The Strategy of Infection as a Criterion for Phylogenetic Relationships of Non-Coli Phages Morphologically Similar to Phage T7

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SUMMARY

Five phages which are morphologically similar to coliphage T7 but attack other host bacteria have been compared to T7 and to its relative, T3, by the following criteria: (a) cross-reactivity with antisera against T7 and T3, (b) DNA base sequence homologies, as determined by the C_{ot} technique, (c) synthesis of two phage-coded enzymes: RNA polymerase and SAMase, (d) patterns of phage-directed protein synthesis, as determined by SDS-polyacrylamide gel electrophoresis followed by autoradiography, (e) SDS-polyacrylamide gel electrophoresis of phage coat subunits. As judged by all these criteria, *Pseudomonas* phage PX3 is not related to T7; thus, morphological similarity was attributed to convergent evolution. The other phages, i.e. *Serratia* phage IV, *Pseudomonas* phage gh-1, *Citrobacter* phage ViIII and *Klebsiella* phage No. 11, were considered to be related to T7 on the basis of similarities in the patterns of phage-coded proteins and because, early after infection, these phages induced, as T7 does, an RNA polymerase which specifically transcribes the DNA of the homologous phage. Phages IV and No. 11 also induced the early synthesis of SAMase (previously only known to occur upon T3 infection). With the exception of phage IV, however, DNA base sequence homologies with T7 or T3 seem to be poor or non-existent. The tested phages, again with the exception of phage IV, did not react with antiserum against T3 or T7.

It is concluded that a particular pattern of phage-directed protein synthesis (as characterized by polyacrylamide gel electrophoresis and enzyme tests) may provide evidence for phylogenetic relationships between phages, even in cases where other criteria, such as genetic recombination, serological cross-reaction, and DNA base sequence homologies, fail to indicate relatedness.

INTRODUCTION

Nothing is known about the phylogenetic origins of bacteriophages. However, during the last three decades, analysis of a large number of phages has made it clear that newly isolated phages can often be assigned to a given natural group of previously known ones, on the basis of such characteristics as morphological similarity, serological relatedness, ability to recombine genetically, or sequence homologies of the corresponding nucleic acids. Thus, with regard to *Escherichia coli* phages with double-stranded DNA, we distinguish such groups as the T-even-related, the λ-related or lambdoids, the T5-related, and, of special concern to us, the T7-related [or, as proposed by Summers (1972), the haptoid] phages.
Each group of phages is characterized by a peculiar 'strategy of infection' (Subak-Sharpe, 1971), a term which encompasses the sum of co-ordinated genetic and biochemical events which are elicited by the invading virus genome and which culminate in the liberation of infective progeny particles.

Thus, the strategy of infection of T7-related phages could be summarized as follows. Upon invasion of a host cell by the phage genome, the host RNA polymerase recognizes promoters located near the conventional left end of the phage DNA and transcribes a segment of about 20% of that molecule. At that point the polymerase is thrown off the template at a transcription termination signal. The segment of the phage genome transcribed by the host RNA polymerase, i.e. the early region, encompasses only a few genes (five, in the case of T7), among which gene 1 is of paramount importance. This gene codes for a polypeptide, with a mol. wt. of about 100,000, which shows rifampicin-resistant RNA polymerase activity. This phage-coded RNA polymerase recognizes only specific promoters located on the phage DNA, and absent on the host DNA (Chamberlin et al. 1970). Since the expression of the early region of the phage genome also results in the inactivation of the host RNA polymerase (for review see Hausmann, 1976), it is thus assured that all transcription is diverted towards expression of the late portion of the phage genome. The crucial early feature of the T7-specific strategy of infection is thus the synthesis of a phage-coded, rifampicin-resistant RNA polymerase.

In this paper we will attempt to show that by characterizing a particular strategy of infection one may be able to produce circumstantial evidence for or against a phylogenetic relationship among two phages, even in cases where the classical criteria, namely morphological similarity, serological cross-reaction, genetic recombination and DNA base sequence homologies, give inconclusive results. As a model system we chose a series of five bacteriophages which, based on morphological criteria, have to be assigned to the group C phages, described by Bradley (1967). This group encompasses phages whose particles display a polyhedral head and a tail which is much shorter than the head diam. The prototype of this group is phage T7, whose particles have a head diam. of 60 nm and a tail length of about 15 nm. The phages chosen for this comparative study were the following: Serratia phage IV; Pseudomonas phage gh-1; Pseudomonas phage PX3; Citrobacter phage ViIII; Klebsiella phage No. 11 (see Table 1). The first two were included in this series because there was further evidence for a phylogenetic relatedness with T7; Serratia phage IV had been shown by Adams & Wade (1954) to cross-react with antisera against T7 or against T3, which is a close relative of T7. And Pseudomonas phage gh-1 encodes a specific RNA polymerase which transcribes only the DNA of this phage (Towle et al. 1975). The other three phages were chosen solely on the basis of their morphological similarity to T7. The fact that T7 only grows on certain strains of Escherichia coli and Shigella, while none of the other phages does, was an indication that their phylogenetic relationship to T7, if any, would be remote in comparison to the group of about a dozen coliphages which are related to T7 (Hausmann, 1976). The absence of a known common host precluded mixed infection experiments for monitoring genetic recombination among the tested phages. Thus, our comparative studies encompassed, as classical criteria for relatedness, the investigation of serological properties and of DNA-DNA hybridization patterns. On the other hand, information on the strategies of infection was sought through enzyme tests for a phage-induced, rifampicin-resistant RNA polymerase and for SAMase (an S-adenosylmethionine-cleaving enzyme characteristic for phage T3), as well as by comparisons of the temporal expression of the banding patterns of phage-coded proteins, as revealed by the method of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and autoradiography (Studier, 1973). Our results suggest
that, by applying these methods routinely in the course of the characterization of new phages, many more distant phylogenetic relationships between phages will be discovered than has been possible up to now and thus throw more light on the problem of phage evolution.

**METHODS**

*Phage and bacteria.* The phage and the corresponding host bacteria used in this work are listed in Table 1. For storage, bacterial cultures were grown in NB medium, mixed with an equal vol. of glycerin and stored in liquid nitrogen. Phage stocks for our work were derived from a single plaque isolated upon arrival of the requested samples. Lysates made from these isolates were concentrated and purified as described by Yamamoto et al. (1970). For general phage work, standard procedures were used (Adams, 1959). For plating *Serratia* phage IV, not more than 10⁷ host cells were poured on a plate, otherwise no plaques would develop. *Pseudomonas putida* was grown at 32 °C, other bacteria at 37 °C. All infection experiments were at 37 °C.

*Media.* NB medium contained, per litre, 10 g nutrient broth (Difco) and 10 g NaCl; this medium was used for growing all host bacteria except *Pseudomonas aeruginosa*, which was grown in TGE-medium, containing, per litre, 5 g Bacto Tryptone (Difco), 2·5 g Yeast Extract (Difco) and 1 g glucose. For incorporation studies with ³⁵S-methionine, the cells were grown in a synthetic medium (S-medium), which contained, per litre, 10 g di-sodium hydrogen phosphate, 4·5 g potassium di-hydrogen phosphate, 1 g ammonium sulphate, 0·5 g sodium citrate, 0·2 g magnesium chloride, 2 g glucose and 40 mg of each amino acid required for protein synthesis, except methionine, which was not added.

*Phage inactivation by antiserum.* Rabbit antisera against T7 or T3 were obtained by weekly subcutaneous injections, for 4 weeks, of 10¹¹ purified phage particles each time; no adjuvant was used. For assaying the activity of an antiserum it was diluted 100-fold into a phage suspension in S-medium, with a titre of 10⁷ particles/ml, and incubated at 37 °C for 10 to 30 min. Every 2 min a sample was withdrawn and titrated in order to determine the K value of the original serum, according to the equation Pt/Po = e⁻K×t/D (Adams, 1959). Po and Pt represent, respectively, the phage titre at the times of serum action zero and t, in min; D is the serum dilution factor; and K is the activity constant for the inactivation reaction (which closely followed first order kinetics until about 95% of the homologous phage were inactivated). K values obtained ranged from 200 to 400. For assaying hetero-
logous activities, antisera were diluted into suspension of the various phages to be tested (10⁷ particles/ml) and samples were withdrawn for titration, as described for the homologous reaction. Heterologous $K$ values ($K_{het}$) were then expressed as a fraction of the corresponding homologous values ($K_{hom}$).

**Enzyme assays.** Phage-induced, rifampicin-resistant RNA polymerase activities were monitored as described by Chamberlin et al. (1970) except that $^{14}$C-UTP (500 ct/min/nmol) was used to label the RNA product. $S$-adenosylmethionine-cleaving activity (SAMase activity) was monitored as described by Gefter et al. (1966). For both types of assays, extracts of phage-infected cells were used as enzyme source (Beier & Hausmann, 1974); for RNA polymerase assays, 20 µg of cell-free extract were added to the assay mixture, 10 µg for SAMase assays.

**DNA-DNA hybridization.** In order to evaluate the relative degrees of genetic homology between the DNA of T7 or T3 and the DNAs of the other phages, the Cot technique of Britten & Kohne (1968) was used. The Cot method is based on the fact that, under appropriate conditions, the extent of re-association observed in a given preparation of denatured DNA depends on the product (Cot) of the DNA concentration (in mol of nucleotides per litre) and the time of incubation (in seconds). The reassociation kinetics of a denatured DNA may conveniently be expressed on a semi-logarithmic plot, where the fraction of re-associated DNA fragments is given on an arithmetic scale on the ordinate and the product of DNA concentration and time of incubation (Cot) on a logarithmic scale on the abscissa. This form of representation yields sigmoidal curves for the renaturation kinetics and allows comparisons over a very wide range of renaturation rates. It also allows an easy graphical determination of the Cot values which correspond to 50% renatured DNA fragments (Cot½ values). Based on the measurements of Cot½ values, our experimental procedure was similar in principle to that of Gelb et al. (1971) for detecting SV40 sequences in virus-transformed cells. First, the renaturation kinetics of a dilute solution (0.05 µg/ml) of $^3$H-labelled T7 DNA (or T3 DNA) was followed. In a second step, a 10- or 20-fold excess of non-radioactive T7 DNA (or T3 DNA, respectively) was added to another sample of the dilute solution of labelled T7 DNA (or T3 DNA) and again the renaturation kinetics followed. It was thus shown, that the renaturation of the labelled DNA was speeded up: the apparent Cot½ values for the renaturation of labelled DNAs were lowered in proportion to the excess of homologous unlabelled DNA added (Fig. 2 and 3). In a third step, unlabelled phage DNAs with only partial sequence homologies were used in renaturation experiments. The capacity of this heterologous DNA, relative to the capacity of homologous DNA, to decrease the apparent Cot½ value of labelled T7 DNA (or T3 DNA) was considered to be a function of the degree of genetic homology between the two DNA species (see also Discussion). In detail, the procedure was as follows: DNA of all phages was obtained by phenol extraction of purified phage suspensions. $^3$H-thymidine-labelled T7 and T3 DNA were prepared from phages grown in $^3$H-thymidine-labelled Escherichia coli B cells resuspended in non-radioactive NB medium. The host cells were labelled as described elsewhere (Issinger & Hausmann, 1973). The DNAs were sheared to fragments with a mol. wt. of about 2 x 10⁸ (i.e. to less than 1/100 genome length) by means of ultrasonic treatment (Branson Sonifier, microtip, intensity setting no. 5). The shearing was done in 2 ml portions of a DNA solution of 50 µg/ml, in dilute saline citrate buffer (0.1% sodium chloride; 0.02% sodium citrate). The mol. wt. of the sheared samples were checked by zone sedimentation in neutral sucrose gradients, according to Burgei & Hershey (1963). The samples of sheared phage DNA were diluted in salmon sperm DNA (used as a carrier), sheared under identical conditions. Appropriate mixtures of sheared DNA samples (see Results) were then denatured (100 °C,
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20 min) and incubated at 68 °C. At different times of incubation, samples (0.2 ml) were placed on water jacketed hydroxyapatite columns at 60 °C (total vol. of column: 2 ml; binding capacity about 1.3 mg DNA) and washed with 3 ml of 0.14 M-phosphate buffer in order to elute single stranded segments. A second washing, with 3 ml of 0.4 M-phosphate buffer, yielded a fraction with double-stranded fragments. The fractions were precipitated with 5% trichloroacetic acid and their radioactivity determined by scintillation counting. The percentage value of renatured DNA corresponding to each incubation time was then plotted against Cot [i.e. the product of DNA concentration (mol of nucleotides/l) and time (s) allowed for renaturation at 68 °C].

Characterization of phage-coded proteins by SDS polyacrylamide slab gel electrophoresis and autoradiography. The experimental layout for polyacrylamide slab gel electrophoresis in the presence of SDS was similar to that described by Studier (1973). Host cells were grown in S medium to a concentration of 3 x 10⁸ cells/ml and were irradiated with u.v. light in 10 ml portions in uncovered Petri dishes (Sylvania germicidal lamp G8T5, 12 cm distance for 2 min with continuous agitation). After 5 min of incubation at 37 °C, phage (10 particles/cell) and 35S-methionine (40 µCi/ml, final concentration) was added to a 3 ml sample of irradiated cells, and incubation continued. At intervals, 0.2 ml samples were poured into tubes containing 25 µg of chloramphenicol and kept at 0 °C. The infected cells were harvested by low speed centrifugation, resuspended in 15 µl sample buffer (0.1 M-tris, pH 6.8; 0.8 M-mercaptoethanol, 2% SDS, 10% glycerin), and heated at 100 °C for 2 min. Samples of 10 µl were applied to the gel pockets of an SDS polyacrylamide gradient slab gel (10 to 18% polyacrylamide), as described by Studier (1973), and subject to electrophoresis under a constant voltage of 150 V for 3 h. The gels were stained, washed and dried (Studier, 1973). The dried gels were autoradiographed for 7 days, by use of Osray T4 X-ray film. For visualizing the bands of phage coat proteins (Fig. 8), purified phage suspensions (10¹² particles/ml) were dialysed against sample buffer and heated at 100 °C for 2 min. A 10 µl portion was then subjected to gel electrophoresis as described. The gel was kept in a staining solution (0.1% Coomassie brilliant blue R250, 40% methanol, 10% acetic acid) for 12 h and placed in a destaining solution (5% methanol, 7.5% acetic acid) for 12 h at 50 °C.

RESULTS

Serological cross-reactivity of anti-T7 and anti-T3 sera

Inactivation experiments with anti-T7 and anti-T3 sera were performed at two different serum concentrations. In one series of experiments, Kₜₗₗ values of 0.7 were chosen. This relatively low serum activity allowed us to measure the inactivation rates which characterize the initially exponential inactivation kinetics. As shown in Fig. 1, Serratia phage IV was the only non-coli phage to react measurably with antisera against T7 or T3. In these experiments, the heterologous inactivation constant of phage IV with anti-T7 serum was about 0.05 (Kₜₗₗ ~ 0.07 Kₜₗₗ). Phage IV reacted with anti-T3 serum with a K value of about 0.25 (Kₜₗₗ ~ 0.36 Kₜₗₗ), i.e. at a rate about five times higher than that corresponding to T7 antiserum. The heterologous K values of anti-T3 serum with regard to T7 was only about one third of the value corresponding to phage IV. Thus, from these experiments it seems that Serratia phage IV is more closely related to T3 than T7 is, although phage IV and T3 share no known common host. (Additional evidence for a close relationship between phage IV and T3 will be discussed later.) The phages which were not inactivated at antiserum concentrations of K ~ 0.7 were also shown not to react at a 20-fold higher serum concentration. Under these conditions, heterologous reaction rates of less than one thousandth of
Fig. 1. Action of anti-T7 (a) and anti-T3 serum (b) on heterologous phages. Diluted samples of rabbit antiserum against T7 or T3 were mixed with phage suspensions (~ 10^7 particles per ml) and incubated at 37 °C. At time intervals the reaction mixtures were assayed for surviving p.f.u. The serum dilution factor was chosen to give a K value of about 0.7 when the serum was assayed with the homologous phage. •—•, T7; □—□, T3; ▲—▲, Serratia phage IV; ■—■, Pseudomonas phage gh-1; ○—○, Pseudomonas phage PX3; △—△, Citrobacter phage VIII; □—□, Klebsiella phage No. 11.

Re-annealing kinetics of the DNAs of T7 and T3 with the DNAs of phages IV, gh-1, PX3, VIII and No. 11

As stated in more detail in the Methods, possible base sequence homologies between T7 or T3 and the other phages investigated here were sought by the C_{ot} technique of Britten & Kohne (1968). As seen in Fig. 2 and 3, the addition of a 10- or 20-fold excess of non-radioactive, homologous DNA reduced the apparent C_{ot1/2} values of the labelled DNAs of T3 or T7 by the expected factor of 10 or 20. On the other hand, the addition of a 20-fold excess of DNA from Citrobacter phage VIII or from Pseudomonas phage PX3 did not interfere with the re-association kinetics of labelled T3 or T7 DNA. Thus, it seems that no base sequence homology exists between these phages and phage VIII or phage PX3. A 20-fold excess of DNA of phage gh-1 had no appreciable effect on the re-association kinetics of ^3H-DNA of T7 but reduced the apparent C_{ot4} of ^3H-DNA of T3 by about 5% of
Non-coli phages related to T7

Fig. 2. Reassociation kinetics of $^3$H-labelled T7 DNA in the presence of non-radioactive DNAs of heterologous phages. Mixtures of sheared, $^3$H-labelled T7 DNA and sheared non-radioactive DNAs (mol. wt. about $2 \times 10^8$) of T7, or heterologous phages, were denatured at 100 °C, in the presence of sheared salmon sperm carrier DNA and incubated at 68 °C. At various times of incubation, samples were placed on hydroxyapatite columns at 60 °C. The fractions of single-stranded DNA were eluted with $0.24$ M-phosphate buffer; then, double-stranded DNA was eluted with $0.4$ M-phosphate buffer. $C_0t$ represents the product of the time of incubation at 68 °C (in seconds) and the concentration of $^3$H-DNA of phage T7 (in mol of nucleotides per litre). Incubation mixtures contained, per ml: $0.05$ µg $^3$H-DNA of T7; $2.5$ mg salmon sperm DNA; one of the following additions: $\circ$, no addition; $\square$, $1.0$ µg T7 DNA; $\triangle$, $0.5$ µg T7 DNA; $\bullet$, $0.5$ µg T3 DNA; $\square$, $1.0$ µg DNA of Klebsiella phage No. 11; $\Delta$, $0.5$ µg DNA of Serratia phage IV; $\blacksquare$, $1.0$ µg DNA of Pseudomonas phage gh-1; $\triangle$, $1$ µg DNA of Citrobacter phage ViIII.

Fig. 3. Reassociation kinetics of $^3$H-labelled T3 DNA in the presence of non-radioactive DNAs of heterologous phages. The experimental layout was as described in the legend of Fig. 2, except that $^3$H-labelled T3 DNA was substituted for $^3$H-labelled T7 DNA. Incubation mixtures contained, per ml: $0.025$ µg $^3$H-DNA of T3; $2.5$ mg salmon sperm DNA; one of the following additions: $\circ$, no addition; $\square$, $0.5$ µg T3 DNA; $\triangle$, $0.25$ µg T3 DNA; $\bullet$, $0.5$ µg T7 DNA; $\Delta$, $0.5$ µg DNA of Serratia phage IV; $\square$, $0.5$ µg DNA of Klebsiella phage No. 11; $\bullet$, $0.5$ µg DNA of Pseudomonas phage gh-1; $\triangle$, $0.5$ µg DNA of Citrobacter phage ViIII.
Fig. 4. Time course of synthesis of phage-directed, rifampicin-resistant RNA polymerase. Growing host bacteria were infected with phage (10 particles per cell) and incubated at 37 °C. At different times, samples of the infected cells were centrifuged. The pellets were resuspended in buffer and the cells disrupted by ultrasound. After removing particulate material by centrifugation, the supernatant extracts were used as a source of enzyme in RNA polymerase tests in the presence of rifampicin (20 μg/ml). DNA from the homologous phage was used as template DNA. (a) Escherichia coli B, infected with phage T7; (b) E. coli B, infected with phage T3; (c) Serratia marcescens 4, infected with phage IV; (d) Pseudomonas putida A. 3.12, infected with phage gh-1; (e) Citrobacter sp. C123, infected with phage viIII; (f) Klebsiella spp. 390, infected with phage No. 11.

The value expected on the assumption of complete homology. DNA from Klebsiella phage No. 11 shifted the C₀t½ values of labelled T3 or T7 DNAs by about 8% of the values expected for fully homologous DNA. With Serratia phage IV DNA the apparent C₀t½ of labelled T3 DNA was shifted by only 20% of the value expected if the same amount of homologous DNA had been used. The addition of phage IV DNA had no appreciable effect on the renaturation behaviour of labelled T7 DNA. For comparison, in the case of ³H-DNA of T7 with excess of T3 DNA (Fig. 2), as well as in the case of ³H-DNA of T3 with excess of T7 DNA (Fig. 3), we found that the shift in apparent C₀t½ values of labelled DNAs was about 30% of the shift observed upon addition of a corresponding excess of homologous DNA.

Rifampicin-resistant RNA polymerase activities after infection by phages IV, gh-1, ViIII, and No. 11

As mentioned in the Introduction, a characteristic feature of the strategy of infection of T7-related phages is the early synthesis of a phage-coded polypeptide (of mol. wt. about 100000) which shows specific RNA polymerase activity depending on the presence of the DNA of the homologous phage as template. Thus, the search for such an RNA polymerase activity was of paramount importance for further characterizing the phages under investigation. For this, growing host cell cultures were infected with phage and further incubated at 37 °C. At various times after infection, samples of these cultures were withdrawn in order
Non-coli phages related to T7

Fig. 5. Time course of synthesis of phage-directed S-adenosylmethionine-cleaving enzyme. The same cell-free extracts of phage-infected host cells, as described in Fig. 4, were used for assaying SAMase activity. (a) Escherichia coli B, infected with phage T3; (b) Serratia marcescens 4, infected with phage IV; (c) Klebsiella spp. 390, infected with phage No. 11.

to prepare cell-free extracts of the infected cells. These extracts were used as enzyme source in RNA polymerase assays in the presence of rifampicin. The DNA used as template was from the same phage which had been used to infect the host cells whose extracts were assayed. The results (Fig. 4) made it clear that, within 2 to 3 min after infection, a sharp rise in rifampicin-resistant RNA polymerase activity occurred in Pseudomonas putida cells infected with gh-1, Serratia marcescens cells infected with phage IV, Citrobacter cells infected with phage VI, and Klebsiella cells infected with phage No. 11. In all cases, the kinetics of RNA polymerase synthesis was virtually identical to that of T7. No new RNA polymerase activity was found to appear after infection of Pseudomonas aeruginosa with PX3, even when assays were made in the absence of rifampicin. In a second series of experiments, heterologous phage DNAs were used as templates in RNA polymerase assays with samples of the extracts which gave the highest activity in the homologous assays (usually the 5 min sample). None of the phage RNA polymerases tested showed any affinity for the DNA of the heterologous phages, except T3 RNA polymerase, which transcribed T7 DNA, and T7 RNA polymerase, which transcribed T3 DNA. In these two cases, we confirmed the previously reported levels of heterologous activity of 10% and 50% respectively (Dunn et al. 1971; Hausmann & Tomkiewicz, 1976).

Phage-coded SAMase activity after infection by Serratia phage IV and Klebsiella phage No. 11

Phage T3 codes for an S-adenosylmethionine (SAM)-cleaving enzyme, SAMase, the product of gene 0.3. This phage gene is the first one to be expressed after infection (Hausmann & Härle, 1971) and acts to overcome the SAM-dependent restriction mechanism of the host (Studier & Movva, 1976). SAMase synthesis is a specific feature of T3 infection and had not been known to occur during the infection cycle of any other phage. Enzyme assays done with extracts of phage-infected cells as putative enzyme sources revealed, however, that Serratia phage IV and Klebsiella phage No. 11 upon infection induce SAMase activity according to the same pattern observed during T3 infection, i.e. a peak of enzyme activity was reached at only 3 to 4 min after infection (Fig. 5). The other phages under investigation showed no detectable SAMase production.
Fig. 6. Time course of synthesis of phage-coded proteins after infection of (a) *Pseudomonas aeruginosa* 2 by phage PX3, (b) *Escherichia coli* B by phage T7. Host bacteria were u.v. irradiated in order to inhibit host protein synthesis. Phage (10 particles per cell) and 35S-methionine were then added and the cultures incubated at 37 °C. At the times indicated, samples were withdrawn. The cells were disrupted and proteins separated electrophoretically (150 V, 3 h), in the presence of SDS, on a 10 to 18% polyacrylamide gradient slab gel. Dried gels were autoradiographed for selective visualization of the bands of phage-coded proteins. Numbers at right indicate corresponding T7 genes. [Our T7 reference type carries a deletion in the o'7 gene region (Hausmann, 1976); therefore no protein band shows at the corresponding position.] The origin is at the top. Uninf., uninfected, irradiated *Pseudomonas aeruginosa* cells (a) were incubated for 25 min in order to evaluate residual protein synthesis by the host. (Similar controls with other uninfected hosts showed no significant residual protein synthesis.)

**Time course of synthesis of intracellular phage-coded proteins, as revealed by SDS polyacrylamide gel electrophoresis and autoradiography**

Phage T7 shows a very characteristic pattern of synthesis of phage-coded proteins (Studier, 1973; Fig. 6b). Shortly after infection, five early proteins are synthesized in the following order (for review, see Hausmann, 1976): gene o'3 product (mol. wt. ~ 10000), gene o'7 product (mol. wt. ~ 40000), gene r RNA polymerase (mol. wt. ~ 10000), a short peptide coded by gene r'1, and a ligase, the product of gene r'3 (mol. wt. ~ 40000). After the appearance of these early proteins, the synthesis of the late proteins starts at about 5 min after infection (37 °C). There are about 25 such late proteins which can be distinguished autoradiographically as individual bands after polyacrylamide gel electrophoresis of appropriately labelled extracts of infected cells (Studier, 1973; Fig. 6b). A comparison of the gel electrophoretic-autoradiographic patterns of phage-coded proteins obtained after infection of our host strains by the corresponding phage revealed striking common features with the T7-specific pattern in the case of *Serratia* phage IV, *Pseudomonas* phage gh-1, *Citrobacter* phage VIII, and *Klebsiella* phage No. 11 (Fig. 7). All these phages directed the synthesis, early in the course of infection, of a protein with a mol. wt. of about 100000. This protein we tentatively consider as homologous to the gene r product of phage T7. Similarly, the early protein of mol. wt. in the range of 40000, whose appearance precedes that of the 100000 mol. wt. protein would be homologous to the gene o'7 product of phage T7. In the cases of *Serratia* phage IV and *Klebsiella* phage No. 11, the first phage-coded protein to appear had
Non-coli phages related to T7

Fig. 7. Time course of synthesis of phage-coded proteins after infection of (a) *Serratia marcescens* by phage IV, (b) *Pseudomonas putida* A-3.12 by phage gh-1, (c) *Citrobacter spp.* Ci23 by phage ViIII, (d) *Klebsiella spp.* 390 by phage No. 11. See legend to Fig. 6 for experimental procedures.

a mol. wt. around 10000; based on these findings, and considering that these two phages were found to code for a SAMase, these proteins are here tentatively considered homologous to the ω-3 gene products of T3 and T7. The topmost band (Fig. 7), corresponding to a late protein of mol. wt. of about 150000, was tentatively considered homologous to the product of T7 gene 16, a coat subunit (see also Fig. 8). Phage PX3, which showed no phage-induced RNA polymerase activity, is also the only phage whose autoradiographic protein banding pattern displayed no obvious similarities with that of T7 (Fig. 6a).
Fig. 8. Banding patterns of phage coat proteins. A sample (c. $10^{12}$ particles) of a purified suspension of particles of each phage was heated in the presence of SDS and then subjected to electrophoresis ($150 \text{ V, 3 h}$) in an SDS polyacrylamide gradient (10 to 18%) slab gel. The protein bands were stained with Coomassie blue. Numbers at the right indicate corresponding genes of T7.

**SDS polyacrylamide gel-electrophoretic banding pattern of particle coat proteins**

The five phage types compared here to T3 and T7 were chosen because of their morphological similarity to T7 under the electron microscope: they have hexagonal heads of about 60 nm in diam. and a stubby tail about 15 to 20 nm long. From these morphological similarities it does not necessarily follow, however, that the patterns of protein subunits composing the phage coats are also similar. Convergent evolution of phylogenetically totally unrelated phage lines could have resulted in superficial morphological resemblance of coats built up of totally different subunits. With this possibility in mind, we separated electrophoretically the coat protein subunits of the phages investigated here (on an SDS polyacrylamide slab gel). As shown in Fig. 8, each phage displayed an individual, unique banding pattern of its coat protein subunits. Nevertheless, the overall similarity of pattern of phages T7, T3, IV, gh-1, ViIII and No. 11 is in clear contrast to the quite different pattern of PX3 coat subunits.

**DISCUSSION**

We think that our findings clearly show that the close morphological similarity between coliphage T7 and Pseudomonas phage PX3 may be best interpreted as an example of convergent evolution and not as a result of a phylogenetic relationship between these two phages. This conclusion is based on the lack of similarity in the subunit composition of the phage coats and the banding pattern of phage-coded proteins, as well as the absence in
Non-coli phages related to Y7

PX3 of the most striking feature of the T7-specific strategy of infection: the early synthesis of a single peptide enzyme with a mol. wt. of about 100,000, endowed with an RNA polymerase activity restricted to the transcription of the DNA of the homologous phage. On the other hand, this, in our opinion, most crucial criterion for phylogenetic relatedness of T7 is fulfilled in the other four non-coli phages investigated here. However, except for the known cross-reactivity in the case of T3 and T7 (Dunn et al., 1971; Hausmann & Tomkiewicz, 1976), we could detect no phage RNA polymerase activity in any heterologous combination (RNA polymerase of one phage with template DNA from another). The common ancestor of these phages which is proposed here obviously existed sufficiently long ago to allow divergent evolution to erase all functional stereospecific complementarity between late promoters on the phage DNAs and the non-homologous phage RNA polymerases. The alternative explanation of a convergent evolution of unrelated RNA polymerases seems extremely unlikely in view of present ideas on the evolution of protein superfamilies (Dayhof, 1976).

Lack of functional stereospecific complementarity was also patent in all serological cross-reaction experiments, except in the case of Serratia phage IV, which reacted with a relatively high $K_{het}$ with anti-T3 serum ($K_{het} \sim 0.36 K_{hom}$) and with lesser intensity with anti-T7 serum ($K_{het} \sim 0.07 K_{hom}$). This suggests a relatively close relationship of this phage to T3. This impression is corroborated by the fact that phage IV, like T3, codes for an early protein with SAMase activity and that denatured phage IV DNA formed heteroduplexes with denatured phage T3 DNA at a relatively high rate, as discussed in the next paragraph, in which the reannealing patterns with heterologous DNAs are considered.

In order to evaluate the relative degree of phylogenetic divergence between the DNAs of two related phages we have measured the rates of reannealing of the heterologous DNAs (leading to heteroduplex formation) and compared these rates to the rate of reannealing of DNA from T3 or from T7 (homologous DNA) treated in the same conditions. For this purpose we have the $C_{st}$ technique of Britten & Kohne (1968), as described in the Methods. Until recently evaluations of base sequence homologies between different DNAs were done mainly by determining saturation levels upon hybridization with corresponding RNA or by measuring the melting temperature ($T_m$) of corresponding DNA heteroduplexes. However, saturation levels do not allow the detection of base substitutions and small deletions or additions distributed randomly through the genome. But this is exactly the situation found if one compares T3 with T7, as has been shown in analyses of phage-coded proteins separated by SDS-polyacrylamide gel-electrophoresis (Hyman et al., 1974), and by electron microscopy of T3 DNA-T7 DNA heteroduplexes (Davis & Hyman, 1971). Therefore, measurements of saturation levels were not considered appropriate for our purpose. However, one could argue that, instead of determining the relative rates of heteroduplex formation, it would be preferable to measure the differences between $T_m$ values of homoduplexes and heteroduplexes ($\Delta T_m$), since the work of other investigators would then be available for comparison. If data on $\Delta T_m$ values gave precise information on the degree of base sequence divergence between two DNAs, such a comparison would indeed be of great value. One first problem in evaluating base sequence homologies by $\Delta T_m$ determinations is that genome regions of low or no homology will not undergo heteroduplex formation in the first place. In such cases, the average value of sequence homology will be systematically overestimated. In addition to this, as was recently pointed out by Britten & Davidson (1976): 'The melting temperature reduction is a good measure of the sequence divergence, but the conversion factor is not accurately known. For this discussion we assume that a reduction of 1 °C in $T_m$ corresponds to about 1 % nucleotide substitution in the two ancestral species lines since
their divergence from a common ancestor.' Their assumption was based on studies with synthetic polynucleotides in which only single base substitutions were present [and which gave results varying by a factor of more than 3 (King & Wilson, 1975)]; the precise effects of random small deletions or insertions have never been subjected to a rigorous study. Since, as mentioned above, many such small deletions (or insertions) have been detected by comparing T3 with T7, and since most spontaneous mutations seem not to be simple base substitutions (e.g. Drake, 1970), it seems to us a fair statement that measurements of ΔTm would not yield more precise information on the actual extent of base sequence homologies between the investigated phages than measurements of the rates of heteroduplex formation, especially if an appropriate comparison with data obtained by still another technique is possible. In the case of T3 and T7, our data can indeed be compared with those of the electron microscopic study of partially annealed T3–T7 heteroduplexes, by Davis & Hyman (1971). These authors examined the extent and position of duplex regions formed along T3–T7 hybrid DNA molecules annealed under conditions of different stringency brought about by varying formamide concentrations. They found continuously varying degrees of homology (ranging from less than 40% to over 90%) along the two compared genomes. From their data we have calculated a weighted average of about 60% genetic homology between the DNAs of T3 and T7. On the other hand, in the present study we found that an excess of unlabelled T3 DNA shifted the apparent C₀ᵗ value of labelled T7 DNA by 30% of the value found by the use of the same amount of excess homologous DNA (Fig. 2) and the corresponding value for the reciprocal experiment (excess non-radioactive T7 DNA plus labelled T3 DNA) was 32%. Thus, in our experimental conditions, in the case of the heterologous system of T3 and T7, a shift in apparent C₀ᵗ value of about 30% of the shift in the homologous systems would correspond to about 60% of base sequence homology, as defined by Davis & Hyman (1971). We would like to emphasize, however, that these authors based their calculations of sequence homologies on the uncertain correspondence factor between ATm and percentage of base substitutions. And, as stated above, this uncertainty is enhanced by the fact that a sizable but unknown fraction of mutational events which accumulated in T3 and T7 since their divergence is not due to single base substitutions. However, in spite of all these uncertainties, we may state, with regard to the non-coli phage DNAs tested, that none had the ability to shift the apparent C₀ᵗ values of either T3 or T7 DNA to the same extent as the DNAs of these two phages in heterologous combination. Thus, by this criterium, none of these phages is as closely related to T3 or T7 as these phages are to themselves; in some cases (see Fig. 2 and 3), where no effect on apparent C₀ᵗ values of T3 DNA and T7 DNA could be measured by adding heterologous DNA, base sequence homologies, if present, seem to be relatively small.

In addition to that, we think that our data on the rates of heteroduplex formation allow some tentative inferences on the nature of the genetic heterologies which characterize the phages we compared to T3 and T7. In our study two extreme kinds of sequence divergence can be anticipated: (1) base substitutions, small deletions and insertions distributed randomly through large sections of the genomes [the results of Davis & Hyman (1971) and of Hyman et al. (1974) suggest this to be the case for T3 and T7]; (2) major changes such as block substitutions or deletions (large relative to the average fragment size, which in our case was about 0.01 of the genome size), leading to no relatedness. The effects of changes of type 1 on the rate of reannealing are not known precisely (a statement which also applies to their effects on ΔTm), but, nevertheless, one may predict that in experiments such as these discussed here, they will lead to a parallel shift of the C₀ᵗ-curves of labelled hetero-
Fig. 9. Computer-simulated time courses of annealing reactions of a labelled DNA preparation in the presence of an excess of unlabelled DNA. The two DNAs share some regions of total base sequence homology and some of total non-homology in the following proportions: (a) 75% homology and 25% non-homology; (b) 50% and 50%, respectively; (c) 25% and 75%, respectively; (d) 100% non-homology; (e) 100% homology. Homologous and heterologous regions are very large relative to the average fragment size of the DNA preparations. The equations for the main graph are as follows:

\[
(d) \quad \frac{C}{C_0} = \frac{1}{1 + KC_0 t};
\]

i.e. an ideal second order reaction where \(C_0\), the initial concentration of homologous DNA, and the constant \(K\), are considered equal to unit; \(C\) is the fraction of labelled DNA remaining single stranded at the time \(t\);

\[
(a) \quad C = \frac{0.75}{1 + 10t} + \frac{0.25}{1 + t};
\]

\[
(b) \quad C = \frac{0.5}{1 + 10t} + \frac{0.5}{1 + t};
\]

\[
(c) \quad C = \frac{0.25}{1 + 10t} + \frac{0.75}{1 + t};
\]

\[
(e) \quad C = \frac{1}{1 + 10t}.
\]

As can be seen from these equations, the excess of unlabelled DNA was 10-fold, i.e. corresponded to the conditions of most of our experiments. In the inset, unlabelled DNA was considered to be present in 100-fold excess in otherwise identical conditions.

In contrast, the effects of changes of type 2 are not immediately predictable. At first glance one might think that biphasic reannealing curves will result, in such a way that a plateau will be found at the percentage level of single strands corresponding to the fraction of the tested DNA which has no counterpart in the related DNA added in excess (this fraction would anneal as if no related DNA was present). In order to examine this question more precisely, we performed computer simulations of reannealing experiments where one DNA probe was in 10-fold or 20-fold excess over the other, i.e. the conditions in which our experiments were done. We tested three different assumptions: (a) both DNAs share a 75% segment of full homology while 25% of the genome have no homology at all; (b) as in (a) but the corresponding proportions are 50% and 50%; (c) as in (a) but the corresponding proportions are 25% and 75%. In no case did a plateau develop (a small tendency to plateau formation could only be observed in simulation experiments with over a 100-fold excess of one DNA species). However, the slopes of the simulated \(C_0 t\)-curves were not shifted in a parallel way, but the shifts were more accentuated in the upper part; this resulted in what we would like to call a diagonal shift (Fig. 9). Since in our experiments all
shifts were clearly parallel, we favour the idea that the genetic divergence among the investigated phages is mainly of type I (i.e. consists mainly of base substitutions and small deletions or insertions). In extreme cases such differences would prevent the detection of any base sequence homologies by means of reannealing experiments. An example of this situation might be Citrobacter phage ViIII, on the one hand, and T3 or T7 on the other. In this case, we have based our hypothesis of phylogenetic relatedness to T7 solely on the apparent similarities in the strategies of infection of these phages, as revealed by the presence of a phage-specific RNA polymerase and the gel electrophoretic patterns of phage-coded proteins.

In order to characterize further the group of T7-related phages, many other phage types will have to be investigated. One interesting question, for instance, refers to the possible phylogenetic relatedness between T7 and such morphologically similar structures as blue-green algae virus LPP-1 (Adolph & Haselkorn, 1972). In addition, in order to allow comparative genetics with T7, hosts for amber mutants of some of these phages should be searched for. Such studies will substantially contribute to the long range goal of establishing a detailed pedigree of what seems to constitute the large and diverse group of T7-like phages. As a result of this, the problems of phage systematics and taxonomy (Bradley, 1967; Wildy, 1971; Fenner, 1976) might also be more easily resolved.

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REFERENCES

Non-coli phages related to T7


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