Physical and Genetic Characterization of Chromosomal Copies of the *Streptomyces coelicolor* Mini-circle

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The two linear, integrated mini-circle copies of *Streptomyces coelicolor* A3(2) were cloned in *Escherichia coli* and their positions on the *S. coelicolor* genetic map were determined. Mini-circle copy A is close to the *argA* locus, 20 kb upstream of the *gyl* operon. Mini-circle copy B is close to the *cysD* locus and is absent from *S. coelicolor* J1501, which has suffered a chromosomal deletion including mini-circle copy B and possibly associated with the *pgl* mutation. The mini-circle copies were not involved in any of the several previously identified physical interactions between the plasmid SCP1 and the *S. coelicolor* chromosome. A new insertion sequence was identified close to the right end of mini-circle copy B in *S. coelicolor* M145. The free mini-circle of *S. coelicolor*, when inserted into an *attP*-deleted derivative of phage ϕ C31, actively integrated this phage into the chromosomes of *S. coelicolor* and *S. lividans* at preferred and secondary sites. The resulting prophages were stably inherited and remained physically intact. No precise excision of prophages from *S. lividans* lysogens carrying insertions at preferred or secondary integration sites was detected: instead, free phages were generated by imprecise excisions. These phages allowed the *in vivo* cloning of segments (> 3 kb in length) of the chromosomal DNA flanking preferred and secondary integration sites. Attempts to delete the preferred integration site by double homologous recombination with a clone carrying flanking DNA sequences and an antibiotic resistance gene were unsuccessful.

INTRODUCTION

The 2.6 kb mini-circle sequence of *Streptomyces coelicolor* A3(2) exists in the *S. coelicolor* genome as two linear, integrated copies (A and B) and also as free mini-circles with a copy number of less than one per ten genomes (Lydiate *et al.*, 1986). Mini-circle functions actively integrate phage KC591 (an *attP*-deleted phage ϕ C31 derivative carrying a cloned free mini-circle) into the genomes of many *Streptomyces* strains. In *S. lividans* this integration is most often at a preferred site but also occurs at secondary sites. The biological properties of the mini-circle, including the mechanism of its integration, the randomness or otherwise of potential host insertion sites and the ability of linear mini-circle copies to excise (precisely or imprecisely), are obviously worthy of investigation.

Several genetic properties of the *S. coelicolor* host of the 2.6 kb mini-circle are likely to involve endogenous transposable elements. Interactions of plasmid SCP1 with various regions of the *S. coelicolor* chromosome caused the formation of highly fertile donor strains and SCP1'

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plasmids (Vivian & Hopwood, 1973; Hopwood & Wright, 1976a; Hopwood *et al.*, 1984). By analogy with the formation of Hfr strains in *Escherichia coli* (Deonier & Hadley, 1980; Guyer *et al.*, 1980) these interactions probably involved transposable elements. Indeed, an insertion sequence has recently been implicated in the integration of SCP1 into the *S. coelicolor* chromosome which yielded the NF fertility type (Kendall & Cullum, 1986). The *pgl* locus of *S. coelicolor* (which is involved in host resistance to infection by phage ϕ C31) is unstable, mutating and reverting at high frequencies (Chinenova *et al.*, 1982). DNA rearrangements might account for this instability and mini-circle copy B was absent from the *pgl* mutant strain J1501 but conserved in all *pgl*⁺ *S. coelicolor* strains investigated, including strain M130, the direct progenitor of J1501 (Lydiate *et al.*, 1986).

The mini-circle sequence of *S. coelicolor* may function as an effective vector for the stable, unit copy cloning of genes in *Streptomyces*. Integration of KC591 into many *Streptomyces* strains (Lydiate *et al.*, 1986) demonstrated the ability of the mini-circle to integrate a 40 kb insert into a wide range of hosts, and the mini-circle-directed integration of pIJ4210 (a pBR327 derivative carrying a mini-circle linearized at the *Hind*III site and a *tsr* gene for thiostrepton resistance) into the preferred site of *S. lividans* (Lydiate *et al.*, 1987) demonstrated that *S. lividans* can be efficiently transformed by mini-circle DNA isolated from *E. coli* and possessing no *Streptomyces* replicon.

Transposable elements that function as effective insertional mutagens have been very useful in molecular investigations of many organisms, facilitating the cloning and genetic mapping of mutated genes. Two derivatives of the 2.6 kb mini-circle, phage KC591 and *E. coli* plasmid pIJ4210, are potential insertional mutagens for the genus *Streptomyces*. They carry antibiotic resistance markers and are actively integrated into *Streptomyces* chromosomes by their cloned mini-circle copies (Lydiate *et al.*, 1986, 1987). However, in order to be useful mutagens these mini-circle derivatives must be able to insert into host genomes at very many different sites.

We report here the cloning of the two linear, integrated mini-circle copies of *S. coelicolor*, together with the mini-circle/chromosome junctions of integrated KC591 prophages. Linear mini-circle copies A and B were located on the *S. coelicolor* genetic map and any involvement of the mini-circle in the generation of *S. coelicolor* donor types or the instability of the Pgl phenotype was investigated. A study of the excision of KC591 prophages from *S. lividans* and attempts to isolate mini-circle-induced mutations in *S. lividans* and to delete the preferred site of mini-circle integration from *S. lividans* are also described.

METHODS

Bacterial strains, plasmids and bacteriophages. *Streptomyces* strains are listed in Table 1. *E. coli* strains F⁻ *lacZ* Δ M15 *recA* (Rüther *et al.*, 1981), ED8767 (Murray *et al.*, 1977) and GM242 (*dam-3 recA rpsL*; M. G. Marinus, personal communication) were used as plasmid hosts. Plasmids and phages are listed in Table 2.

Culture conditions, genetic techniques and transformation. General culture conditions and genetic techniques for *Streptomyces* strains and phages were as described by Hopwood *et al.* (1985). Selenate-resistant mutants were selected on MM-S medium containing glutathione (0.2 mM) and selenate (5 mM) as described by Lydiate *et al.* (1988); 2-deoxyglucose-resistant mutants were selected on NMM medium containing arabinose (25 mM) and 2-deoxyglucose (50 mM) as described by Hodgson (1982). Free phages were isolated from lysogens by inoculating pyrophosphate-washed spores of the lysogens into soft agar overlays seeded with *S. lividans* 1326 spores. The phages released from *S. lividans* TK64::KC591 lysogens were characterized by picking plaques (16 per plate) to soft agar overlays seeded with *S. lividans* 1326 spores, incubating these overnight, treating them with chloroform to kill the remaining 1326 growth and replicating them to R2YE plates seeded with *S. lividans* TK64 spores. After incubation the resulting well-sporulated lawns were replicated to supplemented MM plates containing either thiostrepton or viomycin to select for resistant transductants.

Culture, transformation and colony hybridization of *E. coli* were carried out as described by Maniatis *et al.* (1982).

In vitro manipulation of DNA. Plasmid, phage and chromosomal DNAs were isolated as described by Hopwood *et al.* (1985). All genetic characteristics (particularly those of the unstable donor types) were tested before chromosomal DNA isolation. Endonucleases and DNA ligase were used as recommended by the suppliers. Electro-elution of DNA fragments from agarose gels was as described by Maniatis *et al.* (1982). DNA-DNA hybridizations (including the pBR327 hybridizing standard) were as described by Lydiate *et al.* (1986).

Table 1. *Streptomyces* strains

John Innes stock number	Description*	Reference†
<i>S. coelicolor</i> A3(2) derivatives		
1190	<i>hisA1 strA1 uraA1</i> (SCP1 ⁻ SCP2 ⁺)	A
1889	<i>pgl</i> mutant of A200	B
1915	<i>pgl</i> mutant of 1190	B
1956	<i>cysB6 hisD3 strA1</i> (SCP1 ⁻ SCP2 ⁺)	C
1984	1956 containing SCP1'- <i>cysB</i>	C
2106	<i>pheA1</i> (SCP1 integrated to give stable <i>cysD</i> donor)	D
2147	<i>pgl</i> mutant of A700	B
2293	<i>cysB6 hisD3 strA1</i> (bidirectional donor derived from SCP1'- <i>cysB</i>)	C
2361	<i>pgl</i> mutant of 1956	B
2612	<i>argA1 cysD18 proA1</i> (NF, SCP2 ⁻)	B
A200	<i>ade-v10 pheA1 strA5</i> (SCP1 ⁻ SCP2 ⁺)	A
A317	<i>hisA1 strA1 uraA1</i> (NF)	A
A607	<i>pheA1</i> (transient interaction of SCP1 with the chromosome to give unstable <i>pabA</i> donor)	E
A608	<i>pheA1</i> (SCP1 integrated to give stable <i>pabA</i> donor)	E
A634	<i>argA1 cysA24 proA1 strA1</i> (NF-like donor)	E
A700	<i>argA1 cysD18 proA1</i> (SCP1 ⁻ SCP2 ⁺)	F
DL30	J1501::KC591 (prophage integrated at secondary site C)	G
DL31	J1501::KC591 (prophage replaced mini-circle copy A)	G
DL32	J1501::KC591 (prophage replaced mini-circle copy A)	G
DL33	J1501::KC591 (prophage integrated at secondary site D)	G
DL34	J1501::KC591 (prophage integrated at secondary site D)	G
DL35	J1501::KC591 (prophage not integrated via mini-circle)	G
DL140	1190 mini-circle B::KC590	G
DL142	1190 mini-circle A::KC590	G
DL177	DL31 with additional Arg ⁻ and Cys ⁻ mutations	G
J1501	<i>pgl</i> mutant of M130	H
M107	<i>argA1 cysD18 proA1</i> (SCP1 ⁻ SCP2 [*])	F
M130	<i>hisA1 strA1 uraA1</i> (SCP1 ⁻ SCP2 ⁻)	I
M138	<i>argA1 cysD18 proA1</i> (SCP1 ⁺ SCP2 ⁻)	J
M145	'Wild-type' (SCP1 ⁻ SCP2 ⁻)	J
<i>S. lividans</i> 66 derivatives		
1326	Wild-type (SLP2 ⁺ SLP3 ⁺)	K
DH97	TK64::pIJ983	G
DH102	TK64::pIJ983	G
DL1	TK64::KC591 (prophage integrated at secondary site A)	L
DL3	TK64::KC591 (prophage integrated at secondary site B)	L
DL4	TK64::KC591 (prophage integrated at preferred site)	L
DL5	TK64::KC591 (prophage integrated at preferred site)	L
DL73‡	TK54::KC591 (prophage integrated at preferred site)	
	Dog ^R Vio ^S	G
DL102‡	TK64::KC591 (prophage integrated at preferred site)	
	Sel ^R Vio ^S	G
DL105‡	TK54::KC591 (prophage integrated at preferred site)	
	Sel ^R Vio ^S	G
TK54	<i>his-2 leu-2 spc-1</i> (SLP2 ⁻ SLP3 ⁻)	M
TK64	<i>pro-2 str-6</i> (SLP2 ⁻ SLP3 ⁻)	M

* Symbols for genetic markers as in Hopwood *et al.* (1985). Resistance phenotypes: Dog^R, 2-deoxyglucose resistant; Sel^R, selenate resistant; Vio^S, viomycin sensitive.

† A, Vivian & Hopwood (1970); B, H. M. Kieser, unpublished; C, Hopwood & Wright (1976a); D, Hopwood & Wright (1976b); E, Vivian & Hopwood (1973); F, Bibb *et al.* (1977); G, this work; H, Chater *et al.* (1982); I, Bibb *et al.* (1981); J, M. J. Bibb, unpublished; K, Lomovskaya *et al.* (1972); L, Lydiate *et al.* (1986); M, Hopwood *et al.* (1983).

‡ These strains independently suffered large deletions, each of which removed the left prophage/chromosome junction (Figs 5 and 6).

Table 2. *Plasmids and phages*

Plasmids	Description*	Source†
pBR327	<i>E. coli</i> cloning vector	A
pIJ680	<i>A. Streptomyces</i> plasmid cloning vector	B
pIJ702	<i>A. Streptomyces</i> plasmid carrying the <i>tsr</i> gene	C
pIJ950	pBR327:free mini-circle	D
pIJ952	pBR327:mini-circle copy A	E
pIJ954	pIJ2920:mini-circle copy A (left end)	E
pIJ955	As pIJ954 but with opposite insert orientation	E
pIJ956	pIJ2920:1.45 kb <i>Sst</i> I fragment of DJ1	F
pIJ957	As pIJ956 but with opposite insert orientation	F
pIJ958	pIJ2920:mini-circle copy A (right end)	E
pIJ959	pIJ2920:4.5 kb <i>Bgl</i> II- <i>Sst</i> I fragment of DJ2	F
pIJ961	pIJ2925:mini-circle copy A (right end)	E
pIJ962	pIJ2925:mini-circle copy B (right end)	E
pIJ963	pIJ2922:1.75 kb <i>Pst</i> I- <i>Bam</i> HI <i>hyg</i> gene fragment of pIJ2349	G
pIJ965	pSAE:mini-circle copy A	G
pIJ966	pSAE:mini-circle copy B	G
pIJ981	pIJ2926: <i>S. coelicolor</i> <i>sel/cys</i> cluster	P
pIJ982	pIJ963 with DNA naturally flanking the left end of mini-circle copy A inserted adjacent to the <i>hyg</i> gene	G
pIJ983	pIJ982 with DNA naturally flanking the right end of mini-circle copy A inserted adjacent to the other end of the <i>hyg</i> gene	H
pIJ987	As pIJ962 but with opposite insert orientation	E
pIJ2220	pSAE:mini-circle copy A	I
pIJ2349	pIJ680: <i>hyg</i> gene of <i>S. hygroscopicus</i>	J
pIJ2920	pUC18 derivative with modified poly-linker	K
pIJ2922	pUC18 derivative with modified poly-linker	K
pIJ2925	pUC18 derivative with modified poly-linker	K
pIJ2926	pUC18 derivative with modified poly-linker	K
pSAE	<i>E. coli</i> cosmid cloning vector	L
pUC18	<i>E. coli</i> cloning vector	M
ϕ C31-derived phages		
DJ1	Released from strain DL4, transduced <i>S. lividans</i> to Thio ^R alone at a moderate efficiency	F
DJ2	Released from strain DL4, transduced <i>S. lividans</i> to Vio ^R alone at a low efficiency	F
DJ3	Released from strain DL5, transduced <i>S. lividans</i> to Thio ^R alone at a moderate efficiency	F
DJ4	Released from strain DL5, transduced <i>S. lividans</i> to Vio ^R alone at a low efficiency	F
DJ5	Released from strain DL1, transduced <i>S. lividans</i> to Vio ^R alone at a low efficiency	G
KC300	ϕ C31 <i>attP</i> ⁺ <i>c</i> ⁺ Δ 23::pBR322 Δ W12::vph	N
KC515	ϕ C31 <i>attP</i> ⁻ <i>c</i> ⁺ Δ W17::pBR322::vph::tsr	O
KC590	KC515 carrying the internal 1.82 kb <i>Sst</i> I- <i>Bcl</i> I fragment of the mini-circle (<i>vph</i> gene partially deleted)	D
KC591	KC515 carrying the entire free mini-circle linearized at the <i>Bcl</i> I site	D

* *hyg*, gene conferring hygromycin resistance; Thio^R, thiostrepton resistance; Vio^R, viomycin resistance; *tsr*, gene conferring Thio^R; *vph*, gene conferring Vio^R.

† A, Soberon *et al.* (1980); B, Hopwood *et al.* (1985); C, Katz *et al.* (1982); D, Lydiate *et al.* (1986); E, Fig. 3; F, Fig. 6; G, this work; H, Fig. 7; I, Smith & Chater (1988); J, F. Malpartida, personal communication; K, G. R. Janssen, personal communication; L, Grosveld *et al.* (1982); M, Yanisch-Perron *et al.* (1985); N, Chater *et al.* (1982); O, Rodicio *et al.* (1985); P, Lydiate *et al.* (1988).

RESULTS

Genetic mapping of the integrated mini-circle copies of S. coelicolor

Direct genetic mapping of the integrated mini-circle copies was not possible because they do not confer any known phenotype on their *S. coelicolor* host. Therefore, KC590 prophages (*attP*⁻ *tsr* derivatives of phage ϕ C31 containing an internal segment of the mini-circle) were

Fig. 1. Southern hybridization of a ^{32}P -labelled pIJ950 (pBR322:free mini-circle) probe to DNA samples as indicated. S, pBR327 hybridizing standard (Lydiate *et al.*, 1986) with fragments 27.4, 17.6, 10.1, 7.6, 6.3, 4.4, 3.3, 2.6, 1.85 and 1.4 kb in size. The absence of either the 7.8 kb (mini-circle copy A) or the 12.4 kb (mini-circle copy B) *Bam*HI fragment from the *S. coelicolor* 1190::KC590 lysogens demonstrated prophage integration into one or other of these fragments. (The KC590 prophages have no *Bam*HI sites and yield a 40 kb *Bam*HI fragment.) The retention of the four normal mini-circle-homologous *Pvu*II bands (4.6, 4.3, 4.0 and 3.3 kb in size) by all seven 1190::KC590 lysogens demonstrated that in each case the normal mini-circle/chromosome junctions had been maintained.

integrated into the two linear mini-circle copies of *S. coelicolor* 1190 by homologous recombination (Lydiate *et al.*, 1986, and Fig. 1) and their thiostrepton resistance gene (*tsr*) was used to map the mini-circle copies just as the viomycin resistance gene (*vph*) of KC407 prophages was used to map the IS110 copies of *S. coelicolor* (Chater *et al.*, 1985).

Mini-circle copy A was mapped close to *argA* using a cross involving the SCP1^{NF} fertility factor (Fig. 2a). Consideration of the allele frequencies placed *tsr* either between *proA* and *uraA* (arc 6) or close to *argA*. Since segregation of *tsr* was essentially independent of the segregation of *proA* or of *uraA*, but strongly dependent on the segregation of *argA* (Fig. 2a), the latter position was indicated. This position was confirmed when mini-circle copy A and the *gyl* operon (which also maps close to *argA*: Seno *et al.*, 1984) were found to be separated by only 20 kb in the cloned *S. coelicolor* DNA of pIJ2220 (see below).

Fig. 2. Genetic mapping of integrated *S. coelicolor* mini-circle copies A and B. Triangles mark selected alleles. Numbers round the circles are frequencies of alleles among the 100 recombinants scored and the numbers between the circles identify the seven arcs separating the selected and unselected loci. (a) Strains 2612(KC300) (outer circle) and DL142 (1190 mini-circle A::KC590) (inner circle) were crossed and *hisA*⁺ *strA*¹ recombinants were selected. Segregation of the Thio^R marker (*tsr*) of the KC590 prophage with respect to the *arg*, *ura* and *pro* alleles is indicated in the tabulation. (b) Strains 2612(KC300) (outer circle) and DL140 (1190 mini-circle B::KC590) (inner circle) were crossed and *hisA*⁺ *cysD*⁺ recombinants were selected. The three Thio^S recombinants in (b) all carried the *argA*¹, *proA*¹ and *vph* alleles. The *vph* gene was supplied by the KC300 prophage.

Mini-circle copy B was mapped in the region of *cysD*, also using an *SCP1*^{NF} × *SCP1*⁻ cross (Fig. 2b).

Cloning the integrated, chromosomal mini-circle copies of *S. coelicolor*

Approximately 2 kb of the right end of each chromosomal mini-circle copy together with 6 kb of each adjoining chromosomal segment was cloned in *E. coli* as follows. *E. coli* (F⁻ *lacZ* ΔM15 *recA*) was transformed with a ligation mixture of electro-eluted 8.0–8.6 kb *Sph*I fragments of *S. coelicolor* M130 DNA (some of which contained DNA including most of the mini-circle and chromosomal DNA to the right of the integrated mini-circle copies: Fig. 3) inserted into the *Sph*I site of pIJ2925. Colony hybridization of 240 of the resulting LacZ⁻ clones (using the 1.8 kb *Bcl*I–*Sst*I internal mini-circle fragment as a probe) revealed 12 clones with homology to the mini-circle. Partial restriction mapping demonstrated that two of the clones contained mini-circle copy A with the insert in a single orientation (pIJ961: Fig. 3), six contained mini-circle copy B in one orientation (pIJ962) and four contained copy B in the opposite orientation (pIJ987).

Three cosmid clones each carrying 30–40 kb of *S. coelicolor* M138 DNA inserted into the pSAE vector and containing integrated mini-circle copies were isolated from a library of 500 cosmid clones (Smith & Chater, 1988) probed with the 1.8 kb *Bcl*I–*Sst*I internal mini-circle fragment. One of these clones (pIJ2220) had previously been shown to contain the *S. coelicolor* *gyl* operon (Smith & Chater, 1988). The *gyl* operon was 20 kb to the left of mini-circle copy A in pIJ2220 and the *S. coelicolor* DNA insert of pIJ2220 ended 1.7 kb to the right of copy A (Fig. 3). The two other cosmid clones, pIJ965 and pIJ966, contained mini-circle copies A and B, respectively, but were not mapped in detail.

Mini-circle copy A flanked by *S. coelicolor* chromosomal sequences was subcloned as a 7.8 kb *Bam*HI fragment from pIJ2220 into the *Bam*HI site of pBR327. Recombinant plasmids such as pIJ952 (Fig. 3), where the insert was orientated such that transcription from the vector *tet* promoter read into the right end of the insert, were structurally stable; plasmids with the insert in the opposite orientation suffered deletions in *E. coli*. The left mini-circle/chromosome junction together with 1.5 kb of flanking chromosomal DNA was subcloned as a 1.8 kb *Bcl*I fragment from pIJ952 into the *Bam*HI site of pIJ2920 to form pIJ954 and pIJ955 (Fig. 3).

Fig. 3. Partial restriction maps of the two integrated mini-circle copies of the *S. coelicolor* chromosome: thick lines, mini-circle sequences; thin lines, flanking DNA. For most enzymes, only those cleavage sites closest to the mini-circle, in each of the four flanking regions, have been positioned. The extent of various cloned portions of *S. coelicolor* DNA is indicated by horizontal lines below the restriction maps. Arrows above plasmid numbers indicate the direction of transcription of the vector genes, *lacZ* (pIJ2920 and pIJ2925) or *tet* (pBR327), with respect to the cloned inserts. *, The *KpnI* site in parentheses is not present in *S. coelicolor* but occurs in *S. lividans* (it was mapped in pIJ959 and confirmed by Southern hybridization analysis of *S. lividans* DNA using pIJ954 as probe). †, The arrow indicates one end of the cloned *S. coelicolor* DNA of cosmid pIJ2220. ‡, The 0.97 kb insertion in *S. coelicolor* M145 occurred between the *BamHI* and *BclI* sites flanking the right end of mini-circle copy B.

Similarly, the right mini-circle/chromosome junction was subcloned as a 1.75 kb *BamHI*–*SstI* fragment from pIJ952 into pIJ2920 by *BamHI*–*SstI* replacement to form pIJ958.

Mini-circle copy B and the pgl locus

To investigate the possibility that the excision and reintegration of mini-circle copy B was involved with the instability of the Pgl phenotype the DNA of four *pgl* mutants, together with that of their *pgl*⁺ progenitors (1889 and A200; 1915 and 1190; 2147 and A700; 2361 and 1956) was digested with *BamHI* or with *PvuII* and subjected to Southern hybridization analysis using mini-circle DNA (pIJ950; Table 2) as a probe. All eight strains had normal *S. coelicolor* mini-circle copy A and B bands, indicating that Pgl instability does not involve the mini-circle.

DNA of the same four *pgl/pgl*⁺ pairs, together with that of *S. coelicolor* J1501 and M130 and *S. lividans* 1326, was digested with *SstI* and subjected to Southern hybridization analysis using pIJ962 (Fig. 3) as a probe. Bands corresponding to mini-circle copy B and the 6 kb of flanking chromosomal DNA present in pIJ962 were absent from strain J1501 but present in all other *S. coelicolor* strains tested. This indicated that a deletion extending outside mini-circle copy B (at least to the right) had occurred in strain J1501 and that the mini-circle copy had not excised precisely. The *S. lividans* 1326 DNA contained fragments homologous to the *S. coelicolor* chromosomal sequences flanking the right end of mini-circle copy B; however, these sequences did not function as a preferred site for mini-circle integration in *S. lividans* (Lydiat *et al.*, 1986).

The S. coelicolor mini-circle copies are unchanged in highly fertile donor strains

To investigate whether the mini-circle had promoted any of the known SCP1 integrations responsible for the creation of donor strains (Hopwood *et al.*, 1984), the DNA of these strains

Fig. 4. Southern hybridization of a ^{32}P -labelled pIJ950 probe to DNA samples as indicated. S, pBR327 hybridizing standard (see Fig. 1). The elevated copy numbers of the KC591 prophages compared to mini-circle copy A in strains DL33 and DL34 was indicated by the high intensity of prophage bands (e.g. 3.10 kb *PvuII*) when compared to the analogous mini-circle copy A bands (e.g. 4.02 kb *PvuII*).

(1984, 2106, 2293, A317, A607, A608 and A634: Table 1) together with that of the progenitor strains was digested with *Bam*HI or *Pvu*II and subjected to Southern hybridization analysis using pIJ950 as a probe. Neither mini-circle copy was altered in any of the donor strains tested.

The DNA of *S. coelicolor* M145 (used as a control in the above experiment) showed an altered mini-circle copy B hybridization pattern. Similar Southern analysis of M145 DNA digested with *Bcl*I, *Pst*I, *Sph*I or *Sst*I revealed that this strain carried a 0.97 kb DNA insert (with at least one *Pst*I site) to the right of mini-circle copy B (Fig. 3). This insert had no homology to the 2.6 kb mini-circle and differed from IS110, which is 1.6 kb in length (Chater *et al.*, 1985), and the unnamed 1 kb element of Kendall & Cullum (1986), which had no *Pst*I site.

Integration of phage KC591 into S. coelicolor J1501 directed by the mini-circle

The mini-circle insert of phage KC591 actively integrates this phage into the *S. lividans* chromosome to yield Thio^R lysogens (Lydiate *et al.*, 1986). *S. coelicolor* J1501, which carries only mini-circle copy A (at the site on the *S. coelicolor* chromosome homologous to the preferred

Fig. 5. Two rounds of homologous recombination between mini-circle sequences carried by KC591 and those present in the *S. coelicolor* J1501 genome could account for the replacement of mini-circle sequence A by KC591 prophages in strains DL31 and DL32.

integration site for KC591 in *S. lividans*), was infected with KC591 and the DNA of six of the resulting Thio^R transductants (DL30 to DL35) was digested with *Bam*HI or *Pvu*II and subjected to Southern hybridization analysis using pIJ950 as a probe (Fig. 4).

In two of the J1501::KC591 lysogens (DL31 and DL32) KC591 had replaced mini-circle copy A. Because the mini-circle sequence in KC591 (linearized at the *Bc*II site) is a circular permutation of mini-circle copy A (which is linearized at the mini-circle integration point) this replacement is most easily explained by two successive homologous recombination events (Fig. 5). No free mini-circle DNA was detected in strain DL31 (Fig. 4), and the 2.6 kb mini-circle-homologous *Pvu*II fragment detected in strain DL32 might not have originated from free mini-circles but rather from an unresolved Campbell structure analogous to that depicted in Fig. 5 but in which phage KC591 had integrated via homologous recombination between the short (B/B') regions of homology.

Strains DL30, DL33 and DL34 contained KC591 prophages linearized at (or near) their mini-circle integration points and inserted into the *S. coelicolor* genome at a point removed from mini-circle copy A. The KC591 prophages of DL33 and DL34, which might have the same integration point in the *S. coelicolor* genome, appeared to have a copy number approximately twice that of the chromosomal mini-circle copy A (Fig. 4). The integration of the KC591 prophages of strains DL33 and DL34 into a specific site on a plasmid with a copy number of two per genome would explain these observations. Conceivably the plasmid in question was SLP4 (Hopwood *et al.*, 1983).

The sixth J1501::KC591 lysogen (DL35) exhibited the banding pattern expected of a strain carrying a circular KC591 prophage (Fig. 4). This banding pattern could also be explained by a linear, integrated prophage resulting from the lysogenizing KC591 phage having acquired a copy of an insertion sequence (such as IS110) in the ϕ C31 sequences close to the *tsr* gene and having subsequently become integrated into the host chromosome by homologous recombination with a chromosomal copy of the same element.

Attempt to isolate selenate-resistant (Sel^R) or 2-deoxyglucose-resistant (Dog^R) mutants generated by KC591 integration in S. lividans

Replication of approximately 40000 colonies of independent KC591 lysogens of *S. lividans* TK64 and TK54 to media selecting Sel^R mutants (mutated in genes involved in the early stages of sulphate reduction: Lydiate *et al.*, 1988) or Dog^R mutants (produced by glucose kinase mutation: Ikeda *et al.*, 1984) yielded 10 and 11 isolates, respectively. Southern hybridization analysis demonstrated that each isolate, except the Sel^R mutant DL101, contained a single KC591 copy integrated at the *S. lividans* preferred site. Probing DNA of *S. lividans* DL101 with the cloned *sel/cys* gene cluster (pIJ981) demonstrated that the KC591 prophage of this strain was not present in or near these genes. Thus no KC591-induced, insertional mutations were obtained.

Three of the above isolates (DL73, DL102 and DL105) had not been transduced to Vio^R by KC591, whereas all other isolates were Vio^R as well as Thio^R. The Southern hybridization analysis of these strains, together with the direct analysis of prophage *Sst*II bands (as in Fisher *et al.*, 1987), revealed that all three strains had suffered large deletions which removed considerable portions of the KC591 prophages and chromosomal DNA around the left (*vph*) prophage/chromosome junction.

Excision of the resident prophages from S. lividans TK64 :: KC591 and S. coelicolor J1501 :: KC591 lysogens

The *S. lividans* TK64 lysogens DL4 and DL5 (in which the prophages were integrated at the preferred site) and DL1 and DL3 (in which the prophages were integrated at two different secondary sites) all released free phages at similar frequencies (approximately 0.01 plaques per lysogen spore). The biological properties of 32 newly-excised phages from each lysogen were studied. All these released phages differed from KC591, which transduced *S. lividans* to Thio^R and Vio^R at high frequency. Some 17% of the new phages transduced *S. lividans* to both Thio^R and Vio^R at moderate to low frequencies, 25% transduced it only to Thio^R at a moderate frequency and 5% transduced it only to Vio^R at a low frequency. The remaining phages were unable to transduce *S. lividans* to either Thio^R or Vio^R although the majority were able to transduce *S. coelicolor* to Thio^R or to Thio^R and Vio^R, presumably integrating into the *S. coelicolor* chromosome via recombination between shared mini-circle homology. All four of the initial lysogens released phages of the different kinds.

It was expected that phages transducing *S. lividans* to Thio^R alone had lost the *vph* gene at the left end of KC591 (see the KC591 prophage in Fig. 5), while those transducing *S. lividans* to Vio^R alone had lost the *tsr* gene at the right end of KC591. The DNA of four phages (DJ1, DJ2, DJ3 and DJ4: Table 2) was subjected to restriction mapping and Southern hybridization analysis using probes (Fig. 3) specific for the left end (pIJ954) or right end (pIJ958) of mini-circle copy A; the results are summarized in Fig. 6. To assist in the localization of the points of illegitimate recombination which gave rise to phages DJ1 and DJ2 the 1.45 kb *Sst*I fragment of the former was cloned into the *Sst*I site of pIJ2920 to form pIJ956 and pIJ957 (Fig. 6) and the 4.5 kb *Bgl*II-*Sst*I fragment of the latter was cloned into pIJ2920 by *Bam*HI-*Sst*I replacement to form pIJ959 (Fig. 6).

As expected, the Vio^R transducing phages DJ2 and DJ4 had lost all the mini-circle sequences from the right end of the KC591 prophage together with much of the *tsr* gene and had acquired segments of *S. lividans* chromosomal DNA flanking the left end of the KC591 prophage (3.3 kb and 2.0 kb in length, respectively). Contrary to expectation, the Thio^R transducing phage DJ1 retained an intact *vph* gene and carried *S. lividans* chromosomal sequences from the left end of the KC591 prophage. Since the *vph* structural gene of KC591 lacks its promoter (Bibb *et al.*, 1985), the failure of DJ1 prophages to confer Vio^R on the majority of their *S. lividans* lysogens probably indicates the absence of transcription into the *vph* structural gene from adjacent chromosomal/mini-circle sequences in these lysogens.

Phages DJ2 and DJ4 probably integrated into the *S. lividans* chromosome by homologous recombination between shared chromosomal sequences, but the much more efficient

Fig. 6. Restriction maps of the ends of the KC591 prophages of *S. lividans* DL4 and DL5 lysogens (top line) and regions of the fused left and right prophage ends in the imprecisely excised phages DJ1, DJ2, DJ3 and DJ4. Only *Bgl*II, *Cla*I, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II and *Sst*I sites in KC591 and *Xma*I (X) and the two *Bgl*I (B) sites closest to the left mini-circle/chromosome junction in the flanking chromosomal DNA are shown. All *Sst*II (S) sites are shown. The *Xma*I sites of DJ4 were not mapped. N, sites absent from DJ4; *, sites used in subcloning junction fragments; arrows indicate the direction of transcription of the vector *lacZ* gene in pIJ956, pIJ957 and pIJ959 and of the *vph* gene of phage KC591. Values in parentheses are the distances in kb between marked restriction sites.

integration of phages DJ1 and DJ3 (which carry considerably smaller segments of chromosomal DNA but retain most of the mini-circle sequences) is most easily explained by a residual mini-circle-encoded integration activity, possibly able to act on the left or right mini-circle/chromosome junctions of the KC591 prophage which were retained separately by DJ1 and DJ3, respectively. The failure of DJ1 prophages to express the *vph* gene suggests that they are not integrated at the *S. lividans* preferred site.

The physically less well characterized phages released from strains DL1 and DL3 had a similar range of structures to those derived from prophages integrated at the preferred site. For example, phage DJ5, which was released from strain DL1 and transduced *S. lividans* to Vio^R alone and at a low frequency, had lost the right end of KC591 (to a point between the *Cla*I and *Pvu*II sites of the *tsr* gene) and had acquired 4.0–4.5 kb of *S. lividans* DNA flanking the left end of the KC591 prophage.

In an attempt to isolate *S. lividans* TK64 :: KC591 and *S. coelicolor* J1501 :: KC591 lysogens cured of their prophages, *S. coelicolor* DL31 and *S. lividans* DL1 and DL5 were grown in the absence of antibiotic selection and 1000 spores from the resulting lawn of each strain were inoculated onto complete medium (CM: Hopwood *et al.*, 1985). After incubation, the resulting colonies were replicated to MM plates supplemented with only histidine, uracil and proline and containing thiostrepton and viomycin. All colonies were Thio^R and Vio^R, indicating that none were spontaneously cured of the KC591 prophage.

The experiment was repeated (with strains DL5 and DL31) but the spores were first irradiated with UV to 1% survival in case this might stimulate excision. Nineteen of the 800 DL5 and 22 of the 2600 DL31 colonies tested failed to grow after replication. Ten and 12 of the isolated DL5 and DL31 derivatives, respectively, were weakly Vio^R (they grew on R2YE plates containing

S. lividans chromosome

Fig. 7. Two homologous recombination events between the *S. lividans* chromosome and pIJ983, one in each of the two regions of homology, would result in the preferred mini-circle integration site of *S. lividans* being replaced with the hygromycin resistance gene of *S. hygrosopicus*.

30 μg viomycin ml^{-1} but not on MM plates containing 30 μg viomycin ml^{-1}) while four and three, respectively, were Vio^S (they failed to grow on R2YE plates containing 30 μg viomycin ml^{-1}). Five of the DL5 derivatives were Thio^S and six of the DL31 derivatives had single additional auxotrophic growth requirements for arginine, cystine, methionine or serine while one of the DL31 derivatives (DL177) had a double additional requirement for arginine and cystine. No isolate was completely cured of its KC591 prophage.

Attempted replacement of the S. lividans TK64 preferred mini-circle integration site

The replacement of the *S. lividans* TK64 preferred mini-circle integration site with a hygromycin resistance gene (*hyg*) flanked by cloned segments of chromosomal DNA to the left and to the right of *S. coelicolor* mini-circle sequence A was attempted using homologous recombination. The genomes of *S. coelicolor* and *S. lividans* are very similar and sufficient homology was present to allow recombination between the cloned *S. coelicolor* sequences and the *S. lividans* chromosome.

The replacement construct, pIJ983 (Fig. 7), was made by subcloning the 2.3 kb *KpnI*–*BclI* fragment of the left flanking region in pIJ952 (Fig. 3, sites 5–7) into pIJ963 cut with *BamHI* + *KpnI* (pIJ963 carries the 1.75 kb *PstI*–*BamHI* *hyg* gene fragment of pIJ2349 cloned into pIJ2922 cut with *PstI* + *BamHI*) to give pIJ982, followed by subcloning the 3.9 kb *PstI*–*SphI* fragment of the right flanking chromosomal region in pIJ961 (Fig. 3, sites 16–21) into pIJ982 cut with *PstI* + *SphI*.

Transformation of *S. lividans* TK64 with intact pIJ983 yielded 10^3 hygromycin-resistant transformants per μg of DNA and all the transformants analysed by Southern hybridization were found to contain pIJ983 integrated into the *S. lividans* chromosome via single homologous recombination events involving one or other of the regions of homology between pIJ983 and the *S. lividans* chromosome. pIJ702 gave a control frequency of 10^8 transformants per μg of DNA. Replacement of the preferred integration site with the *hyg* gene of pIJ983 would require two recombination events, one in each of the two homologous segments (Fig. 7).

Two of the *S. lividans* TK64::pIJ983 transformants, DH102, in which the plasmid had integrated via the left segment of homology, and DH97, in which the integration had occurred in the right segment of homology, were studied further. In both strains a second recombination event, between the direct repeats of left and right flanking sequences (present as a result of pIJ983 integration), would either excise the plasmid from the chromosome or delete the preferred integration site. Both events were screened for using colony hybridizations. After two rounds of sporulation on non-selective medium about 1% of colonies of both strains were shown to have lost the *hyg* gene by colony hybridization using a *hyg*-specific probe (the *PstI*–*BamHI* fragment of pIJ2349). A similar frequency was revealed by screening for loss of hygromycin

resistance. However, screening 2000 colonies derived from each strain with a probe specific for the preferred site revealed no colonies that had lost the 3.4 kb region containing the preferred mini-circle integration site of *S. lividans*.

DISCUSSION

The two integrated mini-circle copies of *S. coelicolor* A3(2) were located on the *S. coelicolor* linkage map and were therefore shown to be chromosomal. Mini-circle copy A mapped close to the *argA* locus and was positioned 20 kb upstream of the *gyl* operon on the cloned *S. coelicolor* DNA of pIJ2220. Mini-circle copy B mapped close to the *cysD* locus and was included in a large fragment of chromosomal DNA deleted from strain J1501. This deletion might have been the cause of the *pgl* mutation of strain J1501 (*pgl* also maps close to the *cysD* locus) and it is possible that the chromosomal DNA flanking mini-circle copy B and present on plasmids pIJ962 and pIJ966 will be useful as a molecular probe for the cloning of the *pgl* locus. However, the analysis of a further four *pgl* mutants demonstrated that changes in mini-circle copy B are not obligatorily correlated with changes in the Pgl phenotype.

A 0.97 kb insert was discovered in the chromosomal DNA flanking mini-circle copy B of strain M145. This insert is probably an example of a previously unidentified insertion sequence present in the *S. coelicolor* genome.

The KC591 prophages integrated by the mini-circle into the chromosomes of *S. coelicolor* DL31 and *S. lividans* DL1 and DL5 were stably inherited and structurally stable: they were not lost or rearranged in any of 3000 lysogen colonies tested. UV-irradiation appeared to disturb the KC591 prophages but it is not yet clear whether this UV-induced behaviour is due to mini-circle activation, to activation of the phage ϕ C31 genes of the KC591 prophage, or simply to mutations in the host.

The deletions of the left end of the KC591 prophages together with flanking chromosomal DNA in strains DL73, DL102 and DL105 are analogous to the deletions associated with prophages of KC571 and KC572 derivatives (Fisher *et al.*, 1987) and prophages of pPOD9 (Malpartida *et al.*, 1987). An inadvertent selection for prophages unable to support the growth of spontaneously arising defective virulent derivatives of phage ϕ C31 might account for the isolation of these deleted strains.

There is no evidence for the precise excision of integrated mini-circles: precise excision of KC591 has never been observed. Free phages were easily obtained from *S. lividans* : : KC591 lysogens but, while many of these carried large (> 3 kb) lengths of the chromosomal sequences flanking the prophage, none (< 0.1%) exhibited the behaviour of the original KC591 phage. The large insert of ϕ C31 sequences in the *Bcl*I site of the mini-circle in phage KC591 might have disrupted a function involved in mini-circle excision. The uninterrupted mini-circle copy A of J1501 lysogens DL30 and DL33 might provide *trans*-acting functions to allow the precise excision of their KC591 prophages, yielding free phages identical to KC591 and perhaps leading to strains cured of the phage. The cloning of mini-circle copy A in pIJ952 will allow the construction of a linear mini-circle copy marked with the *tsr* gene and the subsequent testing of its ability to excise and reintegrate when introduced into *S. lividans*.

An effective transposon mutagen for the genus *Streptomyces* would be very useful. One candidate for such a mutagen is the *S. fradiae* transposable element Tn4556 (Chung, 1987). Phage KC591 is a particularly attractive potential mutagen because the imprecise excision of inserted KC591 prophages would allow the direct, *in vivo* cloning of any disrupted genes. However, KC591 was ineffective when used as a mutagen in *S. lividans*. This might have been due to the preponderance of insertions into the *S. lividans* preferred site (95–98% of all TK64 : : KC591 lysogens carried KC591 at the preferred integration site: unpublished data) or to the existence of a limited number of secondary insertion sites precluding the disruption of the target glucose kinase and sulphate metabolism genes by insertion of KC591 prophages. KC591 might be a useful insertional mutagen in *Streptomyces* strains which do not have a preferred site for mini-circle integration.

The attempt to select derivatives of *S. coelicolor* : : KC591 and *S. lividans* : : KC591 lysogens

cured of their KC591 prophages and deleted for the preferred mini-circle integration site was unsuccessful. It was hoped to remove the preferred integration site from *S. lividans* and mini-circle copy A from *S. coelicolor* by homologous exchange with a clone carrying the flanking sequences from both sides of these structures, but the attempts in *S. lividans* proved unsuccessful, perhaps because the region containing the preferred integration site carried functions whose deletion was deleterious to the cell under the conditions used. Such functions would have to lie between the mini-circle copy A integration site and *Pst*I site 16 (Fig. 3) because sequences to the left of the mini-circle integration site were deleted from *S. lividans* strains DL73, DL102 and DL105. However, this experiment did demonstrate that efficient homologous recombination occurred between cloned *S. coelicolor* DNA and the homologous *S. lividans* sequences. Successful attempts to delete segments of *Streptomyces* genomes by homologous exchange have been reported. Eckhardt & Smith (1987) successfully deleted the *S. lividans* β -galactosidase gene using segments of homology 0.7 and 1.2 kb in length cloned in ϕ C31, and Anzai *et al.* (1988) introduced a mutated DNA segment into the step 5 bialaphos biosynthetic gene of *S. hygroscopicus* by putting the *in vitro* altered gene on a *S. hygroscopicus* plasmid, transforming the non-mutant strain and then making and regenerating protoplasts of the transformants. (No mutants were observed after transformation without subsequent protoplasting.)

The nucleotide sequence of the free mini-circle of *S. coelicolor* has been determined (D. J. Henderson, unpublished results), and clones described above (Fig. 3) will allow the nucleotide sequences of the mini-circle/chromosome junctions to be determined and allow the cloning of the unoccupied integration site. Knowing the sequences associated with mini-circle integration should establish the relatedness or otherwise of the mini-circle to other transposable elements such as the well-characterized transposable elements of *E. coli* (Calos & Miller, 1980), the novel Tn1545 element of *Streptococcus pneumoniae* (Caillaud & Courvalin, 1987) and the integrating plasmids of *Streptomyces* and *Nocardia* (Bibb *et al.*, 1981; Madon *et al.*, 1987).

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