Characterization of the temperate actinophage ϕ A7 DNA and its deletion derivatives

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A restriction map of ϕ A7 DNA (46.7 kb) was established for nine endonucleases (*Bcl*I, *Cla*I, *Eco*RI, *Eco*RV, *Hpa*I, *Pvu*I, *Sac*II, *Sph*I and *Xba*I) which cut the phage genome up to 11 times. There were no sites for *Bam*HI, *Bgl*II, *Hind*III, *Pst*I, *Pvu*II, *Sac*I or *Sal*I. ϕ A7 DNA, circularized through its cohesive ends, could integrate into the genome of several *Streptomyces* hosts, to form stable lysogens. Integration occurred by recombination between unique attachment sites on the phage (*attP*) and the host (*attB*) genomes. The *attP* site has been located on the ϕ A7 restriction map. Deletion mutants of ϕ A7 DNA were obtained by selecting for pyrophosphate- or EDTA-resistant clones. The deletions occurred either near the left-hand end of the conventional restriction map, or about 18 kb from the right-hand end, close to, but not affecting the unique *Sac*II site. Together, the deletions defined at least 7.9 kb of DNA (16.9% of the phage genome) non-essential for plaque formation. ϕ A7 DNA was introduced into *S. lividans* protoplasts by liposome-assisted transfection. Since the phage does not adsorb to intact cells of this strain, and therefore does not form plaques, an overlay of *S. antibioticus* spores was used to detect the infectious progeny released by the protoplasts. Using this technique, ϕ A7 could be introduced into *S. antibioticus* with an efficiency of about 6×10^6 p.f.u. per μ g DNA (equivalent to 3×10^{-4} p.f.u. per DNA molecule).

Introduction

Nine actinophages (ϕ A1 to ϕ A9) were isolated from soil on *Streptomyces antibioticus* ATCC 11891, the producer of the macrolide antibiotic oleandomycin (Diaz *et al.*, 1989). One of these phages, ϕ A7, may be a good candidate for the development of cloning vectors. Like most *Streptomyces* phages it belongs to group B of Bradley's morphological classification (Bradley, 1967). It has a polyhedral head (52 nm in diameter) and a long (102 nm), non-contractile tail, ending in a base plate. The phage genome is an approximately 46.7 kb double-stranded DNA molecule with cohesive ends. Hybridization experiments revealed no homology between ϕ A7 DNA and the genomes of the other *S. antibioticus* phages, ϕ C31 (Lomovskaya *et al.*, 1972) or SH10 (Klaus *et al.*, 1981). ϕ A7 has a relatively wide host range, infecting 18 out of 31 *Streptomyces* strains tested. It produced turbid plaques on *S. antibioticus* ATCC 11891, but was unable to lysogenize this host. However, it proved to be temperate in many other strains: *S. albus* P, *S. albus* J1074 (a restriction-less mutant of *S. albus* G; Chater & Wilde, 1980), *S. orchidaceus* NRRL B-2454 and three strains of *S. lavendulae* (NRRL B-1871, NRRL B-2036 and NRRL B-2343). ϕ A7 was heteroimmune with

ϕ A5 and ϕ A8, two other temperate phages isolated from soil samples on *S. antibioticus*, and also with ϕ C31, SH10 and R4 (Chater & Carter, 1979). Like ϕ A7, phages ϕ A5 and ϕ A8 formed stable lysogens in several *Streptomyces* strains, but not in their original host.

ϕ A7 has three properties to make it favourable for utilization as a DNA cloning vector: (i) it has a broad host range, (ii) it is temperate, and (iii) its DNA has cohesive ends, indicating that deletion mutants may be enriched among phages surviving treatment with chelating agents (Parkinson & Huskey, 1971). In this paper we accomplished three other requirements for the use of the phage as a vector: (i) construction of a restriction map, (ii) isolation of viable deletion mutants, and (iii) development of a transfection system which would allow the reintroduction of *in vitro*-manipulated ϕ A7 DNA into *S. antibioticus* ATCC 11891 and other *Streptomyces* strains. We also established the location in the phage DNA of the *attP* site involved in recombination with the host chromosome to form stable lysogens.

Methods

Phages. ϕ A7 was described in a previous paper (Diaz *et al.*, 1989). ϕ C31 wild-type Norwich stock (Lomovskaya *et al.*, 1980) was obtained

from K. F. Chater, John Innes Institute, Norwich, UK. Techniques for phage assay and propagation were as in Hopwood *et al.* (1985).

Bacterial strains. *S. antibioticus* ATCC 11891 and *S. lividans* 1326 were the hosts for propagation of ϕ A7 and ϕ C31, respectively. Both strains were also used in transfection experiments. *S. orchidaceus* NRRL B-2454 and its ϕ A7 lysogen were used for the location of the *attP* site. Culturing of *Streptomyces* strains and isolation of lysogens from bacterial growth within lysis zones were as described by Diaz *et al.* (1989).

Preparation and in vitro manipulation of DNA. Phage DNA was extracted as described by Suárez & Chater (1981) and total DNA from lysogenic and non-lysogenic strains was prepared according to Hintermann *et al.* (1981). Digestion of DNA with restriction enzymes, subsequent analysis by agarose gel electrophoresis and molecular size standards were as in Hopwood *et al.* (1985). Nick translation and Southern blots were also performed as described by Hopwood *et al.* (1985), using hybridization procedure B.

Treatment with chelating agents and isolation of phage deletion mutants. One hundred microlitres of a ϕ A7 phage suspension (about 10^8 – 10^9 p.f.u. ml⁻¹) was added to 900 μ l of EDTA (Na⁺ salt, pH 7.4) or pyrophosphate (Na⁺ salt, pH 7.5) to give a final chelating agent concentration of 100 mM. The mixture was incubated at 37 °C for 30 min. Since surviving phages proved to be resistant to further treatments in the same conditions, they were subjected to one more cycle of inactivation, increasing the concentration of chelating agent to 200 mM and the temperature of incubation to 60 °C. After each cycle the number of viable phages was determined, resistant clones were selected and purified by single-plaque isolations, and their DNA was examined by digestion with restriction enzymes.

Transfection. Transfections of *S. lividans* protoplasts with ϕ C31 (control) or ϕ A7 DNA were performed by the liposome-assisted method developed by Rodicio & Chater (1982), but spores of *S. antibioticus* were used as indicator to detect plaque formation after transfection of *S. lividans* protoplasts by ϕ A7 DNA.

Results and Discussion

Restriction map of ϕ A7 DNA

The genome of ϕ A7 is a linear double-stranded DNA molecule of 46.7 kb, with cohesive ends. Its restriction map for nine endonucleases is shown in Fig. 1(a). The map was obtained using double or triple digestions together with the information about the location of the cohesive ends. In addition, some fragments were subcloned into pBR322 (Bolivar *et al.*, 1977) or into the pUC18 derivative pIJ2921 (Norlander *et al.*, 1983; G. R. Janssen, unpublished results), to confirm the mapping data. The DNA of the phage (propagated on *S. antibioticus* ATCC 11891) contained no sites for *Bam*HI, *Bgl*II, *Hind*III, *Pst*I, *Pvu*II, *Sac*I or *Sal*I, one for *Sac*II, two for *Hpa*I, four for *Cla*I, five for *Pvu*I, six for *Sph*I and *Xba*I, seven for *Eco*RI, ten for *Eco*RV and eleven for *Bcl*II. Comparison of the restriction map of ϕ A7 DNA with those reported for other bacteriophages revealed no similarities.

Mode of lysogenization and location of the *attP* site

The mechanism of lysogenization by phage ϕ A7 was examined by Southern hybridizations. Total DNA from a ϕ A7 lysogen of *S. orchidaceus* was digested with several restriction enzymes and probed with ³²P-labelled ϕ A7 DNA. DNA of the non-lysogenic strain and phage DNA, digested with the same enzymes, were included as controls. Fig. 2(a) shows that the prophage is formed by integration of circularized phage DNA into the bacterial genome, according to the model given by Campbell (1962) for phage λ . Hybridization of ϕ A7 DNA with the DNA of the lysogen digested with *Eco*RV + *Cla*I (Fig. 2a, lane 2) showed the loss of the two bands (*Eco*RV-F and *Eco*RV-G, 2.6 and 2.2 kb respectively) which contain the left and right cohesive ends in ϕ A7 DNA (Fig. 2a, lane 1; see also Fig. 1) and the appearance of a new band (4.8 kb) corresponding to the *cos* fragment, formed by ligation of the cohesive ends. In addition, the phage 13.1 kb *Eco*RV–*Cla*I fragment, in which the integration site (*attP* site) has to be located, is lost in the lane of the lysogen, and two new bands are clearly detected. These bands are probably the two phage DNA–bacterial DNA junction fragments predicted by the Campbell model. In *Bcl*II digestions the number of bands in the DNA of the lysogen (Fig. 2a, lane 5) is as expected if the phage DNA remains circularized and free inside the host. However, since integration was demonstrated by *Eco*RV + *Cla*I digestions, it is possible that it occurs through the *cos* fragment (*Bcl*II-D + A). If so, the fused cohesive ends would not be present in the DNA of the lysogen, and then the large band which appears would be one of the junction fragments. The expected second fragment may hybridize too weakly to be detected (if the *attP* site is very close to one end of the restriction fragment) or may be unresolved in the large band at the top of the gel.

In summary, results of these hybridizations suggest that the *attP* site could be located in the overlapping region between the 13.1 kb *Eco*RV–*Cla*I fragment and the 2.6 kb *Bcl*II-D fragment (which contains the left cohesive end), that is in the 1.9 kb *Eco*RV–*Bcl*II fragment (see Fig. 1). Hybridizations using *Eco*RV, *Cla*I and *Sph*I digestions (data not shown) do not disagree with this hypothesis.

In order to verify the proposed location of the *attP* site, the 13.1 kb *Eco*RV–*Cla*I fragment was subcloned into pBR322, to give pUO1010. A restriction map of the fragment showing the relevant sites is presented in Fig. 2(b). The 8.6 kb *Eco*RV–*Sph*I fragment of plasmid pUO1010 was purified from an agarose gel and used as radioactive probe against DNA of the phage and of the lysogen of *S. orchidaceus*. Digestions with *Eco*RV + *Sph*I clearly demonstrated that the *attP* site is located within

Fig. 1. (a) Restriction map of wild-type ϕ A7 DNA. Letters in the block diagrams are fragment designations in order of decreasing size. The precise position of the *EcoRV*-I, -J and -K fragments was not determined. The DNA contains no sites for *Bam*HI, *Bgl*II, *Hind*III, *Pst*I, *Pvu*II, *Sac*I or *Sal*I. The region established by Southern hybridizations to contain the *attP* site is indicated by the horizontal bar above the map. (b) Location of the deletions in the DNA of phage ϕ A7 and characteristics of the deletion derivatives. The extent of the deletions is indicated by bars below the map (broken lines indicating uncertainty about the exact ends). Lengths of deletions are given in parentheses. C, clear plaques; T, turbid plaques; +, ability, -, inability to lysogenize.

* Four independent deletion mutants with identical structure were obtained. Three gave turbid plaques and retained the ability to lysogenize; one gave clear plaques and could not lysogenize *S. orchidaceus* or *S. albus* P.

the 8.6 kb *EcoRV*-*Sph*I fragment used as probe. Thus, a band the size of the probe appears in the lane corresponding to phage DNA (Fig. 2c, lane 1) but not in the lane of the lysogen (Fig. 2c, lane 2). In the latter the two expected junction fragments are observed. Digestions with *Bcl*I and with *EcoRV* + *Bcl*I allowed a closer location of the *attP* site. In *Bcl*I digestions, according to results shown in Fig. 2(a), the only fragment which is absent in the DNA of the lysogen (Fig. 2c, compare lanes 3 and 4) is *Bcl*I-D. Digestions with

*Bcl*I + *EcoRV* confirm that ϕ A7 *attP* site is located within the 1.9 kb *Bcl*I-*EcoRV* fragment, which is present in the phage DNA but not in the lysogen (Fig. 2c, lanes 5 and 6). Moreover, the fact that only one of the two junction fragments can be observed in both *Bcl*I and *Bcl*I + *EcoRV* digestions strongly suggests that the ϕ A7 *attP* site is very close to the right end of the fragment.

As an additional control the 3.9 kb *Sph*I-*Cla*I fragment from pUO1010 (Fig. 2b) was also probed to DNA of the phage and the lysogen. *Sph*I + *EcoRV* and *Bcl*I

Fig. 2. Location of the *attP* site. (a) Southern hybridizations of digested DNA isolated from ϕ A7 (lanes 1 and 4), a lysogenic strain of *S. orchidaceus* (lanes 2 and 5) and the non-lysogenic strain (lanes 3 and 6). 32 P-labelled ϕ A7 DNA was used as probe. (Fragments smaller than 1 kb, which are not detectable, were observed after longer exposures; data not shown.) (b) Restriction map of the left end of ϕ A7 DNA, showing sites for endonucleases which were relevant for a close location of the *attP* site. The 13.1 kb *EcoRV*-*ClaI* fragment inserted into pBR322 (thick line) and its flanking DNA (dashed lines) are represented. Two probes obtained from the subcloned fragment are indicated by thin lines below the map. (c) Hybridizations of the 8.6 kb *EcoRV*-*SphI* probe to ϕ A7 DNA (lanes 1, 3 and 5) and to DNA of the lysogen of *S. orchidaceus* (lanes 2, 4 and 6). (d) Hybridizations between the 3.9 kb *SphI*-*ClaI* probe and DNAs of the phage (lanes 1 and 3) and the lysogen (lanes 2 and 4). The positions of size standards (kb) are shown. Black circles, fragments containing the cohesive ends; open circles, *cos* fragments; asterisks, fragments carrying the *attP* site; arrows, presumed junction fragments flanking the prophage; asterisks enclosed in a circle, fragments containing both the *attP* site and one of the two cohesive ends.

digestions were used. As expected, identical bands were obtained, irrespective of the source of the DNA (Fig. 2d).

Spontaneous clear-plaque mutants

Spontaneous clear-plaque mutants arose from wild-type ϕ A7 at a frequency of 10^{-4} . DNA extracted from these

mutants displayed the same restriction pattern as the wild-type DNA, indicating that the mutations were not the result of large deletions.

The clear-plaque phenotype occurred not only on strains that could be lysogenized by wild-type phage, but also on *S. antibioticus* ATCC 11891. In this strain, wild-type ϕ A7 forms turbid plaques but not stable lysogens (Diaz *et al.*, 1989). ϕ A7 lysogens of *S. orchidaceus* and *S.*

albus P were resistant to lysis by clear-plaque mutants. Such mutants thus remain susceptible to the prophage repressor, but have lost the ability to establish lysogeny.

Sensitivity to chelating agents

$\phi A7$ clones resistant to chelating agents were selected by passing phages through successive cycles of EDTA or sodium pyrophosphate treatment, as described in Methods. The first treatment (100 mM of chelating agent at 37 °C for 30 min) resulted in a decrease in phage titre of 10^4 -fold and 10^3 -fold for EDTA and pyrophosphate respectively. Surviving phages were resistant to further treatments under the same conditions. Increasing the concentration of the chelating agent to 200 mM and the temperature of incubation to 60 °C only led to a five-fold reduction in the phage titre, with all survivors resistant to a second identical treatment.

Isolation and characterization of viable deletion mutants

In order to detect deletion mutants among pyrophosphate- or EDTA-resistant clones, DNA from more than 100 clones (obtained in four independent experiments, as described above) was examined by *Bst*EII digestion. This enzyme cuts the phage genome at more than 20 sites (Diaz *et al.*, 1989). A total of nine different deletion mutants, $\phi A7\Delta 1$ to $\phi A7\Delta 9$, were identified. The extent of the deletions and their approximate locations in the $\phi A7$ restriction map are shown in Fig. 1 (*b*). They were mapped by digestion of the DNA of each type of mutant with *Bcl*I, *Cla*I, *Eco*RV, *Sac*II and *Sph*I. Between 0.6 and 3.1 kb of DNA was deleted in the mutants. Together they defined at least 7.9 kb of DNA dispensable for plaque formation. Fig. 1 (*b*) also lists the plaque morphology and the ability of the deletion derivatives to lysogenize *S. orchidaceus* and *S. albus* P.

$\phi A7\Delta 1$, $\phi A7\Delta 8$ and $\phi A7\Delta 9$ had deletions located close to the unique *Sac*II site (about 18 kb from the right end of the genome), but all retained this site. These mutants produced clear plaques in all hosts tested (*S. orchidaceus*, *S. albus* P and *S. antibioticus*). All other deletions occurred near the left end of the conventional restriction map. Four of them ($\Delta 2$, $\Delta 3$, $\Delta 4$ and $\Delta 7$) were internal to the *Eco*RV-A fragment. The other two ($\Delta 5$ and $\Delta 6$) affected fragments *Eco*RV-A and *Eco*RV-F, causing their fusion. As pointed out above, the *attP* site is located within the *Eco*RV-A fragment, close to the junction with *Eco*RV-F. However, none of the mutants with deletions in the left region and still capable of producing turbid plaques had lost the ability to form stable lysogens. Other mutants with deletions in the same region formed clear plaques.

All clear-plaque mutants described in this paper were unable to infect $\phi A7$ lysogens, i.e. they were not virulent. Isolation of non-virulent, clear-plaque mutants with deletions in two different regions of the phage genome could indicate the requirement of more than one gene for the establishment of lysogeny (as in the well-characterized situation of phage λ ; Bode & Kaiser, 1965). However, as stated above, clear-plaque mutants were obtained with high frequency from wild-type $\phi A7$. This raises the possibility that spontaneous secondary mutations might be the cause of the clear-plaque phenotype in some of the deletion mutants. In this regard, it should be emphasized that four independent deletion mutants with apparently identical restriction patterns were isolated for $\phi A7\Delta 4$. Three of them produced turbid plaques, whereas one gave clear plaques. Thus, there are two possibilities to explain the latter mutant: (i) the deletion is in fact different, despite the identical restriction patterns; or (ii) it is identical to the three others and a spontaneous second-site mutation led to the clear-plaque phenotype. In order to determine if clear-plaque mutants obtained from $\phi A7$ belong to one or more complementation groups, ability to form stable lysogens after coinfection with pairs of clear-plaque mutants should be analysed. All clear-plaque mutants of $\phi C31$ obtained so far fell into one complementation group. Therefore there is no evidence for an involvement of more than one gene product in the establishment and maintenance of $\phi C31$ lysogeny, although this possibility has not been definitively excluded (Mkrtumian & Lomovskaya, 1972; Lomovskaya *et al.*, 1980).

Existence of a region dispensable for lytic growth close to one end of the DNA molecule seems to be a general property of genomes of *Streptomyces* phages. Apart from $\phi A7$, this fact was previously reported for $\phi C31$ (Chater, 1986), SH10 (Walter *et al.*, 1981), R4 (Ishihara *et al.*, 1982; Morino *et al.*, 1983) and TG1 (Foor *et al.*, 1985). In at least three of these phages the *attP* site is located within ($\phi C31$) or very close to (TG1 and $\phi A7$) the area affected by the deletions. In $\phi C31$, another region dispensable for plaque formation has been detected in the centre of the DNA molecule. Deletions in this region caused a clear-plaque phenotype. Therefore the locations of non-essential DNA in $\phi C31$ and $\phi A7$ genomes roughly coincide.

Transfection with $\phi A7$ DNA

Attempts to transfect *S. antibioticus* protoplasts with $\phi A7$ DNA were not very successful. Although factors known to affect uptake of DNA by *Streptomyces* protoplasts were varied (age of the culture, concentration of glycine), frequencies obtained were always very low (less than 10^2 per μ g of DNA).

Phage ϕ A7 is unable to form plaques on *S. lividans* 1326, a strain for which an efficient transfection system is available (Rodicio & Chater, 1982). It was demonstrated that ϕ A7 does not adsorb to *S. lividans* (L. A. Diaz, unpublished results). We therefore transfected *S. lividans* protoplasts with free ϕ A7 DNA, using spores of *S. antibioticus* as indicator to detect plaque formation by phage particles released from transfected protoplasts. In these conditions, and using the liposome-assisted procedure (Rodicio & Chater, 1982), the efficiency of transfection was 6×10^6 p.f.u. per μ g DNA, equivalent to 3×10^{-4} p.f.u. per DNA molecule (since the M_r of ϕ A7 DNA is 31.2×10^6). These values are similar to those obtained using ϕ C31 DNA to transfect *S. lividans* protoplasts, with spores of *S. lividans* as indicator: 10^7 p.f.u. per μ g DNA or 4.5×10^{-4} per DNA molecule (assuming an M_r of 27.5×10^6 for ϕ C31 DNA; Rodicio & Chater, 1982).

Concluding remarks

Among several actinophages isolated from soil on the oleandomycin producer *S. antibioticus*, the temperate, broad-host-range phage ϕ A7 was selected for its potential advantages as DNA cloning vector (Diaz *et al.*, 1989). The generation of a restriction map of ϕ A7 DNA and the isolation of viable deletion mutants, reported in this paper, have allowed a better assessment of the possibilities of the phage.

Deletions accounted for a total of about 7.9 kb (16.9% of the phage genome), distributed in two different regions. The phage DNA contains a single *Sac*II site close to one of the dispensable regions. However, several attempts to introduce a selectable marker into the unique *Sac*II site of ϕ A7 Δ 2 (the mutant carrying the largest deletion) have failed (L. A. Diaz, unpublished results), suggesting that this site is in an essential region of ϕ A7. Since no other restriction enzyme tested with ϕ A7 DNA has given only one cut, the use of ϕ A7 will require its partial digestion with enzymes which cut several times. The best candidate is *Sph*I, which cuts at least twice in a dispensable region. Experiments are now in progress to introduce a polylinker with recognition sites for enzymes which do not cut the phage genome (*Bam*HI, *Bgl*II, *Hind*III, *Pst*I, *Sac*I and *Sal*I) into ϕ A7 DNA partially digested with *Sph*I. ϕ A7 Δ 6 is being used since the *Sph*I sites known to be dispensable were lost in the mutants carrying the largest deletions (Δ 2 and Δ 3).

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