Pasteurella pestis Bacteriophage H and *Escherichia coli* Bacteriophage ϕ II Are Nearly Identical

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Electron microscopy examination of ϕ II-H deoxyribonucleic acid heteroduplexes, together with polyacrylamide gel electrophoretic analyses of phage ribonucleic acid species and proteins labeled in ϕ II or H-infected cells, demonstrates that *Pasteurella pestis* phage H is nearly identical to coliphage ϕ II.

Phage H was isolated as a virulent phage on *Pasteurella pestis* and is currently used as a clinical test in the rapid identification of the causative agent of bubonic plague (2). (The correct genus name for *Pasteurella* is now *Yersinia*. However for historical reasons we have chosen to retain the name *Pasteurella* in this report.) Molnar and Lawton (11) discovered that H would grow on female but not male strains of *Escherichia coli* and that H is morphologically and physiologically related to some of the other female-specific coliphages. Williams and Meynell (20) showed H and ϕ II to be related because of their low efficiency of plating on certain F⁻ derivatives of *E. coli*. We report here biochemical comparisons which demonstrate, on the macromolecular level, that phage H is nearly identical to the coliphage ϕ II.

MATERIALS AND METHODS

Phage and bacterial strains. In all experiments, the bacterial host was *E. coli* B_s-1/T1 (SY106).

Phage H stocks were obtained from M.P. Oeschger, who had received it from W. D. Lawton (11), and from G. G. Meynell (20). Because there are reported differences in various laboratory strains of ϕ II, stocks of this phage were obtained from four sources: G. G. Meynell, who obtained it from R. Curtiss (20); M. H. Malamy, who obtained it from K. B. Low, who received it from E. Adelberg, who had obtained it at the Pasteur Institute in about 1959 (8); D. A. Monner who received it from J. Monod in 1968 (12); and from E. Wollman (21) through the good offices of B. Bachmann. This last ϕ II was in liquid suspension in a sealed ampoule dated 1953. These strains will be called ϕ IIM, ϕ IIT (8), ϕ IIW (13), and ϕ IIP, respectively.

Propagation and assay of phage. *E. coli* SY106 was grown in TY broth (16 g tryptone [Difco], 10 g

yeast extract, 5 g NaCl, per liter) at 35 to 37 C with shaking. Cells were infected in mid-log phase with phage at a multiplicity of 0.01-0.1. Lysates were concentrated with polyethylene glycol (22) and were purified by banding to equilibrium in CsCl buoyant density gradients. ϕ IIM and ϕ IIT each produced two bands in the gradients (only one of which contained infective phage), whereas ϕ IIW, ϕ IIP, and phage H each produced one sharp infective phage band. All bands were collected, dialyzed against 0.01 M Tris, 0.5 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and titered on *E. coli* SY106 on Luria agar plates by standard phage techniques (1).

Labeling of early RNA. The growth medium was low phosphate medium (LPM) which is TG medium (5), supplemented with dephosphorylated 2% Casamino Acids. The latter component was prepared as follows: 1 ml of 1 M MgCl₂, and then 1 ml of concentrated NH₄OH were added to 100 ml of 10% Casamino Acids (Difco). The MgNH₄PO₄ precipitate which settled out was removed by filtration, and the filtrate was neutralized with concentrated HCl and then sterilized. Cells were grown in LPM at 30 C with shaking to a density of approximately 5×10^8 cells/ml, ultraviolet (UV)-irradiated to 3,600 ergs/mm², and incubated at 30 C for an additional 10 min. Chloramphenicol was then added to 400 μ g/ml, and 5 min later a 2-ml sample of cells was infected with phage at a multiplicity of 10. Three minutes after infection, ³²PO₄⁻³ was added to a final concentration of 100 μ Ci/ml. Ten minutes after infection, the infected cells were poured over crushed ice and harvested by centrifugation. The RNA was extracted by the diethylpyrocarbonate method (18), precipitated overnight with ethanol at -20 C, and resuspended in 200 μ liters of buffer G (0.04 M Tris acetate, 0.02 M EDTA, 0.1% sodium dodecyl sulfate [SDS], pH 7.5) containing 20% glycerol.

Labeling of late RNA. Cells were grown to 5×10^8 bacteria per ml at 30 C in M9 buffer supplemented with 1% Casamino Acids and 1% glucose. At time zero, 5 ml of cells were infected with phage at

a multiplicity of 10; 5 min later, ^{14}C -adenine and ^{14}C -uracil were added to a final concentration of 0.2 $\mu\text{Ci/ml}$ of each; 12 min after infection, the cells were poured over crushed ice. The RNA was isolated as described above.

Labeling of proteins. Cells were grown in M9 medium at 30 C to 4×10^8 bacteria per ml, UV-irradiated to 3,600 ergs/mm², and incubated an additional 20 min at 30 C. Phage were added at a multiplicity of 10 to 20, and 3 min later 0.2 ml of the infected culture was labeled with a ^{14}C -amino acid mixture (final concentration 0.5 $\mu\text{Ci/ml}$). Fifteen minutes after infection, the label was chased for 4 min with 0.2 ml nonradioactive 10% Casamino acids solution, and the cells were chilled and harvested by centrifugation. The cells were resuspended in 0.025 ml lysing buffer (0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue [7]) and boiled for 2 min prior to electrophoresis.

Gel electrophoresis of RNAs and proteins. The RNA samples were analyzed by electrophoresis in 2.5% acrylamide gels containing 0.5% agarose (17) in a slab gel apparatus that is a modification of the one described by Reid and Bielecki (14). Forty-microliter samples of early RNA or 10- μ liter samples of late RNA were layered into the sample wells. Electrophoresis was at 75 v for 2.5 to 3 hr. At the end of the run of the early RNAs, the upper glass plate of the gel apparatus was removed, and the wet gel slab, supported by the lower plate, was covered with plastic film (e. g., Handi-Wrap) and exposed to X-ray film for one-half to several hours, depending on the sample. At the end of the run of the late RNA, the gel was dried, under vacuum over steam heat, and then was exposed to X-ray film for 1 to 5 days.

Proteins were analyzed by electrophoresis in 10 and 20% acrylamide gel slabs (16), using the Tris-SDS system of Laemmli (7). Ten- to twenty-microliter samples were applied per sample well in the stacking gel. Electrophoresis through the 10% gel slab was at 35 v for 14 hr; electrophoresis through the 20% gel slab was at 50 v in the stacking gel, and 100 v in the separating gel for a total of 12 hr. The gel slabs were dried under vacuum over steam heat, and contact autoradiographs were prepared by placing them with X-ray film for 4 days.

Construction of the heteroduplexes. Separated strands of ϕ II DNA and H DNA were prepared by the method of Summers and Szybalski (19) as modified by Davis and Hyman (3). The ϕ II-H heteroduplexes were constructed from the separated strands as previously described (3). In a few cases, the heteroduplexes were prepared starting with intact phage (Summers, Brunovskis, and Hyman, *in press*).

Electron microscopy. The heteroduplexes were mounted in the presence of formamide, which lowers the melting temperature (T_m) of DNA (10), and is used here to obtain denaturing conditions. The isodenaturing procedure has been described in detail (3). The formamide concentrations in the spreading solution and hypophase used here were 30% and 5%, respectively, which yield final conditions equivalent to 39 C below the melting temperature of a 50% guanine plus cytosine DNA (T_m -39 C); also 75% and

45% were used, which give conditions equivalent to T_m -6 C. The electron microscope procedures were those of Davis, Simon, and Davidson (4). For the heteroduplex mapping, 10 molecules were measured. For each molecule, lengths were expressed relative to the total. The length of the single-stranded DNA was corrected using the ratio of the lengths of single-stranded and double-stranded ϕ X174 DNAs mounted under identical conditions.

RESULTS

ϕ II-H heteroduplexes. All eight possible ϕ II-H DNA heteroduplexes among the various ϕ II strains and the two H stocks were constructed and observed in the electron microscope. All such heteroduplexes appeared identical; a sample electron micrograph showing the ϕ II-H heteroduplex is given in Fig. 1. At formamide concentrations equivalent to T_m -39 C, one nonhomology loop appears in the ϕ II-H heteroduplex. The loop starts 46% from one end and extends 0.93% to 53% from the other end. Each single-stranded DNA of the nonhomology loop contains about 300 bases. The limits on the size of an observable nonhomology at these formamide concentrations are that a loop of 50 bases is usually seen, but a loop of 20 is not seen (3). When the ϕ II-H heteroduplex is mounted at T_m -6 C, no additional nonhomology loops are seen. However, because of the problem of lateral aggregation (3), the loop previously seen in every heteroduplex at T_m -39 C appears with a frequency of only one in ten at T_m -6 C. It is therefore possible that loops of 100 to 200 bases might not be detected at T_m -6 C.

Early RNA synthesis. Radioactively labeled phage RNAs were extracted and were analyzed by electrophoresis on polyacrylamide gel slabs. The messenger RNAs in ϕ II or H-infected cells are sufficiently stable to be identified as bands on gels. Early RNAs, i. e., those transcribed by the *E. coli* RNA polymerase, were labeled with $^{32}\text{PO}_4^{-3}$ in phosphate-starved cells that had been treated with UV light and chloramphenicol prior to infection. (The irradiation sufficiently damages the host DNA so that no label enters host RNA, and the presence of chloramphenicol insures that no phage-coded proteins will be produced.) The patterns of early RNA from the four ϕ II infections appeared identical. A sample contact autoradiograph comparing the patterns of ϕ II M and H early RNAs is seen in Fig. 2. The RNAs range in size from about 0.24×10^6 daltons to 1.15×10^6 daltons (W. C. Summers, *unpublished observations*). All but one of the seven bands seen on the early RNA gel

FIG. 1. ϕ II-H DNA heteroduplex. The ϕ II-H heteroduplex was mounted in the presence of formamide, at a concentration equivalent to $T_m - 39^\circ\text{C}$. The grids were stained and shadowed with Pt-Pd (80%-20%). a, A complete ϕ II-H heteroduplex. The arrow points to the single, observable nonhomology loop. b, An enlargement of a ϕ II-H nonhomology loop. Note that the lengths of the two single-stranded DNAs of the nonhomology loop appear to be the same.

migrated identically. There is a difference in the position of the intense band near the middle of the gel; it migrates somewhat faster in the phage H sample. The finding that one RNA

band migrates differently might appear to indicate a major difference in two phage strains. However, analogous experiments with T7 and its various deletion mutants have demon-

FIG. 1B.

strated that an alteration in the early RNA banding pattern of the type seen here is the result of a difference in DNA content so small as to not be detected during electron microscope observation of the DNA heteroduplex. This single band difference has been interpreted as the consequence of the positions of termination and initiation sites relative to the

deleted regions (Summers, Brunovskis, and Hyman, *in press*; Simon and Studier, *personal communication*). Therefore, the difference in the ϕ II and H early RNA patterns may be minor.

Late RNA synthesis. RNAs synthesized late after infection with ϕ II or H were similarly analyzed in 2.5% polyacrylamide gels. Densi-

FIG. 2. Early RNA patterns of ϕ II and H. Electrophoresis was in a 2.5% acrylamide-0.5% agarose slab gel at 75 v for 2.5 hr. The gel was exposed to X-ray film for 3 hr.

tometer tracings of contact autoradiographs are shown in Fig. 3. Again, the ϕ II patterns are identical; the nine reproducible bands which are seen in the ϕ II late RNA gels are also present in the H late RNA gel. These RNAs range in molecular weight, relative to the 16S, precursor 16S, and 23S ribosomal RNAs from uninfected cells, from about 0.5×10^6 daltons to greater than 2×10^6 daltons. The slight differences between the ϕ II and H gels which are present in the positions corresponding to precursor 16S ribosomal RNA and 23S ribosomal RNA are not seen reproducibly in other gels; it is possible, however, that they may represent minor RNA species.

Phage protein synthesis. Finally, ^{14}C -labeled, phage-coded proteins synthesized after ϕ II or H infection were analyzed by electrophoresis in 10% and 20% polyacrylamide Tris-SDS gels. The 10% gel separates polypeptides by molecular weight in the range of about 20,000 daltons to 150,000 daltons (7); the 20%

gel allows observation of polypeptides of molecular weights smaller than 20,000 (16). Cells were irradiated with UV light prior to infection to eliminate host protein synthesis. Autoradiographs of the dried gels are shown in Fig. 4. Within the limits of detection by this system, the proteins of all four ϕ II strains are of the same size. Of the approximately 20 polypep-

FIG. 3. Late RNA patterns of ϕ II and H. Densitometer tracings were made of autoradiographs of 2.5% polyacrylamide gels of the RNAs synthesized 5 to 12 min after infection by ϕ II and H. Migration is right to left and is inversely related to molecular weight. The positions of the RNAs of the uninfected control (23S ribosomal RNA, the precursor 16S ribosomal RNA, and the mature 16S ribosomal RNA) are indicated near the top.

FIG. 4. Patterns of protein synthesis in UV-irradiated *E. coli* SY106 after infection with H and ϕ II. In the 10% polyacrylamide Tris-SDS gel, electrophoresis was at 35 v for 14 hr. In the 20% gel, electrophoresis was at 50 v in the stacking gel and 100 v in the separating gel for a total time of 12 hr. The gels were dried under vacuum over steam heat and exposed to X-ray film for 4 days. The regions where ϕ II and H differ are labeled a to d.

tides that separate clearly on the 10% gels, all but three or four appear identical in phage H and ϕ II. In the 80,000 molecular weight region, the phage H pattern has a slightly smaller band than the corresponding ϕ II band (Fig. 4a); in the 50,000 dalton region, there is a band in the H pattern not found in the ϕ II pattern (Fig. 4b); in the 20,000 dalton region, there is a band found in the ϕ II pattern and not in the H pattern (Fig. 4c); and in the 15,000 to 20,000 dalton region, there is a faint extra band in the H gel pattern (Fig. 4d). Whether these bands all represent different phage proteins or whether some are fragments or incomplete polypeptides is not known. It is clear, however, that the overall pattern of phage protein synthesis

as seen in these gels again emphasizes the near identity of ϕ II and H.

DISCUSSION

The data presented here suggest that *P. pestis* phage H and *E. coli* phage ϕ II are sufficiently similar to be considered minor variants of each other. Their DNAs are close to 99% homologous. The patterns of RNA and protein synthesis also indicate that they are nearly the same.

It is known that host range mutants are readily selected when coliphage ϕ II is propagated on hosts other than *E. coli* B (e.g., various strains of *E. coli* K12) (12). For this reason, phage H (obtained from two sources,

although there was no reason to suspect heterogeneity in H) was compared to four different ϕ II strains (in which there are known heterogeneities). The results were independent of the source of ϕ II or H. These considerations suggest that the differences among the various ϕ II strains are probably the result of point mutations or other small alterations in the DNA coding for the phage tail structures. Interestingly, during the purification of the phage from crude lysates, ϕ II P and ϕ II W, the two ϕ II strains which are known to have been propagated solely in *E. coli* B, each produced one band of infective particles in CsCl buoyant density gradients. The other two ϕ II strains, ϕ II M and ϕ II T, each produced an additional band of noninfective particles, which is reported to consist of phage particles without tails (9).

Previous studies have suggested a relatedness between *Pasteurella* phage and coliphage. Hertman (6) concluded from serological studies that the tails of *P. pestis* phage Y and coliphage T3 possess a common antigen, although T3 does not plaque on *P. pestis*. Molnar and Lawton (11) showed H to be related in morphology and in the physiology of infection to other female-specific phage, including ϕ II. H was found to be particularly similar to ϕ II in its female specificity; the efficiency of plating of both ϕ II and H on certain F^+ strains was 10^{-9} . These authors reported that H and ϕ II differed in that ϕ II adsorbed to both F^- and F^+ strains, but H adsorbed only to F^- strains. However, in view of the wide variability in the plating efficiencies of ϕ II on various F^+ and F^- strains, as reported by Williams and Meynell (20), and Monner and Boman (12), and the near identity of ϕ II and H as reported here, the differences observed by Molnar and Lawton could be the result of the method of propagation of the phage or the particular F^+ strains used.

The techniques used in the present study will not detect most single base pair differences, or other small differences which can produce distinguishable biological characteristics. However, on the biochemical or macromolecular level, *P. pestis* phage H and coliphage ϕ II are nearly identical. These observations question the prudence of using phage H in the clinical identification of *P. pestis* and support the view (15) that *P. pestis* is taxonomically related to the Enterobacteriaceae.

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