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Maturation of bacteriophage 9NA DNA is influenced by the fatty acid composition of the host cell membrane

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Abstract. The fatty acid composition of the membrane of the conditional auxotroph fabB2 can be altered by allowing the cells to grow at non-permissive temperature (37°C) in the presence of a *cis*-unsaturated fatty acid. The phage 9NA, a virulent phage of *Salmonella typhimurium,* can not multiply in fabB2. Synthesis and maturation of the phage DNA are differentially affected by variation in the fatty acid composition of the cell membrane. The replicating DNA associates with the membrane complex, the site of DNA synthesis. The association is comparatively weak in oleic, claidic, palmitoleic, palmitelaidic and linolelaidic acid enriched cells. When the cells are grown in the presence of palmitoleic acid, a large pool of concatemeric phage DNA accumulates in the cytoplasm within 10 min of infection. The conversion of concatemeric DNA to monomeric one i.e., mature phage length DNA, is inhibited in such cells. The presence of concatemeric DNA can be visualized by electron microscope. Such a situation is not observed when the cells are grown in media supplemented with other types of unsaturated fatty acids. The mechanism by which the host cell membrane lipid controls phage development is yet to be worked out.

Keywords. DNA maturation; bacteriophage 9NA; fatty acid auxotroph.

1. Introduction

Bacteriophage 9NA is a virulent phage of *Salmonella typhimurium* (Wilkinson *et al* 1972). Like phage MB78 (Joshi *et al* 1982) it grows on smooth strain of *S. typhimurium.* Unpublished experiments carried out in our laboratory indicated that the phage contains 54 kb long linear, circularly permuted, terminally redundant DNA (unpublished results). Upon infection it does not degrade the host DNA. Phage DNA synthesis starts in association with the host cell membrane. The rate of phage DNA synthesis is maximum within 30 to 40 min after infection. The infected cells start lysing after 50 min. Within 5 min of infection at least seven early phage proteins are expressed. Expression of most of the structural proteins could be seen from 15 min onwards. It has been earlier reported from our laboratory that the fatty acid biosynthetic mutant, fabB2, is a nonpermissive host for the virulent bacteriophage 9NA (Goyal and Chakravorty 1989). Expression of phage

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genes is differentially affected when the host is grown in the presence of different fatty acids. The rate of DNA synthesis was also found to vary quite significantly with variation of fatty acid in the growth media. From the kinetics of DNA synthesis (Goyal and Chakravorty 1989) it was not possible to infer whether there was synthesis of concatemeric DNA, which is a substrate for packaging of the phage DNA into phage head in a headful mechanism resulting in circularly permuted DNA. From the sedimentation pattern the concatemeric DNA can be distinguished from the monomeric one. Hence, the DNA synthesized following infection of the cells grown under different fatty acid supplementation was subjected to sucrose density gradient centrifugation. The present report indicates that the process of maturation of the replicating phage DNA is influenced by the fatty acid composition of the host cell membrane. Further, the observation that the conversion of concatemeric DNA to monomeric one is drastically affected when the host cell membrane is enriched with palmitoleic acid is noteworthy.

2. Materials and methods

2.1 *Reagents*

[^{3}H]thymidine was obtained from Bhabha Atomic Research Centre, Bombay. Fatty acids used for supplementation of media were procured from Serdary Research Laboratories, London, Ontario, Canada. The Spurr reagents such as epoxy resin DER 736, "2-nonen-lyl-l-succinic anhydride (NSA), 2-dimethyl aminoethanol (DMAE), and 4-vinylcyclohexene dioxide (VCD) were obtained from Fluka, Switzerland. Glutaraldehyde was procured from BDH, Poole, England. All other chemicals used were of analytical grade.

2.2 *Bacteria, phage strain and growth media*

S. typhimurium (LT2 strain 18) was originally obtained from Prof. Myron Levine of the Department of Human Genetics, University of Michigan, Ann Arbor, USA. The fatty acid biosynthetic mutant fabB2 was obtained from Prof. Bruce Ames, University of California, Berkeley, USA. The bacteriophage 9NA was obtained from Prof. B A D Stocker of the Lister Institute of Preventive Medicine, London. FabB2 and LT2 cells were grown in minimal medium, composition of which was as follows: one litre of minimal medium (MM) contained K_2HPO_4, 10·5 g; KH_2PO_4, 4·5 g; $(NH_4)_2SO_4$, 1·0 g; sodium citrate $3H_2O$, 0·47 g and MgSO47H2O, 0.1 g. Glycerol or glucose was added at a final concentration of 0·2% as carbon source.

2.3 *Lysis mixture*

Lysis mixture (10 ml) contained 1 ml of 1 M Tris-Cl$^-$, pH 8·0; 2 ml of 0·5 M EDTA, pH 8·5; 0·1 ml of 1 M NaCN; and 0·1 to 0·2ml of freshly prepared lysozyme (10 mg per ml in 0·25 M Tris-Cl$^-$, pH 8·0).

2.4 *Labelling of intracellular DNA*

For pulse labelling of intracellular DNA fabB2 cells ($2{\cdot}6 \times 10^8$ cells per ml) growing exponentially in minimal medium at 30°C without any fatty acid or at 37°C with requisite fatty acid as supplementation were infected with phage 9NA at an m.o.i. of 10. After 10 min [^{3}H]thymidine (20 μCi per ml; sp. activity 18·4 Ci per mol) was added to the cell suspension. Non radioactive (cold) thymidine was added after 2 min to a final concentration of 2 mg per ml for chasing. Samples (0·5 ml) were removed at desired intervals and incorporation was stopped by adding an equal volume of lysis mixture. The lysate was handled extremely gently. Subsequent steps were as described by Botstein (1968). DNA present in these lysates was stable at room temperature for more than a week and there was no loss of trichloroacetic acid precipitable counts.

2.5 *Sucrose density gradient analysis of DNA*

Labelled phage DNAs were analysed from their sedimentation pattern in either neutral or alkaline, 5 to 20% (w/v) sucrose gradients using a Beckman SW 50·1 rotor at 84,000 *g* for 120 min at 25°C as described by Chowdhury and Das (1986). Mature phage length DNA of 9NA was used as marker during sedimentation.

2.6 *Electron microscopy*

For preparation of blocks for electron microscopy exponentially growing cells were infected with phage 9NA at an m.o.i. of 10. At various time intervals 10 ml culture was centrifuged at 6K for 5 min at room temperature in Sorval SS34 rotor. The cells were then fixed in 6% glutaraldehyde in sodium phosphate buffer (0·12M, pH 7·0) at 4°C in dark for 4 h. This was followed by washing of the cells twice with the buffer. The cells were finally resuspended in 1% osmium tetroxide and left overnight in a fume chamber. Next day the black cell pellet was washed with sodium phosphate buffer to remove osmium tetroxide. The cell pellet was then sandwiched in 1·5 to 2% agar. The packed cell pellet was cut into cubes and then stained with uranyl acetate for 2 h. After washing the cubes with 50% ethanol, those were dehydrated with increasing concentration of ethanol and subsequently embedded in Spurr's reagent (Spurr 1969). Thin sections were cut by an LKB (NOVA) ultra- microtome with a Dupont diamond knife. Electron microscopy was carried out in the Institute of Chemical Biology, Calcutta, using Jeol 100 cx transmission electron microscope at 65KV.

3. Results

3.1 *Sedimentation pattern of the DNA replicating in the permissive host LT2*

The formation of concatemeric DNA being a prerequisite for the production of circularly permuted DNA through headful packaging, the site and mode of DNA replication in the permissive host LT2 were first followed. LT2 cells were infected

with phage 9NA at an m.o.i. of 10 and the newly synthesised DNA (10 min after infection) was analysed from its sedimentation pattern in neutral as well as alkaline sucrose gradient. The sedimentation pattern in neutral sucrose gradient (figure la) suggested that the newly synthesised DNA is mostly associated with a fast sedimenting material, a membrane complex (marked as I). Negligible amount of free concatemeric DNA (marked as II) and some mature phage length DNA as shown by arrow (↓) were also detected (figure 1a). After another 17 min the amount of labeled DNA in the membrane complex decreased with a simultaneous increase in the mature phage length DNA (figure lb). DNA from the fast sedimenting complex could be completely chased to the fraction where mature phage length DNA

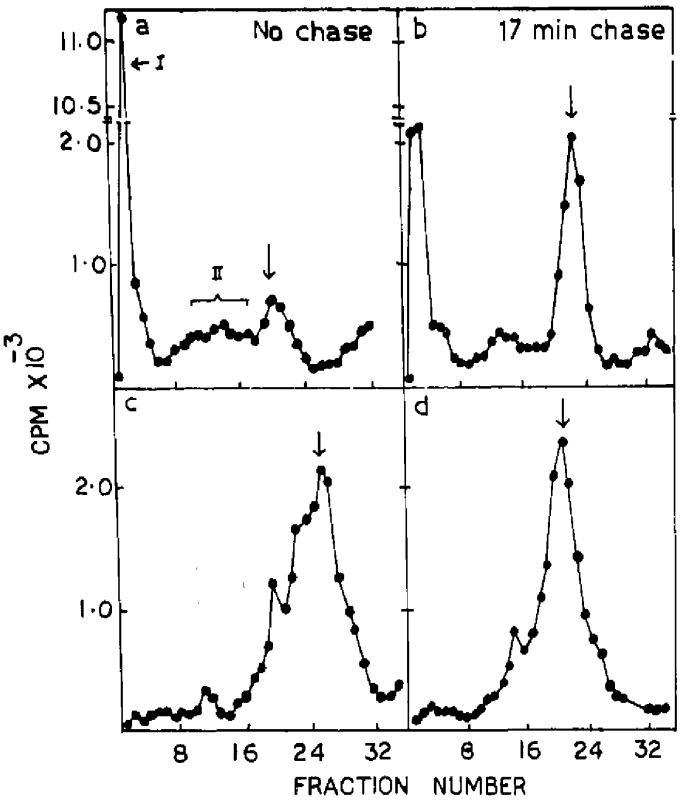

Figure 1. Sucrose density gradient analysis of the phage DNA replicating in permissive hosts.

Exponentially growing LT2 cells were infected with phage 9NA (m.o.i. = 10). The Cells were pulse labelled (10 min after infection) with [^{3}H]thymidine (20 μCi/ml); sp. Activity 184 Ci/m mol) for 2 min. Unlabelled thymidine (2 mg/ml) was added to chase the radioactivity. Samples (0·5 ml) were collected and the reactions were stopped by adding 0·5 ml of lysis mixture. The method of lysis and rest of the experimental protocol were as described by Bolstein (1968). Samples (0·1 ml) were loaded on the top of the sucrose gradients; either neutral (a and b) or alkaline (c and d) and centrifuged for 120 min at 84,000 *g* at 25°C in SW 50·1 rotor of L8M model of Beckman ultracentrifuge. The arrow indicates the position of ^{32}P-labelled 9NA DNA, used as a marker. The sedimentation is from right to left.

sediments (data not presented). That such fast sedimenting fraction is a DNA membrane complex has been confirmed (data not presented) by treating the fraction with proteinase K and running the sample in neutral sucrose density gradient as demonstrated for the bacteriophage MB78 (Khan *et al* 1991).

When the same samples were run on alkaline sucrose density gradient, an interesting change in the sedimentation pattern was observed. No radioactivity was found to be associated with the fast sedimenting fraction (figure 1c and d). Labelled DNA (both phage length and concatemeric) was released from the membrane complex (figure lc). This suggested that the concatemers were also mostly membrane bound and the DNA-membrane complex was alkali labile. After 17 min of chase i.e., 30 min after infection most of the concatemeric DNA was converted to monomer (figure 1d). At this time monomeric DNA could be detected in neutral sucrose gradient also (figure lb) .

3.2 *Sedimentation pattern of the phage DNA replicating in the non permissive host fabB*2 *at 30°C*

When a similar experiment was carried out with the nonpermissive host fabB2 growing at 30°C it was observed that the amount of DNA synthesised was comparatively small (compare figures la and 2a). The newly synthesised DNA was mostly associated with the fast sedimenting fraction (figure 2a) and not much of mature phage length DNA or concatemeric DNA was observed in the gradient (neutral). The fast sedimenting complex also contained some concatemeric DNA. When the samples were chased for a long time (60 min) some concatemeric DNA was released but only a small amount of it was converted to mature phage length DNA (figure 2b). Majority of the newly synthesised DNA, however, remained associated with the membrane although a small amount of concatemeric DNA was also visible in the gradient.

3.3 *Effect of fatty acid supplementation on maturation of phage DNA*

As the main objective was to investigate the effect of fatty acid composition of the cell membrane on the maturation of the phage DNA, fabB2 cells were grown at 37°C in the presence of different fatty acids and the state of the replicating phage DNA in such cells was analysed by neutral sucrose density gradient centrifugation. In oleic acid grown cells the pattern of sedimentation of DNA was somewhat different. The pulse labelled DNA was mostly membrane-bound (figure 2c) as in case of unsuplemented fabB2 (figure 2a). However, remarkable change was noticed when the fate of this DNA was followed. With time the amount of phage length DNA increased considerably and lot of concatemeric DNA was released from the membrane complex (compare figure 2c and d). Release of the replicating DNA from the membrane complex was in sharp contrast to the situation observed in the permissive host LT2 (figure lb) and also fabB2 without supplementation (figure 2b). It seemed that the association of the replicating DNA with the membrane complex was not so stable and consequently the DNA got dissociated from the membrane complex. Although analysis of a number of samples at different times after infection was carried out in every case the results of only one time point have been presented.

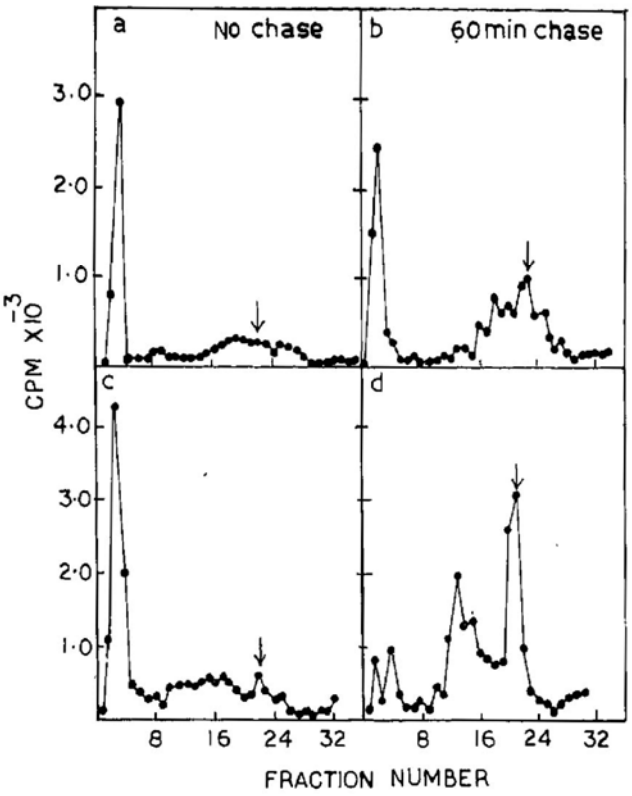

Figure 2. Sucrose density gradient analysis of the phage 9NA DNA replicating in the nonpermissive host fabB2 at 30°C without any supplementation or at 37°C in the presence of oleate.

Cells (fabB2) were grown at 30°C without any supplementation (a and b) or at 37°C in the presence of oleic acid (c and d). Infection of the cells and the rest of the experimental procedures were as described under figure 1. The gradients were of neutral sucrose, (b) and (d) represent samples after 60 min chase.

The pattern of sedimentation of the replicating DNA in cells grown in presence of elaidic acid, palraitelaidic and linolelaidic acid were not much different from that observed in case of oleic acid supplemented cells. In all the cases the rate of DNA synthesis was also much less as compared with that of productive infection (data not presented).

The cells grown in presence of linoleic acid exhibited exactly the same pattern of sedimentation of the phage DNA as seen in case of the permissive host LT2 (figure 3a and b). Not only the rate of DNA synthesis increased but also the association of the newly synthesised DNA with the membrane complex was quite stable and not much of concatemeric DNA was detected in the gradient. Considerable amount of mature phage length DNA could be detected after 60 min of chase suggesting thereby that conversion of concatemeric DNA to monomeric one was not affected. It is rather surprising that although considerable amount of newly syntheisised DNA was converted to monomer no phage particle was produced (data not presented).

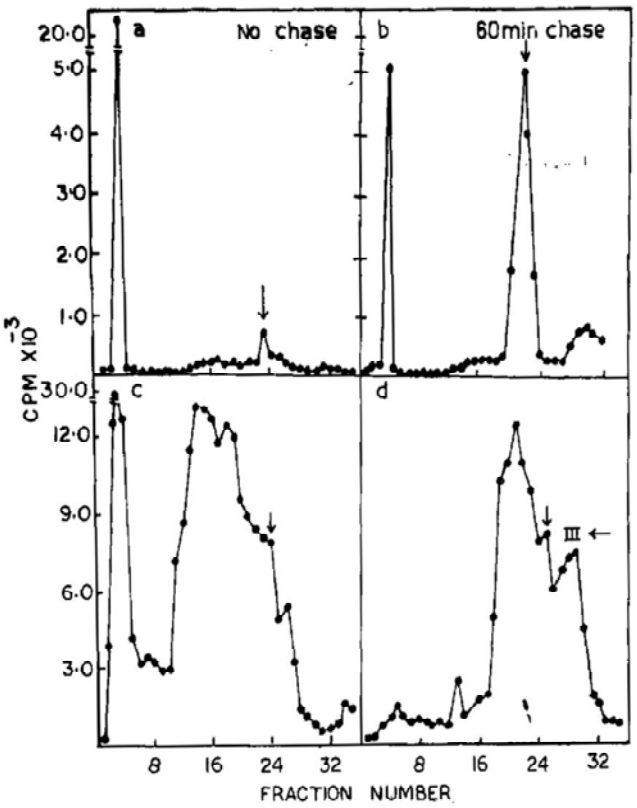

Figure 3. Sucrose density gradient analysis of the phage DNA replicating in fabB2 cells growing in the presence of linoleic acid and palmitoleic acid.

Cells (fabB2) growing exponentially at 37°C in the presence of linoleic acid (a and b) or palmitoleic acid (c and d) were used for experiments. Rest of the conditions were as described under figure 1 with the exception that samples after 60 min of chase have been analysed. The fraction marked as III in (d) represents fragmented DNA.

Effect of supplementation with palmitoleic acid on the maturation of phage DNA was more interesting. Although replicating DNA was found to be associated with the cell membrane a large pool of concatemeric DNA was detected in the gradient (figure 3c). With time concatemeric DNA was released from the membrane complex but there was very little conversion of the concatemeric DNA to monomeric one. Moreover, some DNA seemed to be fragmented as indicated by a slower moving fraction marked as III.

3.4 *Visualization of concatemeric phage DNA through electron microscope*

As large amount of free (not membrane bound) concatemeric DNA was detected in the lysate of palmitoleic acid grown cells (figure 3c and d) ultra thin sections of such infected cells were examined by electron microscope. Concatemeric DNA (threaded structure) was indeed visible in the cytoplasm (figure 4a). A large number of samples collected at different times after infection were examined. No such

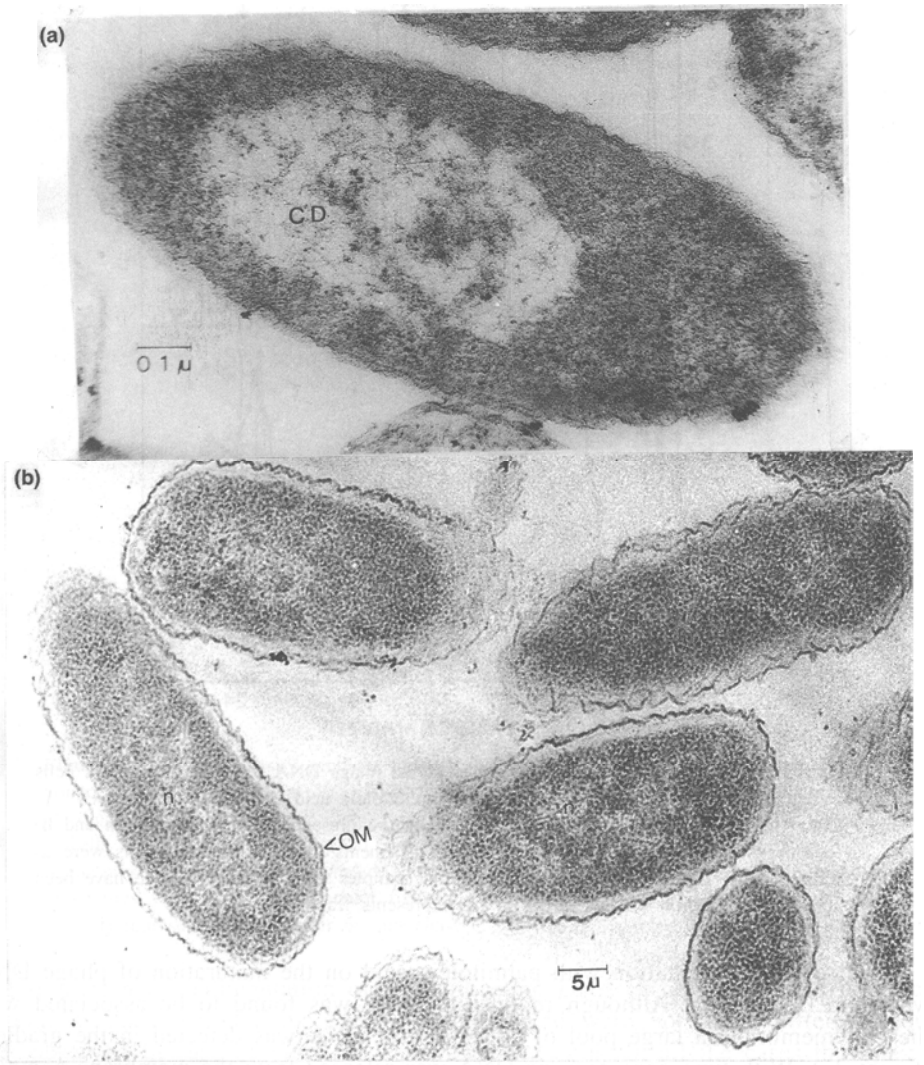

Figure 4. Electron micrograph of a thin section of fabB2 cells grown in the presence of palmitoleate and infected with phage 9NA.

Cells were grown in the presence of palmitoleate at 37°C as described in figure 3. Infection was carried out at an m.o.i. of 10. Cells taken after 10 min of infection (as in figure 3c) were processed for thin section electron microscopy. Concatemeric DNA (thread like structures) has been marked as CD could be seen in infected cells (a). A batch of unsupplemented cells (but phage infected) were similarly processed, (b) No thread like structure could be seen in such cells. OM, Outer membrane; n, nucleoid structure.

structure was seen in phage infected LT2 and fabB2 cells unsupplemented or supplemented with fatty acid other than palmitoleic acid (figure 4b). Similar DNA network has been observed in thin-section electron micrographs of *Vibrio choleras* infected with phage φ 149 (Majumdar *et al* 1984).

4. Discussion

Although the replicon hypothesis (Jacob *et al* 1963) proposes an integral role for the cellular membrane in DNA replication there is hardly any report which deals with the effect of membrane fatty acids on bacteriophage development except that of Labedan (1984) who reported that elaidic acid enriched cell membrane being rigid prevents the injection of T5 DNA into the host cell. The fact that the fatty acid biosynthetic auxotroph fabB2 is a nonpermissive host for the virulent phage 9NA and not for the temperate phage P22 makes it obvious that this strain lacks some functions(s) specific for the development of phage 9NA and not for the temperate phage P22. The latter can grow in fabB2 and supplementation of the medium with any fatty acid does not affect its development (unpublished observations). Earlier it was reported from our laboratory (Goyal and Chakravorty 1989) that although infection, of fabB2 with 9NA is nonproductive, different phage functions are affected to different extent depending on the fatty acid composition of the membrane. Results presented in this paper indicate that maturation of phage 9NA DNA can be influenced by the fatty acid composition of the cell membrane. Further, the association of the replicating DNA with the membrane complex is also influenced by the fatty acid composition of the membrane. The sedimentation of the newly synthesised DNA in the permissive host LT2 follows the same pattern as reported for other *Salmonella* phages (Botstein 1968; Khan *et al* 1991). The effect is remarkable when the cells are grown in presence of palmitoleic acid. The concatemeric DNA does not stay associated with the cell membrane as seen in the normal host LT2. Further, the conversion of concatemeric DNA to monomeric one is blocked, It is only in oleic acid supplemented cells noninfectious viral particles with straight tails and no tail fibre could be detected although the cells do not lyse and continue to grow (Goyal and Chakravorty 1989). Palmitoleate grown cells also become filamentous as a result of phage infection (Goyal and Chakravorty 1989) but no phage particles could be detected in such cells (unpublished observation). All our attempts to isolate mutants that are able to produce phage in this mutant have failed. Thus it became difficult to identify the involvement of any particular phage protein. Moreover, supplementation with different fatty acids resulting in alteration of the membrane fluidity to a different extent (Deb *et al* 1986) affect phage gene expression at different levels (Goyal and Chakravorty 1989) suggesting that it will be difficult to identify any specific process that is inhibited. It has been recently reported by Crooke *et al* (1992) that membrane phospholipid and membrane fluidity plays a great role in rejuvenation of DNA protein which is involved in DNA synthesis. Our observations provide further support to the role of membrane lipid in DNA synthesis and maturation. However, mode of action is yet to be worked out.

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