Advances in VIRUS RESEARCH

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GUIDELINES FOR BACTERIOPHAGE CHARACTERIZATION

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I. Introduction

Bacteriophages or phages occur in a wide range of prokaryotes including bacteria and blue-green algae. With an estimated 1650 isolates studied by electron microscopy, they are the largest viral group described. This group is expanding at a rate of about 130 to 150 virus descriptions per year. These large numbers reflect the relative ease with which phages are isolated and their enormous importance for molecular biology, genetics, and epidemiology. For example, a recent review lists typing sets for about 70 bacterial species and serotypes (Kasatiya and Nicolle, 1978). Although most work has been done on phages of bacteria pathogenic to humans, there is presently a trend toward studying phages of nonmedical and sometimes arcane bacteria.

The isolation of new phages raises problems for investigators, journal editors, and the scientific public alike. Which parameters are important and which methods are error-prone or obsolete? When is a "new" phage actually new and what should be done with it? At present, there is no agreement on parameters, methods, and nomenclature. This has led to widely different, often unsatisfactory phage descriptions and many identical names for different phages. In addition, much effort seems to be devoted to the study of obsolete parameters or properties which, for lack of standard methods, cannot be compared with existing data. Such a situation is detrimental to comparative virology. The purpose of this article is to suggest guidelines for phage characterization and nomenclature and to set forth minimum requirements for their description.

II. Implications of Phage Taxonomy for Phage Description

A. Occurrence and Main Properties of Bacteriophages

Phages occur throughout the bacterial world and have been found in over 80 genera of bacteria and blue-green algae or cyanobacteria. Their hosts include aerobic, anaerobic, budding, gliding, photosynthetic, sporulating, and sulfur bacteria, thermo-, psychro-, and halophiles, spirochetes, mycoplasmas, and rickettsiae. In addition, there are unconfirmed reports of T7-phage-like particles in an eukaryote, the green alga *Chlorella pyrenoidosa* (Moskovets *et al.*, 1970; Tikhonenko and Zavarzina, 1966). Phages have habitats as varied as their hosts. Those of extreme habitats often have evolved a resistance peculiar to that environment and may, for example, be halophiles or psychrophiles. The occurrence and frequency of phages have been the objects of recent reviews which also contain detailed descriptions of phage groups (Ackermann, 1978a,b).

Phages are tailed, cubic, filamentous, or pleomorphic. Cubic phages should more accurately be called polyhedra. Phage nucleic acid is DNA or RNA which may be single- or double-stranded and linear or circular. Some phages contain lipid as part of the envelope or the coat. According to the nature of their nucleic acid and gross morphology, phages have been categorized into fundamental groups (Bradley, 1967; Ackermann and Eisenstark, 1974). The main characteristics of these groups are listed in Table I.

Tailed phages have isometric or elongated heads. Tails are either contractile, long and noncontractile, or very short. About 90% of heads are isometric. These phages vary enormously in their dimensions and in the presence or absence of tail fibers, baseplates, collars, and other appendages. They are both numerous and widespread. New tailed phages are reported every month. About 1550 have so far been described, representing nearly 95% of all known phages.

Cubic, filamentous, and pleomorphic phages seem to be much less frequent and presently number about 100. Among these phages, groups are small and often have only one member. Even the larger groups are of limited occurrence; for example, ΦX-, R17-, and fd-type phages have been found only in enterobacteria or their close relatives such as *Pseudomonas* and *Vibrio*. The *Pseudomonas* phage PM2 is noteworthy for its lipid-containing, complex capsid. The viruses of the recently established PRD1 group occur in *Bacillus* and gram-negative bacteria harboring certain drug-

TABLE I

MAIN PROPERTIES AND APPROXIMATE NUMBER OF MEMBERS IN MAJOR PHAGE GROUPS

Shape	Nucleic acid [a]	Example	Particulars	Number known [b]
Tailed	DNA 2, L	T2	Tail contractile	450
	DNA 2, L	λ	Tail long, noncontractile	800
	DNA 2, L	T7	Tail short	300
Cubic	DNA 1, C	ΦX174	Large, knoblike capsomeres	25
	DNA 2, C	PM2	Lipid-containing capsid	2?
	DNA 2, L	PRD1	Double coat, lipids, pseudotail	4?
	RNA 1, L	R17	—	35
	RNA 2, L	φ6	Lipid-containing envelope	1
Filamentous	DNA 1, C	fd	Long rods	17
	DNA 1, C	MV-L1	Short rods	10
Pleomorphic	DNA 2, C	MV-L2	Lipid-containing envelope	3?

[a] 1, Single-stranded; 2, double-stranded; C, circular; L, linear.
[b] Ackermann (1978a,b).

resistance plasmids. They have a double capsid, the inner one probably being lipoprotein. In addition, empty and damaged particles show a tail-like structure which seems to appear upon nucleic acid ejection. The *Pseudomonas* phage ϕ6 has a lipid-containing envelope and contains three pieces of double-stranded RNA and an RNA polymerase. Filamentous phages include long rods of the fd type and short rods which so far have been found only in *Acholeplasma*. The pleomorphic phages include a few *Acholeplasma* viruses with a flexible, lipid-containing envelope and no detectable capsid.

B. Extant Classification Schemes

Virus taxonomy is based on the properties of the virion and its nucleic acid. The main criteria of the International Committee on Taxonomy of Viruses (ICTV) are particle shape, size, structure, weight, buoyant density, and total composition, and the nature, MW, and composition of the viral nucleic acid (Fenner, 1976; Wildy, 1971). By applying these criteria and using serological and nucleic acid hybridization data, classification schemes for several phage groups were constructed. Only species definition was attempted. The overall results are shown in Table II. Of 1075 phages surveyed, over 60% were categorized into 89 species. This was generally easy with phages containing lipids, unusual bases, or other unique properties. All cubic, filamentous, and pleomorphic phages were

TABLE II
NUMBER OF SPECIES IN CLASSIFIED PHAGES

Phage group	Number of phages		Number of species	Reference
	Surveyed	Classified		
Tailed phages	166	78	20	
Actinophages [a]				Berthiaume and Ackermann, 1977
Of *Agrobacterium-Rhizobium*	128	68	16	Ackermann, 1978c
Of *Bacillus-Clostridium*	192	83	18	Ackermann, 1974, 1978b
Of enterobacteria	375	250	24	Ackermann, 1976
Of gram-positive cocci [b]	116	75	14	Ackermann, 1975
Cubic, filamentous, and pleomorphic phages	98	98	17	Ackermann, 1978a
Total	1075	652	109	

[a] *Streptomyces* and related genera.
[b] *Micrococcus, Staphylococcus,* and *Streptococcus.*

classified, but only half of the tailed phages. For many of the latter, morphology and one-step growth characteristics were the only data available. Other data, especially dimensions, could not be accepted at face value; in addition, few serological and nucleic acid hybridization studies were available.

The fact that 109 phage species can be differentiated reflects the extreme diversification of tailed phages and, probably, a long evolutionary history. Some of these species may be identical with each other; for example, phages of identical and distinctive morphology simultaneously occur in *Bacillus*, *Staphylococcus*, and *Streptococcus* (Ackermann, 1975). Comparative serological and nucleic acid hybridization studies probably will result in some species being combined. On the other hand, only phages of very common bacteria have so far been classified. It is thus likely that many more phage species will be described in the future.

C. Implications for Phage Description

The above-mentioned classification schemes allow some generalizations of practical interest:

1. Morphology often allows instant categorization.
2. Particle weight and nucleic acid content correlate with capsid size, hence the danger of overweighting in numerical taxonomy if all three properties are given equal weight.
3. Tailed phages resemble each other in DNA content (about 50% duplex DNA) and particle buoyant density (about 1.5 gm/ml in CsCl). With respect to these criteria, they appear less diverse than cubic, filamentous, and pleomorphic phages.
4. The particle buoyant density correlates roughly with nucleic acid and lipid content.
5. Except for the presence of unusual bases, phage and host have similar base compositions (Gibbs and Primrose, 1976; see also Guay *et al.*, 1978; Normore, 1978).
6. In phages of the same host, morphologically identical phages are serologically related, and vice versa.
7. Phages are usually genus-specific. Important exceptions are: (a) Phages of enterobacteria where polyvalence is common. (b) Actinophages which sometimes cross genus boundaries (Jones, 1963; Mankiewicz, 1965; Prauser and Falta, 1968). (c) Phages specific for bacteria harboring certain drug-resistance plasmids, namely, enterobacteria, *Acinetobacter*, *Pseudomonas*, and *Vibrio;* they include several cubic DNA phages with double capsids, the cubic RNA phage PRR1, and the filamentous phage PF3 (Bradley, 1974; Bradley and Rutherford, 1975; Olsen and Thomas, 1973; Olsen *et al.*, 1974; Stanisich, 1974; Wong and Bryan, 1978).

8. Serological and nucleic acid hybridization studies are extremely helpful in establishing phage groups.

9. Sensitivity to physicochemical agents must be standardized. Techniques and agents are so varied that most published data are of limited use for comparative virology. The potentially most useful agents are chloroform, heat, and ultraviolet light.

10. Adsorption velocity, latent period, and burst size depend more on host strains and the environment than on the phage itself.

11. The 10 most often investigated properties are, in order, host range, morphology, latent period and burst size, heat sensitivity, adsorption velocity, G + C percentages, nucleic acid MW, phage buoyant density, and overall base composition. A priori, frequently determined properties would seem inherently useful.

D. On Errors and Abuses

Like other papers, those on phages have their share of imperfections which range from misinterpretations to fraud; for example, about 6 years ago, a group of investigators published about 60 *Klebsiella* and *Shigella* phages up to five times under different names. Fortunately, such events are rare, and methodological errors are easy to correct. Before recommending any specific techniques, major sources of errors are discussed.

The most serious failing is to call every new isolate a new virus. This widespread practice would be unacceptable in other branches of virology where proof of identity or uniqueness is usually required. In addition, it is often unclear how many phages were studied and if they were viable. Shortcomings are especially frequent in electron microscopy (Table III); for example, one wonders why descriptions of shadowed phages continue to appear 20 years after the introduction of negative staining. In addition, host ranges are sometimes determined on only five or six bacterial strains.

TABLE III

SOURCES OF ERROR IN ELECTRON MICROSCOPY

Electron microscope, stain, or number of particles measured is not specified.
Phages have not been purified.
Magnification is monitored by inappropriate means (Section IV,A,3) or not at all.
Dimensions of phages are incomplete or not given.
Dimensions are measured on too few particles or on shadowed or positively stained phages.
Descriptions are equivocal or contain errors of interpretation.
Micrographs are of insufficient magnification, out of focus, low in contrast, show image shift or astigmatism, have no scale markers, or are absent altogether.

These shortcomings are easy to detect and ideally should not be overlooked by the editorial boards of scientific journals. Finally, there is no reference collection for phages which collects neotypes and new species, and many of the 1650 phage isolates mentioned above may no longer be available.

Another unfortunate problem is the naming of phages. Since there has been no agreement on nomenclature, simple numerals from 1 to 10, the Greek letter ϕ, and the Latin letter P have enjoyed much favor. The results are many homonyms and a few statistically unlikely accidents; for example, there are two *Bacillus* phages with isometric heads and long, noncontractile tails, one of them being called ϕ3T and the other Tϕ3 (Dean *et al.*, 1976; Egbert and Mitchell, 1967). That some confusion has already been generated may be seen in a recent catalog of viruses (Fraenkel-Conrat, 1974). It lists two phages called N1 and suggests that one of them infects both *Micrococcus lysodeikticus* and *Escherichia coli*.

III. A Tentative Hierarchy of Phage Properties

The ICTV Bacterial Virus Subcommittee was polled on the types and relevance of data suitable for phage characterization. No definitive or precise hierarchy was worked out. Rather, there appeared groups of more or less mutually agreed upon equivalent criteria which reflected the availability or frequency of data as much as their intrinsic importance. The hierarchy proposed here is only for the purpose of obtaining data for phage differentiation. It does not preclude a numerical taxonomy or the use of cryptograms. Criteria may be grouped as follows.

A. High-Level Criteria

These criteria, which are applicable to all phages, describe the virion or its nucleic acid and can be determined precisely and unequivocally. They are listed in Table IV along with the principal methods for their determination. They include the buoyant density of the virion which, although it reflects the composition of the particle, it is commonly used as an independent criterion (Fenner, 1976; Wildy, 1971).

B. Medium-Level Criteria

These criteria include those more useful for one group of phages than another, those for which limited data are available, and those which are considered of lesser value in differentiation: (1) presence and dimensions of organelles such as tail fibers and baseplates; (2) number of pieces of

TABLE IV
HIGH-LEVEL CRITERIA FOR PHAGE DESCRIPTION

Property	Principal techniques
Whole virus	
Size and shape	Electron microscopy
Weight	Sedimentation analysis in buffer or sucrose
Buoyant density	Isopycnic centrifugation in cesium salts or sucrose
Percent of protein	Chemical analysis; approximation by determination of ultraviolet absorbancy
Percent of lipids	Chemical analysis
Nucleic acid	
Type (DNA or RNA)	Acridine orange staining (Section IV,B,2) Alkali denaturation Colorimetric determination of sugars Enzyme sensitivity
Number of strands	Acridine orange staining Chemical analysis of base ratio Determination of buoyant density Thermal denaturation
Conformation (linear or circular)	Electron microscopy
Percent	See "Percent of protein"
MW	Electron microscopy Gel electrophoresis Sedimentation analysis

nucleic acid, base composition, and presence of sugars; (3) number and composition of coat and internal proteins; (4) nucleic acid hybridization and serological data; (5) host range; (6) some genetic properties such as converting or transducing ability; and (7) inactivation by the more frequently determined physical and chemical agents, namely, chloroform, ether, heat, and ultraviolet light.

The number of pieces and base composition are included here because only one phage, the *Pseudomonas* phage ϕ6, has a segmented genome, and because of the already noted parallelism in the base composition of phages and their hosts (Section II,C,5). This does not deny the high value of unusual bases for limited phage groups. In a general way, the value of medium-level criteria depends on the phage group to which they are applied. For example, the filamentous phages of the fd type are distinguished by their high sensitivity to chloroform and sonication and their relative resistance to heat (Marvin and Hohn, 1969). On the other hand, chloroform has little effect on most tailed phages, sonication has rarely

been tested on them, and heat seems to differentiate between individual, related viruses rather than phage groups.

C. Low-Level Criteria

These criteria include host- and environment-dependent properties such as plaque size, adsorption velocity, latent period, burst size, and efficiency of plating. In practice, these are properties to be determined with each phage for purposes of study and purification, but are not useful for comparative characterization. This category may also include rarely determined properties such as the electrophoretic mobility of the phage or tests with generally uniform results, for example, pH sensitivity.

IV. Recommended Methods

A. Electron Microscopy

Electron microscopy is easily performed and frequently allows instant classification of new isolates into morphological groups, thus reducing the need for serological studies. Electron microscopy is thus of paramount importance and should be carried out with great care.

1. Preparation of Phages

Phages from crude lysates must be purified for study of fine structure. A general recommendation is to sediment them twice at 70,000 *g* for 30–60 minutes in 0.1 *M* ammonium acetate (Bradley, 1967). Density gradient-purified and dialyzed phages are best washed too, because dialysis is sometimes insufficient to remove impurities. Fixation may be useful to prevent shrinkage or disruption of particles. Glutaraldehyde fixation (0.25% glutaraldehyde in 0.05 *M* cacodylate buffer, pH 7.4) for 15–60 minutes at room temperature protects the tailed *Xanthomonas* phage XP-12 against uranyl acetate-induced shrinkage (Brown, 1977). OsO_4 fixation before negative staining has also been suggested for phages (Nermut, 1972).

2. Staining

Phosphotungstate (PT) and uranyl acetate (UA) are the most important stains, and both should always be tried. They give satisfactory negative staining; in addition, UA stains variable fractions of DNA-containing capsids positively. Neither stain is perfect; for example, UA

crystallizes easily and causes shrinkage of positively stained phage heads and swelling of protein structures. On the other hand, it enhances tail striations, facilitates the detection of pentagonal capsids, and may be the stain of choice for large, elongated phage heads (Ackermann *et al.*, 1974). PT may disrupt some phages (Barnet, 1972; Schmidt and Stanier, 1965) and should not contain a wetting agent, for example, serum, sugars, or the more recently proposed bacitracin (Gregory and Pirie, 1973). These additives, which indeed give evenly stained preparations, always lead to flattening of the capsid. Shadowing obscures details and alters dimensions. It should be reserved for special techniques such as freeze-etching, or for filamentous phages of the fd type which are usually low in contrast.

3. Magnification

Magnification should be monitored with catalase crystals (Luftig, 1967; Wrigley, 1968). Diffraction grating replicas and latex spheres are suitable for low magnification only; in addition, latex spheres shrink in the beam (Hall, 1966; Luftig, 1967). To minimize the effects of current fluctuations, investigators should take a series of micrographs, for example, a complete film or cassette, in a single session. Each series should include one or two micrographs of catalase, taken at the same magnification as the phage. It is not necessary to mix phages and catalase. Alternatively, T-even phage tails may be used as standards. As calibrated against catalase crystals, they have a length of 113 nm including the baseplate (Ackermann, 1976). Their advantage is that they are relatively small and can be mixed and photographed together with other phages.

4. Dimensions

At least 20 particles per phage should be measured on prints, not projected images, at high magnification (about 300,000 times). It should be reminded that the dimensions of isometric capsids differ by about 15% whether they are measured between opposite sides or apexes. The latter is easier and seems preferable. In any case, the method must be stated. Since capsids are easily deformed, one should measure all three diameters of isometric particles. Phages positively stained with UA should not be measured. Tail lengths should include collars and baseplates.

5. Interpretation and Descriptions

The main problem seems to be the interpretation of the shape of isometric heads, in particular whether they are icosahedra or octahedra. When resting on their sides, both bodies show six-sided outlines and can-