Induction of Bacteriophage from Members of the *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* Serocomplex

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Bacteriophages have been induced from strains in the *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* serocomplex by exposure of cultures to UV light or treatment with mitomycin C. One-sixth of the strains examined, representing all but one of the 31 authenticated serotypes, were found to possess phages lytic for a *Mycobacterium smegmatis* indicator strain. Four single-plaque isolated phages, TM4, TM9, TM10 and TM20, were purified and shown to have a similar morphology on electron micrographs. They had an isometric head of diameter 50–58 nm and a flexible non-contractile tail about 170 nm in length with a terminal bulb. All had an identical buoyant density in CsCl of 1.521 g cm^{-3} and extreme sensitivity to chloroform. The induced phages differed in host range and possessed the unique ability to lyse other members of the serocomplex. Interest in these phages centres on a possible role in mediating genetic interrelationships between members of the serocomplex.

INTRODUCTION

Strains of *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* are grouped together as a biochemically related but serologically distinct complex, termed the MAIS serocomplex (Wolinsky & Schaefer, 1973). These organisms are widespread in nature, and, despite their relatively low virulence, are among the most common of the so-called 'atypical' mycobacteria associated with disease (Wolinsky, 1979). One of the most distinctive features of MAIS organisms is an outer filamentous layer or capsule composed of a unique group of glycopeptidolipids generically known as 'C-mycosides' (Draper & Rees, 1973; Draper, 1974, 1982; Barrow *et al.*, 1980). Serological specificity among members of the MAIS complex is due to variations in the oligosaccharide components of the more polar glycopeptidolipids (Brennan & Goren, 1979; Brennan *et al.*, 1981). The singly glycosylated apolar glycopeptidolipids can serve as the receptor for phage D4 in *Mycobacterium smegmatis* and in an unidentified *Mycobacterium* species designated 1217 (Furuchi & Tokunaga, 1972; Goren *et al.*, 1972). The apolar C-mycoside from *M. scrofulaceum* was capable of inactivating phage D4 to the same extent as that from the other two mycobacteria; however the *M. scrofulaceum* cells did not adsorb the phage and were resistant to lysis by D4 phage (Goren *et al.*, 1972). Indeed, the resistance of members of the MAIS serocomplex to lysis by numerous phages is well documented (Takeya *et al.*, 1959; Buraczewska *et al.*, 1971; Crawford *et al.*, 1981). One possible reason for resistance could be the presence of resident prophages inducing a state of superinfection immunity.

Lysogeny and pseudolysogeny are quite common in the genus *Mycobacterium* and the subject of recent reviews (Rado & Bates, 1980; Grange, 1982). Pseudolysogeny is the term applied to a non-integrated phage carrier state characterized by the spontaneous liberation of phage particles and the segregation of phage-sensitive bacteria (Baess, 1970). Lysogenic bacteria are immune to homologous phage and usually can be induced to produce phages by treatment of the culture with agents which inactivate the phage repressor, such as UV light or mitomycin C. Previous attempts to induce bacteriophage from *M. avium* with UV light have generally been

Abbreviation: MAIS, *Mycobacterium avium*/*Mycobacterium intracellulare*/*Mycobacterium scrofulaceum*.

unsuccessful (Russell *et al.*, 1960), though Buraczewska *et al.* (1971) stated that they had been able to induce phage from 'atypical' mycobacteria by UV irradiation. However, evidence of strain characterization was not presented and the phages induced were only able to lyse saprophytic mycobacteria and some *M. tuberculosis* strains.

The purpose of this investigation was to examine members of the MAIS serocomplex for the presence of inducible bacteriophages to probe the genetic interrelationship of strains in the MAIS complex. This report describes the methods used to induce phages and presents a characterization of four phages which are capable of lysing members of the MAIS serocomplex.

METHODS

Bacteria. All of the strains of *M. avium*, *M. intracellulare* and *M. scrofulaceum* obtained from National Jewish Hospital and Research Center, Denver, Colorado, USA are listed in Table 1. Most are part of an authenticated culture collection (Tsang *et al.*, 1983). The serotype designation of all the MAIS strains was confirmed by TLC of the alkali-stable type-specific polar glycopeptidolipids (Brennan *et al.*, 1982) and by seroagglutination (Tsang *et al.*, 1983). All cultures were started from single colonies which had smooth, opaque, colonial morphology. Strain 607 of *M. smegmatis* (American Type Culture Collection) was used as the indicator organism and for phage propagation.

Media and solutions. Bacteria were routinely grown in 7H11 broth (Cohn *et al.*, 1968) containing 0.05% (v/v) Tween 80. Cells were washed and diluted in 0.85% (v/v) saline containing 0.05% (v/v) Tween 80. Brain heart infusion broth containing 2 mM-CaCl₂ was used for mitomycin C induction and as phage diluent. Tris/HCl (50 mM, pH 9.0) was also used as a phage diluent and buffer. The soft agar overlay technique was used for all phage assays with supplemented Dubois broth base without Tween 80 as the soft agar (Jones & Greenberg, 1978), and 7H11 agar as the hard basal medium.

Phage induction and purification. Exponential phase cultures were diluted twofold in 7H11 broth and incubated at 37 °C with agitation for 36 h. They were centrifuged (4000 g, 10 min), washed once, and resuspended in one-tenth the original volume of saline. The concentrated cell suspension was divided into two aliquots. One was diluted to the original volume with saline and irradiated for 1.5 min with UV light with a maximum emission at 254 nm and flux of 1.05 J m⁻² s⁻¹. After UV exposure the cells were centrifuged and then resuspended in fresh 7H11 broth. The cells for mitomycin C induction were added to the original volume of CaCl₂-containing brain heart infusion broth which contained 2.5 µg mitomycin C ml⁻¹ (Sigma). Cells from both types of induction were incubated in the dark at 37 °C for 24 h. They were then centrifuged and the supernatant filtered through a 0.2 µm pore size filter. Soft agar overlays of the indicator bacteria were spotted with 20 µl of the filtrate or a dilution thereof. The plates were incubated at 37 °C and examined at 24 and 48 h. When well isolated plaques appeared they were picked with a sterile needle and resuspended in a small volume of a fresh broth culture of the indicator organism. After overnight incubation this was centrifuged and the supernatant filtered as above. Serial tenfold dilutions were spotted onto soft agar overlays of the indicator. A well isolated single plaque was again picked, mixed with fresh indicator cells and incubated overnight. The phage suspension was centrifuged and filtered prior to titration. Dilutions of the phage suspension were used to prepare high titre stocks by harvesting soft agar overlays which displayed near confluent lysis.

In some cases high titre phage lysates were purified by banding in CsCl block gradients. The phage suspension was made 10 mM with respect to MgSO₄ and treated for 2 h at 37 °C with DNAase I and RNAase A (Sigma) at 50 µg ml⁻¹. After centrifugation (10000 g, 15 min), the supernatant was dialysed against Tris buffer. A 20 ml sample of the dialysed phage suspension was placed in a nitrocellulose tube and underlaid with 2.0 ml 20% (w/v) sucrose and three bands of CsCl at densities of 1.300, 1.500 and 1.700 g ml⁻¹. The tubes were centrifuged in a SW 27 rotor at 25000 r.p.m. for 4 h at 4 °C and the opalescent phage band collected through the side of the tube and dialysed.

Host range. Strains of mycobacteria were prepared as soft agar overlays and spotted with 20 µl drops of three dilutions (1000 ×, 10 × and 1 ×) of phage. The concentration of phage was adjusted so that the greatest dilution (1 ×) gave 10–20 plaques on *M. smegmatis* overlays.

Electron microscopy. High titre phage lysates were prepared for electron microscopy by centrifugation at 100000 g for 1 h. The pellet was resuspended in one-hundredth the volume of brain heart infusion broth. The phages were allowed to adsorb to carbon-reinforced, Formvar-coated grids and negatively stained with 1.5% (w/v) phosphotungstic acid (pH 7.0). The grids were examined in a Hitachi HU-12 electron microscope operating at 75 kV.

Phage buoyant density. About 8 ml phage lysate was placed in a nitrocellulose tube. Dry CsCl was added until the density was 1.500 g ml⁻¹. The contents were mixed and overlaid with a small volume of 40% (w/w) sucrose. Tubes were centrifuged in a SW 40 rotor at 23000 r.p.m. for 40 h at 18 °C. The obvious phage band was recovered by

Table 1. *MAIS serotypes and strains used for phage induction and host range*

Serotype	Strain	Inducer*	Serotype	Strain	Inducer*
1	11907-300		16	ATCC 15987	
2	14141-1395		17	p-54	UV
3	128 Germany		18	Melnick	
4	135-1079		18	Altmann 4990	
4	6/8	MC	19	Darden	
5	25546-759		19	7-15	
6	34540 Wales		20	TMC 1419	
7	p-49		20	NQ	UV
8	SJB #2		21	2993	
8	9/78/77		22	10409	
9	17484-286		23	CDC 1214	
9	Watson	MC	24	12645	
10	1602-1965	UV	25	72-888	
10	TMC 1461		26	McKenzie	
11	14186-1424		27	Lane 3081	
11	TMC 1462		41	2729 Cardiff	
12	Woodduck	UV	41	Bridge	UV
13	TMC 1466		42	EW 10407	
14	p-39		42	CDC 1198	
15	Simpson		43	Brooks	
16	Yandle-Yandle		43	7-16	

* MC, mitomycin C; UV, ultraviolet light.

aspiration and the refractive index measured on an American Optical Refractometer. The phage buoyant density was calculated using the formula of Brunner & Vinograd (1965).

Phage inactivation with organic solvents. The sensitivity of the various phage preparations to organic solvents was determined with slight modifications of previously described procedures (Bradley & Rutherford, 1975). Bacteriophages were diluted in brain heart infusion broth to a titre of about 2.0×10^7 p.f.u. ml⁻¹ then mixed with redistilled solvent at a lysate:solvent ratio of 3:1. The mixture was gently agitated for 4 h at 37 °C then briefly centrifuged. The aqueous phase from the biphasic mixture was titrated in triplicate. The control consisted of three parts lysate and one part brain heart infusion broth incubated and titrated in like fashion.

RESULTS AND DISCUSSION

Of 30 of the 31 authenticated MAIS serotypes only 7 yielded phages detectable with the *M. smegmatis* indicator strain (Table 1). The UV light exposure generally reduced bacterial viability by 50–90% and was effective at inducing phages capable of lysing the indicator in 5 of the 42 strains tested. Mitomycin C treatment induced phages lytic for *M. smegmatis* in two of the strains (Table 1). However, the strains with UV light-inducible phage did not liberate phage when treated with mitomycin C. Conversely, the strains with mitomycin C-inducible phage did not yield phage upon exposure to UV light.

The plaques which developed on the indicator were approximately 2 mm in diameter and turbid. The number of phages detected after induction was typically about 10^4 p.f.u. ml⁻¹. Plaque formation in the mitomycin C-induced strains was frequently obscured by the presence of a bacteriocin-like clearing, a not unexpected event in view of the presence of bacteriocins in a wide variety of mycobacterial species (Takeya & Tokiwa, 1972). Bacteriocin-like clearing was only observed with undiluted filtrates. Single plaques which developed on the indicator lawn when spotted with filtrate from four different induced cultures were further purified by single-plaque re-isolation. The phages obtained by treatment of strains 6/8 (serotype 4) and Watson (serotype 9) with mitomycin C were designated TM4 and TM9, respectively. Phage TM10 was single-plaque isolated from a UV-induced culture of strain 1602-1965 (serotype 10). Strain NQ (serotype 20) yielded phage designated TM20 upon UV induction. All of the phages were grown to a titre of at least 10^{10} p.f.u. ml⁻¹ on *M. smegmatis*.

Phages from the present study were not inactivated by treatment with DNAase or RNAase. CsCl block gradient purification resulted in a decrease in the phage titre of up to 100-fold. All of the phage preparations used for the block gradient purification gave rise to several bands, but

Table 2. *Lysis of MAIS strains by UV- and mitomycin C-induced phage*

Single-plaque purified TM4, TM9, TM10 and TM20 were used. Test strains were prepared as soft overlays and spotted with 20 μ l of 1000 $\times$, 10 $\times$ and 1 $\times$ dilutions of phage. Concentrations of each phage preparation were adjusted so that the greatest dilution (1 $\times$) gave 10–20 plaques on *M. smegmatis* overlays.

Serotype	Strain	Lysis by phage:			
		TM4	TM9	TM10	TM20
3	128-Germany	+	+	+	+
4	6/8	+	+	+	+
7	p-49	+	+	+	+
17	p-54	+	–	–	+
18	Melnick	+	–	+	+
19	Darden	+	+	+	+
25	72-888	+	+	+	+
41	2719 Cardiff	+	+	+	–
42	EW 10407	+	+	+	+

biologically active phage was located in the lower band. The purified phages were tested for their ability to lyse mycobacteria in the MAIS serocomplex by spot testing. The majority of strains were not lysed by any of the phages (Table 2). About one-fourth of the MAIS strains were sensitive to at least two of the phage isolates and none was sensitive to only one phage. The phages had similar but not identical host range. The four phages could be differentiated by their lytic activity on strains P-54, Melnick and 2729 Cardiff. Phage TM4 lysed all three strains whereas TM9 lysed only one. Phages TM10 and TM20 had non-identical patterns of lysis on two of the three strains. The efficiency of plating of the phage was the same on all strains. There was no apparent correlation of phage sensitivity with serotype and where several strains of a particular serotype were tested they had differing phage sensitivities. There was no indication of restriction of phage growth on any sensitive strain.

Although these phages can be differentiated on the basis of the means of induction and the host range, they are probably derived from a common parent phage, since in *M. smegmatis* lysogenization with either TM4 or TM10 confers immunity to both phages but not to phage D29 (data not presented). Although the phages do not have a wide host range, they are capable of lysing some of the strains from the MAIS serocomplex which were resistant to lysis with the phage used by Crawford *et al.* (1981). We have not yet tested the phage isolates for ability to lyse mycobacteria outside the MAIS complex, so it is possible that some may prove useful in other phage-typing schemes. Such has been the case with a phage from a scotochromogenic acid-fast bacillus termed 33D which could lyse *M. tuberculosis* and *M. bovis* but not *M. tuberculosis* BCG (Buraczewska *et al.*, 1971). All of the phages used in a preliminary attempt to develop a phage-typing scheme for the MAIS serocomplex (Crawford *et al.*, 1981) and most of the phages used for typing *M. tuberculosis* have been isolated from environmental sources. The lysogenic phage induced from members of the MAIS serocomplex could therefore prove useful for phage-typing purposes.

Electron microscopic examination of negatively stained preparations of the phage revealed that all isolates possessed isometric heads and tails (Fig. 1). The head diameter, measured between apices, was 50–58 nm. Each phage had a tail length of about 170 nm. None of the phages had visible base plates or fibres on the tails, but a small bulbous structure was apparent at the distal end of the tails. This identical morphology is not unexpected since the majority of mycobacteriophages described to date have a similar appearance (Rado & Bates, 1980) with the exception of the phages induced from 'atypical' mycobacteria with UV light by Buraczewska *et al.* (1972), which were found to have an elongated head. There was no morphological evidence of phage lipids in any of the phages described herein.

The four TM phages had the same buoyant densities of 1.521 g cm⁻³. The refractive indices of the four preparations varied by less than 0.0002.

The solvent sensitivities of the phages were similar; they were all extremely sensitive to chloroform with an overall decrease in titre of five orders of magnitude. Methanol reduced the

Fig. 1. Electron micrograph of negatively stained phages induced from MAIS strains, and single-plaque purified. (a) Phage TM4, induced with mitomycin C from serotype 4 (strain 6/8); (b) phage TM9 induced with mitomycin C from serotype 9 (strain Watson); (c) phage TM10, UV-induced from serotype 10 (strain 1602-1965); (d) phage TM20, UV-induced from serotype 20 (strain NQ). The bar markers indicate 100 nm.

titre by 10^{-2} . Diethyl ether had a variable effect, reducing the titre by 10^{-1} to 10^{-3} , but not in a reproducible manner. Electron microscopy of the chloroform- and diethyl ether-treated phages showed no obvious change in morphology. Ether seemed to generate more empty-headed phage particles and, with both TM4 and TM9, there were clusters of phages attached by their tails to an amorphous substance (Fig. 2). The lipid solvent sensitivity of mycobacteriophages is widely recognized yet the location of the lipids or their precise function is unknown (Rado & Bates, 1982).

To our knowledge, this is the first report of the induction of phages from the MAIS serocomplex capable of lysing other MAIS strains, and of the induction with mitomycin C of

Fig. 2. Electron micrograph of phage TM4 negatively stained after solvent treatment. (a) Chloroform-treated phage; (b) diethyl ether-treated phage. The bar markers indicate 100 nm.

non-defective phages lytic for any mycobacterial species. A morphological mutant of *M. tuberculosis* BCG has been reported to produce phage-like particles after 2 d growth in media with $2.0 \mu\text{g}$ mitomycin C ml^{-1} . Although these particles did reduce the number of c.f.u. in a broth culture, they did not produce detectable lysis either in broth or on plates (Imaeda & Rieber, 1968).

There are several reports of naturally occurring and experimentally induced lysogenic mycobacteria (Rado & Bates, 1980; Grange, 1982). However, most should more accurately be termed pseudolysogens. Baess (1970) applied this term to mycobacteriophage-host interactions with the following characteristics: the phage DNA did not integrate into the host chromosome; the phages could not be induced by methods which normally induce lysogenic phage; the culture medium contained spontaneously liberated phage particles; and phage-free bacterial segregants appeared in the culture, particularly in the presence of anti-phage serum. Attempts to produce lysogenic or pseudolysogenic *M. avium* or *M. intracellulare* experimentally have been unsuccessful (Russell *et al.*, 1960). These same strains also failed to yield phage upon exposure to UV light. There is but one brief report (Buraczewska *et al.*, 1971) of phage induction with UV light from strains that were classified as avian or 'atypical' mycobacteria, but evidence of strain characterization was not presented and these phages were able to lyse mainly saprophytic mycobacteria or occasionally *M. tuberculosis*.

It was somewhat surprising to find that phage induced from strain 6/8 by treatment with mitomycin C and single-plaque purified on *M. smegmatis* was capable of lysing strain 6/8. This could indicate that a subculture of this strain lost the temperate phage or that this strain is pseudolysogenic with a majority of the cells being sensitive to the phage. The molecular nature of the interaction of phage TM4 with its host cell is unclear but it probably represents a form of pseudolysogeny. A similar situation has been reported with a spontaneously released phage from a strain of *M. bovis* (Tageldin *et al.*, 1981). In a *Bacillus stearothermophilus* strain mitomycin C enhanced the release of a pseudolysogenic phage $\phi\text{u-4}$, which could be detected by plaque formation on the strain from which it was induced (Holmes *et al.*, 1981).

The phages we have induced from members of the MAIS complex should prove useful in understanding the genetics of this clinically important but genetically poorly characterized group of mycobacteria.

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