PHAGE TYPING OF MYCOBACTERIA

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JAMES B. HOELTGEN
1967

PHAGE TYPING OF MYCOBACTERIA

By

James B. Hoeltgen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1967

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks and appreciation to Dr. V. H. Mallmann for her guidance and assistance throughout this study. Appreciation is also extended to Dr. W. L. Mallmann for his advice and counsel.

A special appreciation goes to the author's wife, Kathy, who gave purpose to the desire for intellectual enlightenment.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

No single test or set of tests are available with which many of the mycobacteria isolated from animate and inanimate sources can be identified easily and conclusively. Such tests are needed, particularly for epidemiologic studies of the atypical (anonymous or unclassified) mycobacteria and to establish species identification. In this regard, phage typing has become important in determining the strains, species, and genera of some bacteria. This report constitutes a preliminary study of the susceptibility of some strains and species of Mycobacterium to mycobacteriophage.

LITERATURE REVIEW

Mycobacteria. The genus Mycobacterium contains certain well-defined species which vary from saprophytes to obligate parasites. Historically, the first mycobacterium associated with an infectious disease was the causative agent of Hansen's disease, M. leprae, in 1868. In 1883, M. tuberculosis was first isolated; with this organism Koch formulated the Postulates which have been the sound foundation of infectious diseases. In 1896, M. bovis, and in 1901, M. avium were reported. Species which have been recognized more recently are M. ulcerans, M. marinum, M. kansasii.

As the mortality rate of tuberculosis has declined, an equal decrease in the incidence of disease has not occurred. There has also been an apparent increase in the number of cases from which a well-defined species of mycobacterium could not be isolated. The increase was not real; the number of cases due to M. tuberculosis diminished, but the number of cases due to "atypical" or unclassified mycobacteria increased relatively. The atypical mycobacteria are not drug-selected mutants, although they are generally resistant to therapeutic amounts of streptomycin, paraminosalicylic acid (PAS) and isoniazid (INH). Runyon divided

the atypicals into the following four groups according to the rate of growth at 37°C and pigment production (54):

Group I: M. kansasii, a photochromogen: slow growth, no pigment produced in the dark, yellow pigment produced after short exposure to light.

Group II: Scotochromogens: slow growth, yellow pigment produced in light or in the dark, heterogenous strains.

Group III: Nonphotochromogen: slow growth, no pigment production, heterogenous strains.

Group IV: Rapid growers: growth in five days, many strains.

The mycobacteria range from obligate parasites to saprophytes. Pathogenic mycobacteria are classical intracellular parasites. Many pathogenic mycobacteria are capable of surviving and even multiplying within the phagocytes after ingestion. The histologic hallmark of tuberculosis is the presence of acid fast-organisms in giant cells. Pathogenic mycobacteria vary with respect to the range of hosts they can infect and the sites of infection in the hosts.

Mycobacterial diseases are characterized by their chronicity, latency, induction of delayed sensitivity, and irregular induction of antibody production (2,21). There seems to be no single virulence factor such as an enzyme or exotoxin. The cord factor, a waxy fraction of the cell wall, has been credited with the virulence of the organism, but

this notion is controversial. Lipids undoubtedly contribute to the organism's resistance to phagocytosis and to the inflammatory response which the organism incites.

At the present time there is no generally accepted rapid or even conclusive means for identifying some of the species of mycobacteria (14,24,86,87). Serologic methods are frequently unsatisfactory because of cross reactions, spontaneous cell aggregation caused by hydrophobic characteristics, and the unpredictable presence or absence of antibodies in closed or active cases of tuberculosis (53,76). Chemical tests or combination of tests have been reported as a means of reliable identification (15,25,27,30,46,68,78,79, 80,84,85). Invariably, this reliability has been questioned by others. The differential infectivity of M. tuberculosis, M. bovis, and M. avium for laboratory animals is the only method commonly accepted as reliable. Animal testing is time consuming and expensive. The variable virulence of the atypicals and the unresolved relation of the atypicals to the classical pathogens have discredited the method.

Perhaps the greatest controversy concerns the Group III mycobacteria (42,45,96). The Battey bacillus, one strain of the Group III human pathogens, has little or no virulence for chickens and rabbits. It is indistinguishable from M. avium on the basis of colony morphology, growth at 37^{o}C, or metabolism. There are some differences, although not conclusive in certain chemical tests and the optimal temperature

for growth. Runyon has recently suggested that the name of M. intracellulare be accepted for the Battey bacillus only, but not for other Group III mycobacteria (56).

Tuberculosis or tuberculosis-like disease may be caused by a range of mycobacterial organisms in a variety of domestic animals (33). Some strains of Group III, isolated from tuberculin positive cattle, produced generalized disease and hypersensitivity in experimentally infected calves; others produced limited infections or no infections. Other Group III strains isolated from swine produced limited or no disease in experimental calves; they caused severe disease in swine. Strains of Group III isolated from soil and feed produced neither disease nor hypersensitivity in swine or calves (33). Differentiation among the Group III mycobacteria could not be made on the basis of morphology, cultural characteristics, or cytochemical tests.

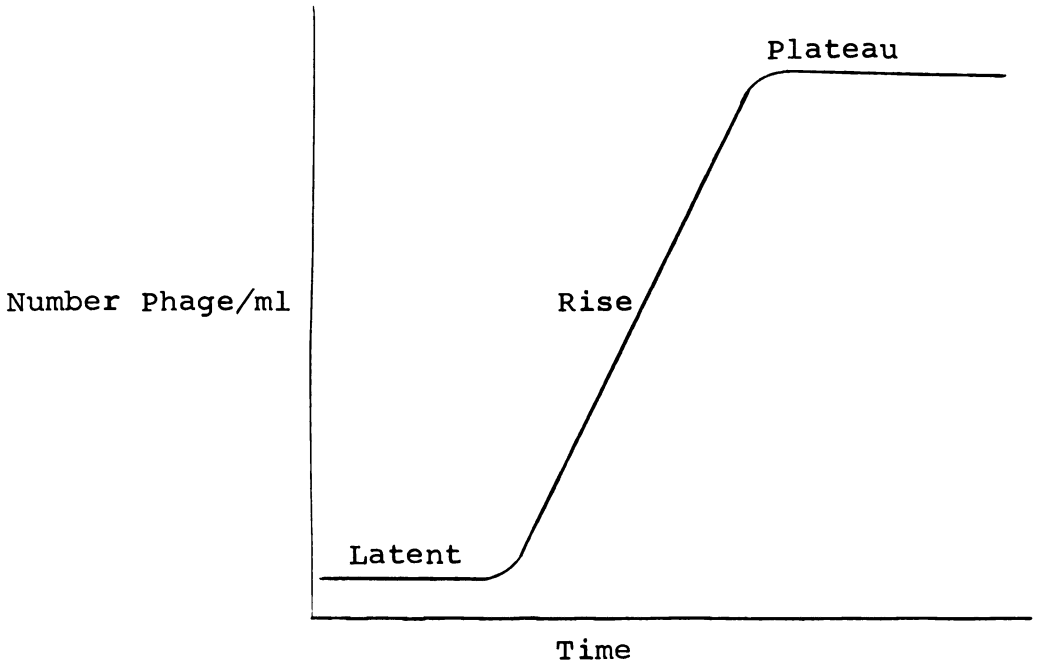
Bacteriophage. Bacteriophage, viruses which infect bacteria, were discovered independently by the British bacteriologist F. W. Twort in 1915, and by the French bacteriologist Felix d'Herelle in 1917. When phage were found which were capable of lysing some species or strains of pathogenic bacteria, a potential therapeutic value was proposed by d'Herelle and subsequently disproved (32). The book "Le bacteriophage; son role dans l'immunite" was written by d'Herelle in 1921.

Twort and d'Herelle independently developed theories on the origin of phage. The origin has not been resolved and is disputed today. One theory, the virus theory, proposes that phage is exogenous and autonomous; the precursor theory proposes that all phage are endogenous.

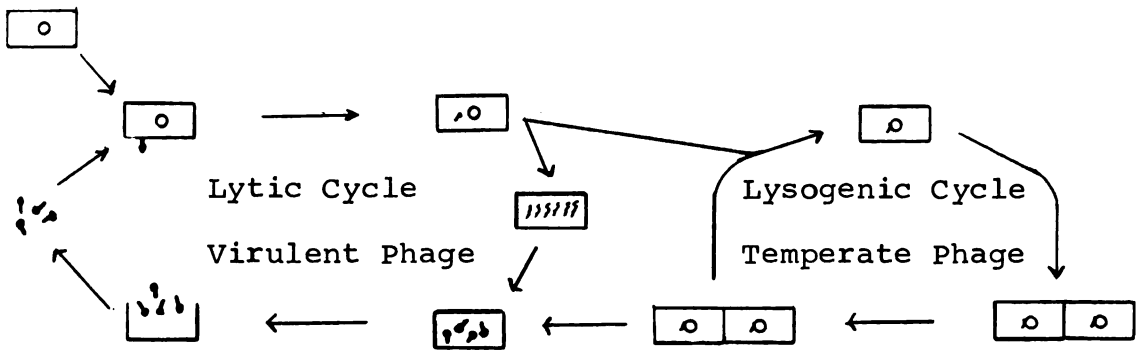
The first mycobacteriophage were isolated from soil by Froman and Bogen in 1953 by a soil enrichment technique (16). Mycobacterial cells were added to a soil sample, the mixture was incubated, and, subsequently, bacteriophage were obtained from filtrates of the soil mixture (9,11,71). It was recently reported that phage can only be isolated from soils that have been contaminated with fertilizer or animal excreta (9,11). Mycobacteriophage have been isolated from the gut of larva, animal feces, and human feces (12,13,37).

In the light of recent publications, bacteriophage can be divided into virulent and temperate phage. Both enter the bacterial cell. The virulent phage induces the lytic cycle consistently after entering the bacterial cell; the temperate phage induces lysis infrequently after entering the bacterial cell. Bacteriophage is adsorbed to the cell wall of a susceptible bacterium and releases lysozyme which digests the cell wall. The phage DNA is then injected into the bacterium. The virulent phage DNA diverts the cell's activities to make more phage DNA and phage protein. In the process known as maturation, the phage protein and the phage

DNA combine to form complete phage particles, and the bacterial cell lyses. The latent period and the burst size for each phage and host system can be determined by the one-step growth experiment. The graph below shows a single-step growth experiment with a typical latent period, rise period, and plateau period (23).



Temperate mycobacteriophage, or some of the components of the mycobacteriophage which enter the cell, attach to the host DNA. The bacterium becomes lysogenized and contains prophage. Prophage only rarely cause the bacteria to produce more mycobacteriophage. The lytic and lysogenic cycles are represented in the following diagram (26):



Bowman and Redmond reported the first naturally occurring temperate mycobacteriophage R1, isolated from M. butyricum (5,6). The mycobacteriophage were induced by ultraviolet light and after a latent period of six hours yielded 10^4 mycobacteriophage particles and a burst size of 20. It was possible to cure, i.e., to free, the bacterial culture of the prophage by exposure to ultraviolet light (7).

There have been few reports of the isolation of mycobacteriophage from naturally occurring lysogenic mycobacteria (36,57,58,59,60,61,62, 90,91). Recently two different mycobacteriophage were isolated from M. jucho (43). In addition to ultraviolet light, streptomycin and hydrogen peroxide have both been used to induce the prophage of lysogenic mycobacteria (50,83). Filtrates of Phage D32 were used to induce M. tuberculosis (H37Rv), and the surviving colonies were lysogenic. Phage induction of the prophage in lysogenic mycobacteria has also been done with other phage and with other lysogenic mycobacteria (82).

The number of mycobacteria that have been found to be lysogenic is relatively few compared to other bacteria. Mycobacteriophage are essentially the same as other bacteriophage, but the long generation time of many of the mycobacteria necessitates some modifications in laboratory techniques (5,9,10,11,17,64,65,66,67,68,69,70). The effect of the high lipid content in the cell wall is not understood. The tendency of many of the mycobacteria to clump make accurate quantitative measurements difficult and, in many instances, impossible.

Phage D28 propagated on M. smegmatis (9626) had a latent period of 70 minutes, a rise period of 30 minutes, and a burst size of 88 (65). The latent period for Phage D29 was 65 minutes, the rise period was 30 minutes, and the burst size was 104 (5). In comparison, many of the T-even coliphage have a latent period of 25 minutes, a rise period of 10 minutes, and a burst size of 200 (1).

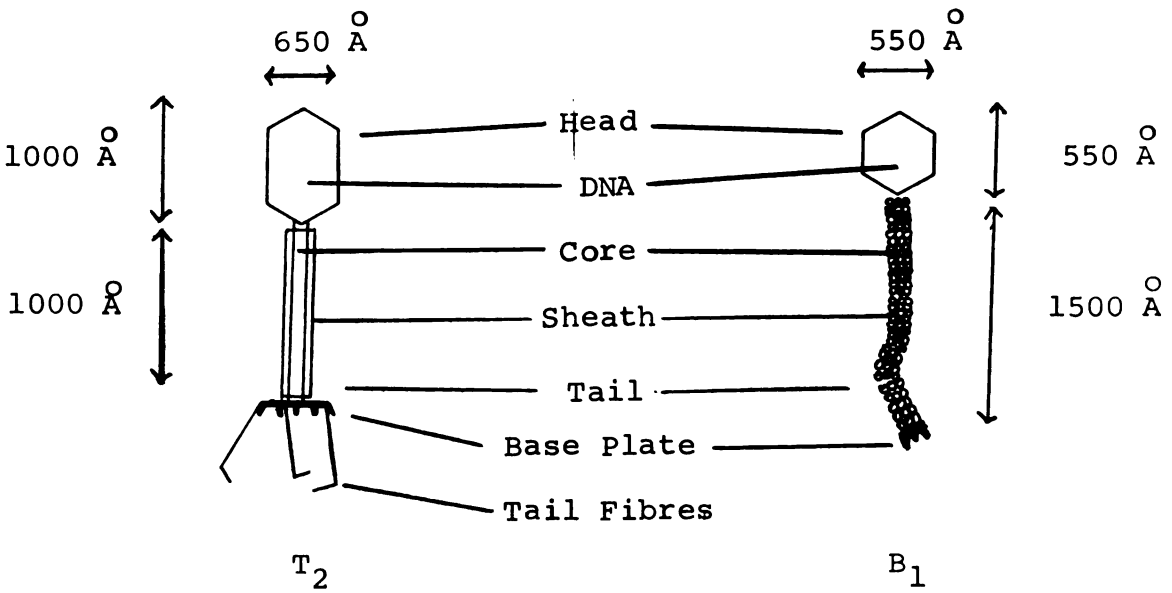
When M. smegmatis (9033) was the host cell for Phage D32, no morphologic changes were seen by electron microscopy during the first 50 minutes after infection with Phage D32 (8,20). Electron dense bodies which closely resembled mature phage heads began to appear in the host cells 70 minutes after infection.

The adsorption rates of mycobacteriophage were very slow. The adsorption rate for a given mycophage varied with the host of the twelve phage; Phage D29 adsorbed fastest to

M. jucho. When the mycobacteriophage were rapidly adsorbed to the host, they were sometimes aborted. This abortion occurred when Phage D29 adsorbed to M. phlei (yoken) (81).

The structure of the mycobacteriophage resemble the structure of other bacteriophage. It is comparable in size, shape, and type of structures, although variations are reported in the length of the tail.

Mycobacteriophage B_1 was 2000 Å long, the diameter of the head was 550Å, and the tail was 1500 Å by 90 Å. The head was icosahedral with six capsomeres on each side. The tail had a sheath surrounding the inner core and was composed of 32 subunits. The tail was noncontractile and the prominent base plates were 100 Å long. Five tail spikes were barely visible. The structure of spikes has not yet been fully described. A comparison of the structure of mycobacteriophage B_1 to coliphage T_2 is represented below (23,73, 74,77).



The mycobacteriophage head is composed of a protein coat surrounding an inner core of DNA. The tail also consists of protein surrounding a hollow core. The DNA of phage is generally double stranded; however, the DNA of coliphage X174 is single stranded. Recently RNA coliphages have been isolated which are structurally more similar to the single stranded DNA phage than to the more common double stranded DNA phage. In their polygonal shape with no visible tail structures, they resemble the plant and animal viruses. They are approximately 225 Å in diameter, which is considerably smaller than the double stranded DNA phage.

Bacteriophage are quite stable, especially in their own lysates. All are stable in a pH range of 5 to 8, some are stable at 4, and others at 10. Phage precipitated by lowering the pH generally retain their infectivity. Free phage are inactivated by compounds such as urea, urethane, mustard gas, halogens, permanganate, ozone, hydrogen peroxide, mercuric ion, thymol, cyanide, and flouride. Some chelating agents such as citrate and triphosphate increase the rate of inactivation of phage. Inactivation of phage by formaldehyde can be reversed by dilution. Glycerine, alcohol, and detergents generally do not inactivate phage.

Phage can be inactivated by sonic vibration and by osmotic pressure, but different phages vary in their relative susceptibility. Inactivation can occur in making dilutions and transfers due to breakage of the tails. Without

intact tails, the phage do not adsorb to the bacterial host cell wall.

Certain ions are necessary for the adsorbtion of the phage to the host cell wall. Divalent cations such as Ca^{++} or Mg^{++} and monovalent cations such as K^{+} and Na^{+} are required in low concentrations. The type and concentration of the cation required depends upon the type of phage. Mycobacteriophage D28 required Ca^{++} and Mg^{++} but not Na^{+} or K^{+}. The adsorbtion rate of mycophage D29 was nonspecifically increased by any of the four cations.

Temperature affects phage in different ways. Heat can inactivate phage by denaturization of protein and follows first order kinetics. The rate of heat inactivation is influenced by the chemical composition of the medium. Phage in saline solution are readily inactivated by heat; in a broth they are many times less susceptible. Mycobacteriophage are stable for months at $4^{o}C$ in their lysates. Phage can be preserved by rapid freezing with liquid nitrogen and by lypholization (31,93).

Temperature may also influence the infectious process. M. avium was resistant to infection by phage at $37^{o}C$, but it was no longer resistant to the same phage at $42^{o}C$ (19).

An unusual role in the disease sarcoidosis has been proposed for mycobacteriophage which lysogenize M. tuberculosis (22,38). When the phage are present and an individual

does not produce antibodies, the phage affect M. tuberculosis so that they do not have cell walls, are not acid fast, and do not induce delayed sensitivity. Instead of tuberculosis, the individual has sarcoidosis (39,40,41,52).

Bacteriophage Typing. The differential susceptibility of different bacteria to a phage or set of phages has contributed significantly to the identification of several genera and species of bacteria, and epidemiologic studies. Many of the procedures used today in phage typing were developed with Staphylococus aureus during the 1950's. Serologic methods divided the staphylococi into a few relatively broad groups, too broad for epidemiological studies (88).

Fish formulated the basis for many of the techniques with which bacteria are identified by their susceptibilities to bacteriophage. Many of the bacteriophage which were first discovered lysed many strains or species of bacteria and were not specific. Other phage or combinations of phage which were more specific were found, either by the isolation of new phage or by the adaptation of old phage to different strains of staphylococci. The routine test dilution and the spot plating method were developed.

Strains of staphylococci retained their original phage susceptibility after growth in mice or on media. Phage typing of the staphylococci permitted their classification into five groups. This system has made a major contribution to epidemologic studies of staphylococci infections.

The classification system of staphylococci by Ripon has been adopted by most laboratories and is based on the serologic relationships and the lytic spectra of the phage (88). Approximately 75% of the staphylococci were lysogenic. The prophage was responsible for the resistance to other phage, and the resistance was specific for a given serologic group.

New phage were developed by adaption or conversion. Adaptions are either mutations which change the host range or modifications induced by the host. A conversion is due to the replacement or alteration by the infecting phage of the prophage initially present in the bacterium. The phage produced is referred to as converted because it now lyses a different serologic type of staphylococci. It has been shown that the plating of concentrated lysates of phage result in production of characteristic prophage rather than the original lysate phage.

Variations in the typing occur, 3% with strong lytic patterns, 50% with partial lytic patterns. There are three possible results in phage typing, although only two are recorded: no lysis, incomplete lysis, and complete lysis. No lysis is recorded as negative; incomplete lysis and complete lysis are recorded as positive. The partial lytic variation is due to incomplete lysis and may become complete lysis or vice versa. Because variation occurs, rigid controls must be employed for exact and reproducible results.

Staphylococcal typing generally includes 21 to 24 phage types, five lytic groups and five serologic types (87). The stock suspensions of phage are obtained after being propagated by the soft agar overlay technique or by the newer cellophane technique. The phage are titrated to determine the routine test dilution (RTD), the highest dilution of phage giving complete lysis. A multiple syringe applicator has been developed which can apply in one hour, routine test dilutions of 26 phage to 300 plates seeded with bacteria to form a lawn. The stock phages should not be propogated on a culture that has somehow been altered. Controls on the typing phage, on its propagating host, and its potency should be included.

The identification of strains and species of the mycobacteria with mycobacteriophage is in the very early stages of development (48,72,75). Relatively few mycobacteriophage have been isolated, and few exhibit host specificity (50). This is not unusual since host specific phage are rare for any genus of bacteria. Large numbers of phage have to be isolated and screened to find host specific strains.

Adaptation has recently been described for the mycobacteriophage, and it has been suggested as a suitable means for the procurement of new phage with greater host specificity (28,35). Either the adapted phage has some change in its genome or it is a new phage which was induced. The phage typing pattern of Phage 101 became more specific for

a species of mycobacteria after serial passage on that species (34).

Another major obstacle in the development of mycobacteriophage typing has been the failure of some investigators to use the routine test dilution (49). If the lysate is too concentrated, non specific lysis occurs, perhaps due to the induction of prophage in the culture or to the presence of contaminating viruses in the lysate at the higher concentrations (50,88).

The method used for typing mycobacteria is essentially the same as the methods described for staphylococci and other general of bacteria. The main difference is that the soft agar overlay technique has not been satisfactory for the preparation of stock phage suspensions. The growth of mycobacteria is inhibited by the soft agar overlay. Plaques may be formed, but poorly. The method most commonly used is to "spot" the routine test dilution of the phage onto a lawn of the mycobacteria (49,50,51). The lawn may have been either freshly seeded or have been grown for several days, depending upon the growth rate of the species or strain of Mycobacterium used. Phage are harvested from lawns of the propagating mycobacteria (previously seeded with the routine test dilution) by flushing the surface of the plate or eluting the phage from agar blocks cut from the area of lysis.

Attempts have been made to type several strains of mycobacteria with mycobacteriophages (3,4,18,28,29,44,48,50). Mycobacterium tuberculosis (H37Rv), M. bovis, M. avium, and the Group III (Battey strain) and a saphrophyte (607) have been typed using mycobacteriophage with limited specific activities (47,54,95). The problem is compounded by the fact that the present classification of many of the mycobacteria is still unresolved. Work has been reported with phage typing of rapid growers (29). The results are somewhat more encouraging.

There is a great need for a reliable method for the identification of mycobacterial isolants. If a phage system can be developed and standardized, it will be a major contribution.

MATERIALS AND METHODS

Media. A 1% dextrose Dubos broth was prepared from Dubos broth base[1] without Tween 80 or enrichment, dispensed into 20 ml screw top tubes in 0.9 ml and 5.0 ml amounts, and autoclaved (15 minutes, 121°C). Dubos-agar was prepared by the addition of 1.0% Bacto agar (Difco) to Dubos dextrose broth and autoclaved (15 minutes, 121°C). A thick layer was poured into Petri dishes. Dubos-Noble agar plates were prepared as described for the Dubos-agar plates, substituting Difco-Noble agar for Bacto-agar. Prepared Lowenstein Jensen medium in tubes was purchased (Difco).

Strains and Cultivation of Mycobacteria. Table 1 lists the mycobacteria used, the laboratory in which they were isolated, and the source of the specimen from which the Mycobacterium was isolated. Stock cultures of the Mycobacterium were maintained on Lowenstein slants at 35°C. Rapid growers, (growth of isolated colonies, 5 days or less) were transferred every two to four weeks; others were transferred every four to twelve weeks. Before seeding with phage, a heavy suspension of cells was used to seed Dubos dextrose

[1]Difco Laboratories, Detroit, Michigan.

Table 1. Origin and source of mycobacterial cultures

Mycobacterium	Laboratory	Source of Specimen
M. tuberculosis		
H37Rv	Redmond	Man
Group I		
M. kansasii	Redmond	Man
Ps88[a]	Redmond	Man
M. Avium		
170-2	M.S.U.[b]	Swine
131-4	M.S.U.	Chicken
132-4	M.S.U.	Chicken
Group III		
50B-0	M.S.U.	Cow
$51C_2$-0	M.S.U.	Cow
$62D_2$-0	M.S.U.	Cow
12100	M.S.U.	Man
P39	Redmond	Man
15 wet	M.S.U.	Inanimate
259-1	M.S.U	Swine semen
$172C_2$-1	M.S.U.	Pig
Group II		
P17	Redmond	Man
P38	Redmond	Man
Rapid Growers		
ATCC 607[c]	Redmond	Inanimate
2118	Redmond	Inanimate

[a]Organism isolated from surgical specimens from human tuberculosis and identified by Ernest H. Runyon.

[b]Tuberculosis Project at Michigan State University.

[c]American Type Culture Collection.

broth. Rapid growers were incubated at 35°C from four to seven days; others, from three to five weeks. The broth cultures were used to prepare lawns for the phage or to add to the phage dilutions for titration.

Source of Phage. Table 2 lists the phage used and the laboratory in which they were isolated, their source, and the propagating Mycobacterium.

Titration of Phage Suspensions. Phage received in agar blocks were eluted in 1.0 ml of Dubos broth overnight at 4°C. Phage were stored at all times at 4°C. The phage were titrated on their designated propagating mycobacteria. Serial ten fold dilutions of the phage were made using 0.9 ml of Dubos broth per tube as diluent. Each dilution was mixed by gentle shaking. One-tenth ml of the propagating host bacteria was added to 0.9 ml of the diluted phage suspension. The suspension was incubated for 15 minutes at hood temperature (approximately 35°C), poured, and spread on a Dubos-agar plate. After being seeded with phage, the rapid growers were incubated at 35°C from 1 to 4 days; others, at 35°C from 7 to 21 days. After incubation, the plates with 30 to 300 plaques were counted and the titer determined by multiplying the number of plaques by the reciprocal of the dilution.

Each phage was diluted to the routine test dilution as determined by the preliminary titration of the original

Table 2. The source and propagating strains of mycobacteriophage used in typing

Phage	Individual From Whom Received[a]	Source	Original Propagating *Mycobacterium*
101A	Manion	Soil[b]	2118
GS4	Redmond	Soil	ATCC 607
LEO	Mankiewicz	Man[c]	ATCC 607
D29	Froman	Soil	ATCC 607
AG1	Redmond	Adapted[d]	*M. kansasii*
GS4E	Redmond	Adapted[e]	*M. tuberculosis* (H37Rv)
DW	Mankiewicz	Man[f]	ATCC 607

[a] R. Manion, Veterans Administration Hospital, Minneapolis, Minnesota. W. Redmond, Veterans Administration Hospital, Atlanta, Georgia and Microbiology Department, School of Medicine, Emory University. E. Mankiewicz, Royal Edward Laurentian Hospital, Montreal, Quebec. S. Froman, Department of Infectious Disease, School of Medicine, University of California, Los Angeles.

[b] Phage 101 adapted to 2118.

[c] Isolated from stool specimen of a sarcoidosis patient.

[d] From a mixture of mycobacteriophage.

[e] From GS4.

[f] Isolated from a stool specimen of a tuberculous patient.

phage suspension on its propagating host. The routine test dilution (RTD) is the highest phage dilution giving confluent lysis.

Phage Production and Storage. The routine test dilution of each phage stock was used to seed its designated propagating host, as described in Titration of Phage Suspensions. After incubation, the surface of the plates with complete or nearly complete lysis were covered with 5.0 ml of Dubos broth and refrigerated (4°C) overnight. The fluid was removed, filtered,[1] and titrated.

The titer of a Phage GS4 suspension was determined after different periods of incubation. Quadruplicate 0.9 ml routine test dilutions of phage GS4 were seeded with 0.1 ml of ATCC 607. After 24, 36, 48, and 96 hours of incubation, the phage was harvested and titrated.

The phage were titrated on strains ATCC 607 and 2118 (rapid growers). The routine test dilutions were determined for the phage capable of lysing these mycobacteria. Routine test dilutions of Phage GS4, LEO, D29, AG1, GS4E, and DW were used to seed ATCC 607; and the routine test dilution of phage 101A was used to seed 2118 for the production of phage stock to be used in typing the mycobacteria. The phage

---

[1]Millipore Filter Corp., HA 0.45u, white grid.

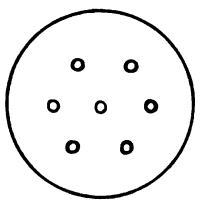
suspensions were filtered, chloroform was added, and the suspension of phage was titrated.

The titer of phage GS4 was determined before and after filtration, centrifugation, and chloroforming. A suspension of Phage GS4 was divided into 3 samples: one sample was filtered, one was centrifuged (2010 x g, 20 minutes), and one was centrifuged (2010 x g, 20 minutes) and then filtered. The three suspensions were then titrated on host ATCC 607. One-tenth ml of chloroform was added to 15 ml of a filtered suspension of Phage GS4 and titrated. Several strains of mycobacteria were tested for their susceptibility to chloroform by adding a heavy inoculum of bacteria to 50 ml of Dubos broth and then adding 0.1 ml of chloroform and plating 1.0 ml on Dubos-agar plates.

The use of stationery and aerated broth cultures (aerated by magnetic agitation) for the production of phage was compared. Six 250 ml Erlenmyer flasks containing 25 ml of Dubos broth were seeded with 0.1 ml of a heavy inoculum of host ATCC 607 and 0.9 ml of the routine test dilution of Phage GS4. Magnetic stirring bars were added to three of the flasks; the flasks were sealed in plastic bags and incubated at 35^{o}C. Those flasks with magnetic stirring bars were aerated with gentle continuous stirring. Flasks with magnetic stirring bars and flasks without magnetic stirring bars were removed from the incubator at 12, 24, and 48 hours.

The cultures were centrifuged at 4°C (2010 x g, 20 minutes), filtered, and titrated.

Spot Typing of the Mycobacteria. Stock suspensions of Phage 101A, GS4, LEO, D29, AG1, GS4E, and DW were used in typing the mycrobacteria. They were filtered, chloroform was added, and they were diluted with Dubos broth to the routine test dilution. The phage were spotted onto the plate according to the following diagram:



One-tenth ml of a heavy inoculum of the rapid growers was spread on duplicate Dubos-agar plates and immediately spotted with one drop of each of the seven typing phages from a 0.5 ml disposable syringe with 26 guage needle. One-tenth ml of a heavy inoculum of the slow growers was spread on duplicate Dubos-agar plates, incubated at 35°C for 7 to 14 days, and spotted with the typing phage. The plates were incubated at 35°C. Rapid growers were observed after three days and slow growers at 7, 14, and 21 days for areas of lysis.

Phage Neutralization Tests. Mice were inoculated subcutaneously with 0.1 ml of either a 10^{-3} dilution of phage GS4 which had been filtered and treated with chloroform (1×10^{9} plaque forming units per ml). The mice were reinoculated after seven days with the same amount of phage. Blood was collected 14 days post inoculation; the serum was prepared and inactivated ($56^{\circ}C$, 30 minutes). Serial two-fold dilutions of the serum were made. One-hundredth ml of a 10^{6} or 10^{8} dilution of phage was added to each serum dilution and the mixtures incubated ($35^{\circ}C$, 30 minutes). One-tenth ml of a suspension of ATCC 607 was added to the suspension and spread on Dubos-agar plates. Normal mouse serum was used in control tests.

RESULTS

The time required for confluent growth on Dubos-agar plates differed among the mycobacteria, as listed in Table 3. The rapid growers ATCC 607 and 2118 had confluent growth after four days; one Group I (Ps88), two Group II (P17 and P38), and Group III (P39), after three weeks; the others, after five weeks.

The results of the preliminary titrations and propagation of mycobacteriophage stock suspensions on the original propagating hosts are listed in Table 4. Eleven of fifteen phages lysed their propagating hosts to some degree. There were 1×10^{9} phage/ml of phage LEO with an RTD of 10^{-5} and 3×10^{7} phage/ml of phage D29 with an RTD of 10^{-4}. The other nine mycobacteriophage which lysed their original propagating hosts to some extent had 1×10^{4} phage/ml or less and RTDs of 10^{0}. When the RTD of each of eleven mycobacteriophages was seeded onto their corresponding propagating hosts, six mycobacteriophage, 101A, GS4, LEO, D29, AG1, and DW, ranged from 1×10^{2} to 2.3×10^{10} phage/ml after harvesting.

Seven mycobacteriophage 101A, GS4, LEO, D29, AG1, GS4E, and DW, lysed either of the rapid growers ATCC 607 or 2118 as listed in Table 5. All seven mycobacteriophage

Table 3. Days required for visible growth of phage propagating mycobacteria in Dubos 1% dextrose broth, 35°C

Mycobacterium	4 Days	3 Weeks	5 Weeks
M. tuberculosis			
H37Rv	None	Very little	Good growth
Group I			
M. kansasii	None	Very little	Good growth
Ps88	None	Good growth	
M. avium			
170-2	None	Very little	Good growth
131-4	None	Very little	Good growth
132-4	None	Very little	Good growth
Group III			
50B-0	None	Very little	Good growth
$51C_2$-0	None	Very little	Good growth
62D-0	None	Very little	Good growth
12100	None	Very little	Good growth
P39	None	Good growth	
15 wet	None	Very little	Good growth
259-1	None	Very little	Good growth
$172C_2$-1	None	Very little	Good growth
Group II			
P17	None	Good growth	
P38	None	Good growth	
Rapid growers			
ATTC 607	Good growth		
2118	Good growth		

Table 4. The amount of mycobacteriophage received[a] and after propagation on the designated *Mycobacterium*[b]

Phage[a]	Host[b]	Stock Phage/ml	Number Phage/ml After Propagation
101A (R)	2118	1×10^4	3×10^8
GS4 (R)	ATCC 607	1×10^3	1.5×10^8
LEO (M)	ATCC 607	1×10^9	8×10^9
D29 (M)	ATCC 607	3×10^7	2.3×10^{10}
AG1 (R)	*M. kansasii*	1×10^2	1×10^3
GS4E (R)	*M. tuberculosis* (H37Rv)	0	0
DW (M)	ATCC 607	9×10^2	1×10^2
DS6A	*M. tuberculosis* (H37Rv)	0	0
AX1	*M. kansasii*	0	0
BM_4Ps	Ps88 (Group I)	10	0
C_3Ps	Ps88 (Group I)	10	0
L_1Ps	Ps88 (Group I)	100	0
BG1	P17 (Group II)	10	0
BG2	P39 (Group III)	1	0
G1	P38 (Group II)	0	0

[a]Stock phage obtained from W. Redmond, (R), Microbiology Dept., School of Medicine, Emory University and E. Mankiewicz, (M), Royal Edward Laurentian Hospital, Montreal, Quebec.

[b]Propagating host designated by laboratory from which phage were received.

Table 5. The amount of phage in stock suspension lytic for mycobacteria ATCC 607 and the amount of phage produced by ATCC 607[a]

Phage	Original Number Phage/ml	RTD[b]	Number Phage/ml Recovered
101A[c]	...	...	...
GS4	1×10^3	10^0	1.5×10^8
LEO	1×10^9	10^{-5}	8×10^9
D29	3×10^7	10^{-4}	2.3×10^{10}
AG1	4×10^8	10^{-3}	1×10^8
GS4E	2.4×10^9	10^{-4}	4×10^9
DW	9×10^2	10^0	1×10^3

[a]Rapid grower.

[b]Subsequently propagated on strain 2118.

[c]RTD: highest dilution of phage giving complete lysis.

lysed ATCC 607; and three mycobacteriophage, D29, LEO, and 101A, lysed 2118 (Table 6). Titers of stock phage suspensions ranged from 9 x 10^2 to 2.4 x 10^9 phage/ml, and RTDs from 10^0 to 10^{-5}. When one RTD of the mycobacteriophages was seeded onto either ATCC 607 or 2118, harvested, and titrated, relatively large amounts of six phage had been produced. There were 1 x 10^8 phage/ml of Phages 101A, GS4, LEO, D29, AG1, and GS4E. Mycobacteriophage DW had a titer of 1 x 10^3/ml. The other eight mycobacteriophage tested, DS6A, AX1, BM_4Ps, C_3Ps, BG1, BG2, and G1, did not lyse either ATCC 607 or 2118.

The titer of phage suspensions increased with the period of incubation up to 48 hr. The titer of the suspension remained constant upon further incubation. The highest titer of a suspension of mycobacteriophage GS4, propagated and titrated on ATCC 607, was obtained after 48 hours of incubation. The amount of phage recovered increased from 24 to 48 hours where it remained constant upon further incubation (Table 7).

Centrifuging reduced the titer of Phage GS4 suspension recovered from and titrated on host ATCC 607. The titer of the centrifuged suspension was 2 x 10^4 phage/ml, whereas the non-centrifuged control was 5 x 10^7 phage/ml.

Chloroform killed all strains of mycobacteria tested and did not inactivate the mycobacteriophage. There were

Table 6. Bacteriophage typing of the mycobacteria

Mycobacterium	Source	Mycophage[a] 101A	GS4	LEO	D29	AG1	GS4E	DW
M. tuberculosis								
H37Rv	Man	-	+	+	+	+	+	+
Group I								
M. Kansasii	Man	+	+	-	-	+	-	-
Ps88	Man	+	-	-	+	+	-	-
M. avium								
170-2	Pig	-	+	-	-	-	-	+
131-4	Chicken	-	+	-	-	-	-	-
132-4	Chicken	-	-	+	-	-	-	-
Group III								
50B-0	Cow	-	+	+	+	+	+	+
$51C_2$-0	Cow	-						
62D-0	Cow	-	+	+	+	+	+	-
12100	Man	-	-	+	+	+	+	+
P39	Man	-	+	+	+	+	-	+
15 wet	Inanimate	-	-	-	+	+	-	+
259-1	Swine semen	-	+	-	+	+	-	+
$172C_2$-1	Pig	-	+	-	+	+	+	+
Group II								
P17	Man	-	-	+	+	+	-	+
P38	Man	-	+	+	-	-	+	-
Rapid growers								
ATCC 607	Inanimate	+	+	+	+	+	+	+
2118	Inanimate	+	-	+	+	-	-	-

[a]Propagating host ATCC 607 for all mycobacteriophage except 101A propagated on 2118.

Table 7. Titers of mycobacteriophage suspensions incubated 24, 36, and 48 hours at 35°C

Phage	Propagated and Titrated On	Number of Phage/ml		
		24	36	48
GS4	ATCC 607	1×10^4	2×10^6	1.5×10^8

5×10^7 phage/ml of GS4 propagated and titrated on ATCC 607 when treated with chloroform; 3×10^7 phage/ml without chloroform treatment.

Aerated broth cultures of mycophage GS4 and LEO had 3×10^2 and 1×10^3 phage/ml respectively. Broth cultures which were not aerated by a spinning magnetic bar had no detectable phage. All three phage tested were propagated and titrated on host ATCC 607. Mycobacteriophage D29 did not propagate in either the broth or the aerated broth cultures.

Phage GS4 when adapted to ATCC 607 showed no significant difference from phage GS4 which was not adapted when both were propagated and titrated on ATCC 607. The unadapted mycobacteriophage GS4 could lyse P38, whereas the adapted mycobacteriophage could no longer lyse P38.

The 18 strains of mycobacteria varied in their susceptibility to seven of the mycobacteriophages (propagated and titrated on ATCC 607 except for 101A propagated and titrated on 2118). The results are listed in Table 6. The three M. avium strains differed in their susceptibility to mycobacteriophages D29, AG1, and to a certain extent to GS4E. Mycobacterium tuberculosis, Group I, Group III, Group II, and the rapid growers were susceptible to mycobacteriophages, D29, AG1, and GS4E, to varying degrees and all were lysed by at least one of the three. The Group III mycobacteria isolated from bovine tissues had the same phage susceptibilities;

the Group III mycobacteria from other sources varied in susceptibility to mycobacteriophages GS4, LEO, and GS4E. The Group III mycobacteria of bovine origin were similar to M. tuberculosis (H37Rv); they were not susceptible to phage 101A; Group 1 and rapid growers were susceptible to phage 101A. They could be distinguished from the other mycobacteria in that the Group III mycobacteria were susceptible to GS4E. Group I, Group II, Group IV, and Group III mycobacteria other than those of bovine origin, were very similar in phage susceptibility. Mycobacteriophage 101A lysed the rapid growers and Group I but not Group III or Group II mycobacteria. Mycobacteriophage LEO did not lyse Group I mycobacteria, but it did lyse the rapid growers. Mycobacteriophage 101A was the least active phage; it lysed only four mycobacteria. No mycobacteriophage lysed all the strains tested. ATCC 607 was susceptible to all seven mycobacteriophages.

Important differential susceptibilities were:

1. Group I mycobacteria (Ps88 and M. kansasii) were the only slow growers lysed by Phage 101A.
2. M. avium (170-2, 131-4, 132-4) was not lysed by Phage D29 or AG1; all Group III mycobacteria were lysed by both.
3. Saprophytic Group III mycobacteria (15 wet and 259-1) and Group III mycobacteria of swine origin (172C-1) were not lysed by Phage LEO; all other Group III mycobacteria were lysed by Phage LEO.

4. Saprophytic Group III mycobacteria (15 wet and 259-1) were not lysed by Phage GS4E; Group III of swine origin (172) was lysed by Phage GS4E.

The phage neutralization titer was greater than 1280; normal serum was negative. The phage control, consisting of phage incubated at 37°C for 30 minutes, had a reduction in titer of three log units.

DISCUSSION

Because the original propagating hosts were largely slow growers and in some instances not very susceptible to their phage, low phage titers were obtained from them. Rapid growers ATCC 607 and 2118 were used to propagate the seven mycobacteriophages used for typing. By adaptation to the rapid growers, considerable time can be saved in obtaining and titrating phage. The host range may be modified, but this does not necessarily reduce the usefulness of the differential susceptibility, once established.

Centrifugation of phage lysates was not regularly used because the titer of the lysate was reduced by centrifugation. Filtration did not reduce the titer, and, in addition, all cells were removed. The tendency of clumping of the mycobacterial cells is often a disadvantage; it is a distinct advantage in membrane filtration.

Chloroform, which is not reportedly used to any extent with mycobacteriophage, appeared to be very satisfactory for the inactivation of any bacterial cells in phage lysates. This was not discussed in any of the pertinent literature read thus far, and no disadvantage to its use is known. It is advisable to exercise all feasible methods of

inactivating the mycobacteria from the standpoint of avoiding possible infections.

More phage was obtained in broth when the medium was agitated during incubation than without. However, enough of the mycobacteriophage for typing could be collected by the Dubos-agar method, and there is less chance of glass breakage, aerosols or other hazardous technique. The necessity of using liquids with mycobacteriophage and the mixing and transferring of those liquids undoubtedly creates aerosols. The route of infection by mycobacteria is the mucous membranes of the respiratory tract. Very small droplets are required. Any method which removes or reduces the probability of an aerosol of viable organisms is desirable. Mycobacteriophage are relatively stable at 4°C for long periods of time. However, after two and one-half months at 4°C in filtered, chloroform-treated lysates, the mycobacteriophage used for typing were no longer capable of lysing the mycobacteria. The means by which they are inactivated is not known. Most probably, the tail proteins or tail structure have been altered or broken, and the phage can no longer adsorb to bacterial cells.

Many of the phages used in typing other genera of bacteria probably have their origin as prophage in those genera. They can generally be induced by several commonly used chemical or physical methods and exposure to infective phage. The prophage may exist in the host as a defective

prophage, lacking genetic information necessary to direct the formation of mature phage particles. The infecting phage may supply the necessary information, and the phage with the genetic material of the prophage is produced (23). This is one explantion for what appears to be a change in the phage's capacity to infect (88).

Few of the mycobacteria have been found to be lysogenic. This could be more correctly stated, very few of the prophage of the mycobacteria have been induced. The lack of lysogeny may be due to the existence of an unusually high proportion of the prophage as defective prophage due to improper methods of induction. The unusually high lipid content in the cell wall of mycobacteria may protect the cell from infection. Directly or indirectly, the repressor which is thought to prevent prophage induction in the bacterial cell may be less readily countermanded. Inducers probably destroy the repressor which leads to prophage development and eventually lysis. In some preliminary studies of induction, not included in this thesis, ATCC 607 was successfully induced by ultraviolet light. This area of research, induction of prophage in mycobacteria, will be pursued further and may provide more specific phage which are needed for typing mycobacteria.

In addition to the phages obtained by isolation from natural sources, phages have been obtained by adaptation which can be used for typing. Their host range is frequently

altered and not necessarily stable. Proper controls must always be included. There may be a slightly different lytic pattern after several transfers on the same host, as was observed with an adapted mycobacteriophage GS4. However, typing phage generally vary up to 15%, which emphasizes the necessity of 'host controls' in any set of typing tests.

Due to the long periods of time needed to propagate an assay mycobacteriophage on slow growers, the work becomes tedious and time consuming. More importantly, there must necessarily be a greater length of time required to obtain results from titration and control plates to determine the RTD. The procedures can be accomplished more effectively and reliably if the phage are adapted so that rapid growers can be used as propagating and control hosts. It has been suggested that this is not adaption due to crossing over between the phage and host genomes but due to the induction of closely related preexisting prophage.

The difference in the susceptibility of M. avium and Group III mycobacteria supports other evidence that M. avium and Group III organisms are not identical. The Group III mycobacteria of bovine origin appear to differ greatly from M. avium, and to a lesser extent from the Group III mycobacteria from other sources. However, the lack of susceptibility of M. avium may be nothing more than the result of the difference in the optimum temperatures for growth.

M. avium strains are not susceptible at 35°C, but they may be at 42°C. This will be investigated further. The lack of susceptibility at 35°C of M. avium does not negate the value of differentiation at 35°C of other mycobacteria. If phage could be isolated from lysogenic M. avium strains which would only lyse M. avium at 42°C and no other mycobacteria at 35°C it would provide an ideal method for the identification of M. avium. Such systems for the other mycobacteria may be possible also.

A large amount of variation does occur in phage typing performed with different lots of phage at different times. This variation was noted for those mycobacteria which were typed over a period of six months. Besides the inherent variation of 15%, which occurs in most of the phage typing systems, additional variation undoubtedly occurs due to variations in the mycobacteria as they are maintained in the laboratory.

Temperature plays an important role in phage specificity, adsorption, and multiplication. A heated block was not used to incubate the phage and mycobacterial mixtures at 35°C for one-half hour prior to plating. This may introduce some variation, although the temperature of the room and bacteriologic hood is relatively constant.

Phage GS4 and the GS4-ATCC 607 system were the most reproducible system tested. Therefore, it was used in the adaptation, centrifugation, chloroform treatment, time, and

induction studies. The results may or may not be applicable to other phage.

An examination of the results of phage typing of the mycobacteria indicate that such studies should be continued. There is no single test or set of tests which are presently satisfactory for the identification of many of the mycobacteria isolated from animate and inanimate sources. If developed, they can contribute significantly to epidemiologic studies. At the present time, the distribution, mode of transmission, and relationships among many of the atypical mycobacteria are not known. It is only known that they can be isolated from a wide range of animate and inaminate sources, that some do produce disease in man and animals, and others are undoubtedly saprophytes. There is no usable method available to differentiate between pathogens and saprophytes. By inference, those isolated from tuberculosis tissues are assumed to be pathogens. Some are, but some may be contaminants. Phage typing may contribute to the present controversy and conflicts.

One of the primary problems of the Tuberculosis Research Project at Michigan State University is one concerned with reliability differentiating between M. avium and Group III mycobacteria, which are potential pathogens for swine but not for calves. By the use of D29 or AG1 and LEO, and then GS4E, this differentiation was possible as follows:

	D29 or AG1	LEO	GS4E
M. avium	-	+	-
Group III - bovine	+	+	+
Group III - swine	+	-	+
Group III - saprophytes	+	-	-

Many more strains and species of mycobacteria will need to be examined for their susceptibility to these and other phage. If the phage system can be developed, phage typing of mycobacteria can be of considerable practical importance, contribute to epidemiologic studies, and provide models for studies on the fundamentals of bacterial host-parasite interactions.

SUMMARY

Mycobacteriophage was propagated on nonpathogenic, rapidly growing mycobacteria. The infectivity of the phage for mycobacteria, for 18 stains or species of mycobacteria, representing M. tuberculosis, M. avium, Group III, Group II, and rapid growers was determined.

By the use of four phage, D29 or AG1, LEO, and GS4E, it is possible to separate M. avium from Group III mycobacteria; Group III mycobacteria of bovine origin from Group III mycobacteria of swine and inanimate origin; Group III mycobacteria of swine origin from Group III of inanimate origin.

LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.

2. Albertson, J. N. 1965. Changing concepts in tuberculosis. The anonymous mycobacteria. Am. Rev. Resp. Dis. 91: 782.

3. Bates, J., and J. Fitzhugh. 1967. Subdivision of the species M. tuberculosis by mycobacteriophage typing. Am. Rev. Resp. Dis. 96: 7-10.

4. Bonicke, R., and D. Stottmeier. 1965. Identifizerung von stammen der species M. borstelense. Klinik Und Er Forschung der Tub. 130: 210-220.

5. Bowman, B. U. 1958. Quantitative studies on some mycobacterial phage host systems. Amer. Rev. Resp. Dis. 79: 52-62.

6. Bowman, B. U., and W. Redmond. 1959. A temperate bacteriophage from M. butyricum. Am. Rev. Resp. Dis. 80: 232-239.

7. Bowman, B. U., and W. Redmond. 1960. Studies on M. butyricum (R1) cured of its prophage. Isolation and factors contributing to an initial failure to support growth of the homologous phage. Bact. Proceed.: 83.

8. Carlberg, D., and G. Jann. 1963. DNA, protein, and inducible enzyme synthesis in phage infected M. smegmatis. Am. Rev. Resp. Dis. 88: 235-239.

9. Cater, J. C., and W. Redmond. 1960. Isolation and studies of phage lytic for mycobacteria. Bact. Proceed.: 83.

10. Cater, J. C., and W. Redmond. 1961. A recombinant of three phage strains lytic for sprophytic mycobacteria. Bact. Proceed.: 164.

11. Cater, J. C., and W. Redmond. 1961. Isolation of and studies on bacteriophage active against mycobacteria. Canad. J. Micro. 7: 697-703.

12. Cater, J. C., and W. Redmond. 1962. Phage active on mycobacteria from stool specimens of tuberculosis patients. Bact. Proceed.: 96.

13. Cater, J. C., and W. Redmond. 1963. Phage isolated from stool specimens. Am. Rev. Resp. Dis. 87: 726-729.

14. Carbon, J., and A. Trujillo. 1963. A comparison of methods for classification of mycobacteria. Am. Rev. Resp. Dis. 88: 546-550.

15. Erlich, H. 1961. Rapid identification of mycobacterial colonies I. The auramine test. Am. Rev. Resp. Dis. 81: 218-225.

16. Froman, S., D. Will, and E. Bogen. 1954. Phage activity against virulent M. tuberculosis. I. Isolation and activity. Am. J. Pub. Health. 44: 1326-1333.

17. Froman, S., M. Lechtman, L. Scammon, D. Will, and B. Eckmann. 1961. Mycobacteriophage lysates as serologic antigens. Am. Rev. Resp. Dis. 83: 901-902.

18. Froman, S., L. Scammon, and C. Hanson. 1961. Phage susceptibility of Battey type of unclassified acid fast bacillus and M. avium isolated from fowl and swine. Am. Rev. Resp. Dis. 84: 118-119.

19. Froman, S., and L. Scammon. 1964. Effect of temperature on the phage susceptibility of strains of M. avium (isolated from fowl). Am. Rev. Resp. Dis. 89: 236-239.

20. Fukai, K., and M. Sellers. 1960. A morphologic study of mycobacteria infected with phage D32. Am. Rev. Resp. Dis. 81: 52-59.

21. Gale, D., E. Lockhart, and D. Reynolds. 1966. Studies of unclassified mycobacteria: II. Survival and dissemination of typical and unclassified mycobacteria in the organs of mice inoculated intraperitonealy or intravenously. Health Lab. Sc. 3: 48-64.

22. Groman, N. 1961. Phage host relationships in some genera of medical significance. Am. Rev. Micro. 15: 153-175.

23. Hayes, W. 1964. The Genetics of Bacteria and their Viruses. John Wiley and Sons, Inc., New York.

24. Hobby, G., W. Redmond, E. Runyon, W. Schaeffer, L. Wayne, and R. Wichelhausen. 1967. A study on pulmonary disease associated with mycobacteria other than M. tuberculosis: Identification and characterization of the mycobacteria. Am. Rev. Resp. Dis. 95: 954-971.

25. Hok, T. 1962. Nicotinamide test for differentiating M. tuberculosis from other mycobacteria. Am. Rev. Resp. Dis. 86: 94.

26. Jawitz, E., J. Melnick, and E. Adelberg. 1964. Medical Microbiology. 103.

27. Jones, W., and G. Kubica. 1963. The differential typing of certain rapidly growing mycobacteria based on their sensitivity to various dyes. Am. Rev. Resp. Dis. 88: 355-359.

28. Juhasz, S. 1963. Phage specific for some mycobacteria. Nature. 200: 804-810.

29. Juhasz, S., and R. Bonicke. 1965. Possible classification of rapidly growing mycobacteria on the basis of their phage susceptibility. Canad. J. Micro. 11: 235-41.

30. Karlson, A. J. 1965. Identification of M. tuberculosis. Am. Rev. Resp. Dis. 91: 801.

31. Meyle, J. S. 1965. Preservation of T_2 phage with liquid nitrogen. Am. Rev. Resp. Dis. 91: 471.

32. Mallmann, W. L. 1931. Studies on bacteriophage in relation to salmonella and pullorum. Agr. Expt. Sta., Mich. State College, Tech. Bull. 109: 1-14.

33. Mallmann, W. L., V. H. Mallmann, M. D. McGavin, and J. A. Ray. 1965. A study of pathogenicity of Runyon Group III organisms isolated from bovine and porcine sources. Am. Rev. Resp. Dis. 92: 82-84.

34. Mallmann, V. H., W. L. Mallmann, and P. Robinson. 1964. Relationship of atypical bovine and porcine mycobacteria to those of human origin. Health Lab. Sc. 1: 11-20.

35. Manion, R., and S. Bradely. 1964. The derivation of new mycobacteriophage typing reagents by propagation on alternate hosts. Am. Rev. Resp. Dis. 89: 764-766.

36. Mankiewicz, E. 1961. Morphological and biological properties of a strain of chromogenic acid fast bacteria and its phage immune variant. J. Gen. Micro. 24: 63-68.

37. Mankiewicz, E. 1961. Mycobacteriophage isolated from persons with tuberculosis and non tuberculosis conditions. Nature. 191: 1416-1417.

38. Mankiewicz, E. 1963. On the etiology of sarcoidosis. Canad. Med. Assoc. J. 88: 593-595.

39. Mankiewicz, E., and J. Beland. 1964. The role of mycobacteriophages and of cortisone in experimental tuberculosis and sardoidosis. Am. Rev. Resp. Dis. 89: 707-720.

40. Mankiewicz, E. 1965. Phage that lyse mycobacteria and corynebacteria and show CPE on tissue culture of renal cells of Cercopithecus aethiops. Canad. Med. Assoc. J. 92: 31-33.

41. Mankieicz, E. 1965. Antigenic components shared by bacteriophages and phage hosts: mycobacteria, corynebacteria, and Hela cells. Growth. 29: 125-139.

42. Mercky, J. 1964. Disease in man associated with the unclassified acid fast bacteria. Am. Rev. Resp. Dis. 89: 460.

43. Mizuguchi, Y. 1965. Lysogenization of Mycobacteria: I. Lysogeny of M. jucho by phage 413-L and 413-S. Biol. Abs. 46: 30323.

44. Murohashi, T., T. Tokunaga, Y. Mizuguchi, and Y. Maruyama. 1963. Phage typing of slow growing mycobacteria. Am. Rev. Resp. Dis. 88: 664-669.

45. Phillips, S. 1965. Atypical pulmonary tuberculosis caused by unclassified mycobacteria. Am. Rev. Resp. Dis. 91: 782.

46. Potriznik, V. 1964. Thioglycollate medium used for the differentiation of unclassified mycobacteria. Am. Rev. Resp. Dis. 90: 160.

47. Redmond, W., and J. Cater. 1960. A phage specific for M. tuberculosis, varieties hominis and bovis. Am. Rev. Resp. Dis. 82: 781-786.

48. Redmond, W., J. Cater, and D. Ward. 1963. Spotting method of phage typing mycobacteria. Am. Rev. Resp. Dis. 87: 257-263.

49. Redmond, W. 1963. The spotting method for phage typing mycobacteria. Am. Rev. Resp. Dis. 87: 294-295.

50. Redmond, W. 1963. Bacteriophages of the mycobacteria. A review. Adv. Tuberc. Res. 12: 191-229.

51. Redmond, W. 1965. Methods used in phage typing the mycobacteria. Personal communication.

52. Redmond, W., and D. Ward. 1965. Relationships between M. tuberculosis (H37Rv) and phage isolated from a sarcoid patient. National Tuberc. Assoc. Annual Meeting - Med. Abs.: 59.

53. Reggiardo, Z. 1964. Antigenic relationships among mycobacteria by agglutination and agglutinin reactions. Amer. Rev. Resp. Dis. 90: 800-803.

54. Rodda, R. 1965. Phage patterns of fast growing mycobacteria. Bil. Abs. 46: 30325.

55. Runyon, E. H. 1965. Pathogenic mycobacteria. Adv. Tuberc. Res. 14: 235-287.

56. Runyon, E. H. 1967. Mycobacterium intracellulare. Amer. Rev. Resp. Dis. 95: 861-865.

57. Russell, R., G. Jann, and S. Froman. 1958. Lysogeny in the mycrobacteria. Bact. Proc.: 39.

58. Russell, R., G. Jann, and S. Froman. 1959. The induction of the lysogenic state in M. tuberculosis (H37Rv). Am. Rev. Resp. Dis. 78: 839.

59. Russell, R., G. Jann, and S. Froman. 1960. Lysogeny in the mycrobacteria. Am. Rev. Resp. Dis. 82: 384-393.

60. Russell, R., G. Jann, and S. Froman. 1963. Lysogeny of the mycobacteria. Am. Rev. Resp. Dis. 88: 528-538.

61. Russell, R., W. Richards, L. Scammon, and S. Froman. 1964. Isolation of a lysogenic Mycobacterium fortuitum from soil. Am. Rev. Resp. Dis. 89: 287-288.

62. Segawa, J., K. Takeya, and M. Sasaki. 1960. Isolation of lysogenic acid-fast bacilli from the soil. Amer. Rev. Resp. Dis. 81: 419-420.

63. Sellers, M., K. Tokuyasu, Z. Price, and S. Froman. 1957. Electron microscope studies of mycobacteriophage. Am. Rev. Resp. Dis. 76: 964-969.

64. Sellers, M., and H. Runnals. 1961. Mycobacteriophage: I. Physiochemical characterization. J. Bact. 81: 442-446.

65. Sellers, M., W. Baxter, and H. Runnals. 1962. Growth characteristics of mycobacteriophage D28 and D29. Canad. J. Micro. 8: 389-399.

66. Sellers, M., and T. Tokunaga. 1966. Further studies of infectious DNA extracted from mycobacteriophages. J. Exp. Med. 123: 327-340.

67. Shadomy, S., S. Froman, and M. Prickett. 1959. Cytology of phage host interactions in M. tuberculosis. Am. Rev. Resp. Dis. 79: 839.

68. Smith, M., and W. Steenken. 1961. Group differentiation of mycobacteria on thioglycolate medium. Am. Rev. Resp. Dis. 84: 447-450.

69. Sula, L, and J. sulova. 1964. The tellurite zonal phenomenon of mycobacterial lysis observed in a BCG strain exposed to mycobacteriophage My F_2/59. Am. Rev. Resp. Dis. 88: 77-79.

70. Sula, L., and J. Sulova. 1965. The phage tellurite zonal phenomenon as a criterion for the differentiation of slowly growing mycobacteria. Bull. Wld. Hlth. Org. 33: 413-417.

71. Takeya, K., and T. Yoshimura. 1957. Isolation of phage active against all types of M. tuberculosis. J. Bact. 74: 540-541.

72. Takeya, K., T. Yoshimura, K. Yamaura, and T. Toda. 1959. Studies on the biological properties of mycobacteriophage. Am. Rev. Resp. Dis. 80: 543-53.

73. Takeya, K., R. Mori, N. Nakashima, M. Koike, and T. Toda. 1960. Light and electron microscope studies of mycobacteriophages and their interactions I. J. Bact. 78: 307-312.

74. Takeya, K., M. Koike, R. Mori, Y. Yuda, and T. Toda. 1960. Light and electron microscope studies of mycobacteriophage interactions II. J. Bact. 78: 313-319.

75. Takeya, K., Y. Zinnaka, K. Yamaura, and T. Toda. 1961. Bacteriophage susceptibility and tuberculin specificity of unclassified mycobacteria. Am. Rev. Resp. Dis. 81: 674-682.

76. Takeya, K., R. Mori, K. Hisatsune, and T. Toda. 1963. A simple hemolysis test for the classification of the mycobacteria. Am. Rev. Resp. Dis. 87: 773-774.

77. Takeya, K., and K. Amako. 1964. The structure of mycobacteriophages. Virology. 24: 461-466.

78. Tarshis, M. 1962. A combined neutral red, serpentine cord, and differential growth test. Am. Rev. Resp. Dis. 86: 749-754.

79. Tarshis, M. 1962. Further investigation on the arylsulfatase activity of mycobacteria. Am. Rev. Resp. Dis. 88: 847-851.

80. Tarshis, M. 1964. A rapid 24 hour micro phenolphthalein sulfatase test (PST) for distinguishing M. avium from Battey bacilli. Am. Rev. Resp. Dis. 90: 455-457.

81. Tokunaga, T., Y. Mizuguchi, and T. Morohashi. 1964. Abortive infection and host killing of mycobacteriophage. Am. Rev. Resp. Dis. 89: 929-932.

82. Tokunaga, T., Y. Mizuguchi, and T. Murchashi. 1964. Phage induction of lysogenic mycobacteria. Am. Rev. Resp. Dis. 90: 431-446.

83. Tokunaga, T., and M. Sellers. 1965. Streptomycin induction of premature lysis of phage infected mycobacteria. J. Bact. 89: 537-538.

84. Urabe, K., and H. Saito. 1964. The urease activity of mycobacteria. Am. Rev. Resp. Dis. 90: 266-277.

85. Wayne, L. 1962. Differentiation of mycobacteria by their effect on Tween 80. Am. Rev. Resp. Dis. 86: 579-581.

86. Wayne, L., J. Doubek, and R. Russell. 1964. Classification and identification of mycobacteria. Am. Rev. Resp. Dis. 90: 588-597.

87. Wayne, L., and J. Doubek. 1965. Classification and identification of the mycobacteria. II. Tests employing nitrate and nitrite as substrate. Am. Rev. Resp. Dis. 91: 738-745.

88. Wentworth, B. B. 1963. Bacteriophage typing of the staphylococci. Bact. Rev. 27: 253-269.

89. White, A., and J. Knight. 1958. Effect of serum and Tween 80 on phage. Am. Rev. Resp. Dis. 77: 134-145.

90. White, A., F. Foster, and L. Lyons. 1961. Alterations of colony morphology of mycobacteria associated with lysogeny. Amer. Rev. Resp. Dis. 84: 118.

91. White, A., F. Foster, and L. Lyons. 1962. Alterations of colony morphology of mycobacteria associated with lysogeny. J. Bact. 84: 815-818.

92. White, A., L. Lyons, and F. Foster. 1963. The effect of serum and Tween 80 on several mycobacterial phages and susceptible mycobacteria. Am. Rev. Resp. Dis. 87: 730-733.

93. Will, D., S. Froman, I, Kransnow, and E. Bogen. 1957. Cultural characteristics and drug and bacteriophage resistance of avian tuberculosis. Am. Rev. Resp. Dis. 76: 435-450.

94. Will, D., S. Froman, Y. Akiyama, and L. Scammon. 1961. Preservation of acid fast bacilli and mycobacteriophage by rapid vacuum drying. Am. Rev. Resp. Dis. 84: 739-743.

95. Vandra, E. 1963. Studies on phage typing the mycobacteria. Acta. Micro. 9: 61-67.

96. Youmans, G. 1963. The pathogenic atypical mycobacteria. Ann. Rev. Micro. 17: 473-494.