THE UNIVERSITY OF CALGARY

CHARACTERIZATION OF Rc-Cal 1, A BACTERIOPHAGE FOR *RHODOPSEUDOMONAS CAPSULATA*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled CHARACTERIZATION OF Rc-Cal 1, A BACTERIOPHAGE FOR RHODOPSEUDOMONAS CAPSULATA in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Rc-Cal 1, a bacteriophage for *Rhodopseudomonas capsulata*, was studied for host range, biophysical parameters, replication, and as a genetic vector. Using the most comprehensive study of *R. capsulata* bacteriophages as a standard, Rc-Cal 1 was found to have a unique *R. capsulata* infectivity pattern.

Biophysically, Rc-Cal 1 phage particles have a buoyant density in CsCl of 1.520 gm/cm^3. The sedimentation coefficient is 374 $S_{20,w}$. Morphologically the phage is polyhedral with a head 49 nm by 70 nm and a non-contractile tail 9 nm by 152 nm. The phage particle gives twenty protein bands on SDS-PAGE. The proteins range from 112,000 daltons to 12,000 daltons with four major bands. The nucleic acid is linear double stranded DNA with a molecular weight of approximately 38 X 10^6 daltons. The average G-C mol % is 71.4% with a good correlation between buoyant density, 1.725 gm/cm^3 in CsCl, and melting point, 85.6°C in 0.1 SSC.

Replication of Rc-Cal 1 gave a burst size of 122. Examination of cells which had been infected with Rc-Cal 1 showed a percentage to be immune to infection and carrying phage. This phenomenon was stable for >100 transfers.

Transduction experiments with two antibiotic markers and a photosynthetic marker were unsuccessful. No transfer took place from the mutants or the wild type cells.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

1.1 Rc-Cal 1

Rc-Cal 1, a bacteriophage which infects *Rhodopseudomonas capsulata*, was isolated by King (1978) from a sewage treatment plant in Calgary, Alberta, Canada. Initial characterization showed Rc-Cal 1 had a polyhedral head and a non-contractile tail. Limited host studies indicated that replication took place only in *R. capsulata* "St. Louis". Replication was most efficient when aerobically grown cells were infected and incubated at 32.5°C. Maximum titres were about 3×10^{10} plaque forming units per millilitre (PFU/ml). Other parameters characterized were temperature sensitivity, chemical sensitivity, and adsorption rates.

1.2 *Rhodopseudomonas capsulata*

Rhodopseudomonas capsulata is a member of the *Rhodospirillaceae* family (Pfennig and Trüper, 1971). Physically these bacteria are Gram negative cells appearing spherical when grown in media below pH 7.0, regularly arranged in chains resembling streptococci. When grown in media above pH 7.0 they are ovoid to rod shaped, 0.5 - 1.2 μm wide and 2 - 2.5 μm long, sometimes up to 6 μm long. Of particular note is the zigzag arrangement of the

cells in chains (Pfennig and Trüper, 1974).

Metabolically, these are phototrophic non-sulfur bacteria which have the ability to grow photoheterotrophically in anaerobic, illuminated conditions and heterotrophically in aerobic, dark conditions (Pfennig and Trüper, 1974). Additionally these bacteria will grow in dark anaerobic conditions using artificial electron acceptors in the medium. Schultz and Weaver (1982) reported fructose was fermented to mixed acids in the dark only in the presence of dimethyl sulfoxide. The mixed acids were oxidized with the reduction of dimethyl sulfoxide to yield energy.

The variety of energy systems with corresponding membrane changes found in rhodopseudomonads provide an ideal model system for study of membrane formation and differentiation (Marrs, 1978). Because the system is easily manipulated by changing the bacterium's environment, analysis of gene expression could be fully developed if suitable genetic vectors, such as bacteriophage transducers, were found.

1.3 RHODOPSEUDOMONAD PHAGES

The motivation for isolating and characterizing phages of photosynthetic bacteria has been to find one capable of transducing the host genome (Wall *et al.*,

1975a). In the early seventies much work was done isolating and characterizing phages which infected rhodopseudomonads (Abeliovich and Kaplan, 1974; Mural and Friedman, 1974; Schmidt et al., 1974; Wall et al., 1975a). There were reports of temperate phages (Mural and Friedman, 1974) but success with transduction has been limited (Marrs, 1978).

In 1968, Freund-Molbert et al. published the first characterization of a rhodopseudomonad phage, RP 1. The host was Rhodopseudomonas palustris 1e5 and the phage replicated during anaerobic and aerobic growth of the host. The phage is icosahedral, 38 to 39 nanometers in diameter, and has a non-contractile tail. Acridine orange stain indicated double stranded DNA (Bosecker et al., 1972) placing the phage in Bradley's Group C (Bradley, 1967).

The second phage characterized used Rhodopseudomonas spheroides for a host (Abeliovich and Kaplan, 1974). RS 1 replicated only in Rhodopseudomonas spheroides strains 2.4.1 and L. Morphologically it is icosahedral, 65 nm in diameter, with a non-contractile tail 60 nm in length containing an end plate with fibers. Particle density is 1.50 gm/cm^3 in CsCl with DNA buoyant density of 1.706 gm/cm^3 in CsCl. The DNA is double stranded with a sedimentation coefficient of 36.5 S and a molecular weight of 3 to 3.5 X 10^7 daltons. Guanine-cytosine mole

percentage (G-C mole %) is 44% calculated by Tm in SSC and 46% calculated by buoyant density in CsCl. Recent work (Donahue et al., 1985) proposes the DNA of RS 1 is in segments of varying lengths.

RØ-1, a temperate phage for Rhodopseudomonas spheroides was briefly described by Mural and Friedman (1974). This phage is polyhedral with a very long non-contractile tail. Nucleic acid is double stranded DNA.

A recent description of a novel temperate phage for Rhodopseudomonas spheroides indicated virulence may be a consequence of its genomic instability (Duchrow et al., 1985). ØRsG1 has a polyhedral head (90 by 46.5 nm) connected with a tail (116 by 9.4 nm), to which a collar was proximally attached. The genome consisted of double stranded linear DNA with cohesive ends and a G-C content of 71.8 mol %. The DNA molecules formed circles in vitro with a mean contour length of 17.18±0.4 μm, which corresponds to 49 kilobase pairs. DNA from virulent ØRsG1 was heterogeneous consisting of two molecules in a ratio of about 1:1. These molecules circularized and had contour lengths of 17.18±0.4 μm and 14.02±0.4 μm corresponding to 49 Kb and 40 Kb respectively. Restriction digest analysis indicated the virulent and temperate genomes were similar and identified an 11.5 Kb EcoRI fragment as carrying the cohesive ends. Because the 49 Kb genomes were indistinguishable, it was suggested the 40 Kb DNA represented the

virulent form of ØRsG1.

The first Rhodopseudomonas capsulata bacteriophage, RC1, was characterized by Schmidt et al., (1974). RC1 was isolated from sewage and has a polyhedral head, sheathed contractile tail and fibers associated with the base plate. Acridine orange stain showed double stranded DNA, placing RC1 in Bradley's Group 1.

An extensive search for bacteriophages infecting Rhodopseudomonas capsulata was made by Wall et al., (1975a). Ninety-five phages were collected from sewage and then categorized by infectivity patterns on 33 strains of Rhodopseudomonas capsulata described by Weaver et al., (1975). The 95 strains were separated into 16 patterns of infectivity. No further characterization except tests for lysogeny and transducing ability were done. These tests proved negative.

1.4 GENE TRANSFER AGENT

A successful transducing agent isolated during the early seventies was GTA (Marrs, 1974). GTA resembles a phage but is smaller than any known transducing phage or any phage of comparable complexity. It is icosahedral, 30 nm in diameter, with short apical spikes. The tail is of variable length and joined to the head by a stain-excluding collar. Tail fibers have been suggested but not

proven. GTA is made up of eight proteins ranging from 13,000 to 40,000 daltons. Total mass is 7.5 X 10^6 daltons with a sedimentation coefficient of 70 - 100 S.

The DNA is linear double stranded (Solioz and Marrs, 1977) with a mass of 3.6 X 10^6 daltons and sedimentation value of 14 - 15 S. Hybridization kinetics with Rhodopseudomonas capsulata DNA and restriction endonuclease digests indicate GTA DNA and host DNA have similar complexities.

GTA has transduced approximately one operon (5 to 10 genes) and will transduce any area of the genome (Yen and Marrs, 1976). This method of generalized transduction has been used in Rhodopseudomonas capsulata to map the genes of carotenoid and bacteriochlorophyll synthesis (Yen and Marrs, 1976); transfer nitrogenase-hydrogenase activity (Wall et al., 1975b); map the photosynthetic region (Taylor et al., 1983); and to transfer the capacity to form bacteriochlorophyll protein complexes into mutants of Rhodopseudomonas capsulata that have lost this capacity (Drews et al., 1976).

1.5 BACTERIOPHAGE CHARACTERIZATION

According to Ackermann et al., (1978) more than 1,650 bacteriophages have been studied by electron microscopy with 130 to 150 additional descriptions being added each

year. As previously discussed, the attraction of bacteriophage studies is the possibility of finding a valuable vector for use in the study of genetics, metabolism, morphology, and epidemiology of the host bacterium. The vast number of descriptions have caused problems in characterization and classification. Until the guidelines published by Ackermann and his colleagues the only characterization scheme was by Bradley (1967) (See Table 1). According to Ackermann et al., (1978), Bradley's outline, though useful, has led to widely different phage descriptions, identical names for different phages and disagreement on parameters, methods and nomenclature to use when characterizing new phages.

In accordance with these problems Ackermann et al. (1978) proposed high, medium and low-level criteria to use in the characterization of bacteriophages. The high level criteria (See Table 2) are applicable to all phages. They describe the virus or it's nucleic acid and can be determined precisely and unequivocally. The medium level criteria include: (1) presence and dimensions of organelles such as tail fibers and base plates; (2) number of pieces of nucleic acid, base composition and presence of sugars; (3) number and composition of coat and internal proteins; (4) nucleic acid hybridization and serological data; (5) host range; (6) some genetic properties such as converting or transducing ability; and (7) inactivation by

the more frequently determined physical and chemical agents, e.g. chloroform, ether, heat and ultraviolet light. These are medium level because they include those criteria more useful for one group of phages than another, those for which limited data are available, and those which are considered of lesser value in differentiation. The low level criteria include host- and environment-dependent properties such as plaque size, adsorption velocity, latent period, burst size, and efficieny of plating.

In this study, characterization of Rc-Cal 1 will be focused towards the high level criteria with additional characterization on host range, genetic properties, and replication cycle.

TABLE 1 Bradley's Scheme for Bacteriophage Classification*

BACTERIOPHAGES	ds-DNA	Group A, tails contractile Group B, tails long, noncontractile Group C, tails short, noncontractile
	ss-DNA	Group D, tail-less, large capsomeres Group F, filamentous
	ss-RNA	Group E, tail-less, small capsomeres

* From Bradley (1967).

TABLE 2. High-Level Criteria for Phage Description*

Property	Principal Techniques
Whole virus	
Size and shape	Electron microscopy
Weight	Sedimentation analysis in buffer or sucrose
Buoyant density	Isopycnic centrifugation in cesium salts or sucrose
Percent of protein	Chemical analysis: approximation by determination of ultraviolet absorbancy
Percent of lipids	Chemical analysis
Nucleic acid	
Type (DNA or RNA)	Acridine orange staining Alkali denaturation Colorimetric determination of sugars Enzyme sensitivity
Number of strands	Acridine orange staining Chemical analysis of base ratio Determination of buoyant density Thermal denaturation
Conformation (linear or circular)	Electron microscopy
Percent	See "Percent of protein"
MW	Electron microscopy Gel electrophoresis Sedimentation analysis

* From Ackermann *et al*. (1978).

2.0 MATERIALS

2.1 CHEMICALS

Standard reagent grade chemicals were used. Restriction endonucleases and DNA molecular weight markers were obtained from Boehringer Mannheim and Pharmacia. Protein molecular weight markers were from Pharmacia. Bacteriological media were from Difco.

2.2 STRAINS

The primary host is Rhodopseudomonas capsulata "St. Louis" (American Type Culture Collection No. 23782). All other strains were obtained from J. Wall, University of Missouri, Colombia, Missouri (See Table 3).

TABLE 3 Bacterial strains used for determining host range

		STRAINS	COLLECTION
Rhodopseudomonas	capsulata	"St. Louis"	ATCC 23782
Rhodopseudomonas	capsulata	6950	J. Wall
Rhodopseudomonas	capsulata	Kb1	J. Wall
Rhodopseudomonas	capsulata	JH1	J. Wall
Rhodopseudomonas	capsulata	SP3	J. Wall
Rhodopseudomonas	capsulata	SP7	J. Wall
Rhodopseudomonas	capsulata	SP8	J. Wall
Rhodopseudomonas	capsulata	SP11	J. Wall
Rhodopseudomonas	capsulata	C4	J. Wall
Rhodopseudomonas	capsulata	LB2	J. Wall
Rhodopseudomonas	capsulata	YW1	J. Wall
Rhodopseudomonas	capsulata	EY3	J. Wall
Rhodopseudomonas	capsulata	LB4	J. Wall
Rhodopseudomonas	capsulata	YW2	J. Wall
Rhodopseudomonas	capsulata	SP18	J. Wall
Rhodopseudomonas	capsulata	SP19	J. Wall
Rhodopseudomonas	capsulata	SP108	J. Wall
Rhodopseudomonas	capsulata	SM3	J. Wall
Rhodopseudomonas	capsulata	MSG	J. Wall
Rhodopseudomonas	capsulata	NP2	J. Wall
Rhodopseudomonas	capsulata	C2	J. Wall
Rhodopseudomonas	capsulata	B10	J. Wall
Rhodopseudomonas	capsulata	$P_{12}P_2$	J. Wall
Rhodopseudomonas	capsulata	$P_{18}M_2$	J. Wall
Rhodopseudomonas	capsulata	MRE	J. Wall
Rhodopseudomonas	capsulata	CC1	J. Wall

3.0 METHODS

3.1 PHAGE MAINTENANCE

3.1.1 MEDIA

R. capsulata "St. Louis" was grown on a synthetic medium (Sojka *et al.*, 1967), designated as RCV by Weaver *et al.*, (1975), containing per litre of deionized H_2O 4 gm D,L-malic acid, 1 gm $(NH_4)_2SO_4$, 0.075 gm $CaCl_2.2H_2O$, 0.2 gm $MgSO_4.7H_2O$, 0.02 gm EDTA, 0.012 gm $FeSO_4.7H_2O$, 0.6 gm KH_2PO_4, 0.9 gm K_2HPO_4, 1 ml trace element solution (1.5 gm $MnSO_4.H_2O$, 2.8 gm H_3BO_3, 0.04 gm $Cu(NO_3)_2.3H_2O$, 0.24 gm $ZnSO_4.7H_2O$, 0.75 gm $NaMoO_4.2H_2O$ per 1000 millitres of deionized H_2O), 0.001 gm thiamine hydrochloride. The pH was adjusted to 6.8 with NaOH before autoclaving. The other media used to grow *Rhodopseudomonas* were RCVB (same as RCV with added biotin, 0.015 mg/L), RCVBN (same as RCVB with added nicotinic acid, 1 mg/L), and PY (Difco Bactopeptone, 3 gm/L, and Difco yeast extract, 3 gm/L).

3.1.2 HOST GROWTH

R. capsulata was grown aerobically for bacteriophage replication in Fernbach flasks filled with one litre of RCV. The inoculated medium was incubated at 32.5°C and

shaken at 200 rpm in a gyrotory shaker-incubator (New Brunswick Scientific Company). Plate cultures (1.5% Difco Bactoagar) were incubated aerobically at 32.5°C. Stock cultures were maintained at -70°C in RCV plus 10% glycerol.

3.1.3 PLAQUE ASSAY

The phage titer is expressed in PFU/ml determined by the soft agar overlay method (Adams, 1959). The phage sample was diluted in sterile RCV and one millilitre of this dilution plus 0.1 ml of early stationary phase cells of Rhodopseudomonas were incubated at 32.5°C for ten minutes. Three millilitres of sterile molten RCV agar (0.5%) at 50°C was added, rapidly mixed and overlayed onto a solid RCV agar (1.5%) plate which had been warmed to 32.5°C. Plaques were counted after the plates had been incubated aerobically at 32.5°C for 24 hours.

3.1.4 PHAGE PURIFICATION

Early log phase, aerobically grown Rhodopseudomonas (approximately 2 -4 X 10^8 cells/ml) were infected with Rc-Cal 1 at a multiplicity of infection (MOI) of two. The infected cells were grown at a decreased aeration rate (125 rpm) for an additional 18 - 24 hours. The lysate was

refrigerated at 4°C for at least 24 hours and all subsequent steps were carried out at 4°C unless otherwise stated.

The lysate was centrifuged at 12,000 g for five minutes and the supernatants pooled. The pellets were resuspended in RCV (10% of the original volume) and shaken for thirty minutes at 200 rpm, room temperature in 2% ($^{v}/_{v}$) chloroform. After centrifugation at 12,000 g for ten minutes the supernatant was added to the lysate supernatant.

Ammonium sulfate was slowly added to the supernatant to give 75% saturation and stirred slowly for three hours. The precipitate was collected by centrifugation at 16,000 g for ten minutes. The lysate was concentrated 50 fold by resuspending the precipitate in an appropriate quantity of phage buffer (RCV medium minus thiamine and malic acid). The suspension was dialyzed against two changes of phage buffer overnight and then treated with deoxyribonuclease (0.001 mg/ml) for one hour at 37°C.

Following nuclease treatment, the crude phage preparation was cleared of large membrane fragments by centrifugation (10,000 g, 10 minutes) and cesium chloride was added to the supernatant until it reached a density of 1.5 gm/cm^3. This solution was centrifuged in a Beckman L3-50 ultracentrifuge using a SW 28 Beckman rotor at 25,000 rpm for 48 hours or a Beckman SW 50.1 rotor at

35,000 rpm for 36 hours. The single band formed during ultracentrifugation was collected by puncturing the bottom of the tube and collecting the appropriate fraction. This fraction was dialyzed against several changes of phage buffer over night.

Further purification of the phage was accomplished by layering the phage on a preformed CsCl gradient and centrifuging for at least 18 hours at 35,000 rpm in a Beckman SW 50.1 rotor. The band was collected, dialyzed against phage buffer and again centrifuged through a preformed CsCl gradient (Abeliovich and Kaplan, 1974).

3.2 WHOLE PHAGE CHARACTERIZATION

3.2.1 ELECTRON MICROSCOPY

Negative staining (Cole and Popkin, 1981) was used to visualize the phage particle. Small drops of purified phage were placed on 200 mesh copper grids coated with formvar. The drops were allowed to dry, then the grid was immersed in electron dense stain (sodium phosphotungstate or uranyl acetate) for thirty seconds. The excess stain was drawn from the grid with filter paper. The air dried grids were viewed in a Hitachi 600 electron microscope with an accelerating voltage of fifty

kilovolts.

3.2.2 BUOYANT DENSITY

Phage particle density was determined by mixing purified phage in phage buffer with CsCl to a density of 1.5 gm/cm^3. This solution was centrifuged at 35,000 rpm for 36 hours and then fractions were collected from the isopycnic gradients as described by Griffith (1976). The refractive index of each fraction was measured on a Jena refractometer and the density determined by the following equation; $p^{25} = 10.8601\eta_D{}^{25} - 13.4974$ (Bruner and Vinograd, 1965). The fractions were assayed for PFU by soft agar overlay and these data were superimposed on the density gradient data.

3.2.3 SEDIMENTATION

The sedimentation coefficient for complete Rc-Cal 1 was determined in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanning system. Rc-Cal 1 was diluted with phage buffer to an O.D. 280 = 1.0 and centrifuged at 14°C, 8,000 rpm for one hour. The sample was scanned every six minutes for determining sedimentation velocity. As internal standards Reo virus Antigen 3 and Reo subviral particles ($S_{20,w}$=630 and

$S_{20,w}$=470, Morgan and Zweerink, 1974) were used. All calculations were done using values presented by Chervenka (1970).

3.2.4 AMINO ACID ANALYSIS

Amino acid analysis was performed at the Protein Sequencing Facility, University of Calgary, Medical Biochemistry Division by D. McKay. An aqueous solution of purified Rc-Cal 1 was dessicated with five nanomoles of norleucine to be used as an internal standard. The dried sample was hydrolyzed with 100 microlitres of 6N HCl plus 0.1% phenol and 0.1% thioglycolic acid. The solution was heated at 110°C for 24 hours *in vacuo*. The quantity of amino acid in the sample was determined by integration and the following equation: amount of amino acid in 50 µl sample = p moles amino acid found X norleucine used / norleucine found. Trytophan and cysteine are destroyed during HCl hydrolysis and were not measured. Aspartic acid and asparagine are converted to the same compound and appear as one peak. The same applies to glutamic acid and glutamine.

3.3 PROTEIN CHARACTERIZATION

The characterization of phage proteins was done by

sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Gradient gels of 7% to 15% acrylamide were used in the Tris-SDS buffer system recommended by Laemmli (1970) with a 3% acrylamide stacking gel. The apparatus was a BRL model V161 vertical gel system with 1.5 mm spacers. Electrophoretic separation was carried out at 15 mA until the tracking dye (bromophenol blue) migrated to 0.5 cm from the gel bottom. The samples contained between 50 - 100 micrograms of protein measured by the Folin phenol assay (Lowry et al., 1951). Intact phage samples were separated into protein subunits by boiling in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 5% β-mercaptoethanol for ten minutes. Sample volume did not exceed fifty microlitres. The gels were fixed in 25% isopropanol : 10% acetic acid overnight and stained with 0.1% Coomassie brillant blue R-250 in 10% acetic acid : 30% methanol. Destaining was done by frequent changes of the dye solvent. Gel storage was in 5% acetic acid.

Protein molecular weights were estimated by co-electrophoresis of protein standards commercially prepared by Pharmacia. These standards included phosphorylase-B 94,000 daltons, bovine serum albumin 67,000 daltons, ovalabumin 45,000 daltons, carbonic anhydrase 30,000 daltons, soybean trypsin inhibitor 20,100 daltons, and α-lactalbumin 14,400 daltons. Rc-Cal 1

protein sizes were estimated from the standard curve established by the markers.

Silver stain was used to detect any proteins below the sensitivity of the Coomassie blue stain (Hitchcock and Brown, 1983). After gel fixation, proteins were oxidized with 10% glutaric dialdehyde for five minutes. Glutaric dialdehyde was washed from the gel with 200 millilitres of distilled water for thirty minutes, repeated eight times. Staining of the proteins was with a solution of silver nitrate (5 ml of 20% $^{w}/_{v}$), ammonium hydroxide (3 ml of 22% $^{w}/_{v}$), sodium hydroxide (28 ml 0.1N) and distilled water (115 ml) for ten minutes. After four, 5 - 10 minute washes with 200 ml of distilled water, the gel was developed for 10 - 20 minutes. Developer consisted of 50 mg citric acid monohydrate and 0.5 ml 37% formaldehyde per litre distilled water. The developer was quenched for one hour with 200 ml distilled water and 10 ml 7% ($^{v}/_{v}$) acetic acid. Gel storage was in distilled water.

Protein quantification was done on Coomassie blue stained gels with a LKB laser densitometer and a Hewlett-Packard 3390A integrator.

3.4 NUCLEIC ACID CHARACTERIZATION

3.4.1 NUCLEIC ACID EXTRACTION

Phage DNA was extracted using the procedure of Maniatis et al. (1982) for isolation of lambda DNA. Purified phage was transferred to a centrifuge tube and EDTA (pH 8.0) added (final concentration 20 mM). Fifty micrograms proteinase K per millilitre and sodium dodecyl sulfate (0.5% final concentration) were added and the solution was mixed gently by inverting the tube several times. The mixed solution was incubated at 65°C for one hour before being extracted by phenol saturated with TE (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). The aqueous phase was separated by centrifugation (1,600 g, five minutes) at room temperature and transferred to a clean tube. Re-extraction was done until no protein precipitate could be seen at the interface. The clean aqueous phase was extracted with 1:1 saturated phenol and chloroform followed by a chloroform extraction. The aqueous phase was dialyzed against three 1000-fold volumes of TE or 0.1 SSC overnight at 4°C. The purity and concentration of the DNA was determined by spectrophotometry (Johnson, 1981).

Bacterial DNA was extracted by the method of Marmur (1961) following the procedure outlined by Johnson (1981). Cells were lysed with 1% SDS and 50 micrograms per millilitre lysozyme at 60°C. Sodium perchlorate was added to the lysate (final concentration 140 gm/L) and then protein was extracted with chloroform : isoamyl alcohol (24:1). The aqueous layer was extracted with ether saturated in TE

and then the ether was evaporated. The crude nucleic acid mixture was treated with ribonuclease (50 μg/ml) at 37°C for ten minutes. Protein was extracted with chloroform and then the DNA was extracted with ether to remove the chloroform. DNA was precipitated with ice cold ethanol and dissolved in 0.1 SSC.

3.4.2 BUOYANT DENSITY

The nucleic acid density was determined by mixing CsCl with 0.1 SSC to a density of 1.685 gm/cc. Fifty microlitres of DNA solution (approximately 17 micrograms of DNA) was added to 4.5 ml of 0.1 SSC - CsCl solution. This solution was centrifuged at 40,000 rpm for 48 hours and then four drop fractions were collected. Ten microlitre drops were measured for refractive index and the remainder of the fraction was diluted with one millilitre of 0.1 SSC. Each fraction was analyzed for DNA by measuring optical density at 260 nm. This curve was superimposed on the density gradient data.

The formula of Woodward and Lebowitz (1980), buoyant density, $\theta=1.6541 + 0.0988$ (G-C mole %), was used to determine the mole fraction of guanine and cytosine.

3.4.3 THERMAL DENATURATION

The thermal denaturation point was determined using the method of Marmur and Doty (1962). A Varian Cary 2290 spectrophotometer with an ethylene glycol-water circulating bath was programmed for a rise in temperature of 1°C/minute. The hyperchromic shift was recorded automatically and the midpoints of this shift (Tm) were used in the formula of Johnson (1981), mole % G-C_x=reference G-C % + 1.99 (Tm_x - Tm_r). Escherichia coli B DNA (Sigma) was used as a reference. All DNA preparations were dialyzed against 0.1 SSC and diluted to an approximate optical density of 0.3 at 260 nm.

3.4.4 SPECTROPHOTOMETRY

The ultraviolet absorbancy ratios proposed by Ulitzer (1972) were used to determine guanine-cytosine mole percentage. E. coli B DNA (Sigma) was used as the reference DNA guanine-cytosine mole percentage (51.3%). E. coli B DNA absorbance ratios at 245 nm/270 nm, 240 nm/280 nm and 240 nm/275 nm were used to plot a line parallel to the composite reference line proposed by Ulitzer. Rc-Cal 1 absorbancy ratios were plotted on the reference line and guanine-cytosine mole percentage was

determined from the x-intercept.

3.4.5 ELECTRON MICROSCOPY

Rc-Cal 1 DNA was prepared for electron microscopy by using the aqueous technique of Davis et al. (1971). In the spreading solution are the following: 0.5 μg/ml DNA, 0.1 mg/ml cytochrome c, 0.5 M ammonium acetate, and 1 mM EDTA (pH 7.5). The hypophase is 0.25 M ammonium acetate and is used to rinse the glass slide before spreading. When the slide is dry, 50 microlitres are spread back and forth across the slide. The film was visualized and compressed by talc dust spread on the hypophase. The film was picked up on 200 mesh carbon coated copper grids and stained for thirty seconds. The stain solution contained 10^{-5} M uranyl acetate in 90% ethanol and was prepared fresh from a stock aqueous solution of 0.001 M uranyl acetate in 0.05 M HCl. Grids were viewed in a Hitachi 600 electron microscope with an accelerating voltage of fifty kilovolts.

3.4.6 RESTRICTION DIGESTS

Restriction digests were carried out in the buffers recommended by Maniatis et al., (1982). Low salt buffer contained 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM

dithiothreitol. Medium and high salt buffers were the same as low salt buffer except they were supplemented with 50 mM and 100 mM NaCl respectively. Depending upon the enzyme, 0.1 volumes of a ten fold strength buffer solution was added to 1 - 5 micrograms DNA, distilled water, and 2 to 8 units of enzyme to make a final volume of 20 - 30 microlitres. Digests were incubated at 37°C overnight. Electrophoresis was in Tris-borate buffer (89 mM Tris, 8.9 mM boric acid, 2.5 mM EDTA, pH 8.8) through 0.8% agarose gels at 15 volts per centimetre of gel. DNA bands were stained with ethidium bromide (2 µg/ml) and viewed under ultraviolet light.

3.5 PHAGE REPLICATION

3.5.1 SINGLE STEP GROWTH CURVE

A single step growth curve was done according to the method of Adams (1959). (This experiment was performed by the author but results, Section 4.2.2, are taken from an identical experiment performed by E. Swedberg). Antibodies were raised against purified Rc-Cal 1 in New Zealand rabbits. One hundred millilitres of Rhodopseudomonas capsulata were grown at 32°C, shaking at 250 rpm. After twelve hours of growth (KU_{66}=22) three millilitres of sterile phage (MOI=1.8) was added to the culture and

allowed to equilibrate for 55 minutes at 32°C, 250 rpm. At the end of the equilibration period 40 millilitres of the cell : phage suspension was placed in a 100 millilitre flask with one millilitre of sterile purified Rc-Cal 1 anti-serum (E. Swedberg, personal communication). This was allowed to mix for ten minutes before diluting. At zero time a 1:2,500 dilution of the cell, phage, and antibody mixture was made to decrease phage inactivation by antiserum from >99.5% to 0%. This dilution was allowed to grow and samples were taken periodically to measure the increase in Rc-Cal 1 infective centres.

3.5.2 HOST RANGE

The ability of Rc-Cal 1 to replicate in other *Rhodopeudomonas capsulata* strains was examined using twenty six strains provided by J. Wall, University of Missouri, Colombia, Missouri (See Table 3). Liquid cultures of all strains were grown photosynthetically overnight. Replication was tested by the soft agar overlay method. Approximately 50 plaque forming units were mixed with the overnight cultures in soft agar and then spread on plates. Plaques were counted after 24 and 48 hours of 32.5°C aerobic incubation.

3.6 GENETICS

3.6.1 MUTAGENESIS

Mutant colonies for transduction experiments were obtained from spontaneous antibiotic resistant mutants (Miller, 1972) and by the tetracyline suicide method for obtaining photosynthetic mutants (Marrs et at., 1980). Spontaneous mutants for antibiotic resistance were screened by spreading cells over RCV-antibiotic plates. The antibiotics used were penicillin G (50 µg/ml), streptomycin sulfate (50 µg/ml), rifampicin (75 µg/ml), novobiocin (20 µg/ml), and nalidixic acid (20 µg/ml). Late log-early stationary phase cells were spread on the agar plate and incubated aerobically at 32.5°C.

Photosynthetic mutants were selected by growing single colony isolates of Rhodopseudomonas capsulata photosynthetically to 50 - 75 KU_{66} in 20 millilitre test tubes filled to the top and screw capped. Tetracycline was added (final concentration 5 µg/ml) and the culture grown for an additional 72 hours. Aliquots of 0.1 ml were spread on plates of RCV and incubated in the dark aerobically for 48 hours. Any colonies which grew were replica plated and grown anaerobically to determine photosynthetic mutants.

Ultraviolet mutagenesis was used to select for

auxotrophic mutants. A two day culture was mixed with with sterile RCV (5 ml:5 ml) in a 90 mm plastic Petri plate and UV irradiated to >99% cell death. The plate was covered and left undisturbed for three hours. The suspension was placed in tubes and allowed to grow in the dark overnight. These cultures were spread on L-broth plates (1 ml/plate) and incubated aerobically and anaerobically. Colonies which grew on L-broth were replicated onto RCV.

3.6.2 TRANSDUCTION

The ability of Rc-Cal 1 to transduce genetic information in Rhodopseudomonas capsulata was tested by standard methods explained in Miller (1972). Rhodopseudomonas cells carrying a genetic marker were grown to early log phase aerobically. The cells were infected with purified Rc-Cal 1 at a MOI of four and grown for an additional 24 hours. The lysate was refrigerated at 4°C overnight, cleared of whole cells by centrifugation and ammonium sulfate precipitated. The concentrated lysate was centrifuged in a cesium chloride isopycnic gradient to clear all chromatophores and small bacterial debris not cleared previously. Cesium chloride was dialyzed away and the combined fractions were filter sterilized. The sterile filtrate was used to infect wild type Rhodopseudomonas

cells for 24 hours. At the end of the infection period these cells were spread on indicator plates to determine if the marker had been transduced. Uninfected wild type cells were also spread on indicator plates to determine spontaneous mutation rate.

4.0 RESULTS

4.1 HOST SPECIFICITY

The ability of Rc-Cal 1 to productively infect other Rhodopseudomonas capsulata stains is shown in Table 4. Four strains beside "St. Louis" (B10, SP3, C4, EY3) produced plaques. The ability of Rc-Cal 1 to infect the five strains was similar. Eight other strains, SM3, MSG, NP2, C2, P12P2, P18M2, MRE, and CC1 were tested against Rc-Cal 1 but not by Wall et al. (1975). No plaques were formed with any of these strains.

4.2 REPLICATION

4.2.1 BATCH REPLICATION

Optimizing the replication of Rc-Cal 1 involved testing a variety of parameters. These parameters were cell growth phase at infection, phage to cell ratio, and time allowed for replication. The optimal replication procedure is - early log phase aerobic cells at a density of 2 to 5 X 10^8 cells/ml, infected at a MOI of 2 to 4 with a replication time of 12 to 24 hours. Maximum yields of approximately 100 fold increase resulted under these conditions. With lower or higher MOI's lower total yields

TABLE 4 Host Range of Rc-Cal 1 Compared to the Sixteen Bacteriophage Types Isolated by Wall *et al.* (1975)[1]

R. capsulata strains	Bacteriophages																
	RC1	RC2	RC3	RC24	RC32	RC44	RC45	RC55	RC75	RC76	RC85	RC86	RC88	RC90	RC91	RC95	Rc-Cal 1[2]
"St. Louis"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B10	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
6950	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
Kb1	+	-	+	-	+	+	-	-	-	+	-	+	-	-	-	-	-
JH1	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
SP3	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+
SP7	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SP8	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
SP11	+	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-
C4	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
LB2	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
YW1	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
EY3	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
LB4	+	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-
YW2	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-
SP18	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
SP19	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
SP108	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

1. Wall *et al.* (1975) tested for phage infection by spotting phage onto lawns of cells, any degree of clearing was scored "+", no effect was scored "-".

2. "+" indicates plaques were seen, "-" indicates no plaques were seen.

were found. Use of older cells (mid log phase - late log phase) also resulted in lowered total yields.

4.2.2 SINGLE STEP GROWTH CURVE

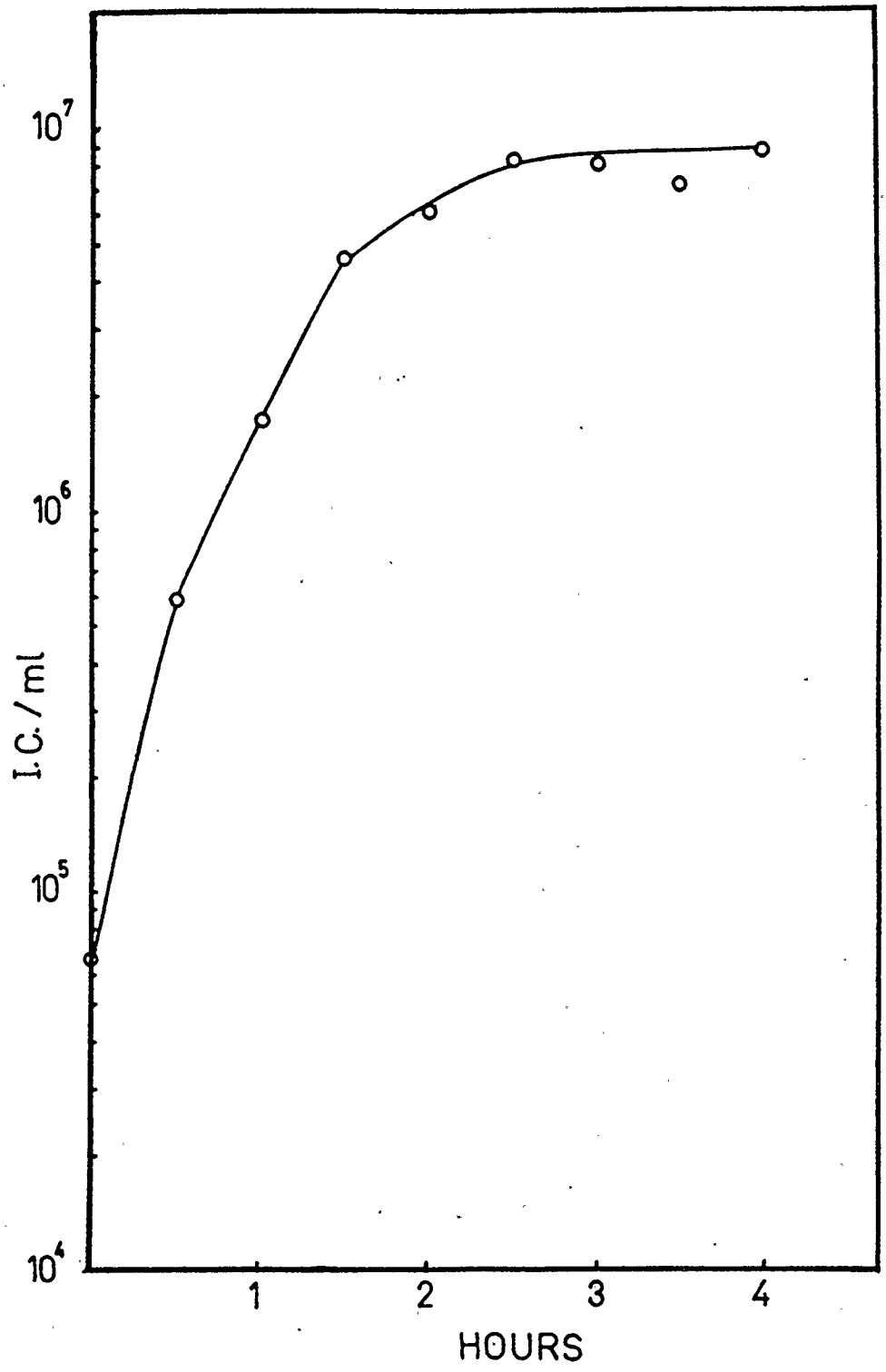
In the single step growth curve experiment (Figure 1, E. Swedberg personal communication) greater than 99.5% of the unadsorbed phage would have been inactivated with the addition of antiserum. The infectious centres (I.C.) at zero time after dilution were 6.7×10^4. This indicates that after one hour of equilibration only 15.5% of the phage had adsorbed. The titre rose over the next 2.5 hours and then leveled off. The average titre over the last four points in Figure 4 is 8.2×10^6 infectious centres. This gives a burst size of 122.

4.2.3 PSEUDOLYSOGENY

At the end of one replication experiment where antiserum had been used to inactivate free phage (>99.5%), the culture was diluted to 10^{-8} and plated. Fourteen colonies was isolated. The colonies were grown up in RCV and tested for infectious centres and the ability to be infected. Fourteen percent of the colonies (2/14) were immune to infection and were releasing phage. These two cultures were diluted 100 fold and allowed to grow up.

FIGURE 1 Single Step Replication Curve of Rc-Cal 1

o = Infectious centres per millilitre (I.C./ml) measured at 30 minute intervals over 4 hours.


10^7
10^6
10^5
10^4
I.C./ml
1
2
3
4
HOURS

They were retested and transferred three times in this manner. After the third transfer they were found to contain 1.1 X 10^9 infectious centres/ml and 1.0 X 10^9 cells/ml (G. Din personal communication). This indicates that persistent infection is stable over many generations of host growth. This phenomena has been tested for >100 transfers and remained stable (G. Din personal communication).

4.3 CONCENTRATION AND PURIFICATION

Characterization of Rc-Cal 1 depended on a high titre of pure phage. The greatest problem in concentrating and subsequent purification of Rc-Cal 1 was separation of host chromatophores from the phage. Chromatophores and phage precipitated at the same concentration of ammonium sulfate. After ultracentrifugation there were often membrane fragments closely associated with the phage band. When this occurred the phage was treated with 2% chloroform followed by ultracentrifugation. A typical concentration profile is seen in Table 5. During concentration 56% of the plaque forming units are lost but concentration is 500 fold.

The phage purity after concentration was assessed by SDS-PAGE. Purified phage proteins were electrophoresed next to a host cell lysate. The host cell lysate was

TABLE 5 Concentration and purification of Rc-Cal 1

STEP	Volume (mls)	PFU/ml	Total PFU	% Recovered
Filtered lysate	1470	4.8×10^{10}	7.1×10^{13}	100%
$(NH_4)_2SO_4$ Ppte	16.2	3.1×10^{12}	5.0×10^{13}	71%
$(NH_4)_2SO_4$ Super	1850	$<10^6$	$<10^9$	<0.001%
CsCl Band	1.3	2.4×10^{13}	3.1×10^{13}	44%

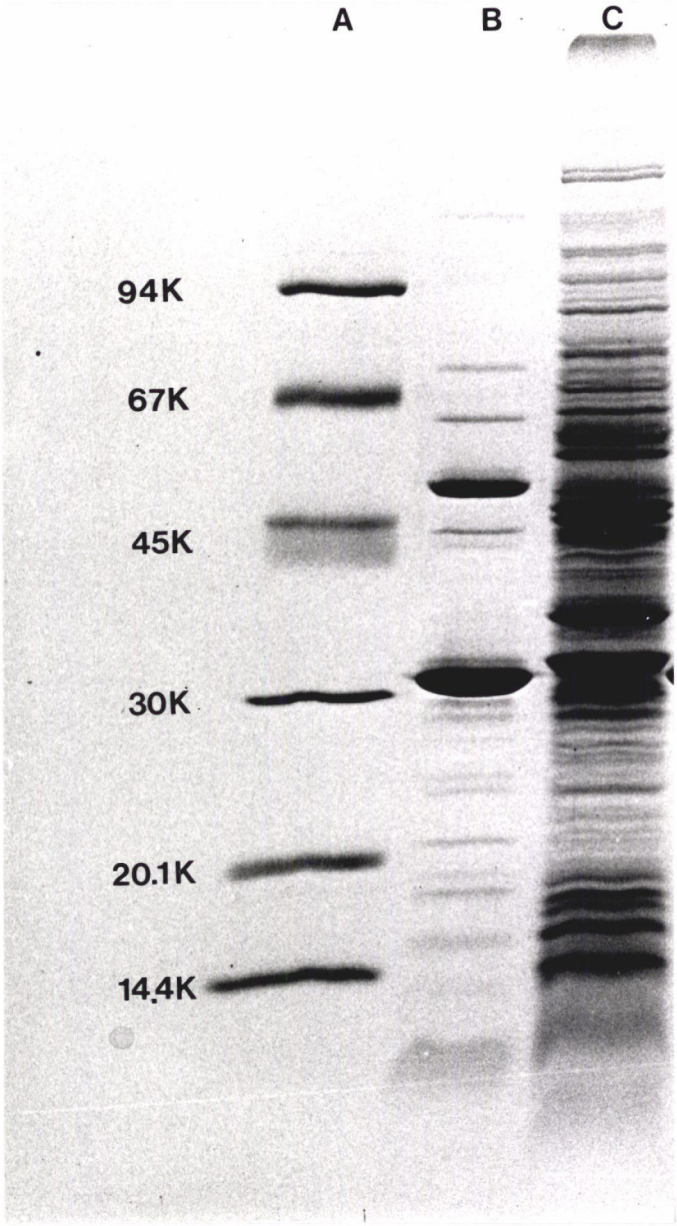
FIGURE 2 Rc-Cal 1 and *Rhodopseudomonas capsulata* on SDS-polyacrylamide (7 - 15 %) linear gradient gel to determine purity of concentrated Rc-Cal 1 from *R. capsulata* contamination.

Lane A is standard protein markers.

Lane B is forty micrograms of Rc-Cal 1 protein.

Lane C is 100 micrograms of French press treated *R. capsulata* cells.

The gel is stained with Coomassie Blue.


A
B
C
94K
67K
45K
30K
20.1K
14.4K

mid-log phase cells broken by a French press at 16,000 lbs/in^2. Figure 2 illustrates the mobilities for Rc-Cal 1 and Rhodopseudomonas capsulata "St. Louis" proteins separated on SDS-PAGE and stained with Coomassie Blue. Additionally, the phage was checked for purity by silver staining of the SDS-PAGE gels. The chemical reaction between silver stain and protein causes differential colouring between proteins. This phenomena was used to check proteins whose mobilities were similar; none of the proteins with similar mobilities stained the same colour.

4.4 WHOLE PHAGE CHARACTERIZATION

4.4.1 MORPHOLOGY

Rc-Cal 1 morphology was examined using sodium phosphotungstate and uranyl acetate to negatively stain the phage particles. The phage particle consisted of an elongated polyhedral head and a long (over twice as long as the head) non-contractile tail. Tail fibers were often seen on Rc-Cal 1 tails, with many in a tripod configuration (Figure 3). Figure 4 was a common occurrence in the electron micrographs and it is possible tail fibers caused phage aggregation. The dimension of the phage head based on measurements from twenty separate

FIGURE 3 Negatively stained Rc-Cal 1

The bar equals 100 nanometers and TF indicates tail fibers. The phage in the lower right hand corner, marked with TF, has tail fibers fully extended in a landing-pod arrangement.

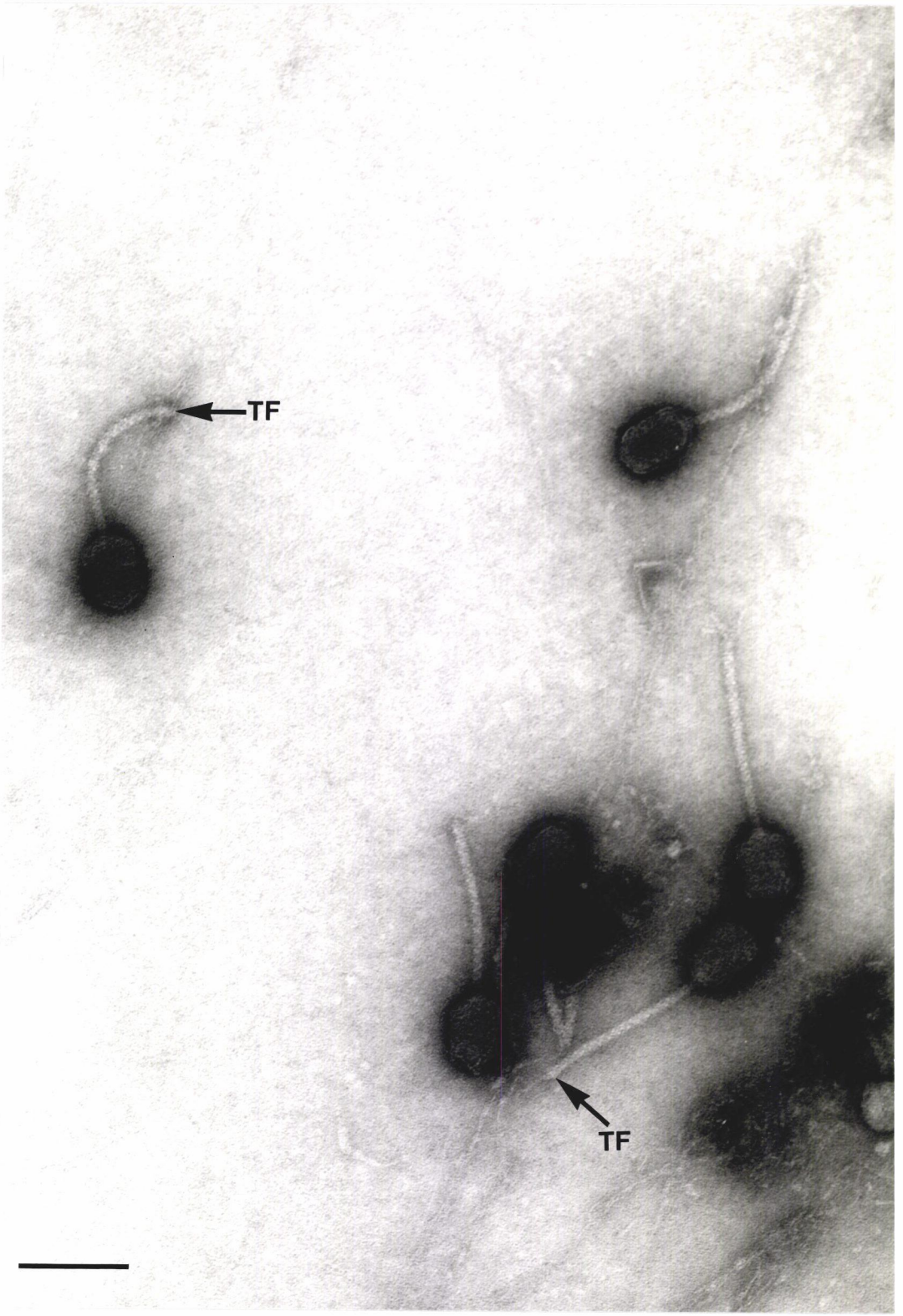
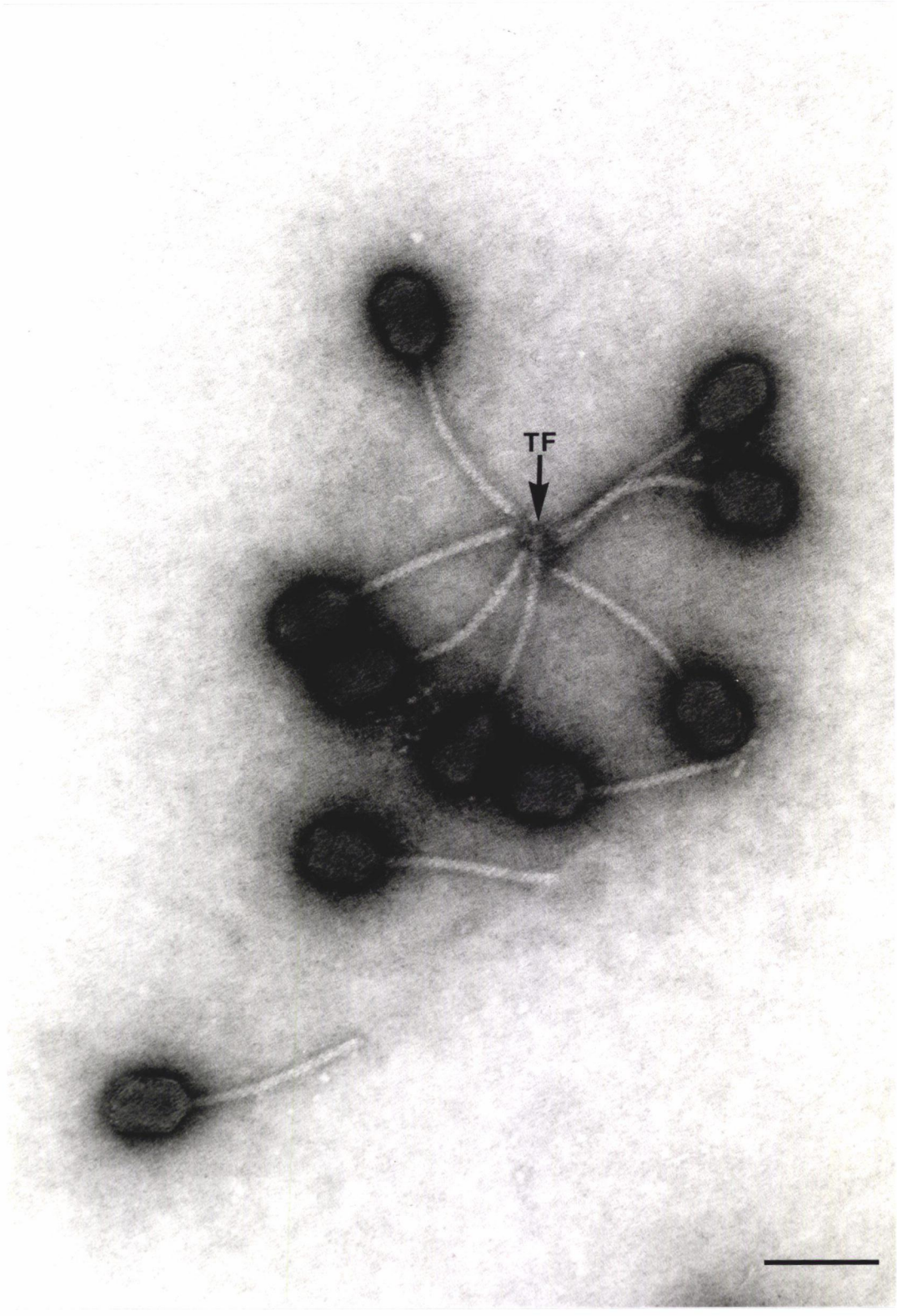

TF
TF

FIGURE 4 Negatively stained Rc-Cal 1

The bar indicates 100 nanometers and TF indicates tail fibers. The rosette arrangement appears to be caused by tail fiber interaction.


TF

phage, was 49 nm wide (range 43-55 nm) by 70 nm long (range 63-77 nm). The tail was 9 nm wide (range 7-11 nm) and 152 nm long (range 137-171 nm). Tail fibers did not appear to have any joints and were of varying lengths. However, there is one example in Figure 3 where the tail fibers appear to have joints in a landing pod type arrangement.

4.4.2 BUOYANT DENSITY

The buoyant density of intact Rc-Cal 1 in a cesium chloride isopycnic gradient is 1.520 gm/cm^3. This is the mean of six experiments whose standard deviation was 0.005 gm/cm^3. The buoyant density determination shown in Figure 5 has the most plaque forming units at a density of 1.522 gm/cm^3. High concentrations of phage ($>2 \times 10^{12}$ PFU/ml) affected the linearity of the density readings. Figure 5 has a total of 8×10^9 PFU and the density readings in linear regression have a correlation of 0.998.

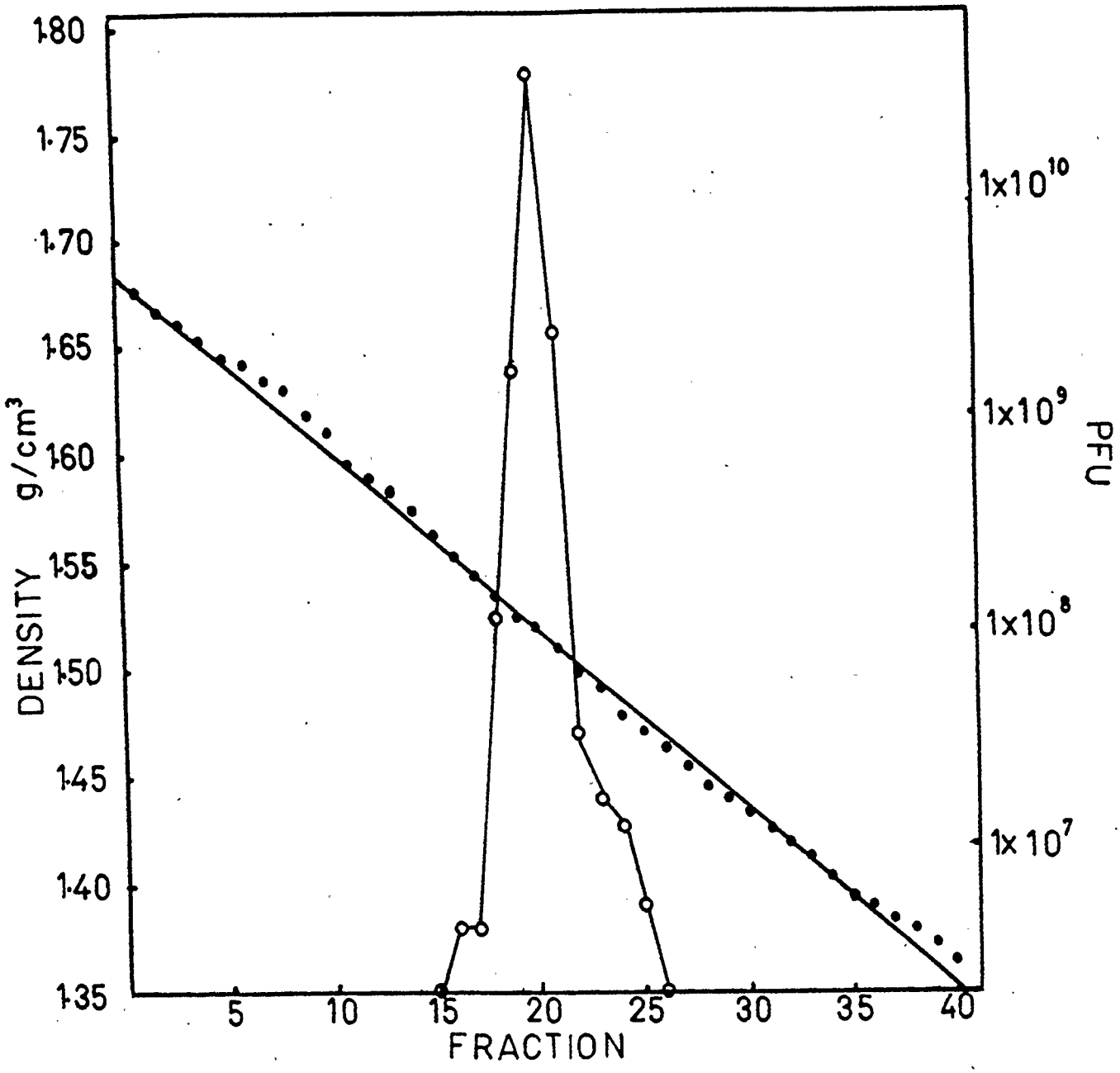
4.4.3 SEDIMENTATION

The sedimentation coefficient of Rc-Cal 1, determined from the mean of three sedimentation velocity experiments, is 374 $S_{20,w}$. The observed sedimentation coefficients were multiplied by the ratio of $S_{20,w}/S_{obs}$ for the Reo

FIGURE 5 Phage Buoyant Density

o = PFU

● = CsCl density (g/cm^3)


1·80
1·75
1·70
1·65
1·60
1·55
1·50
1·45
1·40
1·35
DENSITY g/cm³
1x10¹⁰
1x10⁹
1x10⁸
1x 10⁷
PFU
5
10
15
20
25
30
35
40
FRACTION

virus standards to obtain a $S_{20,w}$ value for Rc-Cal 1. The standard deviation for the three runs was 13 S.

To calculate the molecular weight of Rc-Cal 1 the Svedburg equation (Schachman, 1957) relating molecular weight to the rate of sedimentation and diffusion coefficient was used. Two assumptions had to be made in this calculation because of difficulty in obtaining the proper instruments for measuring partial specific volume and diffusion coefficient. Because Rc-Cal 1 is similar to lambda the values for lambda partial specific volume and diffusion coefficient (Dyson and Van Holde, 1967) were assumed to be reasonably close to the values of Rc-Cal 1. The equation:

$$M = \frac{RTS}{D(1-vp)}$$

where R = universal gas constant
T = absolute temperature
s = sedimentation coefficient
D = diffusion coefficient
v = partial specific volume
p = density
M = molecular weight

gives a molecular weight for Rc-Cal 1 of 64.3×10^6 daltons using the following values; $R=8.135 \times 10^7$ ergs per degree per mole, T=293.2°K, $s=374 \times 10^{-13}$ seconds, $D=1.953 \times 10^{-7}$ cm^2 per second, $v=0.61 cm^3$ per gram, p=1.520 gram per cm^3.

4.4.4 AMINO ACID COMPOSITION

The amino acid composition of Rc-Cal 1 showed the majority of amino acids were non-polar, 51% (Table 6). This indicates the majority of interactions between side chain substituents which stabilize protein structure are hydrophobic. This is in contrast to phages T2 and T3 (Table 6) which have a majority of polar amino acids, 53% and 52% respectively, giving rise to hydrogen bonding and salt linkages between ionic groups. Lambda also has a majority of polar residues, 51% which indicates that possibly the amino acid composition for Rc-Cal 1 is unusual.

4.5 PHAGE POLYPEPTIDES

The polypeptide pattern of purified Rc-Cal 1 (Figure 6) on SDS-PAGE stained with Coomassie Blue indicated two major proteins. Silver staining did not reveal any bands other than those seen with Coomassie Blue stain. On a gel with twenty protein bands identified by laser densitometer tracings (Lane D of Figure 6) four protein bands (those bands with $\geq$2% of the total area, see Table 7) accounted for 91.6% of the protein. The molecular weights of these proteins, determined by linear regression of the six markers (correlation 0.999), are 65,000, 54,000, 31,000,

TABLE 6 Rc-Cal 1 Amino Acid Composition compared with T2, T3 and Lambda

Amino Acid[1]	Rc-Cal 1	T2[2]	T3[2]	Lambda[3]
Alanine	12.2[4]	7.7	9.4	10.3
Arginine	2.6	5.0	6.0	5.6
Asparagine	12.0	11.6	11.5	8.9
Aspartic acid				
Glutamic acid	9.2	11.8	11.3	10.2
Glutamine				
Glycine	15.0	9.4	8.1	8.5
Histidine	1.2	0.9	1.7	0.5
Isoleucine	4.1	6.6	4.7	3.1
Leucine	7.2	5.9	9.4	6.1
Lysine	5.6	6.3	6.0	5.5
Methionine	1.3	2.2	1.9	3.1
Phenylalanine	2.0	5.5	3.4	4.2
Proline	2.9	3.9	4.5	5.6
Serine	7.3	5.3	4.0	7.4
Threonine	9.4	5.9	6.8	8.8
Tyrosine	1.6	6.3	5.1	4.1
Valine	6.5	5.9	6.4	7.4

1. Tryptophan and cysteine were not determined for T2, T3 and Lambda. The tryptophan content of Rc-Cal 1 was 0.3% and the cysteine content was 0.1%.
2. Taken from Fraser (1957).
3. Taken from Villarejo *et al*. (1967).
4. Percentage of total amino acids analyzed.

FIGURE 6 Polypeptides of purified Rc-Cal 1 disrupted by treatment with SDS-mercaptoethanol and electrophoresed on SDS-polyacrylamide (7 - 15%) linear gradient gels.

Lane A has 80 micrograms of protein.

Lane B has 40 micrograms of protein.

Lane C has 20 micrograms of protein.

Lane D has 10 micrograms of protein.

The arrows indicate 20 bands seen by an LKB laser densitometer. The gel is stained with Coomassie Blue. Bands are numbered in direction of migration, top to bottom. Marker molecular weights and mobilities (Lane E) are listed below:

MARKERS

Molecular Weight	Mobility
94,000	4.2 cm
67,000	6.0 cm
45,000	8.2 cm
30,000	10.6 cm
20,000	13.3 cm
14,000	15.1 cm

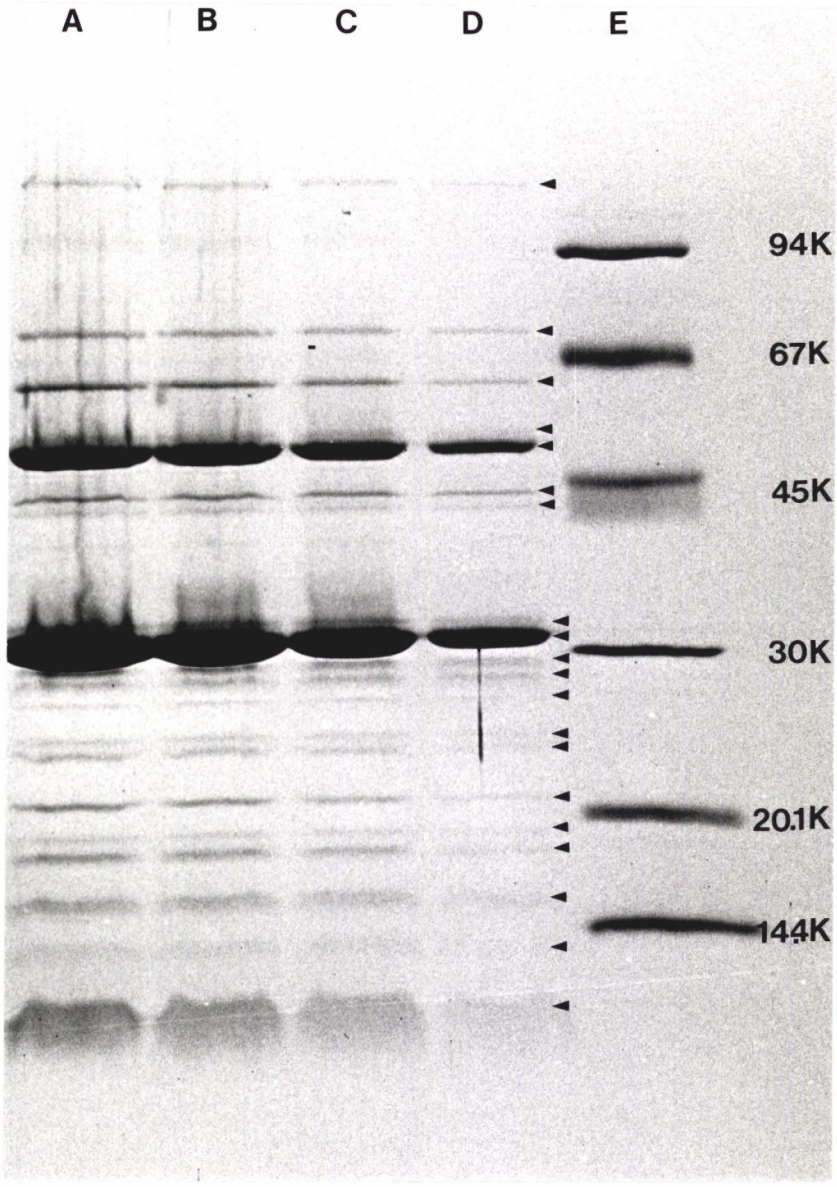

A
B
C
D
E
94K
67K
45K
30K
20.1K
14.4K

TABLE 7 Molecular weights of polypeptides from denatured Rc-Cal 1 on linear gradient SDS-polyacrylamide gels.

Molecular weight was determined from the linear regression of marker mobilities versus the log of the molecular weight. Markers are low molecular weight calibration kit from Pharmacia (See Figure 6).

	Molecular Weight	Mobility	Densitometer Area %	Estimated Total Molecular Weight (Densitometer Area % / 0.1) X MW
1	112,000	3.0 cm	0.1	112,000
2	75,000	5.4 cm	1.9	1,425,000
3	65,000	6.2 cm	2.1	1,365,000
4	56,000	7.1 cm	0.9	504,000
5	54,000	7.3 cm	18.3	9,882,000
6	48,000	8.0 cm	0.3	144,000
7	46,000	8.2 cm	0.1	46,000
8	34,000	10.1 cm	0.1	34,000
9	31,000	10.5 cm	67.6	20,956,000
10	30,000	10.8 cm	0.4	120,000
11	29,000	11.0 cm	0.1	29,000
12	27,000	11.3 cm	0.2	54,000
13	25,000	11.9 cm	1.0	250,000
14	24,000	12.1 cm	0.9	216,000
15	21,000	13.0 cm	0.8	168,000
16	19,000	13.4 cm	0.6	114,000
17	18,000	13.8 cm	0.6	108,000
18	16,000	14.5 cm	0.4	64,000
19	14,000	15.3 cm	0.2	28,000
20	12,000	16.3 cm	3.6	432,000
				Total 36.1 X 10^6

and 12,000. Table 7 indicates the subunits with molecular weights of 31,000 and 54,000 (bands 5 and 9) account for 86% of the protein in the gel. To make an approximate estimate of total protein molecular weight the smallest area by densitometer was used as the denominator with the other protein band area percents as numerator. The sum of these fractions times their respective molecular weights gives an estimated protein molecular weight of 36.1 X 10^6 daltons.

4.6 NUCLEIC ACID CHARACTERIZATION

4.6.1 GUANINE - CYTOSINE MOLE PERCENTAGE

The guanine-cytosine content of purified Rc-Cal 1 DNA was determined by a comparison of three established methods; melting point, buoyant density and ultraviolet spectral absorbancy ratios. Escherischia coli B DNA (Sigma) was used as a control and standard of known guanine-cytosine content (51.3%).

The melting point (Tm) was measured in 0.1 SSC on Rc-Cal 1 DNA from two separate DNA isolations. Figure 7 shows the melting point to be 85.6°C. Using this value in the equation of Johnson (1981) which corrects for experimental conditions the mol % G-C for Rc-Cal 1 is 74.5%.

The buoyant density of Rc-Cal 1 DNA was determined

from four experiments as 1.725 g/cm^3 with a standard deviation of 0.002. The correlation for the linear regression of Figure 8 is 0.998. The buoyant density of 1.725 g/cm^3 equals a guanine-cytosine mole percentage of 71.7% using Woodward and Lebowitz's (1980) formula.

The ultraviolet spectral absorbancy ratios for Rc-Cal 1 DNA were determined at these wave lengths, 245 nm/ 270 nm, 240 nm/280 nm and 240 nm/275 nm. Ulitzer (1972) found the presence of protein up to three times the concentration of nucleic acid did not affect the 245 nm/ 270 nm ratio more than one percent. This ratio, (Figure 9) of the three absorbancy ratios, was assumed the most accurate in case of protein contamination and gave a G-C mol % of 67.9%. The 240 nm/280 nm and 240 nm/275 nm ratios gave G-C mol % of 69 % and 73.3% respectively.

The average G-C mol % for Rc-Cal 1 using the three methods is 71.4%.

4.6.2 RESTRICTION DIGESTS

The use of restriction enzymes to characterize Rc-Cal 1 nucleic acid did not prove very successful. A variety of restriction enzymes were used. Four enzymes with four base recognition sequences, Hha I, Hae III, Alu I, Taq I were used, two of which, Hha I and Hae III, only had guanine and cytosine in the recognition sequence. The

FIGURE 7 Melting Point Determination of purified Rc-Cal 1 DNA with *E. coli* B DNA as a standard in 0.1 SSC.

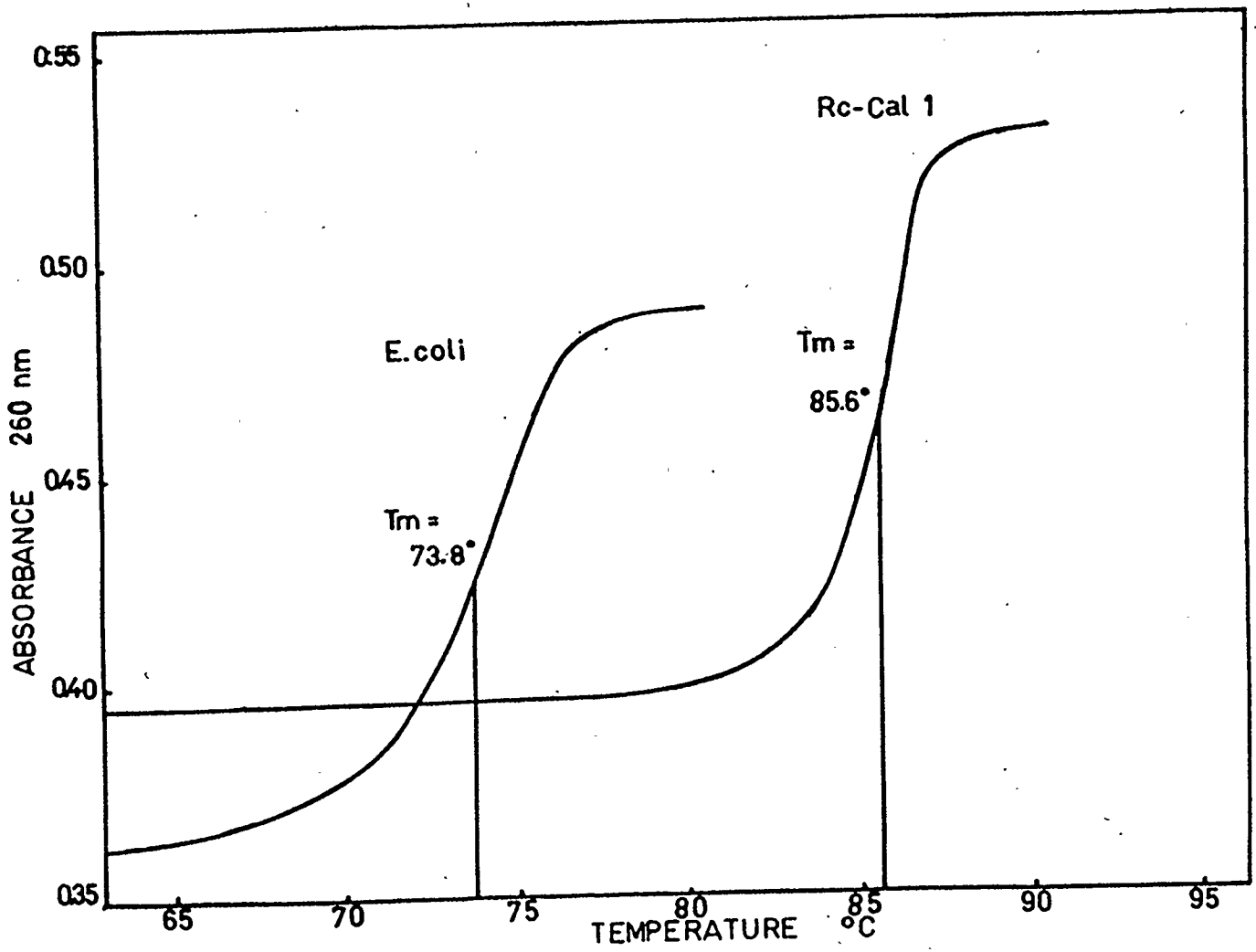

0.55
0.50
0.45
0.40
0.35
ABSORBANCE 260 nm
Rc-Cal 1
E.coli
Tm =
85.6°
Tm =
73.8°
65
70
75
80
85
90
95
TEMPERATURE °C

FIGURE 8 Buoyant Density Determination of purified Rc-Cal 1 DNA.

o = A_{260}

● = CsCl density (g/cm^3)

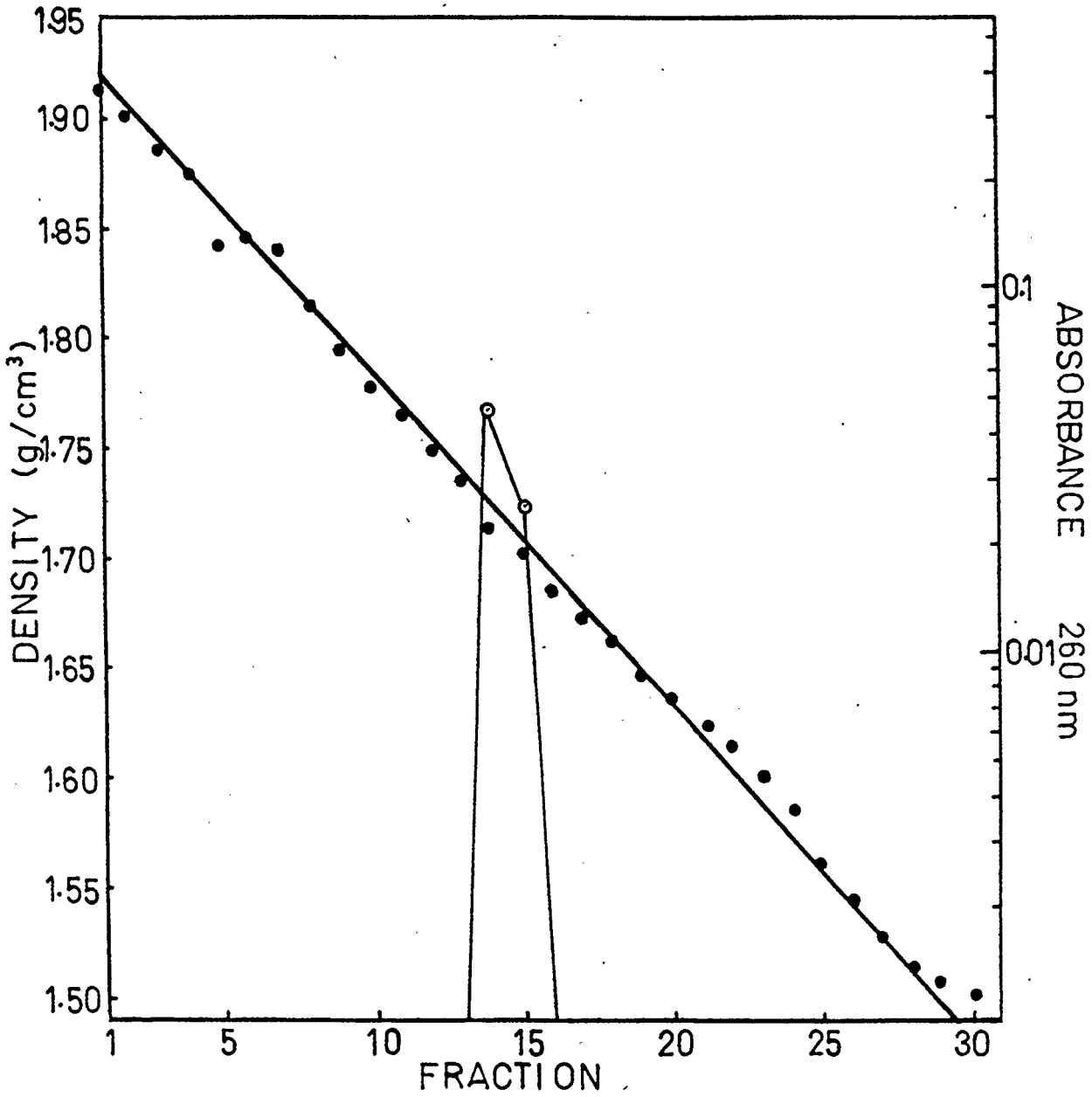
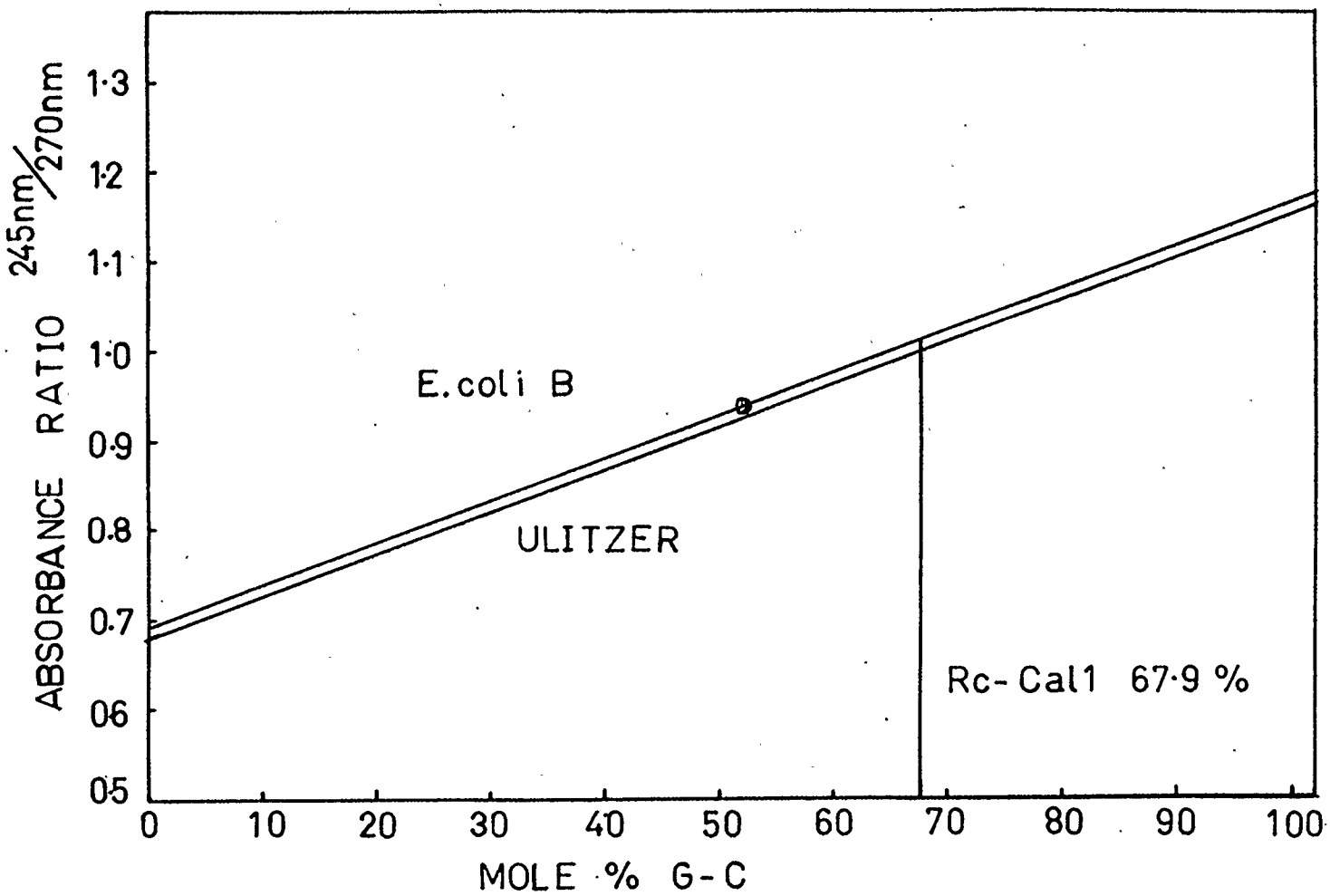

1.95
1.90
1.85
1.80
1.75
1.70
1.65
1.60
1.55
1.50
DENSITY (g/cm³)
0.1
0.01
ABSORBANCE 260 nm
1
5
10
15
20
25
30
FRACTION

FIGURE 9 Spectrophotometric Determination of G-C mole % using the 245/270 absorbancy ratio.

The reference line was determined by Ulitizer (1972). The internal standard was *E. coli* B DNA (o).


ABSORBANCE RATIO 245nm/270nm
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
E.coli B
ULITZER
Rc-Cal1 67·9 %
0
10
20
30
40
50
60
70
80
90
100
MOLE % G-C

high percentage of guanine and cytosine in Rc-Cal 1 would indicate that these enzymes should find recognition sites. No discernible bands were seen. The other ten enzymes used, Bam HI, Kpn I, Sac I, Eco RI, Hinf I, Hind III, Sau 3A, Sal I, Pst I, Pvu I, had six base recognition sequences with random base content. The only discernible bands were seen with Hinf I and Hind III and sometimes the results shown in Figure 10, Lanes B and C were difficult to reproduce. Rc-Cal 1 DNA was repurified by proteinase digestion and phenol extraction to exclude protein interference but the results did not improve. Lambda DNA was mixed with Rc-Cal 1 DNA and they were restricted together. Lambda gave characteristic digests.

In Figure 10 Hind III (Lane B) and Hinf I (Lane C) restrictions of Rc-Cal 1 DNA are shown. In Lane B (Hind III digest) only one intense band is seen with several other brighter areas in the digest which could possibly be bands. This was characteristic of Hind III digests. In Lane C (Hinf I digest) six discernible bands are seen, four very intense and two faint. The four intense bands were always in the same pattern but the faint bands were variable. Lane A shows Rc-Cal 1 DNA unrestricted in the linear form. Lane D shows a commercially prepared (Pharmacia) lambda Hind III digest used for markers. This digest was not heated so some of the <u>cos</u> sites in the 23.1 kilobase pair fragment and the 4.4 kilobase pair fragment

FIGURE 10 Rc-Cal 1 DNA molecular weight determination by 0.3% agarose gel electrophoresis.

Lane A: unrestricted linear Rc-Cal 1 DNA.

Lane B: Hind III restriction digest of Rc-Cal 1

Lane C: Hinf I restriction digest of Rc-Cal 1.

Lane D: commercial (Pharmacia) Hind III restriction digest of lambda.

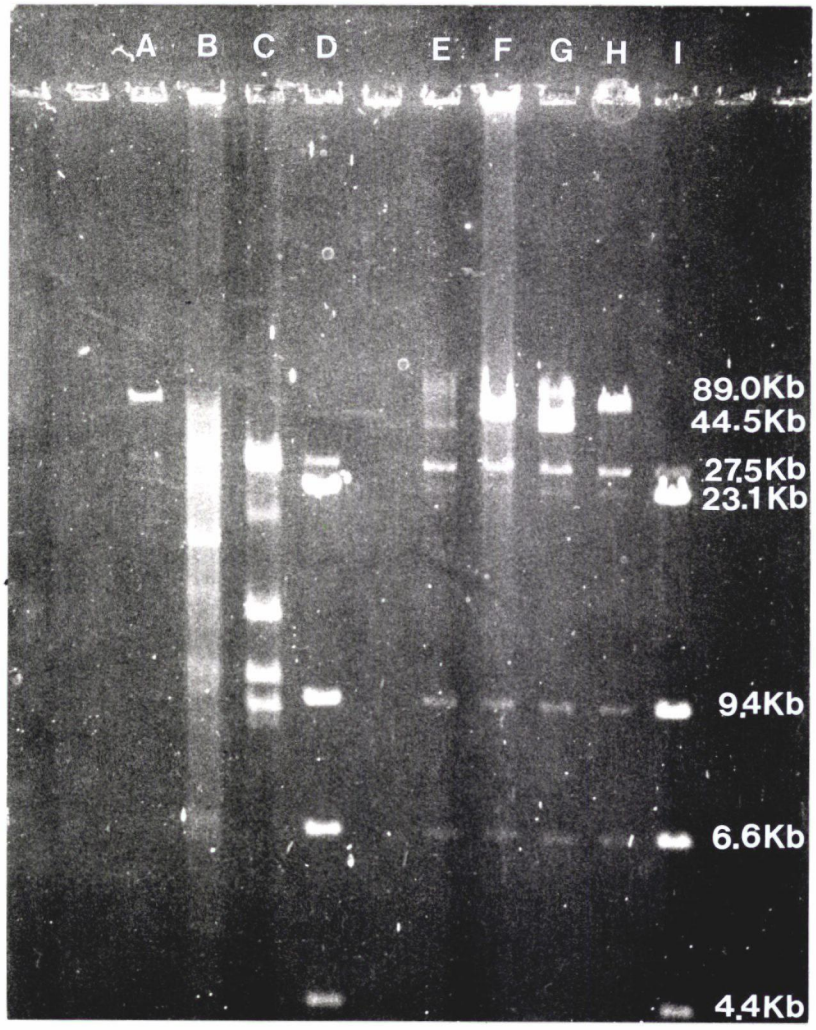
Lane E: T4 DNA ligase preparation of lambda strain EMBL 4. Ligation product is 89 Kb, unligated DNA is 44.5 Kb. Internal markers are Pharmacia Hind III lambda digest.

Lane F: unrestricted linear lambda DNA, strain C1S amber 7, 49.0 Kb. Internal markers are Pharmacia Hind III lambda digest.

Lane G: unrestricted linear lambda DNA, strain EMBL 4, 44.5 Kb. Internal markers are EMBL 4 ligation product, 89.0 Kb, and Pharmacia Hind III lambda digest.

Lane H: unrestricted linear Rc-Cal 1 DNA (proposed 63.0 Kb). Internal markers are Pharmacia Hind III lambda digest.

Lane I: Pharmacia Hind III lambda digest markers: 27.5 Kb, 23.1 Kb, 9.4 Kb, 6.6 Kb, and 4.4 Kb.


A
B
C
D
E
F
G
H
I
89.0Kb
44.5Kb
27.5Kb
23.1Kb
9.4Kb
6.6Kb
4.4Kb

would link, forming a 27.5 kilobase pair marker.

4.6.3 MOLECULAR WEIGHT

Molecular weight determination using restriction fragments of Hinf 1 was also done in 0.3% agarose gels. Previous restriction digests showed there were no detectable fragments smaller than 6,600 base pairs and therefore, for better separation 0.3% agarose gels were used. Using the linear regression of three lambda Hind III fragments as markers (correlation 0.9995) the sum of the intense four fragments (26.1, 13.6, 10.4, 9.1 Kb) gave a size of 59,200 base pairs. The two less intense bands (Figure 10, Lane C) were not included as these were probably partial digestions. Evidence for this was obtained by running a digest with excess enzyme and taking aliquots at 1, 3, 24 and 48 hours. When these were run, seven bands were seen initially followed by six and then four. These four bands were the same intense bands mentioned above giving a size of 59,200 base pairs. This corresponds to a molecular weight of 38.4 X 10^6 daltons, assuming an average molecular weight of 649 daltons per base pair.

Whole DNA electrophoresis indicated a size of 63,000 base pairs ± 3,600 base pairs (Lane H, Figure 10). This was based on eight gels with markers of 89.0 Kb, 49.0 Kb,

and 44.5 Kb. Correlation of these markers in linear regression was 0.98. An example of these gels is seen in Figure 10, Lanes E, F, G and H.

Electron microscopy did not prove to be a satisfactory method for molecular weight determination. According to Davidson and Szybalski (1971) any molecular weight determination of DNA should include an internal standard of known size so variations caused by technique can be accounted for. Obtaining a micrograph of this qualilty was unsuccessful though some micrographs were taken showing molecules of approximately 17.6 microns. Using 192 daltons per angstrom as the mass per unit length (Davidson and Syzbalski, 1971), this gives a molecular weight of 33.8 X 10^6 daltons or 52,100 base pairs.

4.7 GENETICS

Three types of mutants were obtained from the mutagenesis, streptomycin resistant (50 µg/ml), rifampicin resistant (75 µg/ml), and nonphotosynthetic. Two colonies were isolated from each of the streptomycin and photosynthetic mutants and fifteen colonies from the rifampicin mutants. The antibiotic resistant mutants were screened for resistance to 200 µg/ml and remained stable after several tranfers and amplification to 500 ml cultures. The nonphotosynthetic mutants were also

transfered and tested for stability.

Transduction of the streptomycin marker was attempted twice. Wild type phage were grown in the streptomycin resistant cells using the described batch replication procedure except the MOI = 4 to ensure maximum number of infected cells. The lysate was concentrated, purified, and filter sterilized. The sterile phage (3 X 10^9 PFU/ml) infected wild type cells (8 X 10^8 cells/ml) for 24 hours. The culture (approximately 9 X 10^8 cells/ml) were plated on triplicate RCV plates with 200 μg streptomycin/ml. No colonies were found for either attempt. Control plates (wild type cells on 200 μg streptomycin/ml plates) indicated at this concentration there would be no spontaneous mutants. Assuming normal adsorption, (15 - 20%) there was less than one transduced cell in 6 X 10^8 infected cells.

The same procedure was followed for rifampicin mutants. Wild type cells (9 X 10^8 cells/ml) were infected with phage (1 X 10^9 PFU/ml) which had been replicated in rifampicin mutants, concentrated and purified, and filter sterilized. After 24 hours RCV plates with 200 μg rifampicin/ml were spread with approximately 1 X 10^9 cells. No colonies were found. Assuming normal adsorption, there was less than one transduced cell in 6 X 10^8 infected cells.

Nonphotosynthetic mutants (3 X 10^8 cells/ml) infected

with wild type phage (9 X 10^8 PFU/ml) for 24 hours. The culture was spread (10^9 cells/ml) on RCV plates and incubated anaerobically in the light and aerobically. No colonies were seen on the anaerobic plates and a lawn of cells developed on the aerobic plates. Assuming normal adsorption, the genetic material to complement the nonphotosynthetic mutant was transduced less than one in 1.8 X 10^8 infected cells.

Ultraviolet mutagenesis for auxotrophic mutants was unsuccessful. All colonies which grew on L-broth plates also grew when replicated onto RCV. A total of 24 colonies were tested.

5.0 DISCUSSION OF RESULTS

From all published accounts of phage infecting Rhodopseudomonas capsulata there is not one similar to Rc-Cal 1. The most comprehensive comparison available was the report by Wall et al. (1975a). Rc-Cal 1 did not fit into any of the sixteen classes established from infectivity of 95 phage isolates on thirty-three strains of R. capsulata (Table 4). Rc-Cal 1 was unusual in the small number of R. capsulata strains it would infect. Only RC2 infected fewer than Rc-Cal 1. Rc-Cal 1 also was unusual in its ability to infect R. capsulata strain C4. Only one other group, RC3, was able to infect this strain. This would indicate Rc-Cal 1 has a special requirement for infection which only wild type strains and a few others can supply.

The biophysical characterization according to the high level criteria (Table 2) of Ackermann et al. (1978) was generally accomplished. Morphologically, Rc-Cal 1 fits very well into Bradley's (1967) Group B (Table 1). The type species for this group is coliphage lambda (Matthews, 1979). Lambda has an icosahedral head, 54 nm by 54 nm with a tail 140 nm long. A single tail fiber is at the end. Rc-Cal 1 is similar in dimensions except the head is polyhedral and there are three tail fibers in a tripod arrangement.

The tail fibers of Rc-Cal 1 appear to play a role in attachment. The rosette seen in Figure 4 was a common occurrence with many other phage attached at this site in twos or threes. The phage with the landing pod arrangement in Figure 3 was unusual, no other Group B phage exhibit this arrangement. Because this arrangement was seen in only a few micrographs it may be these fibers are very susceptible to mechanical damage. In other micrographs there appeared to be more than three fibers. These are possibly tail fibers folding back on themselves. An example of this is seen in Figure 3 on the upper left phage marked for tail fibers.

In some micrographs there appears to be a protein ring at the junction of the tail and capsid. There is a similar description for ØRsG1 (Duchrow et al., 1985). In micrographs where the ring is more discernible, it appears the neck reduces in diameter as it enters the capsid, similar to lambda (Kellenberger and Edgar, 1971).

A true molecular weight for Rc-Cal 1 was unobtainable because of the lack of a diffusion coefficient and partial specific volume. The estimate of 64.4×10^6 daltons is probably a good rough estimate but relatively slight changes in the diffusion coefficient and partial specific volume cause large changes in the molecular weight. The sedimentation coefficient of lambda ($S_{20,w}$=388) was within the standard deviation of Rc-Cal 1 ($S_{20,w}$=374±13). Lambda

has a molecular weight of 59.5 X 10^6 daltons (Ackermann *et al.*, 1978). Bacteriophage 59, a temperate phage for *Erwinia carotovora* (Kishko *et al.*, 1983), has a very similar morphology to Rc-Cal 1. It's $S_{20,w}$=406±10 and has a molecular weight of 60 X 10^6 daltons. An indirect molecular weight estimate for Rc-Cal 1 can be made by adding the protein molecular weight, 36.1 X 10^6 daltons, and the nucleic acid molecular weight, 38.4 X 10^6 daltons. This combination gives a molecular weight of 74.5 X 10^6 daltons.

The buoyant density of Rc-Cal 1 was well established. Rc-Cal 1 is more dense than lambda and phage 59, 1.52 g/cm^3 versus 1.49 g/cm^3 and 1.50 g/cm^3 respectively. This is due to the increased DNA content of Rc-Cal 1, 38.4 X 10^6 daltons versus 31.9 X 10^6 daltons for lambda and 28.4 X 10^6 daltons for bacteriophage 59. Assuming a buoyant density of 1.300 g/cm^3 for protein (Kishko *et al.*, 1983) and a buoyant density of 1.725 g/cm^3 for Rc-Cal 1 DNA, a buoyant density of 1.52 g/cm^3 for the whole particle would indicate a make up of 48% protein and 52% nucleic acid. The proposed protein molecular weight of 36.1 X 10^6 daltons and nucleic acid weight of 38.4 X 10^6 daltons correlate very well with these percentages. The average make-up of Group B phages is approximately 1:1 (Kishko *et al.*, 1983).

The nucleic acid of Rc-Cal 1 was unusual in several

ways. The molecular weight is high for a phage of its size, as stated above, 6.5 and 10 X 10^6 daltons greater than lambda and phage 59 respectively. In relation to ØRsG1, a phage infecting the same genus, it has 6 X 10^6 daltons more. The G-C mole % is also high when compared to phage from different genera (71.4% compared to lambda 51%) but not when compared to phage of the same genus (ØRsG1 G-C mole % = 71.8). This correlates with the host G-C mole % which, according to Matthews (1979), tailed phages usually do. Rhodopseudomonas capsulata "St. Louis" has a G-C mole % of 68.5 (DeBont et al., 1981).

The difficulty in restriction digests was also unusual. In any of the reports for phage infecting rhodopseudomonads this has not been mentioned. The fairly good correlation between G-C mole % determined by Tm (74.5%) and buoyant density (71.7%) indicates unusual bases are probably not the problem (Mandel and Marmur, 1968). A report by Powell and Davidson (1986) indicated some phage may protect themselves from host restriction by having nucleotide sequences which have a scarcity of restriction sites. It is unusual, however, that the enzymes Hha I and Hae III which have recognition sites of GCGC and GGCC would not give discernible bands.

The polypeptides of Rc-Cal 1 were average in number. Group B phage have between 5 - 23 polypeptides (Matthews, 1979). Rc-Cal 1 has 20 but only four major ones ranging

from 65,000 to 12,000 daltons. Lambda has eleven (Murialdo and Siminovitch, 1971) ranging from 100,000 to 11,000 daltons. The major protein in Rc-Cal 1 has a molecular weight of 31,000 daltons. If lambda serves as a model this would correspond to a head protein. The second major protein had a molecular weight of 54,000 daltons. The second major protein in lambda corresponded to a tail protein.

The protein subunits, of Rc-Cal 1, were unusual in their resistance to denaturation. An attempt to size them by size exclusion chromatography was made, but even after treatment with 6 M guanidine HCl and 8 M urea they tended to aggregate. The amino acid analysis shows no great differences from other phage. The small amount of cysteine present in Rc-Cal 1 could explain the resiliency, if the cysteine residues were used to form disulfide bridges between the protein subunits (Villarejo et al., 1967).

As mentioned in the introduction, one of the main purposes for isolating and characterizing bacteriophage is to find genetic vectors. The lack of success in this study to establish Rc-Cal 1 as a transducer is probably due to small sample size. Stent (1963) states generalized transducers occur at a frequency of about 10^{-5} to 10^{-7} per infective particle adsorbed and specialized transducers at a frequency of 10^{-7} to 10^{-8} per infective particle

adsorbed. The sample sizes in the described experiments indicate for generalized transduction the desired marker would have to be transduced at a frequency of 1 in 20 to 1 in 60 to be seen. In specialized transduction the frequency is much higher, 1 in 2 to 1 in 6. Given the size of the host genome, a definitive statement on the ability of Rc-Cal 1 to transduce can not be made without increasing the sample size of infective particles and the number of markers screened.

The last area of characterization studied was the replication cycle for Rc-Cal 1. In batch replication the optimal parameters were not unusual (Adams, 1959). The single burst experiment indicated unusual characteristics. First, the phage adsorption is poor but production in those infected cells is high, burst size = 122. The single burst measures the mean yield of phage particles per infected bacterium. It would be interesting to do the single cell method of virus multiplication (Adams, 1959) to determine the variance between cells in bacteriophage production. Merriam (1977) reported that single bacteriophage M13 infected cells may produce 1,000 phage per cell division.

Immunity to phage infection, as was seen in 14% of the host colonies tested, is characteristic of lysogeny (Barksdale and Arden, 1974). In these immune hosts Rc-Cal 1 can not be induced as a normal lysogen (King, 1978) but

will continue to release phage and be immune to further infection. This is called persistent infection or the carrier state by Barksdale and Arden (1974). The results indicate that Rc-Cal 1 is very stable in this state. In contrast M13 - infected cells were unstable to prolonged growth under all conditions. Mycoplasma virus MVL2 gave rise to host cells which were persistently infected as a stable heritable trait (Putzrath and Maniloff, 1978). This phage was very similar to persistently infected animal viruses because it is nonlytic, budding through the cell membrane.

Rc-Cal 1 is the only stable pseudolysogenic lytic phage thus far discovered. Because it is so unusual it would be interesting to examine the state closer by DNA-DNA hybridization with the host. Carrier cells should also be examined for extrachromosomal DNA. The conditions which allow this state should also be examined to determine if they are one of the three conditions proposed by Barksdale and Arden (1974) or something novel.

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