Lysogenic Mycobacteria: Phage Variations and Changes in Host Cells

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(Accepted for publication 8 November 1968)

SUMMARY

Lysogenic conversion of mycobacteria by phage Roy results in reciprocal changes in the phase and in the host cells. The temperate phage released after lysogenization differs from the converting phage in its host range. The lysogenic derivatives show marked changes in their colony morphology and in their sensitivity to phagolysis. Some, but not all, of the lysogenic bacteria have suffered losses in enzymic activity. An attempt is made to distinguish between host-determined and phage-determined variations.

INTRODUCTION

Phage-determined changes in colony morphology, enzymic activity and other properties of lysogenized mycobacteria were described in 1961 (Mankiewicz, 1961). White, Foster & Lyon (1962) reported alterations of colony morphology in strain ATCC607 after lysogenization with phages R1 and D32. Russell, Jann & Froman (1963), however, did not detect differences between stock cultures of strain ATCC607 and derivatives of this strain produced by lysogenization with one or other of five phages. Vandra & Takats (1965) lysogenized *Mycobacterium fribergensis* and *M. rabinowitsch* with lytic phages. The staining properties, colony morphology, amidase and catalase activity and synthesis of niacin were similar in both the phage-sensitive parent strains and their lysogenic derivatives. Juhasz & Boenicke (1966) reported 'changes' in colony morphology, and decrease in the growth rate of *M. phlei* F89 after its lysogenization with phage B2. Recently, Jones & White (1968) described the conversion of colony morphology, nitrate reductase activity and Tween hydrolysis of strain ATCC607 associated with its lysogenization by phages D29 and B4.

Changes in bacterial properties obtained with some phage-host systems but not with others could be due to the selective effect of the phage on mycobacterial mutants that pre-existed within the particular bacterial population.

The following experiments were undertaken to demonstrate that, within some pure and homogeneous mycobacterial strains, lysogenization determines host- and phage-variants, while in other phage-sensitive strains no such reciprocal changes occur. This indicates that lysogenization may involve, or determine, different genetic processes in different mycobacterial strains.

METHODS

Mycobacteriophage Roy was isolated in our laboratories from the sputum of a patient with thoracic sarcoidosis. Repeated cultures of sputum from this patient had been negative for mycobacteria. Since its isolation in liquid medium enriched with strain ATCC607 (R) this phage had been propagated on this strain only, and its host range and lytic activity have remained the same. Phage Roy produces 0.8–1.0 mm. clear plaques with regular borders on strains ATCC607 and on TMC1515. At a phage:bacterium ratio of 1:10, its lytic cycle in ATCC607 consists of an absorption period of 30 min. (65% absorption), a latent period of 70 min., a rise period of 30 min. The lytic cycle is completed in approximately 2½ hr. The average burst size is 92. Electron-microscopic studies show this phage to be formed by a head hexagonal in structure measuring 92–94 m μ in length and 53–54 m μ in width. The length of the tail is approximately 250 m μ .

Media for phage study. The RVB liquid medium for the preparation of phage filtrates and the RVA agar medium for phage typing and phage titrations (in r.t.d. and p.f.u.) were prepared and used according to the recommendations of Redmond & Ward (1966).

Table 1. *Mycobacterial strains used in this study*

Species and group	Strain or source
<i>M. tuberculosis</i> , var. <i>hominis</i>	H37RV, Steenken, Trudeau
<i>M. tuberculosis</i> , var. <i>bovis</i>	BCG, Université de Montréal
Atypical Mycobacteria:	
Runyon group I	TMC 1201 TMC 1203 TMC 1204 1518, Royal Edward Chest Hospital
Runyon group II	TMC 1302 TMC 1306 18685, Royal Edward Chest Hospital
Runyon group III	TMC 1403 TMC 1404
<i>M. fortuitum</i>	TMC 1512
<i>M. smegmatis</i>	ATCC 607 TMC 1515
<i>M. fribergensis</i>	E. Vandra, Budapest
<i>M. phlei</i>	E. Vandra, Budapest
<i>M. rabinowitsch</i>	E. Vandra, Budapest
<i>M. stercoïdes</i>	E. Vandra, Budapest
<i>M. lacticola</i>	ATCC 9626
<i>Mycobacterium</i> sp.	ATCC 9033
<i>Mycobacterium</i> sp.	ATCC 4243

TMC = Trudeau Mycobacterial Collection.

ATCC = American Type Culture Collection.

Bacteria. The twenty-one mycobacterial strains included in this study are tabulated in Table 1. Before use, they were plated on RVA agar medium and one colony was selected, suspended in nutrient broth (Difco) and replated on solid medium. This process of colony selection was repeated twice for each strain. Three colonies were selected from the last plating and propagated separately. The three subcultures of each strain were submitted to the tests indicated below, in order to compare their properties and to ascertain the homogeneity and purity of each strain.

The following characters were studied: growth rate in liquid and on solid medium at 37° and at 22°; colony morphology and the conditions for their pigmentation; cord formation and acid fastness in liquid medium; utilization of paraffin as the sole carbon source in Czapek's mineral medium (without sucrose); catalase and peroxydase activity (Tirunaranay & Fischer, 1957), the amidase reactions (Boenicke, 1958), synthesis of niacin (Konno, 1957) and, for bacilli from group IV, the transformation of ammonium ferrocitrate (Tison, Tacquet & Devulder, 1964). Sensitivity to streptomycin, isoniazid and to p-aminosalicylic acid was determined as well as the sensitivity of each strain to phagolysis by phages DS6, G4E, AG1 of Redmond's phage-typing scheme (Redmond, 1963). The presence or absence of an inducible prophage was studied in filtrates of cultures that had been irradiated with u.v. for 30 sec. at 10 cm. distance (15 watt G.E. germicidal tube) or had been exposed to hydrogen peroxide (10%) or Actinomycin-C (10 µg./ml.). Lysis of the mycobacterial strains by phage Roy was determined in the following way: the bacteria were plated on solid medium and incubated for three hours. Droplets of a phage suspension that just produced an area of clear lysis on ATCC607 were deposited on the bacterial lawns. The plates were re-incubated and read after intervals of 48 hr to 10 days. Only complete clearing of the 'spots' was considered as true lysis.

Lysogenization of the mycobacterial strains. Five cultures of each bacterial strain in RVB medium were infected with phage Roy in a phage/cell ratio of 5:1. The infection took place during the exponential growth period of the mycobacteria: the fast growing bacteria were infected after three hr incubation; the slow growing organisms, after 18 hr. The cultures were re-incubated for 3 hr and one ml. samples of the cultures were added to equal volumes of phage antiserum that neutralized 98% of free phage particles. After three hr incubation with the antiserum, the cultures were centrifuged and the sediments were suspended, each in 0.2 ml. of RVB medium. These suspensions were then plated on RVA agar. Colonies produced by bacteria that survived the phage infection were isolated and propagated in RVB medium to which Tween 80 at a concentration of 0.1% was added in order to avoid re-infection by free phage particles. Subcultures on RVA medium and on Loewenstein medium were then submitted to a study of their bacterial properties by means of the above-mentioned tests.

As proposed by Russell, Jann & Froman (1963), temperate phages released by lysogenic mycobacteria are designated by the name of the phage, followed in parentheses by the name of the mycobacterium in which lysogeny was established; lysogenic derivatives are designated by the name of the parent strain, followed in parentheses by the name of the converting phage.

RESULTS

Phage variations. Phage Roy (607) and phage Roy (1515) produce clear spots on mycobacterial strains 607 and 1515. Under experimental conditions for lysogenization, they also produce phage-resistant lysogenic bacteria within these two strains. These release phage particles of the same lytic pattern and produce the lysogenic conversion of the same bacterial strains as did the virulent phage. By its lytic activity, phage Roy is a phage specific for *Mycobacterium smegmatis*.

The nineteen remaining strains resisted phagolysis. Seven strains, however, produced lysogenic colonies after incubation with phage Roy. The number of colonies under-

going lysis after u.v.-induction of the phage-infected population varied from 65 to 90 %. The phage particles released by the lysogenic bacteria showed marked changes in lytic pattern. With one exception, the lytic spectrum had expanded and included, in every instance, the respective host strain. The strains susceptible to lysogenization by temperate phages were the same that were susceptible to the parent phage; efficiency to establish the lysogenic state was, however, much greater for the temperate phages. The lytic spectrum of phage Roy and of the phage variants is summarized in Table 2.

Table 2. *Lytic activity of phage Roy and its host-determinant variants*

Indicator	Phage Roy (607)	(1518)	(1302)	(1306)	(1512)	(Phlei)	(Stercoides)	(Lacticola)
Strain 607	+	+	+	+	+	—	+	+
1515	+	+	+	+	+	—	+	+
1518	—	+	+	+	—	—	—	—
1302	—	—	+	+	—	—	—	—
1306	—	—	+	+	—	—	—	—
1512	—	—	—	—	+	—	—	—
<i>M. phlei</i>	—	—	—	—	—	+	+	—
<i>M. stercoides</i>	—	—	—	—	+	+	+	+
<i>M. lacticola</i>	—	—	—	—	—	—	—	+

Table 3. *Changes in growth rate, colony morphology, pigmentation of colonies and in the microscopic aspect in mycobacteria lysogenized with phage Roy*

Lysogenic derivative of	Growth rate	Colony	Pigmentation	Bacterial morphology
607	Delay	R. S nipples	Earlier	Swollen cytoplasm which lost acid fastness. Heavy beading
1515	Delay	R, S to S	No change	Partial loss of acid fastness
1518	Delay	S	No change	Partial loss of acid fastness
1302	Delay	R, S	Dissociation into pigmented and non-pigmented umbilicated colonies	Partial loss of acid fastness
1306	Delay	R to S	No change	No change
1512	Delay	S	No change	Partial loss of acid fastness
<i>M. phlei</i>	Delay	S	No change	Relative loss of acid fastness
<i>M. stercoides</i>	Delay	S, nipples colonies	No change	Heavy beading, partial loss of acid fastness
<i>M. lacticola</i>	Delay	S	Increase in pigmentation	Heavy beading, partial loss of acid fastness

R = Rough.

S = Smooth.

Changes in bacterial properties associated with lysogenization. Ten mycobacterial variants were isolated from the nine strains lysogenized by phage Roy: two from strain 1302; one from each of the following: 607, 1515, 1518, 1306, 1512, *Mycobacterium phlei*, *M. stercoides* and *M. lacticola*. The lysogenic derivatives shared the ability to produce phage particles, the lytic activity of which has been described previously. They all showed decrease in their growth rate during the first two or three

subcultures. Instead of the rough or wrinkled colonies of most of the parent strains, lysogenic strains produced smooth colonies, some of them heaped or nipped. Microscopically, bacteria from these colonies showed a relative loss in acid fastness that affected mainly the cytoplasm; Much's granula were purple and caused heavy 'beading' of the bacteria. The lysogenic derivatives were resistant to phagolysis by the converting phage and by the phage variants. The colonies of 607 (Roy) and 1515

Table 4. Comparison between strain ATCC607 and strain ATCC607 (Roy) isolated 8 days after phage infection

Character on test	Parent strain	Lysogenic derivative
Colony morphology on RVA agar	Small, wrinkled or rough	Large, smooth, nipped
Pigmentation	Slight, increases with age of culture	Slight, appears immediately
Bacterial morphology and acid fastness	Long granulated rods, 95 % acid fast	Long and short (coccoid) bacilli. Loss of cytoplasm which retains less dye. Large acid fast granules (heavy 'beading')
Growth rate in RVB medium	24 hr at 37° 48 hr at 22°	4-5 days at 37° 9 days at 22°
Paraffin utilization	+++	—
Cord formation	—	—
Amidase reactions:		
Urease	++	++
Pyrazinamidase	++	++
Nicotinamidase	++	++
Acetamidase	±	—
Benzamidase	++	++
Succinamidase	+	+
Niacin synthesis	—	—
Catalase activity	+++	±
Peroxydase	—	—
Nitrate reduction	+	—
Transformation of ammonium ferrocitrate	++	—
Sensitivity to:		
Streptomycin (10-100 mcg./ml.)	Sensitive	Sensitive
Isoniazid (1-10 mcg./ml.)	Resistant	Resistant
PAS (1-100 mcg./ml.)	Resistant	Resistant
Lysis by phage:		
DS 6A	—	—
G 4E	—	—
AG I	+	—
Roy	+	—
Induction by:		
u.v. (30 sec., 10 cm. distance)	—	++
Actinomycin-C (10 mcg./ml.)	—	++
H ₂ O ₂ (10 %)	—	++

(Roy) and of *M. lacticola* (Roy) showed earlier and more pronounced pigmentation than did the respective parent strains. In contrast, strain 1302 (Roy) produced two kinds of colonies: one, pigmented as the parent strain; the other, nonpigmented and umbilicated. The above observations are summarized in Table 3.

The time of appearance of lysogenic derivatives was studied with fast- and slow-growing bacteria. The smooth colonies of 607 (Roy) appeared first in cultures plated 24 hr after infection. They required nine days to attain maturity in these cultures. The slow-growing 1302 (Roy) colonies appeared 10 days after phage infection. Their lag period was 5-6 days. After repeated subcultures, the lag periods were reduced considerably.

Comparison between strain 607 and its lysogenic derivative is summarized in Table 4. In addition to differences in colony morphology (Pl. 1, fig. 1), the microscopic aspect of the bacilli (Pl. 1, fig. 2) and the changes in phagolytic pattern with resistance to phage Roy as well as to phage AG 1, there was a loss of enzymic activity associated with lysogenization. These bacilli were not able to utilize paraffin as their sole carbon source. Their catalase reaction and nitrate reduction tests were negative; ammonium ferrocitrate was no longer transformed. The acetamidase reaction which already was weak in the parent strain, had completely disappeared. There was no change in the sensitivity to antimycobacterial drugs.

The lysogenic derivative of strain 1515 (Pl. 1, fig. 3) showed almost the same changes as strain 607 (Roy): it produced smooth nipped colonies, it had acquired resistance to phagolysis by phage Roy and phage AG 1 and it had lost its nitrate reductase activity. Strain 1518 (Roy) also showed resistance to phagolysis by phage Roy and phage AG 1. In this case the lysogenic conversion was associated with the loss of nicotinamidase and urease activity. The non-pigmented umbilicated colonies of strain 1302 (Roy) (Pl. 1, fig. 4) showed loss of their nicotinamidase, succinamidase and their nitrate reductase activity. Both the parent strain and the lysogenic variant are resistant to phagolysis. No changes in enzymic activity could be detected between the lysogenic strains of 1512, *Mycobacterium phlei*, *M. stercooides*, *M. lacticola* or the pigmented strain of 1302.

DISCUSSION

Variations and 'adaptations' of mycobacteriophages have been reported many times. Among thirty or more phages isolated, in Redmond's laboratories only one had been free of some variations (Redmond, 1963). Russell, Jann & Froman (1963) have shown alterations in host range between lytic phages and their temperate derivatives. These authors suggested that 'some of these alterations may be due to host-controlled modification, while others may be due to the activation of a previously undetected prophage'. The latter mechanism, or recombination, was suggested by observations recently reported on the lytic phenomena of phage Leo propagated on ATCC607 or on strain H₃₇RV as alternate hosts (Mankiewicz & Redmond, 1968).

Failure to demonstrate a prophage in the bacterial parent cells may be due to lack of appropriate techniques or indicator strains, or to the particular state of the prophage. The prophage may be defective or inhibited in the expression of its genes by the action of a repressor present in the bacterial chromosome. Superinfection with phage Roy and recombination, however, may have caused the prophage to become detached from the chromosome.

Phage Roy does not lyse or lysogenize strains of *Mycobacterium tuberculosis* or representative strains from atypical mycobacteria group III. Only one of four strains of photochromogenic mycobacteria was susceptible to lysogenization by this phage. Strain 1518 has recently been isolated in our laboratories and has been classified as a

photochromogen on the basis of photo-induced pigmentation, growth rate, amidase activity, phage-typing and the other above-mentioned tests. Scotochromogenic bacteria, *M. fortuitum* and fast-growing saprophytic mycobacteria appeared to be most susceptible to phage Roy infection.

In their recent study of the conversion of colony morphology and certain enzyme systems of ATCC607 lysogenized with phage D29 or B4, Jones & White (1968) described the appearance of phage-resistant smooth colonies which were lysogenic. Their morphology appears to be quite similar to the colonies of 607 (Roy) and of 1515 (Roy). In contrast to the observations of these authors, strains lysogenized by phage Roy did not revert to the non-lysogenic state nor did they form rough colonies following subculture in media preventing phage readsorption. The high frequency of the loss of the lysogenic state together with the reversion to the formation of R colonies by the 'cured' strain of 607 suggested to Jones and White that the genetic material that controls these changes is found in the extrachromosomal plasmids. This does not seem to apply to 607 (Roy) where the described changes were hereditary and permanent.

Jones & White (1968) reported that the smooth colonies of 607 (D29 or B4) showed increased nitrate reductase activity and a higher degree of hydrolysis of Tween 80. In this study the lysogenic derivative 607 (Roy) showed a reduction in the activity of several enzyme systems including that of the nitrate reductase.

These differences—provided they are not due to technical reasons—may indicate which of the bacterial enzyme systems affected by lysogenization are phage controlled and which are host determined. Similarities in colony conversion suggest that they are independent of the specific phage, while the different effects on the enzymic activity may be determined by the converting or recombinant phage. Further studies along these lines may help to clarify the process of lysogenization and the nature of certain mycobacterial variants.

Thanks are due to Dr V. Pavilanis and the staff of the Department of Virology, Université de Montréal, who performed the electronmicroscopic studies used in this project.

This project was supported in part by grants from the Medical Research Council of Canada and the Muskoka Hospital Memorial Research Fund.

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EXPLANATION OF PLATE

Fig. 1. Conversion from rough to smooth nipped colonies of strain 607 and its lysogenic derivative 607 (Roy). (a) Wrinkled dry colonies; strain 607. (b) Smooth nipped colonies after lysogenization; strain 607 (Roy).

Fig. 2. Microscopic aspect of 607 (Roy) showing large granules and loss of acid fastness of cytoplasm. ($\times 1400$).

Fig. 3. Conversion from rough to smooth nipped colonies of strain 1515 and 1515 (Roy). (a) Rough dry colonies; strain 1515. (b) Smooth nipped colonies after lysogenization; strain 1515 (Roy).

Fig. 4. Dissociated into smooth pigmented and nonpigmented umbilicated colonies of strain 1302 and 1302 (Roy). (a) Round regular colonies; strain 1302. (b) After lysogenization, two types of colonies distinct from those of the parent strain: smooth pigmented and umbilicated non-pigmented colonies; strain 1302 (Roy).

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