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Bacteriophages

Methods and Protocols

Volume 2: Molecular and Applied Aspects

Edited by

Martha R. J. Clokie

Andrew M. Kropinski

Humana Press

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Preface

We are increasingly aware of the many and varied roles that bacteriophages play in microbial ecology and evolution. The implications of bacteriophage–bacteria interactions range from the evolution of pathogenicity to oceanic carbon cycling. However, working with bacteriophages can be difficult due to their small size and specific bacterial host requirements. Written by top international bacteriophage researchers, these volumes pull together a vast body of knowledge and expertise, including almost forgotten classical methods as well as state-of-the-art molecular techniques. It is designed to be a valuable reference for experienced bacteriophage researchers as well as an accessible introduction to the newcomer to the subject.

The books are designed to be modular and are organised in the order in which one would carry out the work. A wide range of projects can be built from these modules by selecting appropriate chapters from each section. Volume 1s Section 1 concerns the isolation of phages from a range of environments. Sections 2 and 3 describe their morphological and molecular characterisation, and present methods for the investigation of their interaction with bacteria. Volume 2s Sections 1–3 are concerned with bacteriophage genomics, metagenomics, transcriptomics, and proteomics. It concludes with chapters on applied bacteriophage biology (Section 4).

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Introduction

Andrew M. Kropinski and Martha R. J. Clokie

The discovery of viruses specific to bacteria (referred to variably as bacteriophages, phages, and bacterial viruses in this volume) is credited to an English bacteriologist, Frederick William Twort *(1)* in 1915 and to a French-Canadian scientist, Felix d'Herelle *(2)* in 1917. It is the latter scientist who probably more accurately recognized what he was dealing with and is responsible for naming these agents of bacterial death. He realized these organisms propagated at the expense of bacteria so named them bacteriophages, which translates as bacterial eaters, "phages" coming from the Greek "phagein" meaning "to eat." He is also responsible for recognizing their potential clinical significance *(3)*.

The first golden age of bacteriophage research ran from the 1930s through to the 1970s and resulted in major discoveries such as the identification of DNA as genetic material and the subsequent deciphering of the genetic code and the discovery of messenger RNA, these breakthroughs led to the birth of the new science of Molecular Biology. This work is described in the book by John Cairns et al. *"Phage and the Origins of Molecular Biology" (4)* and on the excellent American Society for Microbiology Division M (Bacteriophage) homepage (http://www.asm.org/division/M/M.html (thanks to Susan Godfrey, Roger Hendrix, Eric Miller). A summary of some of the major discoveries made during this period is detailed in **Table 1** (the authors apologize for the omission of the impact of many eminent phage biologists).

Following on from this golden age was the 1980s and 1990s, where phages and phage-derived products were essential to the major biotechnological revolution that occurred. Recombinant DNA techniques were developed in which phage played a significant part as primary vectors (filamentous phage *(5)*, λ insertional, and replacement vectors *(6)*) or parts of vectors (promoters [expression vector *(7–9)*], packaging signals [cosmids *(10, 11)* and phagemids *(12)*], integrative signals [integrative vectors *(13–15)*], replicons [phagemids *(16)*], and P1-derived vectors *(17)*]. In addition, they contributed a great variety of enzymes are to be employed today's molecular biology laboratory, including integrases, polynucleotide kinases, DNA ligases, DNA polymerases, RNA polymerases, recombinases, single-stranded DNA binding proteins (SSB), endo- and exonucleases, and even methylases and restriction endonucleases *(18)*.

A good indicator for the amount of interest in bacteriophage research is the number of papers published per year that contain the word "bacteriophage" in their title. This rose steadily from 1950 to 1965 and (**Fig. 1**). There was then a sharp burst of phage publications from 1970 to 1975 followed by a precipitous drop in number. This was due to the unfortunate, lack of interest, and funding for phage biology where many eminent scientists gave up working on phages for more lucrative eukaryotic projects.

Recently, due to an increase awareness of their importance, an interest in bacteriophages has been re-kindled, and an insight into the scale of this renewed enthusiasm can be seen from the huge increase in the number of sequenced phage genomes (**Fig. 1**).

Table 1
Significant experimental observation using bacteriophages

Grouping	Discovery	Year & Reference
Plaque assays		Felix d'Herelle 1917 *(50–52)*
Structure and taxonomy	First EM pictures of phages	T.F. Anderson 1942 *(53)*
	CryoEM	1992 *(54–56)*
	Development of modern taxonomic schemes	1962 *(57–59)*
Composition—general	Phages are composed of protein and DNA	1948 *(60)*
	Isolation of: first lipid-containing phage: PM2	1968 *(61)*
Nucleic acids	Genes are made of DNA	1952 *(62)*
	Introns: type I—T4	*(63–65)*
	Inteins	1998 *(66–69)* [http://www.neb.com/inteins.html]
	Genetic code	1961 *(70)*
	Modified bases: T4 (5-hydroxymethylcytosine)	1953 *(71)*
	tRNA-encoding genes: T4	1972 *(72–75)*
	Restriction and modification: λ	
	a) Phenomenon	1953 *(76)*
	b) Mechanism	1962 *(77)*
	Novel genomes:	
	a) Single-stranded (ss) DNA - øX174	1959 *(78)*
	b) Single-stranded (ss) RNA - f2	1961 *(79)*
	c) Segmented double-stranded RNA - ø6	1973 *(80, 81)*
	d) Phage with terminal proteins - ø29	1971 *(82)*
	Sequence of first:	
	a) ssRNA virus	1976 *(83)*
	b) ssDNA virus	1977 *(84)*
Mutation	rII experiments – T4	1955 *(85, 86)*
	T1 resistance in *E.coli*	1943 *(87)*
Lysogeny and integration	Discovery of lysogeny	1934 *(88–90)*
	Isolation of phage λ	E.M. Lederberg 1951 *(91)*
	Induction	1950 *(92)*

(continued)

Table 1 (continued)

Grouping	Discovery	Year & Reference
	Integration:	
	a) Model	1962 *(93)*
	b) Site-specific recombination	1968 *(94, 95)*
	Repression:	
	a) Model	1961 *(96, 97)*
	b) Experimental evidence	1967 *(98, 99)*
	Integration of phage Mu causes host mutations	1963 *(100)*
	Not all temperate phages integrate:	
	a) P1	1951 *(101, 102)*
	b) Linear prophages - N15	N.V. Ravin 1964 *(103)*
	Lysogenic conversion: a) Toxigenicity – *Corynebacterium diphtheriae* phage B	1951 *(104, 105)*
	b) Serotype: *Salmonella* Anatum phage ε15	1955 *(106, 107)*
Genetic exchange - transduction	P22 and *Salmonella*	1952 *(108)*
	P1 and *Escherichia coli*	1955 *(109)*
	Specialized transduction: λ	1957 *(110, 111)*
	Origin of host DNA in P22 transducing particles	1972 *(112)*
Adsorption and injection	a) penetration of capsule	1979 *(113)*
	b) λ & LamB liposomes	1983 *(114)*
	c) T4 and spheroplasts	1983 *(115)*
	d) T7 DNA uptake requires transcription	2001 *(116)*
Intracellular development	One-step growth curve:	
	a) latent period & burst size	1939 *(117)*
	b) burst size from single cells	1945 *(118)*
	c) eclipse phase	1948 *(119, 120)*
	DNA replication:	
	a) DNA ligase	1967 *(121)*
	b) øX174 – rolling circle	1968 *(122, 123)*
	c) T4 – Okazaki fragments	1969 *(124)*
	d) M13 – RNA primers	1972 *(125)*

(continued)

Table 1 (continued)

Grouping	Discovery	Year & Reference
	e) T7 – visualization & formation of concatemers	1972
	General recombination – λ	1961 *(126--129)*
	Transcription:	
	a) mRNA	1956 *(130--133)*
	b) antitermination	1969 *(134)*
	Protein synthesis:	
	a) SDS gels	1969 *(135)*
	b) discontinuous buffer system	1970 *(136)*
	c) slab gel	1973 *(137)*
	d) ribosomal slippage	1993 *(138)*
	Morphogenesis:	
	a) role of chaperonins	1972 *(139--143)*
	b) cross-linked capsid proteins	1995 *(144,145)*
	c) packaging of φ29 require a small RNA molecule	1987 *(146)*
Phage therapy		F. d'Herelle 1917 *(147)*

From the very steep way in which the slope of the graph of the number of phage genome sequences per year is shooting up, it is quite apparent that we are in a new exponential growth phage of phage research.

There are three main reasons for this renewed interest in bacteriophages. The first is a result of bacterial genome sequencing projects which have revealed that most bacteria are lysogenic for at least one bacteriophage and that phages have played a major role in host genome evolution *(19–24)*. The first project of this kind was the sequencing of the *Haemophilus influenzae* in 1995 which was shown to contain a Mu-like prophage *(25)*. Some bacteria contain many phages in their genomes and pathogenicity is often linked to phage carriage for example the *Streptococcus* group C contain up to 6 phage or prophage-like elements *(26)*. Phages have been shown to encode a range of toxins and gene products that influence their bacterial cells, or even the host in which the bacterium lives (reviewed in *(27)*). An example of the complexity of these interactions can be seen from phages which infect aphid gut bacteria which encode toxins that help bacteria defend the aphid from other invading bacteria *(28)*.

The second reason for the renewed bacteriophage interest is that phage ecologists have shown that soil and water contain between 10 and 100 times more phage particles than bacterial cells, leading to the speculation that the global abundance of phages is probably in the order of 10^{31} *(29)*. Diversity even within phages which infect one bacterial host is also high, for example genomic studies on mycobacterial phages have shown that not only do mycobacterial phage encode genes which are unlike all other genes sequenced thus far, they are also not present in the different phages *(30–35)*. Metagenomic viral studies focusing on the phage in the oceans have also demonstrated the enormous scale of phage

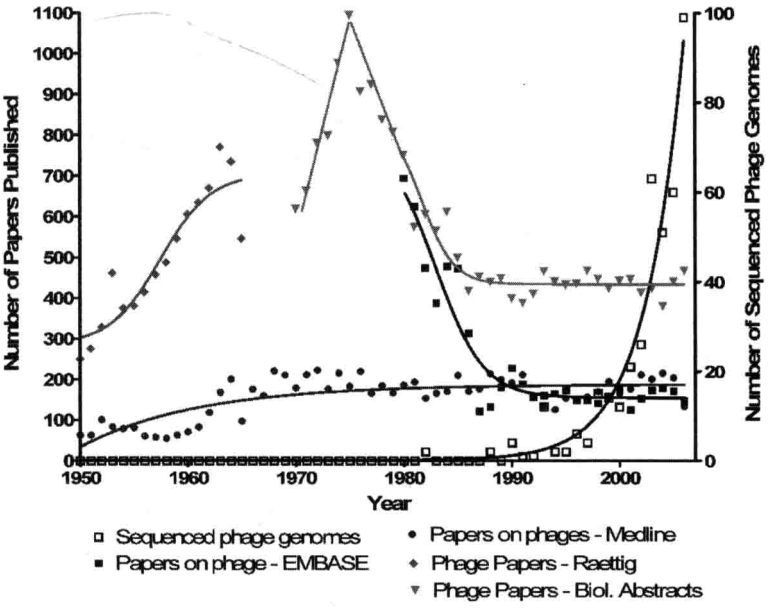

Fig. 1. Publications of bacteriophages and the appearance of phage sequences in GenBank as a function of year. The publication data was derived from four sources: Hansjürgen Raettig for data from 1950 to 1965 *(148, 149)* brought to the editors attention by Hans-Wolfgang Ackermann, and Ovid Medline, EMBASE, and BIOSIS (Biological Abstracts) online literature searches for, respectively, 1950–2006, 1980–2006, and 1969–2006. In the case of Medline and EMBASE, the keyword "bacteriophage" was mapped to subject heading; all subheadings were included; and, the search strategy was focused rather than exploded. With BIOSIS the presence of "bacteriophage or phage" in the title was used to screen scientific articles. NB Although this gives a good indication of phage publications a more detailed analysis is required to tease apart the true number of phage publications, separating those on phage biology from, for example, those on phage typing.

genetic diversity *(29, 36, 37)*. With a raised awareness of phage abundance and diversity has come an appreciation for the consequence of phage action in influencing bacterial population dynamics and evolution and in maintaining essential biogeochemical cycles such as carbon cycling *(38)*. Furthermore, recent genomic and transcriptomic studies have illustrated the extent of interlinked metabolisms of phage and host during a lytic infection for example with bacteriophages which infect cyanobacteria encoding and expressing key photosynthesis gene *(39–42)*.

Finally, but very importantly in terms of phage research are the concerns of the public, governmental healthcare agencies and physicians that something must be done about the growing problem of antimicrobial resistance. This awareness is accompanied by the belated realization that phage therapy, which has been kept alive by the efforts of Eastern European scientists, offers a viable alternative to antibiotic therapy. In Canada, for example, it is now realized that much of the expertise in phage biology has disappeared as a result of retirements and the death of members of the phage community of scientists. The lack of "capacity issues" (i.e., knowledgeable young scientists) has results in the Canadian Institutes for Health Research issuing a call for research grants which will address the potential for using phage as a therapeutic agents. Similarly the same awareness in Europe and the United States has resulted in the number of new bacteriophage research groups increasing and the interest and attendance at bacteriophage conferences is increasing annually. What is apparent, however, is that for bacteriophages to be used therapeutically in countries such

as Europe, Canada and the US, we must properly understand the biology of the interaction between the phage and the bacterial pathogen. We are fortunate to be practicing phage biology in this exciting time where such experimentation is possible.

These volumes are designed to provide the amateur or professional with a step-by-step approach to many of the standard protocols in working with bacteriophages. We include both classical protocols which have been collected before they are forgotten and have to be re-invented, and also state-of-the-art protocols which use many of the latest molecular tools with which to study bacteriophages. It is a complete piece of biology and should take the new comer to bacteriophages from isolating these organisms to characterizing them at every level. It should also equip the experienced phage practitioner wishing to branch out into a new area of phage biology.

Unfortunately with time and space restrictions, it is not possible to be fully comprehensive. When we approached one scientist to contribute to this book he/she replied, "That's microbial archeology. I no longer have access to those laboratory research manuals." For similar reasons, phage immunoelectron microscopy is not covered, nor are the uses of maxi- *(43, 44)* or minicells *(45–49)* for studying phage gene expression.

We are indebted to our authors who have kindly shared their years of experience to make this volume possible. They represent a truly multidisciplinary assemblage of scientists with a huge combined skill set. *Bacteriophages: Methods and Protocols* is a complete piece of biology, laid out in seven sections. Volume 1, Section 1 deals with methods of isolating bacteriophage (and archeophage) from a range of soil or aquatic environments using direct isolation and enrichment approaches. Volume 1, Section 2 covers the characterization of bacteriophages based upon their ability to form plaques, or direct enumeration by fluorescent microscopy or flow cytometry. There is also a chapter on electron microscopy and a further one on classical phage taxonomy. The characterization of host range, adsorption and receptor interaction, and models of plaque development are also considered here. Finally there is a chapter on how to maintain phage stocks once you have them.

Bacteriophage-host interactions (Volume 1, Section 3) includes the construction of mutants using chemical mutagenesis or by recombineering, studies on lysogens, and transduction by temperate and lytic phages. A full scope of genomics is covered in Volume 2, Section 1, from DNA isolation and characterization (PFGE, base composition), through library construction, sequencing, annotation (termini, genes, promoters, terminators) and phylogenetics. Volume 2, Section 2 describes concentrates on transcriptomics and proteomic approaches. These include: mRNA extraction during host infection, quantification of mRNA using real time PCR, and microarray construction. Isolation-independent methods of characterizing phage communities are described in Volume 2, Section 3. Volume 2, Section 4 describes the applied aspects of bacteriophage biology including phage typing, the isolation of lysins, and general and antibody phage display. There is also a final chapter to describe some online resources for phage workers.

To conclude, we hope that you find this book useful and inspiring, and we look forward to the next golden age of phage research.

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