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Control of Lysogeny in Mycobacteria

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Abstract

The transducing mycobacteriophage I3 is a temperate phage which forms turbid plaques of diameter 2–3 mm on the host *Mycobacterium smegmatis* SN2. Mutants of the phage have been isolated which form clear plaques. The complementation analysis using different c mutants have shown that there are at least 3 loci on the phage chromosome involved in lysogenization. In addition, certain host genes also play a critical role in the establishment of lysogeny by I3. Isolation and characterization of the host mutants in which the phage cannot establish lysogeny, have revealed that the cyclic AMP levels of the host determine the lytic or lysogenic response in mycobacteria. One-step growth curve experiments indicate that one cycle of the phage takes 4–5 hours and that the phage liberation is much more asynchronous in wild type phage infection as compared to c mutant infection.

Introduction

The generalised transducing mycobacteriophage I3 isolated in our laboratory (SUNDER RAJ, 1971; SUNDER RAJ and RAMAKRISHNAN, 1970) is a temperate phage which infects several species of saprophytic mycobacteria. The phage has been successfully employed to transduce certain auxotrophic (SUNDER RAJ and RAMAKRISHNAN, 1971) and drug resistance and sensitivity (SAROJA and GOPINATHAN, 1973) markers in *M. smegmatis*. Electron microscopic studies (KOZLOFF *et al.*, 1972) have shown that the phage has a head with regular isometric symmetry and a long contractile tail. The phage DNA is double stranded with a very high G-C composition of 65% similar to that of its host *M. smegmatis* (GADAGKAR and GOPINATHAN, 1976). Presently, attempts are being made to map the genetic structure of the phage 13 chromosome (GOPINATHAN and SAROJA, 1975). As a part of our investigations on the genetics of the mycobacterial system, we have studied some aspects of the interaction of I3 and its host, with particular reference to lysogeny.

Materials and Methods

Organisms and Growth media

Mycobacterium smegmatis SN2 obtained from Dr. R. BÖNICKE, Institute for Experimental Biology and Medicine, Borstel, Germany, was grown in the synthetic minimal medium of YOUMANS and KARLSON (YOUMANS and KARLSON, 1947) containing 0.2% (v/v) Tween – 80.

[1] demised.

Phage lysates were prepared either by confluent lysis on Luria agar plates (LURIA *et al.*, 1957) or by infecting log-phase *M. smegmatis* cultures in Luria broth at a multiplicity of infection of 1–5, and growing in a rotary shaker at 37C after diluting a 100 fold.

Mutagenesis

(1) *Hydroxylamine:* The phage was suspended in potassium phosphate buffer pH 6.0 containing 1 M hydroxylamine and 1 mM EDTA, and incubated for 10 hours and 37C. The reaction was stopped by diluting 100 fold in Luria broth containing 10 mM EDTA.

(2) *N-methyl-N'-nitro-N-Nitrosoguanidine (NTG):* Log phage cells were infected at a multiplicity of infection of 1 and left for adsorption at 37C for 45 min. NTG at a concentration of 500 μg/ml (prepared freshly in acetone) was added and incubation continued. After 45 min the cells were washed free of the mutagen, resuspended in fresh broth and the incubation continued overnight.

Isolation of clear plaque mutants

The phage lysates after mutagenesis were plated out at 37C and the clear plaques were picked. The individual, well-separated plaques were picked and purified through at least three independent steps of single plaque isolation. A total of 27 clear plaque mutants were thus isolated.

Isolation of host mutants

M. smegmatis log phase cells were treated with NTG at a final concentration of 100 μg/ml for 45 min at 37C. Cells were then harvested free of mutagen and incubated overnight at 37C. Mutants which simultaneously lost the ability to ferment the sugars normally used by *M. smegmatis,* viz., lactose, maltose and dulcitol were selected by plating the mutagenised cultures on indicator plates containing the above mentioned sugars and 50 μg/ml of triphenyl tetrazolium chloride. $Lac^-Mal^-Dul^-$ mutants were thus obtained and extensively purified by single colony isolation while checking for the fermentation properties at every step of purification.

Adenyl Cyclase Assay

The $Lac^-Mal^-Dul^-$ mutants isolated were checked for adenyl cyclase activity in cell free extracts by monitoring the conversion of ^{14}C ATP to cyclic AMP (by chromatography). The cell-free extracts were incubated in 50 mM glycine-NaOH buffer, pH 9.75, containing 30 mM $MgCl_2$, 5 mM β mercapto-ethanol and ^{14}C ATP, at 30C for 30 min. The reaction was stopped by adding cold ATP or by heating and the mixture was chromatographed in isopropanol-HCl-H_2O system.

^{14}C ATP (Sp. act. 160 mci/m mole) was a gift from B. B. BISWAS, Calcutta. All other chemicals used were of analytical grade.

Complementation Analysis

Complementation between different c mutants was carried out by spot testing. In these experiments, stocks of individual mutants were diluted to a titer of 10^6–10^7 PFU/ml and 0.05 ml of a mixture of equal quantities of 2 mutants were spotted on preformed lawns of *M. smegmatis* cells. Appropriate controls of individual mutants were always included. The morphology of the lysis area (whether clear or turbid) was observed at the end of 24–30 hours of incubation at 37C. Turbidity caused by growth of bacteria within the zone of lysis was taken as positive complementation while clear zones were taken as negative complementation.

One-step growth curve

The methods employed were similar to those described by ADAMS (1959). Log phase cells were harvested by centrifugation and resuspended in Luria broth without Tween – 80 to give a cell density of 10^8 cells/ml and infected with wild type or clear plaque mutants of the phage at a multiplicity of infection of 1. Adsorption was allowed for 30 min at 0C. The infective centres were separated from the unadsorbed phage either by centrifugation or by the addition of anti-phage serum. These cells were resuspended in fresh Luria broth at a 100 fold dilution and incubated in a rotary shaker at 37C. Aliquots were removed at 20 min intervals and titrated with indicator bacteria.

Results

Isolation and characterization of phage mutants

27 phage mutants defective in the lysogenization of *M. smegmatis*, indicated by the formation of clear plaques have been isolated as described. Table 1 shows the results of complementation analysis using these mutants. From these results, it is obvious that at least three phage loci are involved in the control of lysogeny.

Tab. 1: Complementation groups of clear plaque mutants. Complementation of c mutants were carried out by spot testing as described under methods.

Group I	1, 3, 10, 12, 15, 18, 24
Group II	2, 5, 8, 9, 11, 14, 19, 23
Group III	4, 6, 7, 13, 16, 17, 20, 21, 22, 25, 26, 27

Tab. 2: Fermentation properties of *M. smegmatis* and its mutants.

Strain	Lactose		Maltose		Dulcitol	
	− cAMP	+ cAMP	− cAMP	+ cAMP	− cAMP	+ cAMP
Wild type *M. smegmatis*	+	+	+	+	+	+
Mutant I	−	+	−	+	−	+
Mutant II	−	+	−	+	−	+

One-step growth curve

While the complementation experiments were in progress, one-step growth curve experiments were performed using wild type phage I3 and a randomly picked c mutant. As shown in Figure 1 a single cycle of the phage takes as long as 4–5 hours. This is all the more striking in view of the fact that the normal doubling time of the host bacterium is only 90 min. Wild type I3 takes 40 min longer to complete one growth cycle and has a rise period of 140 min as opposed to 80 min of the c mutant.

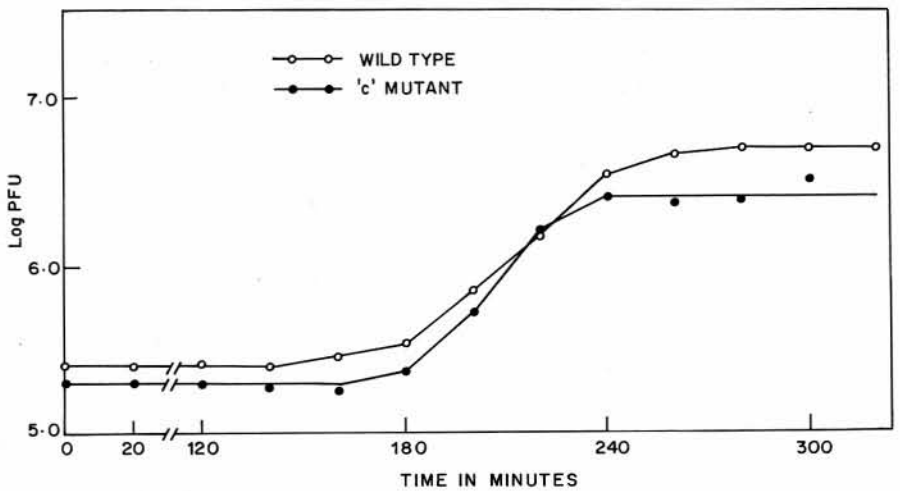

Fig. 1: One-step growth curve of mycobacteriophage I3 – wild type and c mutant. Details of the experimental procedure are described in the text.

Isolation and characterization of host mutants

Starting from *M. smegmatis* as the parent strain, mutants unable to ferment sugars were isolated as described. From Table 2 it can be seen that while the parent strain can utilize lactose, maltose and dulcitol, the two mutants isolated have lost the ability to ferment these sugars simultaneously. Addition of cyclic AMP resulted in the simultaneous recovery to ferment all the three sugars indicating that these mutants are defective in the production of cyclic AMP.

Table 3 shows that cell-free extracts of the two mutants were unable to convert ATP to cyclic AMP, a reaction catalysed by adenyl cyclase. The presence or absence of theophylline, an inhibitor of phosphodiesterase did not alter the results indicating that the lack of cyclic AMP is not due to its rapid degradation.

Behaviour of I3 on host mutants

Table 4 shows the plaque morphology of wild type I3 on the parent and mutant strains of bacteria. The presumably adenyl cyclase mutants were defective in lysogenization but the defect was reversed by the addition of cyclic AMP. Cyclic GMP alone did not reverse the defect nor did it antagonize the reversal by cyclic AMP. Theophylline again had no effect.

Discussion

In the extensively investigated systems such as coliphage lambda and the Salmonella phage P22, the control of lysogeny is now fairly well understood and the involvement of both phage and host genes has been demonstrated. Studies on the phages have also thrown considerable light on the genetics and physiology of the bacteria.

In the case of lambda, three phage genes cI, cII and cIII are involved in the control of lysogeny (KAISER, 1957). cI produces a repressor which is involved both in the establishment and maintenance of lysogeny while cII and cIII products only regulate repressor synthesis. In P22 similarly there are three genes involved in the establishment of lysogeny (LEVINE, 1957) and a fourth locus is involved in the maintenance of lysogeny (GOUGH, 1968).

Tab. 3: Adenyl cyclase activity. The methods used for the enzyme assays are given in the text.

Source of Enzyme	Treatment	cpm after chromatography ATP	cAMP	Total
Wild type *M. smegmatis*	Control (without enzyme)	445	–	445
	Expt. (+ Theo)	240	295	535
	Expt. (– Theo)	330	150	480
Mutant I	Control (without enzyme)	705	–	705
	Expt. (+ Theo)	660	–	660
	Expt. (– Theo)	730	–	730
Mutant II	Control (without enzyme)	532	–	532
	Expt. (+ Theo)	458	–	458
	Expt. (– Theo)	660	–	660

Tab. 4: Plaque morphology of I3 on *M. smegmatis* and its mutants.

Bacteria	Nil	cAMP	cGMP	cAMP + cGMP	Theophylline
Wild type *M. smegmatis*	Turbid	Turbid	Turbid	Turbid	Turbid
Mutant I	Clear	Turbid	Clear	Turbid	Clear
Mutant II	Clear	Turbid	Clear	Turbid	Clear

Results presented here indicate that at least three phage genes are involved in the control of lysogeny in I3. Work is now in progress to determine the actual functions of these genes and the nature of the repressor.

We have also studied the growth of I3 and its c mutants. It is possible to perform one-step growth curve experiments even with the wild type temperate phage because at multiplicities of infection below 5 lysis takes place in preference to lysogeny.

The very slow growth of the phage taking as long as 4–5 hours to complete one cycle is interesting feature of the system. Under the experimental conditions used uninfected bacteria double once every 90 min and how a phage that takes nearly 5 hours to complete one burst can grow on this host is intriguing.

Further, the wild type phage has a much longer rise period than that of the mutant. This can be taken to mean that there is greater asynchrony in the liberation of phage particles in a wild type infection. There could be several reasons for this asynchrony. When we look at the one-step growth curve of a wild type temperate phage we are looking at a system that is potentially capable of lysogenization but has decided to go through lysis. Thus, there would be a residual activity of repressor which could bring about either a delay or asynchrony in phage induced macromolecular synthesis or late gene expression. Macromolecular synthesis and the kinetics of lysozyme synthesis upon phage infection are being studied which may throw some light on this phenomenon.

Bacteria do not normally utilise other sugars when glucose is present. In the absence of glucose, cyclic AMP stimulates the synthesis of enzymes for the metabolism of other sugars (VARMUS *et al.*, 1970, MILLER *et al.*, 1971). Thus, mutants defective in the production of cyclic AMP should also be defective in the utilisation of various sugars. This has been

demonstrated in *E. coli* (PERLMAN and PASTAN, 1969) and the two mutants we have isolated show this to be indeed the case in mycobacteria also. Wild type lambda (GRODZICKER *et al.*, 1972) and P22 (HONG *et al.*, 1971) cannot lysogenize such mutant bacteria. Results presented here indicate that lysogenization by the mycobacteriophage I3 is also influenced by cyclic AMP levels of the host. Cyclic AMP may be a much more general regulator of transcription and thus may be able to regulate repressor synthesis. Further, it is advantageous for the phage to use cyclic AMP levels as a cue to decide between lysis and lysogeny because cyclic AMP levels indicate the nutritional status of the bacteria.

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