ml) to give a multiplicity of infection of 1. Samples (0.05 ml) were removed, diluted in TMG buffer saturated with chloroform, and left to stand for 15 min at 32°C to kill the bacteria.*

**Lysogenization by Rφ6P.** Lysogenization by Rφ6P resulted in conversion to penicillin resistance. At a multiplicity of infection of 1, 80% of the Rφ6P particles adsorbed, whereas only 4 to 5% of the bacterial population became lysogenized (Fig. 1 and 3). Both the adsorption and the lysogenization occurred without detectable loss of viability by the bacterial population, even at a multiplicity of infection of 100.

**Characterization of phage nucleic acid.** Nucleic acid extracted from Rφ6P phage particles gave the staining reaction of double-stranded DNA (3). In a previous communication (28) we reported that freshly extracted phage DNA occurs in a supercoiled configuration, which upon freezing and thawing relaxes to produce an open circular form (Fig. 4). These circles have a mean contour length of 16.5 ± 1.0 μm (10 molecules measured), which corresponds to 33 ± 2 megadaltons (19).

When Rφ6P DNA was subjected to thermal denaturation, the percent guanine plus cytosine, calculated from the melting temperature profile,

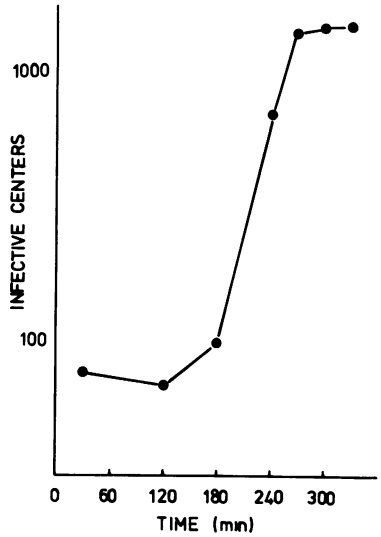


FIG. 2. *Single-step growth of Rφ6P on RS630. An exponential-phase culture of RS630 (0.9 ml, about $10^9$ colony-forming units per ml) was infected with 0.1 ml of a preparation of Rφ6P ($10^{10}$ plaque-forming units per ml) to give an input multiplicity of infection of 1. The phage were allowed to adsorb for 60 min at 32°C. Samples (0.1 ml) of the mixture were removed, diluted 10-fold in anti-Rφ6P antiserum (1/40 dilution in TMG), and incubated for 5 min at 32°C with aeration, and 1-ml samples were removed and assayed for infective centers on RS630.*

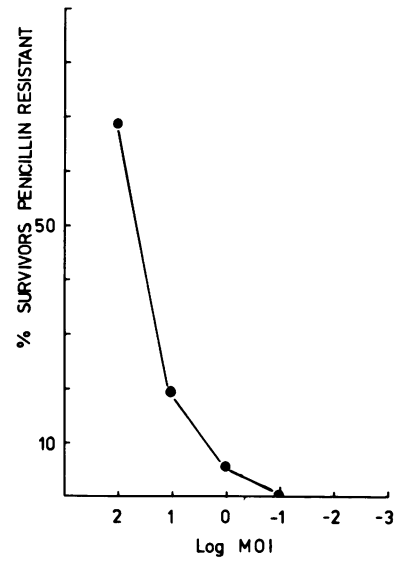


FIG. 3. *Lysogenization of RS630 by Rφ6P. An exponential-phase culture (0.1 ml, $10^7$ colony-forming units per ml) in PYE broth was infected with 0.1 ml of varying dilutions of Rφ6P in TMG buffer at multiplicities of infection (MOI) from $10^{-3}$ to $10^2$ and allowed to adsorb for 60 min at 32°C. Undiluted antiserum (0.05 ml) was added to the mixture, and the culture was incubated for 1 h to allow for expression of penicillin resistance. Samples (0.1 ml) were then diluted and plated on PYE agar, and the percentage of survivors of the penicillin-resistant phenotype was determined by replica plating onto PYE agar containing 1 μg of penicillin per ml.*

was 63.5. As with bacteriophage PM2, the supercoiled nature of the viral genome increased the melting temperature, and it was necessary to shear the phage DNA to produce linear fragments before the melting temperature was determined.

**Prophage induction by mitomycin C.** Rφ6P induction by mitomycin C is depicted in Fig. 5. Considering that there is no lag period due to the infection processes, the time required for phage release after induction agreed with the normal burst time of the phage (Fig. 2). In contrast to mitomycin C, UV irradiation failed to produce detectable induction of the prophage in the same Rφ6P lysogen (data not shown).

**Spontaneous and induced loss of the Rφ6P prophage.** Spontaneous loss of the prophage, as detected by reversion to penicillin and Rφ6P susceptibility, occurred with a frequency of less than 1 in $10^3$. By contrast, 8% of clones reisolated after growth in the presence of 1 μg of mitomycin C per ml appeared cured of the prophage (Fig. 6). All penicillin-susceptible clones were also Rφ6P susceptible. Ten penicillin-susceptible Rφ6P-negative clones were tested for back mutation to penicillin resistance. Since there was no detectable (less than 1 in $10^{11}$) reversion to penicillin resistance, it can be concluded that there was loss of the entire prophage genome.

**Isolation of plasmid DNAs from Rφ6P lysogens.** Genetic evidence presented in this and a previous communication (28) supports the hypothesis that Rφ6P exists as a plasmid in the prophage state. We attempted to isolate plasmid DNA from Rφ6P lysogens (RS601 and RS640) and a nonlysogenic derivative (RS630). DNA samples extracted from these strains were examined in two ways.

First, the entire sample was screened for the presence of plasmid molecules by electron microscopy. Extracts of the lysogenic strains, RS601 and RS604, contained 80 to 90% linear fragments 1 to 5 μm in length. Supercoiled and open circular molecules 33 ± 2 μm (10 molecules measured) in length constituted the remaining 10 to 20% of the sample. An electron micrograph of an open circular form of this plasmid is shown in Fig. 7. In contrast, no plasmid molecules were detected in the nonlysogenic strain, RS630. This is despite the lysogenic and nonlysogenic strains'

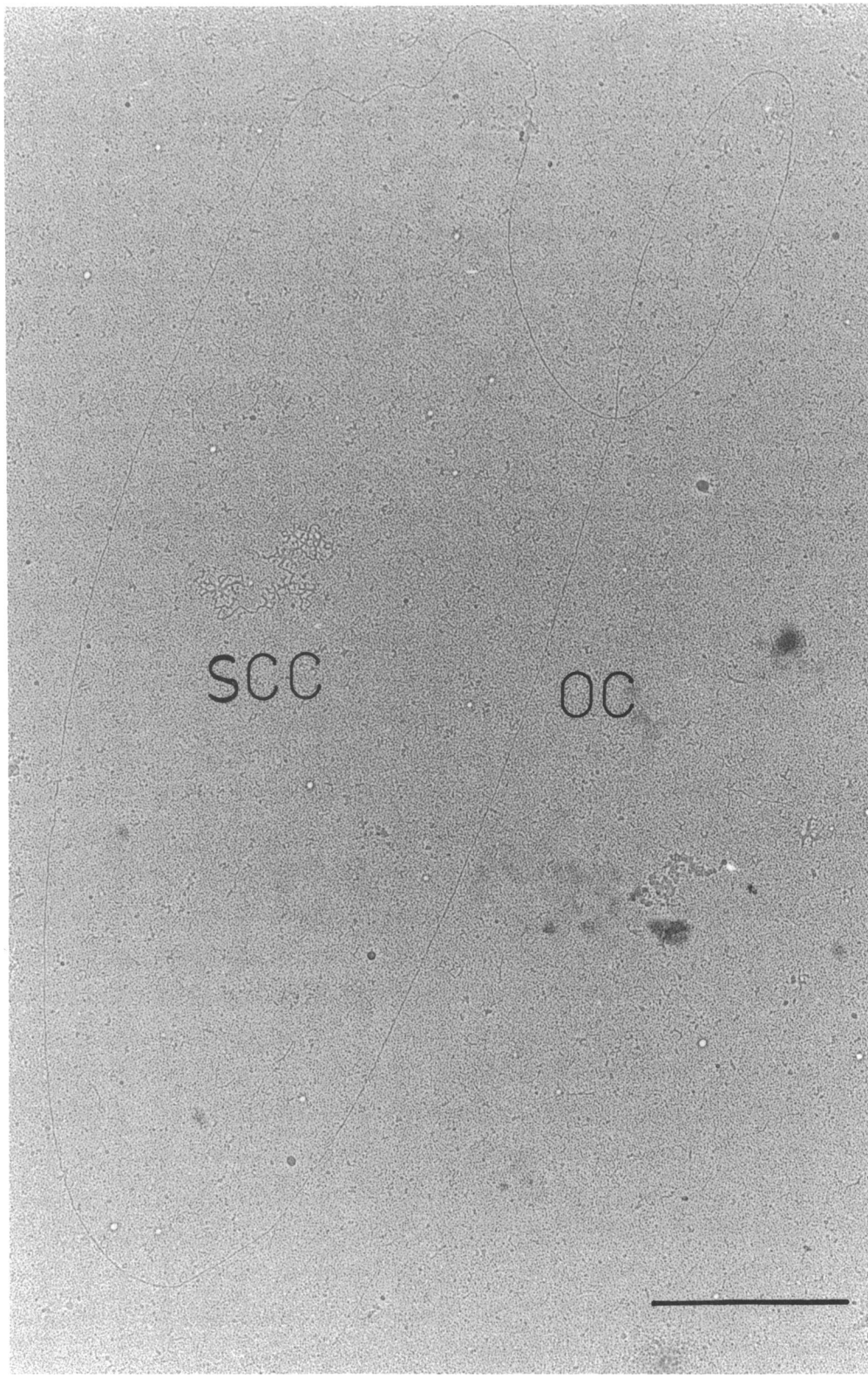


FIG. 4. *Electron micrograph of nucleic acid extracted from free virus particles. Note the open circular (OC) and the densely supercoiled (SCC) molecules. The OC molecule, which has a molecular length of 16.0 μm, was prepared by the method of Kleinschmidt and Zahn (17) as modified by Pemberton and Clark (27). Bar = 1 μm.*

producing equal quantities of linear fragments in 10 separate experiments.

Second, each sample was screened for plasmid DNAs by ultracentrifugation in a 5 to 20% neutral sucrose gradient containing ethidium bromide. After ultracentrifugation the gradient was examined for the presence of fluorescent bands by using long-wave UV irradiation. DNA prepared from lysogenic strains RS601 and RS640 gave a broad upper band and a thin, well-defined lower band, whereas similarly prepared DNA from nonlysogenic RS630 yielded only the broad upper band. Electron microscopic examination showed that each upper band contained only linear molecules, whereas each lower band contained a single size class of supercoiled molecules, 33 ± 2 μm (10 molecules measured for each strain) in length. Since the appearance and disappearance of this 33-μm plasmid seems correlated with the presence and absence of the phage, the simplest conclusion is that Rφ6P exists as a dimeric plasmid in the lysogenic cell. Although there are other possible explanations for these data, covalently closed circular dimers have been found in nonlysogenic *Escherichia coli* infected with lambda (10) and as one of the oligomeric forms of ColE1 (12). In both these instances the dimers are thought to be replicative intermediates.

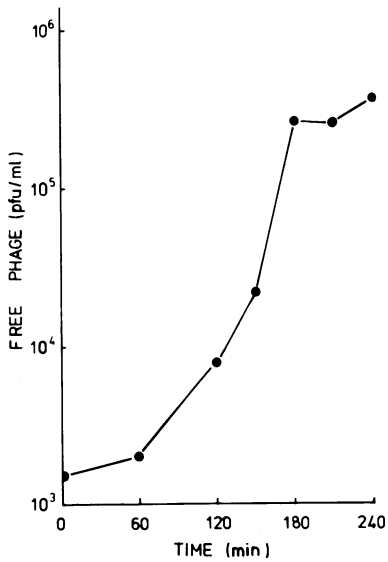


FIG. 5. *Mitomycin C induction of the Rφ6P prophage. Exponential-phase cells of RS601(Rφ6P), about $10^8$ colony-forming units per ml, were exposed to 0.1 μg of mitomycin C per ml in PYE broth for 30 min in the dark. The culture was centrifuged to pellet the cells, which were resuspended in PYE broth to a concentration of about $10^6$ colony-forming units per ml. Samples (1 ml) were removed at the indicated times and assayed for free phage plaque-forming units (pfu) per ml by plating them on a lawn of streptomycin-resistant indicator cells (RS650 str-1), using basal plates of PYE agar supplemented with 5 μg of streptomycin per ml.*

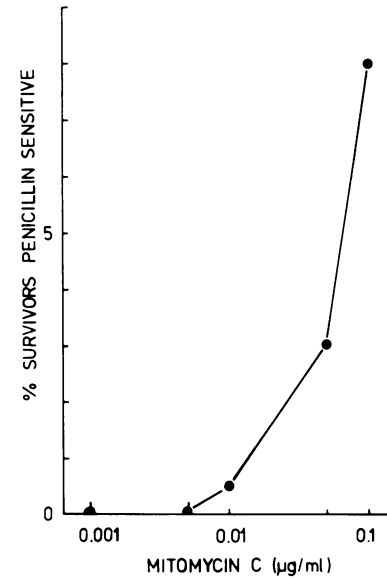


FIG. 6. *Mitomycin C-induced loss of the Rφ6P prophage. An exponential-phase ($10^8$ colony-forming units per ml) culture of RS601(Rφ6P) was inoculated into 5 ml of PYE broth containing 0.05 ml of undiluted anti-Rφ6P antiserum and various concentrations of mitomycin C. After incubation for 24 h at 32°C with aeration in the dark, cultures were stored for a further 24 h at 4°C. The survivors were plated on PYE agar and replica plated onto PYE agar containing 1 μg of penicillin per ml.*

## DISCUSSION

Rφ6P is an unusual biological entity, combining the genetic and biophysical properties of a virus and a drug resistance plasmid. As a bacteriophage it possesses properties which are taxonomically distinct from other bacteriophage types (8). It is only the second reported example of a bacteriophage possessing a supercoiled, circular genome in the viral particle (28). The other example is PM2, a lipid-containing bacteriophage whose natural host is a marine pseudomonad (5–7). There are, however, many differences between PM2 and Rφ6P. These include the morphology of the phage particle, the size of the enclosed viral genome, the guanine plus cytosine content of the DNA, the presence of lipid in the phage particle, and whether the phage is temperate or virulent.

The Rφ6P prophage is stable, as measured by the low level of spontaneous curing of lysogens. In contrast, high-frequency curing is induced by mitomycin C. It was this ability of mitomycin C

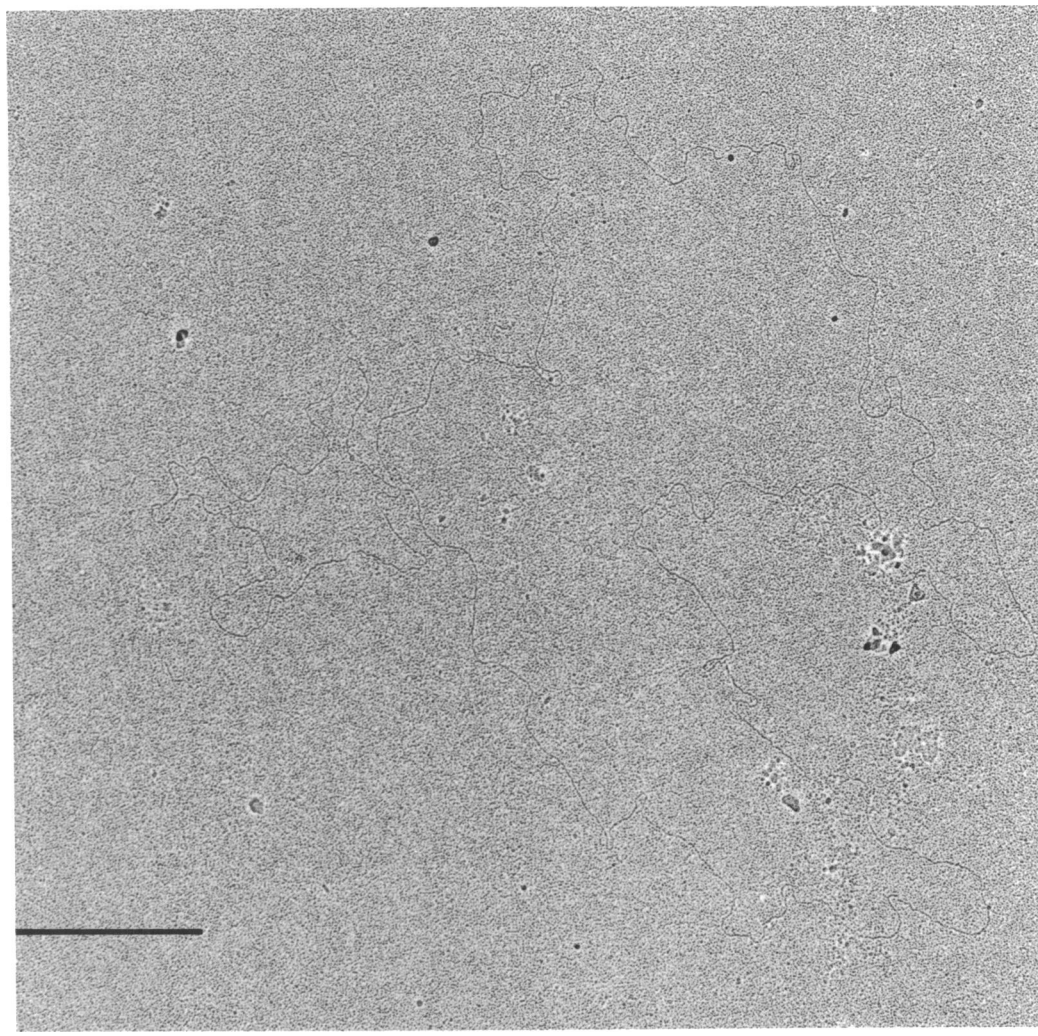

FIG. 7. *Electron micrograph of an open circular molecule 33 μm in length extracted from RS640 by the method of Guerry et al. (14) and prepared for the electron microscope by the method of Kleinschmidt and Zahn (17) as modified by Pemberton and Clark (27). Bar = 1 μm.*

to cure such a stable plasmid that prompted our attempts to isolate a plasmid-free derivative of *R. sphaeroides*.

Wild-type isolates of *R. sphaeroides* have been shown to harbor three plasmid classes (11, 23, 31). One class has a molecular mass of $35 \times 10^6$, whereas the other two classes, each with a molecular mass of $70 \times 10^6$, are separable on the basis of buoyant density. RS601, the wild-type isolate used in our studies, possesses a single molecular-mass species of plasmid DNA. Repeated extractions of RS630, a derivative of RS601, cured of the Rφ6P prophage, failed to yield any detectable plasmid DNA. The conclusion that RS630 is plasmid free is strengthened by the isolation of plasmid DNA from either RS630 lysogenic for Rφ6P or RS630 infected with the wide-host-range R plasmids R68.45 and RP1 (unpublished data). However, there are at least two possible objections to such a conclusion. First, exceptionally large plasmids (greater than 200 megadaltons) may be removed in the precipitation step. Such large plasmids have been detected in strains of *Rhizobium* (24) and *Pseudomonas* (25). Second, RS630 may harbor plasmids with unusual physical properties which prevent their isolation by current methods.

Heffron et al. (15) have concluded that the widespread occurrence of β-lactamase genes results from extensive distribution of a small number of ancestral β-lactamase genes carried on transposable genetic elements. The possibility

that Rφ6P carries a β-lactamase transposon is supported by the isolation of morphologically similar, naturally occurring Rφ6P-type bacteriophages which do not encode a β-lactamase (unpublished data). Further genetic and biophysical studies will be required to determine the nature of both the β-lactamase and its structural gene. With the recent demonstration of conjugation and chromosome transfer mediated by R68.45 (32; unpublished data) and RP1 (unpublished data) and the availability of a plasmid-free strain, the means are available to test this hypothesis.

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