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Characterization of Mycobacteriophage I8 and its Unrelatedness to Mycobacteriophages I1, I3 and I5

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SUMMARY

Homology among the genomes of mycobacteriophages I1, I3, I5 and I8 has been studied. Based on restriction endonuclease cleavage patterns, dot blot hybridization and Southern blot hybridization analysis, the DNAs of phages I1, I3 and I5 have been shown to be homologous and indistinguishable, but entirely different from phage I8. Unlike the others, the I8 genome does not harbour any single-strand interruptions. The DNA is 43 kb in length with limited cyclic permutations and has a G + C content of 54%. The presence of 5-methylcytosine in I8 DNA was indicated from the restriction patterns of *MspI* and *HpaII*. The number of sites and fragment sizes for several restriction enzymes on I8 DNA has been determined. Phage I8 has a replication cycle of 300 min, with a latent period of 180 min, a rise period of 120 min and a burst size of 100. The viability of phage I8 is significantly reduced by treatment with organic solvents.

INTRODUCTION

Although a large number of phages have been isolated for mycobacteria, their genomes have not been characterized in detail. Only the G + C contents and molecular weights of some of the phage DNAs have been reported (Menezes & Pavilanis, 1969; Soloff *et al.*, 1978; Drapier *et al.*, 1978). Recently we have characterized the genome of phage I3 (Reddy & Gopinathan, 1986*a*), a temperate mycobacteriophage. Phage I3 DNA is 135 kb long, circularly permuted and rich in G + C content (67%). The genomic DNA of phage I3 harbours random single-strand gaps in both strands (Reddy & Gopinathan, 1986*b*). For comparative purposes, here we have carried out investigations on the genomes of a few more mycobacteriophages, namely phages I1, I5 and I8, which were isolated in our laboratory (Sunderraj & Ramakrishnan, 1971). Phages I1, I3, I5 and I8 differed from each other in their host range. Further, phage I8 formed clear plaques on all the host strains tested, unlike the others which formed turbid plaques on some of them.

Analysis of restriction endonuclease cleavage patterns (Dean *et al.*, 1976; Chaney *et al.*, 1983), dot blot hybridization (Roberts *et al.*, 1984), and a combination of these two techniques (Cochran & Faulkner, 1983) have been very useful in studying the genomic relatedness among groups of phages and plant and animal viruses. In this paper we report the results of DNA homology studies among mycobacteriophages I1, I3, I5 and I8. Characterization of phage I8 and its genomic DNA are also presented.

METHODS

Growth of bacteria and phages. *Mycobacterium smegmatis* SN2, mycobacteriophages I1, I3, I5 and I8 and *Escherichia coli* K-12 strain C600 were from our laboratory stocks. Phages were propagated on *M. smegmatis* SN2 grown in synthetic medium (Nagaraja & Gopinathan, 1980). *E. coli* was grown in Luria broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2).

DNA extraction and base composition. Phage purification and extraction of DNAs were carried out as described earlier (Reddy & Gopinathan, 1986*a*). The bacterial DNAs were extracted as described by Sadhu *et al.* (1984). Base composition of phage DNAs was determined by thermal melting analysis (Mandel & Marmur, 1968) using a Beckman DU8B spectrophotometer.

One-step growth experiment. A 12 h culture of *M. smegmatis* SN2 (5×10^7 cells/ml) was infected with phage I8 (m.o.i. 0.5) in the presence of 1 mM-KCN and incubated at 37 °C for 10 min for adsorption. The cells were pelleted by centrifugation, resuspended in the original volume of fresh medium, diluted 10^5 -fold and incubated at 37 °C with shaking. Samples were withdrawn at different times and phage titres were determined using *M. smegmatis* SN2 as the plating bacteria.

Organic solvent treatment of the phage. The phage lysate (7×10^{10} p.f.u./ml) was treated with 33% (v/v) of the organic solvents chloroform, ether, benzene and butanol at 37 °C for 45 min. The samples were centrifuged at 4 °C to obtain phase separation, and the organic phase was discarded. The samples were left in Petri plates allowing evaporation of the remaining traces of solvents and the phage titre was determined.

Restriction endonuclease digestion and hybridization procedures. Restriction digestions were carried out as recommended by the suppliers (New England Biolabs) and the DNA fragments were analysed by electrophoresis on agarose gels in 40 mM-Tris-acetate pH 8.0 containing 2 mM-EDTA (Maniatis *et al.*, 1982). The specific DNA fragments were purified from preparative agarose gels by the electro-elution method (Maniatis *et al.*, 1982). Fragments of DNA or intact phage DNA were labelled *in vitro* with [α - 32 P]dCTP by nick translation with *E. coli* DNA polymerase I (Rigby *et al.*, 1977). Southern blot hybridization (Southern, 1975) was carried out at 42 °C in the presence of $6 \times$ SSC, $5 \times$ Denhardt's solution, 50% formamide and 100 μ g/ml of calf thymus DNA. After hybridization, the nitrocellulose filters were washed twice in $2 \times$ SSC containing 0.1% SDS at room temperature and in $0.1 \times$ SSC containing 0.1% SDS at 68 °C, air-dried and exposed to X-ray films at -70 °C for autoradiography. For dot blot hybridization, the binding of DNA to nitrocellulose filters was done as described by Parnes *et al.* (1981). The hybridization conditions were the same as in Southern hybridization.

RESULTS AND DISCUSSION

DNA homology among mycobacteriophages I1, I3, I5 and I8

The homology among the genomes of phages I1, I3, I5 and I8 was examined by restriction endonuclease analysis, dot blot hybridization and Southern hybridization analysis of the restriction fragments.

To compare the restriction patterns, phage I1, I3, I5 and I8 DNAs were individually digested by a variety of enzymes and the products were analysed by electrophoresis on agarose gels. Fig. 1 shows one such gel, where the patterns of *Bgl*III, *Pst*I, *Hind*III and *Bam*HI digests of I1, I3 and I5 DNAs are shown. The restriction patterns of these three DNAs with the enzymes did not show any differences. The cleavage patterns of I1, I3 and I5 DNA with *Xba*I, *Sal*I, *Sma*I and *Kpn*I were also identical, and none were cut by *Eco*RI or *Hpa*I, indicating a great deal of similarity among these genomes. In contrast, the restriction digestion patterns of phage I8 DNA were entirely different from the others (Fig. 2*a*). Enzyme *Eco*RI cleaved I8 DNA seven times, but I3 DNA had no sites for this enzyme (Reddy & Gopinathan, 1986*a*). On the other hand, *Sma*I which cleaved I3 DNA 40 times had no sites on I8 DNA. Differences were also seen in restriction with *Pst*I, *Kpn*I and *Hind*III. Phage I8 is therefore different from the other mycobacteriophages compared here.

The DNA sequence homology of phages I1, I3, I5 and I8 was also studied by cross-hybridization using a dot blot technique (Fig. 3). It is clear from the figure that I3 DNA hybridized well with I1 and I5 but not with I8 DNA (*a*), again showing a great extent of similarity among I1, I3 and I5 genomes. When I8 DNA was used as the probe, it hybridized only to I8 DNA and not the others (*b*) thus showing its distinctness.

Although the restriction endonuclease analysis and dot blot hybridization analysis showed an overall similarity among I1, I3 and I5 DNAs, it is still possible that there are some non-homologous regions among these three genomes which cannot be detected by dot blot hybridization. To clarify this matter, the cross-hybridization analysis was carried out against the restriction fragments of I1, I3 and I5 DNAs. All the *Hind*III and *Bam*HI fragments of I1 and I5 DNAs hybridized with 32 P-labelled I3 DNA, showing homology between I1, I3 and I5 genomes. However, minor differences in the DNA sequence cannot be ruled out with the present data. Under the same conditions 32 P-labelled I8 DNA did not hybridize to any of the I1, I3 and I5 DNA fragments, showing a complete absence of any homologous sequences between I8 and the other genomes.

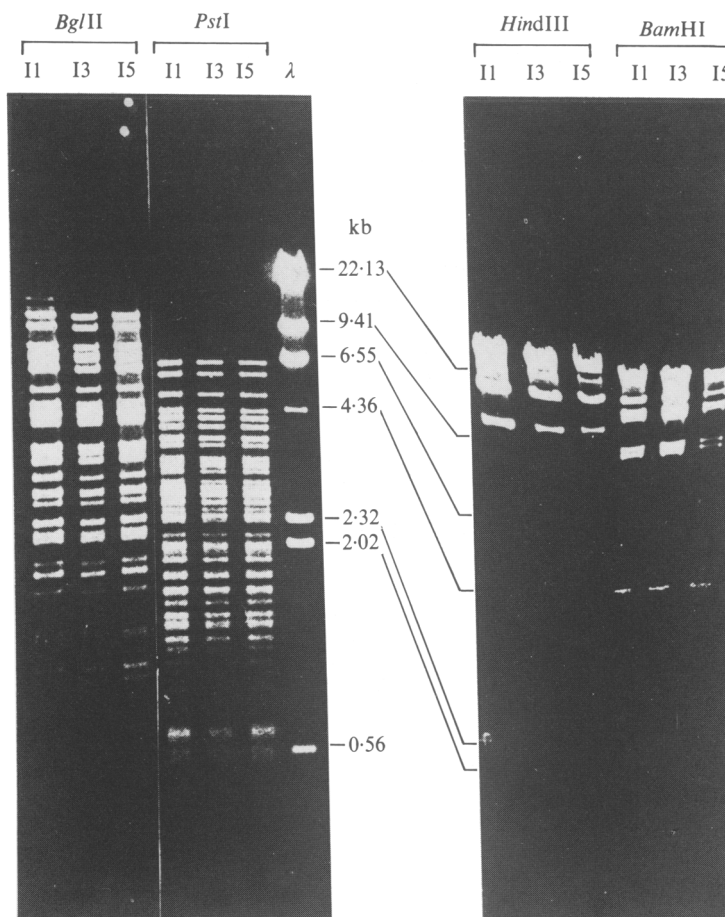


Fig. 1. Restriction endonuclease analysis of phage I1, I3 and I5 DNAs. The *Bgl*II, *Pst*I, *Hind*III and *Bam*HI digests of I1, I3 and I5 DNAs were subjected to electrophoresis on 0.7% agarose gel. Lane λ , λ DNA *Hind*III M_r markers.

Characterization of phage I8 and its DNA

Since the genome of I8 was quite distinct from that of phages I1, I3 and I5, further characterization of phage I8 was undertaken. Characterization of I3, serving as a representative for I1, I3 and I5 has been described elsewhere (Reddy & Gopinathan, 1986*a,b*).

The one-step growth curve of phage I8 on *M. smegmatis* SN2 has been determined. It has a relatively long growth cycle (300 min), comprising an eclipse period of 120 min, a latent period of 180 min, a rise period of 120 min and a burst size of approximately 100. The one-step growth curve of phage I3 was also similar except that its burst size was much lower (Nagaraja & Gopinathan, 1980). Phage I8 showed a loss in titre on treatment with various organic solvents. Maximum inactivation was observed with butanol (10^{-7} survivors) and methanol (10^{-5} survivors). Benzene, chloroform and hexane were much less effective ($>10\%$ survivors). Thus, like other mycobacteriophages (Gope & Gopinathan, 1982; Sellers & Tokunaga, 1970; Soloff *et al.*, 1978), inactivation of I8 by organic solvents suggests the presence of lipids essential for phage viability.

The G+C contents of I1, I3, I5 and I8 DNAs were determined by thermal melting analysis and found to be very similar ($67 \pm 0.3\%$) for the former three phages. At a T_m value for I8 in

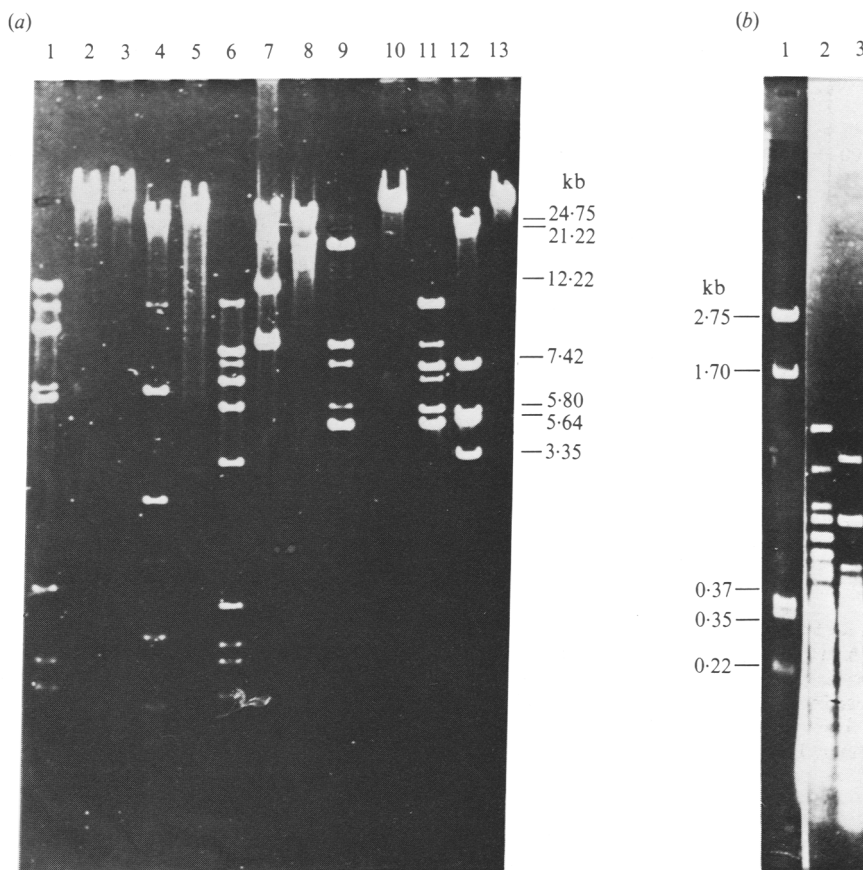


Fig. 2. Restriction endonuclease analysis of phage I8 DNA. (a) Various restriction digests of I8 were analysed on 0.8% agarose gel. Lanes 1 to 6, *EcoRI*, *HindIII*, *XbaI*, *BamHI*, *XhoI* and *SalI*. Lanes 8 to 11, *KpnI*, *BglII*, *SmaI* and *PstI*. Lane 13, undigested I8 DNA. Lanes 7 and 12 contain λ DNA *SmaI* and *EcoRI* *M*. markers. (b) *HpaII* (lane 2) *MspI* (lane 3) digests of I8 and a *HpaII* (lane 1) digest of ϕ X174 DNA were subjected to electrophoresis on 1.8% agarose gel.



Fig. 3. Cross-hybridization among I1, I3, I5 and I8 DNAs by dot blot hybridization. DNA samples (10 μ g each) were denatured with 1 M-NaOH and neutralized with a solution containing 0.5 M-Tris-HCl pH 8.0, 1 M-HCl, 1 M-NaCl and 0.3 M-sodium citrate and spotted on nitrocellulose filters. The filters were washed in $6 \times$ SSC, baked and used for hybridization (conditions given in Methods). The probes used for hybridization were 2 μ g each of (a) I3 and (b) I8 DNA 32 P-labelled *in vitro*. The DNAs in different spots were *E. coli* (used as a control); 2, I3; 3, I1; 4, I5; 5, I8.

0.1 \times SSC of 76.2 $^{\circ}$ C, the G+C content was calculated to be 54% (Fig. 4a). The transition width, defined as the temperature span from 17 to 81% of hyperchromaticity of the profile, was 3.2 $^{\circ}$ C for I8 DNA; that is, much sharper compared to phage λ DNA (Yabuki *et al.*, 1971), and comparable to I3 DNA (Reddy & Gopinathan, 1986a), indicating a random base distribution. The melting of I8 DNA was also monitored at higher resolution (0.2 $^{\circ}$ C intervals) and the first

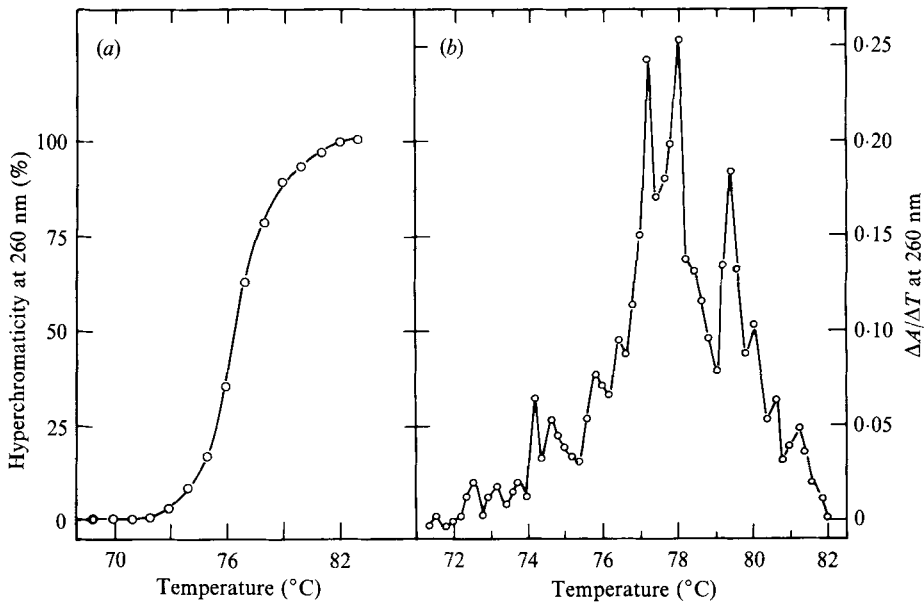


Fig. 4. Thermal melting analysis of I8 DNA. The thermal denaturation of I8 DNA ($1.5 A_{260}/\text{ml}$ of DNA in $0.1 \times \text{SSC}$ containing 0.2 mM-EDTA , pH 7.0) was monitored at (a), 1.0°C and (b), 0.20°C intervals in a Beckman DU8B spectrophotometer fitted with a programmable heating cuvette system. The stabilization time between each temperature rise was 2 min. Using *E. coli* DNA as the standard, the G+C content of I8 DNA was calculated from the expression $\Delta G+C = \Delta T_m \times 2.44$ (Mandel & Marmur, 1968).

derivative plot of the melting data is shown in Fig. 4(b). The number of subtransitions was less in the I8 DNA profile compared to λ DNA (Yen & Blake, 1980). This provided further support for the random distribution of bases in I8 DNA.

In order to check for the presence of methylated bases in I8 DNA, restriction of the DNA with the isoschizomeric enzymes *MspI* and *HpaII* was attempted and the results are presented in Fig. 2(b). *MspI* digested I8 DNA more extensively than did *HpaII*, showing the presence of 5-methylation in the internal deoxycytosine of some of the 5'-CCGG-3' sequences (thus inhibiting *HpaII*). However, phage I3 DNA, grown on the same host, *M. smegmatis* SN2, did not have any methylation at this sequence (Reddy & Gopinathan, 1986a). These results demonstrated that the methylase responsible for I8 DNA methylation was a phage-influenced (coded) function, rather than a host-imposed property. The *MspI* and *HpaII* patterns of I1 and I5 DNAs were identical to I3 DNA patterns (data not shown), another property common to I1, I3 and I5 DNAs.

The restriction digests of I8 DNA were analysed on different concentration agarose gels to resolve all the fragments and the number of sites for various enzymes has been determined. The enzymes *KpnI*, *EcoRI*, *BglII*, *BamHI*, *SalI* and *PstI*, have two, seven, four, seven, nine and six sites respectively. *XbaI* and *SmaI* have no sites on this DNA, indicating either the absence of the corresponding recognition sequence or its methylation. The fragment sizes in various restriction digests of I8 DNA are presented in Table 1. By summation of the fragment sizes of individual enzymes (total of molar fragments plus the largest submolar fragment) the molecular size of I8 DNA was estimated to be a minimum of 43 kb. Not all the submolar fragments of the restriction enzymes were considered in estimating the size of the DNA, because they arise due to cyclic permutations (see below). Thus the genome of I8 is much smaller than that of I3 (135 kb), (Reddy & Gopinathan, 1986a). Further, unlike the DNAs of I3 (Reddy & Gopinathan, 1986b), I1 and I5, denaturation of I8 DNA did not result in fragmentation (data not shown), demonstrating the absence of single-strand interruptions in the latter.

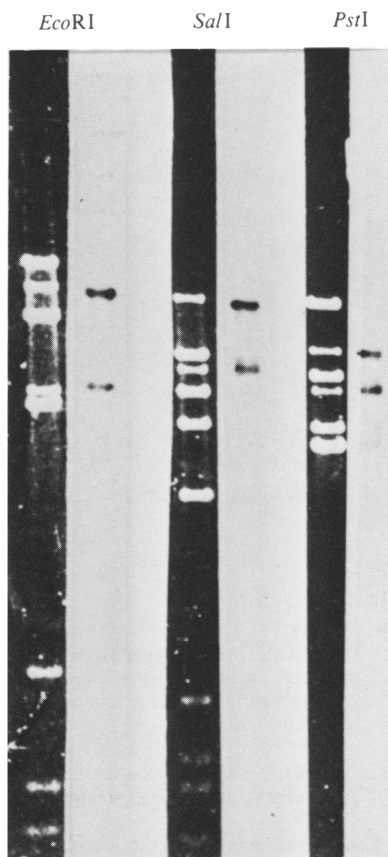


Fig. 5. Circularly permuted nature of I8 DNA. *EcoRI*, *SalI* and *PstI* digests of I8 DNA were subjected to electrophoresis on 0.7% agarose gel, transferred to nitrocellulose filters and hybridized with an I8 *SalI* fragment (11.2 kb submolar) ^{32}P -labelled *in vitro*. The ethidium bromide-stained gels (left) and autoradiograms (right) for each digest are aligned in parallel.

Several of the restriction digests of I8 DNA showed the presence of a few faint (submolar) bands (Fig. 2a). The presence of such faint bands suggests circularly permuted phage DNA. The submolar fragments having homologous sequences should be located at the ends of the DNA molecule, if they represent a limited number of cyclic permutations of the genome (Ramsay & Ritchie, 1980; Jackson *et al.*, 1978). When I8 DNA was 5' end-labelled, only the submolar bands received the label (data not shown) showing that these fragments were located at the ends of the molecule. The 11.2 kb fragment (submolar) of *SalI*-digested I8 DNA was purified from a preparative agarose gel, labelled *in vitro* by nick translation and used as the probe to hybridize to the *EcoRI*, *PstI* and *SalI* restriction fragments (Fig. 5). Apart from self-hybridization, the probe also hybridized with one other faint band (7.62 kb) in the *SalI* digest, as well as with the faint bands (end fragments) present in *EcoRI* and *PstI* digests of I8 DNA. These results showed that the end fragments of I8 DNA had homologous sequences (terminally redundant) and were present in submolar concentrations, a general feature of phage DNAs having a limited number of cyclic permutations.

The extent of submolarity of the faint bands provides a measure of the number of genomic phage DNAs that were derived from a single concatemer (Ramsay & Ritchie, 1980). By quantifying the intensities of the fragments of *SalI*, *PstI* and *BglII* digests of I8 DNA with a laser microdensitometer, it was found that two to three molecules of I8 DNA were formed from a concatemer. We believe that as in some other circularly permuted phage DNAs (Ramsay &

Table 1. Restriction digestion of phage I8 DNA

Fragment	Size (kb) of fragment			
	<i>Bgl</i> II	<i>Eco</i> RI	<i>Sa</i> I	<i>Pst</i> I
1	18.50	12.80	11.20*	11.30
2	8.40	11.30*	8.20	8.60*
3	7.62*	9.20	7.62*	7.62
4	6.10*	6.70*	7.00	7.20*
5	5.5†	6.20	6.00	6.00
6		2.80	3.30	5.60†
7		2.10*	2.60	1.60*
8		1.80*	2.25*	
9			2.10*	
10			1.80*	
Total	51.62	52.90	52.12	53.52

* Faint (submolar) band.

† Doublets.

Ritchie, 1980), phage I8 DNA packaging also proceeds by a headful mechanism initiated from a unique site on concatemers of limited length.

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REFERENCES

- CHANEY, S. M. J., WARREN, K. G., KETTYLS, J., ZBITNUE, A. & SUBAK-SHARPE, J. H. (1983). A comparative analysis of restriction enzyme digests of the DNA of herpes simplex virus isolated from genital and facial lesions. *Journal of General Virology* **64**, 357–371.
- COCHRAN, M. A. & FAULKNER, P. (1983). Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. *Journal of Virology* **45**, 961–970.
- DEAN, D. H., ORREGO, J. C., HUTCHISON, K. W. & HALVORSON, H. O. (1976). New temperate bacteriophage for *Bacillus subtilis*, ρ11. *Journal of Virology* **20**, 509–519.
- DRAPIER, J. C., QUETIER, F. & PETIT, J. F. (1978). Characterization of Mycobacteriophage ATCC 11759. Unusual physicochemical properties of its DNA. *European Journal of Biochemistry* **91**, 163–170.
- GOPE, M. L. & GOPINATHAN, K. P. (1982). Presence of lipids in mycobacteriophage I3. *Journal of General Virology* **59**, 131–138.
- JACKSON, E. N., JACKSON, D. A. & DEANS, R. J. (1978). *Eco*RI analysis of bacteriophage P22 DNA packaging. *Journal of Molecular Biology* **118**, 365–388.
- MANDEL, M. & MARMUR, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods in Enzymology* **12B**, 195–206.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory.
- MENEZES, J. & PAVILANIS, V. (1969). Properties of mycobacteriophage C2. *Experientia* **25**, 1112–1113.
- NAGARAJA, V. & GOPINATHAN, K. P. (1980). Requirement for calcium ions in mycobacteriophage I3 DNA injection and propagation. *Archives of Microbiology* **124**, 249–254.
- PARNES, J. R., VELAN, B., FELSENFELD, A., RAMANATHAN, L., FERRINI, U., APPELLA, E. & SEIDMAN, J. G. (1981). Mouse β2-microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. *Proceedings of the National Academy of Sciences, U.S.A.* **78**, 2253–2257.
- RAMSAY, N. & RITCHIE, D. A. (1980). A physical map of the permuted genome of bacteriophage T1. *Molecular and General Genetics* **179**, 669–675.
- REDDY, A. B. & GOPINATHAN, K. P. (1986a). Characterization of genomic DNA of mycobacteriophage I3. *Current Microbiology* (in press).
- REDDY, A. B. & GOPINATHAN, K. P. (1986b). Presence of random single-strand gaps in mycobacteriophage I3 DNA. *Gene* **44**, 227–234.
- RIGBY, P. W. J., DIECKMANN, M., RHODES, C. & BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237–251.
- ROBERTS, I. M., ROBINSON, D. J. & HARRISON, B. D. (1984). Serological relationships and genome homologies among geminiviruses. *Journal of General Virology* **65**, 1723–1730.
- SADHU, C., DUTTA, S. & GOPINATHAN, K. P. (1984). Influence of formamide on the thermal stability of DNA. *Journal of Biosciences* **6**, 817–821.

- SELLERS, I. & TOKUNAGA, T. (1970). Inactivation of mycobacteriophages by lipid solvents. In *Host-Virus Relationships in Mycobacterium, Nocardia and Actinomycetes*, pp. 134-143. Springfield: Charles C. Thomas.
- SOLOFF, B. L., RADO, T. A., HENRY, B. E. & BATES, J. H. (1978). Biochemical and morphological characterization of mycobacteriophage R1. *Journal of Virology* **25**, 253-262.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.
- SUNDERRAJ, C. V. & RAMAKRISHNAN, T. (1971). Genetic studies in mycobacteria: isolation of auxotrophs and mycobacteriophages for *Mycobacterium smegmatis* and their use in transduction. *Journal of the Indian Institute of Science* **53**, 126-140.
- YABUKI, S., FUKE, M. & WADA, A. (1971). The fine structures in melting curves of deoxyribonucleic acids of bacteriophage lambda. I. *Journal of Biochemistry* **69**, 191-207.
- YEN, W. S. & BLAKE, R. D. (1980). Analysis of high-resolution melting (thermal dispersion) of DNA. *Methods. Biopolymers* **19**, 681-700.

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