Heat-stable and Density Mutants of Phages T1, T3 and T7

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SUMMARY

The coliphages T1, T3 and T7 rapidly lost infectivity when incubated at 60° in saline-citrate medium. From the survivors of heat-inactivated wild-type phage stocks heat-stable (st) mutants were isolated, which all had a reduced buoyant density. The st mutant phages of T3 and T7 contained 3 to 5% less DNA than their wild-type parent, while no loss of DNA could be detected for T1 st mutants. This correlation between heat stability and reduced density was confirmed by showing that a mutant of T3 selected for its lighter density had also mutated to heat-stability.

INTRODUCTION

Wild-type phage T5 loses infectivity on incubation at elevated temperatures in saline-citrate medium (Adams & Lark, 1950; Lark & Adams, 1953). From the surviving phage, mutants have been isolated which are heat-stable (Adams & Lark, 1950; Hertel, Marchi & Müller, 1962; Rubenstein, 1968). These heat-stable mutants (called st mutants) are less dense than wild-type phage (Lark, 1962; Hertel et al. 1962; Rubenstein, 1968). Rubenstein (1968) showed that the reduced density is associated with a reduced DNA content of the st phage particle; the most heat-stable mutants are the lightest and have lost most DNA. This interesting correlation between mutation to heat stability and reduced density and DNA content in T5 suggested to us that the simple process of selecting heat-stable mutants might be a general method, applicable to a variety of phages, for isolating variants with altered DNA contents. Such a method allowing the isolation of viable phages with smaller DNA molecules would permit studies of the size, distribution and nature of the apparently non-essential parts of the genome of this group of phages. For these reasons we have investigated whether the ability to sport heat-stable mutants is a general property of several phages, and whether such mutants also consequently have decreased densities and smaller DNA molecules. If heat-stability results from the deletion of part of the DNA molecule, the selection of heat-stable mutants should also select for particles with a reduced density and less DNA.

In this paper we present results obtained with the coliphages T1, T3 and T7. These phages were chosen because their DNA molecules, like those of T5, have non-permuted base sequences (Olligs, 1967; Thomas & MacHattie, 1967). It has been suggested that the maturation of phage DNA molecules in some cases involves the excision of phage-equivalent lengths of DNA from larger DNA structures known as concatemers (Streisinger, Emrich & Stahl, 1967; Thomas, Kelly & Rhoades, 1968). For phages with non-permuted DNA molecules the unit lengths of DNA would be expected to be cut from concatemers by a mechanism which recognizes specific terminal nucleotide sequences (Thomas et al. 1968). If this is so, the deletion of DNA which does not include those specific sequences would lead to the production of phage particles with less DNA and a lower density than their wild-type.
Phage and bacteria. The wild-types of phages T1, T3 and T7 and their mutants were grown and assayed on *Escherichia coli* strain B.

Isolation of heat-stable mutants. The experimental technique adopted was to expose wild-type phage populations to successive heat inactivation cycles. Following each heat inactivation the surviving phage population was grown to give a further high titre stock. Preferential heat inactivation of the parent phage would progressively enrich the proportion of any heat-stable mutants among the survivors.

For each inactivation cycle the phage suspension (about \(5 \times 10^9\) p.f.u./ml.) was diluted 100-fold into 2 \(\times \) SSC (0.3 M-NaCl, 0.03 M-sodium citrate) and incubated at 60° to reduce the infectivity to about 1% of the initial titre. One ml. of the inactivated phage suspension was added to 5 ml. culture of *Escherichia coli* B (\(5 \times 10^8\) cells/ml.) growing at 37° in H-broth (Krieg, 1959) and the culture incubated until lysis was complete. This process was repeated 8 to 10 times. From the last lysates of each phage type, 20 to 50 plaques were isolated and diluted to a titre of \(10^8\) p.f.u./ml. in 2 \(\times \) SSC; samples were removed before and after incubation at 60° for 1 hr. Part of each sample was assayed for infectivity. A plaque stock showing an enhanced heat stability was given an *st* symbol and plaque isolation number and used to prepare a high-titre phage lysate.

Growth and purification of radioactive phages. Cultures of *Escherichia coli* B were grown with aeration at 37° in synthetic TCG medium (Kozinski & Szybalski, 1959). At a density of \(10^8\) cells/ml. the medium was supplemented with either \([3H]\)thymidine (10 \(\mu\)c/ml.) or \([32P]\)orthophosphate (4 \(\mu\)c/ml.) and further incubated to a titre of \(3 \times 10^8\) cells/ml. Phage was then added to an m.o.i. of 2 p.f.u./cell for T1 or \(0.25\) p.f.u./cell for T3 and T7, and the infected cultures incubated for one hr at which time lysis usually occurred spontaneously. Routinely a few drops of chloroform were then added and air was bubbled through the culture until lysis was complete. The phage particles were immediately purified and concentrated by two cycles of differential centrifugation with low speed runs at 7,000 \(g\) for 10 min. and high speed runs at 50,000 \(g\) for 30 min. Phage pellets were resuspended in phosphate buffer pH 7 (Hershey & Chase, 1952) by gentle mechanical shaking, and the suspensions stored at 4°.

Assay methods. Phage infectivity was assayed on nutrient agar plates by the top-layer method (Adams, 1959). Radioactivity was counted in a Nuclear Chicago scintillation counter. Liquid samples were dried on 2.5 cm. squares of Whatman GF83 glass fibre paper and each square suspended in 10 ml. liquid scintillant (4 g. 2,5-diphenyloxazole and 0.05 g. 1,4-bis[2-(5-phenyloxazolyl)]benzene/l. of toluene). Fractions from both caesium chloride and sucrose gradients were usually collected directly on glass fibre paper.

Extraction of DNA molecules. Unbroken DNA molecules were extracted by rolling equal volumes of purified phage suspension and freshly distilled water-saturated phenol on a test-tube roller with the tubes rotating about their long axis at 75 rev./min. The details of this method were described by MacHattie et al. (1967). The extracted DNA was dialysed 4 to 5 times against a large excess of 0.05 m-NaCl, 0.01 m-tris, pH 8.0 to remove residual phenol and stored at 4°.

Density gradient centrifugation. Anhydrous CsCl was dissolved in H-broth at a concentration of 45.1% (w/v) and a density of about 1.5 g./ml. Three ml. volumes of CsCl containing 0.1 to 0.2 ml. of phage suspension were overlaid with paraffin oil and centrifuged in the SW39 rotor of a Spinco Model L ultracentrifuge. After centrifugation at 25,000 rev./min. for 20 hr at 20° the gradients were unloaded by collecting fractions dropwise from the bottom of the centrifuge tube through a No. 18 steel syringe needle held in a rubber stopper.
Heat-stable and density mutants of phage

Refractive indices of undiluted gradient fractions were read at 25° immediately after collection. Usually the gradients showed a linear variation in refractive index. Densities of phage particles were calculated from the linear refractive index curves using the empirical relationship of Thomas & Berns (1961). Because of the relatively small density differences between wild-type and st mutant-phage particles, the buoyant densities of st phage were always determined relative to their wild-type in density gradients containing both phage types. For this calculation the centre of a unimodal band of radioactivity in a density gradient was determined by a method first described by Rubenstein (1968). Peak fractions are selected to obtain a symmetrical distribution of label and to include about 80% of the peak material. The band centre is calculated by summing the products of the counts in each fraction and the distance of that fraction from one end of the gradient and dividing this product by the total number of counts in the selected fractions. For a given peak of label the band centre is the same regardless of whether the distance is measured from the top or the bottom of the gradient. This method was used by Rubenstein to calculate the average distance that DNA molecules sediment in a sucrose gradient, but we have found that it is also suitable for equilibrium density gradients.

Sucrose gradient centrifugation. The sedimentation of native DNA molecules was conducted according to the methods of Burgi & Hershey (1963). Differentially labelled mixtures of wild-type or wild-type and st mutant DNA were layered on 5 m. linear gradients of 5 to 20% (w/v) sucrose dissolved in 0·1 M-NaCl 0·05 M-phosphate, pH 6·8. The gradients were centrifuged at 20° in the SW 65 rotor of a Spinco Model L ultracentrifuge. After centrifugation the gradients were unloaded by the method described above for density gradients. The average distance that a band of DNA had sedimented was again calculated by the method of Rubenstein (1968). From several independent determinations on a given pair of DNAs, the average distance that DNA-2 had sedimented relative to DNA-1 (D2/D1) was used to calculate the relative molecular weights (M2/M1) from the relationship: D2/D1 = (M2/M1)0·35 (Burgi & Hershey, 1963). The molecular weights of the wild-type DNAs were taken from those listed by Thomas & MacHattie (1967) and used to calculate the molecular weights of the st mutant DNA molecules.

RESULTS

The isolation of heat-stable mutants

The wild-types of phages T1, T3 and T7 were inactivated when heated at 60° in 2 × SSC (Fig. 1). After exposure of these wild-type phages to a series of 8 to 10 successive heat inactivation cycles, 30 to 50% of the phages isolated from the final lysates of each phage type showed an increased heat-stability. From the heat inactivation curves of several heat-stable (st) mutants derived from each parent phage, the greatest difference in inactivation rates was between T3+ and its st mutants (Fig. 1). T1st mutants showed the least relative change in heat-stability.

Density gradient analysis of the heat-stable mutants

The wild-type phages were routinely labelled with 3H or 32P and the st mutants with 32P. The 3H and 32P-labelled phages were then centrifuged in pairs in the same density gradient. When the phages were identical, except for the label, they banded at identical positions in a density gradient (Figs. 2a, 3a, 4a). However, whenever the pair of phages consisted of the wild-type and one of the several st mutants isolated, the st mutant invariably banded at a lower density (Fig. 2b to 4b). The st mutants of T3 showed the largest density reduction while T1st mutants were only marginally less dense than T1+ (Table 1).
Sucrose gradient centrifugation of wild-type and st mutant DNA molecules

The similar behaviour of T1, T3 and T7 to that of T5 regarding mutation of heat-stability and reduced density led us to look for differences in the molecular weights of wild-type and st mutant DNA molecules. The sedimentation behaviour of wild-type and st mutant DNA molecules.

Fig. 1. The kinetics of heat inactivation of the wild-type and some st mutants of phages T1, T3 and T7. Phage suspensions were incubated at 60° in 2 x SSC and at intervals samples were assayed for infectivity.

Fig. 2. The CsCl density gradient distributions of T1+ and T1st mutant phage particles. (a) Mixture of [3H]T1+ and [32P]T1+. 105, two-drop fractions were collected. (b) Mixture of [3H]T1+ and [32P]-T1st12; 110, two-drop fractions were collected. The bottom of the gradient is to the left. --- ---, 3H label; ----, 32P label.
Heat-stable and density mutants of phage was examined by centrifuging differentially labelled DNA mixtures in the same sucrose gradient (5 to 20% (w/v) sucrose pH 6.8). When intact molecules isolated from the wild-type and st mutants of phages T3 and T7 were sedimented through sucrose gradients, the st mutant DNA always sedimented more slowly than the corresponding wild-type DNA (Table 2). Control mixtures of wild-type DNA differing only in their label sedimented at

Fig. 3. The CsCl density gradient distributions of T3+ and T3st mutant phage particles. (a) Mixture of [3H]T3+ and [32P]T3+; 73, three-drop fractions were collected. (b) Mixture of [3H]T3 and [32P]T3st16; 67, three-drop fractions were collected. The bottom of the gradient is on the left. ———, [H] label; ——— [32P] label.

Fig. 4. The CsCl density gradient distributions of T7+ and T7st mutant phage particles. (a) Mixture of [3H]T7+ and [32P]T7+; 75, three-drop fractions were collected. (b) Mixture of [3H]T7 and [32P]T7st15; 70, three-drop fractions were collected. The bottom of the gradient is on the left. ———, [H] label; ———, [32P] label.
identical rates (Table 2). The slower sedimentation of T3 and T7 st DNA indicated that these DNA molecules had smaller molecular weights (Burgi & Hershey, 1963). This conclusion only assumes that mutation to heat-stability did not alter the configuration of the st DNA molecule. The calculations of molecular weights indicated that for T3 st mutants there was an apparent deletion of about 5% of the DNA molecule, while for T7 st mutants the amount of DNA lost was about 3% (Table 2).

The sedimentation profiles of T1 + and T1 st DNA molecules overlapped almost completely showing no detectable difference in sedimentation rate.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Density in CsCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 +</td>
<td>1.494 ± 0.001 (9)</td>
</tr>
<tr>
<td>T1 st12</td>
<td>1.493 ± 0.001 (3)</td>
</tr>
<tr>
<td>T1 st31</td>
<td>1.493 ± 0.000 (3)</td>
</tr>
<tr>
<td>T3 +</td>
<td>1.502 ± 0.001 (5)</td>
</tr>
<tr>
<td>T3 st11</td>
<td>1.496 ± 0.001 (2)</td>
</tr>
<tr>
<td>T3 st16</td>
<td>1.496 (1)</td>
</tr>
<tr>
<td>T3 st18</td>
<td>1.495 ± 0.002 (2)</td>
</tr>
<tr>
<td>T7 +</td>
<td>1.500 ± 0.001 (3)</td>
</tr>
<tr>
<td>T7 st15</td>
<td>1.497 (1)</td>
</tr>
<tr>
<td>T7 st18</td>
<td>1.497 (1)</td>
</tr>
<tr>
<td>T7 st26</td>
<td>1.497 (1)</td>
</tr>
</tbody>
</table>

Each value is the average of the number of independent measurements given in parentheses.

**Table 2. Relative sedimentation rates and molecular weights of wild-type and st mutant DNA molecules**

<table>
<thead>
<tr>
<th>DNA-1*</th>
<th>DNA-2*</th>
<th>D2/D1 †</th>
<th>M2/M1</th>
<th>M1$\times 10^{-6}$</th>
<th>M2$\times 10^{-6}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 +</td>
<td>T1 +</td>
<td>1.0001 ± 0.0001 (4)‡</td>
<td>1.000</td>
<td>31.2</td>
<td>31.2</td>
</tr>
<tr>
<td>T1 +</td>
<td>T1 st12</td>
<td>1.0002 ± 0.0002 (4)</td>
<td>1.000</td>
<td>31.2</td>
<td></td>
</tr>
<tr>
<td>T1 +</td>
<td>T1 st19</td>
<td>1.0011 ± 0.0071 (2)</td>
<td>1.003</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>T1 +</td>
<td>T1 st31</td>
<td>1.0006 ± 0.0006 (3)</td>
<td>1.002</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>T3 +</td>
<td>T3 +</td>
<td>0.9999 ± 0.0002 (3)</td>
<td>0.999</td>
<td>23.3</td>
<td>23.3</td>
</tr>
<tr>
<td>T3 +</td>
<td>T3 st11</td>
<td>0.9979 ± 0.0003 (3)</td>
<td>0.944</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td>T3 +</td>
<td>T3 st16</td>
<td>0.9810 ± 0.0010 (4)</td>
<td>0.945</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td>T3 +</td>
<td>T3 st17</td>
<td>0.9856 ± 0.0013 (4)</td>
<td>0.959</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>T3 +</td>
<td>T3 st18</td>
<td>0.9827 ± 0.0020 (4)</td>
<td>0.951</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>T7 +</td>
<td>T7 +</td>
<td>1.0001 ± 0.0002 (4)</td>
<td>1.000</td>
<td>24.7</td>
<td>24.7</td>
</tr>
<tr>
<td>T7 +</td>
<td>T7 st15</td>
<td>0.9889 ± 0.0005 (3)</td>
<td>0.969</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>T7 +</td>
<td>T7 st18</td>
<td>0.9876 ± 0.0003 (3)</td>
<td>0.958</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td>T7 +</td>
<td>T7 st26</td>
<td>0.9903 ± 0.0013 (3)</td>
<td>0.973</td>
<td>24.0</td>
<td></td>
</tr>
</tbody>
</table>

* DNA types sedimented as a mixture through a particular sucrose gradient.
† Average of the ratios of the distances that DNA-2 had sedimented relative to DNA-1.
‡ Each average based on the number of independent determinations given in parentheses.
§ Mol. wt. of DNA-1.
|| Mol. wt. of DNA-2.

The non-revertibility of T3 and T7 st mutants

Stocks of T3 st16 and T7 st15 were each centrifuged in a density gradient and the fractions from the heavy side of the band were pooled and the phages regrown. (The selected heavy fractions included the first fraction on the heavy side of the peak down to the fraction containing about 1% of the peak infectivity.) This process was repeated six times. The final phage stocks were labelled with $^{32}$P, mixed with their corresponding [H]tritiated parent st mutant and centrifuged in a density gradient to compare their densities. There was no
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difference in either case. Our inability to isolate phage particles with a greater buoyant density following extensive selection from the heavy side of density gradient bands of T3 and T7st mutants suggested that full or partial reversion did not readily occur.

Direct selection of density mutants

Since density mutants of T1, T3 and T7 existed and could be isolated by heat selection, we thought it should also be possible to isolate them by the direct, but more laborious process of repeatedly selecting phage particles from the light side of a density gradient band of wild-type phage. A T3+ stock was centrifuged in a CsCl gradient and the fractions on the light side of the peak were pooled and grown to a high-titre stock. This second lysate was in turn banded in a density gradient and the light fractions isolated and regrown. After five selection cycles, a plaque was picked from the fifth lysate and the density of the phages compared to that of T3+. The selected T3 phage showed a uniform shift to a lighter density very similar to that of the T3 st mutants. This light phage stock was heat-stable to about the same degree as the T3 st mutants obtained by direct heat selection. Mutation to heat-stability and lighter density were thus correlated and a consequence of the same process.

DISCUSSION

Our results demonstrate that the ability to mutate from the heat-sensitive wild-type to a heat-stable form is a general property of phages T1, T3 and T7. Previously this had been shown for T5 (Adams & Lark, 1950) and more recently for phage λ (Parkinson & Huskey, 1970). For all five phages mutation to heat-stability is correlated with a reduction in the density of the phage particle and, with the exception of T1, the reduced density has been demonstrated to result, as predicted, from the loss of some DNA from the phage particle. The failure to detect reversion of T3 and T7st mutants supports the deduction from the sedimentation data which indicate that st mutants have lost DNA sequences.

The T1st mutants show only a slight density reduction compared to T1+ phage. The difference, however, is reproducible and was observed throughout a series of eleven experiments comparing the density of T1+ phage with each of four T1 st phages examined. The sedimentation rates of T1+ and T1st DNA molecules were virtually identical. Therefore we cannot distinguish between the possibility that the two DNA types have identical molecular weights or that T1st DNA molecules are shorter by a small amount not resolved by sedimentation analysis. (Since molecular weight and sedimentation rate are logarithmically related, two DNAs with molecular weights differing by 2 % would differ only by 0.7 % in their sedimentation rate. Smaller molecular weight differences would not be distinguishable by this method.)

Our findings, taken together with those of Rubenstein (1968) and Parkinson & Huskey (1970) suggest that for phages with non-permuted DNA molecules the isolation of heat-stable mutants can be used as a standard method for selecting viable phages with reduced densities. The procedure is simpler and quicker than the direct method of selecting density mutants from density gradients.

The st mutants of each phage type were isolated by repeated heat selection from a single initial wild-type stock. Thus it is possible that the st mutants isolated from a given phage type are clonally related. In this study we were interested in trying to establish the existence of heat-stable mutants and were not concerned with their possible genetic relation, so did not attempt to ensure that each st mutant was of independent mutational origin. Simple modification of the heat selection technique would permit the isolation of genetically distinct st mutants.
Why there should be the observed correlation between heat-stability and DNA content is not fully understood. Heat inactivation causes the DNA to be released from the particles of several phages including T5 (Lark & Adams, 1953), T7 (Freifelder, 1965), λ (Parkinson & Huskey, 1970), T1 and T3 (D. A. Ritchie, unpublished results). In fact, we find that for T1, T3, T7, T2, T4 and P22 the DNA is released as full-length molecules. But why the DNA release mechanism should be so sensitive to small variations in DNA complement we cannot yet explain.

As yet there is no information about the location of the deleted regions in the st phages of T3 and T7. We are attempting to identify which regions of the DNA are missing and whether the DNA is deleted from only one or several different locations in a given DNA molecule.

These deleted sequences must be genetically non-essential, at least for growth in *Escherichia coli*. Such regions might represent duplicate phage genes or genetic information also carried by the host cell, or perhaps phage functions which are not always required for vegetative growth, such as the rII gene products of phage T4. The situation is clearer in temperate phages, for it is known that DNA regions involved with lysogeny can be deleted without hindering lytic functions. No doubt this is why as much as 23% of the λ DNA molecule can be deleted (MacHattie & Thomas, 1964).

The five phages capable of producing heat-stable density mutants all have DNA molecules with non-permuted DNA sequences (Olligs, 1967; Thomas & MacHattie, 1967), and for several of them a concatenated structure has been implicated in the DNA replication process (Smith & Skalka, 1966; Salzman & Weissbach, 1967; Thomas *et al.* 1968). A suggested mechanism for cutting non-permuted DNA molecules from concatemers involves the recognition of specific terminal nucleotide sequences (Thomas *et al.* 1968) so that the deletion of DNA would lead to the excision of shorter DNA molecules. For phages with permuted DNA sequences, such as the T-even phages, it has been suggested that phage-sized DNA molecules may be excised from concatemers by a reaction which recognizes a fixed length of DNA and not specific DNA sequences (Streisinger *et al.* 1967; Thomas *et al.* 1968). In this case, a deleted DNA region would be compensated for by the DNA molecule having a longer terminal repeat region (Streisinger *et al.* 1967; Kvelling, 1969). Thus for permuted phages, mutation to heat-stability and reduced density, if it does occur, may not reflect a change in the size of the DNA molecule but some other mechanism. Our preliminary results with T2 and T4, to be the subject of a further paper, show a different pattern from that with the T-odd phages; repeated heat selection (15 to 16 successive heat-inactivation cycles) has revealed phages with only a marginally increased heat-stability, compared to the parent phage. The major findings with the T2 and T4 heat-selected phages relevant to the present discussion are the following: (1) the DNA content is very similar or identical to the parent phage, (2) the phage particles show a bimodal density distribution with 10 to 30% banding with the parent phage, the remainder being of very light density, (3) both density bands contain fully infectious phages, (4) the two density types are genetically identical since the progeny of phages taken from either density band reproduce the bimodal density pattern, (5) osmotic shock evidence with the T4 heat-selected phages indicates that the configuration of the capsid protein subunits is altered and that these changes result from a single reversible mutation.

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