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STUDIES OF BACTERIOPHAGES INDUCED FROM *STREPTOCOCCUS CREMORIS* STRAIN R_1: IS R_1 A DOUBLE LYSOGEN ?

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Genetics at Massey University, New Zealand.

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ABSTRACT

Early studies on *Streptococcus cremoris* strain R_1 suggested that it was polylysogenic. Later, it was reported that its induced lysates contained bacteriophages (phages) of two types which were believed to differ in their morphology, buoyant densities, immune specificities and in their responses to heterologous antiphage sera. Further work on the strain did not reproduce the above observations, but did often give results which were consistent with it being a double lysogen. This project was an in-depth investigation of phages induced from R_1, in an attempt to establish the single or double lysogenic nature of the strain.

Mid-log phase R_1 cells were harvested, washed with homologous antiphage serum and induced to lyse with ultraviolet light (UVL). The resulting phage lysates were analysed on caesium chloride (CsCl) density gradients. Though the OD_{254} (optical density at 254 nm) scans of the gradients detected the presence of only one phage band, p.f.u. (plaque forming unit) profiles of the gradient fractions on indicator strains R_1C and 368 revealed, in addition to the main phage peak, several minor p.f.u. peaks (termed satellite and shoulder peaks) as possible manifestations of different phage types in the R_1 lysates. Further CsCl density gradient analyses of phage stocks and pooled phage fractions of these minor p.f.u. peaks showed that the latter phages were identical with those of the main phage peaks of mean buoyant density of 1.485 g/ml.

Further characterization of the phages recovered from the CsCl gradients by neutralization tests with homologous antiphage serum confirmed the existence of only one serological phage type in the R_1 lysates. Final verification of the unity in phage type in R_1 lysates came from SDS-gel electrophoreses of the phages recovered from the different p.f.u. peaks and from lysates, which showed the largely identical gel patterns of their protein components. Host-specificity tests of the phages provided the last piece of evidence for the conclusion that R_1 is a single lysogen, harbouring only one prophage in its genome. Review of past electron-microscopic studies

of R_1 lysates substantially support this conclusion. In fact, reconstruction of R_1 by lysogenization of a cured strain (R_1C) yielded a strain (R_1r) which closely resembled the original in lysogenic properties.

From the data collected in the course of this work, it was inferred that 368 lysates possibly contained defective phages. An attempt was made to cure 368 of its supposedly defective prophage in the hope of providing a 'cleaner' strain for studying the host-induced variation observed in the R_1C-368 system. Though possible cured derivatives were obtained, they did not prove to be an improvement over the parental strain 368 with respect to their efficiency of plating for R_1 phages.

Finally, phage mutant isolation and recombination experiments were attempted in the hope of gaining an insight into the lysogenic system operating in the R_1 cells. Using UVL and nitrous acid (HNO_2) mutagenesis on the temperate $\phi r_1/R_1C$ induced from R_1, about 75 independently arising clear plaque-forming mutants were isolated for mapping experiments. Pairwise crosses between the UVL and HNO_2^- induced mutants were performed by coinfecting R_1C cells. Though far from conclusive, the preliminary results obtained indicated a general low occurrence of turbid-plaqued (wild type) phage recombinants, and hence a low frequency of recombination.

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SECTION ONE

INTRODUCTION

In our simplicity, in an attempt to understand, recognize and assimilate mentally the infinite variations in life forms that characterize Nature, we generalize by classifying like forms together into discrete groups. The consequence is the constant reshuffling of groups within the classification schemes to keep pace with time and peace among the majority of workers (Jordan & Burrows, 1948). The following classification, however, is adopted for the bacterial strain under study here, that is *Streptococcus cremoris* strain R_1 (Niven, *et al*, 1957; Deibel & Seeley, 1974):

Kingdom:	Procaryotae
Division:	Scotobacteria
Class:	The Bacteria
Order:	Eubacteriales
Family:	Streptococcaceae
Genus:	Streptococcus
Species and strain:	*Streptococcus cremoris* R_1.

Trivial though it may seem, controversy still exists as to which kingdom the bacteria rightfully belong. In the eighth edition (1974) of Bergey's Manual of Determinative Bacteriology, bacteria are assigned to the new kingdom Procaryotae. In the same text, under the newly suggested division Scotobacteria (that is procaryotes indifferent to light) and class The Bacteria, lies the order Eubacteriales which carries the *Streptococcus cremoris* R_1 under the family Streptococcaceae and the genus Streptococcus.

This genus was first described by Billroth in 1874 as 'globular microorganisms growing in chains' (McCarty, 1973). About a decade later, Rosenbach identified the latter as 'Streptococcus' (Zinsser, 1964). Subsequent years witnessed a rapid accumulation of data on the genus, particularly on species which were pathogenic in man and domestic animals. Some of the microbes held responsible for the souring of

milk also have long been found to fall within this genus.

Individual streptococcus cells vary from spherical to ovoid in shape depending on the species, with size ranging from 0.4 - 1.0 μ in diameter (Burrows, 1968). In a growing culture, the individuals become elongated along the axis of the chain with subsequent cell divisions all occurring in one plane, perpendicular to the chain axis. The daughter cells thus formed usually do not become severed, resulting in chains characteristic of the genus. The strength of cell-to-cell attachment is to some degree a species or strain characteristic, and the chain length tends to be inversely related to the adequacy of the culture media and growth conditions (McCarty, 1973). The actual cell contact was reported to be the uncleaved new cell walls which were synthesized equatorially (Cole & Hahn, 1962).

Though variations in Gram-stains have been observed (Gebhardt & Anderson, 1959; Dring & Hurst, 1969), streptococci are generally considered Gram-positive. They are all nonsporogenous and non-motile, with an occasional occurrence of motile strains in Group D being the exceptions. Some strains may be capsulated (MacKie & McCartney, 1960).

The minimal nutritional requirements of this genus are generally complex. Though most species are facultative anaerobes with a few being micro-aerophilic or obligately anaerobic, they all derive their energy primarily from the fermentation of sugars with dextrorotatory lactic acid as the predominant end product (that is homofermentative). Presence of oxygen or other hydrogen acceptors may alter the end products normally formed.

Classification within this genus has presented many difficulties (Lancefield, 1969). Early physiological studies recognized the existence of varieties, and a systematic approach was attempted in 1903 when Schottmüller proposed that the different varieties then observed be classified on the basis of their ability to hemolyze erythrocytes. In 1919, Brown introduced the terms alpha (α), beta (β) and gamma (γ) to describe the three types of hemolytic reactions generally observed on blood-agar plates. In 1937, combining the hemolytic responses with

TABLE I SOME PHYSIOLOGICAL TESTS USED TO DIFFERENTIATE THE STREPTOCOCCI [1]

Early divisions.	Examples of present known group or species.	Growth at:		Growth in media containing:			Growth initiation at pH 9.6.	Heat tolerance (60°C for 30 min).	Strong reduction of litmus milk.	NH_3 from peptone.
		10°C.	45°C.	0.1% methylene blue (in milk).	6.5% NaCl.	40% bile.				
Pyogenic	*Str. pyogenes*	-	-	-	-	-	-	-	-	+
	Str. dysgalactiae	-	-	-	-	-	-	-	ND	ND
	Str. equi	-	-	-	-	-	-	-	-	+
	Str. zooepidemicus	-	-	-	-	-	-	-	-	+
	Str. equisimilis	-	-	-	-	-	-	-	-	+
	Group G streptococci	-	-	-	-	-	-	-	-	+
	Group E streptococci	-	-	-	-	-	-	-	-	+
	Str. anginosus	-	d	-	-	d	-	-	-	+
	Str. agalactiae	-	-	-	-	+	-	-	-	+
	Str. sanguis	-	d	-	-	+	-	d	-	d
Viridans	*Str. acidominimus*	-	-	-	-	ND	-	-	ND	ND
	Str. mitis	-	d	-	-	-	-	-	ND	ND
	Str. salivarius	-	+	-	-	-	-	-	-	-
	Str. uberis	+	+	-	-	-	-	+	ND	ND
	Str. thermophilus	-	+	-	-	-	-	+	-	-
	Str. equinus	-	+	-	-	+	-	d	-	-
	Str. bovis	-	+	-	-	+	-	+	-	-
Lactic	*Str. cremoris*	+	-	d	-	+	-	d	d	-
	Str. lactis	+	-	+	-	+	-	d	+	+
Enterococcus	*Str. faecalis*	+	+	+	+	+	+	+	d	+
	Str. faecium	+	+	+	+	+	+	+	-	+

[1] + = most (90-100%) strains positive.
- = most (90-100%) strains negative.
d = some strains positive, some negative.
ND = no or insufficient data.

physiological properties, Sherman was able to recognize four main divisions designated Pyogenic, Viridans, Lactic and Enterococcus, each containing several groups or species (see Table I) (Abd-El-Malek & Gibson, 1948). These divisions, aside from segregating the virulent pyogenic streptococci and saprophytic lactic forms from the rest, offer little assistance in species differentiation within the genus.

Primarily through the efforts of Lancefield in 1933, the pyogenic and enterococcus divisions were further sub-divided into five major serological groups designated Groups A to E, based on the distinct carbohydrate antigens of their cell envelopes (see Table II). Since then these dominant so-called 'C' substances, which constitute approximately 10% of the cell dry weight (McCarty, 1973), have been demonstrated to be rather specific and common among the streptococci. Further work resulted in the establishment of Groups F and G by Lancefield and Hare in 1935, Groups H and K by Hare in the same year, and L and M by Fry three years later. In 1943, Mattick and Shattock assigned the lactic streptococci to Group N, followed by the recognition of Group O by Boissard and Wormald in 1950. Subsequent studies ended in further findings of Groups Q, R, S, and T.

Currently, the collective use of the physiological and serological procedures offer the most definitive approach in species differentiation of the genus (refer to Tables I & II). In the 1974 edition of Bergey's Manual of Determinative Bacteriology, only 21 species are recognized with 5 possessing no demonstrable group-specific antigen. Though a total of about 17 serological groups have been worked out over the years, no species designation has yet been suggested for some of them. Though still far from completion, the present classification system of streptococci nevertheless provides us with the much needed framework within which work on this genus may systematically proceed.

The milk-souring microbes of the 'lactic' group in Sherman's early divisions are generally characterized by having low minimum and maximum growth temperatures, strong reducing action and a marked tolerance to methylene blue. These features, supplemented by other

TABLE II GROUP CLASSIFICATION OF THE STREPTOCOCCI

Group.	Group - specific hapten: Chemical nature.	Cellular location.	Hemolysis.[2]	Usual habitat.	Examples of recognized species.
A	Rhamnose-N-acetyl-glucosamine polysaccharide.	Cell wall	β	Man	*Str. pyogenes*
B	Rhamnose-glucosamine polysaccharide.	Cell wall	β,α,γ	Cow, man	*Str. agalactiae*
C	Rhamnose-N-acetyl-galactosamine polysaccharide.	Cell wall	β	Animals,man	*Str. equisimilis*
			β	Animals	*Str. zooepidemicus*
			β	Horse	*Str. equi*
			α	Cow, sheep	*Str. dysgalactiae*
D	Glycerol teichoic acid containing D-alanine and glucose.	"Intracellular" between the cell wall and membrane (Smith & Shattock, 1964)	γ	Man, animals	*Str. faecalis* sub-sp. *faecalis* sub-sp. *liquefaciens* sub-sp. *zymogens*
			α	Man, animals	*Str. faecium*
			α	Animals,man	*Str. bovis*
			α	Horse	*Str. equinus*
			β,α	Pig	*Str. suis*
E	Rhamnose polysaccharide	Cell wall	β	Milk, pig	*Str.* species

Table II continued

F	Rhamnose and a glucopyranosyl-N-acetyl-galactosamine tetrasaccharide.	Cell wall	β,α,γ	Man	*Str. anginosus*
G	Rhamnose-galactosamine polysaccharide.	Cell wall	β	Man, dog	*Str.* species with large colony-formation
H	Rhamnose polysaccharide.	Cell wall	β,α	Man	*Str. sanguis*
K	Rhamnose polysaccharide.	Cell wall	γ	Man	*Str. salivarius*
L	ND[3]	ND	β,α	Man, dog,pig	*Str.* species
M	ND	ND	β,α	Man, dog	*Str.* species
N	Glycerol teichoic acid containing D-alanine and galactose phosphate (Elliot, 1963).	"Intracellular" between the cell wall and membrane (Smith & Shattock, 1964)	α,γ	Milk, dairy products.	*Str. lactis* sub-sp. *diacetylactis* (or *Str. diacetilactis*)
			α,γ	Milk, dairy products.	*Str. cremoris*
O	ND	ND	β,α	Man	*Str.* species
Q	ND	Cell Wall (Smith & Shattock, 1964)	α	Chicken,man, animals	*Str. avium*
R	ND	ND	β,γ	Pig, man	*Str.* species
S	ND	ND	ND	ND	*Str.* species
T	ND	ND	ND	ND	*Str.* species
?[4]	ND	ND	α,γ	Milk, cow	*Str. uberis*

Table II continued

None[5]	Ribitol teichoic acid with choline phosphate (species-specific hapten).	Cell Wall	β, α	Man, animals	*Str. pneumoniae*
None	ND	ND	α	Cattle	*Str. acidominimus*
None	ND	ND	α	Man	*Str. mitis*
None	ND	ND	α	Milk, dairy products.	*Str. thermophilus*

[2] *e.g.* β = most (90-100%) strains β-hemolytic.
β, α, γ = some strains β-hemolytic, some α- and some γ-hemolytic.
[3] ND = no or insufficient data.
[4] There are controversies as to which group *Str. uberis* belongs.
[5] None = no group antigen isolated.

characteristics of the organisms, outlined rather clearly the early boundaries and identity of the group. In 1943, Mattick and Shattock proposed the assignment of the lactic group to Group N which was then comprised of two generally accepted species, namely *Str. lactis* and *Str. cremoris* (Yawger & Sherman, 1937; Briggs & Newland , 1952). Together with *Str. diacetilactis*, whose species rank has been debated since Swartling's first proposal in 1951, these three Group N members (see Table III) (Briggs, 1952; Sandine, *et al*, 1959; Sandine, *et al*, 1962; Reddy, *et al*, 1969) have far out-paced all other streptococci in economic importance. They acquired commercial distinction through their use as 'starters' in the dairy industries.

Starters may be classified, according to the species present, as follows (Reiter & Møller-Madsen, 1963):

1. Single-strain starters
   a) *Str. lactis* or *Str. cremoris*
   b) *Str. diacetilactis*
2. Mixed-strain starters
   a) *Str. lactis* or *Str. cremoris* or both
   b) *Str. lactis*, *Str. cremoris* and *Leuconostoc cremoris*
   c) *Str. lactis*, *Str. cremoris*, *Str. diacetilactis* and *Leuc. cremoris*
   d) *Str. lactis*, *Str. cremoris* and *Str. diacetilactis*
   e) Several strains of *Str. diacetilactis*.

The most popular starters used in Europe and U.S.A. today are of types 1a,2a and 2b. Types 2c and 2d have been slow in gaining recognition, while types 1b and 2e are not normally employed. Recently there is a preference for *Str. cremoris* strains, particularly in Cheddar cheese manufacturing, owing to the fact that the former are less likely to give fruity or bitter defects.

Since early 1900, when cultures of lactic streptococci were first used as starters in the dairy industry, workers involved in the manufacture of cheese had often experienced difficulties with the variations in acid production exhibited by some starter cultures.

TABLE III DIFFERENTIAL PHYSIOLOGICAL CHARACTERISTICS OF GROUP N STREPTOCOCCI

Physiological reactions.		*Str. lactis.*	*Str. cremoris.*	*Str. diacetilactis.*
Origin of isolation.		milk	milk	Spontaneously soured potato mash (Briggs, 1952)
Growth at 40°C.		+	-	+
Growth in presence of:	(4% NaCl.	+	-	+
	(0.3% methylene blue ((in milk).	+	d[6]	ND[7]
	(pH 9.2.	+	-	-
Litmus milk at 21°C.[8]		F	F	S
NH_3 produced in 4% peptone.		+	-	ND
Utilization of citrate.		-	-	+
Hydrolysis of:	(arginine.	+	-	d
	(hippurate.	d	-	ND
Production of diacetyl and CO_2 in single-strain milk culture (Sandine, *et al*, 1962).		low	low	high

Table III continued

Physiological reactions.		*Str. lactis.*	*Str.cremoris.*	*Str.diacetilactis.*
Fermentation of:[9]	(dextrin.	A	-	A, +, -
	(glycerol.	-	-	+, -
	(inulin.	+, -	-	+, -
	(maltose.	A	-	A
	(mannitol.	-, A	-	A, -
	(rhamnose.	+, -	-	+, -
	(sucrose.	-, A	-	-
	(salicin.	A	-	A
	(trehalose.	A, -	-	ND
	(xylose.	+, -	-	+, -
Aroma production in single-strain culture.		-	-	+

[6] d = some strains positive, some negative.
[7] ND = no or insufficient data.
[8] F = reduction in 8 hours, acid coagulation in 24 hours.
S = reduction in 8 hours, acid coagulation in 24 hours for some strains and 48 hours for others.
[9] A = growth with acid production for most (80-100%) strains.
+ = growth without acid or gas production for most (80-100%) strains.
- = no growth for most (80-100%) strains.
-,A = some strains do not grown, some grow with acid production.
etc.

Particularly puzzling were instances in which a starter, having functioned satisfactorily for some time, suddenly underwent a dramatic loss in activity (termed starter failure). The frequent association between aeration of milk media with starter failures led to the suggestion that cellular metabolic systems were inhibited by atmospheric oxygen (Whitehead & Wards, 1933; Whitehead & Cox, 1934). Though Hadley and Dabney had reported the existence of phages active against lactic streptococci employed as starters as early as 1926, it was not until about a decade later in 1935 that Whitehead and Cox managed to relate the occasional presence of virulent phages in starter cultures to starter failures.

Especially in large Cheddar cheese factories, the problem of starter failures is of serious economic concern (Hull, 1977b; Lawrence, 1978; Lawrence, *et al*, 1978). The immediate remedy taken to overcome the problem was to prevent phage contamination in the fermentation mixtures, a task which later proved to be impractical (Whitehead, 1953). The alternative, from the practical point of view, was to screen for phage-resistant starter strains. Though some success has been attained by rotational use of several phage-resistant starter strains, one must admit that the relief gained is only temporary. The real issue lies in the origin of these virulent phages in starter mixtures.

Hunter first reported in 1947 the occurrence of phage-carrier states of some strains of *Str. cremoris*. These pseudo-lysogenic strains were often partially immune to attacks by other phage types, and they could be'cured' of the 'symbiotic' phage through repeated single-colony isolation (Hunter, 1947; Barksdale & Arden, 1974). In 1949, Reiter reported the possible existence of lysogeny in the lactic streptococci. Subsequent work revealed that a high proportion of lactic strains appear to be lysogenic for a variety of phages (Lawrence, *et al*, 1976; Reiter, *et al*, 1976). Besides providing the most probable explanation for the origin of phages in starter mixtures, such common occurrence of lysogeny further undermines the rationale for the use of phage-resistant starter strains (Williamson & Bertaud, 1951; McKay, *et al*, 1973; Koroleva, *et al*, 1978; Terzaghi & Terzaghi, 1978).

Genetic studies of lysogeny in starter strains would appear to be most promising in the long run for providing a solution to the problem of phage-related starter failures. In 1973, McKay and Baldwin demonstrated the presence of phage particles in UVL-induced lysates of *Str. lactis* C2 under the electron-microscope, but they were unable to demonstrate plaque formation by the phages. In 1975, Park and McKay showed that 9 out of 12 American commercial starters (comprised of *lactis* and *cremoris* strains) tested were either UVL or mitomycin C inducible. Although presence of phage particles in the lysates of two *Str. cremoris* strains were observed under electron-microscope, their attempts to identify indicator strains were unsuccessful. Of the 87 lactic streptococcal strains screened by Kozak and co-workers in Poland for lysogens in 1973, only 5 of 46 *Str. lactis* strains, 2 of 24 *Str. diacetilactis* strains and none of 17 *Str. cremoris* strains were found to be lysogenic as inferred from plaque forming ability of their lysates. The general lack or absence of suitable and reliable indicators possibly accounts for such a low frequency of lysogeny found by Kozak's group above, especially for *Str. cremoris* strains (Heap & Limsowtin, 1978). Even though demonstrable lysis from induction did not always occur, electron-microscopic examinations of the lysates have been reported to reveal either intact phage particles or phage fragments (Huggins & Sandine, 1977).

Perhaps one of the best demonstrations of lysogeny in *Str. cremoris* was given by Lowrie in 1974. From UVL and mitomycin C inductions of the cheese starter, *Str. cremoris* strain R_1, Lowrie obtained lysates which plated on three closely related *Str. cremoris* strains, namely US_3, AM_1 and its derivative SK_{11}. Being the best indicator (that is giving the highest plaque counts), AM_1 was hence used as the indicator for R_1-induced phages. It should be noted that the so-called AM_1 here was in fact a derivative of the original AM_1, and was accordingly given a new strain designation 368 (Terzaghi & Terzaghi, 1978). However, a point of importance in Lowrie's work was the apparent low phage yield of 1 plaque forming unit (pfu) on 368 per 10 induced cells. Besides a reflection on the inadequacy of the indicator (the efficiency of plating of 368 was also very dependent on the physiological states of the culture), the low yield observed could also possibly be attributed to the

adsorption of induced phages to cell debris, or more importantly, to the concurrent induction of other phages which are defective or noninfective on 368. In other words, one must think of the possibility of R_1 lysates being a mixture of phage types, or that R_1 is a poly-lysogen.

In 1976, Georghiou succeeded in isolating a cured derivative (that is cured of its resident prophage(s)) of strain R_1 by locating UVL - noninducible clones among the 10^{-5} survivors of UVL - irradiated log phase R_1 cells. This strain, designated R_1C, was found to be a good indicator (as expected) for the phages in induced R_1 lysates, yielding plaque counts of a factor of 10^2 - 10^4 higher than that observed on indicator 368. During the course of her work, Georghiou obtained evidence which suggested a probable mixture of two phage types in R_1 lysates, an idea first expressed by Lowrie two years before. Her electron-microscopic examinations of an UVL-induced R_1 lysate revealed phage particles which were interpreted to consist of 2 morphological types, collared and non-collared. In a similarly induced R_1 lysate run to equilibrium on a CsCl density gradient (at 24,000 rpm for 36 hours), the gradient fractions when titrated on indicator strains R_1C and 368 gave 2 peaks of p.f.u. at buoyant densities of, respectively, 1.50 g/ml and 1.47 g/ml. Phage stocks prepared from the 2 peaks, on further CsCl runs, yielded again 2 peaks of p.f.u. on the two hosts at similar relative buoyant densities. Neutralization tests of the 2 apparent phage types with the same heterologous antisera gave different responses. In addition, they were inferred to confer different specific immunity in their respective prophage states as judged from spot-test experiments. In summary, it was concluded that the R_1 lysates contained phages of 2 types: a collared phage of buoyant density 1.50 g/ml and a collarless form of buoyant density 1.47 g/ml, which also differed serologically as well as in immune specificity. In other words, R_1 was believed to be a double lysogen.

Although follow-up work by Terzaghi and Cleaver (personal communication) did not reproduce all the above results, their observations did point in the same direction. That is, the CsCl run (at 30,000 rpm for 48 hours) of their UVL-induced R_1 lysate gave a double-peaked OD_{254}

profile. Unfortunately the 2 OD peaks detected were so close that they were collected within a single fraction. Subsequent plating profiles on indicators R_1C and 368 gave a single peak of p.f.u. at buoyant density of 1.48 g/ml.

Hence the question remains: Is R_1 a double lysogen? How related, or different, are the 2 induced phage types if the above is true? Before the R_1-R_1C system can serve as a useful case study of lysogeny in starter strains, the above queries need to be resolved. My current work is an attempt to answer these questions.

Briefly, log phase R_1 cells were collected, pre-washed with homologous antiphage serum and UVL induced to lyse. The phages from the resulting lysates were characterized by the following methods:

1) CsCl density gradient;
2) Serological tests;
3) SDS-gel electrophoresis, and
4) Host-specificity tests.

The results obtained conclusively established the presence of only one phage type in the R_1 lysates, and that *Str. cremoris* strain R_1 is a single lysogen.

SECTION TWO

MATERIALS

1. Bacteria.

All the lactic streptococcal bacteria used in this work are listed in Tables VIII and IX, and are currently maintained in this laboratory. They were initially from the Culture Collection Unit of the New Zealand Dairy Research Institute, Palmerston North.

The main strains of concern are *Str. cremoris* strains R_1, R_1C, R_1r, 368 and 368(r_1) (refer to Figure 33; see Table IV). Strain R_1, introduced into this country in 1917, is presently a commercial cheese starter. Its lysogenic nature was suggested by Lowrie in 1974. R_1C is a derivative strain of R_1 which is cured of its parental prophage, isolated by Georghiou in 1976. R_1r is R_1C re-lysogenized by $\phi r_1/R_1C$. 368 (which was the indicator strain AM_1 in Lowrie's 1974 paper) is a slow milk-coagulating derivative strain of the original AM_1, a commercial cheese starter (Terzaghi & Sandine, 1975; Terzaghi & Terzaghi, 1978). 368(r_1) is strain 368 lysogenized by $\phi r_1/368$.

R_1C and 368 are the indicator strains used here for all induced phages.

Escherichia coli strain AB3013 is the indicator for $\phi\lambda$ included as an internal density marker in all CsCl runs.

2. Bacteriophages.

Phages under study here are all originally from UVL-induction of log-phase R_1 cells (refer to Figure 33).

In general, the phage induced from R_1, designated ϕr_1, form turbid plaques on indicator R_1C and small clear plaques on indicator 368. When propagated on either indicators R_1C or 368, ϕr_1 will be designated $\phi r_1/R_1C$ and $\phi r_1/368$ respectively. ϕr_1-UV/R_1C and ϕr_1-NA/R_1C are clear-

plaqued mutants of the temperate $\phi r_1/R_1C$ obtained from, respectively, UVL and nitrous acid mutagenesis of the latter. The ϕr_1-UV1/R_1C, used in the preparation of antiphage serum A/S r_1-UV1/R_1C, is one of the UVL-induced clear plaque-forming mutant.

3. Media.

3.1. M17 media.

These are improved growth media for lactic streptococci and their bacteriophages, developed by Terzaghi and Sandine in 1975.

3.1a. M17 broth.

	0.5% lactose-M17 broth.	"lac$^-$"-M17 broth.[a]
Distilled water	900 ml	900 ml
Polypeptone(-Peptone)	5 g	5 g
Phytone(-Peptone)	5 g	5 g
Yeast extract	2.5 g	2.5 g
Ascorbic acid ($C_6H_8O_6$)	0.5 g	0.5 g
β-disodium glycerophosphate[b]	19 g	19 g
Beef extract	5 g	5 g

↓

Dissolve.

↓

Add sterilized

1M $MgSO_4(.7H_2O)$ solution[c]	1 ml	1 ml
20% (w/w) lactose solution[d]	25 ml	none

↓

Top up to 1 litre with distilled water.

Mix well and distribute into 100 ml lots, and sterilize for 15 min at 15 lb pressure (in pressure cooker).

a 1% lactose-M17 broth = 0.5 ml 20% (w/w) lactose solution + 9.5 ml "lac$^-$"-M17 broth (*i.e.* M17 broth containing no lactose).

b It is used for buffering the growth media.

c All chemical solutions are made with deionized water. If required sterile, they are sterilized for 10 min at 10 lb pressure.

d 10 g lactose dissolved in 40 ml distilled water, and sterilize for 20 min at 10 lb pressure.

3.1b. M17 top-agar and M17 paltes.

	M17 top-agar.	M17 plates.
Distilled water	900 ml	950 ml
Agar (Davis)	4.5 g	10 g
Polypeptone	5 g	5 g
Phytone	5 g	5 g
Yeast extract	2.5 g	2.5 g
Ascorbic acid	0.5 g	0.5 g
β-disodium glycerophosphate	19 g	19 g
Beef extract	5 g	5 g
	↓	↓
	Ensure all are dissolved and well-mixed, especially the agar.	Autoclave (15 min at 15 lb pressure, at 121°C), mix well and cool to 47°C.
	↓	↓
Add sterilized		
1M $MgSO_4(.7H_2O)$ solution	1 ml	1 ml
1M $CaCl_2(.6H_2O)$ solution	none	10 ml
20% (w/w) lactose solution	25 ml	25 ml
	↓	↓

Top up to 1 litre with distilled water.
Mix well and distribute into 100 ml lots, and sterilize for 15 min at 15 lb pressure.

↓

Pressure cook for 5 min at 15 lb pressure to melt, and maintain at 47°C for use.

Mix and pour plates (at about 20 ml per plate).
Stand plates for 24 hours at room temperature, to set and dry out the excess moisture, before use.
Store in air-tight container in cold room (5°C) if necessary.

3.2. BHI (Brain Heart Infusion) media.

These are standard growth media for *Escherichia coli* strains and their bacteriophages.

	3.7% BHI broth.[e]	3.7% BHI top-agar.	3.7% BHI plates.
Distilled water	1 litre	1 litre	1 litre
BHI broth	37 g	37 g	37 g
Agar	none	4.5 g	12 g

↓ ↓ ↓

Dissolve and mix well, and distribute into 100 ml lots.
Sterilize for 15 min at 15 lb pressure.

Ensure all are dissolved and well-mixed, particularly the agar.

↓

Distribute into 100 ml lots, and sterilize.

↓

Pressure cook for 5 min at 15 lb pressure to melt, and maintain at 47°C for use.

Autoclave (15 min at 15 lb pressure, at 121°C).

↓

Mix and pour plates.
Stand plates for 24 hours at room temperature, to allow setting and drying out excess moisture, before use.
Store in air-tight container in cold room if necessary.

e Unless stated w/w or v/v, otherwise % calculations are based on w/v.

4. Other solutions.

4.1. Standard saline citrate (SSC).

Deionized water	1 litre
NaCl	8.78 g (0.15 M required)
Tri-sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	4.41 g (0.015 M required)

↓

Distribute into 100 ml lots, and
sterilize for 10 min at 10 lb pressure.
It is supplemented with a final 0.001 M $MgSO_4$ before use.

4.2. 1% Peptone.

1 litre distilled water +
10 g Bacto-peptone.

↓

Dissolve (the solution should be clear), and
distribute into 300 ml lots.
Sterilize for 20 min at 10 lb pressure.

↓

Keep in cold room.
Used as chilled 1% peptone for blending cells.

4.3. Phosphate-buffered saline, pH 7.
Used for UVL irradiation of bacteria.

Deionized water	1 litre
$Na_2HPO_4.12H_2O$	17.91 g (0.05 M required)
KH_2PO_4	3.40 g (0.025 M required)
NaCl	8 g (0.8% required).

4.4. Irradiation buffer.

Used for UVL irradiation of phages.

Phosphate buffer *f*	1 litre
1% gelatin solution	0.25 ml
0.5 M $MgSO_4(.7H_2O)$ solution	0.08 ml
0.1 M $CaCl_2(.6H_2O)$ solution	0.5 ml

f Phosphate buffer = 1 litre deionized water +

0.6 g $Na_2HPO_4.12H_2O$ (0.0017 M required) +
0.3 g KH_2PO_4 (0.0022M required).

4.5. Stock solutions for nitrous acid mutagenesis of phages.

4.5a. Acetate buffer, pH 4.65.

Deionized water	900 ml
1 M acetic acid *g*	51 ml
1 M sodium acetate ($CH_3COONa.3H_2O$)	49 ml

g 1 M acetic acid = 12 g glacial acetic acid (17.6 M) dissolved in 200 ml deionized water.

4.5b. 2 M sodium nitrite solution.

13.8 g $NaNO_2$ dissolved in
100 ml deionized water.

4.5c. Phosphate buffer, pH 7.2.

Deionized water	1 litre
$Na_2HPO_4.12H_2O$	7 g (0.02 M required)
KH_2PO_4	3 g (0.022 M required)
NaCl	4 g (0.07 M required)

↓

Keep in cold room, and
used as chilled phosphate buffer.

4.6. Tris buffer (0.05 M Tris pH 7.2).

50 ml of 0.2 M Tris (hydroxymethyl) aminomethane +
44.2 ml of 0.2 M HCl, and
diluted with deionized water to a final volume of 200 ml.

↓

For 0.05M Tris pH 7.2, add

NaCl	2.4 g (0.2 M required)
1 M sodium azide ($NaNO_3$)	0.2 ml (0.001 M required)

↓

This is the buffer used in making CsCl solutions for density gradient equilibrium runs.

4.7. Stock solutions for SDS-gel electrophoresis.
Solutions are best stored in cold room, except for SDS sample buffer, BPB tracking dye, Isopropoanol stain and Soaking solution which are kept at room temperature.

4.7a. Lower Tris buffer (1.5 M Tris.HCl, pH 8.8, 0.4% SDS).

Deionized water	70 ml
Trizma base (<u>i.e.</u> recrystallized Tris (hydroxymethyl) aminomethane)	18.17 g

↓

Add 12 M HCl to lower pH of solution to 8.8.
Add 4 ml 10% (w/w) SDS (sodium dodecyl sulfate).
Top up to 100 ml with deionized water.

↓

pH is checked and adjusted if necessary.
(This buffer is 4x its final concentration in the gel).

4.7b. Upper Tris buffer (0.5 M Tris.HCl, pH 6.8, 0.4% SDS).

Deionized water	70 ml
Trizma base	6.06 g

↓

Lower pH of solution to 6.8 with 12 M HCl.
Add 4 ml 10% (w/w) SDS.
Top up to 100 ml with deionized water.

↓

pH is checked and adjusted if necessary.
(This buffer is 4x its final concentration in the gel).

4.7c. 20% acrylamide + 0.8% methylene-bis-acrylamide.

Deionized water	70 ml
Acrylamide	20 g
N,N'-Methylene-bis-Acrylamide	0.8 g

↓

Dissolve and mix for 30 min or
until the solution returns to room temperature.
Top up to 100 ml with deionized water.

↓

The solution is filtered through Whatman #1 filter paper before use.

4.7d. 30% acrylamide + 1.6% methylene-bis-acrylamide.

Deionized water	70 ml
Acrylamide	30 g
N,N'-Methylene-bis-Acrylamide	1.6 g

↓

Dissolve and mix for 30 min or
until the solution returns to room temperature.
Top up to 100 ml with deionized water.

↓

The solution is filtered through Whatman #1 filter paper before use.

4.7e. Ammonium persulfate.

0.2 g ammonium persulfate dissolved in
2 ml deionized water.
This solution is made just before use.

4.7f. SDS sample buffer.

Upper Tris buffer	25 ml
2-mercaptoethanol ($HS.CH_2.CH_2OH$)	10 ml
SDS	6 g

↓

Top up to 100 ml with deionized water.
(This buffer is 4x concentrated, and is diluted as such with sample or water before boiling and loading on gels).

4.7g. BPB tracking dye.

0.05 g bromphenol blue (BPB) dissolved in
40 ml glycerol.

↓

Top up to 50 ml with deionized water.
(This tracking dye is diluted 10x with sample or water before boiling and loading on gels.
The glycerol increases the density of the samples and helps to hold the latter down in the wells.)

4.7h. Tris-glycine reservoir buffer, pH 8.3.

Deionized water	2 litres
Trizma base	6.07 g
Glycine	28.8 g
SDS	2 g

↓

Store in cold room.

4.7i. Isopropanol stain.

Deionized water	650 ml
Isopropanol	250 ml
Glacial acetic acid	100 ml
Coomassie brilliant blue R.	0.4 g

4.7j. Destaining solution (10% (v/v) acetic acid).

10 ml acetic acid +
90 ml deionized water.
Fresh solution is made each time it is needed.

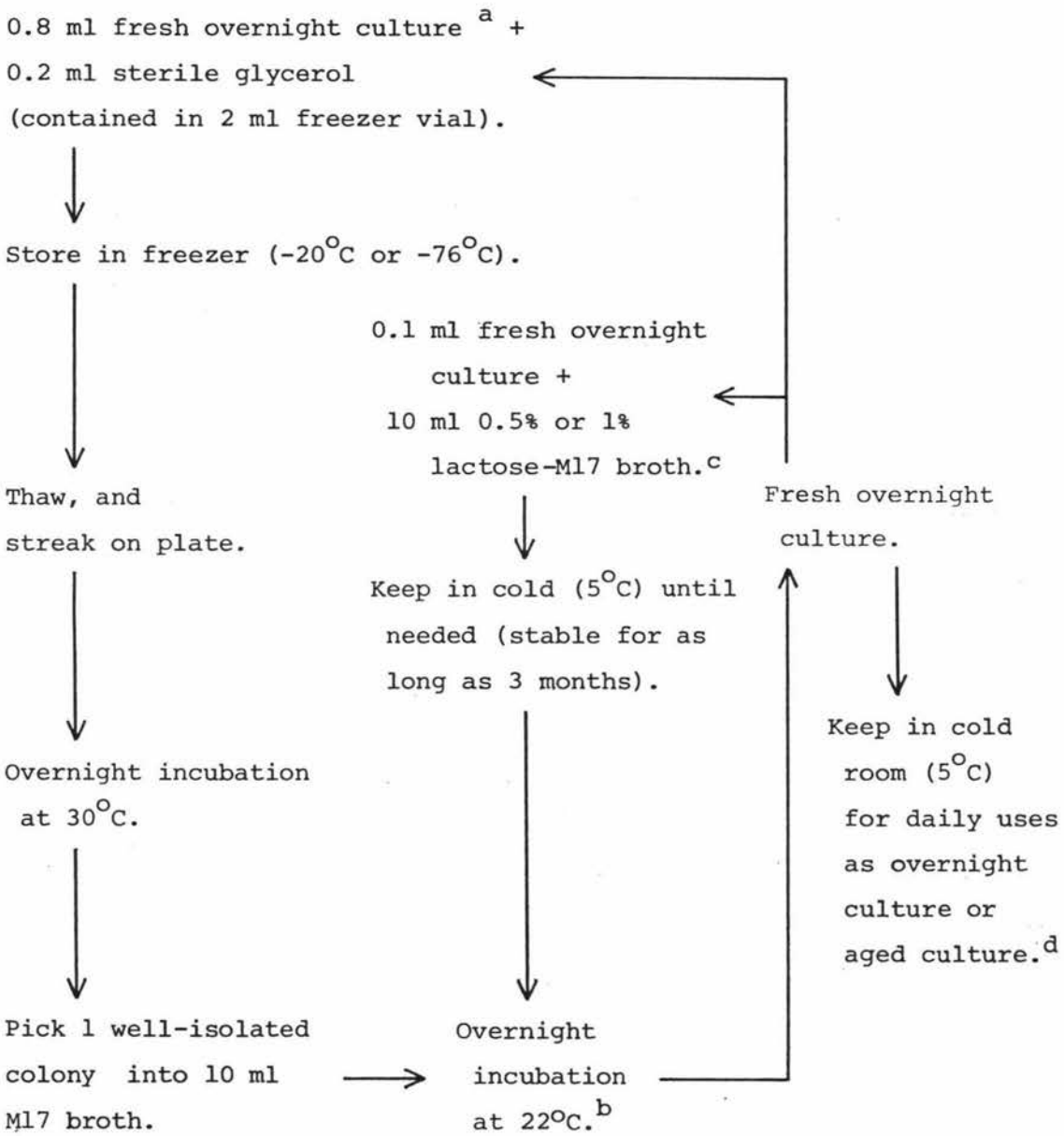
4.7k. Soaking solution (25% (v/v) methanol - 5% (v/v) glycerol).

Deionized water	700 ml
Methanol	250 ml
Glycerol	50 ml.

SECTION THREE

EXPERIMENTAL PROCEDURES

1. Maintenance of lactic streptococcal bacteria.



[a] Overnight = 16 hours.
Glycerol is not necessary when cultures are stored at -76°C, but is essential when storage is at -20°C (its use is to prevent large crystal formation inside the cells, hence reducing cell deaths).

b Some strains showing slow growth are incubated for 40 hours instead. The 22°C incubation temperature is aimed to prevent over saturation (physiological) of the cells.

c This 1% inoculum is always made first before the fresh overnight culture is used for other purposes.

d Overnight culture refers to cultures grown in 0.5% lactose-M17 broth. These cultures are discarded after a week.
Aged culture refers specifically to 1% lactose-M17 broth culture, which may be used for up to 1½ weeks as the so-called '11-day aged culture' (Terzaghi & Terzaghi, 1978).

2. Assay, isolation and propagation of lactic streptococcal phages. (Adams, 1959).

Phage assay.

0.05 ml 1 M $CaCl_2(.6H_20)$ solution [e] +
0.1 ml overnight/aged indicator/host bacterial culture +
0.1 ml phage sample [f] +
2.5 ml M17 top-agar (added after 5 min allowance for phage adsorption), and
plate.[g]

↓

Incubate overnight at 22°C.

↓

Plaque count: units of phage titre are expressed as pfu/ml (*i.e.* plaque forming units per ml).

↓

Isolation of a phage.

The plaque is stabbed with the wire,[h] and is diluted, plated and incubated (as above).

1 ↓ 2 ↓ 3 |

10-30 plaques/plate is considered best in this purification process.

3 ↓

Plaques in this plate should be homogeneous (as evidence of single phage type isolated).

↓

Phage stock making.

10 ml M17 broth +
1 well-isolated plaque +
0.1 ml host bacterial culture +
0.1 ml 1 M $CaCl_2$ solution.

↓

Incubate for 7 hours at $22^{o}C$.

↓

Bench centrifugation to sediment cells and debris.[i]

↓

Assay phage stock (the supernatant).
(Stock titre obtained is usually 10^{8}-10^{9} pfu/ml.)

↓

Keep at $5^{o}C$ (stocks remain stable in this state for as long as 7 months at least. Stocks can be kept in freezer for long-term storage.) (Whitehead, 1958; Keogh & Pettingill, 1966).

e Ca^{++} is needed for adsorption and multiplication of lactic streptococcal phages (Shew, 1949; Lowrie & Pearce, 1971; Sozzi, *et al*, 1966).

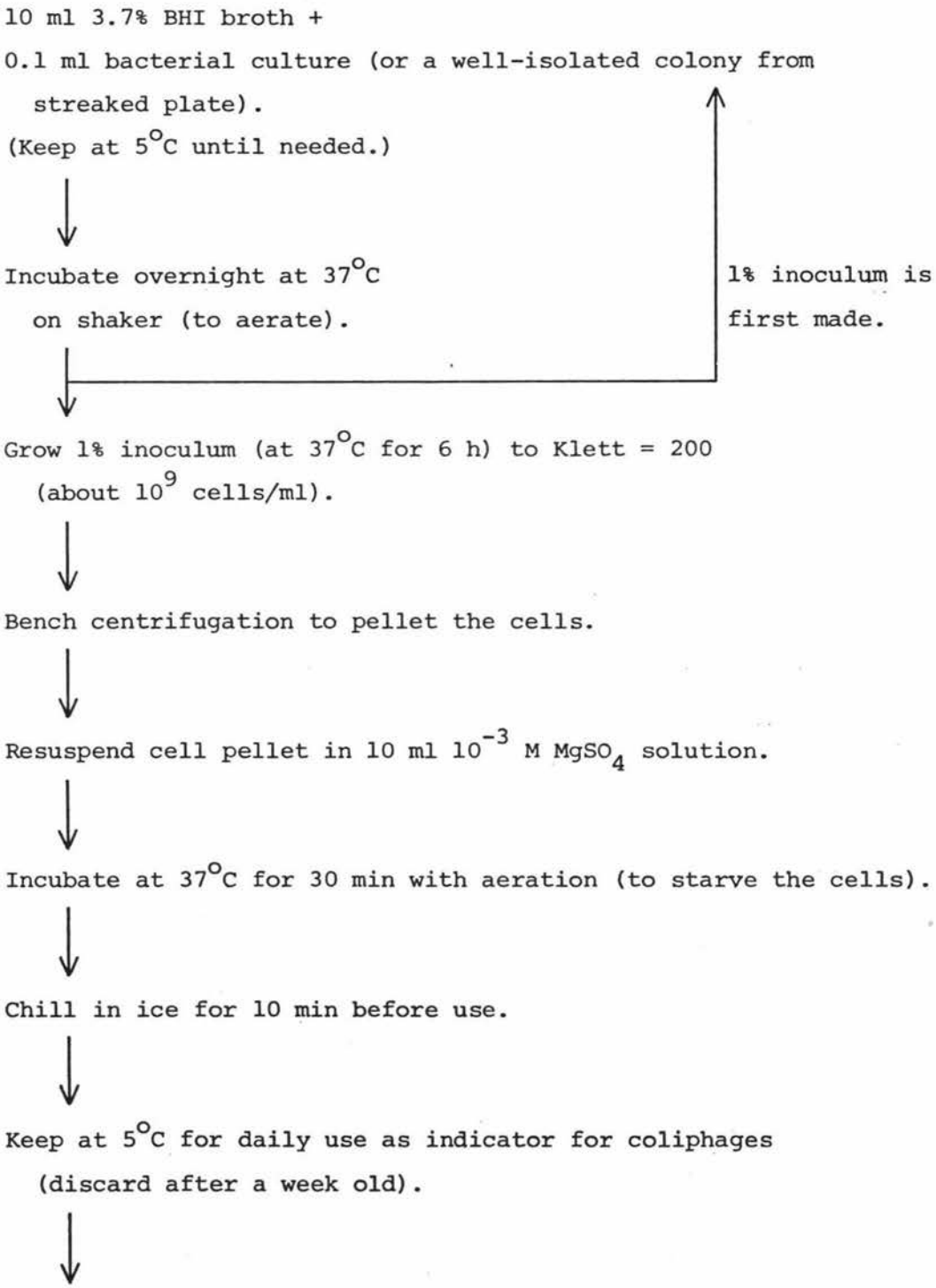
f For dilutions of lactic streptococci and their phages, 10% (v/v) M17 (i.e. 10 ml M17 broth + 90 ml sterile distilled water) is used as diluent.
0.05 ml sample into 5 ml diluent = 10^{-2} dilution;
0.1 ml sample into 0.9 ml diluent = 10^{-1} dilution.
Count of 100-300 plaques per plate is deemed ideal for assay.

g Plating tubes are maintained at $37^{o}C$ in Multi-blok heater.

h About 10^{6} pfu is picked when a plaque is stabbed with the wire.

i Sedimentation of particles in a volume of 10 ml or less is done on bench-top clinical type centrifuge, run at maximum speed (about 2,000 x *g*) for 15 min. This process is simply referred to as "bench centrifugation" in the text.

3. Preparation of indicator *Escherichia coli* strains for assay of coliphages.



Assay of øλ.

0.05 ml 1 M $MgSO_4(.7H_2O)$ solution +
0.1 ml prepared culture of *E. coli* AB3013 +
0.1 ml phage sample [j] +
2.5 ml BHI top-agar, and
plate.

↓

Incubate overnight at 37°C.

↓

Plaque count.

[j] For dilutions of *E. coli* and their coliphages, SSC (+ 10^{-3} M $MgSO_4$) is used as the diluent.

4. Measurement of growth kinetics in broth cultures of lactic streptococci.

Sample.[k]

9 ml M17 broth +
0.09 ml overnight culture
(contained in BL spectrometer tube).

Blank.[l]

9 ml M17 broth
in BL spectrometer
tube.

Growth kinetics of the broth culture at 30°C is followed through its OD_{600} profile.

[k] Sample is a 1% inoculum.

[l] The blank is used for calibration of the spectrometer (Spectronic 20 (Bausch & Lomb) spectrometer).

5. UVL irradiation of bacteria.

Preparation of cells.

Mid-log phase culture.
(Incubate 1% inoculum at 30°C for 5 or 6 hours).[m]

↓

Bench centrifugation to pellet the cells
(at maximum speed for 15 min).

↓

Blending of cells.
(Cells are resuspended in 100 ml chilled 1% peptone, and blended at low speed (using Waring blendor) for 7 min (with a 5 sec break after each minute) in the cold.)[n]

↓

Sorvall centrifugation to pellet the cells.[o]

↓

Resuspend cells in phosphate-buffered saline to a titre of about 10^7 diplococci/ml.

↓

UVL irradiation.[p]

Spread 7 ml cell sample into a glass petri-dish of 14 cm diameter (the sample depth is about 3 mm).

All manipulations in these steps are done under dim yellow light to prevent photoreactivation.

↓

UVL irradiate the sample (at 23°C) for various time lengths.

↓

Samples are removed at regular intervals, diluted and plated (using soft-agar overlay method).

↓

Incubate plates at 30°C
in the dark (to prevent photoreactivation)
for 40 h (to allow sufficient time for formation
of visible colonies).

[m] For strains R_1, R_1C and R_1r, the incubation time is about 5 h, and for 368 and 368(r_1) it is about 6 hours (the OD_{600} reading = 0.4-06, and cell count = 10^7-10^8 chains/ml (using a Petroff-Hausser counting chamber)). Average chain length is about 16 cells.

[n] Mainly 2-celled chains are observed in blended cultures. Viable cell loss through blending is between 30-50% (±10%).

[o] Sedimentation of particles in volume of 100 ml or more is done using a Sorvall RC2-B centrifuge, run at 9,000 rpm (about 13,000 x *g*) for 15 min at temperature 5-15°C. This process is simply referred to as "Sorvall centrifugation" in the text.

[p] The intensity of the UVL is one øT4 hit/3 sec.

6. UVL irradiation of phages.

Phage stock (in M17 medium and of titre 10^{8-9} pfu/ml).

↓

Dilute stock at least 10^{-2} into irradiation buffer[q] (to a titre of 10^7 pfu/ml or less).

↓

Spread 4 ml phage sample into a glass petri-dish of 9 cm diameter (giving sample depth of about 3 mm).

↓

UVL irradiate the sample (at 23°C) for various time lengths.

↓

Samples are removed at regular intervals, diluted and plated.

↓

Incubate plates overnight at 26°C in the dark.

↓

Work under dim yellow light to safe-guard against photo-reactivation.

Plaque count and/or

phage isolation

[q] The essential dilution of at least 10^{-2} is to reduce the protein concentration (hence reducing absorption of UVL) of the stock medium to enable deeper penetration by UVL.

7. Nitrous acid mutagenesis of phages.

Phage stock (in M17 medium).

↓

Dilute stock at least 10^{-3} into prewarmed (37°C) acetate buffer of pH 4.65.[r]

↓

Stand for 10 min at 37°C.

↓

Add 1 ml prewarmed (37°C) 2 M $NaNO_2$ solution to 9 ml phage sample (final concentration of 0.2 M Na nitrite is required).

↓

At regular intervals, samples are removed and immediately diluted (at least 10^{-1} to terminate the HNO_2 activity) into chilled phosphate buffer.

↓

Samples are further diluted (with 10% M17 diluent), and plated.

↓

Incubate plates overnight at 26°C.

↓

Plaque count and/or
phage isolation.

r The 10^{-3} (at least) dilution is to replace the M17 medium of the phage stock with acetate buffer of pH 4.65.

8. UVL induction of bacteria.

Culture.

Mid-log phase culture.

↓

Bench centrifugation to pellet the cells.

↓

Antiserum washing of cells (to remove free phages).

Resuspend cells in 10 ml diluted antiphage serum.
(for R_1, R_1C and R_1r cells, the antiserum used is A/S r_1-UV1/R_1C at k = 0.66/min, and for 368 and 368(r_1) cells, the antiserum is A/S r_1/368 at k = 0.26/min.)^s

Incubate for 10 min at 37°C.

↓

Bench centrifugation to pellet the cells.

1↓ | 2

Resuspend cells in 10 ml diluent (to remove the antiserum).

2↓

Bench centrifugation to pellet the cells.

↓

Blending of cells.

Cells are blended (in chilled 1% peptone solution) for 7 min.

↓

Sorvall centrifugation to pellet the cells.

↓

Resuspend cells in 8 ml 10^{-3} M $MgSO_4$ solution
(cell count of 10^8 diplococci/ml is usually observed).

↓

UVL irradiation.

Spread 4 ml cell sample into a glass petri-dish of 9 cm diameter (forming a 3 mm layer).

↓

UVL irradiate the sample (at 23°C) for 15 sec
(swirl after every 5 sec).

↓

Work under dim yellow light.

Transfer 3 ml irradiated sample +
6 ml M17 broth
into a spectrometer tube.

↓

Incubate sample and control[t]
at 30°C in the dark.

Lysis is followed by measuring the absorbance at 600 nm (on a Bausch & Lomb spectronic 20 spectrometer) at ½ h intervals.

↓

Bench centrifugation to remove cells and debris.

↓

Lysate (the supernatant).

[s] A/S r_1-UV l/R_1C is antiserum made against phage ør_1-UV l/R_1C, and A/S r_1/368 is antiserum against ør_1/368.

[t] The control is either 3 ml unirradiated sample + 6 ml M17 broth or 3 ml irradiated R_1C sample + 6 ml M17 broth.
Both the sample and the control are calibrated against a blank (which is 3 ml 10^{-3} M $MgSO_4$ solution + 6 ml M17 broth).

9. CsCl density gradient equilibrium run.[u]

Phage stock or lysate.[v]

↓

Bench centrifugation to sediment cells and debris (at maximum speed for 25 min).

↓

Ultracentrifugation of the stock/lysate to pellet the phages (using Beckman Model L5-50 Ultracentrifuge, rotor type SW 50.1 of 5 ml tube capacity): run at 30,000 rpm (about 108,000 x *g*) for 2 hours at temperature 5-20°C.

↓

Carefully discard the supernatant without disturbing the phage pellet.[w]

Immerse the phage pellet slowly and gently (to prevent breakage of the phages) in 0.2 ml CsCl solution (of density 1.48 g/ml and pH 7.2), and stand overnight in cold room to resuspend the phages.

↓

Shake up the phage suspension.

Add 0.1 ml ϕλ stock (of titre 10^7 pfu/ml) as internal density marker.

Then add in 4 ml CsCl solution (of density 1.48 g/ml, pH 7.2) slowly.

↓

Equilibrium ultracentrifugation (using rotor type SW 50.1): run at 30,000 rpm for 48 hours at temperature 5-20°C.

↓

Raise the running temperature to that of the room about 3 hours before the end of run, and ensure that the brake is off.

↓

Fractionation of the gradient (collecting 0.1 ml per fraction) is done with OD_{254} tracing.[x]

↓

Gradient check.[y]

↓

0.5 ml M17 broth is added to each fraction, and the fractions are kept in cold room for further analysis.

[u] This is simply referred to as CsCl run in the text.

[v] Titre of at least 10^8 pfu/ml is necessary to enable reading of the OD_{254} profile of the gradient.

[w] As M17 medium tends to interfere with OD_{254} reading, as much of the supernatant as possible is removed at this step.
Care is taken not to lose the phage pellet (which appears as an opaque mass) as it often slides along the wall (in contrast to T4 phage pellet which seldom does) when disturbed.

[x] ISCO density-gradient fractionator, recorder and optical units are used here.
Organic chase solution (Fluorinert) of density 1.93 g/ml is used.

[y] Refractive indices of the fractions collected are measured using an Atago Abbe refractometer, and their respective densities are extrapolated from a standard graph. The curve of Density against Fraction is then plotted to check the quality of the gradient formed.

10. Neutralization of phages by antiphage serum.[z]

0.4 ml 10% M17 diluent +
0.1 ml diluted antiserum.

↓

Stand at 37°C for 10 minutes.

↓

Add 0.5 ml phage sample.

↓

Samples are removed at regular intervals and immediately diluted (at least 10^{-2} to terminate antiserum activity), and assayed.

z The antiserum refers to either A/S r_1-UV1/R_1C used at a final k = 0.66/min or A/S r_1/368 at k = 0.26/min.
Dilutions of antiserum are done with 10% M17 diluent.
Preparations of the above antisera were by Terzaghi and Cleaver. Briefly, 500 ml of each phage stock were made. The phages were pelleted (centrifuged for 2 h at 30,000 rpm in Beckman 35 rotor). The resulting phage pellets were each resuspended overnight (in the cold room) in 0.3 ml M17 broth. The phage suspensions were then filtered through 0.22 µm millipore filters.
The phages were further purified on sucrose gradients (10-30% (w/w) in 0.05 M Tris pH 7.2, run for 100 min at 30,000 rpm in Beckman SW 41.Ti rotor), collected (about 10^{12} pfu per sample), concentrated and emulsified in an equal volume (0.6 ml) of Freund's Complete Adjuvent. 0.5 ml was injected intramuscularly into each hind leg of an immunologically fresh rabbit. The rabbits were re-inoculated at 4 weeks, but replacing Complete with Incomplete Adjuvent this time. About 40 ml blood were siphoned from the heart of each rabbit 2-week later(that is when the test samples collected at 10 days showed high antiserum activity against the homologous phages).
The fresh blood was left to stand at 37°C for 3 h, shaken to loosen the clots from the container (glass) walls and kept overnight in the cold. The antiserum was removed the following day, bench centrifuged (at maximum speed for 10 min) and distributed into 1 ml lots. Their neutralization constants (or k values) were determined, and they were stored at -5°C.

11. SDS-gel electrophoresis of phage proteins.

Preparation of phage stock

100 ml M17 broth +
10^{5-6} pfu of the phage sample +
1 ml overnight host culture +
1 ml 1 M $CaCl_2$ solution.

↓

Overnight incubation at 22°C.

↓

Sorvall centrifugation to sediment cells and debris.

↓

Assay the stock.[aa]

(10 ml sample is reserved for other analyses).

↓

Phage purification (See page 39)

Preparation of phage lysate

Mid-log phase culture.

(12 10 ml-cultures are done individually).

↓

Bench centrifugation to pellet the cells.

↓

Resuspend cells in 3.5 ml 10^{-3} M $MgSO_4$ solution.

↓

Transfer sample into glass petri-dish (of 9 cm diameter).

↓

Work under dim yellow light.

UVL irradiate the sample for 20 sec (swirl after every 5 sec).

↓

Transfer sample into 6 ml M17 broth.

↓

Incubate overnight at 30°C in the dark.

↓

Pool all 12 samples and Sorvall centrifuge to remove cells and debris.

↓

Assay the lysate.

(10 ml sample is reserved for other analyses).

↓

Phage purification

Phage stock or lysate (from above).

↓

Sorvall centrifugation to remove cells and debris.

↓

Ultracentrifuge the stock/lysate to pellet the phages [ab]
(run at 30,000 rpm (about 100,000 x *g*)
for 5 hours at temperature 5-20°C).

↓

Retain the phage pellet.[ac]
Resuspend the phages in 2 ml 0.05 M Tris pH 7.2
(+ 0.2 M NaCl).[ad]

↓

CsCl step-gradient ultracentrifugation [ae]
(run at 40,000 rpm for 3 hours at temperature 17-25°C).

↓

Fractionation of step-gradient with OD_{254} tracing.

↓

Phage band (or fractions) at the interphase of densities 1.4-1.5 g/ml are pooled (about 1 ml total) as the purified phage sample.[af]

↓

Dialysis [ag]

Dialyse the phage sample against 1 litre distilled water in cold for 24 hours (with constant stirring).

↓

Preparation of phage proteins [ah]

Dialysed phage sample +
0.01 ml DNase [ai]
(contained in plating tube).

↓

Freeze-thaw 9 times (to disrupt the phage particles) to enable DNA digestion. [aj]

↓

Stand at 37°C for 10 min.

↓

Lyophilize the sample in freeze-drier.

↓

Dissolve the protein residue in 0.03 ml SDS-BPB mixture.
(SDS-BPB mixture = 0.08 ml distilled water +
0.03 ml SDS sample buffer +
0.012 ml BPB tracking dye).

↓

Heat the sample in boiling water for 5 min, and allow to cool.

↓

The sample (*i.e.* phage proteins) is now ready for gel run.
(It is kept at -70°C; it is re-heated for 3 min before use.)

↓

SDS-gel electrophoresis (see page 42)

Preparation of gel

Plate assembly.

The glass plates (*i.e.* the back plate and the notched front plate) and spacers (of 1.5 mm thickness) [ak] are assembled and sealed (using white petroleum jelly), and clamped onto the gel-stand.

↓

Pouring running gel.

10% gel is found to be the best; the size of the gel is 11 x 11 x 0.15 cm.

Stock Solutions.	10% running gel (in ml).	Stacking gel (in ml).
Lower Tris buffer	5	-
Upper Tris buffer	-	1.25
20%-0.8% acrylamide	10	-
30%-1.6% acrylamide	-	0.75
Distilled water	5	3
Fresh ammonium persulphate	0.05	0.015
TEMED al	0.01	0.005

Each gel run requires about 20 ml running gel and 5 ml stacking gel.

The stock solutions (at room temperature) are added and mixed in the above order, with ammonium persulphate and TEMED added just before pouring (the latter are catalysts which promote polymerization of the acrylamide (which is carcinogenic)).

Pour in the gel mixture up to 2 cm from the top, and carefully overlay with distilled water (to level and to permit polymerization of the gel at the interphase). It is then left to stand for ½ hour.

Discard the distilled water and replace with 4x diluted lower Tris buffer (to prevent drying), and stand overnight in the cold before use.

↓

Pouring stacking gel.

Allow sufficient time for the running gel (stored in the cold) to equilibrate with the room temperature.

Stock solutions (at room temperature) are mixed, and 0.5 ml gel mixture is used to rinse the top of the running gel (after pouring off the buffer) and discarded, and the rest of the gel mixture is then poured in.

Immediately work in the comb to 1 cm depth, making sure that no air bubbles are trapped anywhere. Allow ½ hour for gel to polymerize.

Remove the comb carefully, and rinse each well with distilled water.

(Stacking gel is poured just before loading on the samples).

↓

SDS-gel electrophoresis

The gel assembly is dismounted from the gel-stand, the bottom spacer is carefully removed (without disturbing the gel and the side spacers), and is then mounted onto the (greased) electrophoresis apparatus.

Fill the bottom and top reservoirs with Tris-glycine reservoir buffer. Using a syringe fitted with a bent needle, rinse each well of the stacking gel with the reservoir buffer and remove any air bubble from the bottom edge of the running gel.

Label the wells and load the samples with micro-syringe.. The sample size used ranges from 0.005-0.035 ml.

↓

The electrophoresis apparatus is then connected to the power supply (the bottom reservoir is positive and the top reservoir negative).

The gel is run at constant current and minimum voltage. It starts with a current of 12 mA for about 1½ hours or until the BPB tracking dye moves into the running gel (whichever comes first), before being increased to 20 mA for the rest of the run.

(When 2 gels are run simultaneously on the same power supply, the starting current would be 24 mA followed by 40 mA). am

The run is terminated when the tracking dye is about 1 cm from the bottom of the gel.
(Actual running time averages about 4 hours).

↓

Staining the gel

The gel assembly is removed from the electrophoresis apparatus. Lying on the back plate the right spacer is carefully removed (without disturbing the gel). The glass plates are then pried apart steadily from the right bottom corner. The plate carrying the gel is then held, gel side down, over a plastic container holding about 200 ml isopropanol stain, and the gel is gently peeled off the plate into the stain.

The gel is soaked in the isopropanol stain for about 7 hours (on a slow shaker).

↓

Destaining the gel

Drain off the staining solution and rinse the gel with distilled water. Destain the gel in 300 ml 10% (v/v) acetic acid overnight on shaker. Replace with fresh destaining solution the next day and soak again overnight (the protein bands should be distinctly visible at this stage, otherwise further changes of destaining solution are necessary).

↓

Storage of wet gel

Gels can be stored in the wet state in cold
either in 10% (v/v) acetic acid
or in 25% methanol - 5% glycerol soaking solution
(the methanol in the latter tends to shrink the gel).
Photographs of gels are usually taken at this stage.

↓

Gel drying

If gels were required dry, they are first soaked overnight in soaking solution (the methanol in the solution helps to prevent cracking of the gel during drying, and the glycerol helps in adhering the dried gel to the paper support).

Briefly, the drying procedure is as follows:-
The gel is supported on 3 pieces of chromatography paper (cut to the size of the gel) and is placed on the vacuum gel dryer. A piece of plastic film (cut to slightly bigger size than that of the gel) is placed over the gel, and the whole upper surface of the gel dryer is then covered with a rubber sheet which is sealed to the dryer when vacuum is applied. The dryer is then rested, on the gel side, on a Multi-blok heater (at about $70^{\circ}C$) and the gel is left to dry for 24 hours.

When dry, the gel (together with the paper support) is wrapped in plastic film and mounted on cardboard, and labelled.

aa Titre of stock or lysate should be at least 10^8 pfu/ml to be considered worth proceeding with the experiment.

ab Using Beckman L5-75 ultracentrifuge, rotor type 35 with 6 x 96 ml tube capacity.

ac The process of decanting off the supernatant and retaining the phage pellet can be difficult as the latter tends to slide.

ad Slowly and gently immerse the phage pellet in the Tris buffer, and stand overnight in the cold to resuspend the phages. Shake up before use. (Titre of this phage concentrate is usually 10-fold that of its initial stock or lysate.)

ae This step proves to be very important in the purification process. Rotor type used is SW 41.Ti (of 6 x 12 ml tube capacity); 1 ml each of CsCl solutions of densities 1.7, 1.6, 1.5, 1.4 and 1.3 g/ml are carefully layered into a step-gradient, and finally top up with the phage sample. With CsCl density as high as 1.7 g/ml, the running temperature must not go below $17^{\circ}C$ as CsCl crystals may form!

af Titre of at least 10^9 pfu/ml is needed for running on gel electrophoresis.

ag Dialysis tubings are kept in 50% ethanol in the cold. Before use, appropriate lengths are cut, rinsed (with distilled water) and checked for leaks.

ah It is important that the final ion concentration in the phage protein sample is low, since high ion content tends to smear the track with proteins during gel electrophoresis.

ai DNase used is Deoxyribonuclease 1 Sigma (i.e. Deoxyribonucleate oligonucleotide-hydrolase) from beef pancreas. It is dissolved in SSC at concentration of 1 mg/ml, and stored in freezer.

aj Freezing is usually done in methanol bath (about -20°C) and thawing out in warm water (about 70°C). After the freeze-thaw process, the sample solution should appear relatively clear and nonviscous.

ak It is important that the glass plates are extremely clean; after each use, they are washed with lab-detergent, rinsed with hot water, distilled water, and left standing to drip-dry. They are acid-washed whenever necessary. The spacers and combs just go through ordinary washing.

al TEMED = N,N,N',N''-tetramethyl-ethylenediamine.

am 20 mA is about the maximum current permissible without warming up the gel considerably.

12. Spot tests for testing susceptibility of bacterial strains to phages.[an]

Plate seeding.

0.05 ml 1 M $CaCl_2$ solution +

0.1 ml overnight/aged bacterial culture (to be tested) +

2.5 ml top-agar, and

overlay on plate.

↓

Allow sufficient time for setting of top-agar.

↓

Spot testing.

Phage samples are spotted on the seeded plates (allow time for the spots to soak in).

↓

Incubate plates overnight at 22°C.

↓

Examinations of plates.

[an] This is usually referred to as spot tests in the text.

13. Experiments on curing of *Str. cremoris* strain 368.[ao]

Culture.

Mid-log phase culture or
aged (2-day) culture.

↓

Bench centrifugation to pellet the cells.

↓

Antiserum washing of cells.

Resuspend cells in 10 ml diluted A/S r_1/368 (at k=0.26/min) and incubate at 37°C for 10 min.

↓

Bench centrifugation to pellet the cells.

1 ↓ | 2

Resuspend cells in 10 ml diluent.

2 ↓

Bench centrifugation to pellet the cells.

↓

Blending of cells.

Resuspend cells in 100 ml chilled 1% peptone solution and blend for 7 min.

↓

Sorvall centrifugation to pellet the cells.

↓

Resuspend cells in phosphate-buffered saline to the required cell count of about 10^8 diplococci/ml. (Viable cell titre is also determined).

↓

UVL irradiation.

Spread 7 ml cell sample into a glass petri-dish (of 14 cm diameter).

↓

UVL irradiate the sample for 25-100 sec (shake after every 5 sec).

↓

0.1 ml irradiated sample is spread (using a sterilized glass rod) on M17 plate containing no Ca^{++}; 10 plates are done.

Work under dim yellow light.

↓

Incubate plates at $30^{\circ}C$ in the dark for 40 hours.

↓

Isolation of UVL-noninducible derivatives (see page 48)

and

Isolation of 368 lysate-sensitive derivatives (see page 49)

Isolation of UVL-noninducible derivatives

100 colonies are picked and transferred into 0.2 ml aliquots of M17 broth distributed in wells of depression trays.

↓

Incubate at 30°C for 3 hours (to produce log phase cells).

↓

A sample from each culture is streaked on a master plate.

↓

The cultures are then UVL irradiated for 20 sec (swirl after every 5 sec).

Add 0.5 ml fresh M17 broth to each culture.

(These manipulations are carried out under dim yellow light).

↓

Incubate the cultures at 30°C in the dark for 7 hours.

↓

Incubate the plates overnight at 30°C.

↓

Master plates (keep in cold).

↓

Screening of cultures: cultures which exhibit (comparatively) heavy growth are re-streaked from master plates.

↓

Incubate streaked plates overnight at 30°C.

↓

A well-isolated colony in each plate is picked and re-streaked.

↓

Incubate streaked plates overnight at 30°C.

↓

A well-isolated colony in each plate is picked and sub-cultured (*i.e.* the colony + 10 ml M17 broth and incubated for 40 h at 22°C).

↓

Test isolates for curing based on

(1) sensitivity to 368 lysate, and

(2) UVL inducibility.

Isolation of 368 lysate-sensitive derivatives

Overlay the original plates with UVL-induced 368 lysate.

(*i.e.* 0.05 ml 1 M $CaCl_2$ solution +
0.2 ml 368 lysate +
3 ml top-agar, and
overlay on the plate (swirl the soft-agar round several times before setting to smear the colonies).)

↓

Incubate plates overnight at 22°C.

↓

Examinations of colonies; colony showing any sign of suspect lysis is picked and streaked on fresh plate.

↓

Incubate streaked plates overnight at 30°C.

↓

A well-isolated colony is picked and re-streaked on fresh plate.

↓

Incubate streaked plates overnight at 30°C.

↓

A well-isolated colony is picked and sub-cultured.

↓

Test isolates for curing.

ao "Cure" in this context has an operational definition of "deprive a lysogenic cell of its resident phage"; hence an overnight culture of cured derivative is (UVL) noninducible and is sensitive to its parental phage lysate.

14. General procedure used in phage recombination experiments.

Preparation of R_1C cells.

Overnight R_1C culture.

↓

Bench centrifugation to sediment the cells.

↓

Resuspend cells in 100 ml chilled 1% peptone and blend for 7 min.

↓

Sorvall centrifugation to pellet the cells.

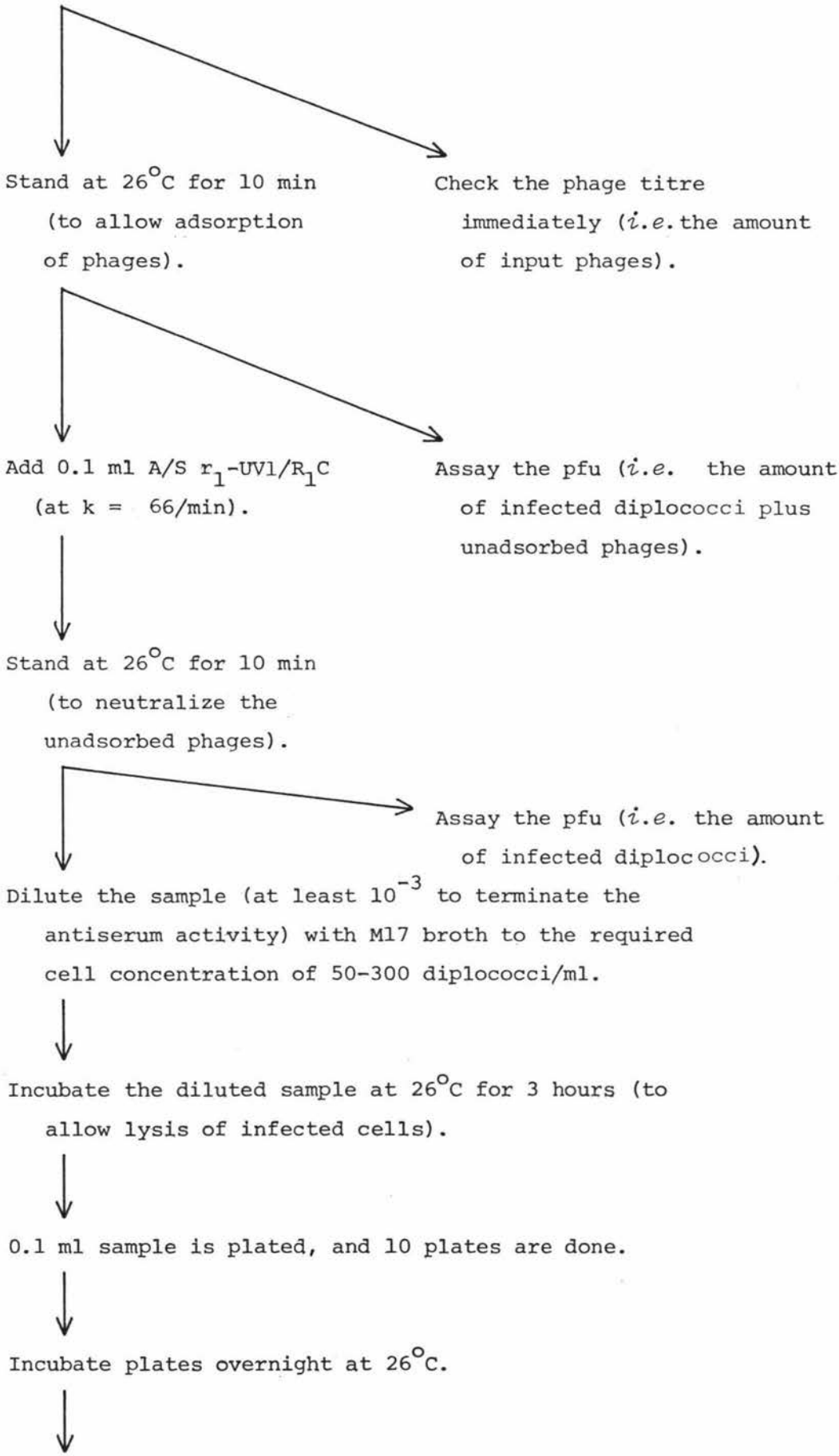
↓

Resuspend cells in 3 ml phosphate-buffered saline (Cell count = viable titre = 10^9 diplococci/ml are usually obtained).

↓

Phage recombination experiment.

0.35 ml ϕr_1-UV/R_1C sample (of 5×10^9 pfu/ml) +
0.35 ml ϕr_1-NA/R_1C sample (of 5×10^9 pfu/ml) +
0.05 ml 1 M $CaCl_2$ solution +
0.35 ml prepared R_1C cells (of 5×10^8 diplococci/ml).[ap]


Stand at 26°C for 10 min (to allow adsorption of phages).
Check the phage titre immediately (i.e. the amount of input phages).
Add 0.1 ml A/S r1-UV1/R1C (at k = 66/min).
Assay the pfu (i.e. the amount of infected diplococci plus unadsorbed phages).
Stand at 26°C for 10 min (to neutralize the unadsorbed phages).
Assay the pfu (i.e. the amount of infected diplococci).
Dilute the sample (at least 10-3 to terminate the antiserum activity) with M17 broth to the required cell concentration of 50-300 diplococci/ml.
Incubate the diluted sample at 26°C for 3 hours (to allow lysis of infected cells).
0.1 ml sample is plated, and 10 plates are done.
Incubate plates overnight at 26°C.

Screen plates for turbid plaques (*i.e.* wild type recombinant phages).

ap Required ratio of numbers of ϕr_1-UV/R_1C (in pfu) to ϕr_1-NA/R_1C (in pfu) to R_1C cells is 5 : 5: 1.

SECTION FOUR

RESULTS AND DISCUSSIONS

1. *Some characteristics of the lactic streptococcal bacteria.*

The main strains of concern in this work are *Str. cremoris* strains R_1, R_1C, R_1r, 368 and 368(r_1) (see Table IV & Figure 33). Morphologically they are indistinguishable from one another: their colonies on agar plate appear white, small and round, and when viewed under ordinary light-microscope they are seen as long chains of ovoid cells elongated in the direction of the chain.

Since log phase cells are essential for induction, instead of the usual incubation temperature of 22°C, optimum growth temperature of 30°C was used in induction experiments to shorten the incubation time. Figure 1 shows the difference in growth rates between the representative strains at 30°C, with strain R_1 being a faster grower than 368 and 368(r_1). One may infer from the identical growth rates of the latter strains that growth curves of R_1C and R_1r would approximate that of R_1. In order to maintain consistency, mid-log cultures were always used for UVL induction; for strains R_1, R_1C and R_1r, they are represented at OD_{600} = 0.55, and at OD_{600} = 0.5 for strains 368 and 368(r_1). In other words, incubation time of 5 and 6 hours at 30°C would give mid-log cultures for the former and latter strains respectively (see Figure 1).

It is seen that strain 368(r_1) exhibits an identical growth rate to that of its parental strain 368. Such a harmonious and stable phage-host association in an 'artificial' lysogen may reflect the ease with which lysogeny could arise in nature, which in turn probably explains such widespread occurrence of lysogens in these bacteria.

TABLE IV SOME DISTINGUISHING CHARACTERISTICS OF *STR. CREMORIS* STRAINS R_1, R_1C, R_1r, 368 AND 368(r_1)

Features	*Str. cremoris* strains:				
	R_1	R_1C	R_1r	368	368(r_1)
Origin of strain (see Figure 33).	a commercial cheese starter	cured derivative of R_1	relysogenized form of R_1C	a slow milk-coagulating derivative of AM_1	lysogenized form of 368
Growth rate of 1% broth culture at 30°C (see Figure 1).	fast	fast	fast	relatively slow	relatively slow
Response on UVL induction (see Figures 3 & 4).	lysis	no lysis	lysis	retarded culture growth	lysis
Phage tests: Plating efficiency of $\phi r_1/R_1C$.	nil	high	nil	low	nil
Phage tests: Plating efficiency of $\phi r_1/368$.	nil	low	nil	high	nil
Phage tests: Plaque morphology of both $\phi r_1/R_1C$ and $\phi r_1/368$.	no plaque formation	big turbid plaque	no plaque formation	small clear plaque	no plaque formation

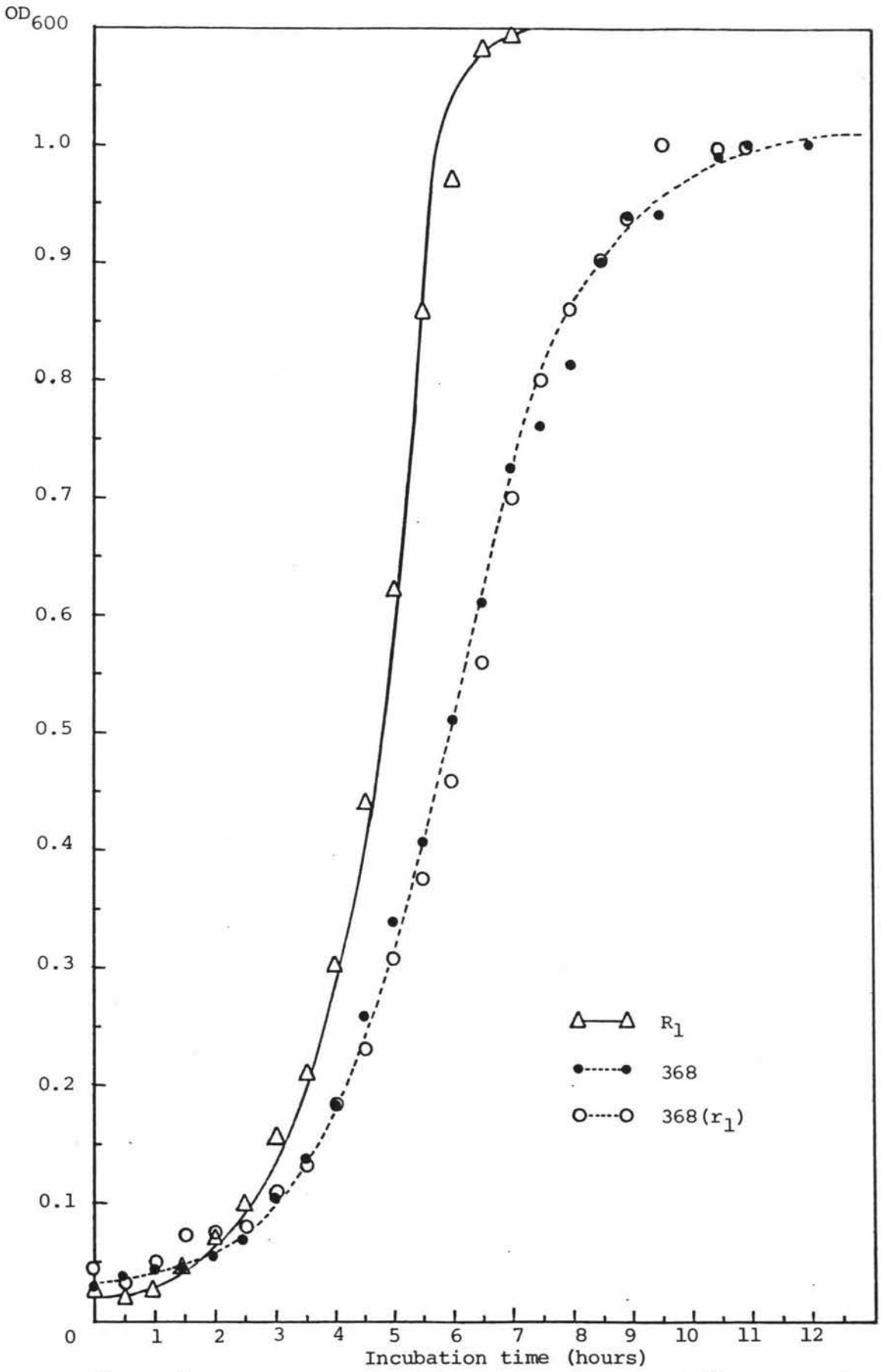


Figure 1. Growth curves of 1% broth cultures of *Str. cremoris* strains R_1, 368 and 368(r_1) at 30°C.

2. *Ultraviolet light irradiation of lactic streptococcal bacteria.*

The chain-forming habits of these bacteria pose a problem when determining their viable cell titres. A way to overcome this obstacle is to blend the cells (Martley, 1972). Examination of blended samples under ordinary light-microscope revealed mainly 2-celled chains. Though the chain counts of blended cultures from Petroff-Hausser counting chamber agreed well with the colony counts from agar-overlay method, one was uncertain about the viability of the cells in a diplococcus: that is whether a colony on an agar plate arose from just one or both cells of the diplococcal chain. As shown in Figure 2, a multi-hit UVL survival curve suggests an average chain length of 2.5 cells in blended samples. This means that both the cells in a diplococcal chain were viable. The constant presence of 3 and 4-celled chains in the blended cultures probably accounts for the 0.5 cell discrepancy observed. The figure also shows the difference in the UVL inactivation kinetics between strain 368 and its lysogenized derivative 368(r_1). The apparent higher UVL sensitivity of the latter could possibly be due to the presence of a prophage (Barksdale, 1959).

3. *UVL induction of Str. cremoris strains R_1, R_1r, 368 and 368(r_1).*

The induction curves of the above strains are shown in Figures 3 and 4. The lysate from Figure 3, referred to as R_1 lysate A in the text, was propagated and used in early preparations of the antiphage sera and in UVL and nitrous acid mutagenesis (Figures 30 & 31). The R_1 lysate from Figure 4A, referred to as R_1 lysate B, was used mainly in CsCl analysis run (Figure 13).

In all induction experiments the unirradiated samples were used as control, except for R_1 in Figure 4A where unirradiated and irradiated R_1C samples were used instead. A noticeable feature common in all the induction profiles is the apparent lack of lag phase, presumably due to the use of log phase cells.

R_1C is a derivative strain of R_1; as evidenced in Figure 4A, the former is cured of its parental prophage (Georghiou, 1976). The observed reduction in growth rate and final cell density of the irradiated R_1C sample relative to its unirradiated ones were probably

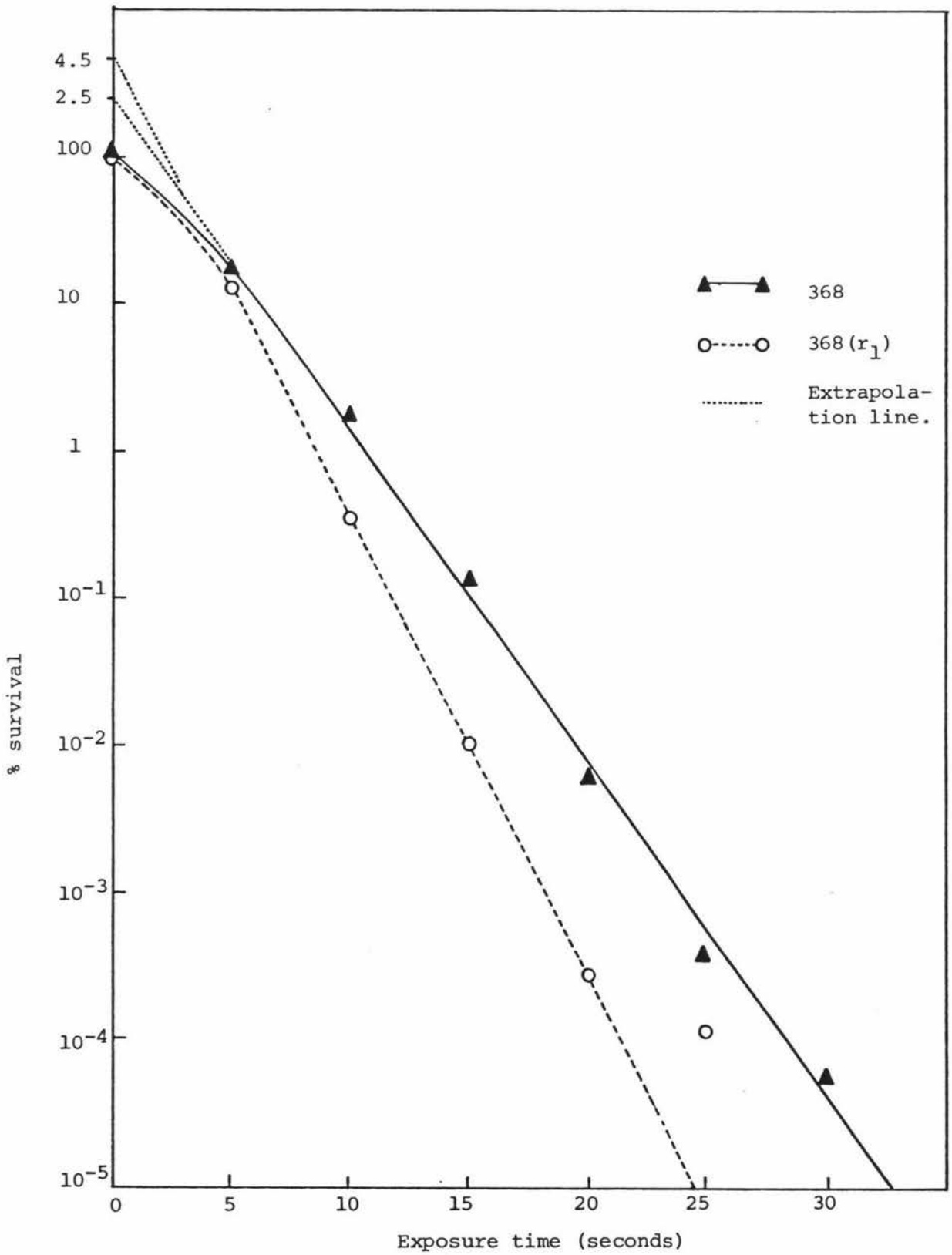


Figure 2. UVL survival curves of *Str. cremoris* strains 368 and 368(r_1).

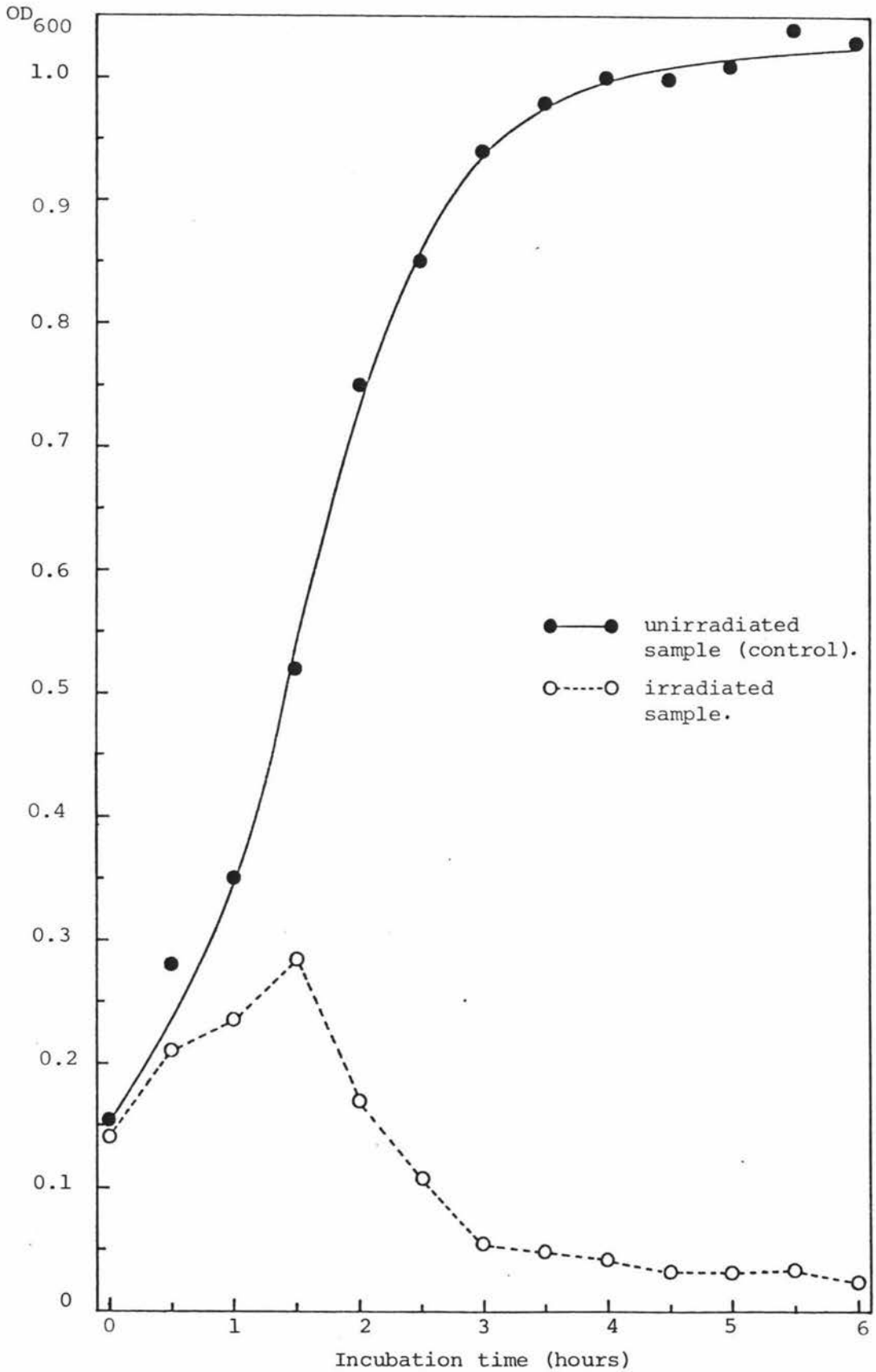


Figure 3. First UVL induction curve of *Str. cremoris* strain R_1.
(This lysate is referred to as R_1 lysate A in the text.)

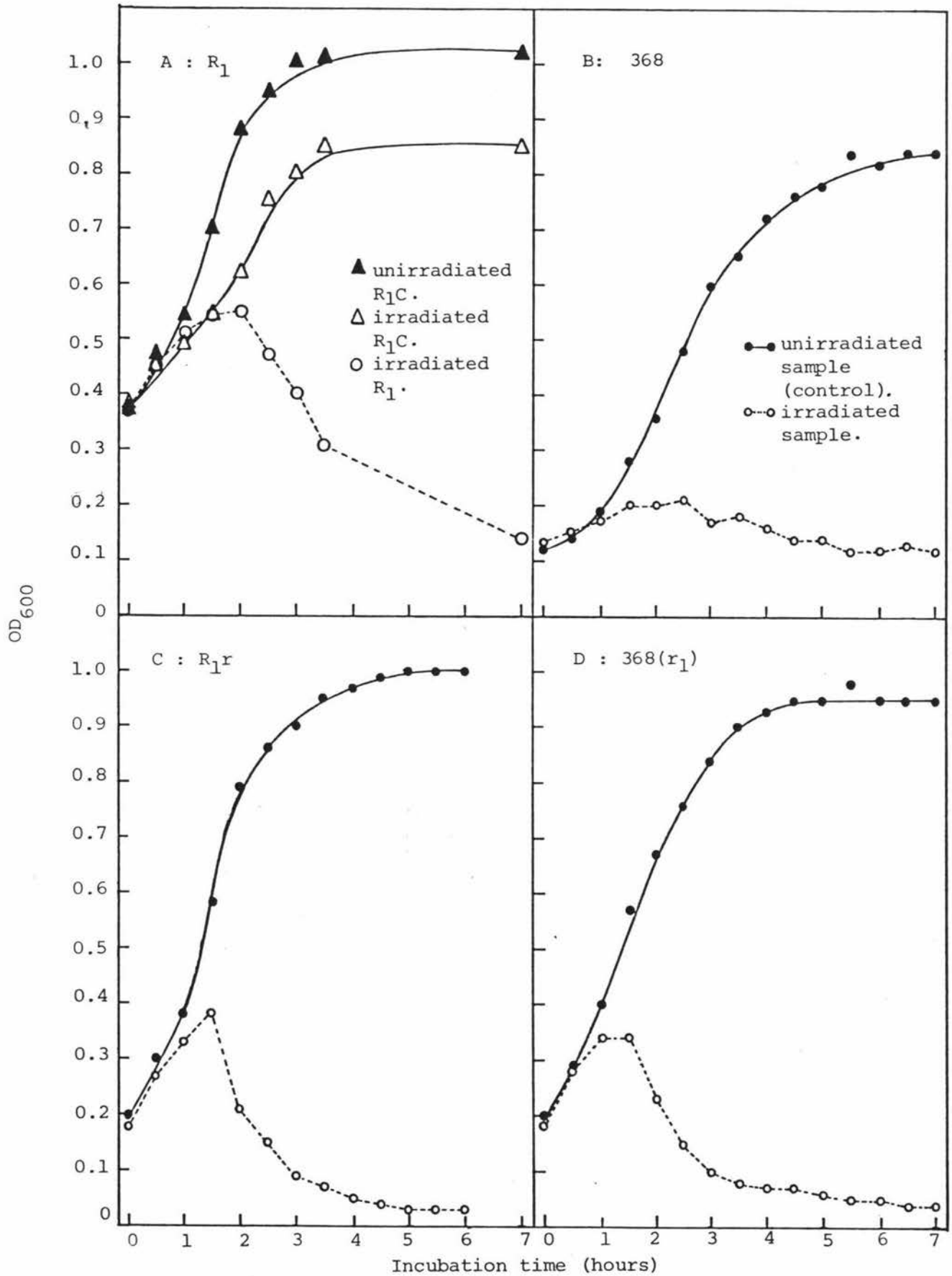


Figure 4. UVL induction curves of *Str. cremoris* strains R_1, R_1r, 368 and 368(r_1).
(The R_1 lysate obtained in Figure 4A is referred to as R_1 lysate B in the text.)

due to the mutagenic effects of UVL. In contrast is the induction response of strain 368 in Figure 4B which falls intermediate between that of irradiated R_1C and R_1 samples. The fact that it can be lysogenized by $\phi r_1/368$ to give $368(r_1)$ implies that it is not lysogenic for ϕr_1; its induction profile suggests it to be UVL inducible for an unknown entity — bacteriocins, killer particles or possibly a phage heteroimmune to ϕr_1 (Bradley & Dewar, 1966).

Figure 3 represents a typical UVL or mitomycin C induction profile of a lysogenic bacteria; it shows the contrast between the pronounced lysis of the UVL-treated cells and the undisturbed growth of the control (Jacob & Wollman, 1953; Levine, 1961). A point of interest with respect to the irradiated samples is the ever present rise in OD_{600} in the first 1½ - 2 hours of incubation preceding lysis. This is particularly obvious in the case of the induction response of strain $368(r_1)$ with reference to 368 seen in Figures 4D and 4B respectively. The significance of these early cell divisions in relation to the induction of the prophage is unknown.

With the onset of lysis was the immediate drop in OD_{600}, accompanied by increase in viscosity of the medium as phages were released. Since the cells have been pre-washed with homologous antiphage sera, the resulting lysates would contain only induced phages. Tabulated in Table V are the numbers of induced phages per cell lysed for the various lysates. Considering an average burst size of 35 pfu per infected cell (at 30°C) for lactic streptococci (Keogh, 1973), the yields obtained for the lysates are indeed very low, corresponding to that reported by Lowrie (1974). Two immediate questions come to mind, namely :-

> Is such low yield of infective phages an inherent feature of these induced lysates?
>
> Or is it that the lysates, particularly R_1 lysate, contains a mixture of perhaps 2 phage types with the majority phage type being inactive and nonadsorbable on indicators R_1C and 368?

The experiments that followed were designed to decide on the two possibilities.

TABLE V DATA ON UVL INDUCTION OF *STR. CREMORIS* STRAINS R_1, R_1r, 368 AND 368(r_1) (Refer to Figures 3 & 4)

Data	Lysates of *Str. cremoris* strains:				
	R_1 lysate A	R_1 lysate B	R_1r lysate	368 lysate	368(r_1) lysate
Initial cell titre (cfu*/ml).	3.4×10^8	5.9×10^8	6.3×10^8	3.3×10^7	5.7×10^8
Cell titre after 15 sec UVL irradiation (cfu/ml).	7.9×10^6	3.8×10^7	1.3×10^7	1.2×10^5	1.7×10^5
Percentage survival of cells.	2.3%	6.4%	2.1%	0.4%	0.03%
Calculated cell density (cells/ml.OD_{600}).	1.6×10^9	1.1×10^9	2.3×10^9	1.7×10^8	2.1×10^9
Change in OD_{600} during lysis (unit OD_{600}).	0.26	0.41	0.35	0.09	0.30
Estimated number of cells lysed per ml (cells/ml).	4.1×10^8	4.6×10^8	8.0×10^8	1.5×10^7	6.3×10^8
Phage titre of lysate obtained (pfu/ml).	3.6×10^8	1.0×10^9	3.8×10^9	no indicator available	1.8×10^8
Theoretical number of induced phages per cell lysed (pfu/cell).	0.9	2.2	4.8	-	0.3
Assuming an average burst size of 35 pfu/ infected cell (Keogh, 1973), the approximate percent phage yield per induced cell.	2.6%	6.3%	14%	-	0.9%

* cfu = colony forming unit.

A state of understanding and organization has not yet been attained in the classification of lactic streptococcal phages. Most characterization of the latter was based on features such as host-range (Henning, *et al*, 1968a; Chopin, *et al* , 1976), morphology (Bauer, *et al* , 1970; Terzaghi, 1976; Tsaneva, 1976), serology (Wilkowske, *et al* , 1954a & 1954b; Jarvis, 1977) and their various combinations (Jarvis, 1978). Using these approaches, the lactic streptoccoal phages have been classed into a variety of groups by different workers. The question of how similar or different the phages are within each group remains to be resolved. In investigating the possibility that strain R_1 is a double lysogen, where the probability of the two resident phages being very different is low, the above general criteria may be insufficient to differentiate the two phage types if present. Hence the following sequence of analyses are employed to establish the identity of the phage(s) induced from R_1 (Stetter, 1977):

(a) Electron-microscopic studies;
(b) CsCl density gradient analysis;
(c) Serological tests;
(d) SDS-gel electrophoresis; and
(e) Host specificity.

4. *Electron-microscopic studies of phage lysates and stocks.*

This is by far the most straight-forward method for differentiating phage types (Williams, 1953; Bradley & Kay, 1960). Morphologically, the lactic streptococcal phages comprise a heterogeneous group of bacterial viruses: their head structures range from prolate to common isometric ones of various sizes, the occasional presence of a neck or collar, their noncontractile tails of varying widths and lengths, and which usually terminate in a small base plate or knob (Keogh & Shimmin, 1974; Terzaghi, 1976; Huggins & Sandine, 1977).

Since electron-microscopic examinations of R_1 lysates have been adequately covered (Lowrie, 1974; Georghiou, 1976; Terzaghi, personal communication), no similar work is repeated in this study.

Morphologically the phages are of one type: they possess an isometric head of about 47 nm diameter, a cross-striated tail which is noncontractile and flexible, measuring 120 nm by 9 nm approximately, and which ends in a small base plate. Their general structure resembles that of coliphage λ. They belong to Group B of Bradley's classifications of phages and contain double-stranded DNA (deoxyribonucleic acids) as their genetic materials (Bradley, 1967; Henning, *et al*, 1968b).

However, it must be mentioned here that in some preparations of R_1 phages for electron-microscopic work, where the former were propagated either lysogenically or lytically, a collar structure was occasionally discernible on some phage particles in electron-micrographs. Since such a feature was observed both in lysates as well as in pure phage stocks (Georghiou, *et al*, manuscript in preparation), it seems unlikely that there are two phage types as was once believed (Georghiou, 1976), distinguished by the presence or absence of a collar. Owing to the fragility of collar structure and the difficulty in resolving it under electron-microscope, one would infer from the above observation that R_1 phages are in fact collared. Further, the collar in coliphage T4 has been shown to be controlled by a single "non-essential" gene (Wood & Revel, 1976), and in this context the presence or absence of a collar alone may not be sufficient proof of identity or nonidentity of phages. Hence the overall conclusion arrived at electron-microscopic studies is that there is only one morphological phage type, most probably collared, in R_1 lysates.

In reference to the data on the number of induced phages per cell lysed estimated for the various lysates in Table V, the comparatively low value obtained for 368(r_1) lysate was possibly due to impairment of the host cells (i.e. 368 cells) by UVL as shown in Figure 4B. However, such consistent findings in low yields of infective phage particles in all the lysates (see Table V) and as estimated from different sources (Lowrie, 1974) suggest the possibility that it is an inherent feature of the induced lysates of these strains.

5. *Caesium chloride density gradient analysis of R_1 phages.*

As stated in the Introduction, the early CsCl runs of R_1 lysates by Georghiou (1976) gave two peaks of pfu on indicators R_1C and 368: a major peak on 368 at a buoyant density of 1.470 g/ml and a minor peak on R_1C at 1.500 g/ml (same position as coliphage λ used as an internal density marker). CsCl runs of pure phage stocks of the two peaks identified them to be of two phage types of different buoyant densities and host-specificities (Georghiou, 1976). Other CsCl gradient runs of R_1 phages by different workers gave similar results in their OD_{254} profile and in their pfu profiles (Terzaghi, personal communication).

Titrations of gradient fractions on overnight cultures (*i.e.* 0.5% lactose-M17 broth cultures incubated 16 hours at 22°C) of heterologous hosts (see legend of Figure 5) gave erratic phage titres and irreproducible pfu profiles. This finding probably accounts for some of the unexplained observations noted in the early CsCl runs of R_1 phages where fresh 0.5% lactose overnight cultures were used. Aging (at 5°C) of 1% lactose-M17 broth cultures of heterologous hosts has been shown to increase their efficiency of plating to near, but not exceeding, that of the homologous hosts (Terzaghi & Terzaghi, 1978). Such aged heterologous cultures also proved to be consistent in their plating efficiency and gave reproducible pfu profiles of gradient fractions. However, it must be noted that their pfu profiles may shift up or down the titre scale depending on the age of the cultures; for instance, the 12-day aged 368 culture used in Figure 5 gave a pfu profile closer to the pfu profile of the homologous host R_1C than that given by the 2-day aged 368 culture used in Figure 6. In other words, the relative positions of the pfu profile of the heterologous host to that of the homologous host on the titre scale are characteristics of the bacteria and not the phages.

Besides the high production of incomplete phages seen as phage fragments and ghosts in electron-micrographs of induced lysates, the low yield of 1 to 5 infective phages per cell lysed estimated for the latter (see Table V) could perhaps partly be explained by high incidence of inactive and nonadsorbable phage particles which were untitratable. This possibility, however, was annulled by the detection of only one OD_{254} peak in CsCl runs of the lysates which corresponds precisely

with the main pfu peaks of R_1C and 368 (Figures 5, 11, 12 & 13). One may argue for the alternative likelihood of both the infective and noninfective phage particles being banded as a single OD_{254} band; however, the mean value of 2×10^{11} phage particles (of T-even phages)/ $cm^2.OD_{260}$ determined by Luria *et al* (1951) as compared to 4.4×10^{11} - 3.4×10^{12} pfu/$cm^2.OD_{254}$ estimated here for the main pfu peaks of the induced lysates (see Table VI) makes unnecessary the hypothesis of additional undetected phage types. We thus shall assume that the low phage yield per induced cell is an inherent property of the system, and that the phage titre expressed as pfu/ml is a direct measure of the total amount of phage particles in the sample.

The low 0.08 - 5.8% of phage recovered in CsCl runs of both lysates and pure phage stocks (see Table VI) is similar to the high phage loss of 90.3 - 99.37% (Table VI) seen with the included standard λ phage. This implies that such high loss of phage is characteristic of these CsCl runs, the cause of which is probably osmotic shock of the phage particles when diluting the latter out of CsCl solutions.

As mentioned in footnotes of Table VI, there are variable differences in data between sets of CsCl runs. For instance, in the Set 1 and Set 7 CsCl runs, the data obtained for the samples within each set are very similar but differed between the sets (see Table VI). In addition, there are also systematic differences between early sets of CsCl runs (*i.e.* Sets 1-4) and runs done later in the series (*i.e.* Sets 5-7), especially in respect to data on percentages of phage recovery (see Table VI). These differences stemmed from variations and modifications in experimental procedures.

The CsCl run of R_1 lysate A (UVL induction shown in Figure 3) is depicted in Figure 5. The OD_{254} tracing of the gradient showed the presence of only one phage band. But, when the gradient fractions were titrated on the homologous host R_1C, the resulting pfu profile revealed the presence of two pfu peaks: a satellite peak, designated Peak AII, and a major peak, Peak AI, which corresponds to the OD_{254} band (see Figure 5). They have a buoyant density of, respectively, 1.425 g/ml and 1.487 g/ml relative to that of coliphage λ (an internal density marker) of

TABLE VI DATA ON CsCl DENSITY GRADIENT ANALYSIS OF R_1 PHAGES [a]

Sample	Titre of lysate or stock (pfu/ml)	Amount of phage input (pfu)	Amount of phage recovered in the gradient fractions (pfu)	Percentage of phage recovered in the run (%)	Extrapolated buoyant density of the phage (g/ml)	Estimated phage titre per unit absorbancy at 254 nm (pfu/cm^2. OD_{254})	Percentage of coliphage λ recovered in the run (%)
R_1 lysate A (Figure 5)	3.5×10^8	1.8×10^9	2.9×10^6	0.16	1.487	3.4×10^{12}	2.1
Peak AI/R_1C stock (Figure 6)	9.0×10^9	4.5×10^{10}	1.6×10^8	0.36	1.482	7.7×10^{11}	1.8
Peak AI/368 stock (Figure 7)	8.0×10^8	4.0×10^9	1.6×10^7	0.40	1.482	7.7×10^{12}	2.2
Peak AII/R_1C stock (Figure 8)	8.0×10^9	4.0×10^{10}	1.9×10^8	0.48	1.482	6.5×10^{11}	2.0
Peak AIII/R_1C stock (Figure 9)	1.0×10^9	5.0×10^9	2.0×10^7	0.40	1.483	9.3×10^{12}	2.7
Peak AIII/368 stock (Figure 10)	9.0×10^8	4.5×10^9	1.2×10^7	0.27	1.482	not done	1.5
R_1r lysate (Figure 11)	3.8×10^9	1.9×10^{10}	1.6×10^7	0.08	1.487	1.8×10^{12}	0.83
368(r_1) lysate (Figure 12)	1.8×10^8	9.0×10^8	1.0×10^6	0.11	1.487	4.4×10^{11}	1.1
ør_1-UV1/R_1C stock (Figure 22)	9.0×10^9	4.5×10^{10}	1.4×10^8	0.31	1.482	3.3×10^{12}	0.63
R_1 lysate B (Figure 13)	1.0×10^9	5.0×10^9	5.9×10^7	1.2	1.490	8.5×10^{11}	8.3
Peak BI fractions (Figure 14)	2.0×10^6	1.0×10^7	5.8×10^5	5.8	1.490	not done	5.3
Peak BII fractions (Figure 15)	9.0×10^4	4.5×10^5	1.1×10^4	2.4	1.489	not done	6.3
Peak BI/R_1C stock (Figure 16)	7.0×10^9	3.5×10^{10}	9.4×10^8	2.7	1.485	6.3×10^{11}	8.3
Peak BI/368 stock (Figure 17)	2.0×10^9	1.0×10^{10}	1.8×10^8	1.8	1.483	2.5×10^{12}	7.3
Peak BII/R_1C stock (Figure 18)	6.0×10^9	3.0×10^{10}	1.0×10^9	3.3	1.483	5.0×10^{11}	8.7
Peak BII/368 stock (Figure 19)	1.0×10^9	5.0×10^9	1.4×10^8	2.8	1.482	1.3×10^{12}	9.7

[a] Notes: Phage samples which were run simultaneously are as followed:

Set 1 - R_1 lysate A, R_1r lysate and 368(r_1) lysate.
Set 2 - Stocks of Peak AI/R_1C, Peak AI/368 and Peak AII/R_1C.
Set 3 - Stocks of Peak AIII/R_1C and Peak AIII/368.
Set 4 - ør_1-UV1/R_1C stock.
Set 5 - R_1 lysate B.
Set 6 - Fractions of Peak BI and Peak BII.
Set 7 - Stocks of Peak BI/R_1C, Peak BI/368, Peak BII/R_1C and Peak BII/368.

Though there are uniformities of data within sets of run, there are slight differences in values between sets due to technicalities.

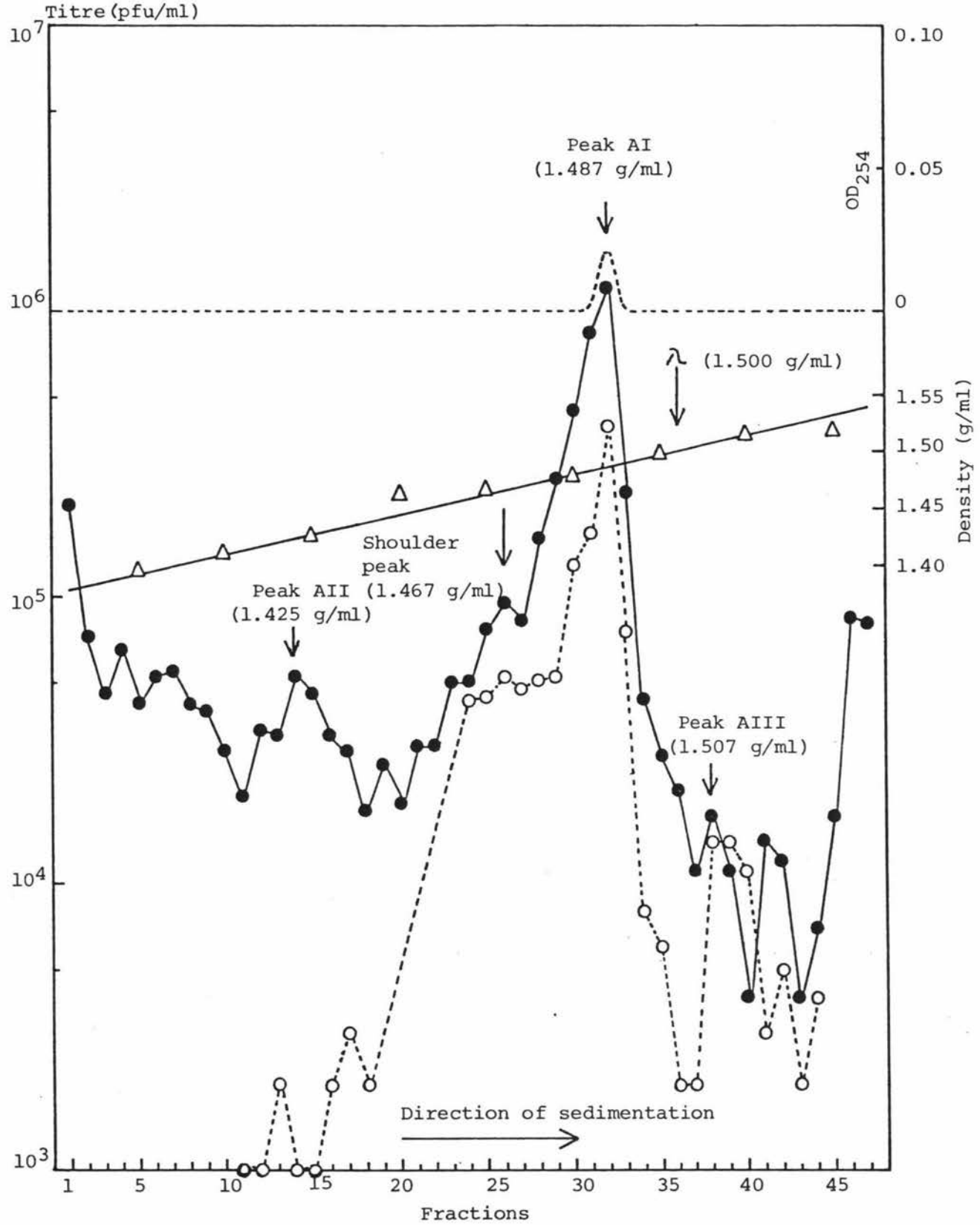


Figure 5. CsCl run of R_1 lysate A.

●—● : p.f.u. profile on homologous host.

○·····○ : p.f.u. profile on aged heterologous host.

△—△ : Density gradient normalized to a buoyant density of 1.500 g/ml for coliphage λ.

····· : OD_{254} profile.

Notes: For R_1 and R_1r lysates and for phages propagated on R_1C, the homologous and heterologous hosts are, respectively, R_1C and 368.
For 368(r_1) lysates and for phages propagated on 368, the homologous and heterologous hosts are, resepctively, 368 and R_1C.
Aging of heterologous hosts may range from 2-12 days.

1.500 g/ml. When the gradient fractions were further titrated on aged heterologous host 368, besides the expected Peak AI, the pfu profile obtained revealed another suspect satellite peak, Peak AIII, at a buoyant density of 1.507 g/ml. Though Peak AI is represented on both the pfu profiles, Peak AII only appeared on the pfu profile of the homologous host, hence implying a lighter phage type which was host-specific to indicator R_1C. Conversely, Peak AIII stood for a heavier phage type which was host-specific to indicator 368. In short, with these observations the notion that there are more than one phage type present in the R_1 lysate seems plausible.

Phages from each peak were then isolated and propagated on their respective hosts, and the resulting pure phage stocks were further analysed on CsCl runs as

Peak AI/R_1C stock (Figure 6),
Peak AI/368 stock (Figure 7),
Peak AII/R_1C stock (Figure 8),
Peak AIII/R_1C stock (Figure 9), and
Peak AIII/368 stock (Figure 10).

As shown in Figures 6-10, the OD_{254} tracings of the gradients detected only one phage band. Titrations of the respective gradient fractions on both homologous and aged heterologous hosts revealed only one pfu peak which corresponds precisely with the OD_{254} band; they all fall within the buoyant density range of 1.482 - 1.483 g/ml irrespective of the origins of the phage stocks. That is to say the phages that were originally banded as satellite Peaks AII and AIII were in fact of the same phage type as Peak AI phages (refer to Figure 5). This fact is further supported in serological tests of the respective phage stocks (see Figure 25). In other words, the satellite pfu Peaks AII and AIII are inferred to be artifcats.

One may notice from the foregoing CsCl runs that such satellite peaks of pfu seemed to appear only in the R_1 lysate. Are such peaks found only in R_1 lysates or are they found in induced lysates in general? To answer this question requires CsCl analyses of R_1r and $368(r_1)$ lysates.

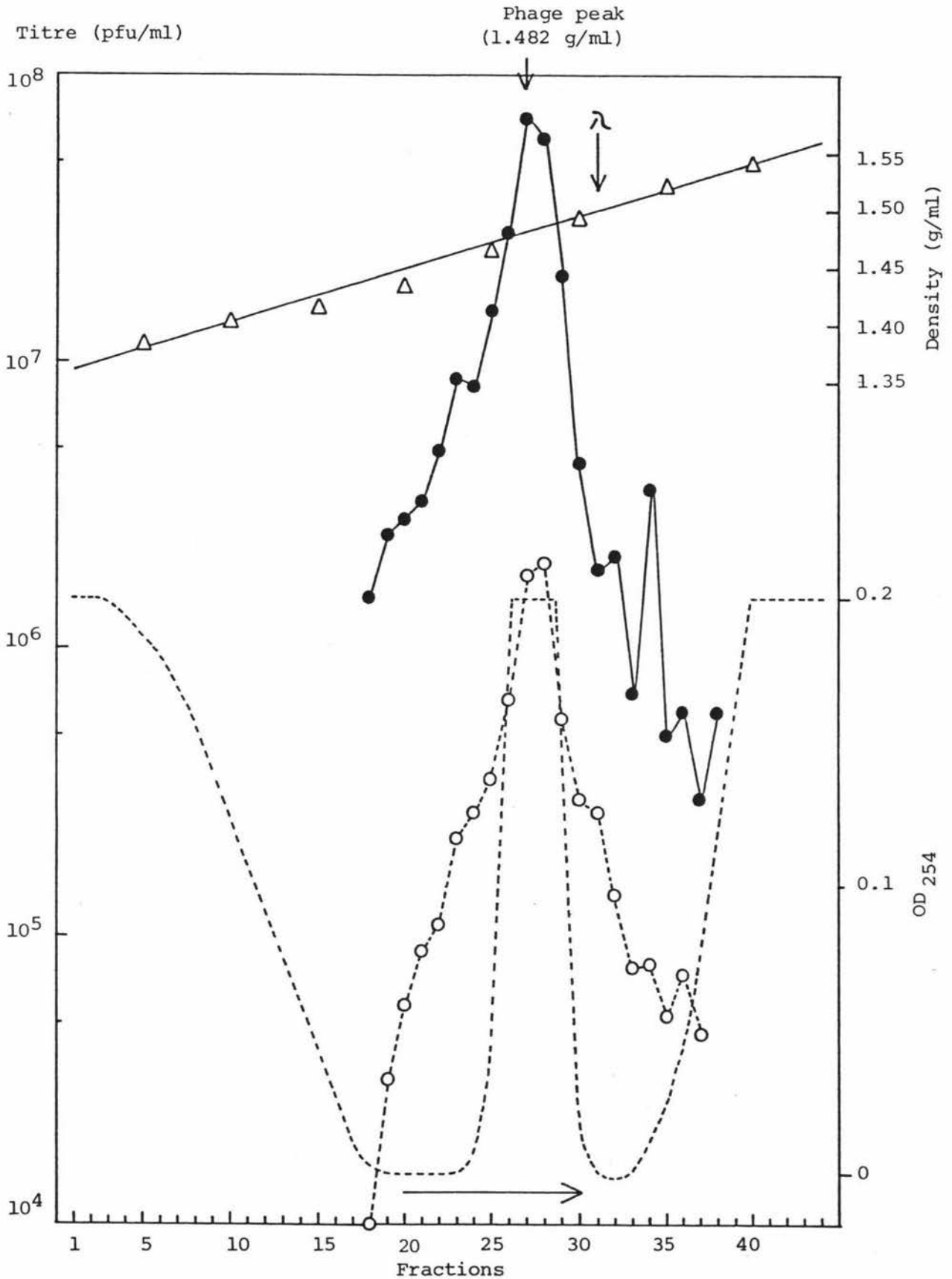


Figure 6. CsCl run of Peak AI/R_1C stock from R_1 lysate A. (Symbols are as for Figure 5.)

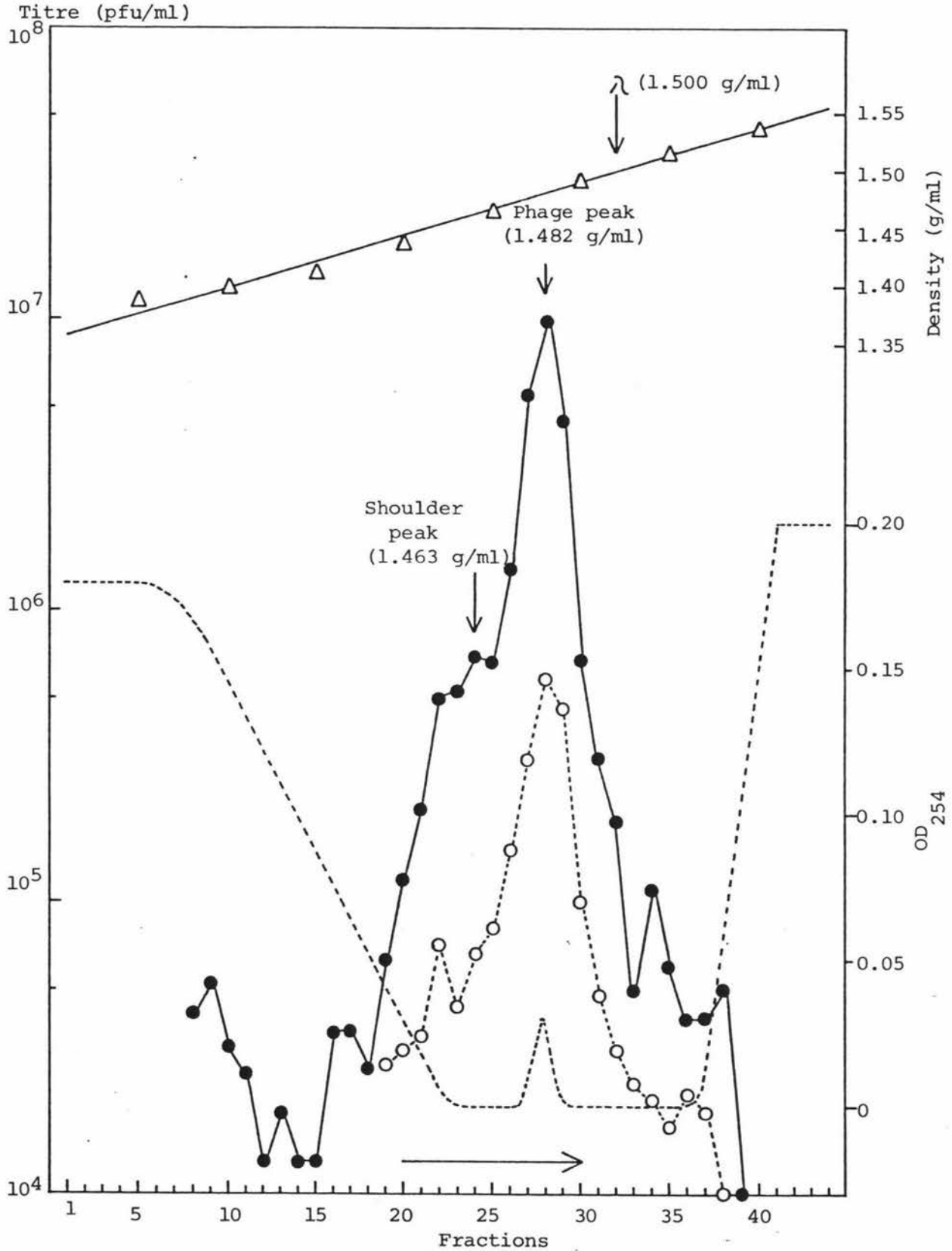


Figure 7. CsCl run of Peak AI/368 stock from R_1 lysate A. (Symbols are as for Figure 5.)

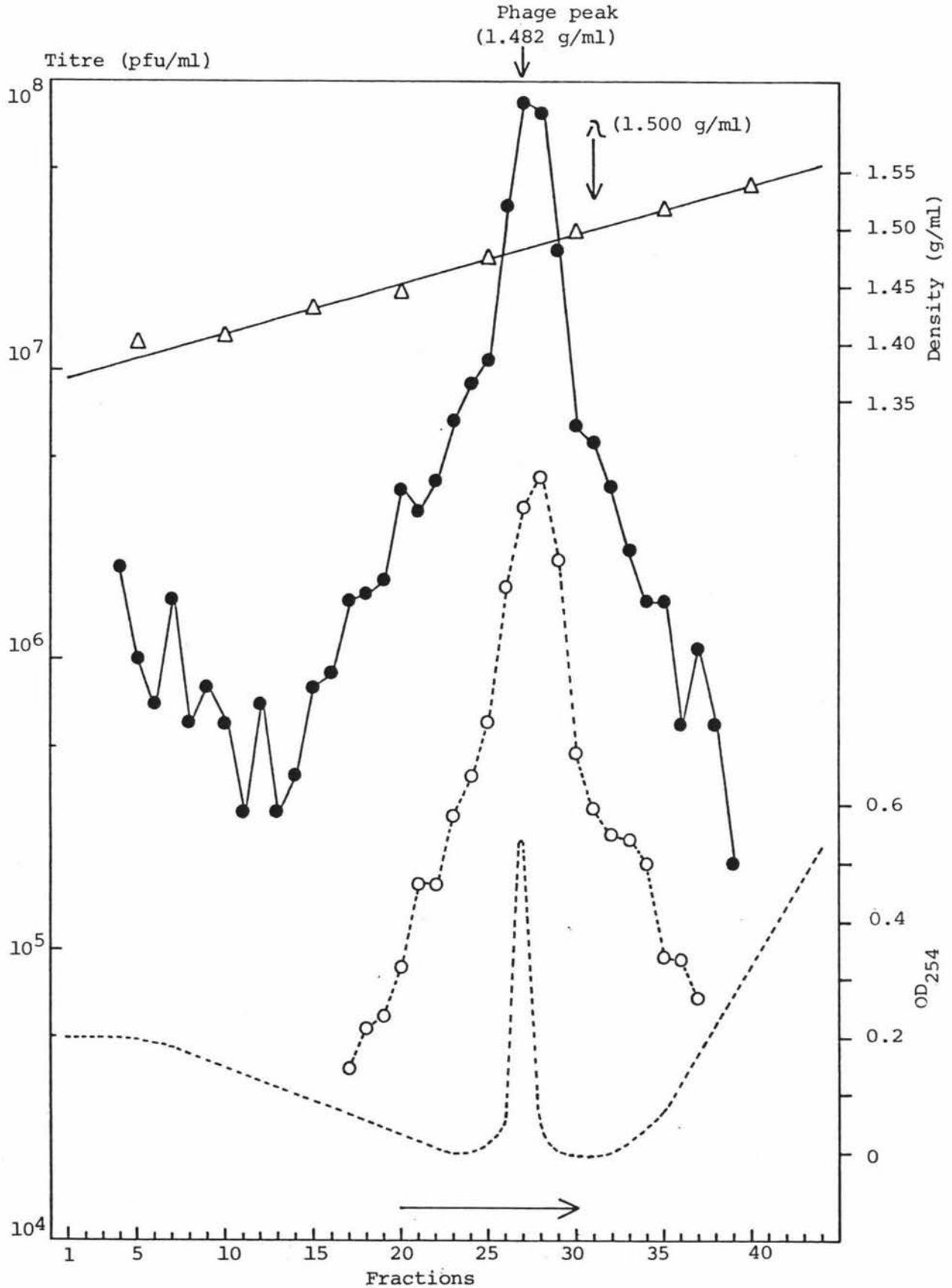


Figure 8. CsCl run of Peak AII/R_1C stock from R_1 lysate A. (Symbols are as for Figure 5.)

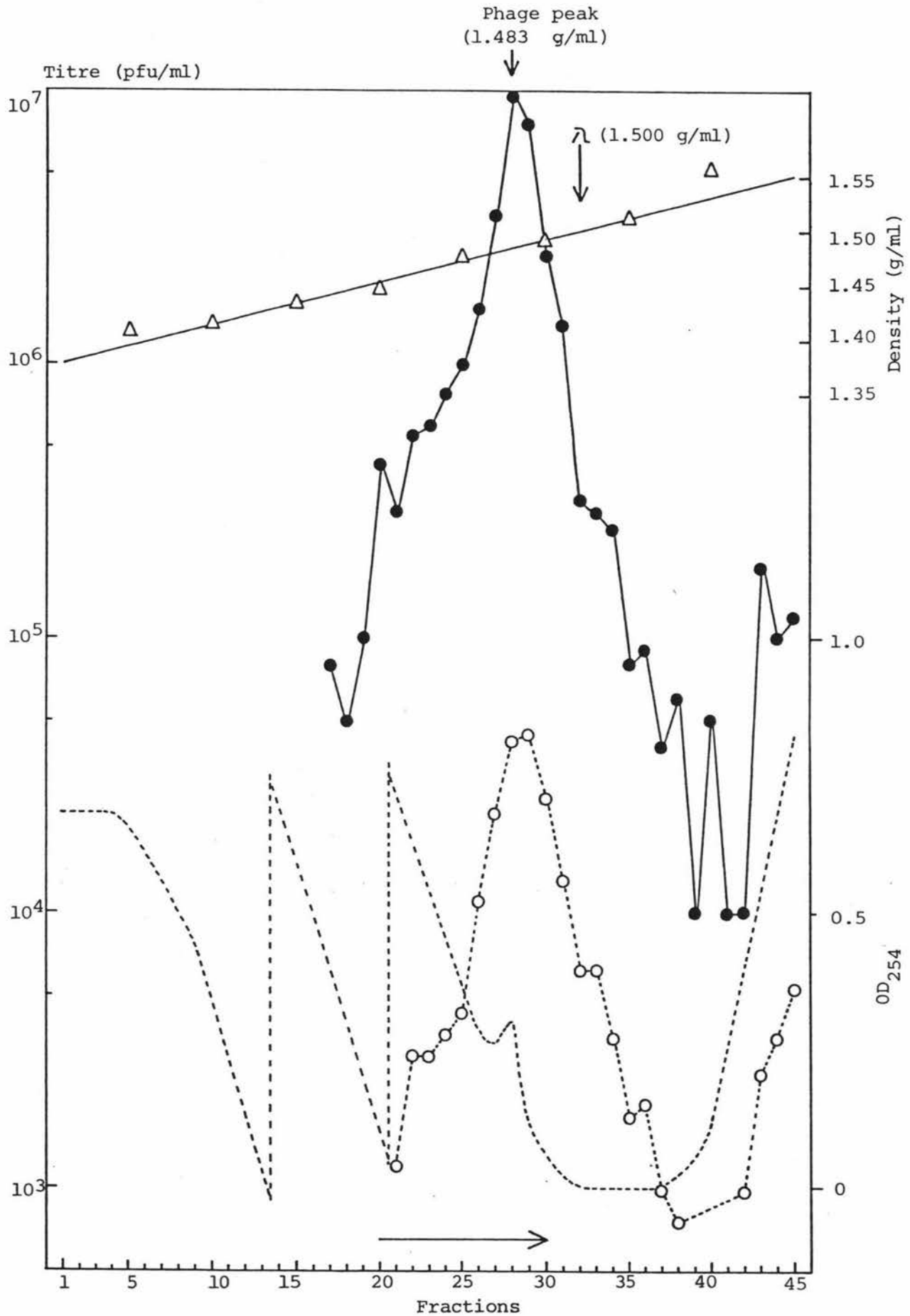


Figure 9. CsCl run of Peak AIII/R_1C stock from R_1 lysate A.
(Symbols are as for Figure 5.)

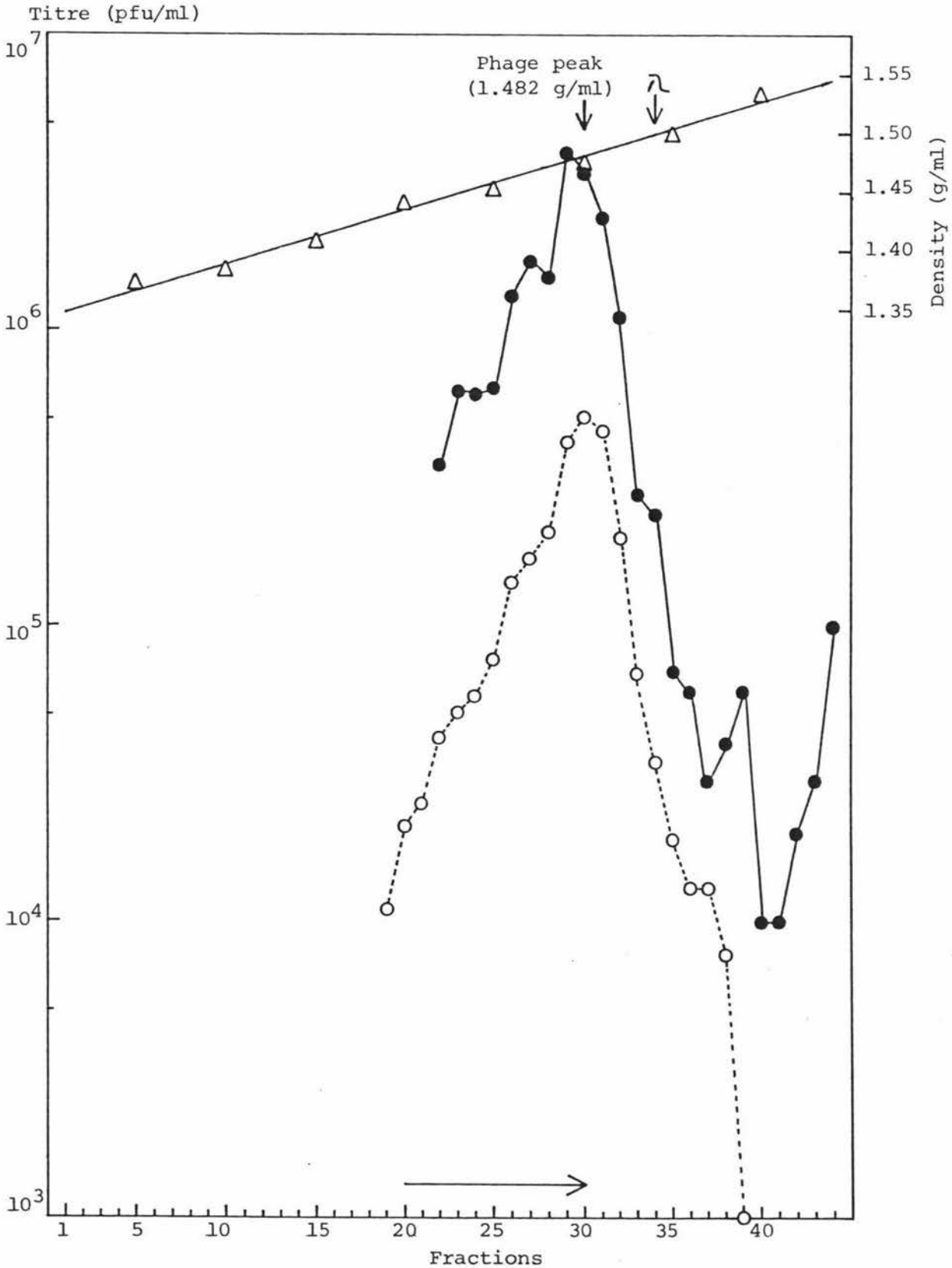


Figure 10. CsCl run of Peak AIII/368 stock from R_1 lysate A. (Symbols are as for Figure 5.)

Strain R_1r is R_1C relysogenized by $\phi r_1/R_1C$, and hence its induced lysate should contain only phages of one type. Shown in Figure 11 is the CsCl run of the lysate obtained in Figure 4C. As depicted, the OD_{254} scan of the gradient detected only one phage band which corresponds to the main pfu peaks on both the homologous and aged heterologous hosts of density 1.487 g/ml. However, close inspection of the pfu profile of the homologous host located a suspect satellite peak at density of 1.405 g/ml (see Figure 11).

Before considering the results of CsCl run of $368(r_1)$ lysate, the following points about the 368 lysate need mentioning first. No indicator could be located for 368 lysate (UVL induction shown in Figure 4B) from among the strains listed in Table VII. When the lysate was concentrated and analysed in a CsCl run, the OD_{254} scan of the gradient could detect no phage band. Direct platings of the gradient fractions on aged R_1C and SK_{11} (see Figure 33) gave occasional plaque formation. Judging from the UVL induction response of 368 in Figure 4B and the behaviour of its lysate in a CsCl gradient, it is possible to conclude that 368 lysate contains no phage-like particles. As stated, strain $368(r_1)$ is 368 lysogenized by $\phi r_1/368$. An induced lysate of $368(r_1)$ would therefore contain the input phage type plus the original 368 lysate. Since the latter entity has been shown to be inert in most assays, thus only the former is expressed in titrations of the lysate.

Figure 12 depicts the CsCl run of $368(r_1)$ lysate obtained in Figure 4D. The OD_{254} tracing (not shown) of the gradient showed a discernible phage band which coincides with the main Peak CI. Aside from this main peak of buoyant density of 1.487 g/ml, the pfu profile on homologous host 368 located satellite peaks at densities of 1.390 g/ml (Peak CII), 1.427 g/ml (Peak CIII) and 1.515 g/ml (Peak CIV) (see Figure 12). The identity of all the phages at Peaks CI to CIV was shown by neutralization tests of their respective phage stocks against homologous antiphage serum (see Figure 27). Hence from the foregoing results on CsCl runs of R_1r and $368(r_1)$ lysates, it is seen that satellite peaks of pfu are manifested not only in R_1 lysates but in induced lysates in general. This fact lends further support to the artifactual nature of the satellite peaks.

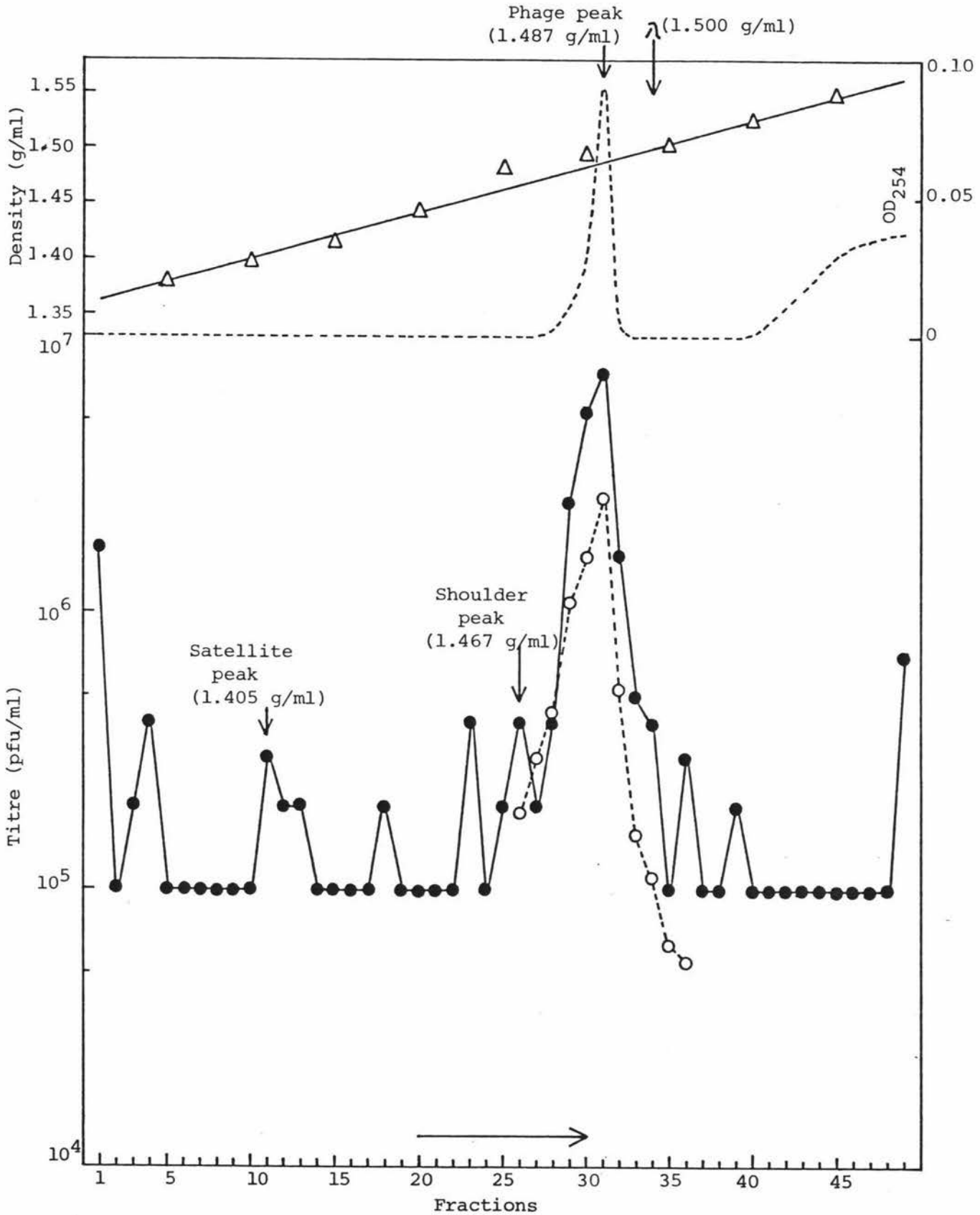


Figure 11. CsCl run of R_1r lysate.
(Symbols are as for Figure 5.)

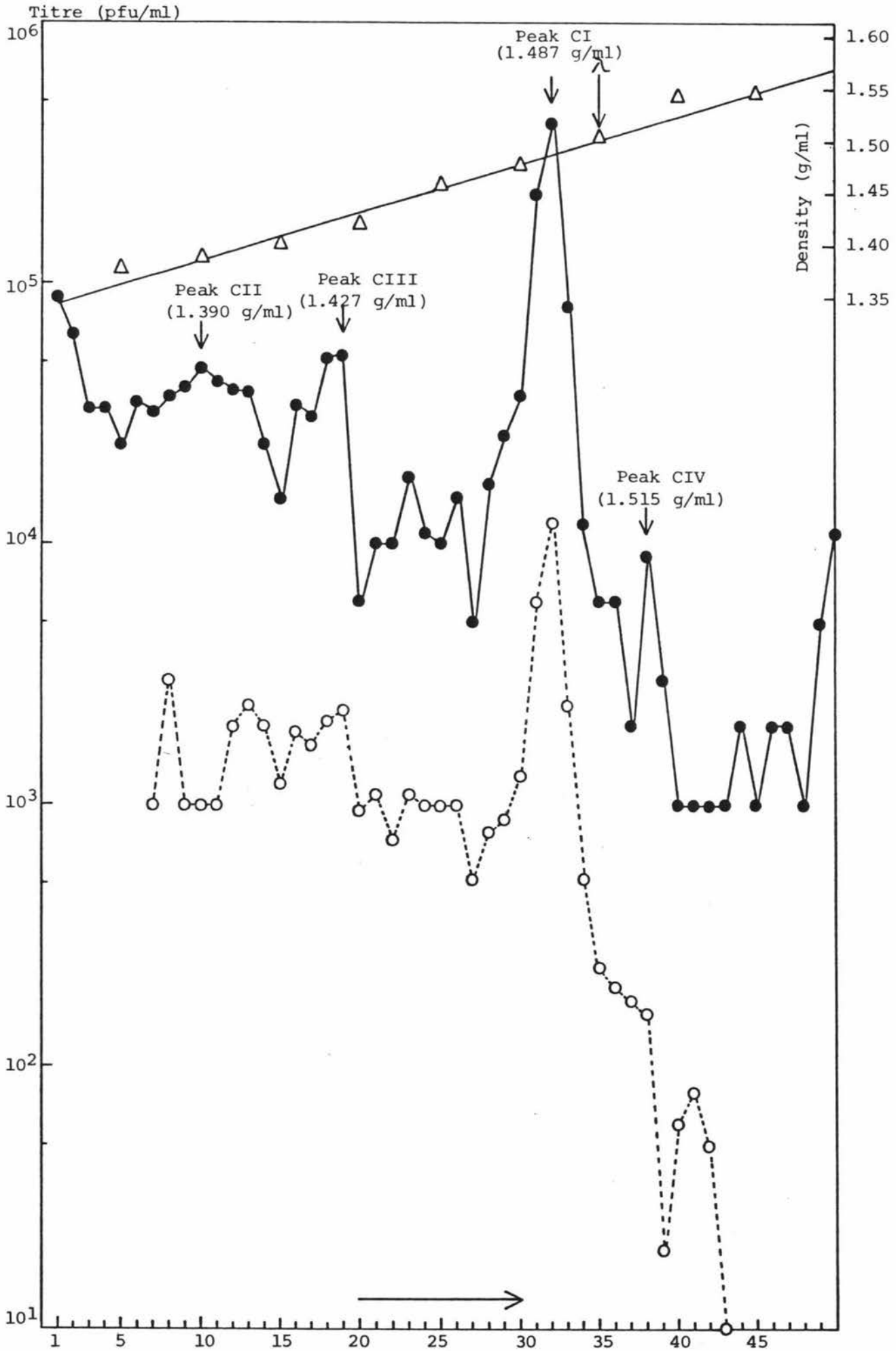


Figure 12. CsCl run of 368(r_1) lysate.
(Symbols are as for Figure 5.)

One feature which has escaped our attention in the foregoing discussions is the presence of a "shoulder peak" (in contrast to satellite peak) in some samples of the CsCl runs (see Figures 5, 7 & 11). Such a "shoulder peak" is clearly manifested in CsCl run of R_1 lysate B (UVL induction depicted in Figure 4A) shown in Figure 13. The OD_{254} scan of the latter gradient could detect only one phage band which coincides with the main pfu Peak BI at a density of 1.490 g/ml. However, titrations of the gradient fractions on homologous host R_1C revealed the presence of the shoulder peak, Peak BII, at density of 1.468 g/ml together with a suspect satellite peak at density of 1.398 g/ml. This shoulder peak appears even more prominently in the pfu profile using the aged heterologous host 368 (Figure 13. See also Figure 5).

In contrast to satellite peaks which appear generally only in induced lysates and at varying densities, the shoulder peak has been located in CsCl runs of both induced lysates (Figures 5, 11 & 13) and pure phage stocks with propagating hosts R_1C as well as 368 (Figures 22 & 7 respectively). They all fall consistently within the buoyant density range of 1.463 - 1.468 g/ml (see Table VII). In fact, such shoulder peak (of mean buoyant density of 1.466 g/ml) together with the main pfu peak (of mean buoyant density of 1.485 g/ml) resemble, respectively, the pfu peak on 368 (of density 1.470 g/ml) and the pfu peak on R_1C (of density 1.500 g/ml) reported by Georghiou (1976). These findings warrant in-depth investigations of the main Peak BI and shoulder Peak BII in the CsCl run of R_1 lysate B (see Figure 13) as possible manifestations of two phage types in the R_1 phages.

The immediate reactions to the above observations are: Could Peak BI and Peak BII represent a case of head dimorphism as reported for *Pseudomonas syringae* ϕ12B, phages P1, P2 and P22, a Staphylococcus phage and *Streptococcus lactis* ϕ3ML (Bradley, 1963; Bradley, 1967)? Or could phages of shoulder Peak BII be morphologically similar but of less DNA content than that of Peak BI (Fukuda & Yamamoto, 1972), for instance delection mutants? Or could Peak BII possibly represent satellite phages as exemplified by phages P4-P2 system (Six & Klug, 1973; Six, 1975; Shore, *et al*, 1978)?

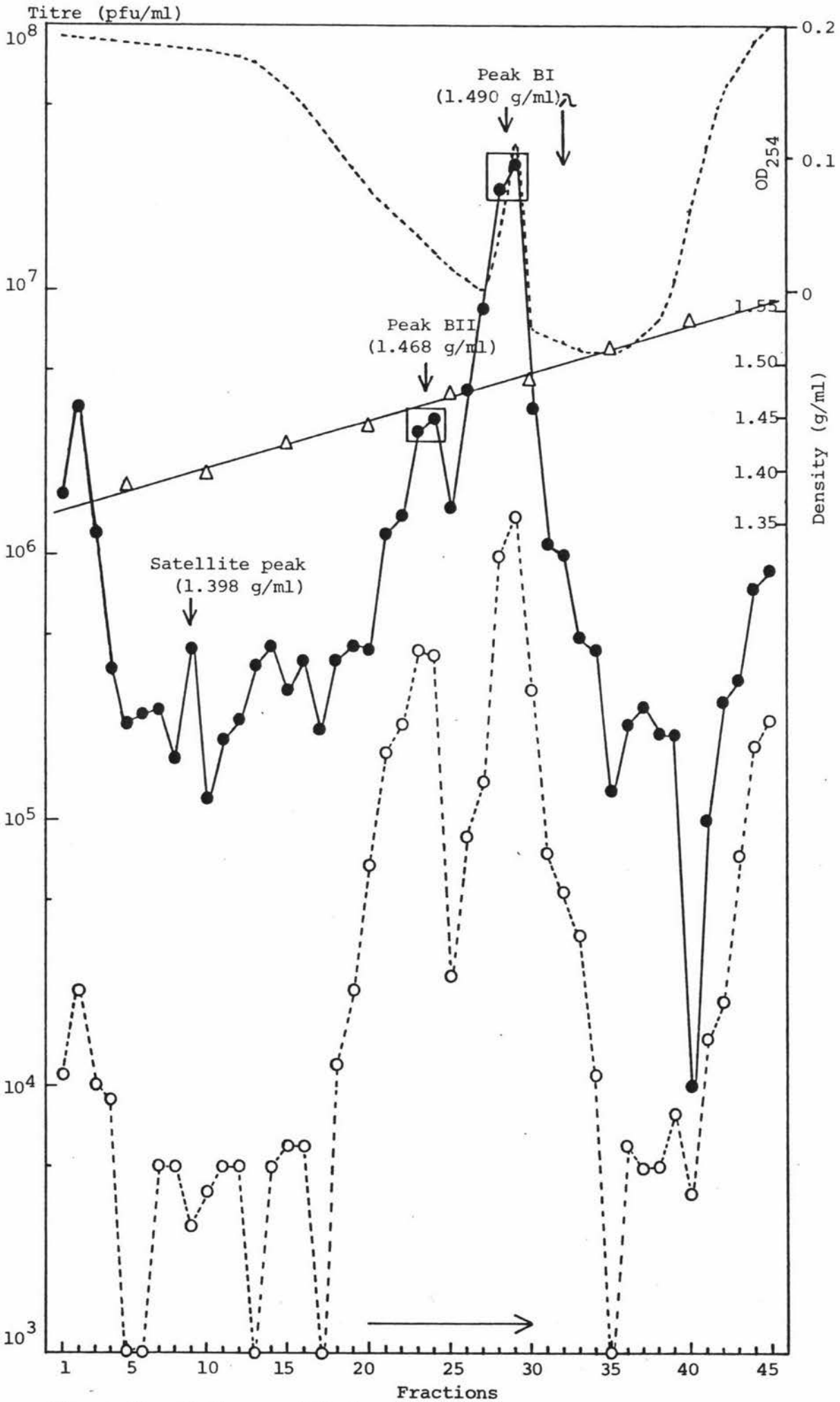


Figure 13. CsCl run of R_1 lysate B.
(Symbols are as for Figure 5.)

To resolve these possibilities the following CsCl runs were carried out:

Pooled fractions of Peak BI (Figure 14),
Pooled fractions of Peak BII (Figure 15),
Peak BI/R_1C stock (Figure 16),
Peak BI/368 stock (Figure 17),
Peak BII/R_1C stock (Figure 18) and
Peak BII/368 stock (Figure 19).

Except for Peak BI and Peak BII fractions whose phage titres were too low to be discernible optically, the OD_{254} scans of the above gradients could detect only one phage band. The pfu profiles of the respective gradient fractions on both homologous and aged heterologous hosts revealed only one pfu peak of density range 1.482 - 1.490 g/ml irrespective of the origins of the phage samples (see Figures 14-19). The fact that direct re-runs of Peak BII fractions (*i.e.* without any propagation of the phages) on a fresh gradient gave identical results to that of Peak BI fractions confirmed unequivocally the identity of the phages of both the peaks, at least with respect to their buoyant density (see Figures 14 & 15). It hence ruled out the possibilities of head dimorphism, deletion mutants or satellite phages in R_1 lysates.

To further characterize the phage preparations, the following methods were employed:

(i) Serological tests of phages with homologous antiphage sera, and
(ii) SDS-gel electrophoresis of whole phage proteins.

6. *Serological tests of R_1 phages against antiphage serum A/S r_1-UV1/R_1C.*

Antiserum test offers both a convenient and specific means of examining protein species. It is used here mainly to test the identity of the phage tail proteins which constitute the adsorption apparatus of the phage partciles, which results in the eventual inactivation of the homologous phage. Hence a measure of the rates of inactivation between

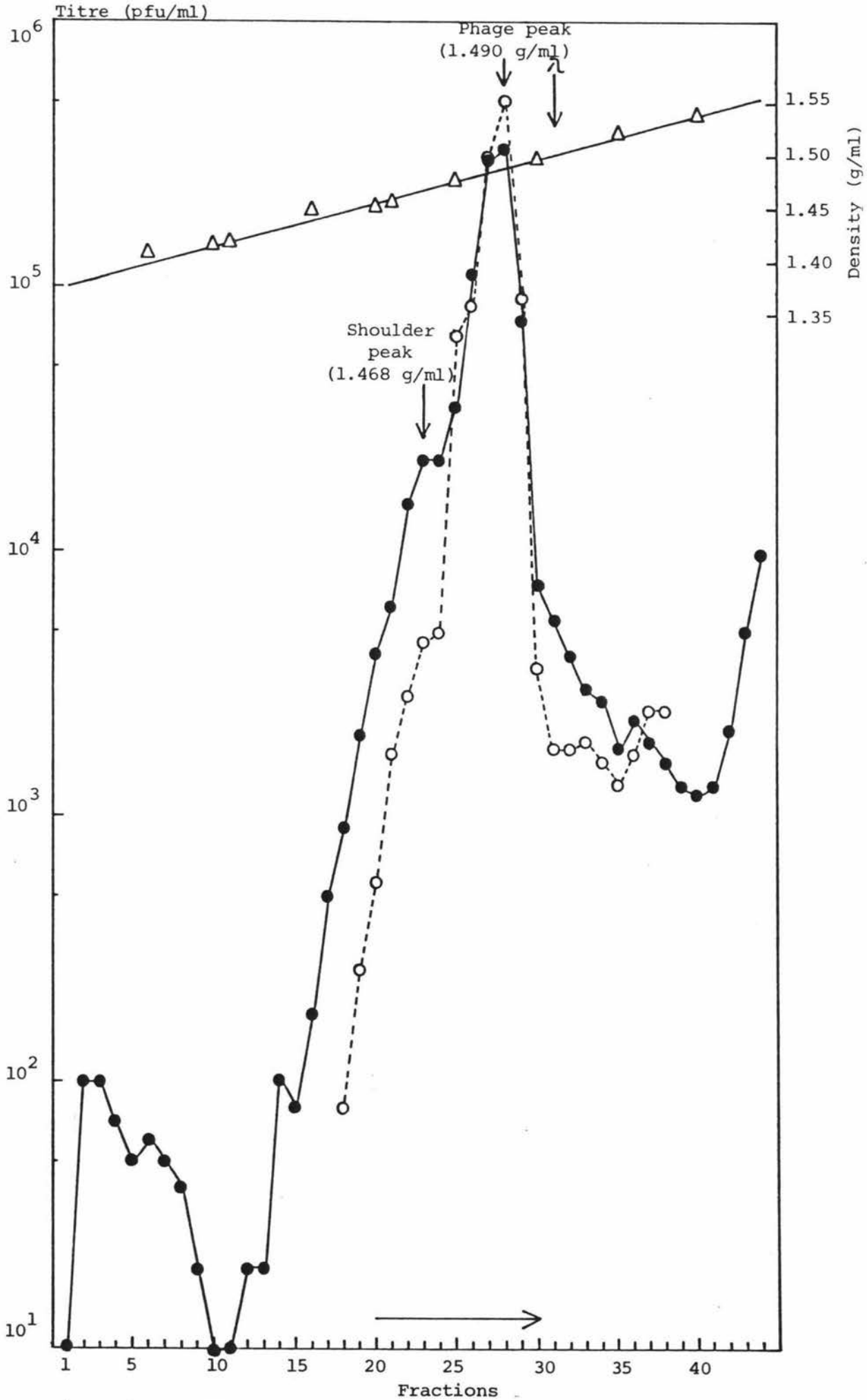


Figure 14. CsCl run of Peak BI fractions from R_1 lysate B. (Symbols are as for Figure 5.)

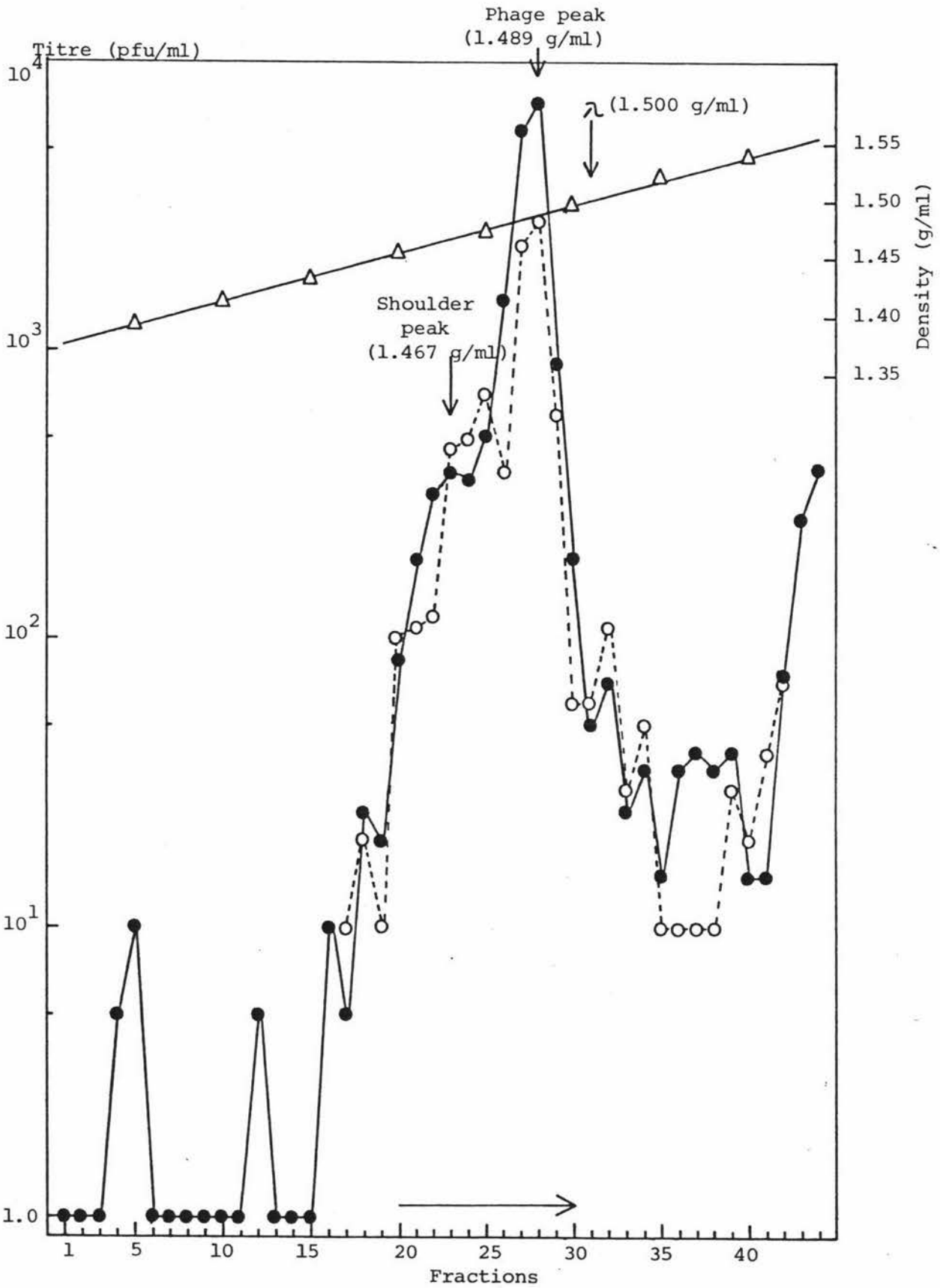


Figure 15. CsCl run of Peak BII fractions from R_1 lysate B.
(Symbols are as for Figure 5.)

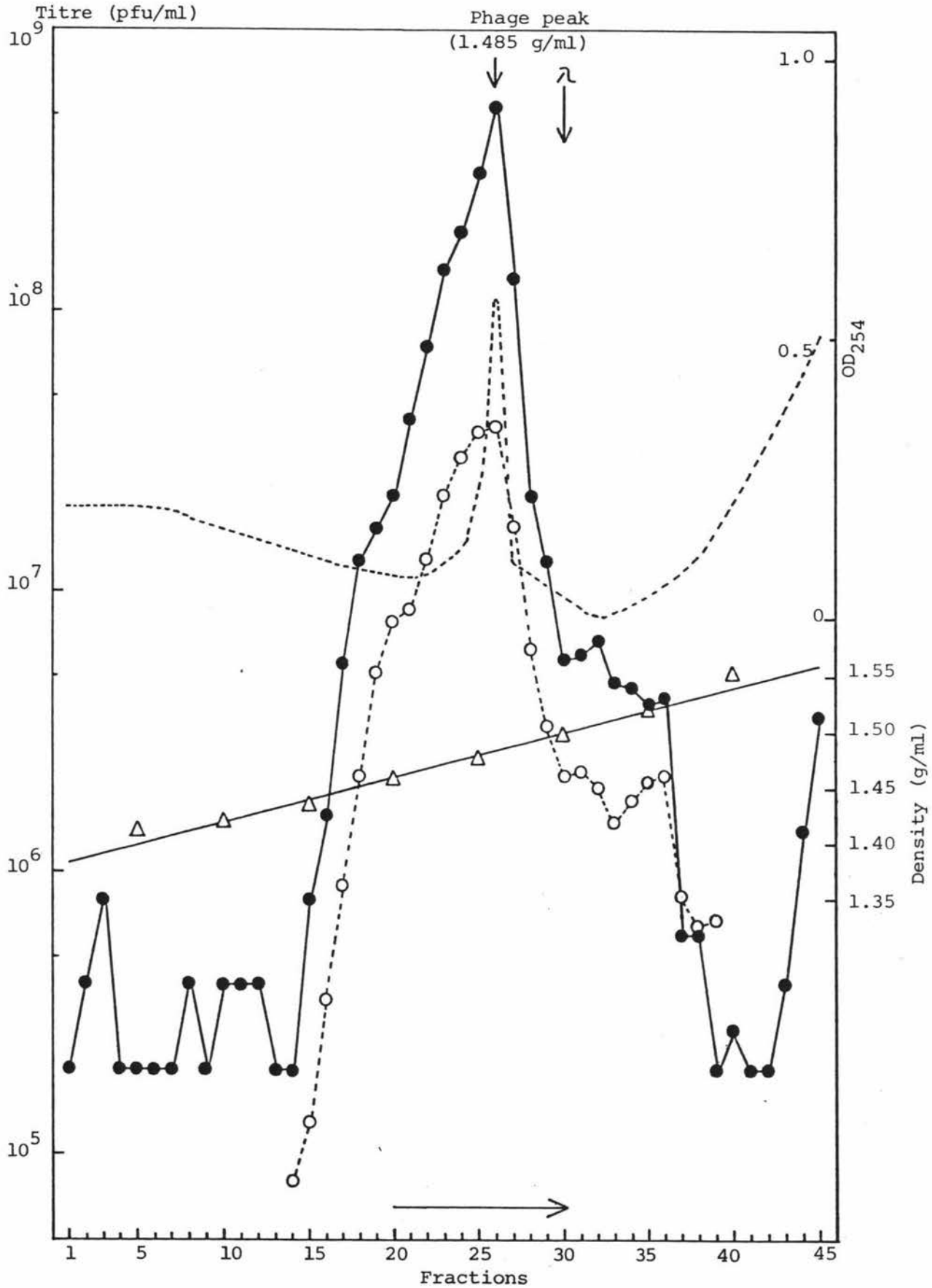


Figure 16. CsCl run of Peak BI/R_1C stock from R_1 lysate B.
(Symbols are as for Figure 5.)

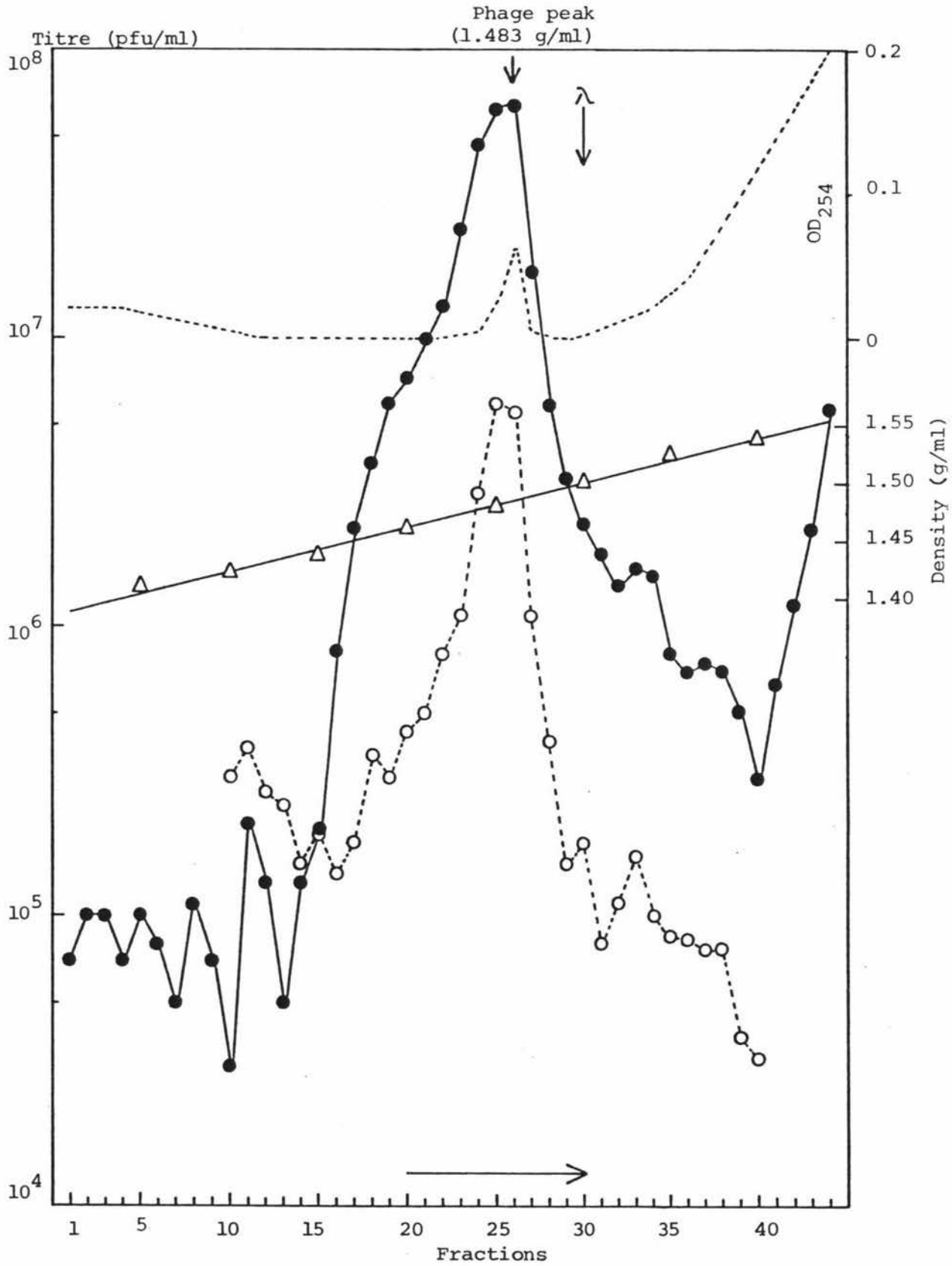


Figure 17. CsCl run of Peak BI/368 stock from R_1 lysate B.
(Symbols are as for Figure 5.)

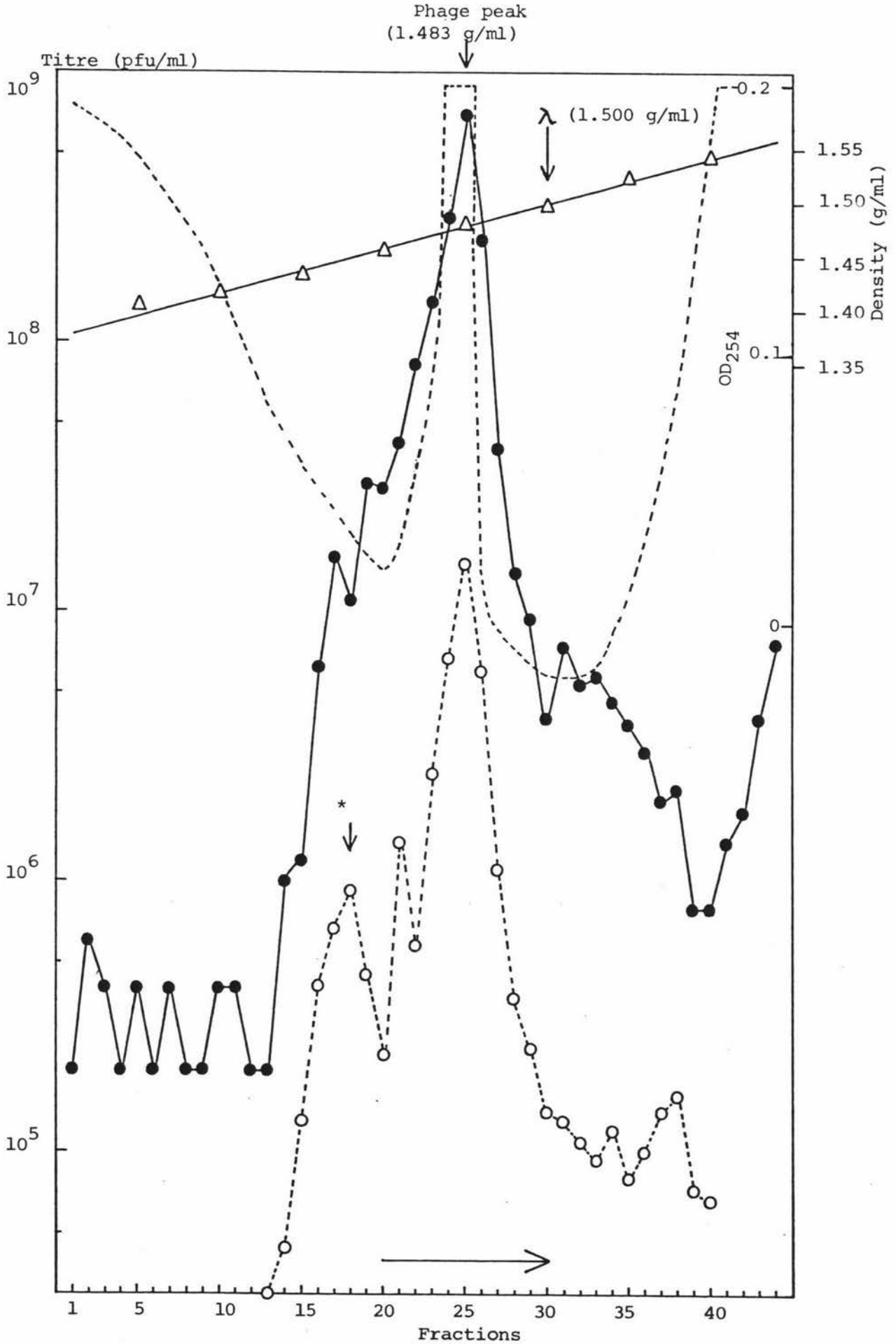


Figure 18. CsCl run of Peak BII/R_1C stock from R_1 lysate B. (Symbols are as for Figure 5.)

* Atypical result due to variations in aged culture of heterologous host 368.

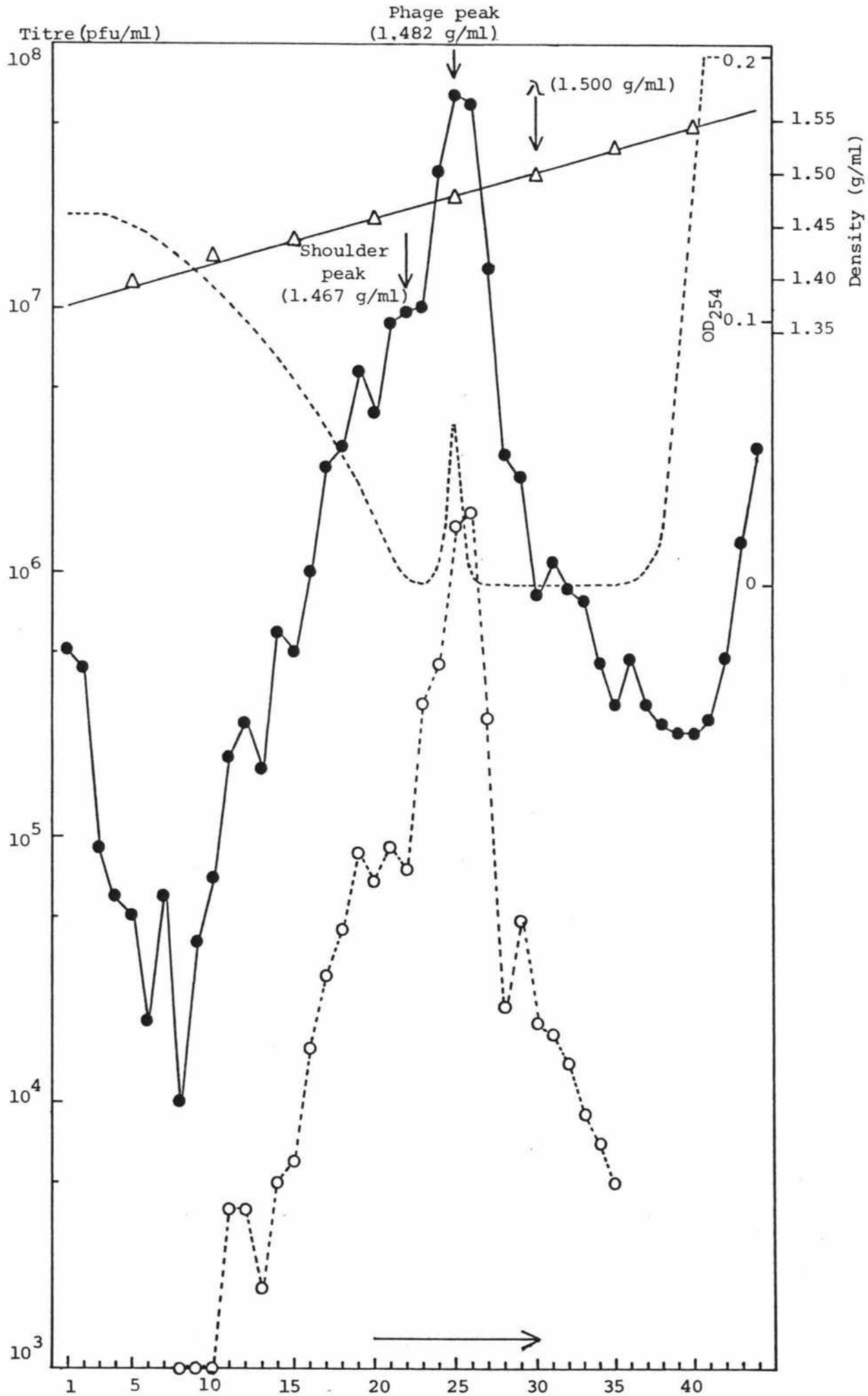


Figure 19. CsCl run of Peak BII/368 stock from R_1 lysate B.
(Symbols are as for Figure 5.)

TABLE VII OCCURRENCE OF SHOULDER PEAK IN CsCl RUNS OF R_1 PHAGES.

Sample of CsCl run	Extrapolated buoyant density of the shoulder peak (g/ml)
R_1 lysate A (Figure 5)	1.467
Peak AI/368 stock (Figure 7)	1.463
R_1r lysate (Figure 11)	1.467
R_1 lysate B (Figure 13)	1.468
Peak BI fractions (Figure 14)	1.468
Peak BII fractions (Figure 15)	1.467
Peak BII/368 stock (Figure 19)	1.467
ϕr_1-UV1/R_1C stock (Figure 22)	1.464

different phage samples using the same antiphage serum would give an indication of their serological relatedness.

As mentioned before, $\phi r_1/R_1C$ is a temperate phage which forms turbid plaques on indicator R_1C (see Table IV). In order to acquire high enough titres for preparations of antiphage serum, an UVL-induced clear-plaqued mutant, designated ϕr_1-UV1/R_1C (see subsection 10), was used. Antiphage serum made against this phage is referred to as A/S r_1-UV1/R_1C in the text, and A/S $r_1/368$ refers to antiphage serum prepared against $\phi r_1/368$. These two antiphage sera were prepared because of early observed differences between phages propagated on R_1C and on 368. However, their antigenic identity is shown in Figures 20 and 21 by reciprocal testing with the two antisera.

In Figure 20, it can be seen that $\phi r_1/R_1C$ is antigenically identical to its clear-plaqued mutant, ϕr_1-UV1/R_1C; in fact, the profiles of their CsCl runs (not shown for $\phi r_1/R_1C$) were identical (Figure 22). Further, the apparent serological identity of $\phi r_1/R_1C$ and $\phi r_1/368$ as inferred from Figures 20 and 21 supports the notion of host-induced variation of the phages in the R_1C-368 system rather than a deficiency in adsorption (see subsection 8 & Table X).

Since one is here testing for possible differences between the tail proteins of phages isolated from satellite peaks, shoulder peaks and main peaks of phage lysates and stocks in CsCl gradients, it is necessary to first check the homogeneity of the phage used to raise the A/S r_1-UV1/R_1C.

Shown in Figure 22 is the CsCl analytical run of ϕr_1-UV1/R_1C stock; as seen, besides the main peak of pfu which corresponds to the OD_{254} phage band at density of 1.482 g/ml, a shoulder peak is apparent at density of 1.464 g/ml. But, when the phages from the respective peaks were tested directly with A/S r_1-UV1/R_1C as shown in Figure 23, they are seen to be identical, at least serologically. In other words, ϕr_1-UV1/R_1C stock is antigenically homogeneous, and will be used as the standard in all serological tests that follow. As shown in Figure 24, there is no noticeable difference in inactivation kinetics of

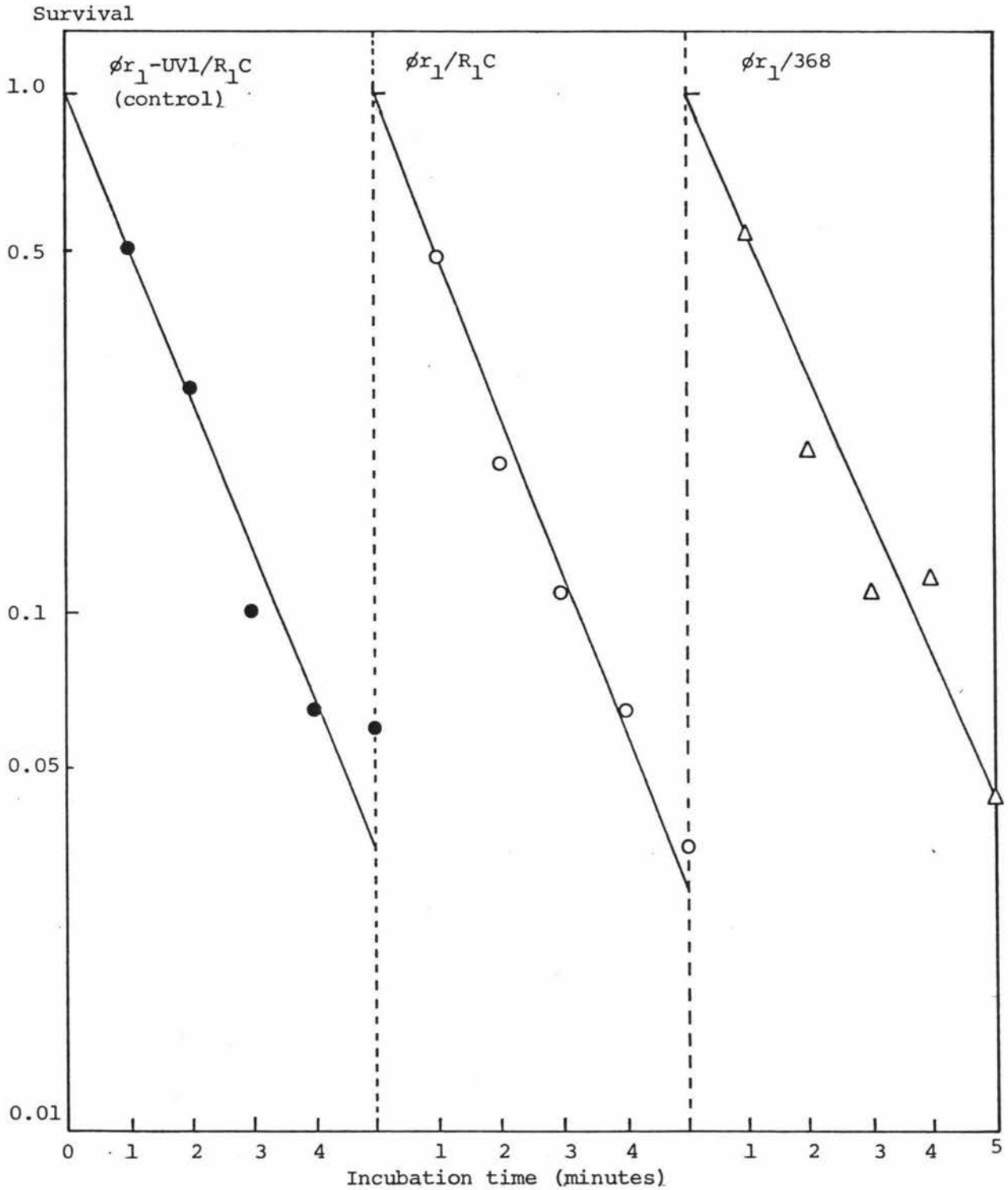


Figure 20. Neutralization of $\phi r_1/R_1C$ and of ϕr_1/368 by A/S r_1-UV1/R_1C at 30°C.

(Notes: From the graph, the neutralization constant of undiluted A/S r_1-UV1/R_1C with respect to the homologous phage is estimated to be k = 660/min.
This antiphage serum is always used at a final dilution of $1:10^3$.)

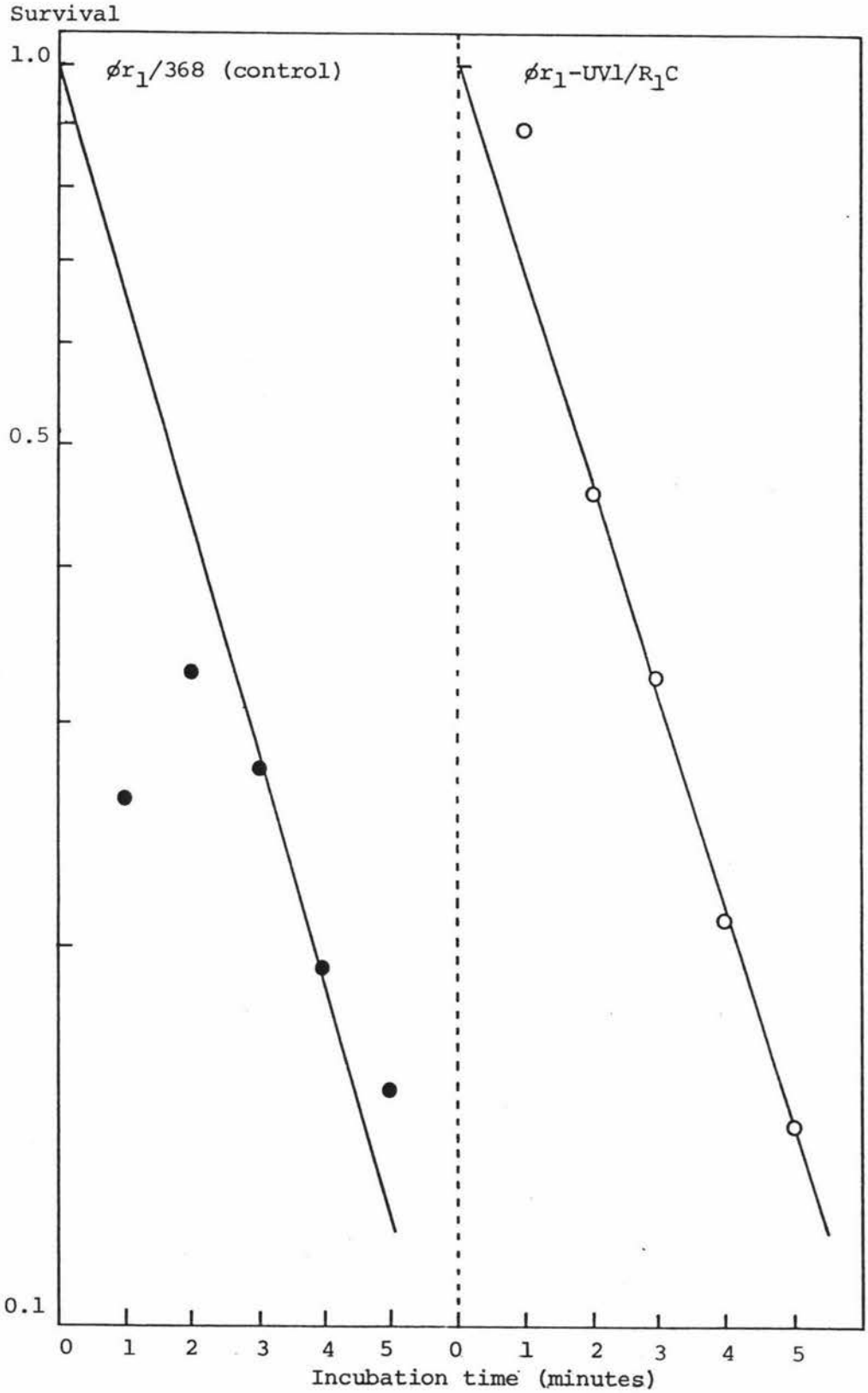


Figure 21. Neutralization of ϕr_1-UV1/R_1C by A/S r_1/368 at 30°C.

(Notes: The neutralization constant of undiluted A/S r_1/368 with respect to the homologous phage is estimated from the graph to be k = 2600/min. This antiphage serum is always used at a final dilution of $1:10^4$.)

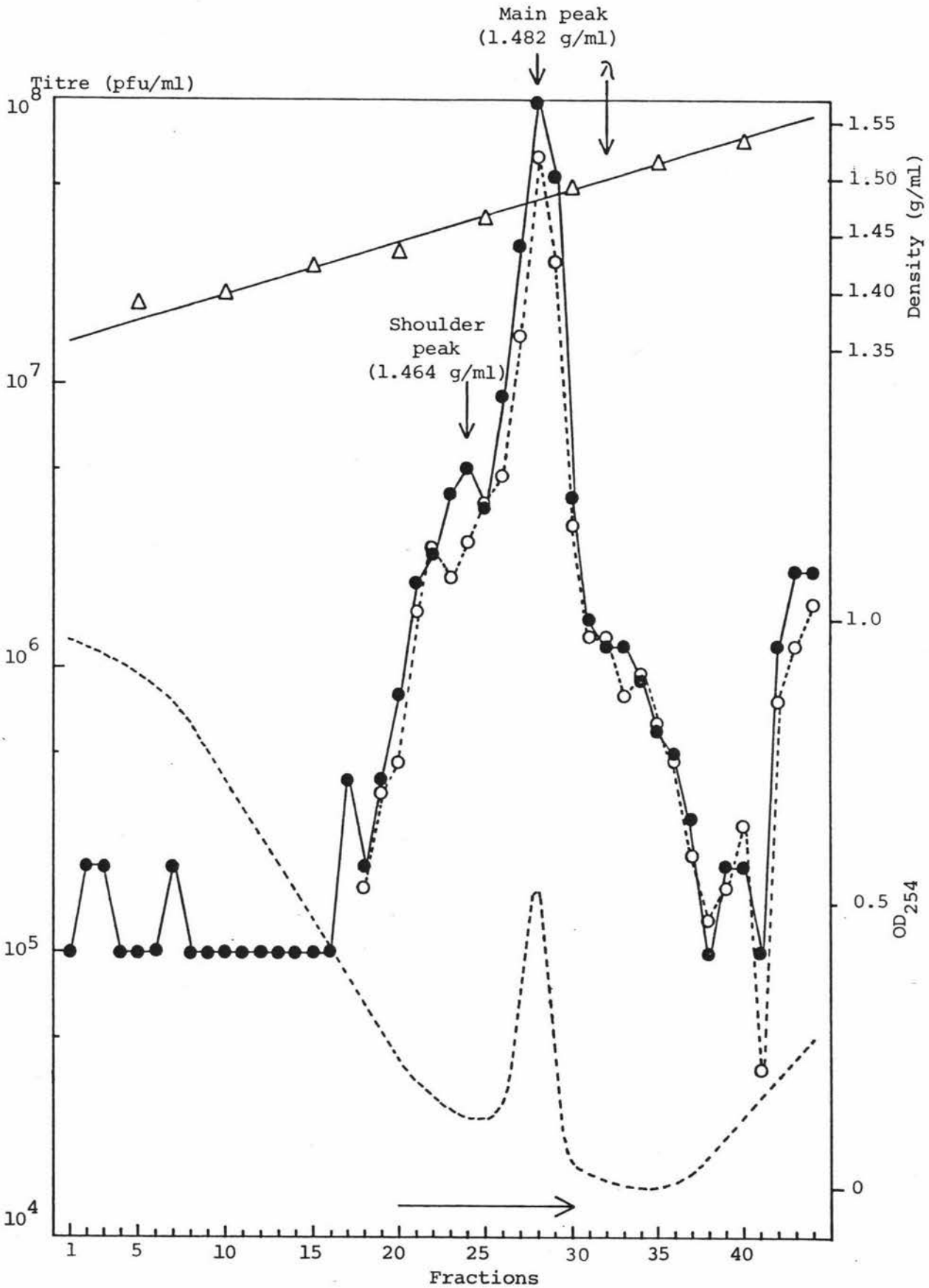


Figure 22. CsCl run of ϕr_1-UV1/R_1C stock.
(Symbols are as for Figure 5.)

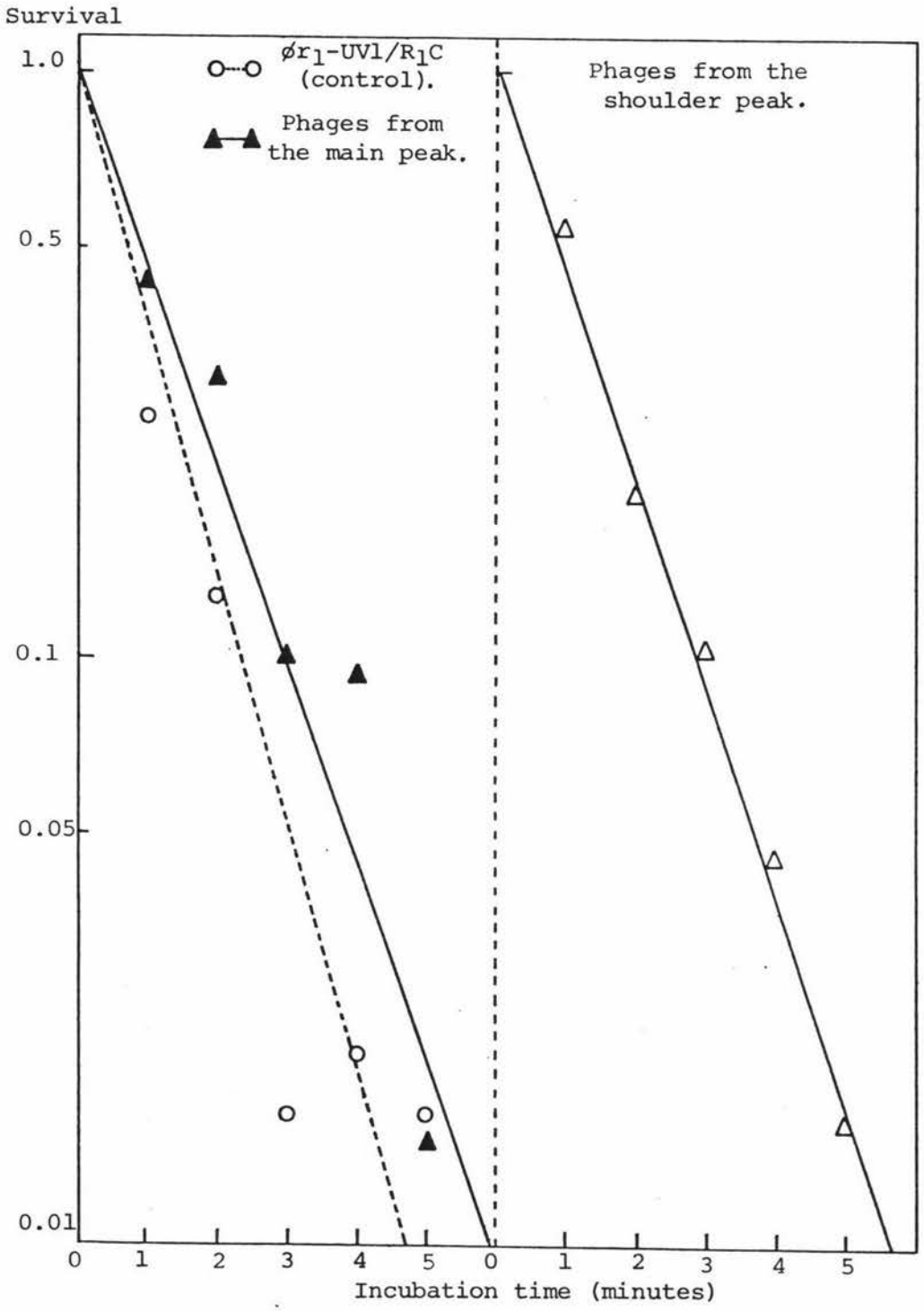


Figure 23. Neutralization of phages from CsCl run of $\text{ør}_1\text{-UV1/R}_1\text{C}$ stock by A/S $r_1\text{-UV1/R}_1\text{C}$ at 30°C.

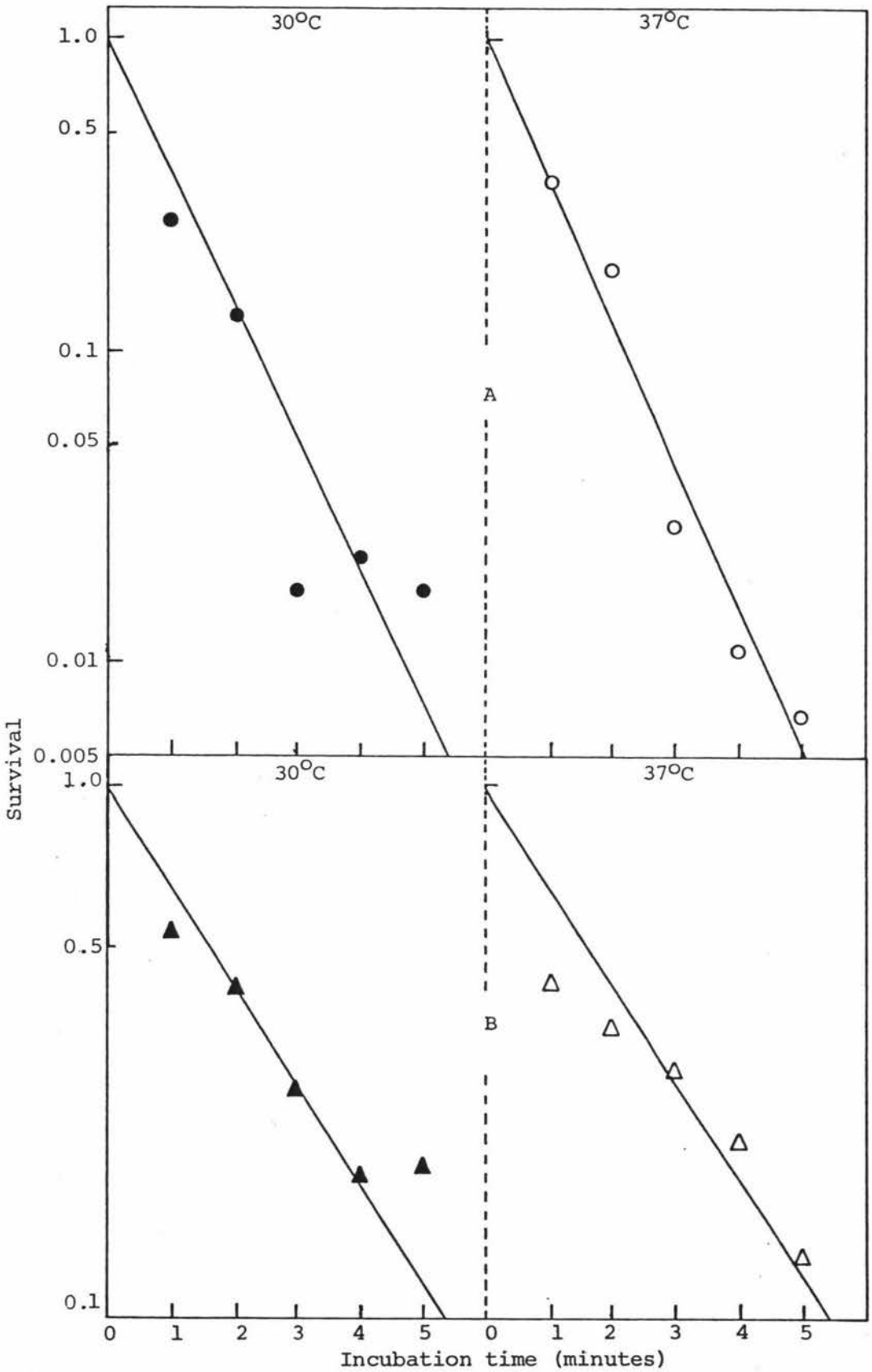


Figure 24. Neutralization kinetics of phages by homologous antiphage sera at 30°C and at 37°C.

A. Inactivation of ϕr_1-UV1/R_1C by A/S r_1-UV1/R_1C (at k = 0.66/min).

B. Inactivation of ϕr_1/368 by A/S r_1/368 (at k = 0.26/min).

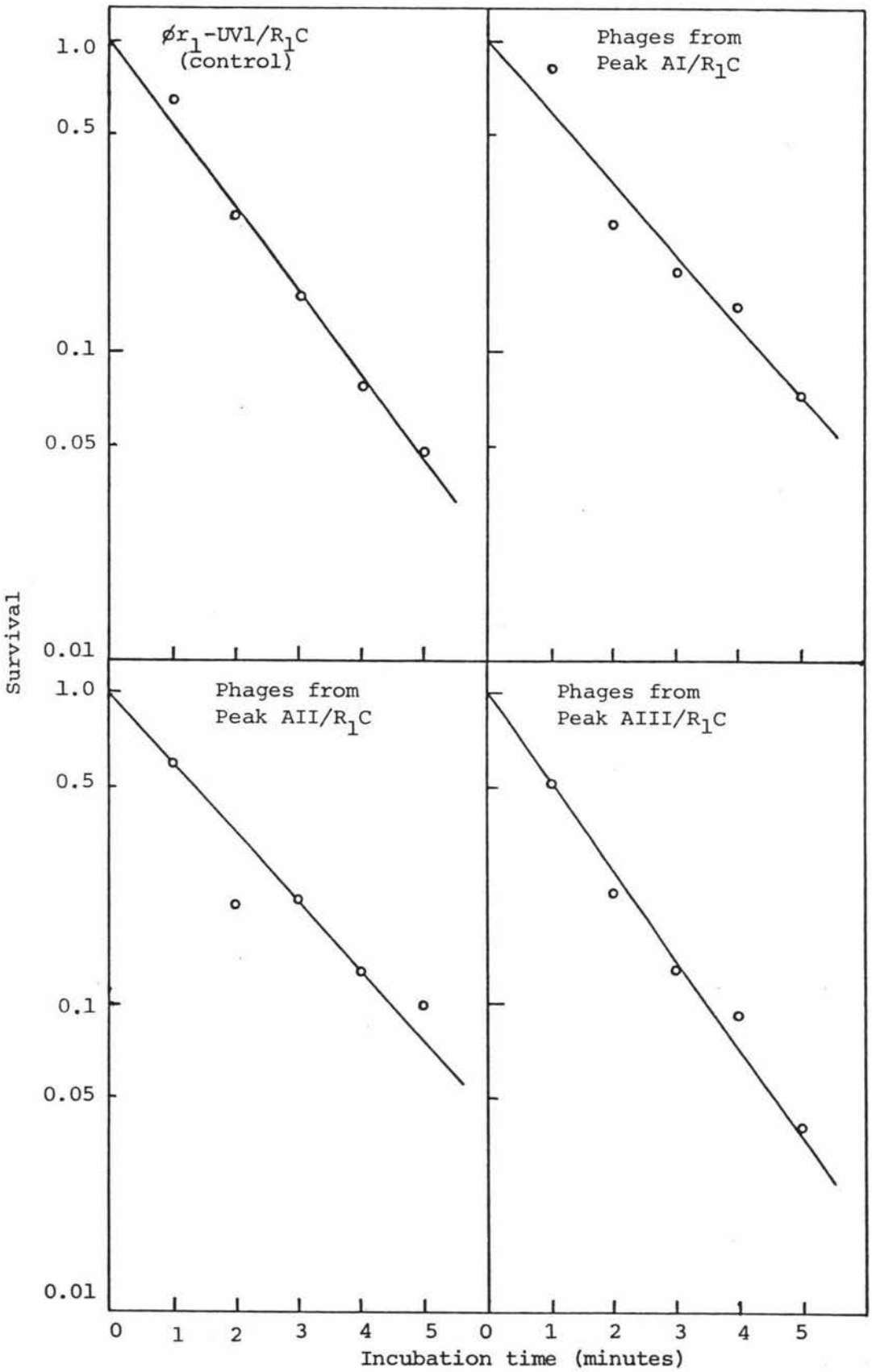


Figure 25. Neutralization of phages from CsCl run of R_1 lysate A by A/S r_1-UV1/R_1C at 30°C.

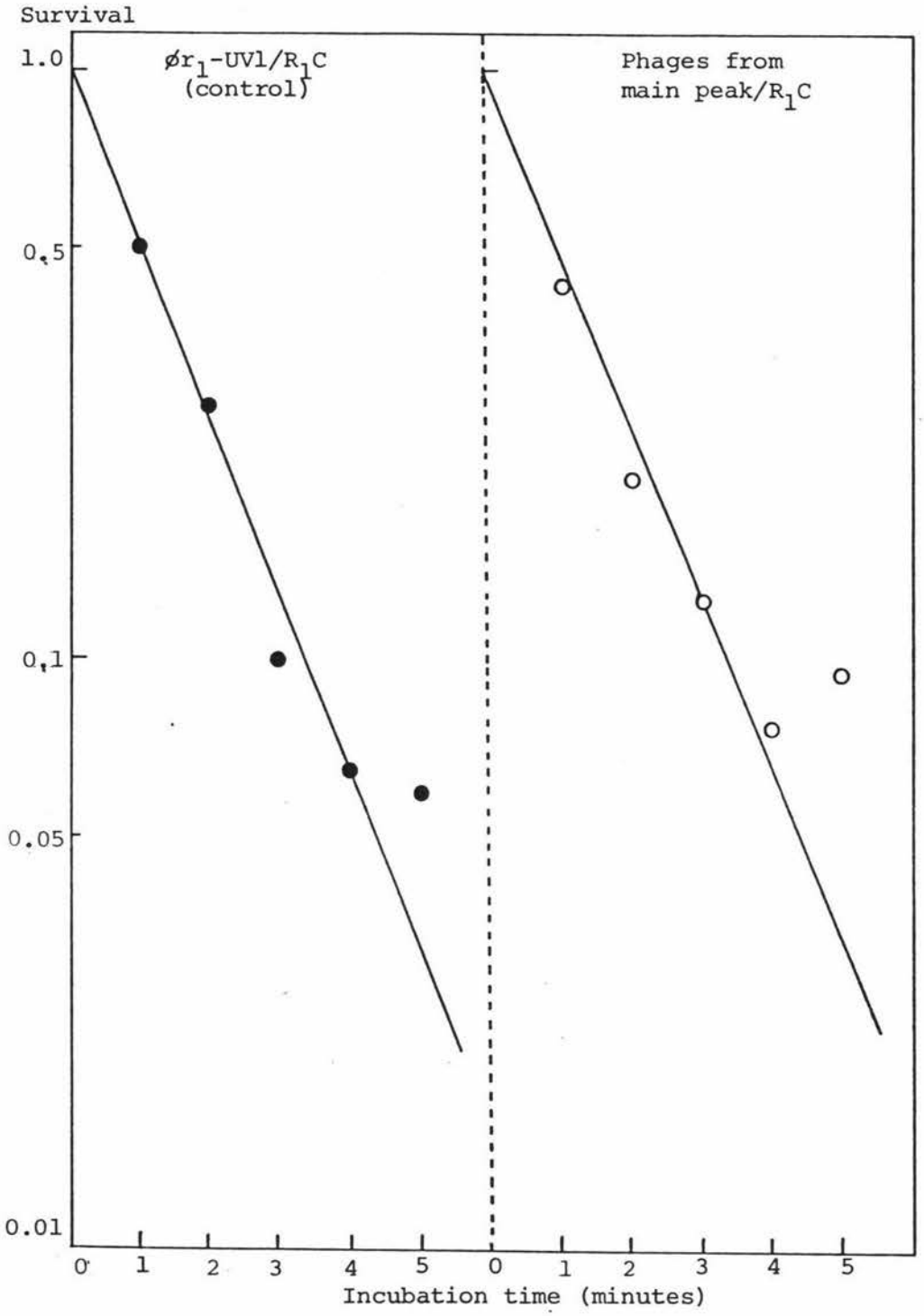


Figure 26. Neutralization of phages from CsCl run of R_1r lysate by A/S r_1-UV1/R_1C at 30°C.

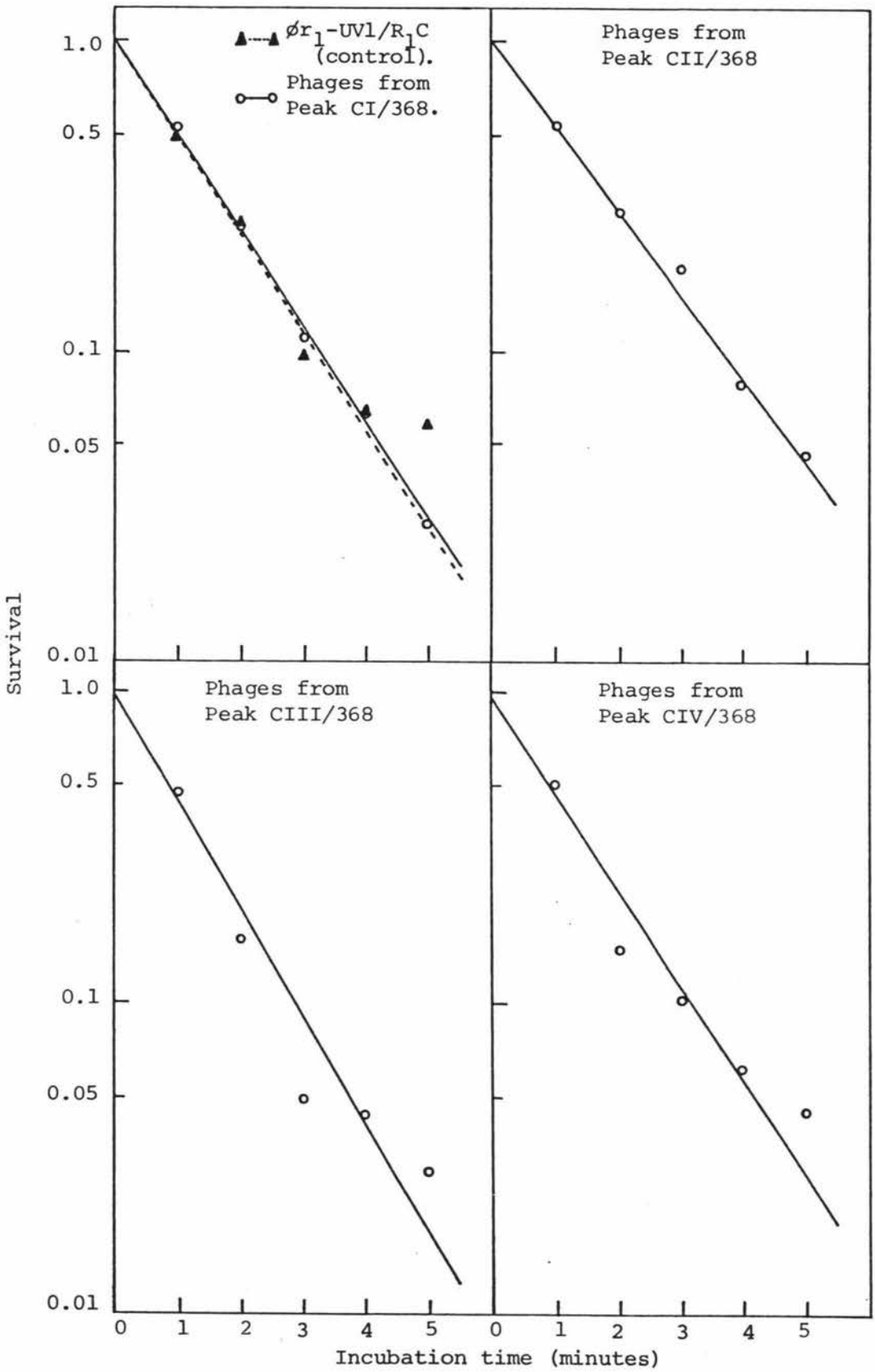


Figure 27. Neutralization of phages from CsCl run of 368(r_1) lysate by A/S r_1-UV1/R_1C at 30°C.

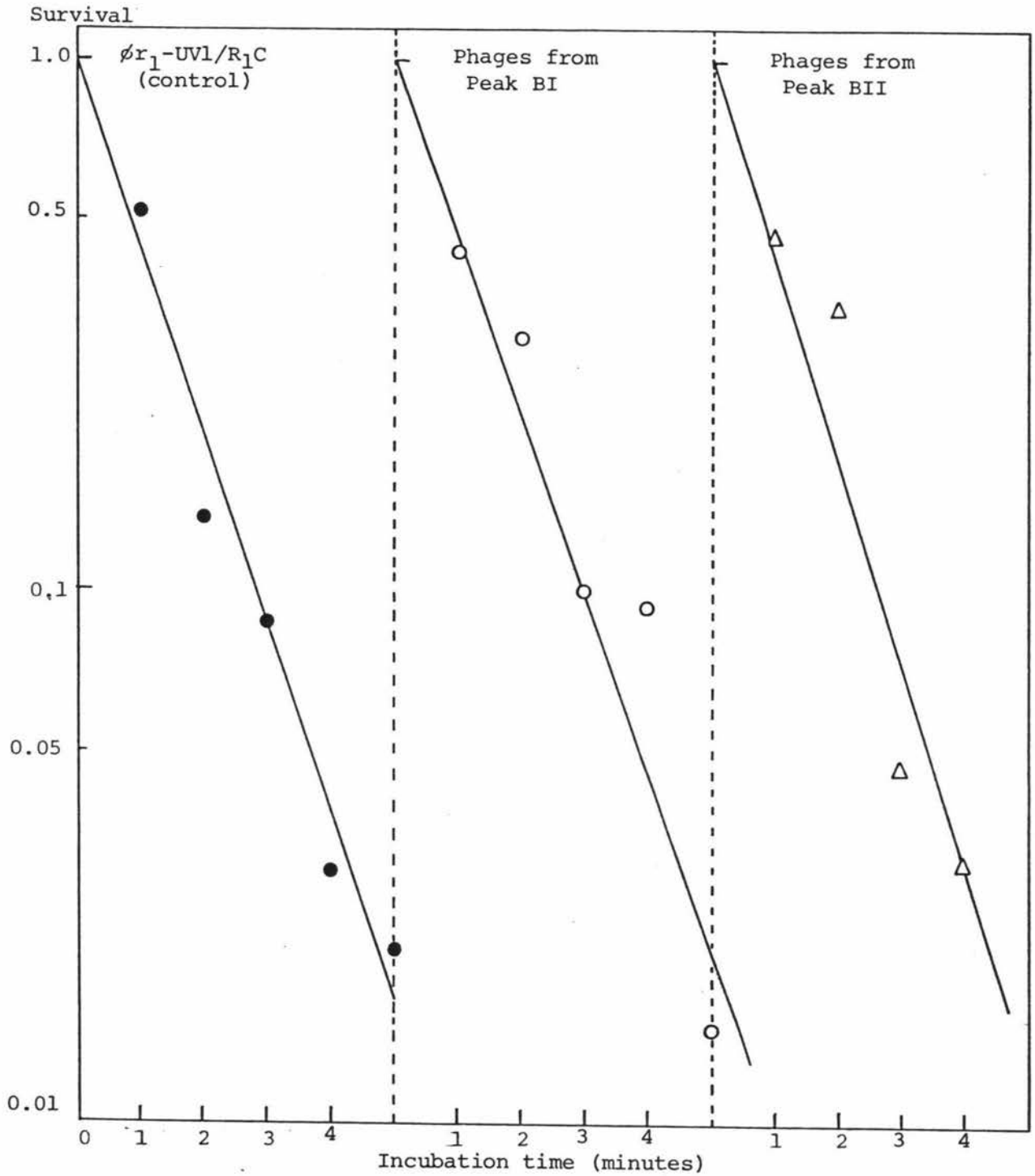


Figure 28. Neutralization of phages from CsCl run of R_1 lysate B by A/S r_1-UV1/R_1C at 37°C.

phages by their homologous antiphage sera done at different incubation temperatures commonly used in this work.

Figure 25 shows the antiserum tests of phages isolated from CsCl run of R_1 lysate A. As seen, the serological identity of the phages from the different peak fractions adds weight to the argument that the satellite Peaks AII and AIII (see Figure 5) are artifacts. The same may be said of satellite Peaks CII, CIII and CIV in CsCl run of 368(r_1) lysate (see Figure 27). We have already seen in Figures 11 and 12 that the phages induced from strains R_1r and 368(r_1) have a buoyant density of 1.487 g/ml, very close to that of the original lysogenizing phage which averages 1.485 g/ml; their serological idenity is here confirmed in Figures 26 and 27. In other words, the same input phage type is regained on induction of both strains R_1r and 368(r_1) (Mankiewicz, *et al*, 1969).

As shown in Figure 28, phages at Peaks BI and BII in CsCl run of R_1 lysate B (see Figure 13) are antigenically identical; that is to say, there is no apparent difference in tail structure of the two phage samples. However there is, one may argue, a possible difference in other structural proteins of the two phage particles. To check this possibility entails the SDS-gel electrophoretic analysis of their structural proteins as is next described.

7. *SDS-gel electrophoresis of R_1 phage proteins.*

Gels were tried at concentrations of 7.5, 10 and 15%, and at a 5-15% gradient. Of all, the best resolution of protein bands was achieved in 10% gels as are shown in Plates A to C. Coliphage T4 was included as a standard in all runs (Vanderslice & Yegian, 1974; Terzaghi, personal communication).

In Plate A, the phage stocks Peak BI/R_1C, Peak BII/R_1C, Peak BI/368 and Peak BII/368 were each propagated from a purified plaque. As inferred from the idenity in their gel patterns, with the exception of a few variations in the intensity of the bands, the

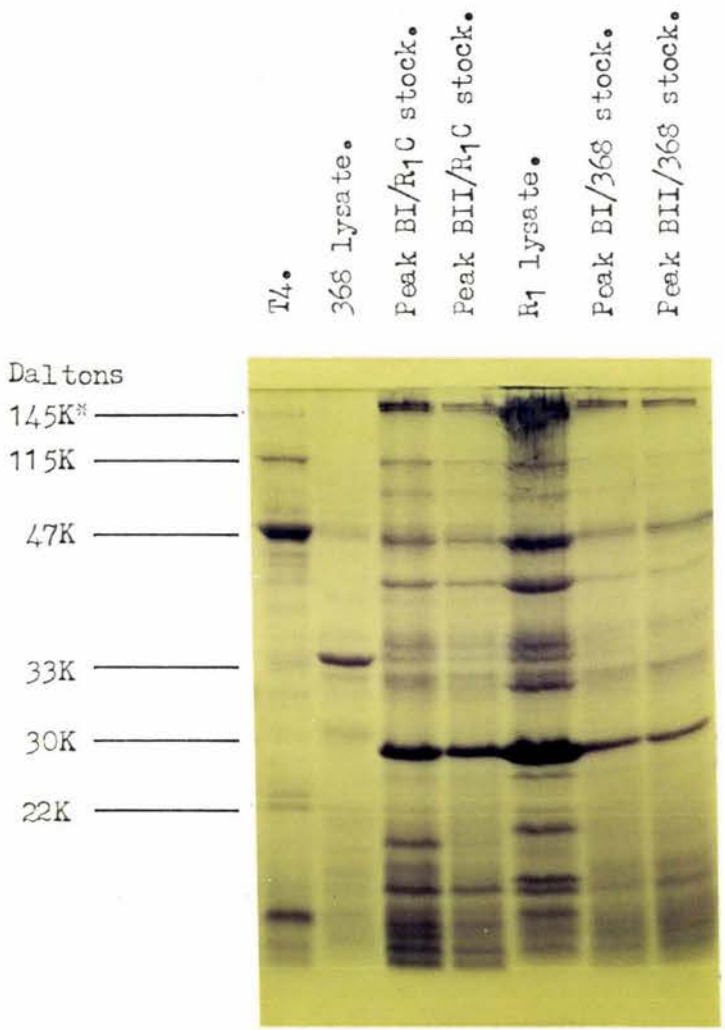


Plate A. SDS-gel electrophoresis of phage stocks and lysates.

* 1K = 1,000.

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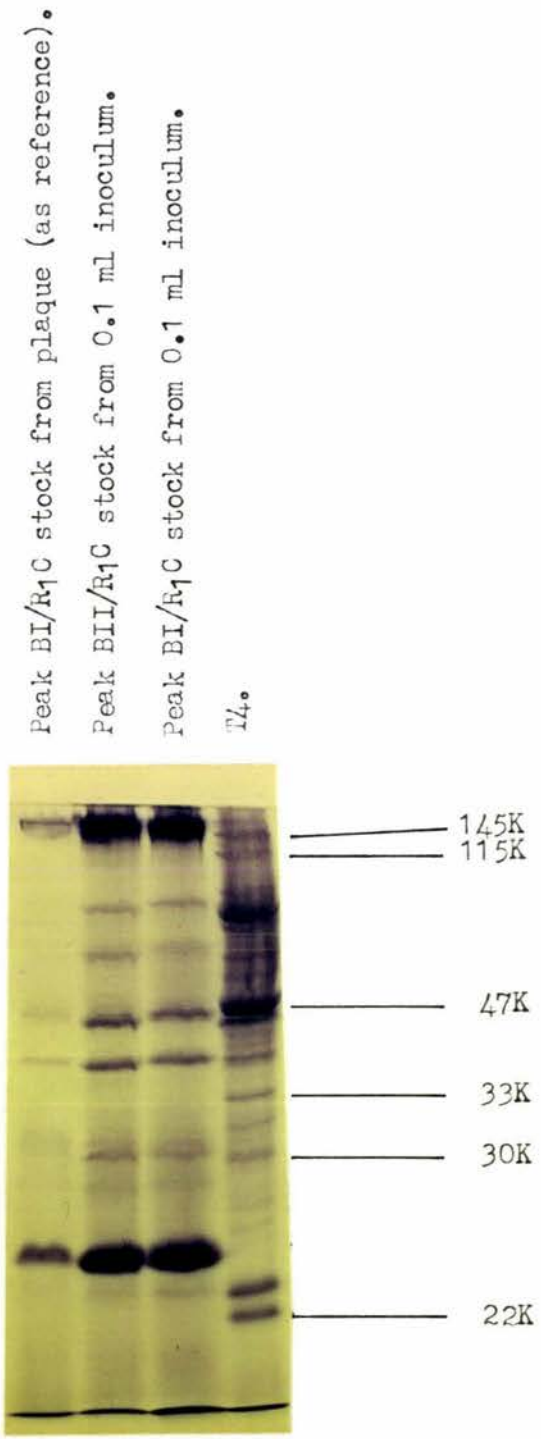

Plate B. SDS-gel electrophoresis of phages isolated from CsCl run of R_1 lysate B. (See Figure 13.)

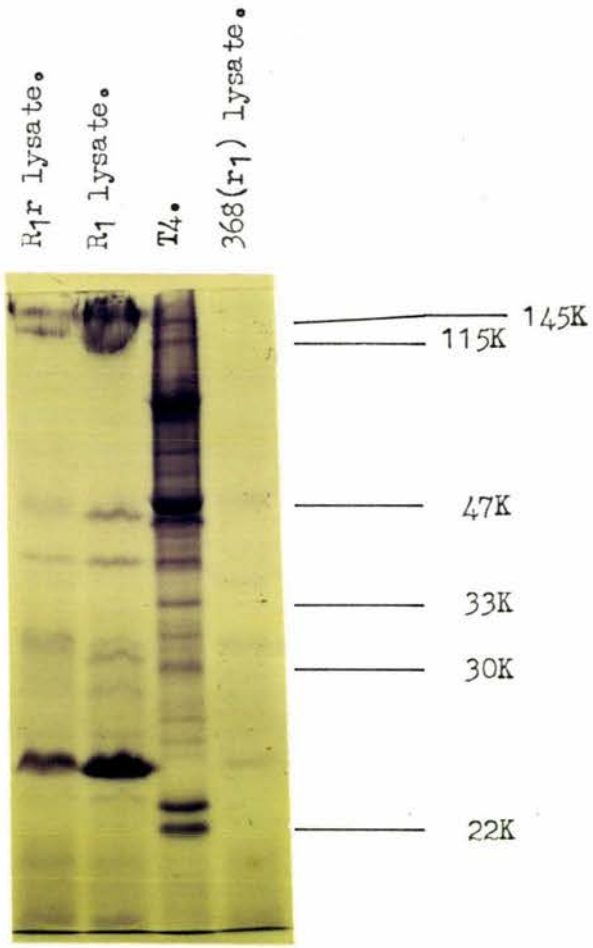

Plate C. SDS-gel electrophoresis of UVL induced lysates.

phages at shoulder Peak BII and at main Peak BI in CsCl run of R_1 lysate B (see Figure 13) are identical, irrespective of the propagating hosts used. To safeguard against the theoretical possibility of picking the same phage-type plaques in the above phage preparations, separate stocks of Peak BI/R_1C and Peak BII/R_1C were prepared from 0.1 ml inoculum of, respectively, Peak BI and Peak BII fractions, and were further analysed in SDS-gel as shown in Plate B. As seen in the latter plate, there is no discernible difference in gel patterns between Peak BI and Peak BII phages, irrespective of how the stocks were prepared. In other words, there seems to be only one phage type in the R_1 lysate. This inference is confirmed by the overall identity in gel patterns of the newly prepared R_1 lysate and the phage stocks in Plate A (with the exception of 3 bands at the low molecular weight end). Further verification of the single lysogenic nature of strain R_1 comes from the identity in protein bands between R_1 and R_1r lysates as shown in Plate C.

Minor variations in the positions and/or band densities noted above are considered to be experimental artifacts, as have been observed in the T4 standards (compare Plate A and Plates B or C).

However, the gel pattern of the 368 lysate differs considerably from that of either R_1 lysate or phage stocks as clearly seen in Plate A. The very finding of protein bands in the 368 lysate and their occasional resemblance to bands of the phage stocks imply the presence of phage-related entities in the former lysate. With regards to the gel pattern of 368(r_1) lysate in Plate C, the bands are too faint to be of analytical value.

It is not inconceivable that similarity in protein species may not necessarily reflect similarity in functions. This idea is envisaged in the different groupings of the same lactic streptococcal phages based on host-range and serological tests (Nichols & Hoyle, 1949; Wilkowske, *et al*, 1954a & 1954b). As a final check on the physical identity of the Peak BI and Peak BII phages, host-specificity tests of the latter were next performed.

8. *Host-specificity tests of R_1 phages on different strains of lactic streptococci.*

All the bacterial strains tested in Tables VIII and IX were each grown from 1% inoculum in 0.5% lactose-M17 broth, incubated at 22°C for 16 hours. Some, which showed slow growth, were incubated for 40 hours instead. That is, in the context of this work, they are said to be overnight cultures (in contrast to aged cultures); as such variability in their plating efficiency, as exemplified by overnight cultures of R_1C and 368 noted above, are anticipated. Further, it should also be noted that 368 and SK_{11} are derivative strains of AM_1 (Lowrie, 1974; Terzaghi & Terzaghi, 1978; see Figure 33). With the exception of ML_8 which is a *Str. lactis* strain, the rest are *Str. cremoris* strains.

If one were screening for indicator strains for lysates of R_1 and R_1r from Table VIII, at first glance it would seem that strains R_1C and R-G4 would best serve the purpose. But when the lysates were titrated individually on the respective strains, no plaque formation was obtained in R-G4 seeded plates, though titres of R_1 and R_1r lysates on R_1C were found to be, respectively, 4×10^8 and 4×10^9 pfu/ml. Such ambiguous response in spot tests as exemplified by strain R-G4 is also shared by strain SK_{11}, and is perhaps a phenomenon of phage killing.

An attempted search for indicators for the 368 lysate in Table VIII located two possible candidates, namely E_8 and SK_{11}. However, no plaques were obtained when the lysate was plated on aged cultures of the two strains as well as on R_1C. Even the picking and re-plating of the plaques from the SK_{11}-seeded plate (*i.e.* plaques of 368 lysate spot-tested on SK_{11} in Table VIII) gave no further plaque formation. Since plaques from 368 lysate have been obtained (for instance on SK_{11} and R_1C in CsCl run of the lysate mentioned in subsection 5), coupled with the UVL induction response of 368 (see Figure 4B; Keogh & Shimmin, 1969), and the presence of phage-type proteins in SDS-gels of the lysates, it is possible that 368 lysate contains defective phages.

In Table IX, it is seen that phages at Peak BI and Peak BII fractions share identical host specificity. This is confirmed by the identity in host specificity of Peak BI/R_1C and Peak BII/R_1C stocks,

TABLE VIII SPECTRUM OF LYTIC RESPONSE OF LACTIC STREPTOCOCCAL STRAINS TO DIFFERENT INDUCED LYSATES [b]

Bacterial strains:	Lysates:			
	R_1 lysate (4×10^8 pfu/ml)	R_1r lysate (4×10^9 pfu/ml)	368 lysate ($\geq 1 \times 10^2$ pfu/ml)	368(r_1) lysate (2×10^8 pfu/ml)
104	-	-	-	-
108	-	-	-	-
130	-	-	-	-
130JD	-	+	-	-
158	+	+	-	-
158-x-milk	-	+*	-	-
166	+	+	-	-
166-G7	-	-	-	-
168	-	-	-	-
240	-	-	-	-
286	-	-	-	-
316	-	-	-	-
368	+	+	-	+++
368(r_1)	+*	+*	-	-
386	-	-	-	-
398	+	+	-	-
402	+	+	-	-
AM_1	-	+	-	+++
AM_2	-	+	-	-
E_8	-	-	-	-
G-P	-	+*	-	++
H_1	-	-	-	-
H_2	-	-	-	-
ML_8	-	-	-	-
R_1	-	-	-	-
R_1C	++	++	-	-
R_1r	-	-	-	-
R-G4	++	++	-	+++
SK_{11}	-	-	+	+
SK_{11}-G	-	-	-	-
114	-	-	-	-

[b]
- : no observable lysis.
+ : discernible lysis with occasional few plaque formation.
++ : distinct lysis, sometimes with plaques.
+++ : heavy lysis.
* : confirmed lysis from without.

TABLE IX SUSCEPTIBILITY OF LACTIC STREPTOCOCCAL STRAINS TO PHAGES ISOLATED FROM CsCl RUN OF R_1 LYSATE B [c] (Refer to Figure 13)

Bacterial strains	Phage Samples: Peak BI fractions (1×10^6 pfu/ml)	Peak BII fractions (5×10^4 pfu/ml)	Peak BI/ R_1C stock (2×10^8 pfu/ml)	Peak BI/ 368 stock (2×10^7 pfu/ml)	Peak BII/ R_1C stock (2×10^8 pfu/ml)	Peak BII/ 368 stock (2×10^7 pfu/ml)
104	-	-	-	-	-	-
108	-	-	-	-	-	-
114	-	-	-	-	-	-
130	-	-	-	-	-	-
130JD	-	-	-	-	-	-
158	-	-	-	-	-	-
158-x-milk	-	-	-	-	-	-
166	-	-	+	-	+	-
166-G7	-	-	-	-	-	-
168	-	-	-	-	+*	-
240	-	-	-	-	-	-
286	-	-	-	-	+*	-
316	-	-	-	-	-	-
368	-	-	++	+++	++	+++
368(r_1)	-	-	+*	+*	+*	+*
386	-	-	-	-	-	-
398	-	-	-	-	-	-
402	-	-	-	-	-	-
AM_1	-	-	-	++	-	++
AM_2	-	-	-	-	-	-
E_8	-	-	-	-	-	-
G-P	-	-	-	-	-	-
H_1	-	-	-	-	-	-
H_2	-	-	-	-	-	-
ML_8	-	-	-	-	-	-
R_1	-	-	-	-	-	-
R_1C	+++	++	++	+	++	+
R_1r	-	-	-	-	-	-
R-G4	-	-	+	++	+	++
SK_{11}	-	-	-	+	-	+
SK_{11}-G	-	-	-	-	-	-

[c] Symbols are as for Table VIII.

and of Peak BI/368 and Peak BII/368 stocks. The apparent variabilities observed between the three pairs were due to differences in phage titres and in the different propagating hosts used (as discussed below). In other words, phages of Peak BI and Peak BII are identical in respect to host range. The similarity in host specificities between R_1 and R_1r lysates and stocks of Peak BI/R_1C and Peak BII/R_1C further supports the single-lysogenic nature of strain R_1.

In summary, phages induced from R_1 (designated ϕr_1) can plate on R_1C and at a lower e.o.p. (efficiency of plating) on 368 (see Table X). ϕr_1, which were propagated lytically on R_1C, were referred to as $\phi r_1/R_1C$, and likewise for $\phi r_1/368$. R_1r and $368(r_1)$ are strains R_1C and 368 lysogenized, respectively, by $\phi r_1/R_1C$ and $\phi r_1/368$ (see Figure 33). Carrying it one step further, ϕr_1, $\phi r_1/R_1C$, $\phi r_1/368$ and phages induced from R_1r and $368(r_1)$ are, in retrospect, very similar in respect to their phenotypic features such as morphology (Georghiou, *et al*, manuscript in preparation), buoyant density (see subsection 5) and protein constituents (see subsections 6 & 7). An exception to the rule is given in Table VIII by the differences in lytic spectra between $368(r_1)$ lysate and lysates of either R_1 or R_1r. These are even more pronounced between phages propagated on R_1C and 368 shown in Table IX. This observed phenomenon of host-induced modification - restriction (see Table X) by strains R_1C and 368 (Georghiou, 1976) has been demonstrated to be physiologically labile by Terzaghi and Terzaghi (1978). In fact, the use of aged indicator cultures for better plating of heterologous phages, as evidenced in this work, originated from the latter finding.

The host-induced variation of ϕr_1 in R_1C-368 system is portrayed in Table X (Luria, 1953; Arber, 1965; Revel & Luria, 1970; Arber, 1971). A point to note is the frequent observed variability in EOP of a stock on the same overnight culture of heterologous host; the cause of such variation is not known. In general, the EOP on the heterologous host 368 proves to be more variable and relatively lower than on the heterologous host R_1C; the reason could perhaps be due to additional restriction or interference imposed by the defective prophage present in 368 cells (Lederberg, 1957; Bertani, 1958; Tomizawa & Ogawa, 1967; Matsubara, 1972; Behnke & Malke, 1978a & 1978b).

TABLE X EFFICIENCY OF PLATING (EOP) OF PHAGES ON *STR. CREMORIS* STRAINS R_1C AND 368 [d]

Phage [e]	EOP		Phage [e]	EOP	
	R_1C	368		R_1C	368
ϕr_1 (induced from 368(r_1))	$< 1.0 \times 10^{-8}$	1.0	ϕr_1 (induced from R_1)	1.0	2.0×10^{-7}
$\phi r_1/R_1C$	1.0	1.0×10^{-7}	$\phi r_1/368$	1.5×10^{-5}	1.0
$\phi r_1.R/368$	1.5×10^{-5}	1.0	$\phi r_1.3/R_1C$	1.0	2.5×10^{-5}
$\phi r_1.R.3/R_1C$	1.0	3.0×10^{-7}	$\phi r_1.3.R./368$	1.5×10^{-5}	1.0
$\phi r_1.R.3.R/368$	1.5×10^{-5}	1.0	$\phi r_1.3.R.3/R_1C$	1.0	4.0×10^{-5}
$\phi r_1.R.3.R.3/R_1C$	1.0	3.7×10^{-5}	$\phi r_1.3.R.3.R/368$	2.0×10^{-5}	1.0

[d] The same overnight cultures of R_1C and 368 were used throughout, and the determinations of EOP were all done in one plating.

[e] *e.g.* '$\phi r_1.R.3.R.3/R_1C$' means that the phage ϕr_1 has been propagated on R_1C, 368, R_1C and 368 in that order, with R_1C as its current propagating (*i.e.* homologous) host.

An attempt to cure 368 of its putative defective prophage (as described in the text section) proved unsuccessful. As deduced from Table IX, perhaps the use of AM_1, SK_{11}, R-G4 or their non-lysogenic derivatives in place of 368 above may offer a 'cleaner' system. Or even perhaps the replacement of temperate ϕr_1 by a clear plaque-forming phage (for instance ϕr_1-UV1/R_1C) may provide a better system for a case study of the above observed host-induced variation so prevalent in lactic streptococci (Collins, 1956; Pearce, 1978).

9. *An attempt at curing of Streptococcus cremoris strain 368.*

An attempt was made here to obtain a cured derivative of 368 which could serve as an indicator strain for phages present in the 368 lysates. Also, by eliminating from the former its resident defective prophage, it was hoped to increase its plating efficiency for R_1 phages, and perhaps to improve on 368 in providing a more efficient system (*i.e.* R_1C- cured 368 system) for studying the host-induced variation noted above.

The technique employed was basically the same as that developed by Georghiou (1976); that is to UVL irradiate log-phase 368 cells (blended) to about 10^{-5} survival (*i.e.* about 25 seconds UVL exposure) (see Figure 2), and to screen for cured colonies among the surviving fraction. Many variations and modifications of the original technique were tried (though only the standard basic features are outlined in Experimental Procedures) on log-phase as well as on aged 368 cells, and with UVL doses ranging from 25 to 100 seconds. Though no 368 lysate-sensitive variants were located, suspected UVL insensitive derivatives were found in 2-3% of the survivors. These supposedly 'cured' derivatives were isolated and further tested for curing based on

(a) their UVL inducibility, and

(b) their sensitivity to parental 368 lysate.

The UVL inducibility tests of two isolates of the 'cured' 368 are shown in Figure 29A. With reference to the UVL induction response of parental strain 368, it is apparent that the test responses of the two isolates are less UVL sensitive than the former. In other words, it seems that the two isolates may represent cured derivatives of 368. However, when their sensitivity to parental 368 lysate were examined (using both

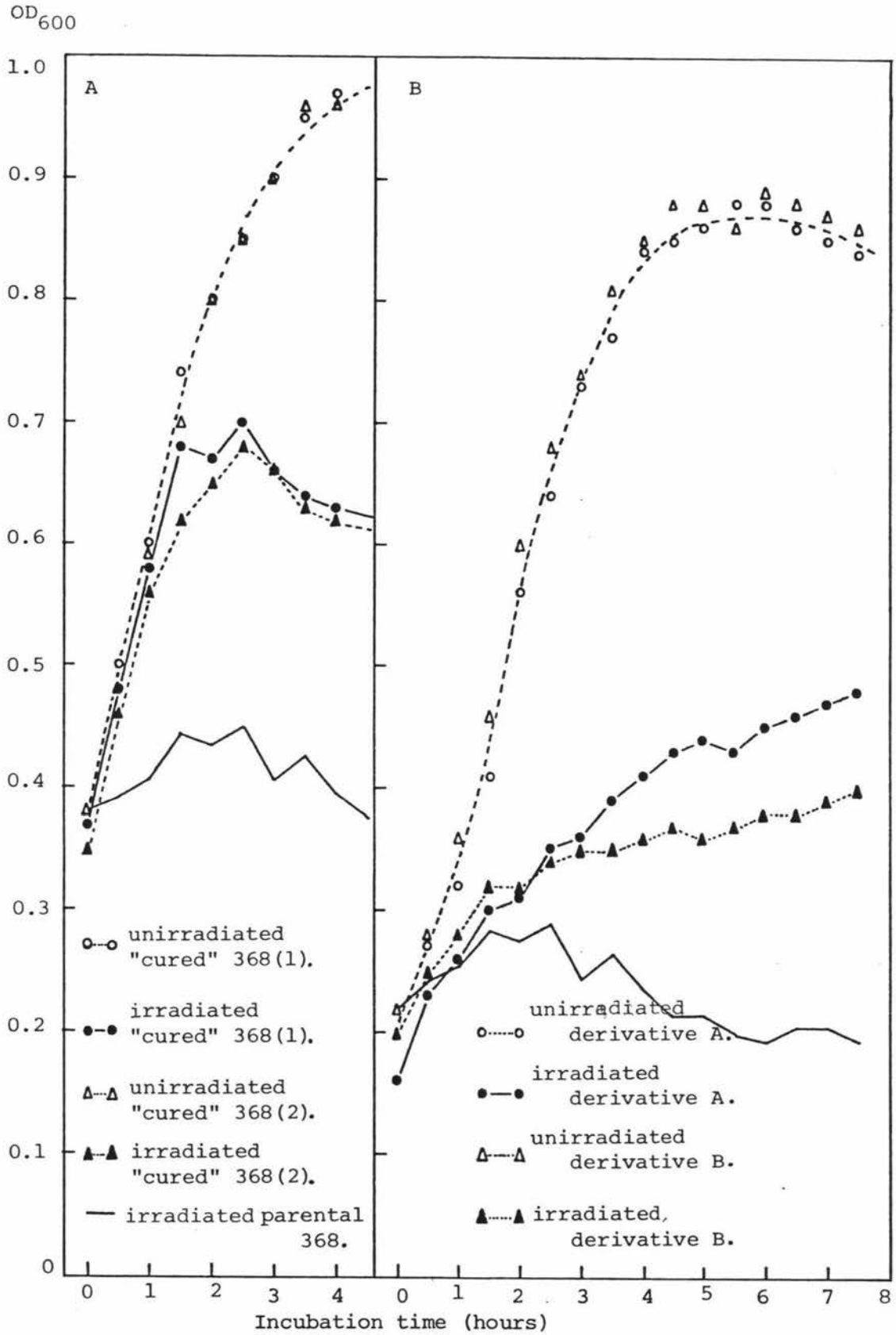

Figure 29. UVL inducibility tests of 'cured' derivatives of *Streptococcus cremoris* strain 368.
A. 'Cured' derivatives of *Str. cremoris* strain 368.
B. New derivatives from 'cured' 368(1).

their overnight and aged cultures), no discernible cell lysis nor plaque formation were observed. These results come as no surprise since 368 lysates were inferred above to be comprised of defective phages. The isolates were next tested in respect to their efficiency of plating of $\phi r_1/R_1C$ and $\phi r_1/368$; though inconclusive, the results obtained gave no positive indication of better plating efficiency of the isolates over that of parental 368. In fact, if there was any difference at all it would be for the worse.

To check if further UVL treatment of the 'cured' 368 isolates could produce the desired derivative, 'cured' 368(1) (refer to Figure 29A) was further subjected to curing as described in Experimental Procedures. Again, about 2-3% of the survivors were found to be relatively insensitive to UVL irradiation; two of these isolates were further tested for curing as shown in Figure 29B. As seen in the figures, the latter isolates (both the irradiated and the unirradiated samples) showed varied growth from that of their parental 'cured' 368(1). With regards to their efficiency of plating for $\phi r_1/R_1C$ and $\phi r_1/368$, no apparent difference was found between the primary and the seconary isolates.

10. *Ultraviolet light and nitrous acid mutagenesis of phages, and phage recombination experiments.*

From the foregoing sections on electron-microscope studies, CsCl density gradient analysis, serological tests, SDS-gel electrophoresis and host-specificity tests, it has conclusively been shown that *Str. cremoris* strain R_1 is a single lysogen, harbouring a single prophage in its genome. The ease with which an artificial lysogen such as R_1r (representing approximately the original R_1) can be constructed made suitable the study of lysogeny in these strains. In addition, the fact that strain R_1 has been commercially employed as a cheese starter favours it as a model strain for a case study of lysogeny in lactic streptococci of industrial importance. Therefore, an attempt was made at mapping experiments with the hope of gaining some insights into the lysogenic system of R_1 (Lieb, 1953a & 1953b).

A pure stock of temperate $\phi r_1/R_1C$ was made from R_1 lysate A obtained in Figure 3. When the phage was plated (*i.e.* on homologous

host R_1C) and incubated overnight at different temperatures, no plaque formation was obtained at 33°C and 36°C, while turbid plaques were observed at 22°C, 26°C and 30°C (Sozzi, *et al*, 1978). Of the latter, 26°C was chosen for use as a standard incubation temperature because it gave relatively big plaques with uniform turbidity. With reference to the lysogenic system of øλ-*E. coli* used here as a working model (in which the λ cistrons cI, cII and cIII located in the immunity region are responsible for the establishment and maintenance of lysogeny), C mutants of temperate ør_1/R_1C were collected by screening for clear-plaqued variants at 26°C (Kaiser, 1957; Levine, 1957). Since the spontaneous background mutation rate of the latter was found to be less than 1×10^{-7}, UVL and nitrous acid mutagenesis were employed (Bowen, 1953; Drake, 1970).

Shown in Figures 30 and 31 are the inactivation curves and the rates of induced clear-plaqued mutants of, respectively, UVL and nitrous acid treated stocks of temperate ør_1/R_1C. During the first 20 seconds of UVL irradiation, the induced mutation rate (if any) was less than 1×10^{-7} survivors; similarly, the rate was less than 1×10^{-6} for the first 2 minutes of HNO_2 treatment. Overall, about 45 UVL-induced and 30 HNO_2-induced mutants were collected, designated respectively, ør_1-UV1/R_1C to ør_1-UV45/R_1C and ør_1-NA1/R_1C to ør_1-NA30/R_1C. However, it should be mentioned that none of these mutants could plate on R_1 cells. In fact, a UVL dose range of 60-120 seconds and HNO_2 treatment for up to 18 minutes on ør_1/R_1C could produce no plaque-former on R_1, at least at a rate of less than 1×10^{-6} survivors. Even further treatment of the clear-plaqued ør_1-UV1/R_1C by UVL and HNO_2 (as shown in Figure 32) could produce no phage mutant capable of growing on R_1 cells. In other words, no phage mutant was obtained which was insensitive to prophage immunity. Also, a point to note in Figures 30-32 is that ør_1-UV1/R_1C is more resistant and more susceptible to, respectively, UVL and HNO_2 treatment than its parental ør_1/R_1C.

All the 75 clear-plaqued mutants isolated above (unfortunately) formed indistinguishable plaque types. Collectively, their plaques (*i.e.* on R_1C) were clear, big, round and sharp-edged, and all gave rise to a halo on the third day of incubation; their yields in stocks were usually 10-100 fold higher than that given by their parental ør_1/R_1C.

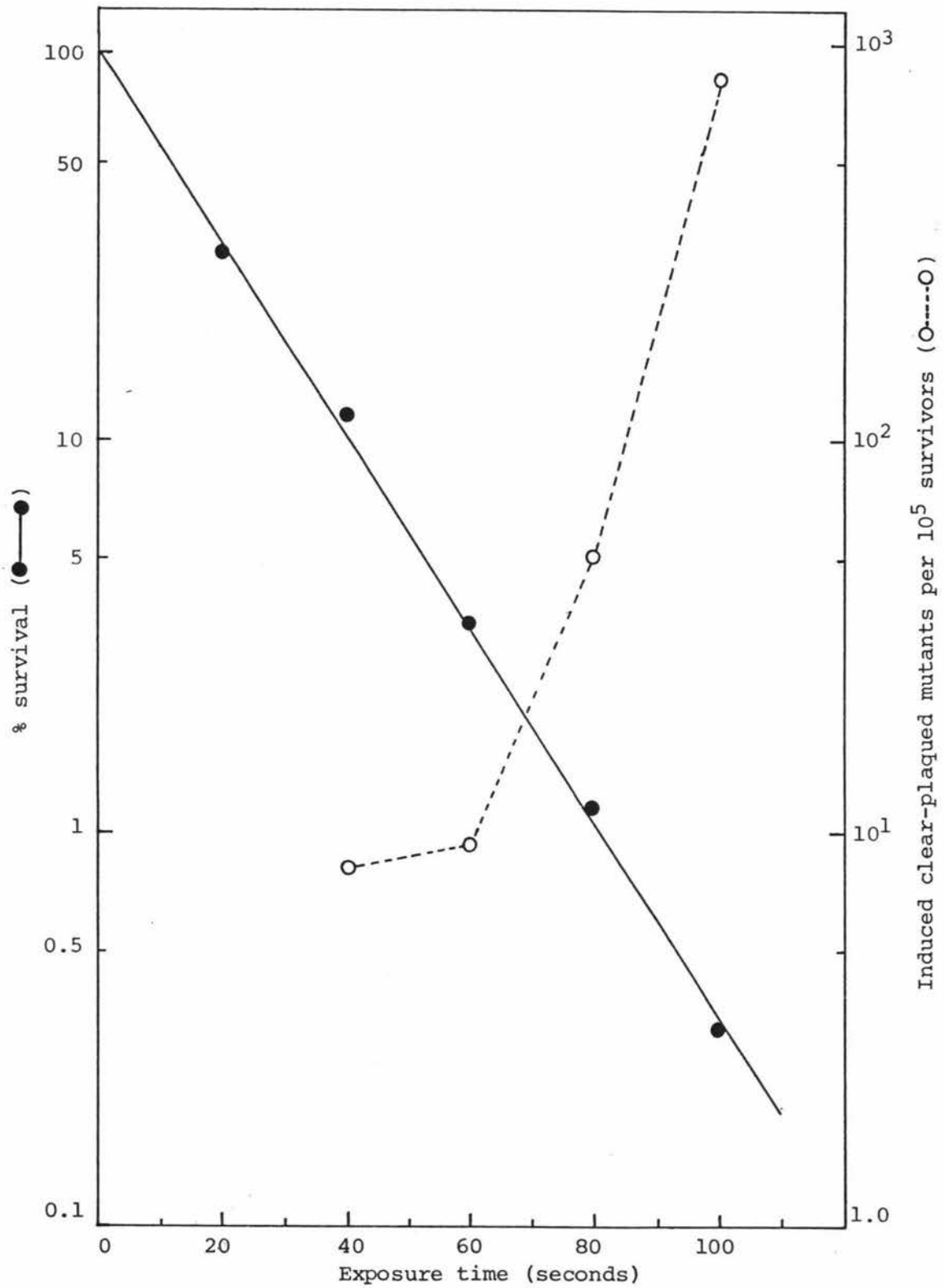

Figure 30. UVL irradiation of temperate $\phi r_1/R_1C$.
(Average one lethal hit per 17 seconds.)

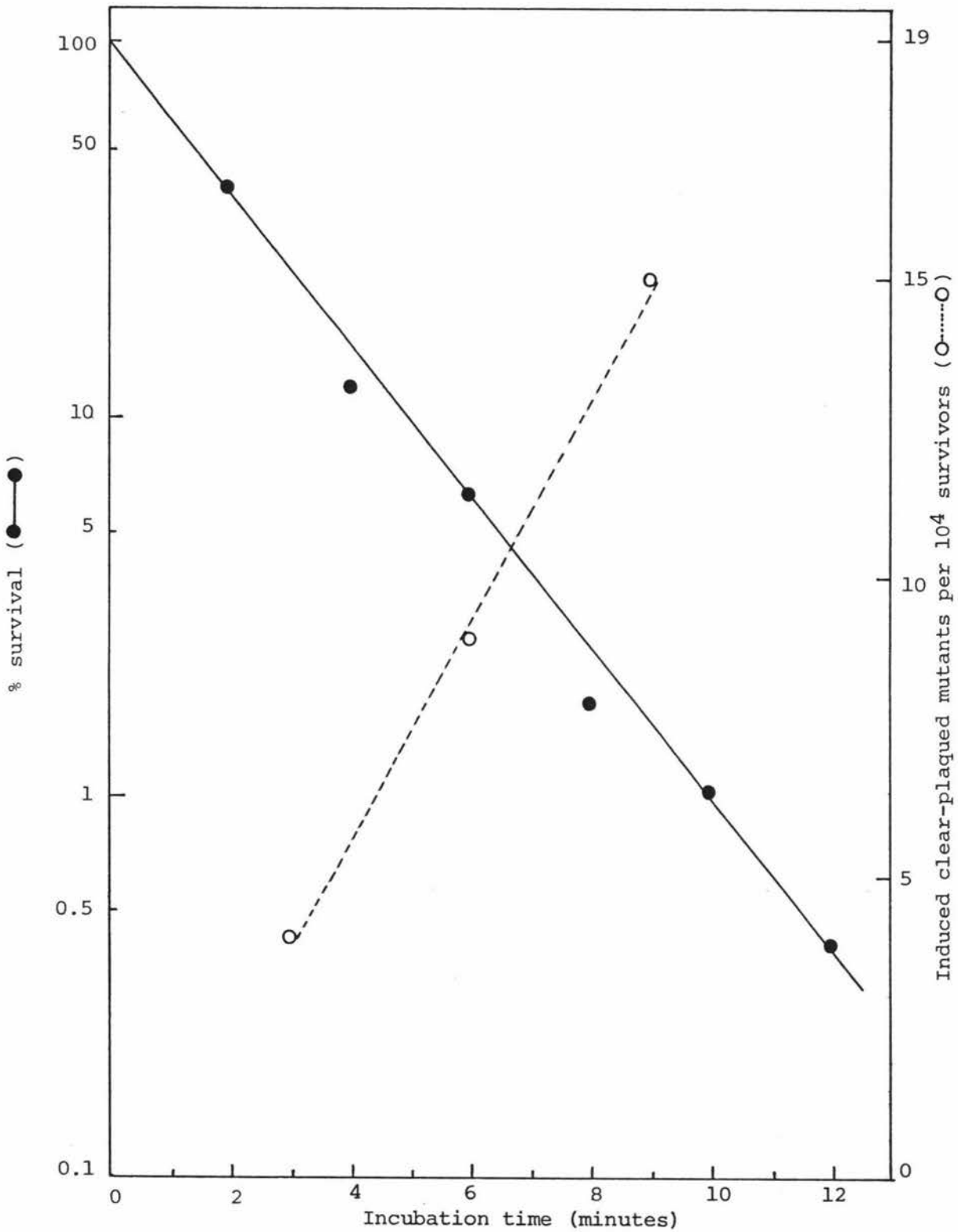

Figure 31. Nitrous acid treatment of temperate $\phi r_1/R_1C$. (Average one lethal hit per 2.1 minutes).

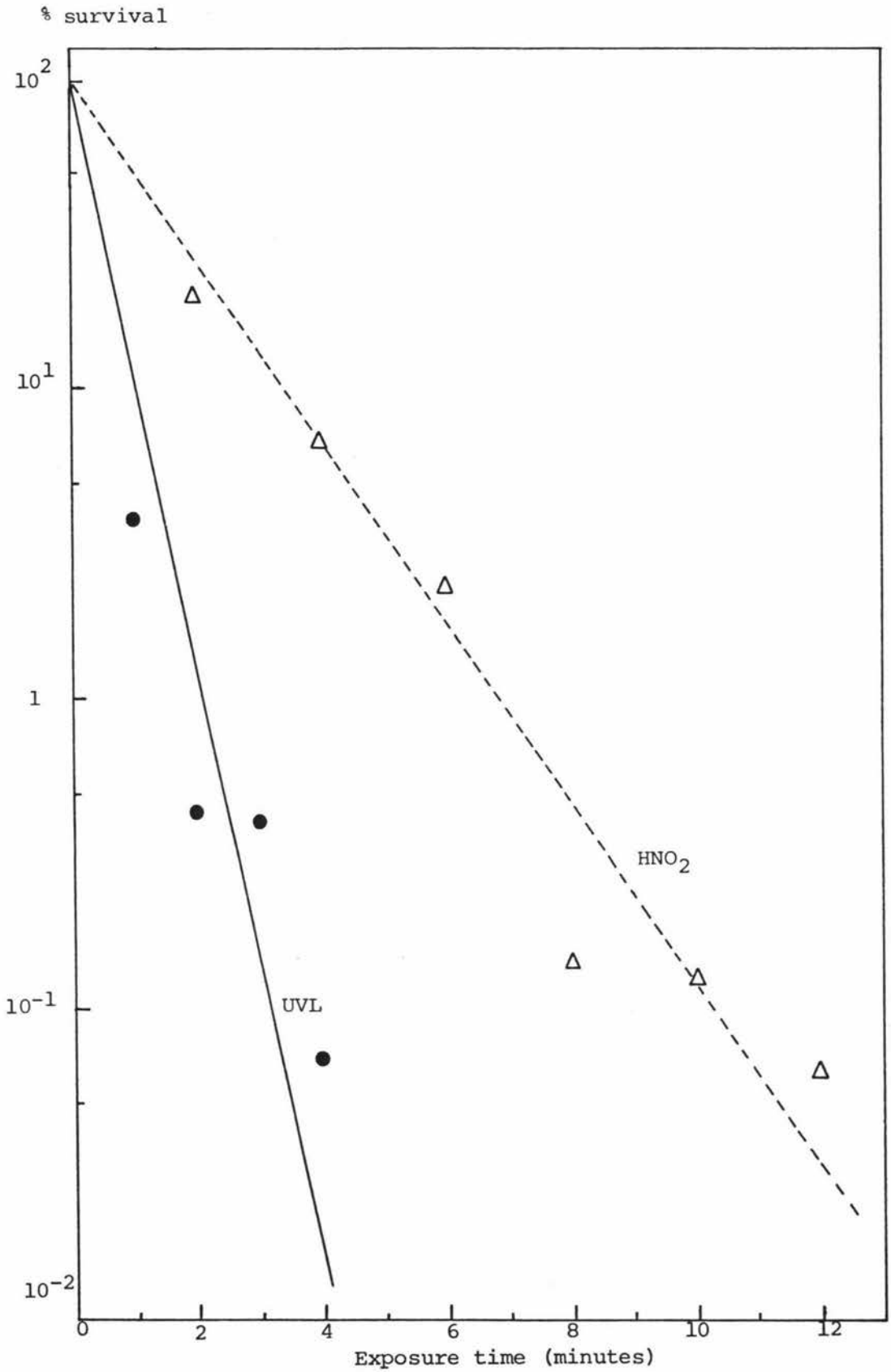

Figure 32. UVL and HNO_2 survival curves of the clear plaque-forming mutant, ϕr_1-UV1/R1C.
(Average one lethal hit per 24 sec for UVL and 1.5 min for HNO_2.)

All these mutants were isolated from varying mutagenic doses; for UVL, the exposure time ranges from 40-120 seconds, and 3-9 minutes for HNO_2 mutagenesis.

The general method used in the following recombination experiments is outlined in Experimental Procedures. Pairwise crosses between UVL and HNO_2 mutants were done and the progeny phages were screened for wild-type recombinants which formed turbid plaques on R_1C. Though not all of the mutant collection were tested, the results obtained indicates a general low frequency of wild type recombinants, at a rate of less than 1×10^{-5}. Occasionally, turbid-plaqued formers were scored at a frequency of about 1 per 10^4 progeny phages which could well reflect the reversion rate.

As a concluding remark, it needs to be mentioned that the inconclusive results noted above are not necessarily a true reflection of the ϕr_1-R_1C lysogenic system. At this stage, the immunity system operating in these strains remains unknown despite the initial objective of this work to elucidate it.

SECTION FIVE

CONCLUSION

Temperate phages can perpetuate in nature in either of two ways: they can exist lysogenically in a bacterial population or exhibit lytic growth in infected cells (Bertani, 1953). Besides its dependence on the genotypes of the phage and the host cell, the frequency of lysogenization is also under the strong influence of environmental factors and the physiological state of the cells (Lieb, 1953a; Lwoff, 1953; Bertani & Nice, 1954). During the process of lysogenization, the phage genome is believed to become inserted into the bacterial chromosome by a recombinational event (Campbell, 1962), and the phage thus assumes the prophage state and is perpetuated as an integral part of the host cell. Further, the existence of polylysogeny has also been demonstrated; it was found to occur in coliphage λ in *E. coli* K12 (Arber, 1960). For instance, mixed infection of λ and λb2 permitted the insertions of their genomes at the same site in the host chromosome (Zichichi & Kellenberger, 1963; Campbell, 1965). Similarly in *E. coli* K12 which was lysogenic for λ, the superinfecting λ genome was integrated into the resident prophage (Calef, *et al*, 1965). However, double lysogenization is not limited to only one site in the host chromosome: mixed infection of a strain of *E. coli* K12 with two λb2$^+$ exhibited insertions of the phage genomes in which the resulting prophages were located at two different attachment sites (Campbell, 1965). Appleyard (1954) constructed a polylysogenic strain which carried a total of six recognizable prophage alleles by super-infecting a strain of *E. coli* K12 (containing a defective λ prophage) with a weak virulent λ mutant. Such phenomena are not unique to coliphage λ but has also been observed for the coliphage P2, if not at a higher frequency. In fact, independent genetic studies have revealed the existence of at least three chromosomal sites where P2 prophage establishment can occur. In addition, polylysogeny is not limited to the carriage of a single type of phage. Rountree (1949b) has described a staphylococcal strain which carried two serological phage types; the same author has also reported a coagulase-positive strain of Staphylococcus carrying as many as five distinct phages (Rountree, 1949a). In general, though

polylysogeny is hard to achieve in the laboratory, nonetheless bacterial strains lysogenic for more than one phage type do exist in nature. Upon induced or spontaneous lysis, doubly lysogenic bacteria have been reported to liberate phages of both kinds plus their recombinants (Whitfield & Appleyard, 1958; Arber, 1960).

In light of the above, it is not unconceivable that the phenomenon of polylysogeny extends into the genus Streptococcus, particularly in lactic streptococci where lysogeny seems to be a rule rather than an exception. The strain of interest here is *Str. cremoris* strain R_1, and the initial questions asked (in the Introduction) are:

> Is R_1 a double lysogen?
>
> How related, or different, are the two induced phage types if the above is true?

Two additional questions were further raised in the Results and Discussions, *viz*

> Is the observed low yield of infective phages per induced cell an inherent feature of the lactic streptococcal lysates?
>
> Or is it that the lysates, particularly R_1 lysates, contain a mixture of perhaps two phage types with the majority phage type being inactive and non-adsorbable on indicators R_1C and 368?

Keogh (1973) found that the average burst size of lytically grown lactic streptococcal phages at 30°C ranged from 2-105 pfu per infected cell. The number of infective phage per induced cell, that is 0.9-2.2 pfu/cell lysed, as estimated for R_1 lysates in Table V is therefore not exceptional. The fact that such low phage yields, which vary from 0.3 - 4.8 pfu/cell lysed (see Table V), were obtained for lysates of R_1, R_1r and 368(r_1) irrespective of the inducing agents used (either UVL or mitomycin C as in Lowrie, 1974) suggest that it is an inherent feature of the induced lysates of these strains.

A probable cause for such observed low phage yields is poor phage assembly and the production of incomplete phages so commonly

found in induced lysates. In fact, R_1 lysates examined in electron-micrographs were seen to be comprised mainly of phage fragments and ghosts (Lowrie, 1974); the proportion of intact phage particles, presumably infective phages, was calculated to be approximately 15% of the lysate. Assuming an overall mean burst size of lactic streptococcal phages (Keogh, 1973) to be 35, the proportion of infective phage yield in R_1 lysates is estimated in Table V to be 2.6 - 6.3%, which agrees reasonably well with that calculated from electron-micrograph above. This very result means that the titre here expressed as pfu/ml of a R_1 lysate is probably a true representation of the total amount of intact phage particles. In other words, R_1 lysates probably do not contain inactive and nonadsorbable phages; this notion was verified in CsCl runs of R_1 lysates which manifested no detectable stray OD_{254} phage bands besides the main ones. Conversely, it is justifiable to say that all intact R_1 phages plate on either indicators R_1C or 368.

Referring back to the original question: Is R_1 a double lysogen? To re-phrase it: Do R_1 bacteria harbour two prophages in their genomes? Or, does induced R_1 lysate contain a mixture of phages? As a working guideline, R_1 can be doubly lysogenic in two ways:

(a) the two prophages, or the phages released, are similar; and
(b) the two prophages are distinctly different.

The electron-microscopic resolution of only one morphological phage type in R_1 lysates (Lowrie, 1974; Georghiou, *et al*, manuscript in preparation) eliminates the second possibility of the two prophages being distinctly different. One is then left with the occurrence of satellite and shoulder peaks in CsCl runs of R_1 lysates as possible manifestations of a second phage type very similar to that of the main peak. However, further CsCl analyses and serological tests confirmed the identity of phages from satellite and main peaks, suggesting the artifactual nature of these satellite peaks. Their appearance in CsCl runs of R_1r and $368(r_1)$ lysates indicated that their occurrence was not unique to R_1 lysates, hence excluded them as a manifestation of a different phage type in R_1 lysates. Phage aggregation

(Parmelee, *et al*, 1949) or adsorption to cellular debris (Bradley, 1963; Oram & Reiter, 1968; Watanabe, *et al*, 1977) could perhaps partly account for the existence of such observed satellite peaks.

Phages from the shoulder peak of a CsCl run of an R_1 lysate have been exhaustively examined in CsCl runs, serological tests, SDS-gel electrophoresis and host-specificity tests, and the results obtained show that they are not a different phage type from that of the main peak. The fact that the shoulder peak was manifested in CsCl runs of both induced lysates as well as pure phage stocks further suggests that it is an artifact.

Hence in reply to the original query, it is here concluded that R_1 is a single lysogen, harbouring only one prophage in its genome; the induced phage, ϕr_1, has a mean buoyant density of 1.485 g/ml.

Finally, I shall close the chapter with the summary flow-chart of the bacteria and phages which have made their appearance in this work, together with the schemes employed in their analyses which lead to the final deduction of the single lysogenic nature of *Streptococcus cremoris* strain R_1.

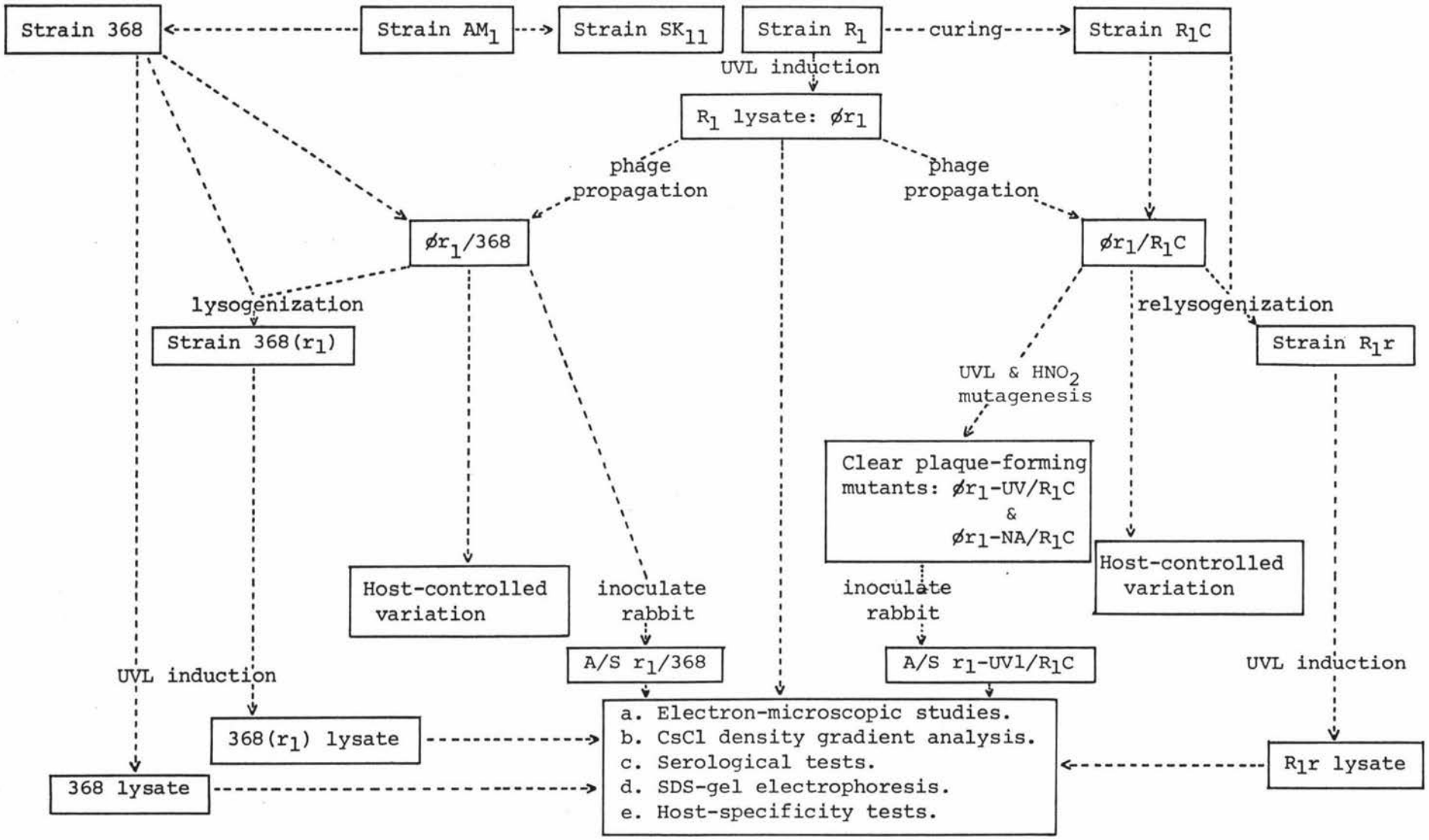

Figure 33. Flow-chart summary of bacteria and phages, and the scheme of analyses used.

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