Host range of 14 mycobacteriophages in *Mycobacterium ulcerans* and seven other mycobacteria including *Mycobacterium tuberculosis* – application for identification and susceptibility testing

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The host range of well-characterized mycobacteriophages, such as D29 and TM4, has been determined, together with that of more recently isolated mycobacteriophages, in *Mycobacterium ulcerans*, *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG, *Mycobacterium avium*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Here, a set of virulent phages for *M. ulcerans*, a pathogen with a dramatic increase of incidence over the last decade, is demonstrated. In this work, a mycobacteriophage replication assay was adapted for the identification and rifampicin-susceptibility testing of *M. ulcerans*. Mycobacteriophages have generated a number of useful tools and enabled insights into mycobacterial genetics. With regard to the neglected pathogen *M. ulcerans*, the findings presented in this work allow the application of a large range of phage-based vectors and markers. The potential of phage therapy can now be evaluated for this extracellular pathogen.

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INTRODUCTION

Mycobacteriophages have proved to be useful genetic tools for the manipulation of pathogenic mycobacteria. The construction of conditionally replicating mycobacteriophages based on phage TM4 and D29 has enabled knock-out mutants to be generated in many mycobacterial species (Bardarov *et al.*, 1997; Harris *et al.*, 1999; Rybníček *et al.*, 2003). Furthermore, mycobacteriophages are used as diagnostic markers to improve and expedite the recognition of pathogenic mycobacteria in patient samples, as well as the drug resistance of these pathogens. Commercial kits based on phage technology are now available (Alcaide *et al.*, 2003; Muzaffar *et al.*, 2002). In addition, TM4 and D29 luciferase reporter phages have been constructed for the same purpose (Jacobs *et al.*, 1993; Pearson *et al.*, 1996). Broxmeyer *et al.* (2002) demonstrated that mycobacteriophages have potential as antimycobacterial agents for infections caused by *Mycobacterium avium* and *Mycobacterium tuberculosis* when using a non-pathogenic mycobacterium as a delivery system to intracellularly located bacteria. Over 250 mycobacteriophages have been described, and typing schemes have been developed for many mycobacterial species (Crawford & Bates, 1984).

For the successful application of phages, the knowledge of their host range is a prerequisite. However, data on the host range of the recently isolated mycobacteriophages are rare and have often been communicated orally. This led us to examine 14 mycobacteriophages for their ability to infect and replicate in several fast- or slow-growing mycobacteria, including *M. tuberculosis*, *Mycobacterium ulcerans* and *M. avium*. The complete nucleotide sequences of eight of the phages used in this study are available through GenBank (Pedulla *et al.*, 2003). The determination of the host-range for *M. ulcerans* may help to improve our understanding of the pathogenesis of this organism, as well as the diagnosis of this neglected pathogen. The slow-growing *M. ulcerans* causes Buruli ulcer, a serious ulcerating skin disease common in many tropical countries (Thangaraj *et al.*, 2003). This major mycobacteriosis represents the third most common mycobacterial infection in immunocompetent patients (Weir, 2002). To date, the accepted treatment is surgical resection of infected skin followed by grafting. However, efforts are being made to evaluate the potential of drug treatment in larger clinical trials (Espey *et al.*, 2002). It is likely that future treatment schemes will be based on the antituberculous drug rifampicin. However, rifampicin-resistant *M. ulcerans* mutants have already been isolated from experimental mouse lesions after monotherapy with this drug (Marsollier *et al.*, 2003). This led us to adapt phage-based rifampicin-susceptibility tests to *M. ulcerans*.

Abbreviation: FAS, ferrous ammonium sulfate.

As with other mycobacterial diseases, there are no routine serological tests available for Buruli ulcer. Primary cultures for the diagnosis of *M. ulcerans* may take 6–8 weeks for a positive result. There is a need for affordable alternative diagnostic methods. Phages used for the rapid identification of a given mycobacterium from patient samples lack specificity. They can be useful if they show a superior sensitivity to an examination by acid-fast smear microscopy in a setting in which PCR detection is not available or not affordable. Investigations of the phage-based detection of *M. tuberculosis* from sputum samples have given contradictory results (Alcaide *et al.*, 2003; McNerney *et al.*, 2004; Muzaffar *et al.*, 2002). In contrast to staining and PCR methods, phage assays can detect viable mycobacteria only, which is the principle of susceptibility tests. Here, we show that specificity for the detection of *M. ulcerans* can be enhanced by splitting the sample and pre-incubating aliquots at 41 °C and 32 °C for 24 h. *M. ulcerans* cells incubated at the higher temperature do not allow phage replication and detection, whereas a mycobacterium with a different optimal growth temperature, such as *M. tuberculosis*, will give a positive result at both temperatures.

METHODS

Bacterial strains, mycobacteriophages, media and culture methods. The mycobacterial strains used in this work are listed in Table 1. All strains were grown in Middlebrook 7H9 broth (Difco) enriched with 10% OADC (oleic acid, BSA, dextrose and catalase) (Difco) in 175 cm² tissue-culture flasks (Falcon) at 37 °C, except for *M. marinum* and *M. ulcerans*, which were grown at 32 °C.

Rifampicin-resistant *M. ulcerans* isolates were generated by plating 5×10^{10} c.f.u. of a clinical isolate from Ghana on Middlebrook 7H10 plates containing 10 µg rifampicin ml⁻¹ (Sigma).

Except for phage TM4, the primary isolation of all mycobacteriophages used in this study employed *Mycobacterium smegmatis* as host. Mycobacteriophage D29 was isolated from soil by Froman *et al.* (1954). Doke *et al.* (1960) isolated the temperate phage L5 from cultures of *M. smegmatis*. TM4 was isolated from an *M. avium* culture after treatment with mitomycin C (Timme & Brennan, 1984). Bx2 was isolated from environmental samples from the Bronx Zoo by the William R. Jacobs laboratory at the Albert Einstein College of Medicine, New York. The remaining phages were isolated in Graham F. Hatfull's laboratory at the University of Pittsburgh, except for phage Che8, which was isolated in Chennai, India. The phages are listed in Table 2.

Growth of mycobacteriophage. All mycobacteriophages were propagated in *M. smegmatis* mc²155, as described elsewhere (Sarkis & Hatfull, 1998). In brief, 250 µl of cells grown to an OD₆₀₀ of 1.0 was combined with approximately 10⁵ phage particles and 3 ml molten top agar (4.7 g Middlebrook 7H9 broth base, 7 g Bacto agar (Difco) in 900 ml distilled water enriched with 25 ml 40% glucose and 10 ml 0.1 M CaCl₂). This mixture was poured on 7H10 agar plates containing 10% OADC and incubated overnight at 37 °C. The plates were covered with 5 ml phage buffer (10 mM Tris, pH 7.5, 1 mM MgSO₄, 70 mM NaCl) for 4 h at 4 °C, and phage particles were harvested by transferring the buffer into a sterile container. After passing the phage suspension through a 0.2 µm pore-size filter, the phage titre was determined using a standard spot test. Using this method, titres >10¹⁰ p.f.u. ml⁻¹ were reached for all phages.

Host-range determination. Mycobacteria were grown to an OD₆₀₀ of 1.0. If necessary, clumps were dispersed by passing the bacterial suspension several times through a 25-gauge needle. A volume of 500 µl was added to 3 ml top agar containing 1 mM

Table 1. Mycobacterial strains used in this study

Species	Strain	Reference or source
<i>M. tuberculosis</i>	H37Rv	ATCC*
<i>M. tuberculosis</i> complex, n=5	Clinical isolates	BNI†
<i>Mycobacterium bovis</i>	BCG Pasteur	ATCC
<i>M. avium</i>	3746/02	Fz-Borstel‡
<i>M. avium</i>	701	P. L. Small, UTK§
<i>M. avium</i>	702	P. L. Small, UTK
<i>M. scrofulaceum</i>	1320	P. L. Small, UTK
<i>M. scrofulaceum</i>	1315	P. L. Small, UTK
<i>M. ulcerans</i>	1615 (ATCC 35840)	Isolated in Malaysia, ATCC
<i>M. ulcerans</i>	1615 mycolactone ⁻ mutant	P. L. Small, UTK; Stinear <i>et al.</i> (2004)
<i>M. ulcerans</i> , n=15	Clinical isolates	All endemic continents, BNI
<i>M. ulcerans</i>	RifR (rifampicin-resistant)	BNI (this work)
<i>M. marinum</i>	ATCC 927	ATCC
<i>M. marinum</i>	565	P. L. Small, UTK
<i>M. fortuitum</i>	1529	P. L. Small, UTK
<i>M. chelonae</i>	1543	P. L. Small, UTK
<i>M. smegmatis</i>	mc ² 155	BNI; Snapper <i>et al.</i> (1990)

*American Type Culture Collection.

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Table 2. Host range of 14 mycobacteriophages in eight mycobacterial species

1, *M. smegmatis* mc²155; 2, *M. tuberculosis* H37Rv; 3, *M. tuberculosis* 371; 4, BCG Pasteur; 5, *M. avium* 701; 6, *M. avium* 702; 7, *M. avium* 3746/02; 8, *M. scrofulaceum* 1320; 9, *M. scrofulaceum* 1315; 10, *M. ulcerans* 1615; 11, *M. ulcerans* 1615M; 12, *M. ulcerans* M18 (Ghana); 13, *M. ulcerans* S12 (Ghana); 14, *M. marinum* ATCC 927; 15, *M. marinum* 565; 16, *M. fortuitum* 1529; 17, *M. chelonae* 1543. P, Plaque formation.

Phage	Mycobacterial strain																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Barnyard	P																
Black	P																
Raspberry																	
Bxz2	P	P	P	P	P	P	P	P		P	P	P	P			P	P
Che8	P																
D29	P	P	P	P	P	P	P	P		P	P	P	P				
L5	P	P	P	P	P	P	P	P		P	P	P	P			P	P
Omega	P																
PBI1	P				P				P	P							
PG2	P								P	P						P	P
Rosebush	P																
SB1	P																
Cooper	P															P	P
TM4	P	P	P	P						P	P	P	P				
Wildcat	P								P	P						P	P

CaCl₂ and poured onto 7H10 agar plates enriched with 1 mM CaCl₂ and OADC. For each phage, a serial dilution was prepared in phage buffer. Twenty-microlitre volumes of the original stock and of each dilution were pipetted onto the bacterial lawn, and the spots were allowed to dry completely. Plates were incubated for 4 days for the fast-growing mycobacteria, and for up to 6 weeks for the slow-growing strains, at the optimal temperature for the individual strain. All experiments were performed in triplicate.

Phage amplification assay. Fifteen rifampicin-sensitive *M. ulcerans* clinical isolates (confirmed by standard agar dilution tests) from the major endemic regions and one rifampicin-resistant isolate were grown in Middlebrook 7H9 broth enriched with 10% OADC. Five 1.5 ml screw-capped microcentrifuge tubes were loaded with 1 ml of each culture and centrifuged at 5000 r.p.m. The supernatant was discarded and the pellet resuspended in 1 ml of fresh Middlebrook 7H9 broth supplemented with 10% OADC. Of the five tubes, one was incubated at 41 °C and the second at 32 °C for 24 h. In the three remaining tubes, rifampicin was added at 1, 5 and 10 µg ml⁻¹, and incubation took place at 33 °C for 24 h. From each sample, 50 µl was transferred to new 1.5 ml tubes, and 50 µl aliquots of phage D29 at 10⁷ p.f.u. ml⁻¹ were added. The D29 phage stock had been diluted beforehand in 7H9 broth with 10% OADC and 2 mM CaCl₂. Phage infection was allowed to take place for 90 min; temperatures were the same as in the prior 24 h incubation period. Inactivation of exogenous phage particles was achieved by the addition of 2 mM ferrous ammonium sulfate (FAS, Carl Roth) (McNerney *et al.*, 1998). After vigorous vortexing and incubation of the samples for 5 min at room temperature, 20 µl of each aliquot was spotted on *M. smegmatis* mc²155 top agar indicator plates. The presence of plaques after an overnight incubation at 33 °C was taken

to indicate a *M. ulcerans*-positive or rifampicin-resistant isolate. The test was performed in the same manner with five clinical isolates of the *M. tuberculosis* complex.

Electron microscopy. A CsCl-purified stock of mycobacteriophage D29 was prepared as described previously (Sarkis & Hatfull, 1998). D29 (10⁹ p.f.u.) was incubated for 30 min with 10⁷ c.f.u. of *M. ulcerans* 1615 at 37 °C. A 5 µl drop of the sample was placed on a freshly glow-discharged, collodion and carbon-coated grid. After 1 min, the grid was washed with 10 mM ammonium acetate, and then stained for 30 s with 0.75% uranyl formate. Samples were examined and photographed with a Hitachi H800 scanning transmission electron microscope operating at 100 kV.

RESULTS AND DISCUSSION

The results for the plaque formation on the tested mycobacterial species are given in Table 2. For each mycobacterium tested, only the observation of individual plaques at high dilutions of the phage solution was used as a proof for growth support. Plaque formation that takes place only at the lowest dilutions is often due to 'killing from without'. This phenomenon occurs when a very high phage : cell ratio leads to lysis of the bacteria without productive phage infection (Sarkis & Hatfull, 1998).

Six mycobacteriophages did not form plaques on any species other than *M. smegmatis*, the mycobacterium which was used for their primary isolation from environmental samples. A cluster of four phages, namely D29, TM4, L5 and Bxz2, seemed to have the broadest host range, with at least three of these phages forming plaques on all of the slow-growing species, except for *M. marinum* and one strain of *M. scrofulaceum*. The genomes of these four phages have detectable sequence similarity, which might be one reason for their shared host range (Pedulla *et al.*, 2003). CaCl₂ is important for the infection of a number of mycobacteriophages. In our experiments, the CaCl₂ concentration was maintained at 1 mM. It is possible that different concentrations alter the host range of some phages.

Host range in *M. ulcerans* and *M. marinum*

The host-range spectrum obtained for *M. ulcerans* was similar to that of *M. tuberculosis* and *Mycobacterium bovis* BCG. This opens the possibility to apply phage-based diagnostic tools, as well as vectors for improved transformation, to this important pathogen. D29 seems to be a good choice for mycobacteriophage assays for the detection of *M. ulcerans* in patient samples or to investigate the drug resistance of mycobacterial isolates. This phage proved to be lytic in *M. ulcerans* species which originated from four different continents. *M. ulcerans* shows genetic heterogeneity and variable phenotypic characteristics among strains of different geographic origins (Portaels *et al.*, 1996). Even so, the lytic phenotype of D29 is stable in *M. ulcerans*, as seems to be the case for most strains of *M. tuberculosis* (Banaiee *et al.*, 2003; McNerney *et al.*, 2004).

Additionally, phage therapy could be considered for *M. ulcerans* infections. In Buruli ulcer, mycobacteria are

typically found extracellularly, where they might be immediately accessible by lytic phages. Again, phage D29 might be the best candidate for this purpose, since the plaques are relatively large and adsorption of phage particles seems to be efficient (Fig. 1).

M. ulcerans produces the polyketide-derived macrolide mycolactone, which is required for the tissue damage that characterizes Buruli ulcer (George *et al.*, 1999; Mve-Obiang *et al.*, 2003). Although mycolactone is a major component of the cell surface of *M. ulcerans*, it does not seem to be involved in the binding of phages or the injection of phage DNA, since the avirulent mycolactone-negative mutant 1615M showed the same host range as the wild-type strain.

Interestingly, none of the tested phages formed plaques on two strains of *M. marinum*. In the case of TM4 and D29 at least, this was most likely due to an intracellular inhibition of phage replication, rather than a receptor–receptor binding protein mismatch, since shuttle plasmids based on these two phages are able to transfect *M. marinum* (Rybníček *et al.*, 2003). This restriction of phage growth in *M. marinum* might be of advantage to phage-derived diagnostic tools for the rapid identification of *M. ulcerans*. The observation of plaque formation in *M. ulcerans*, but not in *M. marinum*, allows the discrimination of these two pathogens which share the ability to cause dermal diseases and have a common optimal growth temperature of 32 °C. However, additional *M. marinum* strains need to be tested to confirm this phenotype.

Host range in mycobacteria other than *M. ulcerans* and *M. marinum*

Except for Bx2, none of the recently isolated phages formed plaques on *M. tuberculosis* or *M. bovis* BCG, showing that phages isolated in the fast-growing *M. smegmatis* are often

confined to this mycobacterium or other fast-growing species. It is likely that environmental samples, for example from compost, may contain additional phages, and the use of slow-growing species such as *M. bovis* BCG should be considered when searching for wild-type phages with a broader or different host spectrum.

Interestingly, our three *M. avium* strains, as well as strains of the closely related *M. scrofulaceum*, were resistant to TM4, and this differs from observations made by others (Foley-Thomas *et al.*, 1995). Since TM4 was induced from *M. avium*, it is possible that the restriction of replication observed in this mycobacterium is due to a lysogenic state of TM4 which confers superinfection immunity. Also, *M. avium* and *M. scrofulaceum* showed differing host ranges at the subspecies level, which was not observed in the other mycobacterial species tested. For example, Bx2 formed plaques on *M. avium* 701 and 702, but not on *M. avium* 3746/02. Also, phage D29 formed plaques on *M. scrofulaceum* 1320, but not on *M. scrofulaceum* 1315. This failure to form plaques on all isolates of one species impedes the use of mycobacteriophage detection assays for the *M. avium*–*M. scrofulaceum* complex, and the possibility of such a phenotype should be kept in mind when using tools based on this phage. It has been stated elsewhere that phage D29 shows a very broad host range, infecting a wide variety of slow-growing mycobacteria, with the exception of *M. avium* (McNerney *et al.*, 2004). Again, this differs from our observations. D29 readily infected all three *M. avium* isolates tested. Hatfull & Sarkis (1993) describe the ability of phage L5 to infect some substrains of *M. avium*. It is likely that phage D29 shows a similar phenotype; the fact that our *M. avium* strains allowed L5 and D29 growth supports this hypothesis.

However, in the fast-growing strains, a remarkable observation was plaque formation of phage L5 but not of D29 in the *M. fortuitum*–*chelonae* complex. These two phages are very closely related, with extensive detectable DNA sequence similarity (Ford *et al.*, 1998; Pedulla *et al.*, 2003). Also, D29 is a homo-immune relative to L5.

Phage amplification assay for detection of rifampicin-resistant *M. ulcerans* and discrimination between *M. ulcerans* and *M. tuberculosis*

Several protocols for phage-based susceptibility tests and identification of *M. tuberculosis* from patient samples have been published (Albert *et al.*, 2002; Butt *et al.*, 2004; Simboli *et al.*, 2005). These protocols were successfully adapted to *M. ulcerans*. The fact that the growth of *M. ulcerans* as well as the phage replication in this host is strongly restricted to temperatures below 35 °C can be used for differentiation between *M. ulcerans* and *M. tuberculosis*, both of which cause dermal disease. Plaque formation on indicator plates after a 24 h incubation period at both 33 °C and 41 °C indicates a *M. ulcerans*-negative sample, whereas plaque formation on the 33 °C sample only will be *M. ulcerans* positive (Table 3,

Fig. 1. Phage D29 adsorbed to *M. ulcerans* 1615.

Table 3. Phage amplification test

Incubation time prior to the plaque test was 24 h. P, Plaque formation; (P), few plaques; NT, not tested. See also legend of Fig. 2.

Strain	Incubation at 32 °C	Incubation at 41 °C	1 µg rifampicin ml ⁻¹	5 µg rifampicin ml ⁻¹	10 µg rifampicin ml ⁻¹
<i>M. ulcerans</i> , n=15	P		(P)		
<i>M. ulcerans</i> Rif ^R	P		P	P	P
<i>M. tuberculosis</i> , n=5	P	P	NT	NT	NT

Fig. 2). In two *M. ulcerans* isolates, some plaques were observed after the 24 h incubation period at 41 °C. Here, increasing the incubation time to 48 h improved the performance of the test, with no plaques visible on the indicator plate. Also the rifampicin-susceptibility test correlated well with results from rifampicin agar-dilution tests. In the rifampicin-sensitive isolates, plaque formation was decreased in a concentration-dependent manner: no plaques were visible at 10 µg rifampicin ml⁻¹. The rifampicin-resistant isolate *M. ulcerans* Rif^R showed plaque formation at all three concentrations. We found the performance of the phage amplification test to be easy and fast, giving positive results within 48 h. The test performed equally well when using *M. ulcerans* colonies picked from Loewenstein–Jensen agar (data not shown). This test might be an alternative to

expensive diagnostic tools that are dependent on the supply and storage of complex reagents. Particularly in acid-fast-positive patient samples, the rapid temperature-sensitive-phage amplification test may differentiate between *M. ulcerans* and other mycobacteria, having major consequences concerning the management of the disease. A range of slow-growing mycobacterial species should be tested before the test is evaluated using samples such as swabs or skin biopsies. A laboratory performing phage-based tests could also improve its diagnostic performance with respect to tuberculosis, which is usually of high incidence in Buruli ulcer endemic regions.

In summary, we have shown that the mycobacteriophages D29, Bxz2, L5 and TM4 have a broad, and to some extent similar, host range among the slow-growing mycobacteria. With Bxz2, we present an additional virulent phage for *M. tuberculosis*. The data obtained for *M. ulcerans* open new possibilities for the diagnosis and genetic manipulation of this pathogen, whereas *M. marinum* seems to be broadly resistant to phage replication. To our knowledge, this has been the first systematic investigation of the phage host range in these two organisms.

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Fig. 2. Phage-based susceptibility and identification test. Before the addition of phage D29, *M. ulcerans* and *M. tuberculosis* strains were pre-incubated as indicated below. The plaque pattern allowed discrimination between rifampicin-resistant and -sensitive isolates (lines A3–A5 and lines B3–B5), and between *M. ulcerans* and *M. tuberculosis* (lines A1–A2, B1–B2, C1–C2). Line A, rifampicin-sensitive *M. ulcerans* isolate. A1, 24 h pre-incubation at 32 °C; A2, 24 h pre-incubation at 41 °C; A3, 24 h pre-incubation with 1 µg rifampicin ml⁻¹; A4, 5 µg rifampicin ml⁻¹; A5, 10 µg rifampicin ml⁻¹. Line B, rifampicin-resistant *M. ulcerans* Rif^R, pre-incubation of B1–B5 like A1–A5. Line C, *M. tuberculosis* complex, pre-incubation of C1–C2 like A1–A2. D1, phage only with 2 mM FAS; D2, phage only.

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