

Characterization and Comparison of the DNAs of the Three Closely Related Bacteriophages gd, ge and gf with the Genome DNA of the Hydrogen-oxidizing Host Strain *Pseudomonas pseudoflava* GA3

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SUMMARY

The double-stranded (ds)DNAs of the three closely related temperate *Pseudomonas pseudoflava* bacteriophages gd, ge and gf were studied biochemically and biophysically. The GC content of the DNA was $67.4 \pm 0.5\%$ and differed only slightly from that of the host *P. pseudoflava*. By electron microscopic length measurements a mol. wt. of 26.1×10^6 to 26.7×10^6 was calculated for the three bacteriophage DNAs. Homogeneity of the bacteriophage DNAs was further demonstrated by specific cleavage with restriction endonucleases *EcoRI* and *HindIII*. It was concluded that the three homo-immune bacteriophages are identical. The genome size of the host *P. pseudoflava* GA3 was 3.7×10^9 as calculated from optical renaturation rate measurements with *Xanthomonas pelargonii* reference DNA. The bacteriophage gd genome thus amounts to 0.7% of the chromosome of this bacterial host.

INTRODUCTION

Since site-specific endonucleases are available, temperate phages are playing a role as molecular vehicles for cloning of foreign DNA in bacteria (Murray & Murray, 1974). Considering the trend of applying these techniques to non-coliform bacteria of different physiological types, there might be more interest in temperate phages of the obligately or facultatively chemoautotrophic soil bacteria. Among the chemoautotrophs, which can derive their carbon from CO₂ via the Calvin cycle and generate their energy from respiration of inorganic substrates, the hydrogen bacteria are specialized for the oxidation of molecular hydrogen.

For the hydrogen-oxidizing soil bacterium *Pseudomonas pseudoflava* (Auling *et al.* 1978) temperate bacteriophages have been isolated (Auling, 1975; Auling *et al.* 1977) capable of infecting *P. pseudoflava* under both heterotrophic and autotrophic conditions. Three of these phages (gd, ge and gf) proved to be homo-immune and were found to be similar in many respects (Auling, 1978). This paper presents the characterization of the DNAs of these phages and a comparison with the DNA of the host *P. pseudoflava*.

METHODS

Bacteria, bacteriophages and growth conditions. The phages were propagated on their original *P. pseudoflava* host strains (gd on GA3, ge on GA4 and gf on GA5). High-titre phage stocks were prepared and purified as described by Auling (1978). *Xanthomonas pelargonii* ICPB P121, kindly provided by J. De Ley as a reference for the mol. wt. determination of the bacterial host genome, was grown on Nutrient Broth (NB; Difco Laboratories, Mich., U.S.A.) as nutritionally complex medium.

Preparation of phage and bacterial host DNA. CsCl-purified phage samples with a titre of about 2×10^{10} p.f.u./ml were used for preparation of phage DNA. Volumes of phage (5 to 10 ml) with an A_{260} of 3 to 5 were stabilized before extraction of DNA by a two-step dialysis against 1 l of 10 mM-potassium phosphate buffer, pH 7.0, in order to avoid premature lysis of phages and leakage of bacteriophage DNA. Gentle disruption of phage protein coat was obtained by heating phage samples in 0.25% SDS for 5 min at 60 °C, after which time the blue opalescence disappeared. The phage DNA was extracted with phenol (Mandell & Hershey, 1960). The aqueous extract (10 to 20 ml) was dialysed at 4 °C against 3 l of tris-HCl buffer, pH 8.0, in the following order: 1 l of 20 mM-tris plus 1 mM-NaCl plus 2 mM-EDTA, 1 l of 20 mM-tris plus 10 mM-NaCl plus 2 mM-EDTA and 1 l of 10 mM-tris plus 1 mM-EDTA. The phage DNA was concentrated by high speed centrifugation (150000 g, 4 °C, 15 h), resuspended in 0.5 ml of 10 mM-tris-HCl buffer plus 1 mM-EDTA, pH 8.0, and stored at 4 °C. Purified DNA of λ c₈₅₇ S7 bacteriophage was provided by M. Reh. *Neurospora crassa* mitochondrial (mt)-DNA was isolated from the wild-type strain EM 5256 (Strain 424, Fungal Genetic Stock Center, Arcata, Calif., U.S.A.) as described by Bernard *et al.* (1976).

Bacterial DNA was prepared as described (Auling *et al.* 1980). DNA concentration was calculated assuming that a solution with A_{260} of 1 contains 50 µg DNA/ml (Hereford & Rosbash, 1977).

DNA melting curves. Samples of concentrated phage DNA and purified host DNA were dialysed against 1 × SSC buffer, pH 7.0. Thermal denaturation was carried out in a Pye Unicam SP 1800 spectrophotometer in 1 × SSC buffer, pH 7.0 by the method of Mandel & Marmur (1968) as described by Baumgarten *et al.* (1974). *Escherichia coli* K12 DNA ($T_m = 90.6$ °C; De Ley, 1970) was used as control DNA in each determination.

Determination of the buoyant density of DNA. Buoyant density determinations were performed in a Spinco model E analytical ultracentrifuge equipped with a Kel-F centre-piece. Buoyant density was determined either by using a second DNA of known density in the same run or by absolute determination with the isoconcentration point method using the fact that after centrifugation to equilibrium the buoyant density at the isoconcentration point equals the buoyant density of the solution before centrifugation (Ifft *et al.* 1961; Szybalski, 1968). The reference DNA was extracted from the *Escherichia coli* bacteriophage λ c₈₅₇ S7. The value of the buoyant density was taken from the wild-type bacteriophage λ ($\rho = 1.7093$ g/ml; Kropinski, 1974). About 1 µg/ml of DNA (final concentration) was centrifuged in 5.8 M-CsCl in 1 mM-EDTA plus 10 mM-tris-HCl buffer, pH 8.0, for 22 h at 25 °C and 44000 rev/min. Buoyant density was calculated according to Schildkraut *et al.* (1962).

Electron microscopy of bacteriophage DNA. Electron microscopy of bacteriophage DNA was performed as described by Mayer *et al.* (1973). Electron micrographs were taken with a Philips EM 301 electron microscope at primary magnifications between 7000 and 15000 calibrated with a cross-lined grating replica.

Specific fragmentation of bacteriophage DNA with EcoRI and HindIII restriction endonucleases. Digestion with restriction endonuclease EcoRI (Miles GmbH, Frankfurt, West

Germany) was performed in a reaction mixture of 100 μ l containing 100 mM-tris-HCl, pH 7.5, 50 mM-NaCl, 10 mM-MgCl₂, 2 μ g DNA and 1 μ l of enzyme stock solution. The digestion was completed after 20 min incubation at 37 °C. Digestion with *Hind*III (Miles) was performed in 10 mM-tris-HCl, pH 7.5, 50 mM-NaCl, 10 mM-MgCl₂ and 0.1 mg/ml bovine serum albumin at 37 °C.

Gel electrophoresis of restriction fragments was performed in 0.5% agarose (MC1 SeaKem, Biomedical Division of Marine Colloids, Inc., Rockland, Me., U.S.A.) containing 36 mM-tris-HCl, pH 7.8, 30 mM-sodium phosphate, 1 mM-EDTA, for 16 h at 4 °C, 30 V, in glass tubes of 1 cm diam. Gels were stained in an aqueous solution of ethidium bromide (0.5 μ g/ml) and photographed with Kodak-Panatomic X-film through an orange filter.

Determination of the genome size of the host bacterium. A combination of the method of Gillis *et al.* (1970) and of the procedure of Bradley (1973) was developed for optical determination of the reassociation kinetics of DNA in sodium phosphate buffer. For reduction of temperature, formamide was added to the adjusted DNA samples, giving a final solvent concentration of 25% (v/v) formamide and 0.5 M-sodium phosphate, pH 7.0. The denaturation-renaturation procedure as described in detail by Auling *et al.* (1980) was repeated five to six times for each sample.

Abbreviations. ICPB = International Collection of Phytopathogenic Bacteria (Davis, Calif., U.S.A.; T_m = mid-point of thermal denaturation of DNA; SSC = standard saline citrate buffer (150 mM-NaCl, 15 mM-sodium citrate, pH 7.0).

RESULTS

Melting temperatures and buoyant densities of phage and host DNAs

The melting curves obtained from samples of bacteriophage gd DNA and of *P. pseudoflava* GA3 DNA are shown in Fig. 1. The curves are very similar and have the shape characteristic of dsDNA molecules. The absorbance of the phage DNA did not increase below 85 °C, also indicating the lower mol. wt. of the phage DNA compared to the host DNA. The steeper melting curve of the phage DNA relative to the bacterial DNA sample may also result from greater homogeneity in base pair distribution in the smaller DNA of the phage. The overall hypochromicity was approx. 35%. Similar melting profiles were observed for the other phage and host DNAs. Chemical similarity between phage and host DNA is also revealed by density-gradient equilibrium sedimentation analysis resulting in a difference in density between ge DNA and GA4 DNA of only 0.002 g/ml. An average buoyant density of 1.727 g/ml was measured for the bacteriophage DNAs by the isoconcentration point method as well as in runs with reference DNA. A mixture of all three bacteriophage DNAs banded in one peak in an isopycnic CsCl density gradient (Fig. 2). Both from buoyant density and from thermal denaturation studies nearly identical average % GC ratios were obtained for phage and host DNAs by calculation according to Schildkraut *et al.* (1962) and with equation 10 of De Ley (1970). The data are summarized in Table 1.

Electron microscopy of bacteriophage DNAs

Electron microscopy of the *P. pseudoflava* bacteriophage DNAs revealed circular (Fig. 3) as well as linear molecules. Comparison of contour lengths of both configurations of the DNAs of the three phages gd, ge and gf showed no significant differences. Linear and circular DNA molecules of double and triple contour lengths were also observed, but the portion of the molecules with triple contour length was relatively small. The circular DNA molecules as well as the linear ones with double and triple contour length could be eliminated by short pre-treatment with heat (5 min at 70 °C) followed by rapid cooling in ice before preparing the droplets for electron microscopy. When the cooling step after heat treatment was

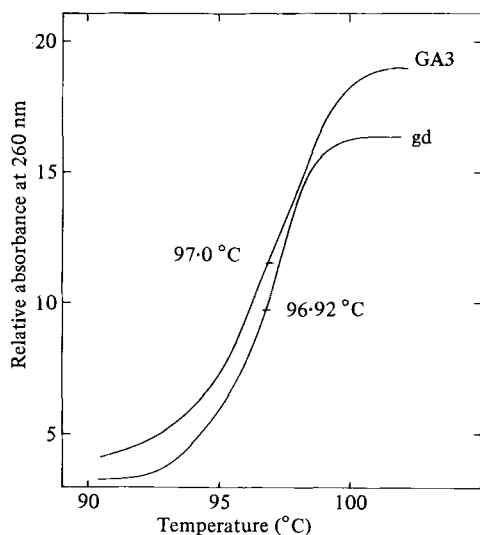


Fig. 1

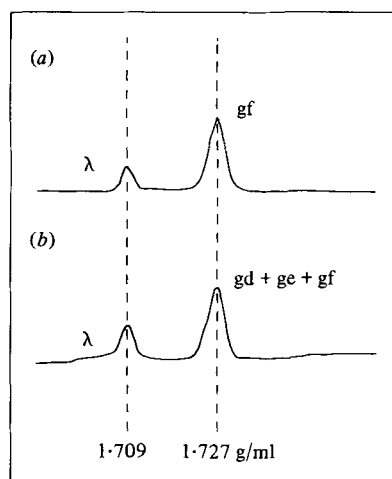


Fig. 2

Fig. 1. Thermal denaturation of bacteriophage gd DNA and *P. pseudoflava* GA3 host DNA in $1 \times$ SSC. Values for absorbance at 260 nm were corrected for thermal expansion.

Fig. 2. Microdensitometer tracings of u.v. photographs of *P. pseudoflava* bacteriophage DNA from CsCl equilibrium centrifugations: (a) gf DNA and λ -DNA; (b) a mixture of gd DNA, ge DNA and gf DNA, centrifuged together with λ -DNA as density reference.

Table 1. *Bacteriophage and host DNA base composition, calculated from average buoyant densities and T_m values*

Source of DNA	Buoyant density	%GC	T_m value	% GC
Phage gd	1.727	67.9	96.9	67.1
Phage ge	1.727	67.9	96.8	66.9
Phage gf	1.727	67.9	96.8	66.9
<i>P. pseudoflava</i> GA3	—	—	97.0	67.3
<i>P. pseudoflava</i> GA4	1.725	66.5	96.7	66.6
<i>P. pseudoflava</i> GA5	—	—	96.6	66.4

omitted, circular DNA molecules reappeared with time. For the calculation of the mol. wt. of the phage DNAs, the measured contour lengths (in μm) of DNA preparations at ionic strength 0.2 were multiplied by the factor 2.07×10^6 (Lang, 1970). The measured length values and calculated mol. wt. are summarized in Table 2.

Preliminary electron microscopic studies of partially denatured gd DNA revealed no circular permutation of the bacteriophage genome (F. Mayer, unpublished results).

Fragmentation analysis of bacteriophage DNAs with restriction enzymes

When DNA samples of the three CsCl-purified *P. pseudoflava* bacteriophages were analysed on 0.5% agarose gels before digestion with restriction enzymes, unexpected heterogeneity was found after staining with ethidium bromide: two clear bands, lying close together, could be seen, and a less distinct as well as a rather faint band were detected at regions of considerably higher mol. wt. (Fig. 4a).

Fingerprint analysis by digestion with restriction endonucleases *Eco*RI (Fig. 5) and *Hind*III resulted in identical fragmentation patterns in the agarose gel electrophoresis for the three phages gd, ge and gf. The *Hind*III-treated samples exhibited only two distinct bands at nearly the same position in the gels (Fig. 4b). Five major and two minor bands

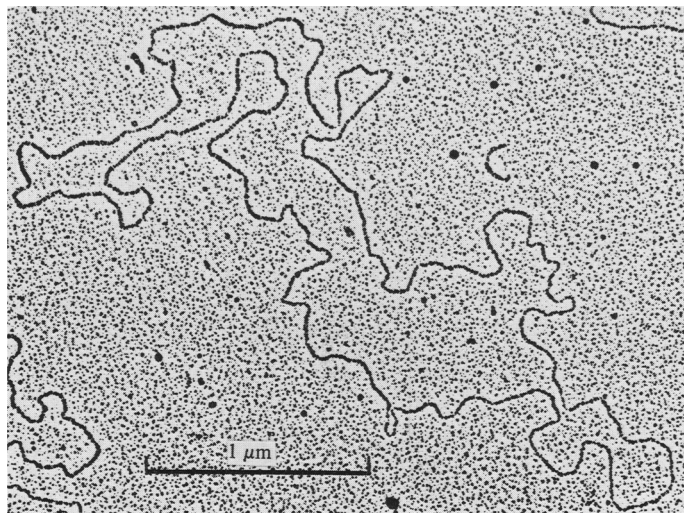


Fig. 3. Electron micrograph of a circular molecule of bacteriophage gd DNA. Metal shadowing, $\times 35000$.

Table 2. *Electron microscopic length measurement of the DNAs of the P. pseudoflava bacteriophages gd, ge and gf**

Phage	Measured contour length (μm)	Calculated mol. wt.
gd	$12.91 \pm 2.6 \%$	$26.7 \times 10^6 \pm 2.6 \%$
ge	$12.66 \pm 2.5 \%$	$26.1 \times 10^6 \pm 2.5 \%$
gf	$12.81 \pm 2.4 \%$	$26.5 \times 10^6 \pm 2.5 \%$

* Fifteen molecules were measured for calculation of mol. wt. of gd DNA and ten molecules for both ge and gf DNA.

were found after digestion of the three phage DNAs with *Eco*RI (Fig. 4c). When the *Eco*RI-digested samples were heated (5 min at 70 °C) and rapidly cooled on ice prior to separation on agarose gels, one of the distinct bands ($\overset{\circ}{C} + \overset{\circ}{E}$) disappeared almost completely and the intensity of the two otherwise faint bands ($\overset{\circ}{C}$, $\overset{\circ}{E}$) was enhanced to the level expected for a specific fragmentation of normal stoichiometry (Fig. 4d). By double-digestion of the bacteriophage DNA with both restriction enzymes, cleavage of the large *Eco*RI fragment A into two smaller fragments (A'_1 and A'_2) was obtained (Fig. 4e).

Approximate determination of the mol. wt. of *P. pseudoflava* bacteriophage DNA was tried by analytical agarose gel electrophoresis. Mitochondrial (mt)-DNA from *Neurospora crassa* (wild-type 5256), gd DNA and λ -DNA were digested by *Eco*RI and separated on parallel gels. The electrophoretic mobilities of the gd DNA fragments (with omission of that band we assumed to represent the annealed fragments $\overset{\circ}{C} + \overset{\circ}{E}$) were plotted against the log of the known mol. wt. of the *Eco*RI fragments of the reference DNAs (λ -DNA: data from Allet *et al.* 1973; *N. crassa* mt-DNA: data from Bernard *et al.* 1975). By summation of the graphically determined mol. wt. of the *Eco*RI fragments of gd DNA (Fig. 6) a total mol. wt. of 23.8×10^6 was calculated with λ -DNA as reference, and 29.7×10^6 with *N. crassa* mt-DNA as reference.

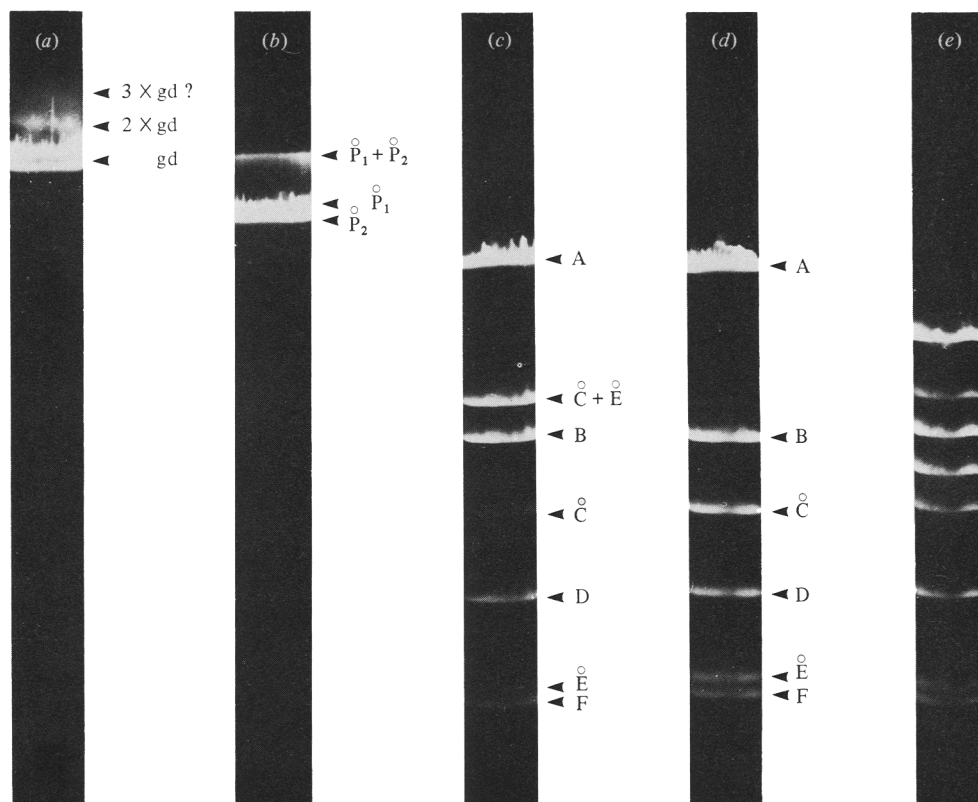


Fig. 4. Analytical agarose gel electrophoresis of bacteriophage gd DNA, before and after treatment with restriction endonucleases. (a) Unfragmented gd DNA: the two bands designated gd represent the linear and circular configuration of the monomeric DNA molecule; (b) gd DNA fragmented by *Hind*III into $\bar{P}_1$ and $\bar{P}_2$; (c) gd DNA fragmented by *Eco*RI into A, B, C, D, E and F; (d) gd DNA fragmented by *Eco*RI, heated for 5 min at 70 °C and rapidly cooled in ice before separation on agarose gels; (e) *Eco*RI and *Hind*III double-digest of gd DNA, compared to digestion of gd DNA with *Eco*RI alone (c). In (e) the large *Eco*RI fragment A has disappeared and two new bands named A'_1 and A'_2 are to be seen due to the digestion of *Eco*RI fragment A. The bands $\bar{P}_1$, $\bar{P}_2$, C and E represent restriction fragments possessing one of the 'sticky ends' of the undigested gd molecule. The bands C+E, and $\bar{P}_1+\bar{P}_2$ arise by binding of fragments due to the complementary sequences of the cohesive ends.

Determination of the genome size of the bacterial host DNA

The mol. wt. of the bacterial host DNA was determined from initial optical renaturation rate measurements (Gillis *et al.* 1970) with the formamide modification described by Auling *et al.* (1980) by comparison of the apparent renaturation rates of *P. pseudoflava* GA3 DNA to *X. pelargonii* ICPB P121 DNA. Gillis *et al.* (1970) described the haploid genome of *X. pelargonii* as having a mean GC content of 66.5% and a mol. wt. of 2.4×10^9 . As both bacteria had a similar mol. % GC no corrections for differences in the GC content were necessary. From six independent experiments the total mol. wt. of the haploid genome DNA of the bacteriophage host *P. pseudoflava* GA3 was determined as 3.7×10^9 .

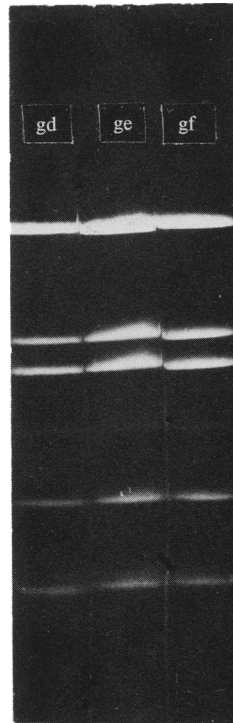


Fig. 5. Fragmentation patterns of the DNAs of the *P. pseudoflava* bacteriophages gd, ge and gf after digestion by restriction endonuclease *EcoRI*.

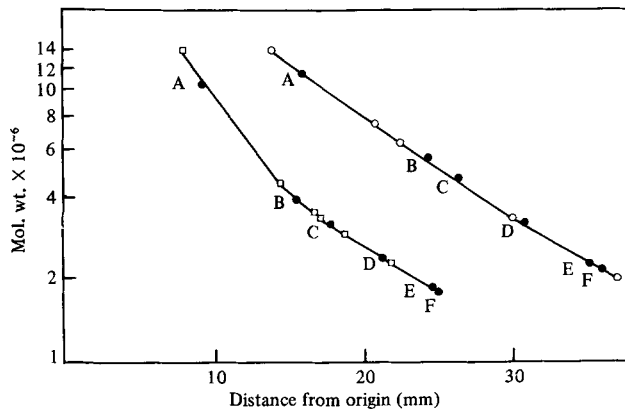


Fig. 6. Calibration graph for approximate mol. wt. determination of bacteriophage gd DNA by agarose gel electrophoresis. Mol. wt. of *EcoRI* fragments of gd DNA (●), λ -DNA (□) and *N. crassa* mt-DNA (○) plotted against electrophoretic mobility in agarose gels.

DISCUSSION

The biophysical and biochemical studies on the DNAs of the three *P. pseudoflava* bacteriophages gd, ge and gf indicated a nearly perfect agreement on many essential properties. This homology of the three bacteriophage nucleic acids is revealed by the results summarized in Table 1 and Table 2 and by the identical agarose patterns after digestion with *EcoRI* (Fig. 5) and with *HindIII* restriction enzyme. Taken together with the data on the properties of the bacteriophage protein coats and on their biological relatedness (Auling, 1978; Auling

et al. 1977), one can conclude that the three *P. pseudoflava* bacteriophages gd, ge and gf are identical.

The bacteriophage DNAs were found to have a GC content very similar to that of the respective host DNAs (Table 1). The GC contents calculated from thermal denaturation and from buoyant density studies strongly suggest that no unusual bases or sugars are present in the three bacteriophage DNAs (Mandel & Marmur, 1968). Even with the modified regression equations derived specially for bacteriophage DNAs by Kropinski (1974), only a small discrepancy of about 3 % in the GC content was obtained from both methods.

The dsDNAs of the three bacteriophages gd, ge and gf should have cohesive ends like the lambdoid phages, as different configurations of the phage DNA molecules were observed by electron microscopy of untreated samples. By using conditions which ensure the melting of short stretches of hydrogen bonding, different configurations were reduced to one linear form. The most likely explanation is the presence of 'sticky ends' in the phage genome. Base pairing of the cohesive ends of the same DNA molecule would result in the formation of a monomeric circle, and linear and circular DNA molecules of double and triple contour length would be formed by base pairing of the cohesive ends of different DNA molecules. The unexpected heterogeneity of the undigested phage nucleic acids which had been isolated from CsCl-purified phage can also be explained by the presence of cohesive ends in the phage genome DNAs. In addition, some aspects of the fingerprint analyses from the experiments with restriction enzymes *EcoRI* and *HindIII* can be explained by the presence of 'sticky ends' in the bacteriophage DNA molecules, e.g. the effect of heating on the mobility and number of bands produced by *EcoRI* (Fig. 4c, d).

It may be useful for further studies that the cleavage of the phage DNAs by *HindIII* resulted in only two fragments of similar size. The enzyme cuts the bacteriophage DNAs in the middle of the molecule and its recognition site lies on the large *EcoRI* fragment A.

Determinations of the bacteriophage genome size were tried by three independent methods. The mol. wt. observed by graphic determination from CsCl equilibrium banding experiments of gd DNA was unexpectedly low and was therefore neglected. Mol. wt. values derived by this type of density gradient experiment are substantially lower than those obtained by other methods (Eigner, 1968). Summation of the mol. wt. of *EcoRI* fragments of bacteriophage gd DNA calibrated on analytical agarose gels with reference DNA fragments gave two different values for the mol. wt. of the *P. pseudoflava* bacteriophage DNA: 23.8×10^6 and 29.7×10^6 . This discrepancy might result from the difficulty in calibrating the larger DNA fragments in 0.5 % agarose gels. The mol. wt. calculated on the basis of length determination of the bacteriophage DNAs in the electron microscope (26.1×10^6 to 26.7×10^6) lie between the two values obtained by analytical agarose gel electrophoresis. We consider the values obtained by electron microscopy to represent the real mol. wt. of the DNA of the *P. pseudoflava* bacteriophages gd, ge and gf. The mol. wt. of the *P. pseudoflava* bacteriophage DNAs are close to those obtained from a variety of other bacteriophages, e.g. from T₇ of *E. coli*, P22 of *S. typhimurium*, $\phi 105$ and SPP-1 of *B. subtilis*, 13 vir of *Proteus* and PL 25 of *Providencia* (MacHattie & Thomas, 1970). From the mol. wt. of their DNAs the phages gd, ge and gf should have about 30 genes.

As the independently determined genome size of the bacterial host *P. pseudoflava* GA3 was 3.7×10^9 , one can calculate that the phage genome amounts to 0.7 % of the host genome. Until now, the status of the *P. pseudoflava* bacteriophages after lysogenization of the host (either prophage or plasmid) has remained uncertain.

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