Morphological and Molecular Characterization of Several Actinophages Isolated from Soil Which Lyse *Streptomyces cattleya* or *S. venezuelae*

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Several lytic and lysogenic actinophages were isolated from soil samples infected with *Streptomyces cattleya* and *S. venezuelae*. The morphologies and some biological properties of the phages, and the physico-chemical characteristics of their DNAs, were compared. Electron micrographs indicated that all the phage heads were of an icosahedral form, but head size and length of the tail varied. Two of the phages had a broad host range; the other isolates could lyse only a limited number of species. The molecular sizes of the phage DNAs were between 32.2 and 98.5 kb as estimated by electron microscopy and restriction enzyme analysis. The same study also indicated that one of the DNA species contained cohesive ends. The G + C content of the DNAs ranged between 45.1 and 74.2 mol % as estimated from melting studies. Sedimentation velocity experiments implied that several of the phage DNAs were probably heavily glycosylated or methylated. These modifications might explain the partial or slow digestion of some of the DNAs by several of the 23 restriction enzymes tested. Protoplasts of the appropriate *Streptomyces* strains could be efficiently transfected with phage DNA in the presence of 25% (w/v) polyethylene glycol (mol. wt 6000).

INTRODUCTION

Actinophages have been isolated and investigated for a variety of reasons, including: for the problems they cause in the fermentation industry, as taxonomic tools for typing of streptomycetes (Bradley *et al.*, 1961) and in genetic studies (Lomovskaya *et al.*, 1980). The progress in the development of phage vectors has resulted in actinophages becoming of increasing interest in recombinant DNA technology (Chater, 1980; Chater *et al.*, 1982; Lomovskaya *et al.*, 1982).

The usefulness of a bacteriophage as a vector in recombinant DNA technology depends on its DNA and the phage host range. Ideally, the phage should have a wide host range, a genome which can be deleted for some non-essential regions, and target sites for several common restriction enzymes. It must also be possible to transfect the phage DNA or its recombinant molecules. With these ideas in mind we have screened several soil samples for actinophages which could lyse strains of the industrially-important *Streptomyces cattleya* or *S. venezuelae* and we have performed a morphological and molecular characterization of the isolates obtained. In addition, the possibility of transfection was tested using polyethylene glycol (PEG) (Suarez & Chater, 1980; Isogai *et al.*, 1980).

METHODS

Organisms. Phages from soil samples were isolated using *Streptomyces cattleya* NRRL 8057 and *S. venezuelae* ETH 14630 as indicator strains. The host range of the different phages isolated was checked for the following

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Streptomyces strains: *S. ghanaensis* Hoechst 5/1; *S. ederensis* ATCC 15304; *S. geysiriensis* ATCC 15303; *S. bambergeniensis*; *S. coelicolor* M130 (John Innes Institute, Norwich Collection); *S. lividans* 1326 (John Innes Institute, Norwich Collection); *S. clavuligerus* NRRL 3585; *Streptomyces* Y5633 (FH 1426); *S. glaucescens* (ETH 566); *S. tendae*; *S. aureofaciens*; *S. lipmanii* ATCC 27357; *S. olivaceus* ATCC 21379; *S. griseus*; *S. diastaticus* NRRL 2650; *S. antibioticus* ATCC 8663; *S. aurantiogriseus* ATCC 27435; *S. pristinaespiralis* CBS 914.69; *S. rimosus* CBS 938.68; *S. loidensis* ATCC 11415; *S. erythreus*; *S. numidans* R760; *S. griseosporus* ATCC 27435; *S. osteogriseus* NRRL 2558; *S. virginiae*; *S. graminofaciens* ATCC 12705.

Media and buffers. *Streptomyces* strains were cultivated on Penassay medium (Antibiotic medium III, Oxoid) or RGMC which contained (g l⁻¹): tryptone, 10; yeast extract, 1; NaCl, 8; glucose, 1; MgCl₂, 1; CaCl₂ · 2H₂O, 0.3. For phage propagation the strains were grown on phage medium (Korn *et al.*, 1978). Phages were collected, stored or diluted in phage buffer, i.e. (g l⁻¹): bacto-peptone, 1.0; CaCl₂ · 2H₂O, 0.74; pH 7.2. Phage DNA was stored in TE-buffer composed of 1 mM-EDTA and 10 mM-Tris, pH 7.5. For protoplast production *Streptomyces* mycelium was grown in medium S (Okanishi *et al.*, 1974) supplemented with 1% glycine. Protoplasts were stabilized in medium P and regeneration was on R2YE-medium (Chater *et al.*, 1982).

Isolation of *Streptomyces* phages. To detect the possible presence of phages in soil samples, 100–200 g samples were incubated for one week in phage medium containing mycelial fragments (10⁷ c.f.u. ml⁻¹) of the indicator strain, i.e. *S. venezuelae* or *S. cattleya*, either at room temperature (with shaking at intervals) or at 30 °C (with shaking at 120 r.p.m.). After incubation, the samples were centrifuged (5 min, 3000 g) and then either sterilized by membrane filtration, or treated with a few drops of chloroform to kill all viable cells.

The supernatant solutions were investigated for the presence of *Streptomyces* phages using the soft agar overlay method. A 1 ml sample of a homogenized overnight culture of a susceptible strain (10⁷ c.f.u. ml⁻¹) grown in liquid phage medium was mixed with 0.1 ml of the supernatant solution to be investigated and 2 ml of soft phage medium agar (at 45 °C). This suspension was subsequently poured onto plates of phage medium. The homogenized mycelium suspension could be stored at 4 °C for at least one week.

Phages were detected by the appearance of plaques in the bacterial lawn after overnight incubation at 30 °C. Distinct phages could be distinguished by differences in plaque morphology, size and turbidity. To purify different phage types, serial replating from single plaques was carried out.

One phage (P23) used in the experiments was obtained from Dr F. Korn, Technische Hochschule, Darmstadt, FRG.

Preparation of high titre lysates. To obtain phage suspensions of high titres, confluent plate lysates were prepared by plating 10⁴–10⁵ p.f.u. per plate and incubating overnight. Phages from these plates were collected by addition of 5 ml phage buffer and soaking at room temperature for 30 min. Shaking-flask lysates were prepared by the addition of 10⁵ p.f.u. to 200 ml cultures containing homogenized mycelium (10⁷ c.f.u. ml⁻¹). Incubation was carried out at 30 °C and 120 r.p.m. for 40 h. Bacteria were removed by centrifugation (10 min, 4000 g). Phage preparations were sterilized by treatment with chloroform.

Purification of phages and isolation of phage DNA. Phage particles were concentrated by centrifugation at 150000 g for 1 h. Phage pellets were resuspended in a minimal volume of phage- or TE-buffer, and treated with a small amount (10 µg ml⁻¹) of DNAase and RNAase (1 h, 37 °C). Purification was achieved by CsCl gradient centrifugation (5.5 g CsCl in 7 ml TE-buffer) at 100000 g for 20–24 h. After collecting the phage band, which was grey-white in colour, CsCl was removed by dialysis against TE-buffer.

DNA was extracted using phenol saturated with 0.1 M-Tris/HCl buffer, pH 8.0. After gentle mixing and centrifugation in an Eppendorf centrifuge for 3 min, the aqueous phase containing phage DNA was removed and dialysed against TE-buffer.

Identification of temperate phages. Temperate phages could be distinguished from non-temperate phages by the appearance of lysogenic strains in the centre of the plaques. These lysogenic bacteria were immune to superinfection by the same phage. Furthermore, in a culture of a lysogenic strain, free phage particles would be detected, since lysogenic strains would spontaneously go into the lytic cycle at a low level. This transition to the lytic cycle could be increased by exposure to UV light (260 nm; germicidal lamp, Sylvania G8T5, USA).

Host range. Host range was determined using a qualitative spot test (0.1 ml phage suspension of 10¹⁰ p.f.u.) on lawns of different strains. If plaques appeared the strain was considered to be susceptible to the particular phage.

Protoplast formation and transfection. Mycelial fragments grown for 20 h in medium S containing 1% glycine were treated with lysozyme (10 mg ml⁻¹) in medium P for 15–45 min to induce protoplast production (Okanishi *et al.*, 1974). Protoplasts were separated from mycelial debris by low speed centrifugation (700 g, 5 min) and they were subsequently washed in medium P to remove lysozyme.

To transfect protoplasts, 5–10 µg phage DNA was added to about 10⁸ pelleted protoplasts followed by the addition of 0.5 ml of a solution of 25% (w/v) PEG, mol. wt 6000, in 0.01 M-CaCl₂ at pH 8.0, with 0.05 M-glycine/NaOH. After 1 min, 0.5 ml medium P was added, and the protoplasts were centrifuged and washed once more with 1 ml medium P. Transfection was observed by plaque formation in the lawn of bacteria regenerated from protoplasts on R2YE-medium.

Determination of G + C content of DNA. This was estimated both by buoyant density (θ) determination and by

determination of the thermal denaturation temperature (T_m). Buoyant density measurements were carried out according to Pühler *et al.* (1979); T_m values were estimated in 0.015 M-NaCl and 1.5 mM-sodium citrate (Marmur & Doty, 1962) using a double beam Beckman UV 5230 spectrophotometer at 260 nm with a thermocouple in the sample cuvette for temperature reading. The molar percentage base composition can be derived from sedimentation velocity studies using the formula $G + C = 10.12\theta - 16.74$ (Woodward & Lebowitz, 1980) and from thermal denaturation studies according to the equation $G + C = (T_m - 49.9)/0.48$ (Mandel & Marmur, 1968).

Electron microscopy of phages and phage DNA. Phage particles were examined after negative staining (Valentine *et al.*, 1968). For phage DNA the methods of Burkardt *et al.* (1978) were used.

Isolation of extrachromosomal DNA of *Streptomyces* spp. Mycelium of *Streptomyces* spp. cultivated in 50 ml medium S for 48 h was harvested by centrifugation (10 min, 26000 g), homogenized and washed twice in TE-buffer containing 10% (w/v) sucrose. As described by Wohlleben & Pühler (1982), washed mycelium was resuspended in 5 ml LI (containing 50 mM-glucose, 10 mM-CDTA, 25 mM-Tris, pH 8.0) supplemented with 10 mg lysozyme ml⁻¹, and the suspension was incubated for 30 min at 30 °C. Following protoplast formation 10 ml of an alkaline SDS solution (1% SDS in 0.2 M-NaOH) was added and gently mixed. This suspension was kept on ice for 5 min. After addition of 7.5 ml 3 M-potassium acetate, pH 4.8, the lysate was left on ice for a further 60 min. Proteins and chromosomal DNA were removed by centrifugation (25000 g, 15 min) and plasmid DNA was precipitated by the addition of 0.6 vol. 2-propanol. After 30 min at room temperature, the DNA was collected by centrifugation (25000 g, 15 min) and dried at 60 °C for 10 min. The DNA was further purified by buoyant density gradient centrifugation in CsCl (7 g in 7 ml) containing ethidium bromide (8.5 mg ml⁻¹). After centrifugation (85000 g, 45 h) the DNA band was visualized under UV light (300 nm), and was removed using a syringe. The ethidium bromide was extracted by isoamyl alcohol saturated with NaCl; CsCl was removed by dialysis against TE-buffer.

Restriction enzyme analysis of phage DNA. Phage DNA was incubated with 23 different restriction enzymes, using the assay conditions described by the manufacturers. Results of digestion were determined by agarose gel electrophoresis (1% agarose in Tris/acetate buffer, pH 7.8; 40 mM-Tris, 10 mM-sodium acetate, 1 mM-EDTA). Electrophoresis was carried out at 8 V cm⁻¹ for 2.5 h. After staining with ethidium bromide, DNA bands were visualized on a UV transilluminator at 300 nm and photographed on Polaroid film.

The presence of cohesive ends was investigated by electron microscopic analysis (open circular molecules which are formed spontaneously during the droplet preparation indicate complementary single-stranded ends) as well as by comparison of staining intensities of different fragments of restricted DNA in samples subjected to heating (70 °C) and rapid cooling with fragments not treated in this way. When cohesive ends are present, non-heated samples contain more fused sticky ends; heated (followed by rapid cooling) samples display more unfused cohesive ends. In addition, the sum of the lengths of unfused sticky ends should be equal to the length of the fragment containing fused cohesive ends.

Fragment sizes were determined by comparison of the electrophoretic mobility of the fragments with that of coliphage λ c1857S7 (Miller, 1972) fragments obtained after digestion with *EcoRI*, *HindIII* and the combined digestion *EcoRI* + *HindIII*.

RESULTS AND DISCUSSION

Isolation and morphological characterization of phages

Phage particles were detected in four out of the six soil samples tested. No real differences were observed in isolation efficiency between shaking cultures at 30 °C or standing cultures at room temperature. Using differences in plaque morphology and lysogeny as criteria, nine phage types could be isolated (Table 1). High phage titres, i.e. 10¹⁰–10¹² p.f.u. ml⁻¹, were obtained from confluent plate lysates.

Electron microscopic investigations of the different phages showed that all the phage heads were of an icosahedral form, but phage head size and tail length varied considerably (Table 1). Some of the phages (e.g. CPC) had a very long tail (Fig. 1*b*). Another type (CRK) had a very short tail and, in contrast to most of the other phages, showed no base plate (Fig. 1*a*). The extremely short tail of CRK might explain the formation of rather stable conglomerates giving rise to three separate phage bands after CsCl gradient centrifugation.

Host range

The host ranges of the different phages were examined by plaque formation on 27 different strains. All the phages except P23 and CPC had a relatively narrow host range; VWB could lyse

Fig. 1. Electron micrographs of different *Streptomyces* phages (a) CRK, (b) CPC, (c) VWB, (d) CTK. The bar markers represent 0.1 μ m.

Table 1. Morphological characteristics of different *Streptomyces* phages

Phage	Plaque morphology			Phage dimensionst (nm)				Base-plate-like structure
	Size* (mm)	Transparency	Edge	Head		Tail		
				L	W	L	W	
CPC	1.0-2.0	clear	sharp	104.1	74.4	330.3	11.9	+
CRK	1.5-3.5	clear	sharp	57.2	49.1	25.2	13.4	-
CT	2.5-4.5	clear	sharp	54.4	51.7	98.6	10.8	+
CPT	1.2-3.0	turbid	sharp	59.5	53.5	103.8	9.0	+
CTK	1.2-3.0	turbid	sharp	51.5	46.0	103.0	8.3	+
CWK	1.8-4.0	clear	sharp	45.3	42.5	106.8	10.1	+
VWB	0.1-0.8	clear	sharp	58.0	54.0	208.3	11.1	+
VPI	1.2-3.0	clear	~diffuse	81.8	71.4	303.5	14.1	-
VW3	1.0-3.0	clear	~diffuse	78.8	71.4	315.6	13.5	-
P23†	1.0-2.5	clear	sharp	97.0	90.0	313.3	18.7	+

* After 20h incubation.

† L, length; W, width.

‡ Obtained from Dr F. Korn (see Methods).

only one strain of the species tested (Table 2). The species *S. olivaceus*, *S. lipmanii*, *S. erythreus* and *S. clavuligerus* were most susceptible to the different phages isolated.

Fig. 2. Influence of UV irradiation (260 nm) on the release of phages from lysogenic strains. Lysogenic bacteria were grown for 24 h in phage medium and collected by centrifugation. After washing, respectively 0.5×10^7 , 2.0×10^7 and 1.0×10^7 c.f.u. ml⁻¹ of *S. venezuelae* VWB (■), *S. cattleya* CT (□) and *S. cattleya* CPT (●) were irradiated and the suspensions were tested for the presence of free phages.

Table 2. Physical characteristics of *Streptomyces* phages and their host range

Phage	Size (kb)		Host range
	From restriction enzyme analysis	From EM measurements	
Virulent			
P23	98.5 ± 14	ND	<i>S. venezuelae</i> , <i>S. cattleya</i> , <i>S. ghanaensis</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i> , <i>S. ederensis</i> , <i>S. geysiriensis</i> , <i>S. bambergensis</i> , <i>S. coelicolor</i> , <i>S. lividans</i> , <i>Streptomyces</i> Hoechst 5633, <i>S. glaucescens</i> , <i>S. tendae</i> , <i>S. aureofaciens</i> , <i>S. lipmanii</i> , <i>S. griseus</i> , <i>S. antibioticus</i> , <i>S. rimosus</i> , <i>S. loidensis</i> , <i>S. erythreus</i> , <i>S. numidans</i> , <i>S. griseosporus</i> , <i>S. graminofaciens</i>
VPI (= VW3)	95.4 ± 11	95.84 ± 4.85	<i>S. venezuelae</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i> , <i>S. lipmanii</i>
CPC	—	74.19	<i>S. cattleya</i> , <i>S. ghanaensis</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i> , <i>S. ederensis</i> , <i>S. geysiriensis</i> , <i>S. bambergensis</i> , <i>Streptomyces</i> Hoechst 5633, <i>S. glaucescens</i> , <i>S. griseus</i> , <i>S. rimosus</i> , <i>S. erythreus</i>
CRK	38.5 ± 2	32.2 ± 0.7	<i>S. cattleya</i> , <i>S. olivaceus</i> , <i>S. griseus</i>
CWK	38.3 ± 2	37.87 ± 1.3	<i>S. cattleya</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i> , <i>S. lipmanii</i> , <i>S. antibioticus</i> , <i>S. erythreus</i> , <i>S. griseosporus</i>
Temperate			
VWB	46.0 ± 3.9	41.94 ± 0.95	<i>S. venezuelae</i>
CT	52.5 ± 1.2	39.59 ± 1.39	<i>S. cattleya</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i> , <i>S. lipmanii</i> , <i>S. antibioticus</i> , <i>S. aurantiogriseus</i> , <i>S. erythreus</i> , <i>S. griseosporus</i>
CPT (= CTK)	40.2 ± 0.5	40	<i>S. cattleya</i> , <i>S. ghanaensis</i> , <i>S. olivaceus</i> , <i>S. clavuligerus</i>

ND, Not done; —, could not be estimated.

Isolation and properties of lysogenic strains

As indicated in Methods, temperate phages could be recognized by the development of lysogenic bacteria at the centre of the plaques. Four of the ten different phages investigated were identified as temperate, viz. CPT, CTK, CT and VWB. Lysogenic strains of *S. cattleya* containing CPT, CT or CTK were immune to superinfection by CPT, CT or CTK and also by CWK, but they were still susceptible to CPC or P23. Strains of *S. venezuelae* containing temperate VWB were susceptible to P23, and also to VPI and VW3.

Spontaneous transition from the temperate stage to the lytic cycle occurred at a rate of 1–5% of the number of viable cells. Induction of the lytic cycle could be enhanced by exposure (1–5 s) of the lysogenic strain to UV (260 nm). Longer irradiation decreased the number of viable phages (Fig. 2).

Preliminary experiments to investigate whether the prophages were integrated in the chromosome or existed as an extrachromosomal element (as for PI phage of *S. fradiae* phage SF1: Chung, 1982) indicated that they were probably integrated in the chromosome, because no extrachromosomal DNA could be observed. For the detection of extrachromosomal DNA, 48 h old mycelium of a lysogenic *Streptomyces* species grown in medium S was lysed and DNA was extracted according to the method of Wohlleben & Pühler (1982). This method has been proven to be efficient for the isolation of plasmid-like DNA in streptomycetes. In the case of temperate phages (CT, CPT or CWK), no extrachromosomal DNA was observed after CsCl centrifugation. For the VWB lysogenic *S. venezuelae* strain, which also contained the plasmid pSVH₁ (12.6 kb) (W. Wohlleben & A. Pühler, unpublished observations), only the latter plasmid but no other extrachromosomal DNA band was observed. These results proved the utility of this procedure for the isolation of plasmid DNA. However, if the prophages were present as a low copy number plasmid or as linear plasmids (Hirochika & Sakaguchi, 1982), this method might be unsatisfactory.

Influence of Ca²⁺ and Mg²⁺ on plating efficiency of phages CPT, CRK and VWB

The efficiency of plaque formation of phage particles was dependent on the Ca²⁺ and Mg²⁺ concentrations in the medium for the three phages studied: CPT and CRK on *S. cattleya* and VWB on *S. venezuelae*.

On phage medium agar containing increasing concentrations of Ca²⁺ and/or Mg²⁺ (as MgSO₄ · 7H₂O), the optimum concentration of Ca²⁺ was different for different phages (Table 3). Mg²⁺ was not required for plaque formation when the medium contained Ca²⁺. It could only partially replace Ca²⁺ in the case of VWB or CRK. In combination with Ca²⁺ there was no effect of Mg²⁺ on the plating efficiency for the different phages investigated.

From these results, it was concluded that the Ca²⁺ requirement for plaque formation varied for different phages and that, in fact, the Ca²⁺ concentrations in the phage medium had to be adjusted for each type of phage.

Protoplast formation and transfection

For many *Streptomyces* spp., protoplast formation can be induced by the action of lysozyme, when mycelium is grown in a medium containing glycine (Okanishi *et al.*, 1974). For *S. cattleya* and *S. venezuelae*, lysozyme was also effective for protoplast formation. After 15–30 min incubation in medium P containing 10 mg lysozyme ml⁻¹ the mycelia were almost entirely converted to protoplasts. Regeneration of protoplasts occurred effectively on R2YE-medium. The number of osmotically stable cells in the protoplast preparation was investigated by osmotic shock treatment in distilled water. For *S. cattleya* protoplast preparations, the number of osmotically stable cells was, on average, less than $1 \times 10^{-3}\%$ of the total number of viable cells, i.e. about 10^9 c.f.u., and for *S. venezuelae* this number was less than $1 \times 10^{-1}\%$.

Efficient transfection of *Streptomyces* protoplasts is possible by treatment of the protoplasts with PEG in the presence of phage DNA (Suarez & Chater, 1980; Isogai *et al.*, 1980). Transfection could be observed by plaque formation in the regeneration plates after 48 h regeneration, or by soaking regeneration plates with 5 ml phage buffer and testing the latter suspension for the presence of phages. We carried out transfection experiments for all the phages isolated, using 5–10 µg DNA and 25% (w/v) PEG (mol. wt 6000). Using *S. cattleya* or *S. venezuelae* as host strain, transfectants could be obtained with all the phages. The number of infective centres ranged between 1 in 10^4 and 1 in 10^5 protoplasts as estimated by counting the number of plaques on the transfection plates. This number was in good agreement with other transfection experiments described (Krügel *et al.*, 1980; Suarez & Chater, 1980; Isogai *et al.*, 1980).

G + C content and molecular weight estimations of DNA

The base composition of DNA was calculated from buoyant density determinations and the results were compared with estimates derived from thermal denaturation experiments. Such comparison was made because values obtained by equilibrium centrifugation may be influenced

Table 3. Influence of Ca^{2+} and Mg^{2+} on plating efficiency of phages CPT, CRK and VWB

Phage	Plating efficiency (p.f.u. ml ⁻¹)									
	Ca^{2+}					Mg^{2+}				
	0 mM	2.5 mM	5.0 mM	10 mM	20 mM	40 mM	80 mM	10 mM	5 mM + 5 mM	
CPT	<10 ⁷	<10 ⁷	1.3 × 10 ⁷	1 × 10 ⁹	1.25 × 10 ⁹	1.45 × 10 ⁹	6 × 10 ⁸	<10 ⁷	1 × 10 ⁹	
CRK	6.5 × 10 ⁸	6.8 × 10 ⁹	8.5 × 10 ⁹	6.5 × 10 ⁹	1.5 × 10 ⁹	6 × 10 ⁷	<10 ⁷	9 × 10 ⁸	8 × 10 ⁹	
VWB	<10 ⁷	7 × 10 ⁸	2.7 × 10 ¹⁰	3.5 × 10 ¹⁰	7 × 10 ⁹	2 × 10 ⁷	<10 ⁷	5.3 × 10 ⁸	2.5 × 10 ¹⁰	

Table 4. *Estimation of G + C content of actinophage DNA by melting temperature (T_m) and buoyant density (θ) determinations*

Phage DNA	T_m (°C)	Mol % G + C of DNA*	θ (g cm ⁻³)	Mol % G + C of DNA†
P23	72.10	46.2	1.7068	53.2
VP1 (= VW3)	71.55	45.1	1.7070	53.5
CPC	71.78	45.6	1.7053	51.8
CRK	76.11	54.6	1.7155	62.1
CWK	82.59	68.1	1.7299	76.7
VWB	83.16	69.3	1.7304	77.2
CT	83.45	69.9	1.7297	76.4
CPT (= CTK)	85.52	74.2	1.7300	76.2

* Based on $G + C = (T_m - 49.9)/0.48$.† Based on $G + C = 10.12\theta - 16.74$.

by glycosylation or methylation of the DNA (Szybalski & Szybalski, 1977). Consequently, differences between the results obtained from both methods suggest that DNA might be modified.

Except for the temperate phages and for CWK, the G + C content of the phage DNA (Table 4) was quite different from that of streptomycetes, which ranges between 69 and 76 mol % (Cross & Goodfellow, 1973). The base composition of all the virulent phages except CWK ranged between 45 and 54 mol % as calculated from T_m . This G + C content was rather low in comparison to other data in the literature (Lomovskaya *et al.*, 1980; Chater, 1980). However, the results were confirmed by both buoyant density and T_m determinations. On the other hand, the G + C content of the temperate phages was between 70 and 74 mol % (on the basis of T_m calculations) and these values were in much better agreement with the base composition of streptomycetes.

The lack of full agreement between the base compositions obtained by the two methods might suggest modification of the phage DNA. A further indication of the presence of modified DNA was obtained from restriction enzyme analysis (see below).

The molecular weights of the phage DNAs were estimated by electron microscopic length determination as well as by summation of fragments obtained after digestion with restriction endonucleases (Table 2). CRK and CWK had a relatively small genome; three others, P23, CPC and VP1, had much larger genomes. Results obtained by the two methods were in good agreement for all the phage DNAs except CRK and CT. In these instances restriction enzyme analysis gave remarkably higher genome lengths than obtained by electron microscopic measurements. These differences could be due to the partial digestion of highly modified phage DNA thus giving rise to an overestimation of the genome lengths.

For comparison, the smallest actinophage genome described so far is 36 kb (Stuttard & Dwyer, 1981), whereas the largest is 99 kb (Lomovskaya *et al.*, 1980).

Restriction endonuclease analysis of DNA from different phages

The DNA of the phages was digested with 23 restriction enzymes (Table 5). A first conclusion which could be drawn was that VP1 was identical to VW3, and CPT to CTK, although they were isolated from totally different samples. Furthermore it became apparent that some of the phage DNAs were probably highly modified. For example, the DNA of CPC and VP23 was not cut by *Sau3AI*, which might imply the methylation of cytidine in this recognition sequence (Huang *et al.*, 1982). The presence of 5-methylcytosine might explain the non-activity of *Bam*HI or *Bgl*II, not only for CPC or VP23, but also in the case of CT and CWK.

The partial or slow digestion of the phage DNA (indicated in Table 5 by + or ?) might suggest that the phages isolated contained methylated or glycosylated DNA, or DNA with unusual base composition, interfering with the binding of the restriction enzymes. These modifications might be caused partially by the host cell machinery, since, after cloning fragments of CPT (ligated to

Table 5. Restriction enzyme analysis of different phage DNAs

Enzyme	Sequence†	No. of fragments obtained after digestion of phage DNA‡							
		CPC	CRK	CT	CPT (= CTK)	CWK	VWB	VP1 (= VW3)	VP23
<i>AluI</i>	AG↓C★T	D	D	D	D	D	D	D	D
<i>BamHI</i>	G↓GAT★C°C	-	5	-	2	-	>10	-	-
<i>BclI</i>	T↓G★ATCA	-	3	-	10	-	>12	15	-
<i>BglII</i>	A↓G°ATCT	-	7	-	2	-	4	>15	-
<i>BsrEII</i>	G↓GTNACC	>12	>12	11	16	11	15	>10	>15
<i>Clal</i>	AT↓CG★AT	>15	11	6	8	6	2	>15	>15
<i>EcoRI</i>	G↓A★ATTC	+?	8	5?	4/5?	6?	2	>10	>15
<i>EcoRV</i>	GAT↓ATC	-	8	5	5	5	4	>15	>15
<i>HindII</i>	GTPy↓Pu★A°C	+	>12	+	>10	>10	>12	+	+
<i>HindIII</i>	★A↓°AG°CTT	-	-	-	-	-	3	2	>15
<i>HpaI</i>	GTT↓A★A°C	2?	3	-	2?	-	-	8/10	8
<i>HpaII</i>	°C↓★CGG	D	D	D	D	D	D	D	D
<i>KpnI</i>	GGTAC↓C	-	3	-	+	-	>10	6/8	>15
<i>PstI</i>	CTGCA↓G	-	-	-	-	+	>15	-	-
<i>SacI</i>	GAGCT↓C	-	-	-	>15	-	>15	-	-
<i>SalI</i>	G↓T★CGAC	-	6?	-	+?	-	>8	-	-
<i>Sau3AI</i>	↓G°ATC	-	D	D	D	D	D	D	-
<i>SmaI</i>	CC★C↓GGG	-	-	>10	>15	>10	≥10	-	-
<i>SphI</i>	GCATG↓C	-	-	8	8	4	10	-	-
<i>SsrI</i>	GAGCT↓C	-	8	12	>10	10	>15	-	-
<i>SsrII</i>	CCGC↓GG	-	-	D	D	D	>10	-	-
<i>XbaI</i>	T↓CTAG★A	6	-	-	-	-	-	>15	-
<i>XhoI</i>	C↓T★CG★AG	-	-	7	>12	>10	-	-	-

† Key: ★, Methylation inhibits the enzyme; °, no inhibition of the enzyme by methylation; ↓, indication of the point of cleavage.

‡ D, DNA digested in several small fragments; +, partial digests.

pBR322 or pACYC184) in *Escherichia coli* HB101, MM294 or in a *dam*⁻ strain (J. Anné, W. Wohlleben & A. Pühler, unpublished observations) phage DNA was digested without difficulty by *EcoRI*, *KpnI* or *SalI*; phage DNA isolated from *Streptomyces* spp. was almost completely resistant to these restriction enzymes.

Modification of phage DNA was, however, probably not caused exclusively by the host, but largely inherent to the DNA itself, provided it was replicating in a host containing the enzymes required for DNA methylation or glycosylation or for delivery of unusual purine or pyrimidine bases. An indication that this assumption was probably correct is the observation that a particular phage, when grown on different *Streptomyces* spp., yielded DNA with the same pattern of resistance to certain restriction endonucleases, and no new bands appeared in the gels.

The presence of cohesive ends was investigated using digested DNA. The density of the bands in the gels was compared in samples without and with heating and rapid cooling of the digested DNA. Of all the phage DNAs investigated, only VWB showed DNA bands in the gel with variation in intensity (compared to non-heated samples) after heating and rapid cooling. In addition, the sum of the lengths of the smaller fragments which showed increased intensity after heating, as a result of increased amount of non-fused cohesive ends, was equal to the length of the fragments with reduced intensity, i.e. which contained the fused cohesive ends (data not shown). The presence of cohesive ends was also demonstrated by electron microscopy. Circular DNA molecules (20 out of 52 molecules) were observed for VWB (Fig. 3). Circular DNA molecules were not observed for any of the other samples.

The DNA of phage CPT was investigated in more detail, because it was noticed that after complete digestion and subsequent gel electrophoresis two or three bands always stained less intensively. This phenomenon was not caused by partial digestion nor did these bands contain fragments of fused or non-fused sticky ends. Attempts to construct a restriction map revealed that the appearance of the dense bands may have been due to the occurrence of different phage

Fig. 3. Electron micrograph of a closed circular phage DNA molecule of *S. venezuelae* phage VWB. The bar marker represents 1 µm.

lengths present as a result of the DNA packaging mechanism (headful mechanism). This is currently being investigated further.

Finally, when comparing the restriction endonuclease profiles of the different phage genomes, it is remarkable that the phages with the broadest host range contained DNA which was more resistant to endonuclease activity (Table 5). Therefore, for the construction of wide host range phage cloning vehicles, it might be necessary to insert into the phage genome one or more target sites for several commonly used restriction enzymes.

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