Pseudolysogeny in *Mycobacterium diernhoferi* ATCC19341

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Lysogeny is characterized by the stable integration of the bacteriophage genome (the prophage) with the bacterial chromosome (Jacob & Wollman, 1959). There are however a number of host-phage relationships, collectively referred to as pseudolysogeny, in which the prophage appears to coexist in a temperate state with the host but without the occurrence of a stable inheritable union of their respective genomes. The literature concerning pseudolysogeny has been reviewed by Baess (1971).

In a form of pseudolysogeny referred to as the carrier state (Hayes, 1968), the prophage is carried within the bacterium as an episome-like unit which does not replicate in sequence with the bacterial chromosome. Thus, in contrast to true lysogeny, the prophage is only transmitted to a minority of the progeny. However, in some bacteria the phages undergo replication with lysis of the host and reinfection of surrounding phage-free bacteria, thus maintaining the presence of the phage in the culture. The cycle of curing and reinfection may be blocked by culturing the strain in the presence of an antiserum or other substances capable of neutralizing the extracellular phages. Strains of bacteria exhibiting pseudolysogeny of the carrier-state type are detectable by observing the spontaneous appearance of phage plaques when cultured on suitable solid media.

Beck (1965) described a strain of *Mycobacterium fortuitum*, referred to as strain C.U. This strain was found to be pseudolysogenic by Baess (1971) who isolated phage BK5 from the spontaneously-appearing plaques. Subsequently the phenomenon was observed in two other strains of *M. fortuitum*, ATCC23029 (I. Baess, personal communication) and ATCC 23041 (Nordström & Grange, 1974). In addition a form of pseudolysogeny was observed in *Mycobacterium smegmatis* ATCC607 after the experimental infection of this strain with phage L1 (Tokunaga & Sellers, 1970). It was therefore of interest to determine whether pseudolysogeny occurred naturally in mycobacterial species other than *M. fortuitum*. This paper reports the occurrence of pseudolysogeny in *Mycobacterium diernhoferi* ATCC 19341, which was observed during a study of 100 rapidly growing mycobacteria representing twelve different species.

METHODS

The bacteria. One hundred rapidly growing mycobacteria were obtained from the collection of mycobacteria at the School of Pathology, The Middlesex Hospital, London. These strains represented twelve species: *Mycobacterium chelonae*, *M. diernhoferi*, *M. duvalii*, *M. engbaekii*, *M. flavescens*, *M. fortuitum*, *M. gilvum*, *M. neoaurum*, *M. phlei*, *M. rhodesiae*, *M. smegmatis* and *M. vaccae*. The identity of all strains had previously been established in taxonomic studies using immunodiffusion analysis (reviewed by Stanford, 1973).

Detection of pseudolysogeny. A loopful of bacteria from a culture on Lowenstein Jensen medium was added to 1 ml of nutrient broth in a small bottle containing some glass beads. The bottle was shaken on a vortex mixer until a smooth bacterial suspension was obtained. A soft agar overlay was prepared by melting 7 ml of nutrient agar containing: glycerol,

3 % (w/v); calcium chloride, 0.001 M; agar, 0.8 % (w/v). After cooling the molten agar to 50 °C, 0.2 ml of bacterial suspension was added and the mixture poured on to a base of 1 % nutrient agar (i.e. 1.0 %, w/v, agar) in a Petri dish and allowed to solidify. Plates were incubated at 32 °C for 10 days and were inspected at intervals for the spontaneous appearance of phage plaques.

Isolation of bacteriophage. One loopful of *M. diernhoferi* ATCC19341 was added to 10 ml nutrient broth containing 0.001 M-calcium chloride and incubated at 32 °C for 5 days. The bacteria were sedimented by centrifugation at 1500 g for 15 min in a bench centrifuge and the supernatant was passed through a 0.22 µm membrane filter. The filtrate was prepared for electron microscopy as described previously (Grange & Bird, 1975).

Determination of bacteriophage host range. A culture filtrate of *M. diernhoferi* ATCC19341, which had been found to produce a zone of confluent lysis when a drop was spotted on to the homologous strain, was spotted on to soft agar overlays containing the other rapidly growing strains used in the study. The plates were incubated at 32 °C for 10 days and examined at intervals for lysis.

Production of antiserum. Antiserum to *M. diernhoferi* ATCC19341 was raised in a rabbit according to the method described by Stanford & Beck (1968) and Stanford & Gunthorpe (1971).

Isolation of cured variants. *Mycobacterium diernhoferi* ATCC19341 was inoculated into 2 ml amounts of antiserum (50 %, v/v) in nutrient broth and incubated with occasional shaking to disperse clumps of bacteria. Portions (0.2 ml) of the cultures were subcultured into fresh antiserum every 3 or 4 days for a total of six passages. Cultures obtained from four separate series of passages were examined for the presence of phage plaques. Two of the cultures were found to be free of phage.

Re-establishment of pseudolysogeny in the cured variants. The two phage-free variants described above were inoculated on to nutrient agar and single colony isolates obtained. These were inoculated into the culture filtrate containing the phage. After 3 days' incubation at 32 °C, subcultures were made on to Lowenstein Jensen medium and subsequently examined to determine whether pseudolysogeny had become re-established.

Cultural, biochemical and serological investigations. The morphology of the colonies of *M. diernhoferi* ATCC19341, the cured and the reinfected variants were examined on 1 % nutrient agar. All variants were examined by immunodiffusion analysis (Stanford & Beck, 1968; Stanford & Gunthorpe, 1971) using the antiserum described above. The variants were also tested for nitrataase activity (Bönicke, 1962) and production of acid from carbohydrates (Gordon & Smith, 1953).

RESULTS

Spontaneously-appearing phage plaques were only observed on one of the 100 strains examined, viz. *M. diernhoferi* ATCC19341. When cultured in a soft agar overlay this strain showed numerous minute turbid plaques. Two variants which did not produce such plaques were obtained after six passages in 50 % (v/v) homologous antiserum. Pseudolysogeny was re-established in single colony isolates of these variants by culturing them in a broth culture filtrate containing the phage.

The broth culture filtrate of *M. diernhoferi* ATCC19341 produced zones of confluent turbid lysis when spotted on to overlays containing the homologous strain and the variants described above. No lysis was produced on any of the other 99 strains, which included four other strains of *M. diernhoferi*. Electron microscopy showed that the phage has a long noncontractile tail (Fig. 1). This phage is designated Mx4.

Fig. 1. Bacteriophage Mx4 isolated from *M. diernhoferi* ATCC19341.

The original strain of *M. diernhoferi* ATCC19341, the two cured variants and the two reinfected variants were identical in their colonial morphology, rate of growth, nitrate reductase activity, utilization of carbohydrates and antigenic structure as determined by immunodiffusion analysis. The variants all produced smooth, slimy, glossy colonies on nutrient agar, reduced nitrate, and produced acid from arabinose, fructose, glucose, inositol, mannitol, mannose, trehalose and xylose, but not from dulcitol, erythritol, galactose, lactose, raffinose, rhamnose and sorbitol. On immunodiffusion analysis all variants were identical to the original strain, the antigenic structure of which has been described by Stanford & Wong (1974).

DISCUSSION

Mycobacterium diernhoferi is a rapidly growing mycobacterium which has been isolated mainly from the environment of cattle (Bönicke & Juhasz, 1965). It has been shown to be a distinct species on the basis of numerical analysis (Bönicke, 1966; Kubica *et al.* 1972) and immuno-diffusion serology (Stanford & Wong, 1974).

The pseudolysogenic nature of *M. diernhoferi* ATCC19341 was demonstrable by the observation of minute phage plaques appearing spontaneously on the strain and by the isolation of cured variants after several passages in homologous antiserum. The re-establishment of pseudolysogeny in the cured variants showed that these variants were not mutants resistant to phage infection. Furthermore, the use of single colony isolates in the reinfection studies indicates that the phenomenon of spontaneously appearing phage plaques was not due to the original culture being a mixture of strains.

No cultural, biochemical or serological variation was detectable between the original

culture and its cured and reinfected variants. Similarly Baess (1971) failed to detect any difference between the pseudolysogenic *M. fortuitum* strain C.U. and its cured variant, whereas phage-induced variation is readily detectable on the establishment of true lysogeny in mycobacteria (Juhasz, 1970; Wenzel, 1972; Grange & Bird, 1975). Although it is possible that phage conversion does not occur in pseudolysogenic strains it is likely that any phage-associated variation would be masked by the excess of phage-free bacteria in the culture.

The mechanism of pseudolysogeny of the carrier-state type is at present poorly understood. Although it appears that the prophage exists within the host as an autonomously replicating episome, it is not known whether the prophage remains constantly in an extrachromosomal location or whether a phase of integration with the bacterial genome may occur. From studies on the experimental infection of *M. smegmatis* by phage L1 (Toku-naga & Sellers, 1970) it was suggested that this host-phage relationship was of an intermediate type between pseudolysogeny and true lysogeny. Thus although phage L1 appeared to establish a lysogenic relationship with its host, this relationship broke down at a high frequency resulting either in lysis or curing of the host bacterium, the cured bacteria then being susceptible to reinfection.

It is clear from surveys of the literature (Hayes, 1968; Baess, 1971) that studies on the various forms of pseudolysogeny have lagged far behind those on true lysogeny and virulence. This is perhaps unfortunate as a greater knowledge of pseudolysogenic states in bacteria might help to explain certain aspects of latency and persistence in mammalian host-virus relationships.

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