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Bacteriophages of *Rhodopseudomonas spheroides*: Isolation and Characterization of a *Rhodopseudomonas spheroides* Bacteriophage

AHARON ABELIOVICH AND SAMUEL KAPLAN

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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A DNA-containing bacteriophage, designated RS1, infecting *Rhodopseudomonas spheroides* 2.4.1, has been isolated from sewage. The buoyant density of RS1 in CsCl equilibrium centrifugation is 1.50 g/cm^3, and the buoyant density of RS1 DNA is 1.706. The phage possesses a polyhedral head, approximately 65 nm in diameter, and a tail 60 nm long. When grown on aerobic cells, RS1 has a latent period of 120 min and an average burst size of 20. When grown on anaerobic cells, RS1 has a latent period of 150 min, and a burst size similar to that observed during aerobic infection. The adsorption rate constant of RS1 to aerobic cells is 1.2×10^{-9} ml/min, and 0.58×10^{-9} ml/min to anaerobic cells. Adsorption of RS1 to *R. spheroides* requires the presence of divalent cations.

Rhodopseudomonas spheroides belongs to the group of nonsulfur purple bacteria, which can grow photosynthetically under anaerobic conditions, or heterotrophically under aerobic conditions. To date, there is only one published report on the isolation of a bacteriophage specific for any of the nonsulfur purple bacteria; Freund-Molbert et al. (7) described a bacteriophage infecting *Rhodopseudomonas palustris*.

Because of the unique ability of this group of organisms to exist under two quite distinct physiologic conditions, and the subcellular differentiation which accompanies these alternative physiologic states, the isolation and examination of specific bacterial viruses might reveal rather unique patterns of control. For example, the effect of light and oxygen, already shown (4, 13) to be of special importance in determining the differentiation process in *R. spheroides*, seems to play a major role in phage development and infection as shall be described later. In addition, the general lack of mechanisms for gene exchange within this group of organisms (B. Marrs has recently described a genetic system for *R. capsulata* strain "St. Louis," [B. Marrs, Proc. Nat. Acad. Sci., in press]) in itself, provides sufficient impetus for the isolation and characterization of *R. spheroides* bacteriophage.

MATERIALS AND METHODS

Organisms and media. *Rhodopseudomonas spheroides* 2.4.1 was supplied by R. Y. Stanier, *R. spheroides* "L" was obtained from J. Lascelles, and *R. spheroides* M29:5 (met$^-$leu$^-$) was from W. R. Sistrom. *R. palustris* ATCC 17002, *R. capsulata* KBl, and *R. gelatinosa* strain 2150 were from N. Pfennig. *R. capsulata* "St. Louis" was from H. Gest. *Rhodospirillum rubrum* strain Ha was obtained from N. Pfennig, and *Rhodospirillum rubrum* ATCC 11170 was obtained from G. Ordal.

All *Rhodopseudomonas* strains were grown aerobically on Sistrom's medium A (12) supplemented with 0.2% Casamino Acids (Difco). Aerobic and anaerobic cultures were prepared as described by Fraker and Kaplan (5). The *Rhodospirillum* strains were grown on a medium (N. Pfenning, personal communication) containing KH_2PO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.04%), NaCl (0.04%), NH_4Cl (0.05%), $CaCl_2 \cdot 2H_2O$ (0.005%), Na succinate (0.2%), yeast extract (0.1%),Fe citrate (5 mg/liter), ethanol (0.05%), Na thioglycolate (0.02%), and 10 ml per liter of media of a trace elements solution containing $ZnSO_4 \cdot 7H_2O$ (10 mg), $MnCl_2 \cdot 4H_2O$ (3 mg), H_3BO_4 (30 mg), $CoCl \cdot 6H_2O$ (20 mg), $CuCl_2 \cdot 2H_2O$ (1 mg), $NiCl_2$ $6H_2O$ (2 mg), and $Na_2MoO_4 \cdot 2H_2O$ (3 mg), dissolved in 1 liter. The medium was brought to pH 6.7 after all additions.

Isolation of phage DNA, and determination of its molecular weight and base composition. RS1 (*R. spheroides* phage 1) DNA was prepared according to the method described by Thomas and Abelson (15), dialyzed overnight against SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and stored in SSC at 4 C. Base composition of RS1 DNA was calculated from its buoyant density in CsCl (9) and from its thermal denaturation curve. Thermal denaturation and renaturation were determined employing the methods described by Marmur and Doty (10) on samples of 15 μg of DNA per ml in SSC, by using a Beckman Acta III spectrophotometer, programmed for automatic temperature control. λ_{vir} DNA was used as reference.

Equilibrium centrifugation of phage DNA in

CsCl. Equilibrium centrifugation of phage DNA was performed in a Beckman model E analytical ultracentrifuge, by using *Tetrahymena* A 16672b DNA (ρ 1.683) as reference. Refractive index of samples was measured with an Abbe refractometer. The refractive index of the sample of phage DNA (2 μg of DNA in 1/10 SSC solution) was brought to 1.3443 (ρ = 1.6991) with solid CsCl. Centrifugation was for 16 h at 44,000 rpm at 25 C. UV absorption photographs were made, and scanned with a microdensitometer (Mk IIIb, Joyce Loebl and Co. Ltd, England). The density of the DNA was calculated by the method of Sueoka et al. (14). The molecular weight of RS1 DNA was determined employing two methods: (i) from the sedimentation coefficient derived from sucrose gradient centrifugation, as described by Freifelder (6), by using [^{3}H]adenine-labeled λ_{vir} DNA (λ_{vir} was grown on *E. coli* C600 in L broth) as a marker. RS1 DNA was labeled with $^{32}PO_4$ by growing *R. spheroides* cells in the presence of 5 μCi of PO_4 per ml in the standard medium. λ_{vir} was kindly provided by Dale Kaiser. Method (ii) was from the rate of reassociation of sheared, melted DNA, also employing λ_{vir} DNA as a marker, according to the method described by Britten and Kohne (2). For calculation of the molecular weight by the second method we assumed the absence of repetitious DNA sequences in RS1 DNA.

DNA concentrations. DNA concentrations were determined by the method of Ceriotti (3) and from their absorbance at 260 nm, assuming an absorbance coefficient of 20 per mg of DNA/ml.

Protein determinations. Proteins were determined by the method of Lowry et al. (8).

Phage antiserum. Antiserum against phage RS1 was prepared by injecting rabbits with a series of three subcutaneous injections of purified phage suspension in Freund complete adjuvant (0.5 ml of 10^{12} PFU/ml + 0.5 ml of adjuvant), once every 2 weeks. Serum used in these experiments was prepared after 4 weeks.

Adsorption rate and one-step growth experiments. The adsorption rate and one-step growth curve were determined by the methods described in Adams (1).

Materials. CsCl was a product of A. D. Mackay Inc., N. Y.

Carrier-free $^{32}PO_4$ was purchased from New England Nuclear Corp., Boston, Mass. [2,8-^{3}H]adenine was from ICN Pharmaceutical Inc., Cleveland, Ohio.

RESULTS

Source of the bacteriophage RS1. RS1 was isolated from material collected from a pig farm sewage oxidation pond. The sewage was treated with chloroform and centrifuged at 12,000 $\times$ *g* for 10 min. Samples (100 ml) were mixed with an equal volume of double-strength medium, and inoculated with 10^8 (total) cells of *R. spheroides* 2.4.1. The enrichment cultures were incubated aerobically for 72 h at 30 C, treated with chloroform, and centrifuged at 12,000 $\times$ *g* for 10 min. The supernatant was plated with aerobically grown *R. spheroides* (2 ml of supernatant and 10^8 cells per plate) and incubated aerobically at 30 C. Five to ten clear plaques per plate appeared within 24 h. A single plaque was picked and was used for the preparation of viral stocks for further characterization. Figures 1 and 2 show the typical appearance of RS1 in negatively stained preparations. In all preparations, approximately 0.1% of the phages appeared quite different (Fig. 3), but we were unable to separate the two forms, and it is not clear whether this is the same or a different phage.

Growth and purification of phage. An aerobic culture of *R. spheroides* was allowed to grow to a cell concentration of 5 $\times$ 10^8/ml, and phage were added to a final MOI (multiplicity of infection) of 0.5. The infected culture was then incubated for 8 to 12 h with vigorous shaking to maintain adequate aeration until maximal clarification of the cultures occurred. Chloroform was then added, and bacterial debris was removed from the lysate by centrifugation at 16,000 $\times$ *g* for 10 min. The phage was pelleted by a 4-h centrifugation at 19,000 rpm in a Spinco type 19 rotor at 4 C, and resuspended in growth medium. The suspension was mixed with solid CsCl to give a final density of 1.50 g/cm^3, and was centrifuged to equilibrium in a SW 50.1 rotor at 35,000 rpm for 24 h at 25 C. Fractions were collected dropwise from the bottom of the tube and their refractive index was measured. The pooled phage fractions were dialyzed overnight against 1 liter of medium. Figure 4 shows the coincidence of infectivity, optical density, and DNA across a typical CsCl gradient prepared as described above.

Host specificity. Purified phage RS1 preparations were tested for their ability to form plaques on the following bacterial strains: *R. spheroides* 2.4.1, *R. spheroides* "L," *R. spheroides* M29:5, *R. palustris* ATCC 17002, *R. capsulata* Kbl, *R. capsulata* "St. Louis", *R. gelatinosa* strain 2150, *Rhodospirillum rubrum* ATCC 11170, and *Rhodospirillum rubrum* Ha. RS1 replicated only in *R. spheroides* 2.4.1 and in *R. spheroides* L, forming clear plaques. Infection of *R. spheroides* M29:5 with RS1 results in what might be described as an abortive infection (1), since infection of the cells on agar plates at MOI greater than 1 results in confluent lysis, whereas infections at MOI less than 1 do not produce plaques. All further experiments were performed with *R. spheroides* 2.4.1.

Adsorption and one-step growth. RS1, at either low or high MOI, adsorb readily to aero-

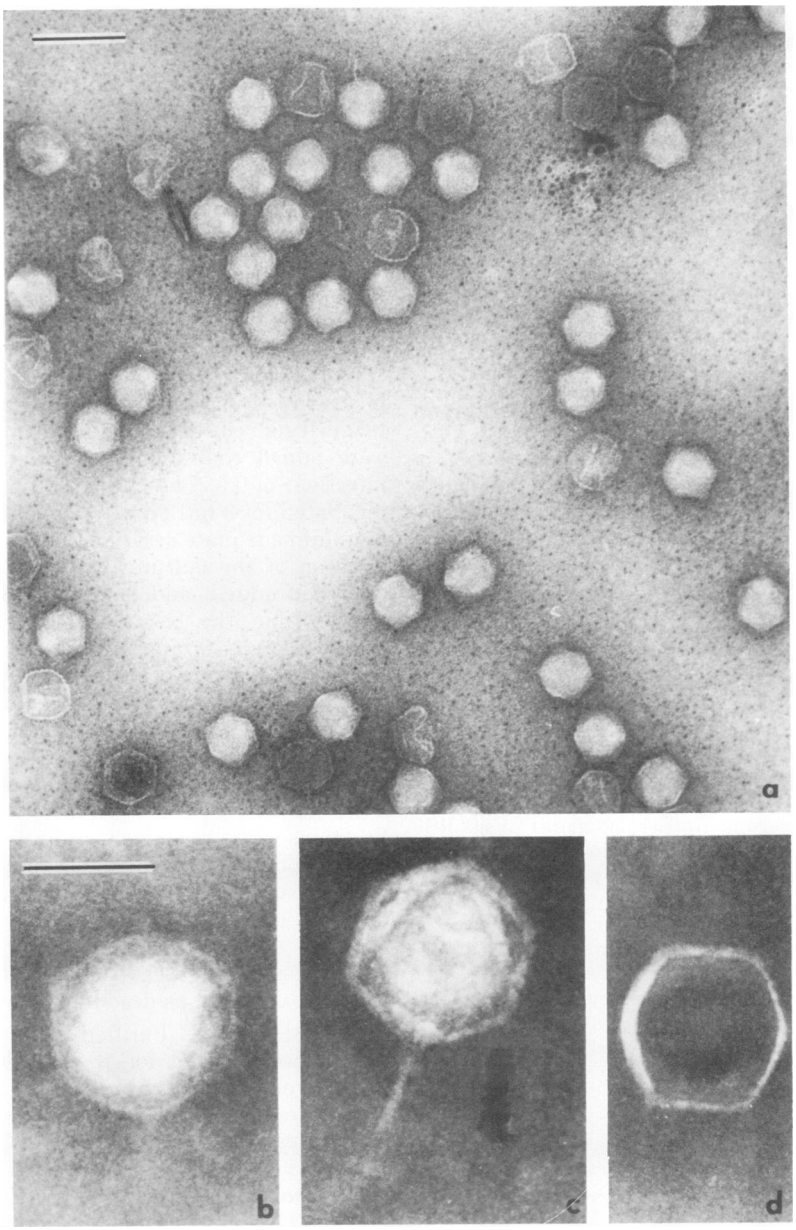

FIG. 1. *Electron micrograph of RS1 particles. Negative stain with 1% ammonium molybdate. In (a) the scale represents 100 nm, (b-d) scale represents 50 nm.*

bically grown *R. spheroides* 2.4.1, (Fig. 5), with 99% of the added phage particles being adsorbed to the cells within 12 min. The infected cells can be later detected as infected centers on plates. On the other hand, when anaerobic cells were infected at low MOI, only 7 to 12% of the phage particles could be detected as infected centers on aerobic indicator host cells, and the recovery of free phage particles from the incubation mixture varied from 20 to 70% in different experiments. These results indicate that only a small and variable fraction of the input phages that adsorb at low MOI to anaerobic cells result in infected centers. However, at MOI of > 10, all anaerobic cells give rise to infected centers.

Adsorption of RS1 to both aerobic and anaer-

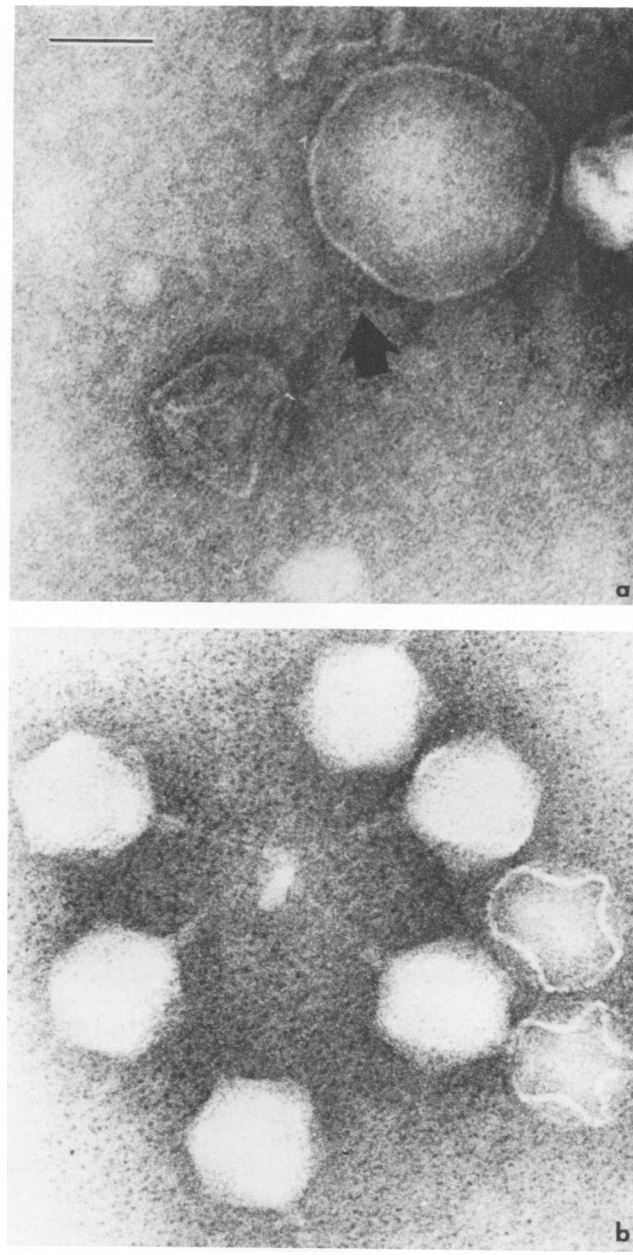

FIG. 2. *Electron micrograph of RS1 particles. (a) Arrow points to base plate and tail fibers. Scale represents 50 nm. (b) Clustered RS1 particles. Scale same as (a).*

obic cells requires the presence of the divalent cations, Mg^{2+} or Ca^{2+}. We observed, using increasing quantities of either cation, an increase in adsorption, reaching a plateau value of 100% adsorption with 5×10^{-3} M Mg^{2+} or 10^{-3} M Ca^{2+}. The adsorption rate constant was calculated by the equation $K = 2.3/B + x\log P_0/P$ (1), and was found to be 1.2×10^{-9} ml/min for aerobic cells, and 0.58×10^{-9} ml/min for anaerobic cells.

A typical one-step growth curve is shown in Fig. 6. The latent period is 120 min for aerobic cells, and 150 min for anaerobic cells. The burst size in both cases is 15 to 20 PFU.

Buoyant density of RS1, RS1-DNA, and G-C content. The buoyant density of the phage

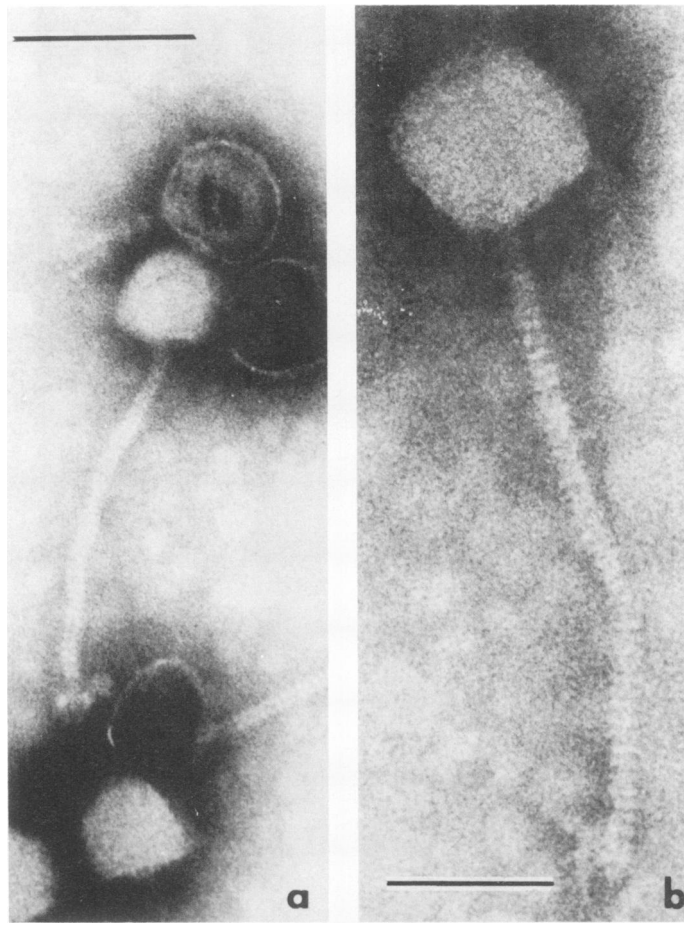

FIG. 3. *Electron micrograph of unidentified phage particles found at concentration of approximately 0.1% in purified preparations of RS1. (a) Bar represents 100 nm. (b) Bar represents 50 nm.*

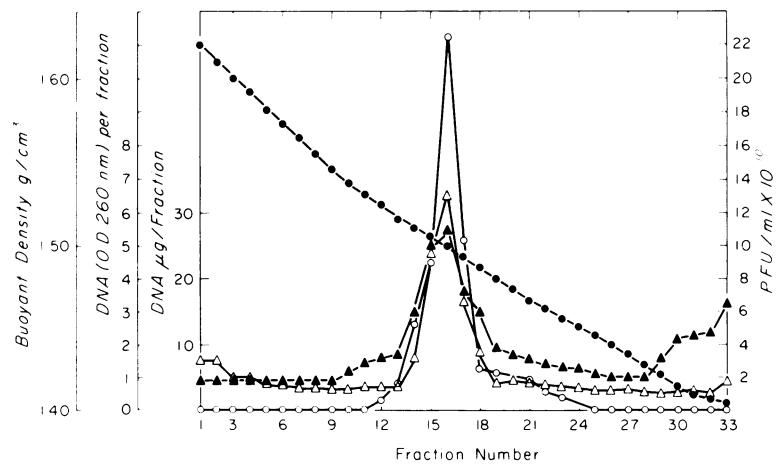

FIG. 4. *Purification and buoyant density determination of RS1 by equilibrium density gradient centrifugation in CsCl. Centrifugation was for 24 h at 35,000 rpm in a Spinco SW50.1 rotor. Symbols: ○, infectivity; ▲, DNA; △, O.D. 260 nm.*

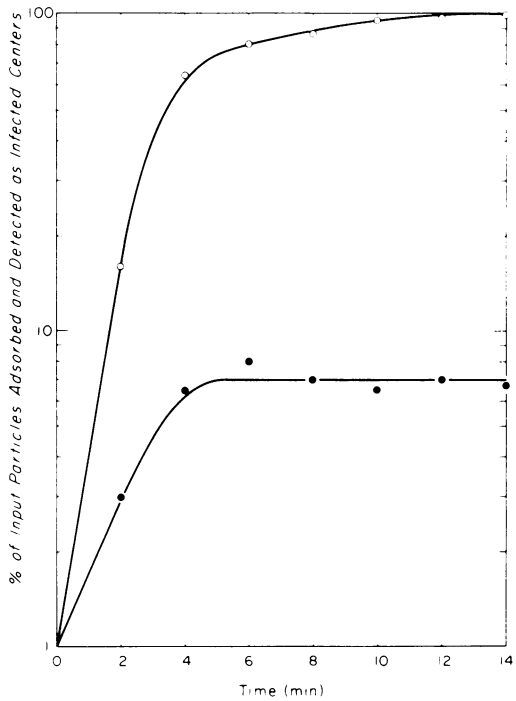

FIG. 5. *Adsorption and plaque formation of RS1 to aerobic and anaerobic cells. Phage were added to exponential phase cells (10^9/ml) at a multiplicity of 0.05. Samples (0.1 ml) were transferred to 1 ml of medium containing diluted (1:20) phage antiserum, incubated 10 min, and centrifuged at 10,000 rpm for 10 min in the cold. The pellet was assayed for infective centers by using aerobically grown cells as indicator. The number of free phage particles remaining in the supernatant of the incubation mixture was determined at the end of the incubation period. Symbols: O, aerobic cells; ●, anaerobic cells.*

particles was determined by measuring the refractive index at the peak of infectivity in a preparative CsCl gradient (Fig. 4). Ultracentrifugation in such a preparative gradient gives an apparent density of 1.50 for RS1 particles. The buoyant density of the phage DNA was calculated to be 1.706 g/cm^3, relative to *Tetrahymena* A1667 2b DNA, corresponding to a guanine plus cytosine (G + C) content of 46% (14). The T_m of RS1 DNA in SSC is 87.5 C (Fig. 7), corresponding to a G + C value of 44% (10). The fact that both methods employed for determining a G + C content yielded essentially identical results is strong evidence for the absence of significant quantities of any unusual bases in RS1 DNA.

Molecular weight of RS1 DNA. S_{20} value of RS1 DNA was observed to be 36.5 from sucrose gradient sedimentation, by using λ_{vir} DNA (S_{20} = 34.4 ± 0.3) as a reference. The molecular weight of RS1 DNA was calculated from these values by the equation $S1/S2 = (M_1/M^2)^{0.38}$ (6) to be 3.5×10^7.

Assuming the absence of repetitious DNA sequences in RS1 DNA, the number of base pairs per genome was calculated according to the method of Britten and Kohne (2) from the reassociation curve of sheared, thermally denatured DNA, by using λ_{vir} DNA as reference (Fig. 8). The similarity of 1/2 Cot values of λ DNA and RS1 DNA suggests a molecular weight of 3.0×10^7 for RS1 DNA.

DISCUSSION

RS1 is a bacteriophage composed of a polyhedral head approximately 65 nm in diameter, with a tail which is 60 nm long and which contains at the end of the tail an end plate with fibers. The nucleic acid was identified as a

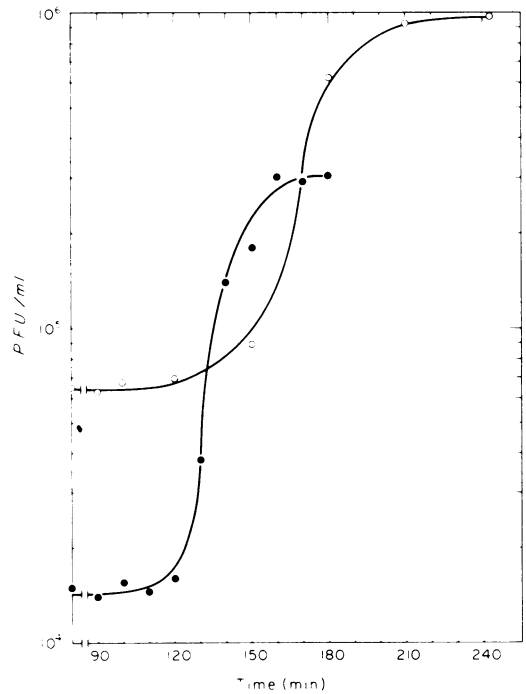

FIG. 6. *One-step growth curve for RS1 grown on aerobic and anaerobic cells. Adsorption was allowed to continue for 15 min at 30 C followed by the addition of diluted antiphage serum (1:20). The suspension was incubated for 10 min at 30 C and centrifuged at 10,000 rpm for 10 min. The cells were then resuspended in medium, diluted, and incubated at 30 C. Samples (0.1 ml) were removed at appropriate time intervals for plating. MOI for aerobic cells was 0.01; MOI for anaerobic cells was 10. Symbols: ●, aerobic cells; O, anaerobic cells.*

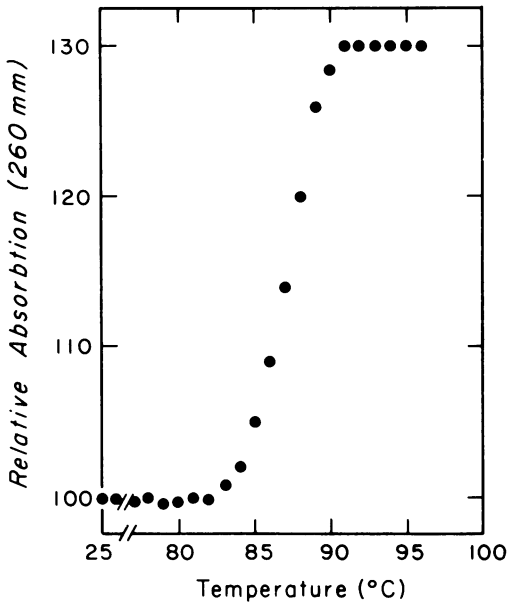

FIG. 7. *Thermal denaturation of RS1 DNA in SSC buffer, pH 7.0.*

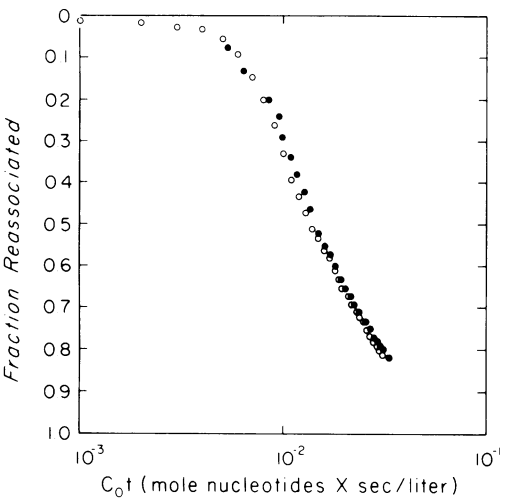

FIG. 8. *Reassociation of thermally denatured double-stranded DNA from RS1 and λ_{vir} in SSC buffer pH 7.0. The DNA was sheared by 20 passes through a no. 26 hypodermic needle (○) RS1 DNA, (●) λ_{vir} DNA.*

double-stranded DNA with a molecular weight of 3.0×10^7 to 3.5×10^7, and the G-C content was calculated to be 46 mol% from measurement of its bouyant density, and 44 mol% from its thermal denaturation. The average nucleotide composition of RS1 DNA is thus very different from that of its host, which has a G-C content of 67% (11). RS1 showed a very narrow host range. Of all strains tested, RS1 was capable of replicating only in *R. spheroides*. In the case of *R. spheroides* M29:5 there appears to be some form of host restriction. Infected cells did lyse, but did not form progeny that could be recovered from liquid medium, nor did they form plaques. It is also evident that some form of physiologic specificity exists, since anaerobic *R. spheroides* cells are to a large extent immune to infection by RS1. It is possible that this partial immunity of the anaerobic cells represents some changes in the structure or composition of the surface of the cells, and that these changes cause difficulties in the adsorption or penetration process, or both. As *R. spheroides* natural habitat is generally anaerobic, this partial resistance to infection by RS1 might have some ecological significance. However, once a successful infection has occurred, the burst size of the phage progeny is similar to that after infection of aerobic cells, indicating that once the phage DNA has successfully penetrated anaerobically grown cells it has no difficulties in producing a phage progeny in anaerobic cells.

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