Host Modification and Restriction with a Mycobacteriophage Isolated from a Pseudolysogenic *Mycobacterium chelonae*

By WILBUR D. JONES, JR, AND JOSEPH GREENBERG

*Mycobacteriology Branch, Center for Disease Control, Public Health Service,
U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333, U.S.A.*

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SUMMARY

A pseudolysogenic *Mycobacterium chelonae* and its phage $\phi 630$ are described. Phage $\phi 630$ is the first mycobacteriophage reported to be resistant to the non-polar solvents chloroform, dioxan and diethyl ether. The phage had a latent period of 75 min, a rise period of 90 min and a burst size of 51. Evidence is presented for host modification and restriction. Phage $\phi 630A$, grown on host strain *M. chelonae* F-630 Rg, plated on the alternative host *M. smegmatis* ATCC607 with an efficiency of plating (e.o.p.) of 10^{-5} . Phage $\phi 630B$, grown on host *M. smegmatis*, plated with an e.o.p. of 10^{-5} on the alternative host F-630 Rg. Phages $\phi 630A$ and $\phi 630B$ adsorbed equally well on their alternative hosts and on their indicator host strains. The progeny of plaques from initial platings on the alternative host, when grown in the alternative host, exhibited a marked reduction in e.o.p. on their original host.

INTRODUCTION

In contrast to other phage-bacterial systems, little is known about the mycobacteriophage-mycobacterial host relationship. Bowman (1958) was the first to characterize quantitatively the virus-host relationship in the mycobacteria. Lysogens of *Mycobacterium smegmatis* were characterized by a high frequency of 'cured' cells in the bacterial population and a high titre of phage present in the supernatant fluids of the cultures (Jones & White, 1968; Hubáček, Kupkova & Mohelská; 1970; Baess, 1971). The virus-host relationship in the above systems has the characteristics of pseudolysogeny as defined by Lwoff (1953).

Methods for phage typing isolates of *M. tuberculosis* are currently being investigated in a series of co-operative studies (Rado *et al.*, 1975). The phage typing of *M. tuberculosis* could more easily be understood and the methods more easily standardized, if some basic information were available concerning the phage-host relationships. Redmond (1963) and Marion & Bradley (1964) suggested that phages adapted to alternative mycobacterial hosts should be used for phage typing. Nördström & Grange (1974) used adapted phages in typing of the species *M. ranae*. Rado, Bates & Fitzhugh (1976) and Jones & Greenberg (1976) presented evidence suggesting that host modification and restriction of the phages plays a significant role in phage typing of *M. tuberculosis*. Because of technical problems that exist in working with *M. tuberculosis* and the time required to obtain results from such slow-growing mycobacteria, the availability of a rapidly growing mycobacterial system that could be used to develop techniques and methods and could be applied to *M. tuberculosis* would be a definite advantage (Rado *et al.*, 1976; Jones & Greenberg, 1976).

The present investigation describes the characteristics of a pseudolysogenic isolate of

M. chelonae and its phage $\phi 630$. Evidence is presented for host modification and restriction of the phage, using *M. smegmatis* ATCC607 as the alternative host.

METHODS

Mycobacteria and phages. *Mycobacterium chelonae* strain F-630($\phi 630$) produced plaques on a soft agar lawn of itself. If grown in 7H9 broth containing Tween 80, the culture produced smooth F (SmF) and rough F (RgF) colony types in the ratio of 4:1 when streaked on agar media for colony determination (Jones & Kubica, 1965). After extensive single colony purification the SmF colony types continued to produce RgF variants and to liberate phage, whereas the purified RgF colony types were stable and did not produce phage. A single 'cured' RgF isolate was selected and designated as F-630Rg. A mutant of F-630Rg which was resistant to dihydrostreptomycin sulphate at $1000 \mu\text{g ml}^{-1}$ was isolated and designated as F-630RgSM^r. *Mycobacterium smegmatis* ATCC607, hereafter referred to as 607, was also used as a host strain.

The phage isolated directly from F-630($\phi 630$) and replicated on F-630Rg as the indicator host strain was designated as phage $\phi 630A$. After phage $\phi 630A$ was adapted to replicate on 607 as the indicator host strain, it was designated as phage $\phi 630B$.

Media. Dubos oleic agar (BBL) supplemented with Dubos oleic albumin complex (Difco) (OAA agar) was used for colony determination, for colony forming unit assays, and as the hard basal medium for phage assays. The 7H9 broth medium for bacterial growth was Middlebrook 7H9 broth (Difco), supplemented with 0.05% (v/v) Tween 80 (Atlas Power Company) and Middlebrook ADC enrichment (Difco).

Before use in the phage tests, the bacteria grown in 7H9 broth were washed by centrifuging the cultures at $5000 \text{ rev. min}^{-1}$ for 15 min, resuspending the pellet in heart infusion broth (Difco) supplemented with 0.02 M-calcium chloride (HI broth), and recentrifuging. The bacterial pellets were then resuspended in tempered (37°C) HI broth. Phage assays were performed according to the soft agar overlay method (Adams, 1959). Soft agar overlays were prepared with Dubos broth base w/o Tween 80 (Difco) supplemented with 0.7% (w/v) agar. The above media were all prepared and used as previously described (Jones & White, 1968). All bacterial cultures were grown at 37°C .

Bacterial and phage assays. Assays for colony forming units (c.f.u. ml^{-1}), phage assays (p.f.u. ml^{-1}), adsorption tests and one-step growth tests were performed as described previously (Jones & David, 1971). Single burst experiments were done as described by Adams (1959). Specific phage $\phi 630A$ antisera were prepared and used as described previously (Jones & White, 1968). When the methods of Adams (1959) were used, the *K* value of the phage antiserum was 88.

Sensitivity to non-polar solvents. Phages $\phi 630A$ and $\phi 630B$ were exposed to the non-polar solvents chloroform, dioxan and diethyl ether for 10 min, as described previously for mycobacteriophage D29 (Jones, David & Beam, 1970), and were then plated to detect p.f.u.

Electron microscopy. Lysates of phages $\phi 630A$ and $\phi 630B$ were prepared and examined with a GEC/AEI EM801 electron microscope as described by Grange & Bird (1975).

Tests for modification and restriction. The efficiencies of plating (e.o.p.) of phages $\phi 630A$ and $\phi 630B$ on their indicator and alternative host strains were determined by the method of Adams (1959). Adsorption tests were performed for both the indicator host strain and the alternative host strain for each phage. Changes in the lytic activity of the phages were followed by picking single plaques to both host strains.

Fig. 1

Fig. 2

Fig. 1. Colony forming units (—) and plaque forming units (---) produced by *M. chelonae* F-630(φ630) in heart infusion broth containing Tween 80. Samples were removed at intervals and assayed for c.f.u. ml⁻¹ and p.f.u. ml⁻¹. Phage assays were done with the indicator strain F-630 RgSM^r in soft agar plated on OAA agar containing dihydrostreptomycin sulphate at 1000 µg ml⁻¹.

Fig. 2. Colony forming units (—) and plaque forming units (---) produced by *M. chelonae* F-630(φ630) in heart infusion broth. Other details as in legend to Fig. 1.

RESULTS

Pseudolysogenic state of F-630(φ630)

Since mycobacteria have a tendency to clump, the purification of single colonies is more difficult than with other bacteria. Serial transfer of single colonies had to be done at least six times on each isolate before stable RgF colony types that were free of phage could be obtained. The SmF colony type could not be obtained in the pure SmF form and consistently produced RgF colony variants and phage.

To investigate the production of phage in relation to c.f.u., a 3-day-old 7H9 broth culture of F-630(φ630) was thoroughly washed by centrifuging five times. After each centrifugation, the bacterial pellet was resuspended in HI broth. After the final wash the culture was divided into two parts; one part contained HI broth, and the other HI broth with 0.05% Tween 80 to prevent readsorption of phage. The two cultures were incubated at 37 °C. Samples were removed at intervals and assayed for c.f.u. ml⁻¹ and p.f.u. ml⁻¹. The phage assays were done with F-630 RgSM^r as the indicator host and OAA agar containing dihydrostreptomycin sulphate at 1000 µg ml⁻¹. The data obtained for HI plus Tween 80 test culture are shown in Fig. 1. The c.f.u. counts gradually increased over the 24 h test period. Phages appeared and rapidly increased in numbers between the 2 h and 3 h samplings

Fig. 3

Fig. 3. One-step growth curve of phage $\phi 630A$ on its indicator host strain F-630 Rg. Phage assays were done with strain F-630 Rg SM^r in soft agar plated on OAA agar containing dihydrostreptomycin sulphate at $1000 \mu\text{g ml}^{-1}$.

Fig. 4

Fig. 4. Electron micrograph of mycobacteriophage $\phi 630A$. Bar marker represents 40 nm.

and continued to increase slightly over the next 4 h. The data obtained for the HI broth test culture are shown in Fig. 2. In contrast to the culture containing Tween 80, both the c.f.u. and p.f.u. assays of the culture containing only HI broth fluctuated over the 24 h test period; however, there was a correlation between the decreases and increases in the c.f.u. and p.f.u. counts. In addition, although the number of phages released initially was smaller in HI broth than in HI broth plus Tween 80, the final phage titre in HI broth was 10^6 p.f.u. ml^{-1} , whereas in HI broth plus Tween 80 the final titre was only 5×10^3 p.f.u. ml^{-1} .

Biological properties of phages $\phi 630A$ and $\phi 630B$

One-step growth tests with phage $\phi 630A$ on its indicator host strain F-630 Rg (Fig. 3) showed that the phage had a latent period of 75 min, followed by a rise period of 90 min. The burst size of phage $\phi 630A$ on F-630 Rg was 51. Phage $\phi 630A$ antiserum had a K value of 88 against both phages $\phi 630B$ and $\phi 630A$. Phages $\phi 630A$ and $\phi 630B$ were not inactivated by chloroform, dioxan or diethyl ether. Electron microscopy of phage $\phi 630A$ showed a hexagonal head of approximately 60 nm diam. and a tail approximately 11 nm in diam. and 260 nm in length (Fig. 4). Phage $\phi 630B$ was similar in morphology to phage $\phi 630A$.

Evidence for modification and restriction

Phage $\phi 630A$, grown on *M. chelonae* strain F-630 Rg, plated with an e.o.p. of 1.2×10^{-5} on *M. smegmatis* strain 607. Plaques of phage $\phi 630A$ which formed on strain 607 were transferred to 607 again; the phage replicated on the 607 host was designated as phage $\phi 630B$. With the e.o.p. of phage $\phi 630B$ on its indicator host 607 designated as 1, the e.o.p. on F-630 Rg was 1×10^{-5} . Adsorption tests were performed to determine if the low e.o.p. of

Table 1. Adsorption of phages $\phi 630A$ and $\phi 630B$ to their indicator and alternative host strains

Adsorption was measured by the decrease in phage titre in the supernatant fluids of samples taken at 0 and 15 min. Results are averages of four plates per test.

Phage	Time (min)	Strain 607		Strain F-630 Rg	
		0	15	0	15
$\phi 630A$		6.0×10^5	3.1×10^4	6.0×10^5	3.0×10^4
$\phi 630B$		6.0×10^5	2.9×10^4	6.0×10^5	3.0×10^4

phage $\phi 630A$ on 607 and phage $\phi 630B$ on F-630Rg were the result of the failure of the phages to adsorb to their alternative host strains. Both phages adsorbed equally well on both host strains (Table 1).

Plaques of $\phi 630A$ on 607 and $\phi 630B$ on F-630Rg were transferred using sterile toothpicks to lawns of both F-630Rg and 607. A second transfer of individual plaques was made to lawns of F-630Rg and 607. In addition, plaques were transferred from lawns of F-630Rg containing plaques of phage $\phi 630A$ and lawns of 607 containing plaques of phage $\phi 630B$. Phage $\phi 630A$ picked directly from a lawn of F-630Rg would not lyse 607. Phage $\phi 630B$ picked directly from a lawn of 607 would not lyse F-630Rg. Surviving plaques of phage $\phi 630A$ on lawns of 607 lysed both F-630Rg and 607 on first transfer; however, on second transfer these phages would lyse only 607. Surviving plaques of phage $\phi 630B$ on F-630Rg lysed lawns of both F-630Rg and 607, but on second transfer these phages would lyse only lawns of F-630Rg.

DISCUSSION

The pseudolysogenic *M. chelonae* strain F-630($\phi 630$) had the same general characteristics as *M. smegmatis* ($\phi D29$) reported by Jones & White (1968). The phage-producing colonies were of the SmF colony type, and the cured, non-phage-producing colonies were of the RgF type. In 7H9 broth containing Tween 80, the ratio of Rg to Sm colony types was 4:1. Using HI broth with and without Tween 80, it was demonstrated that the high titre of phage in the supernatant fluids was dependent on the phage replication on the susceptible cells in the bacterial population. White & Knight (1954) demonstrated that Tween 80 effectively blocks adsorption of the phages to their host cells. The fluctuation of the c.f.u. count and its relationship to phage adsorption and release has been described by Jones & David (1971), who used phage D29 and *M. smegmatis* ATCC607.

Mycobacteriophages $\phi 630A$ and $\phi 630B$ were resistant to the nonpolar solvents chloroform, dioxan and diethyl ether. This differs from findings with other mycobacteriophages, including phage D29, which are inactivated by these solvents (Bowman, 1969; Jones *et al.*, 1970; Sellers & Tokunaga, 1970; Bowman *et al.*, 1973; Jones, 1973). With this exception, the biological characteristics of phages $\phi 630A$ and $\phi 630B$, such as burst size, one-step growth cycle, the low *K* value of the phage antiserum and morphology, were similar to those of other mycobacteriophages.

Mycobacteriophages $\phi 630A$ and $\phi 630B$ plated with an e.o.p. of 10^{-5} on their alternative host strains. Adsorption studies demonstrated that both phages adsorbed equally well on their indicator and alternative host strains. These observations ruled out lack of adsorption as a possible explanation of the low e.o.p. on the alternative host strains. The fact that phage $\phi 630A$ antisera neutralized phages $\phi 630A$ and $\phi 630B$ with equal efficiency is taken as evidence that $\phi 630A$ and $\phi 630B$ are the same phage. The transfer of single plaques of phage

$\phi 630A$ from its indicator strain F-630 Rg, and of phage $\phi 630B$ from its indicator strain 607, demonstrated that, under these conditions, phage $\phi 630A$ lysed only F-630 Rg and phage $\phi 630B$ lysed only 607. The initial transfer of the surviving plaques of the two phages on their alternative hosts resulted in the lysis of both F-630 Rg and 607 strains; however, a second single plaque transfer resulted in the loss of lytic activity of either phage on its original indicator host strain. The capacity of the phages to adsorb to both host strains, and the loss of the lytic activity after one transfer, are characteristic of host modification and restriction (Arber & Dussoix, 1962; Arber, 1965; Hayes, 1968). The system described here requires two transfers. To obtain modified phage plaques, the plaques must first be transferred from lawns of the alternative host containing high concentrations of plated phages. The lysis of both host strains by the phages on first transfer from the alternative host lawn is probably the result of the carry-over of both modified and unmodified phages. The first transfer serves as a purification of the modified phages which is reflected in the second step.

With the rapidly-growing system described in this report as a model system, studies are in progress to investigate host modification and restriction in more detail and the role it plays in the phage typing of mycobacteria.

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