Characterization of FP22, a large streptomycete bacteriophage with DNA insensitive to cleavage by many restriction enzymes

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Bacteriophage FP22 has a very broad host range within streptomycetes and appeared to form lysogens of *Streptomyces ambofaciens* ATCC 15154. FP22 shared strong cross-immunity and antibody cross-reactivity with bacteriophage P23, but not with seven other streptomycete bacteriophages. FP22 particles had a head diameter of 71 nm and a tail length of 307 nm. The FP22 genome was 131 kb, which is the largest bacteriophage genome reported for streptomycetes. The G + C content of the genome was 46 mol% and restriction mapping indicated that FP22 DNA had discrete ends. NaCl- and pyrophosphate-resistant deletion mutants were readily isolated and the extent of the deletions defined at least 23 kb of dispensable DNA in two regions of the genome. The DNA was not cleaved by most restriction endonucleases (or isoschizomers) which have been identified in the streptomycetes, including the tetranucleotide cutter *MboI* (GATC).

Introduction

Many *Streptomyces* spp. produce type II restriction endonucleases (Roberts, 1988), and analyses of bacteriophage–host interactions have established that the expression of restriction endonucleases in streptomycetes can block infection by bacteriophages that contain the corresponding cleavage sites in their DNA (Chater & Wilde, 1980; Lomovskaya *et al.*, 1980; Cox & Baltz, 1984; Chater, 1986). Many streptomycete bacteriophages lack cleavage sites for subsets of restriction endonucleases produced by streptomycetes (Cox & Baltz, 1984; Anné *et al.*, 1984; Foor *et al.*, 1985; Greene & Goldberg, 1985; Chater, 1986; Kuhn *et al.*, 1987; Diaz *et al.*, 1989), and broad-host-range streptomycete phages, on the average, form plaques on about 50% of hosts tested (Cox & Baltz, 1984; Matsushima & Baltz, 1989; McHenney & Baltz, 1989). The strong correlation between the expression of restriction and the blockage of plaque formation for certain bacteriophages suggested that about 80% of *Streptomyces* species express significant restriction (Cox & Baltz, 1984).

Bacteriophage FP22 was isolated from soil by enrichment on *Streptomyces fradiae* and *Streptomyces griseofuscus* (Cox & Baltz, 1984). FP22 and several other broad-host-range streptomycete bacteriophages have been used in several studies to characterize *in vivo* restriction in streptomycetes (Cox & Baltz, 1984; Matsushima *et al.*,

1987; Matsushima & Baltz, 1989; McHenney & Baltz, 1989). From these studies, it was determined that FP22 had a double-stranded DNA genome, formed plaques on over 70% of streptomycete species or strains tested, including *Streptomyces coelicolor* A3(2) and *Streptomyces lividans* 66, and plated at high efficiency on several strains which produce well-characterized restriction endonucleases.

Since FP22 had a very broad host specificity and appeared to be able to avoid restriction barriers in *Streptomyces* spp., we are interested in exploring it as a possible vehicle to transfer DNA in streptomycetes. We report here the characterization of FP22 and the isolation of deletion mutants which might be used to construct FP22-based cloning vectors.

Methods

Media and culture conditions. The actinomycete strains were grown in TS broth and fragmented by homogenization and ultrasonic vibration as described by Baltz (1978). NC broth and NC agar were as described by Cox & Baltz (1984). Bennett's medium (Waksman, 1961) was supplemented with 2 ml Czapek's mineral mix per litre. Czapek's mineral mix contained per litre: 100 g KCl, 100 g MgSO₄·7H₂O and 2 g Fe₂(SO₄)₃·7H₂O. BC agar comprised Bennett's medium plus 4 mM-Ca(NO₃)₂. Cells were grown on AS-1 agar (Baltz, 1980) for spore formation.

Bacteria and bacteriophages. The actinomycete strains used in this study are listed in Table 1 or in the text. The bacteriophages used included FP4, FP22, FP43, FP50, FP60, FP61 (Cox & Baltz, 1984), P23 (Anné *et al.*, 1984), VP11 (Dowding, 1973) and R4 (Chater & Carter, 1979). All bacteriophage lysates were prepared on *S. griseofuscus* on NC or BC agar plates as described previously (Cox & Baltz, 1984). FP22sm3 was a spontaneous mutant isolated on *S. fradiae*. NaCl-resistant mutants were isolated after incubating FP22 particles for 3–4 h at 44 °C in NC broth containing 0.2 M-NaCl and 0.4 mM- $\text{Ca}(\text{NO}_3)_2$, then plating survivors on NC agar plates. NaCl-resistant mutants were also incubated in 1 mM-tetrasodium pyrophosphate (PP_i) and 100 mM-Tris (pH 7.5) at 44 °C for 10–60 min to test for resistance to PP_i .

Molecular techniques. Bacteriophage λ DNA was purchased from New England Biolabs. FP22 and P23 DNA was isolated by the small-scale phage preparation method of Hopwood *et al.* (1985). Total cellular DNA of *S. ambofaciens* was isolated according to Fishman & Hershberger (1983). FP22 and P23 were nick-translated with [^{32}P]dCTP, and Southern hybridizations were carried out as described by McHenney & Baltz (1988).

Determination of guanine plus cytosine (G + C) content. G + C content was determined from the melting temperature (Marmur & Doty, 1962; Dove & Davidson, 1962) using a Gilford spectrophotometer and thermo programmer with bacteriophage λ DNA as a control.

Electron microscopy. Electron microscopy was carried out on negatively stained FP22 phage particles in 1% (w/v) aqueous uranyl acetate.

Restriction digestion. Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Labs, Boehringer Mannheim Biochemicals, Amersham, United States Biochemical or Promega. All enzymes that did not cleave FP22 DNA under standard conditions were retested with higher enzyme concentrations and longer incubations, and were tested for activity on λ DNA alone. All streptomycete enzymes or isoschizomers which failed to cleave FP22 DNA were also incubated with λ DNA mixed with FP22 DNA. In every case, λ DNA was cut to completion, while FP22 DNA was not cleaved.

Gel electrophoresis. Restriction fragments less than 48 kb were resolved by standard methods (Sambrook *et al.*, 1989). Fragments larger than 50 kb were resolved by FIGE (Carle *et al.*, 1986) using the DNASTAR PULSE system (DNASTAR Inc., Madison, WI, USA). The gels were run at 90 V, with a 15 min prerun in a forward direction, followed by alternating 4 s forward, 2 s reverse pulses for 64 h. Ligated λ DNA (Carle & Olson, 1984) was run as standards.

Anti-FP22 serum. This was prepared as described by McHenney & Baltz (1988). Antiserum titre was determined by incubating appropriate dilutions of antiserum in NC broth with FP22 and determining the first-order inactivation rate constant, $K = -\ln[N/N_0] \cdot t^{-1}$; where N/N_0 is the surviving fraction of p.f.u. at time t (min).

Results

General properties of FP22

FP22 formed small plaques (0.5–1 mm diameter) on mycelial fragments of *Streptomyces fradiae* after 1 d incubation at 34 °C, and the plaques developed turbid edges (but not turbid centres) after 3 d incubation (Fig. 1a, c). Mutant plaques of FP22 were observed (0.05%)

Fig. 1. Plaque morphology of FP22 and a large clear-plaque mutant FP22sm3. FP22 and FP22sm3 were plated on *S. fradiae* ATCC 19609 mycelial fragments and incubated at 34 °C. (a) FP22, 24 h; (b) FP22sm3, 24 h; (c) FP22, 72 h; (d) FP22sm3, 72 h. The bar in (a) represents 5 mm.

that appeared normal after 1 d incubation but continued to expand in size, resulting in 3–5 mm clear plaques after 3 d (see FP22sm3, Fig. 1b, d). When an FP22sm3 plaque expanded into a wild-type plaque, it did not lyse the cells in the turbid edge. FP22 and FP22sm3 gave indistinguishable small (0.5–1 mm) turbid plaques with clear centres on *S. griseofuscus* that expanded in size with continued incubation. Both wild-type and mutant phages formed turbid plaques on *S. ambofaciens*. Where tested, the efficiency of plating (e.o.p.) of FP22 was not changed by using spores instead of mycelial fragments (Table 1).

Electron microscopic analysis indicated that FP22 had a hexagonal (presumably icosahedral) head of about 71 ± 6 nm maximum diameter and a tail about 307 ± 22 nm long. The tip of the tail had a short spike (not shown). The morphology of FP22 particles falls under type B1 of Ackerman & Eisenstark (1974). The DNA of FP22 had a G + C content of ~ 46 mol% as determined by melting temperature (T_m).

Cox & Baltz (1984) showed that FP22 DNA lacked cleavage sites for several restriction enzymes produced by streptomycetes and formed plaques on most of the corresponding strains. We tested the e.o.p. of FP22 on six other strains known to produce restriction endonucleases. FP22 formed plaques on the producers of *Sal*DI (*Nru*I), *Sal*CI (*Nae*I) and *Sca*I, but did not form plaques on the *Sal*HI (*Mbo*I) and *Spa*I (*Xho*I) producers (Table

Table 1. FP22 plaque formation on *Streptomyces* strains

Strain	Relevant phenotype or property	Source or reference*	FP22 e.o.p.†
<i>S. albus</i> H	Produces <i>Sal</i> HI (<i>Mbo</i> I)	ATCC 12757; Roberts (1988)	$<4 \times 10^{-7}$
<i>S. albus</i> C	Produces <i>Sal</i> CI (<i>Nae</i> I)	ATCC 21290; Roberts (1988)	8×10^{-2}
<i>S. albus</i> D	Produces <i>Sal</i> DI (<i>Nru</i> I)	ATCC 21132; Roberts (1988)	1×10^{-2}
<i>S. albus</i> subsp. <i>pathocidicus</i>	Produces <i>Spa</i> I (<i>Xho</i> I)	KC 5-0166; Roberts (1988)	$<4 \times 10^{-7}$
<i>S. ambofaciens</i>	Relatively non-restricting	ATCC 15154; Cox & Baltz (1984); Matsushima & Baltz (1985)	0.1‡
<i>S. ambofaciens</i> KC16	Putative FP22 lysogen	This report	$<5 \times 10^{-8}$
<i>S. caespitosus</i>	Produces <i>Sca</i> I	Roberts (1988)	1×10^{-5}
<i>S. fradiae</i> C373.0	Produces tylosin, expresses several restriction systems	ATCC 19609; Matsushima <i>et al.</i> (1987)	0.2
<i>S. fulvissimus</i>	Produces <i>Sfu</i> I (<i>Asu</i> II)	ATCC 27431; Veitinger <i>et al.</i> (1990)	2×10^{-7}
<i>S. griseofuscus</i> C581	Non-restricting	ATCC 23916; Cox & Baltz (1984)	1.0‡
<i>S. lividans</i> 66	Relatively non-restricting	NRRL 15826; Hopwood <i>et al.</i> (1985)	1×10^{-5} ‡

* ATCC, American Type Culture Collection, Rockville, Maryland, USA; NRRL, Northern Regional Research Laboratory, Peoria, Illinois, USA.

† All platings were done with FP22 lysates from *S. griseofuscus*.

‡ Similar e.o.p. values were obtained on spores or mycelial fragments.

Table 2. Plaque formation by bacteriophages on *S. ambofaciens* strains and inactivation of bacteriophages by anti-FP22 serum

Bacteriophage	Relative e.o.p. on <i>S. ambofaciens</i> *		Relative inactivation rate‡
	ATCC 15154 (wild-type)	KC16 (putative lysogen)	
FP4	2.1	0.01	0.09
FP22	0.1	$<5 \times 10^{-8}$	1.00
FP43	0.1	0.1	0.18
FP50	0.7	0.5	<0.01
FP60	0.02	0.01	0.61
FP61	0.42	7×10^{-7}	0.18
P23	0.2	$<5 \times 10^{-8}$	1.06
R4	1.4	0.3	0.17
VP11	1.4	0.2	0.10

* Lysates were prepared on *S. griseofuscus*, and e.o.p. was determined relative to titres obtained on *S. griseofuscus*.

† First-order inactivation rate constants (*K*) were calculated using appropriate dilutions of antiserum. The relative inactivation rate is the ratio of the mean *K* value obtained for a given phage from two independent determinations to the mean obtained from four determinations of the FP22 *K* value. The *K* value for FP22 was 1350 min^{-1} .

1). FP22 plated on the producer of *Stu*I (*Asu*II) at a low efficiency (Table 1) in spite of the fact that FP22 DNA has 22 *Asu*II sites. However, FP22 phage grown on *Streptomyces fulvissimus* was not modified for *Sfu*I restriction since it replated on *S. fulvissimus* at similar low efficiency. FP22 did not form plaques on *Actinoplanes teichomyceticus* (ATCC 31121), *Amycolatopsis orientalis* (ATCC 19795), *Arthrobacter* sp. (NRRL 3381),

Micromonospora rosaria (ATCC 29337), *Micromonospora* sp. 71-1225 (Seno & Hutchinson, 1986), *Nocardia lurida* (ATCC 14930), *Nocardia* sp. (NRRL 8156), *Saccharopolyspora erythraea* (NRRL 2338) or *Saccharopolyspora hirsuta* (ATCC 27875 and NRRL 12045).

To isolate lysogens, FP22 was plated on *Streptomyces ambofaciens* at a m.o.i. >10 . After 16 d at 34 °C, FP22-resistant colonies were picked and patched on NC agar, then incubated at 34 °C for 6 d. The cells were tested for resistance to FP22 by cross-streaking against FP22 and for phage release by replica plating onto lawns of *S. griseofuscus*. Several isolates were retested for phage release on NC agar and in NC broth after growth to stationary phase. Putative lysogens (e.g. KC16) remained resistant to FP22 and continued to release p.f.u.

The e.o.p. of several streptomycete bacteriophages was determined on the wild-type *S. ambofaciens* and one putative lysogen KC16 (Table 2). FP22 plated at an e.o.p. of 0.1 on wild-type *S. ambofaciens*, but of $<5 \times 10^{-8}$ on KC16. Six bacteriophages (FP4, FP43, FP50, FP60, R4 and VP11) plated at high efficiencies on KC16 and ATCC 15154. Bacteriophages P23 and FP61, on the other hand, plated at high efficiency on ATCC 15154 and at $<5 \times 10^{-8}$ and 7×10^{-7} , respectively, on KC16, suggesting that P23 and FP61 may be homoimmune to FP22. Three other putative *S. ambofaciens* FP22 lysogens did not support plaque formation by FP22, but supported plaque formation by FP4, R4 and VP11 at e.o.p. values similar to those observed on KC16.

Several streptomycete bacteriophages were treated with anti-FP22 serum, and first-order inactivation rate constants were determined (Table 2). FP50 was not inactivated by anti-FP22 serum; R4, FP4, FP43, VP11

Fig. 2. Sensitivity of FP22 strains to inactivation by NaCl or PP_i treatment. (a) FP22 incubated in 0.2 M-NaCl at 44 °C (see Methods) then plated on *S. fradiae*. ○, Total p.f.u.; □, large clear plaques. (b) FP22 strains incubated as in (a) then plated on *S. griseofuscus*. ○, FP22; □, FP22sm5; △, FP22sm1; ●, FP22sm2; ■, FP22sm4. (c) FP22 strains incubated in 1 mM- PP_i and 100 mM-Tris (pH 7.5) at 44 °C then plated on *S. griseofuscus*; symbols are as in (b).

and FP61 were inactivated at much slower rates than FP22 (9–18%); FP60 was inactivated at a relatively high rate (61%); and P23 was inactivated at a rate essentially identical to that observed with FP22.

Deletion mutants

When FP22 was treated with 0.2 mM-NaCl at 44 °C at low Ca^{2+} concentration, it was inactivated with a progressively declining slope (Fig. 2a). The proportion of phages displaying a large clear mutant plaque phenotype on *S. fradiae* increased from about 0.05% to about 45% over a 4 h treatment period. Several large clear plaque mutants (*sm2* and *sm4*) and several other mutants (*sm1*, *sm5*, *sm10*, *sm21* and *sm40*) surviving a 4 h NaCl treatment were isolated and verified to be more resistant to NaCl than wild-type FP22 (Fig. 2b).

Exposure to the chelating agent tetrasodium pyrophosphate (PP_i) is lethal to most *cos*-packaging bacteriophages (Stuttard, 1989). Deletion mutants of those bacteriophages are often resistant to PP_i exposure (Chater, 1986). Therefore, the NaCl-resistant mutant phages were also tested for resistance to PP_i (Fig. 2c). Most mutants were as sensitive to PP_i as wild-type FP22. However, FP22sm1 was partially resistant and FP22sm5 and *sm21* were highly resistant to PP_i treatment. Spontaneous large clear plaque mutant FP22sm3 displayed normal sensitivity to NaCl and PP_i treatment (data not shown), indicating that the large clear plaque phenotype does not always correlate with NaCl resistance.

DNA homology relationships between FP22 and P23

After *Mlu*I restriction digestion, FP22 DNA shared at least three bands (3.2, 2.1 and 1.2 kb) that comigrated with DNA from bacteriophage P23 (Fig. 3a). To determine if these bacteriophages shared DNA homology, we transferred the DNA bidirectionally to two nylon filters and probed each blot with nick-translated P23 or FP22 DNA (Fig. 3b and c, respectively). Labelled DNA from each phage showed no hybridization to λ DNA (data not shown) but only slight homology to DNA of the other phage. The FP22 D, E and F bands (5.4, 4.7 and 4.6 kb, respectively) showed considerably less hybridization to nick-translated FP22 DNA than smaller and larger bands (Fig. 3c). Label incorporation by nick-translation is less efficient at the ends of large DNA molecules (Sambrook *et al.*, 1989); therefore, the reduced radiolabel in bands D, E and F suggested that they may be at the ends of FP22 DNA (see restriction mapping below).

Nature of the ends of FP22 DNA

The fact that FP22 mutants resistant to NaCl or PP_i had acquired deletions suggested that FP22 may have discrete ends (Chater, 1986; Stuttard, 1989). The possible presence of cohesive ends of FP22 DNA was analysed by three methods: ligation, annealing and DNA polymerase I labelling. FP22 and λ DNA were incubated with DNA ligase (Carle & Olson, 1984) and analysed by FIGE. λ DNA ligated to form dimers and trimers, but no evidence for ligation of FP22 DNA was

Fig. 3. Cross-homology of FP22 and P23 DNA. Phage DNA cut with *Mlu*I and run on an agarose gel (a) was transferred to nylon membranes and probed with P23 (b) or FP22 (c) DNA labelled by nick-translation. Asterisks indicate bands identical in size between FP22 and P23. Neither P23 nor FP22 showed hybridization to λ DNA (data not shown). The scales (in kb) on the left (a) and right (b and c) are λ *Hind*III and λ *Xho*I digests.

seen with undigested samples or with ligation mixes digested with *Aat*II, *Alw*NI, *Avi*II, *Cla*I or *Mlu*I. Increasing the ligase concentration 400-fold had no effect. For annealing, duplicate samples of FP22 DNA cut with *Eco*RV or *Mlu*I and λ DNA cut with *Nru*I were incubated in 1 M-NaCl at 37 °C for 90 min. One sample was heated for 10 min at 80 °C, then both samples were loaded onto an agarose gel (Kuhn *et al.*, 1987). *cos*-containing λ *Nru*I fragments were efficiently annealed at 37 °C, but dissociated after heating to 80 °C. However, in FP22 digests, no new bands were observed on agarose gels. FP22 and λ DNA was incubated with the Klenow fragment of DNA polymerase I and nucleoside triphos-

phates with [32 P]dCTP (Sambrook *et al.*, 1989), cut with several restriction endonucleases, then analysed for incorporation of label after gel electrophoresis. Radiolabel was observed in two bands of FP22 DNA in each case; however, the intensity of the radiolabel was substantially less than that observed with bacteriophage λ controls. Therefore, although FP22 has discrete ends, none of the three methods used indicated that the ends are cohesive.

Restriction endonuclease cutting pattern

Cox & Baltz (1984) suggested that FP22 DNA lacked recognition sequences for several restriction endonucleases produced by streptomycetes. To further explore the distribution of recognition sites for streptomycete-produced and other restriction endonucleases, we incubated FP22 DNA with many restriction endonucleases that recognize hexanucleotide palindromic sequences containing six, four, two or zero G·C base pairs. FP22 DNA isolated from *S. griseofuscus* was cut by two of seven enzymes that recognize hexanucleotide sequences containing only G·C base pairs; by 12 out of 23 enzymes that recognize hexanucleotide sequences containing four G·C and two A·T base pairs; by 14 of 17 enzymes that recognize hexanucleotide sequences containing two G·C and four A·T base pairs; and by two of three enzymes that recognize hexanucleotide sequences containing only A·T base pairs (Table 3). FP22 DNA was cut by only 3 of 14 hexanucleotide palindromic cutters known to be produced by streptomycetes.

The apparent avoidance of certain hexanucleotide palindromic restriction sites recognized by streptomycete restriction endonucleases was also supported by the patterns of cutting obtained with enzymes that recognize degenerate palindromic sequences and interrupted palindromes. FP22 DNA was cut by 13 of 14 enzymes that recognize degenerate palindromic sequences, including several for which palindromic subsets were not observed in FP22 DNA (data not shown). For example, *Ban*II (GRGCTC), which cut FP22 more than 25 times, can cut at four sequences: two palindromic (GAGCTC, GGGCCC) and two non-palindromic (GAGCCC, GGGCTC). Since *Sac*I (GAGCTC) and *Apa*I (GGGCCC) did not cut FP22 DNA, then all cleavage by *Ban*II occurred at non-palindromic sites. *Ava*I (CYCGRG) also cuts FP22 DNA many times, whereas the palindromic subsets (CTCGAG and CCCGGG) were not cut.

FP22 was also cut by 12 of 13 enzymes that recognize interrupted palindromes, including several that interrupt streptomycete restriction endonuclease cleavage sites not observed in FP22 (data not shown). For example,

Table 3. *In vitro* restriction digestion of FP22 DNA

	Enzyme*	Recognition sequence	Cuts in FP22 DNA†	Enzyme*	Recognition sequence	Cuts in FP22 DNA†
6 GC	‡ <i>Sma</i> I (<i>Xma</i> I)	CCCGGG	0	<i>Bss</i> HII	GCGCGC	13
	§ <i>Sac</i> II	CCGCGG	0	<i>Nar</i> I	GGCGCC	1
	<i>Eag</i> II	CGGCCG	0	<i>Apa</i> I	GGGCCC	0
	§ <i>Nae</i> I	GCCGGC	0			
4 GC	<i>Mlu</i> I	ACGCGT	8	§ <i>Sac</i> I	GAGCTC	0
	<i>Eco</i> 47III	AGCGCT	18	§ <i>Sph</i> I	GCATGC	0
	§ <i>Stu</i> I	AGGCCT	0	<i>Bam</i> HI	GGATCC	0
	<i>Pml</i> I	CACGTG	13	<i>Nhe</i> I	GCTAGC	18
	§ <i>Pvu</i> II	CAGCTG	8	‡ <i>Kpn</i> I (<i>Asp</i> 718I)	GGTACC	15
	<i>Nco</i> I	CCATGG	0	§ <i>Sal</i> I	GTCGAC	0
	<i>Pvu</i> I	CGATCG	0	§ <i>Apa</i> LI	GTGCAC	19
	<i>Avr</i> II	CCTAGG	17	<i>Bsp</i> MII	TCCGGA	0
	<i>Spl</i> I	CGTACG	13	§ <i>Nru</i> I	TCGCGA	0
	§ <i>Xho</i> I	CTCGAG	0	<i>Avi</i> II	TGCGCA	9
	§ <i>Pst</i> I	CTGCAG	0	<i>Bal</i> I	TGGCCA	14
	<i>Aat</i> II	GACGTC	5			
2 GC	<i>Spe</i> I	ACTAGT	12	<i>Bsp</i> HI	TCATGA	>25
	<i>Bgl</i> II	AGATCT	0	<i>Xba</i> I	TCTAGA	>20
	§ <i>Sca</i> I	AGTACT	0	§ <i>Bcl</i> I	TGATCA	0
	<i>Hind</i> III	AAGCTT	>30	<i>Eco</i> RV	GATATC	14
	<i>Cla</i> I	ATCGAT	18	<i>Xca</i> I	GTATAC	17
	<i>Nsi</i> I	ATGCAT	11	<i>Hpa</i> I	GTTAAC	10
	<i>Nde</i> I	CATATG	15	<i>Sna</i> BI	TACGTA	11
	<i>Afl</i> II	CTTAAG	>25	§ <i>Asu</i> II	TTCGAA	22
	<i>Eco</i> RI	GAATTC	>30			
0 GC	<i>Ssp</i> I	AATATT	21	<i>Dra</i> I	TTTAAA	0
	<i>Ase</i> I	ATTAAT	17			

* All enzymes shown recognize six-base palindromic recognition sequences. Data on the frequency of cleavage of FP22 DNA with restriction enzymes recognizing six-base interrupted and six-base degenerate palindromic sites and four-base palindromic sites is available upon request.

† The number of cuts in FP22 DNA was based on estimates of band numbers based on 0.8% agarose gel electrophoresis. Single bands smaller than 500 bp or multiple bands of approximately the same size may not have been resolved. Numbers with a '>' indicate the presence of multiple unresolved bands.

‡ Enzymes in parentheses are isoschizomers which are affected differently by methylation of the target site (Nelson & McClelland, 1989) but gave the same cleavage pattern. Isoschizomers *Bst*NI and *Eco*RII, *Bst*XI and *Mfi*I, *Hpa*II and *Msp*I, *Mbo*I and *Sau*3A1 also gave identical cleavage patterns on FP22 DNA.

§ Restriction endonuclease specificities which are found in *Streptomyces* spp. (Roberts, 1988; Leung *et al.*, 1989; Veitinger *et al.*, 1990).

whereas there were no sites in FP22 DNA for *Nco*I (CCATGG) and *Nae*I (GCCGGC), there were many sites for *Bst*XI (CCAN₆TGG), *Pfi*MI (CCAN₅TGG) and *Bgl*I (GCCN₅GGC).

FP22 DNA had >60 to >100 sites for eleven restriction enzymes that recognized tetranucleotide palindromic sequences (data not shown). However, FP22 DNA was not cut by *Mbo*I, *Sau*3A1 and *Dpn*I, all of which recognize GATC or G^mATC.

Restriction map and size of FP22 DNA

Table 4 shows a summary of sizes of DNA fragments generated by several restriction endonucleases. The

summation of fragment sizes ranged from 125 to 135 kb and averaged 131 kb. The restriction map of FP22 is shown in Fig. 4(a). Most of the deletion mutants map in the first 20 kb of the phage genome. The location of several deletions is shown in Fig. 4(b). The *sm2* and *sm4* mutants gave large clear plaque phenotypes. Two mutants (*sm1* and *sm15*) containing larger deletions also affected plaque morphology: *sm1* gave a partial clear phenotype, whereas *sm15* gave turbid but larger than normal plaques. The *sm5*, *sm21* and *sm40* mutants had turbid plaques similar to the wild-type. The *sm40* deletion was located at least 10 kb downstream of the other deletions (Fig. 4a).

Fig. 4. Restriction map of FP22 and deletion mutants. (a) The sizes of the restriction fragments are given in Table 4. The solid bar indicates the region of the FP22 genome containing deletions enlarged in (b). The open bar indicates the region containing the 5.0 kb *sm40* deletion. (b) The approximate extent of six deletions, *sm1*, *sm2*, *sm4*, *sm5*, *sm15* and *sm21*, is indicated (—). Plaque morphology of FP22 mutants on *S. fradiae* at three days: 0.5–1.5 mm plaques with turbid edges (*sm5*, *sm21*, *sm40*); 2–3 mm plaques with turbid edges (*sm15*); 1 mm semi-turbid plaques (*sm1*); 3–5 mm clear plaques (*sm2* and *sm4*). Restriction enzymes are: A, *AspI*; Av, *AviIII*; C, *ClaI*; E, *EcoRV*; H, *HpaI*; M, *MluI*; X, *XbaI*. The scales at the bottom of (a) and at the top of (b) are in kb from the left end of FP22 DNA.

Table 4. Size estimates of restriction enzyme cleavage fragments of FP22 DNA

Fragment	Size of fragments (kb)* obtained after digestion with:							
	<i>HpaI</i>	<i>AviII</i>	<i>PvuII</i>	<i>MluI</i>	<i>AatII</i>	<i>AlwNI</i>	<i>AspI</i>	<i>NarI</i>
A	35	55†	82†	57†	56†	62†	115	125
B	25	15.7	12.0	41	44	23	13.5	10.0
C	19.5	14.0	11.0	12.7	13.8	20	0.5	
D	12.5	11.7	10.2	5.4	12.7	10.0		
E	10.3	11.6	4.8	4.7	3.7	9.6		
F	6.9	10.4	3.9	4.6	2.2			
G	6.0	4.7	3.8	3.3				
H	4.4	2.0	3.1	2.2				
I	4.2	1.9	2.4	1.3				
J	2.8	1.6						
K	2.7							
Total size (kb)	129	129	133	132	132	125	129	135
Mean size (kb)‡	131							

* The size of fragments > 50 kb was determined by FIGE.

† The log of the size (kb) of λ standards and their mobility gave a sigmoidal relationship below about 90 kb in the FIGE analysis. Therefore, the size of FP22 fragments between 50 and 90 kb was less accurate than that of larger and smaller fragments. These numbers were determined from at least two trials, and standard error in kb for each fragment was: *AviII* A, 0; *PvuII* A, 1.4; *MluI* A, 2.8; *AatII* A, 1.4; *AlwNI* A, 1.2.

‡ Uncut FP22 DNA ran at 134 kb on FIGE; standard error in kb was 2.5.

Discussion

Bacteriophage FP22 is the largest streptomyces bacteriophage yet described, having a genome size of 131 kb. Unlike its hosts and most other streptomyces plasmids and bacteriophages, which have a high G+C content, FP22 had a G+C content of 46 mol%. FP22 also has the largest host range of bacteriophages that we have studied (Cox & Baltz, 1984; Matsushima & Baltz, 1989; McHenney & Baltz, 1989). Since a high percentage of streptomyces appear to express restriction systems (Cox & Baltz, 1984; Matsushima & Baltz, 1989), it seemed possible that the low G+C content of FP22 might be associated with its ability to avoid certain restriction systems. We carried out an extensive restriction enzyme cleavage analysis of FP22 DNA, and noted that FP22 DNA was cut by 41% of enzymes recognizing G+C-rich hexanucleotide sequences and by 75% of enzymes recognizing A+T-rich hexanucleotide sequences. Of these, FP22 DNA was cut by only 21% of streptomyces enzymes that recognize hexanucleotide palindromic sequences, but was cut by 81% of other enzymes. FP22 was cut many times by most restriction endonucleases that recognize tetranucleotide sequences, but was not cut by *Mbo*I or *Sau*3AI or seven other enzymes and isoschizomers containing GATC in their recognition sequences (*Bam*HI, *Bcl*I, *Pvu*I, *Bgl*II, *Bst*YI, *Mfl*I, *Xho*II). An isoschizomer of *Mbo*I has been identified in *Streptomyces albulus* (Roberts, 1988), but FP22 did not form plaques on this strain (Table 1). The frequency of cleavage by enzymes recognizing interrupted or degenerate palindromes was very high and therefore supported the notion that FP22 DNA specifically lacks certain palindromic restriction enzyme recognition sequences, mainly those corresponding to streptomyces restriction endonuclease specificities.

The failure of some enzymes to cleave FP22 DNA was not due to DNA methylation. FP22 DNA was not cut by *Dpn*I, which cuts at G^mATC, so it does not contain 4-methyladenine in this context. Six pairs of isochizomers that are affected differently by methylation (Nelson & McClelland, 1989) gave identical cleavage patterns on FP22 DNA (Table 3), indicating that the two most common forms of DNA methylation, *N*⁵-methylcytosine and *N*⁶-methyladenine, are not widely distributed in FP22 DNA. Also, since FP22 has many sites for restriction endonucleases not observed in streptomyces, it does not contain a modified base insensitive to restriction endonuclease cutting, and does not express a general mechanism to neutralize the effects of restriction endonucleases. Several kb of FP22 DNA have been cloned in *Escherichia coli*, and the cloned segments were not cleavable by *Mbo*I, *Sau*3AI, *Dpn*I, *Sal*I, *Sac*I, *Sph*I or *Sac*II (J. Larson & C. Hershberger,

personal communication). Also, the 1.8 kb of FP22 DNA which has been sequenced (B. Dehoff, P. Rosteck & C. Hershberger, personal communication) contained no sites for any enzyme which failed to cleave FP22 DNA.

Since FP22 DNA has a G+C content of roughly 50 mol%, a randomly occurring hexanucleotide site should occur about 33 times per genome [i.e. $(0.25)^6 \times 130$ kb]. The probability of finding zero sites for a particular enzyme based upon a Poisson distribution is $\sim 10^{-13}$. A randomly occurring tetranucleotide should occur over 500 times per FP22 genome and the random probability of finding zero sites is $< 10^{-200}$.

The results of the restriction enzyme analysis and probability calculation strongly suggest that FP22 has evolved specifically to minimize the presence of sites for restriction endonucleases produced by streptomyces, thus permitting it to carry out a productive life cycle in a very high percentage of streptomyces, perhaps 70% (Cox & Baltz, 1984; Matsushima & Baltz, 1989; McHenney & Baltz, 1989). A mutational loss of specific restriction sites could be facilitated by the unusual distribution of base pairs in most streptomyces and their plasmids and bacteriophages. Streptomyces have a G+C content of >70 mol%, and have an average distribution G+C content of 70:50:91 mol% in position 1:2:3 of codons (Bibb *et al.*, 1984; Seno & Baltz, 1989). Most streptomyces restriction endonucleases recognize hexanucleotide sites high in G+C content, which have at least two G·C base pairs separated by two base pairs. Because of the 91% probability of finding G or C at position 3 in codons of streptomyces, the G·C base pairs separated by two nucleotides should occur frequently in third positions of adjacent codons. Thus a mutational process that favoured G·C→A·T transitions or G·C→A·T transversions at third positions of codons could eliminate many restriction sites by neutral mutations. If the progenitor of FP22 was a bacteriophage of relatively high G+C content, then the 46 mol% G+C observed in FP22 is consistent with this notion. It is not apparent if spontaneous mutation processes that might lower G+C content, such as deamination of cytosine to uracil (or *N*⁵-methylcytosine to thymine) to give G·C→A·T transitions (Baltz *et al.*, 1976; Duncan & Miller, 1980) or depurination of guanosine residues followed by random insertions (Loeb, 1985), coupled with strong selection, would be sufficient to drive this process. Alternatively, FP22 may encode a mutagenic enzyme system that selectively eliminates certain sequences.

Another apparently related phage, P23 (Korn *et al.*, 1978), has a similar morphology to FP22, also has a broad host range and a large genome with low G+C content, and, like FP22, lacks most restriction sites recognized by

streptomycete restriction endonucleases, including Sau3AI (Anné *et al.*, 1984). We showed that P23 is inactivated by anti-FP22 serum and does not form plaques on an apparent FP22 lysogen of *S. ambofaciens*. FP22 and P23 share a few bands of similar size after restriction endonuclease cleavage; however, DNA cross-homology was weak. Bacteriophages CPC (Anné *et al.*, 1984) and ϕ A1 (Diaz *et al.*, 1989) also have broad host ranges and large genomes with low G+C content, and lacking many streptomycete restriction endonuclease cleavage sites. This supports the notion that certain streptomycete bacteriophages have evolved to avoid restriction barriers by a mechanism that reduces the G+C content.

Although DNA polymerase I labelling and annealing experiments did not suggest that FP22 DNA had cohesive ends, restriction mapping studies indicated that it had discrete ends. This conclusion was further supported by the facile isolation of mutants resistant to NaCl or NaCl and PP_i, which contained internal deletions. Restriction mapping of the mutations verified that they contained the same terminal restriction fragments as wild-type FP22 and therefore packaged less DNA in head particles than FP22. The basis for the NaCl resistance is not known, but may be due to increased osmotic stability of phage heads containing a less than normal amount of DNA. Alternatively, certain deletions may cause the production of phage particles with altered head or tail components that are more stable to NaCl treatment.

Analysis of several deletion mutants identified a 6 kb region required for the normal (small, turbid edge) plaque phenotype on *S. fradiae*. Deletions over part of this region resulted in large clear, large turbid edge or small semi-turbid plaques. The basis for the differences in plaque morphology on *S. fradiae* has not been explored. These and other deletions also defined a region of at least 23 kb of DNA which was dispensable for plaque formation.

FP22 may have several important applications. Since its host range appears to be limited to *Streptomyces* spp., it can be used in conjunction with other streptomycete phages to rapidly classify new soil isolates as streptomycetes. Since FP22 DNA lacks sites for most type II streptomycete restriction endonucleases, it might be used to identify streptomycete strains that produce other types of restriction endonucleases, or its DNA might be used as an *in vitro* substrate to help classify restriction endonucleases from other micro-organisms. Finally, since at least 23 kb of FP22 DNA is dispensable, it could be developed as a vehicle to introduce relatively large integrated plasmids, cloned chromosomal genes or transposable elements into a wide range of streptomycetes. Because of the broad host range of FP22, FP22-

based vectors might be used to shuttle DNA between a wide range of well-characterized academic strains (e.g. *S. lividans* and *S. coelicolor*), industrial strains (e.g. *S. fradiae* and *S. ambofaciens*), and poorly characterized wild isolates from soil.

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