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THE NATURE AND INCIDENCE OF LYSOGENY IN *MYCOBACTERIUM FORTUITUM*

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PLATES XIII AND XIV

LYSOGENY is a common and widespread phenomenon amongst bacteria but has been infrequently reported in the genus *Mycobacterium* (Bönicke, 1969). However, the facts that mycobacteriophages may be readily isolated from the environment and lysogeny easily established in the laboratory suggest that the carriage of bacteriophage may be much commoner among mycobacteria than has previously been demonstrated.

Recently the species *M. fortuitum* (*ranae*) has been subjected to detailed studies to determine the nature and significance of its intraspecific variation (Grange and Nordström, 1974; Grange and Stanford, 1974; Pattyn *et al.*, 1974). On the basis of the variation occurring within this species it was concluded that the variants arise by deletional mutation from a so-called common progenitor type (Grange, 1973; Stanford and Grange, 1974). A few strains, however, were found to possess anomalous properties not readily explicable on the basis of simple mutational derivation from other strains. It was considered that such strains might owe their anomalous properties to the lysogenic state. Studies on the bacteriophage typing of *M. fortuitum* (Grange and Nordström, 1973; Nordström and Grange, 1974) demonstrated a close correlation between lysis by a group of phages termed the *Tara* phages and the ability of the strains to utilise inositol. A few inositol-utilising strains were resistant to lysis by the *Tara* phages, a possible example of superinfection immunity due to lysogeny.

It was considered, therefore, that the strains of *M. fortuitum* included in these investigations might be a suitable group for a study of the incidence and

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nature of lysogeny in the genus *Mycobacterium*. In this study, observations on 28 strains of *M. fortuitum* are reported.

MATERIALS AND METHODS

Bacteria. Twenty-eight strains of *M. fortuitum* were obtained from the collection of mycobacteria at the School of Pathology, the Middlesex Hospital Medical School, London; 26 of these have been described in a previous study (Pattyn *et al.*, 1974). The two additional strains, nos. 638 (ATCC23029) and 650 (ATCC23041), were included as examples of pseudo-lysogenic mycobacteria (Nordström and Grange, 1974). The collection numbers and the origin of the 28 strains are shown in table I.

Bacteriological investigations. The cultural, biochemical and antigenic properties of the strains have been described previously (Grange and Stanford, 1974; Pattyn *et al.*, 1974). All strains were examined for susceptibility to lysis by bacteriophage BK4 and for inositol

TABLE I
Source and characteristics of the 28 strains of Mycobacterium fortuitum

Strain no.*	Source†	Immuno- diffusion serotype	Biotype‡	Inositol§	DNAase	Lysis by phage BK4
4	NCTC (no. 8573)	I	A	+	—	+
81	NCTC (no. 2891)	I	A	—	—	—
491	Middlesex Hospital, clinical isolate	I	A	—	—	—
638	ATCC (no. 23029)	I	A	—	+	—
2076 (576)	IMT	I	A	—	—	—
1811 (676)	IMT	I	A	—	—	—
1900 (679)	IMT	I	A	—	—	—
1954 (680)	IMT	I	A	—	—	—
2040 (681)	IMT	I	A	+	—	—
19 (799)	Statens Seruminstitut	I	A	+	—	C
20 (800)	Statens Seruminstitut	I	A	+	+	C
516 (843)	Statens Seruminstitut	I	A	+	—	C
927 (844)	ATCC (no. 6841)	I	A	+	—	C
108	Dr J. Marks, Cardiff (no. 16575)	II	C	+	—	C
109	Dr J. Marks, Cardiff (no. 18519)	II	C	+	+	+
2042 (577)	IMT	II	B	—	—	C
487	Clinical isolate, Zaire	III	C	+	—	++
1812 (677)	IMT	III	C	+	—	C
5	NCTC (no. 2006)	IV	C	+	—	+
33	Strain C.U. (Beck, 1965)	IV	C	+	+	—
650	ATCC (no. 23041)	IV	C	+	+	—
411 (862)	Statens Seruminstitut	IV	C	+	+	—
630	ATCC (no. 23021)	V	B	—	—	—
632	ATCC (no. 14467)	V	B	—	—	—
31 (802)	Statens Seruminstitut	V	B	—	—	—
800 (675)	IMT	V	B	—	—	—
928 (806)	Statens Seruminstitut	V	B	—	—	—
1828 (678)	IMT	V	B	—	—	—

* Strains from the Institut de Medicine Tropicale, Antwerp and the Statens Seruminstitut, Copenhagen, are referred to by the collection numbers of these Institutes. Numbers in brackets are the collection numbers of the School of Pathology, The Middlesex Hospital, London.

† NCTC = National Collection of Type Cultures; ATCC = American Type Culture Collection; IMT = Institut de Medicine Tropicale, Antwerp.

‡ Bönicke (1966).

§ + = Utilised; — = not utilised.

|| C = confluent lysis at RTD (routine test dilution); ++ = confluent lysis at 10 RTD; + = confluent lysis at 100 RTD.

utilisation by the modified carbohydrate-nitritase test (Grange and Nordström, 1973). Production of deoxyribonuclease was determined by the method of Mankiewicz and Tamari (1972).

Bacteriophage. Phage I3 was adapted to immunodiffusion-serotype-III strains of *M. fortuitum* as described previously (Nordström and Grange, 1974) to give phage I3A. Phage I3 had been purified by two single plaque passages before adaptation and its appearance was checked by electronmicroscopy (fig. 1). Antiphage serum was prepared against phage I3 propagated on its host strain *M. smegmatis* SN2 in Sauton's medium containing calcium chloride 1 µg per ml. The phage suspension was separated from bacteria by passage through a 0.22-µm membrane filter and titrated on the host strain to determine the phage titre. A rabbit received 1 ml of phage suspension, containing 10⁸ plaque-forming units, intravenously twice weekly for 3 weeks and was bled after a further 2 weeks. The neutralising effect of the antiphage serum on phages I3 and I3A was determined; 0.5-ml amounts of phage suspensions containing approximately 10⁸ plaque-forming units per ml were incubated with an equal quantity of neat and 10-fold dilutions of the antiserum for 1 h before titration. The phage suspensions were also incubated with normal rabbit serum as controls.

Detection of pseudolysogenic strains. Pseudolysogeny of the "carrier-state" type (Hayes, 1968) was detected by observing the spontaneous appearance of occasional phage plaques on the host strains (Baess, 1971). The strains of *M. fortuitum* were incorporated in soft-agar overlays prepared from nutrient broth supplemented with calcium chloride, 10 mg per litre and solidified with Oxoid Agar no. 1, 0.7% (w/v). The plates were examined for plaques after incubation at 32°C for 4 days.

Detection of phages able to lyse susceptible indicator strains. The 28 strains were inoculated into 10-ml amounts of nutrient broth and incubated at 32°C. for 7 days. The culture media were separated from the bacteria by passage through 0.22-µm membrane filters and each filtrate was spotted on to cultures of the other strains incorporated in soft-agar overlays as described above. The overlays were examined for lysis after incubation at 32°C for 4 days.

Electronmicroscopy. The strains selected for electronmicroscopy (shown in table II) were inoculated into 50-ml amounts of nutrient broth. After incubation at 32°C for 5 days, the majority of bacteria were removed by centrifugation at 3000 r.p.m. in a bench centrifuge. The supernates were spun for 1 h at 45,000 *g* in a MSE Superspeed 50 ultracentrifuge. The supernates from this centrifugation were discarded and the pellets kept at 4°C. Within 1 h

TABLE II
Strains of M. fortuitum selected for examination by electronmicroscopy

Strain no.	Reason for selection
4	Negative allantoinase; resistance to lysis by <i>Tara</i> phages
109	DNAase; resistance to lysis by <i>Tara</i> phages
487	Serotype III strain
491	Negative allantoinase and urease
2042 (577)	Inositol non utilising serotype-II strain
650	Previously undescribed pseudolysogenic strain
1812 (677)	Serotype-III strain with serotype-II variants
2040 (681)	Resistance to lysis by <i>Tara</i> phages
20 (800)	DNAase; mucoid colonies
411 (862)	DNAase; supposedly cured of phage BK5
5	Serotype-IV strain
81	Control. Co-type strain of <i>M. ranæ</i>
927 (844)	Control. Co-type strain of <i>M. fortuitum</i>
632	Control. Type strain of <i>M. peregrinum</i>
19 (799)	Control. Serotype-I strain
108	Control. Serotype-II strain

of centrifugation the pellets were resuspended in 0.1 ml of distilled water. Two drops of each suspension were mixed on a clean glass slide with an equal volume of 4% ammonium molybdate solution, adjusted to pH 6.8, with 0.1N ammonia solution, and the mixture dropped on to Formvar carbon-coated Smethurst New 200 grids. After 15 s, excess fluid was removed and the grids were allowed to dry before being examined in a GEC/AEI EM801 electron microscope.

Results

Bacteriological investigations. The immunodiffusion serotypes, the biotypes based on carbohydrate utilisation (Bönicke, 1966), utilisation of inositol as detected by the modified carbohydrate-nitritase test, susceptibility to lysis by phage BK4 (Grange and Nordström, 1973), and DNAase production are shown in table I. DNAase production, found by Mankiewicz and Tamari (1972) to be a property of some lysogenic mycobacteria, was detectable in strains 33, 109, 411, 638 and 650, which produced zones of clearing of 12 ± 2 mm in the DNA containing agar.

In a previous study (Pattyn *et al.*, 1974), a close correlation between serological, cultural and biochemical properties of *M. fortuitum* was found. However, strain 4 did not hydrolyse allantoin, strain 491 did not hydrolyse allantoin or urea, and strain 2042 differed from the other serotype-II strains in its inability to utilise inositol. It was considered that these anomalous strains, together with those producing DNAase and the inositol-utilising strains resistant to lysis by phage BK4, might owe their properties to the lysogenic state. These strains, listed in table II, were examined by electronmicroscopy; strains showing no characteristics suggestive of lysogeny were included as controls. These strains were a co-type strain of *M. fortuitum*, no. ATCC 6841 (no. 927), a co-type strain of *M. ranae*, no. NCTC2891 (no. 81), the type strain of *M. peregrinum*, no. ATCC14467 (no. 632), a serotype-I strain (no. 19), and a serotype-II strain (no. 108).

Strains of serotypes III and IV are culturally and biochemically indistinguishable (Grange and Stanford, 1974) and were included in the electronmicroscopic study to determine whether the serological difference between these strains was due to phage. Strain 1812 was originally classified as a serotype-II strain (Pattyn *et al.*, 1974) but has subsequently been found to be a mixture of biochemically identical bacteria of serotypes II and III, the former being derivable from the latter.

Detection of pseudolysogeny. Phage plaques appeared spontaneously on strains 33, 638, and 650. The phages in strains 33 and 638 have been described previously and are designated respectively BK5 (Baess, 1971) and BK6 (Baess, personal communication). The phage carried by strain 650 is referred to as phage Mx1 and is shown in fig. 2.

Detection of phages causing lysis of sensitive strains. The culture filtrate of strain 4 produced plaques on strain 1954. This phage, designated Mx2, has been referred to briefly by Nordström and Grange (1974) and is shown in fig. 3.

Electronmicroscopy. In addition to the phages referred to above, morphologically complete phages were observed in cultures of strains 20 and 411.

The phage liberated by strain 20, shown in fig. 4, did not lyse any of the strains of *M. fortuitum* used in this study, but lysed *M. smegmatis* strains ATCC607 and ATCC23011. This phage is designated Mx3. Strain 411 is a variant of strain 33 cured of phage BK5, and is referred to as strain C.U.4 (Baess, 1971). The phage in this strain is morphologically identical to that in strain 411.

Culture supernates of strains 109 and 487 and the serotype-III form of no. 1812 contained occasional whole but defective-looking phages as shown in fig. 5. These strains also liberated bundles of structures resembling phage tails, as shown in figs 5 and 6. The culture supernates of strain 2040 contained unassembled phage subunits, mainly heads.

In addition to the phage tail-like units referred to above, many culture supernates contained much larger tubular structures, as shown in fig. 7. In some cases these structures exceeded the lengths of the bacterial cells.

The occurrence of bacteriophage described above is summarised in table III. No phages or phage subunits were observed in the culture filtrates of strains 5, 19, 81, 108, 491, 632, 927, 2042 and the serotype-II form of strain 1812.

Adaptation of phage I3 to a serotype-III strain. Bacteriophage I3 (Sundar Raj and Ramakrishnan, 1970) differs from all other mycobacteriophages so far examined in its possession of a contractile tail (Kozloff *et al.*, 1972). The morphology of phage I3A, obtained by the adaptation of the purified phage I3 to *M. fortuitum* strain 487, is shown in fig. 8. Phage I3A has a similar head to that of phage I3 (fig. 1) but the tails of the two phages are quite different, that of phage I3A being non-contractile and twice the length of that of phage I3.

Antiserum against phage I3 caused complete inhibition of phage I3 at a dilution of 1 in 10 and a 1000-fold reduction of plaque-forming ability at a

TABLE III
Strains of M. fortuitum found to be associated with bacteriophage

Strain no.	Associated with anomalies in				DNAase	Type of lysogeny
	colonial morphology	enzymic activity	antigenic structure	lysis by <i>Tara</i> phages		
4	—	+	+	+	—	Liberates phage Mx2
33	—	—	—	+	+	Pseudolysogenic, phage BK5
638	—	—	—	—	+	Pseudolysogenic, phage BK6
650	—	—	—	+	+	Pseudolysogenic, phage Mx1
20 (800)	+	—	—	—	+	Complete phage*
411 (862)	—	—	—	+	+	Complete phage*
109	—	—	—	+	+	Defective phage*
487	—	—	+	—	—	Defective phage*
1812 (677)	—	—	+	—	—	Defective phage*
2040 (681)	—	—	—	+	—	Defective phage*

* On electronmicroscopy.

dilution of 1 in 100. The antiserum caused no reduction of the plaque-forming ability of phage I3A. Normal rabbit serum has no significant effect on the plaque-forming ability of either phage suspension.

DISCUSSION

Of the 28 strains included in this study, 10 were found to be associated with bacteriophage. One strain liberated a phage that lysed a sensitive indicator strain, three were pseudolysogenic, two liberated morphologically complete phages that did not lyse any of the strains used in this study and four liberated defective phages. The frequency of bacteriophage observed in this study is much higher than that previously reported in the genus *Mycobacterium*. It is, however, considered probable that similar studies on other species of mycobacteria would reveal a similar high incidence of lysogeny.

Three of the four strains carrying defective phage also liberated bundles of fibres which may be phage tails. Care is required in the identification of such structures, because other fibrous and tubular structures occur in association with mycobacteria (Imaeda, Kanetsuna and Galindo 1968; Draper and Rees, 1973). The fact that the fibrous structures in the culture filtrates of strains 109, 487 and 1812 occur together with defective-looking whole phages suggests that they are phage-associated. The larger tubular structures present in many of the culture filtrates (fig. 7) are not considered to be associated with phage and are similar in appearance to the structures surrounding cells of *M. lepraemurium* (Draper and Rees, 1973).

The lysogenic nature of the strains subsequently shown to liberate whole or defective phages had been predicted on the basis of anomalies not readily explicable by mutational derivation from a common progenitor type of the species. On the other hand, "normal" strains, including type strains of *M. fortuitum*, *M. perigrinum* and *M. ranae*, did not liberate phages demonstrable on electronmicroscopy. It is therefore concluded that the carriage of phage contributes significantly to variation within the species *M. fortuitum*. The phage-associated properties observed in this study correspond to changes produced by experimental lysogeny. Properties that may be modified by the establishment of lysogeny include enzymic activity (Bönicke and Juhasz, 1970; Wenzel, 1972), colony morphology (Jones and White, 1968; Bönicke and Saito, 1970), rate of growth and nutritional requirements (Juhasz, 1968) and cell-wall-agglutination serotype (Jones and Beam, 1969). Strains 491 and 2042, although differing from the other strains of their respective serotypes, were not found to be lysogenic and almost certainly owe their anomalous properties to mutation.

The serotype-III form of strain 1812 was found to liberate defective phage and tail-like units, whereas the serotype-II form, although retaining the biochemical properties of its parent strain, liberated no such phage. This strongly suggests that immunodiffusion serotype may be modified by the establishment of lysogeny. It has been observed that strain 4 (NCTC8573), the host strain of phage Mx2, differed from the other serotype-I strains when tested with

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FIG. 1.—Phage I3 propagated on *Mycobacterium smegmatis* strain SN2. Electromicrograph (EM). $\times 150,000$.

FIG. 2.—Phage Mx1 from the pseudolysogenic strain 650 (ATCC23041). EM. $\times 95,000$.

FIG. 3.—Phage Mx2 from strain 4 (NCTC8573). EM. $\times 95,000$.

FIG. 4.—Morphologically complete phage liberated by strain 20. EM. $\times 95,000$.

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FIG. 5.—Morphologically defective phage from serotype-III strain 1812. Some phage tail-like structures are also present. EM. $\times 95,000$.

FIG. 6.—A bundle of phage tail-like structures from strain 109. EM. $\times 95,000$.

FIG. 7.—Tubular structures in the culture supernate of strain 1812. EM. $\times 63,000$.

FIG. 8.—Phage I3A, obtained by adaptation of phage I3 to serotype-III strain 487. EM. $\times 150,000$.

its homologous antiserum (Stanford and Gunthorpe, 1969). This may represent another example of phage-mediated antigenic variation.

The ability of mycobacteriophages to adapt to strains of a number of different species is well recognised; and it has been suggested that, in some cases, such adaptation is due to genetic recombination between the adapting phage and defective phage in the new host (Mankiewicz and Redmond, 1968; Juhasz and Bönicke, 1970). In the present study, evidence is presented that the adaptive modification of phage I3 to phage I3A by passage on a serotype-III strain of *M. fortuitum* is due to recombination of the genome of phage I3 with a prophage in this strain. The purification of phage I3 before adaptation makes it highly unlikely that phage I3A was obtained by selection of a minority population from phage I3. Variation by mutation cannot be definitely ruled out, but the observed changes are too great and constant to be readily explicable on this basis. Thus, genetic recombination between phage I3 and prophage in the new host is the most probable explanation of the adaptive process.

In view of the frequency of lysogeny in *M. fortuitum*, and the role of phage in determining variation within this species, serious consideration should be given to the occurrence and significance of phage in mycobacteria of greater clinical importance; in particular, the possible role of phage in the determination or modification of the virulence of the organisms should be considered. Although there is unlikely to be such a dramatic association between pathogenicity and the lysogenic state as in the case of *Corynebacterium diphtheriae* (Freeman, 1951), minor phage-induced changes may modify the host response to challenge by a mycobacterium. It is perhaps of relevance that the only strain of *M. smegmatis* ever incriminated as a cause of disease in man was found to be lysogenic (Juhasz, Harize and Gelbart, 1970). However, the question whether pathogenicity in *M. tuberculosis*, *M. ulcerans* and *M. leprae* is associated with lysogeny remains yet to be answered.

SUMMARY

Ten of 28 strains of *Mycobacterium fortuitum* (*rauae*) were found to be associated with bacteriophage; three were pseudolysogenic, one liberated a phage that lysed a sensitive indicator strain, two liberated morphologically complete phages that did not lyse any of the strains used in this study and four liberated morphologically defective phages. The lysogenic and defectively lysogenic strains showed anomalies in cultural, biochemical and antigenic properties and in susceptibility to superinfecting phages. In view of the high frequency of lysogeny found in *M. fortuitum*, the role of bacteriophage in the variation of properties, including pathogenicity, of mycobacteria of greater clinical importance merits further consideration.

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