The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome

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The linear plasmid pPZG101 of *Streptomyces rimosus* R6 was restriction mapped with the enzymes *Asel*, *BfrI*, *DraI* and *XbaI*. It is 387 kb in size and the ends are inverted repeats of at least 95 kb in length. Twenty spontaneous morphological variants and seventeen auxotrophic mutants were screened for changes in the plasmid. Two strains were found that had lost all plasmid sequences. Four strains had integrated parts of the plasmid into the chromosome. Restriction analysis suggested that at least three of the integrated strains had retained free plasmid ends. If it is assumed that the chromosome of *S. rimosus* R6 is linear, this might be explained by replacement of one or both chromosome ends by a plasmid end. One strain, which overproduced oxytetracycline, carried an enlarged linear plasmid of 1 Mb in size that had acquired chromosomal sequences from the oxytetracycline biosynthesis cluster.

Keywords: *Streptomyces rimosus*, linear plasmid, oxytetracycline biosynthesis genes, plasmid prime

INTRODUCTION

Linear plasmids are common in *Streptomyces* (Kinashi *et al.*, 1987; Hershberger *et al.*, 1989). In the best-investigated cases, the ends are inverted repeats and have a protein bound to the 5' ends of the DNA (Hirochika *et al.*, 1984; Keen *et al.*, 1988; Kinashi & Shimaji-Murayama, 1991; Chen *et al.*, 1993), which is thought to act as a primer for replication (Salas, 1991). Sakaguchi (1990) has defined a class of elements with inverted repeats at their ends called invertrons, which includes linear plasmids, *Bacillus* phage ϕ 29, adenovirus and yeast killer plasmids. The terminal inverted repeats in *Streptomyces* plasmids vary between 44 bp and over 80 kb (Chen *et al.*, 1993; Kinashi *et al.*, 1991). Linear plasmids of sizes between 12 kb and over 500 kb have been reported in *Streptomyces* (Keen *et al.*, 1988; Kinashi *et al.*, 1987). The designation 'giant linear plasmid' is often used to describe the larger linear plasmids; however, there is no evidence for any essential differences between such large plasmids and smaller linear plasmids.

The sex factor SCP1 of *Streptomyces coelicolor* A3(2), which is a linear plasmid of about 350 kb (Kinashi & Shimaji-Murayama, 1991), can integrate into the chromosome (Vivian & Hopwood, 1973). Analysis of one integrant showed that most of SCP1 was present, but that the free ends had been lost (Hanafusa & Kinashi, 1992). SCP1' plasmids carrying various chromosomal genes have also been reported (Hopwood & Wright, 1976). SCP1 carries genes for biosynthesis of the antibiotic methylenomycin (Wright & Hopwood, 1976), but in other linear plasmids in *Streptomyces* there is little evidence for the encoding of antibiotic biosynthesis genes.

Streptomyces rimosus is used commercially to produce oxytetracycline, and genetic studies have been carried out by several laboratories. Linear plasmids have been detected in three *S. rimosus* strains (Chardon-Loriaux *et al.*, 1986; Rathos *et al.*, 1989; Cullum *et al.*, 1991). In the last case, we reported the presence of a linear plasmid in *S. rimosus* R6, a strain where a variety of genetic markers and a genetic map are available (Alačević *et al.*, 1978). It is unknown whether this plasmid, which we have named pPZG101, plays a role in conjugational fertility. In another *S. rimosus* strain conjugational fertility has been attributed to two genetically identified plasmids, SRP1

Abbreviations: OTC, oxytetracycline; PFGE, pulsed-field gel electrophoresis.

(Friend *et al.*, 1978) and SRP2; the existence of SRP2' plasmids carrying oxytetracycline (OTC) resistance gene(s) was also reported (Rhodes *et al.*, 1984). Two resistance genes have been reported in this strain; they flank the 24 kb biosynthesis gene cluster (Butler *et al.*, 1989; Binnie *et al.*, 1989).

In this paper, we report the restriction map of the linear plasmid pPZG101 in *S. rimosus* R6 and the presence of homologous plasmids in two other *S. rimosus* strains. We also screened various classes of mutants to detect interaction of the plasmid with the chromosome (i.e. integration or prime formation).

METHODS

Strains, media and culture conditions. *Streptomyces rimosus* R6 derivatives were obtained from the culture collection of the PLIVA Research Institute, Zagreb. The auxotrophic mutant strains 127, 235, 502, 521, 531, 570, 584, 586, 596, 601, 605, 609, 612, 613 and 615, which are all derivatives of the parent strain 65, are described by Pigac & Alačević (1979). Strains 554 and 593 (this study) are MNNG-induced riboflavin-requiring (*rib-12*) and thymine-requiring (*thy-5*) auxotrophic derivatives of strain 500 (Gravius *et al.*, 1993). The 20 spontaneous morphological variants used were all the variants studied by Gravius *et al.* (1993), with the exception of MV3 and MV32, which were omitted because of their high reversion frequencies. *S. rimosus* strain R7 (ATCC 10970) was obtained from the American Type Culture Collection. *S. rimosus* strain M4018 (Butler *et al.*, 1989) and GLW220 (a derivative in which the whole OTC cluster is carried on the genetically-defined plasmid SRP2 as an SRP2', Iain Hunter, personal communication) were kindly provided by Iain Hunter, University of Glasgow, UK. Plasmid pGLW106, which carries the *otrA* gene on a 2.5 kb *Bgl*II-*Bam*HI fragment (Butler *et al.*, 1989) cloned into plasmid pPFZ34, was kindly provided by Iain Hunter. *Escherichia coli* HB101(pUT1954) (Reynes *et al.*, 1988) was used as a source of the *otrB* gene for DNA hybridization.

Methods for propagation and maintenance of *Streptomyces* stocks, and the complete agar medium (CM) (Hranueli *et al.*, 1979), minimal agar medium (MM) (Alačević *et al.*, 1973) and the complete pre-cultivation liquid medium (CPM) (Pigac *et al.*, 1982) used, were described previously.

DNA manipulation. Total DNA from *Streptomyces* strains was isolated using the 'Kirby mix' method (Hopwood *et al.*, 1985). Plasmid DNA from *E. coli* was purified by caesium chloride/ethidium bromide gradient centrifugation (Sambrook *et al.*, 1989). DNA digestion with restriction enzymes, agarose gel electrophoresis, Southern blotting, labelling of DNA probes with digoxigenin, hybridization and detection of bands were carried out as described by Gravius *et al.* (1993).

Pulsed-field gel electrophoresis (PFGE). DNA was prepared from mycelium immobilized in agarose blocks as described by Gravius *et al.* (1993). Restriction digests were carried out with *Ase*I (Biolabs), *Bfr*I (Boehringer), *Dra*I (Biolabs) and *Xba*I (Pharmacia) using the enzyme supplier's recommended buffer. A Bio-Rad CHEF DRII gel apparatus was used with running conditions selected to give optimal resolution in the molecular mass regions of interest (see figure legends). *Saccharomyces cerevisiae* chromosomes or lambda ladders were used routinely as markers. However, the sizes for fragments under 500 kb were recalculated using a correction for the high G + C content based on ladders of the *S. rimosus* phages RP2 and RP3 (Gravius *et al.*, 1994). Linear plasmid DNA was prepared by running un-

digested total DNA in a low-melting-point agarose (Incert agarose, FMC Biozym) gel in 1 × TAE (0.04 M Tris/acetate, 0.001 M EDTA, pH 8.0) buffer, using 200 V for 20 h with a ramped pulse time of 60–100 s. For preparation of probes, the whole gel was stained in ethidium bromide and slices of gel containing plasmid DNA were cut out. The DNA was purified by melting and phenol extraction (Sambrook *et al.*, 1989). When the DNA was to be digested, the appropriate gel slices were identified by staining one track with ethidium bromide. The gel slices were then incubated in 300 µl of the appropriate restriction buffer at 4 °C (2 × 3 h). Digestion was performed using 200 µl buffer and 100 U restriction enzyme for 24 h at 37 °C.

RESULTS

Restriction mapping of the plasmid

Previous work had shown the presence of a plasmid DNA molecule in *S. rimosus* R6-500 that migrated in pulsed-field gels like a linear molecule of about 370 kb (Cullum *et al.*, 1991). The plasmid DNA was sensitive to exonuclease III (B. Gravius, unpublished results), which also supported the presence of a linear molecule. DNA of the linear plasmid was isolated by running undigested total DNA of *S. rimosus* R6 on a low-melting-point agarose pulsed-field gel and excising the gel slices containing the plasmid band. These slices were digested with the rarely cutting restriction enzymes *Ase*I, *Bfr*I, *Dra*I and *Xba*I and subjected to PFGE (Fig. 1a). Initially, difficulties were encountered in mapping the plasmid because of a systematic error due to the faster migration of G + C-rich fragments than lambda DNA ladder fragments of equivalent size; this problem was solved by use of G + C-rich markers (see Methods). *Dra*I gave two fragments of

Fig. 1. (a) Restriction digests of pPZG101. Gel slices containing plasmid DNA were digested with different restriction enzymes and subjected to a second round of PFGE. 1, lambda ladder, 2, *Ase*I, 3, *Bfr*I, 4, *Dra*I, 5, *Xba*I. The pulse programme was 24 h, ramp of pulse times 3–40 s. (b) Southern transfer of the gel from (a) hybridized with digoxigenin-labelled DNA from the *Ase*I-B fragment of pPZG101. Detection by light emission.

Fig. 2. Restriction map of pPZG101. In the linear map (upper part of figure) the 11 restriction sites are numbered and the enzymes are indicated by: A (*AseI*), B (*BfrI*), D (*DraI*) and X (*XbaI*). In the block diagram (central part of figure) the restriction fragments are denoted by letters and the sizes are given in kb.

200 kb and 187 kb (Fig. 1a, track 4), whose sum is the size of the undigested plasmid; there was an additional weak band of 387 kb visible corresponding to undigested plasmid. Thus, there is a single *DraI* site in the plasmid.

The other three enzymes gave either two or three fragments, whose sum was less than that of the size of the undigested plasmid. Double digests of these enzymes with *DraI* (data not shown) revealed that the unique *DraI* site was within the largest restriction fragment in each case. The simplest explanation for the discrepancies in the sums of the sizes of restriction fragments is that the smaller fragments were present as double bands. This was supported by the following experiments.

The three *AseI* fragments are of sizes 200 kb, 64.5 kb and 29 kb respectively (Fig. 1a, track 2). The 64.5 kb *AseI*-B band was eluted from a gel, labelled with digoxigenin and used as a hybridization probe against a Southern transfer of a gel with restriction digests with the four enzymes (Fig. 1b). Both *DraI* fragments hybridized strongly (Fig. 1b, track 4), compatible with the idea that the *AseI*-B band was a doublet. Similarly, the *AseI*-C fragment also hybridized to both *DraI* fragments (data not shown). A partial *AseI* digest (data not shown) contained additional fragments of about 95 kb and 230 kb, which were interpreted as fusions of the *AseI*-B + C and *AseI*-A + C bands respectively. This suggested the *AseI* map in Fig. 2, with long terminal inverted repeats of over 90 kb.

XbaI gave two fragments, of 217 kb and 85 kb (Fig. 1a, track 5). The *AseI*-B fragment hybridized to the 85 kb *XbaI* fragment (Fig. 1b, track 5). Double digests with *AseI* and *XbaI* (data not shown) located the *XbaI* sites within the 29 kb *AseI*-C fragment, confirming the positions shown in the map (Fig. 2).

Digests with *BfrI* gave a similar pattern to those with *XbaI* (Fig. 1a, track 3), but showed an additional weak partial digest band corresponding in size to a fusion of the

BfrI-A + B fragments. The *AseI*-B fragment hybridized to the *BfrI*-B fragment, but also revealed an additional *BfrI*-C fragment of 7 kb (Fig. 1b, track 3), which was not visible in the gel (Fig. 1a, track 3). An *AseI*-*BfrI* double digest (data not shown) localized the *BfrI* site in the *AseI*-C fragment. These data gave the map for *AseI*, *BfrI*, *DraI* and *XbaI* shown in Fig. 2.

Strains lacking a cytoplasmic linear plasmid

Twenty spontaneous variants isolated during a study on genetic instability (Gravius *et al.*, 1993) were screened by PFGE for loss of pPZG101 plasmid molecules. One of the variants (MV25) had lost the plasmid band (see Fig. 5a, track 3). A further variant (MV17) contained a larger plasmid (see Fig. 5a, track 4) that will be described further below.

When *AseI* digests were carried out with the original *S. rimosus* R6 strain, after a prerun to remove the plasmid DNA, a Southern blot showed no hybridization with pPZG101 (data not shown), indicating that the R6 chromosome had no homology to pPZG101. In contrast, restriction digests of total DNA of MV25 (not subjected to a prerun) showed hybridization with DNA of pPZG101 (Fig. 3b, tracks 4, 6 and 8). This, and the lack of a visible plasmid in undigested MV25 samples, suggested that the plasmid had integrated into the chromosome of MV25. Comparison of *AseI* digests of MV25 with the parent strain (Fig. 3b, tracks 3 and 4) shows that bands indistinguishable in size from the *AseI*-B and *AseI*-C fragments are still present in MV25. Further restriction digests showed that both the *BfrI*-B and *BfrI*-C fragments (Fig. 3, tracks 5 and 6) and the *XbaI*-B fragment (tracks 7 and 8) seem to be conserved in MV25. Thus, for all three restriction enzymes, the fragments of the plasmid which make up the long inverted terminal repeats (Fig. 2) seem to be retained in MV25; these data suggest strongly that

Fig. 3. (a) PFGE of total DNA from parent strain *S. rimosus* R6-500 (+) and spontaneous variant MV25 (V) digested with the restriction enzymes indicated. Sc, *S. cerevisiae* chromosomes; λ , lambda ladder. The pulse programme was 30 h, ramp of pulse times 4–35 s. (b) Southern transfer of the gel of (a) hybridized with digoxigenin-labelled DNA from pZG101.

Fig. 4. (a) PFGE analysis of *AseI*-digested DNA of mutants. WT, *S. rimosus* R6-500 parent strain; λ , lambda ladder; Sc, *S. cerevisiae* chromosomes. The pulse programme was 28 h, ramp of pulse times 60–130 s. (b) Southern transfer of the gel of (a) hybridized with digoxigenin-labelled DNA from pZG101.

at least one free plasmid end is present in the integrated form. However, for each enzyme, the largest (central) fragment is not conserved in MV25. In the cases of *BfrI* and *XbaI* a single new fragment of 100 kb appears (Fig. 3b, tracks 6 and 8), whereas two new fragments of 690 kb and 480 kb are seen in *AseI* digests (track 4; the size estimates were obtained from a gel using a pulse program to resolve higher molecular mass fragments; data not shown).

Seventeen auxotrophic mutant strains were also screened and five of them did not contain a plasmid band. Total DNA of these strains was analysed with *AseI* and *XbaI*, as

for MV25 (data not shown). Two of the strains R6-554 (*rib-12*) and R6-593 (*thy-5*) showed no hybridization with a pZG101 probe, proving total loss of the plasmid. The other three strains, R6-612 (*tty-2 pan-2 otc-187*), R6-613 (*tty-2 pan-2 otc-188*) and R6-615 (*his-9 pan-2 otc-190*), showed hybridizing fragments (Fig. 4b, tracks 7, 8, 9), suggesting plasmid integration into the chromosome. R6-612 and R6-613 resembled MV25 in that the fragments corresponding to the plasmid ends seemed to be conserved (i.e. *AseI*-B, *AseI*-C; Fig. 4b, tracks 7 and 8; *XbaI*-B, data not shown). The fourth strain, 615, showed a different hybridization pattern lacking the *AseI*-B and *AseI*-C fragments (Fig. 4b, track 9). All four integrated

Fig. 5. (a) PFGE analysis of undigested DNA of spontaneous variants. Sc, *S. cerevisiae* chromosomes; WT, *S. rimosus* R6-500 parent strain. The pulse programme was 32 h, ramp of pulse times 60–100 s. (b) Southern transfer of the gel from (a) hybridized with an *otrB* probe. (c) Southern transfer of the gel from (a) hybridized with digoxigenin-labelled pPZG101 DNA.

strains showed different *AseI* restriction patterns from each other.

While examining the auxotrophic mutants, it was noticed that the plasmid was smaller than in the isolate used for restriction mapping (Fig. 2). All the auxotrophic mutants with cytoplasmic plasmid were derivatives of R6-65 and it is likely that an increase in the size of the plasmid has occurred between R6-65 and the newer derivative R6-500. We named this smaller plasmid pPZG102 (as present in strain 601). In *AseI* digests, pPZG102 shows an *AseI*-A fragment of 213 kb (Fig. 4, tracks 2, 3, 4), which is slightly larger than that in pPZG101 (track 1). This would be explained if pPZG102 did not carry long inverted repeats, i.e. it would have only two *AseI* sites, generating three single fragments.

A derivative of the linear plasmid carrying OTC genes

One variant strain (MV17) carried a plasmid DNA molecule (named pPZG103) of about 1 Mb instead of the 387 kb pPZG101 (Fig. 5a, track 4). This strain produces more OTC than the parent strain and this overproduction is unstable (Gravius *et al.*, 1993). It therefore seemed possible that pPZG101 had acquired chromosomal sequences from the OTC region. This was tested by hybridizing a Southern transfer with a probe containing the *otrB* OTC-resistance gene which is part of the biosynthesis cluster (Butler *et al.*, 1989). Fig. 5(b) (track 4) shows that pPZG103 hybridizes with the *otrB* probe whereas pPZG101 shows no signal (track 2). Similarly, pPZG103, but not pPZG101, hybridized to an *otrA* probe (data not shown). DNA of pPZG101 was eluted from a PFGE gel, labelled with digoxigenin and also used as a hybridization probe. Fig. 5(c) (track 4) shows that

pPZG103 hybridizes to pPZG101. Thus, the 1 Mb pPZG103 is a derivative of pPZG101 carrying chromosomal sequences from the OTC region.

When DNA from MV17 was digested with *AseI*, after a prerun to remove pPZG103 sequences, no differences in comparison to the parent strain could be seen (data not shown). In particular, when the *otrB* gene was used as a hybridization probe, the expected hybridization to a single 795 kb fragment (Gravius *et al.*, 1993) was seen. Thus, an unaltered copy of the OTC region is probably still present on the chromosome. When total DNA (not prerun) of the strain was digested with *XbaI* and, after PFGE, was used for Southern hybridization with the *otrB* gene, only one fragment of 415 kb hybridized (data not shown), which was identical in size to that of the parent strain (Gravius *et al.*, 1993). Thus, the whole of this fragment seems to be present in an unaltered form in pPZG103. The 415 kb *XbaI* fragment showed an increased intensity, suggesting that pPZG103 was present at a copy number several times that of the chromosome. When an *AseI* digest of total DNA of MV17 was hybridized with an *otrB* probe, there was strong hybridization to a fragment of 640 kb and weaker hybridization to the 795 kb chromosomal band (data not shown). When labelled DNA from pPZG101 (data not shown) was used as a probe, fragments of sizes corresponding to the plasmid ends (i.e. the *AseI*-B and *AseI*-C fragments) hybridized, but no other 'junction fragments' could be detected.

Detection of homologous plasmids in other *S. rimosus* strains

Two other *S. rimosus* strains, R7 (ATCC 10970, Alačević *et al.*, 1973) and M4018 (Butler *et al.*, 1989) were screened

for the presence of plasmids. Both contained linear plasmids slightly smaller (312 kb) than that in *S. rimosus* R6 (data not shown). GLW220, a derivative of M4018 that contains a genetically defined plasmid SRP2' carrying the OTC biosynthesis genes, was also screened. In GLW220 (data not shown), instead of the 312 kb band there were a major band of 950 kb and two weaker bands of 1200 kb and 680 kb. This suggests that the linear plasmid corresponds to SRP2 and that a similar event may have occurred as in MV17.

Total DNA from strain R7 (i.e. consisting of chromosomal and plasmid DNA) was digested with *AseI* or *DraI* and subjected to PFGE (data not shown). Southern transfers of the gels were hybridized with digoxigenin-labelled DNA from pPZG101 (data not shown). Three *AseI* fragments of 218 kb, 64.5 kb and 29 kb hybridized – the two smaller fragments were indistinguishable in size from the pPZG101 *AseI*-B and *AseI*-C fragments. *DraI* fragments of 187 kb, 41 kb and 36 kb hybridized, the largest fragment being indistinguishable in size from the pPZG101 *DraI*-B fragment. These data suggest that the plasmid in R7 probably has one end similar to the right-hand end of pPZG101 (Fig. 2), but that the other end differs.

DISCUSSION

We constructed a restriction map of the 387 kb linear plasmid pPZG101 from *S. rimosus* R6-500 (Fig. 2). The ends of the plasmid were long inverted repeats of at least 95 kb. Long inverted repeats have also been observed in the linear plasmid SCP1 of *S. coelicolor* A3(2) (Kinashi *et al.*, 1991), but the example of plasmid SLP2 of *S. lividans* 66 (inverted repeat of 44 bp; Chen *et al.*, 1993) shows that long inverted repeats are not a universal property of linear *Streptomyces* plasmids. It appears that the plasmids in *S. rimosus* R7 and older *S. rimosus* R6 derivatives have shorter inverted repeats (if any) than the mapped plasmid. It is likely that the long inverted repeat seen in pPZG101 has arisen during the strain development programme. One possible mechanism to generate the longer repeat would be a recombination event between two copies of a hypothetical linear plasmid ancestor aligned in opposite orientation; such a single cross-over recombination event has been observed between two linear plasmids in *Rhodococcus* (Kalkus *et al.*, 1993).

The detection of OTC-producing strains lacking plasmid sequences shows that pPZG101 is not essential for OTC synthesis. Although six of thirty-seven mutants tested had part of the plasmid integrated in the chromosome or lacked plasmid sequences altogether, it seems unlikely that the changes in the plasmid are related to the mutant phenotypes; the high frequency of changes probably just reflects plasmid lability. It is likely that pPZG101 is homologous to the sex factor SRP2 in another *S. rimosus* strain (Rhodes *et al.*, 1984). The availability of cured strains of *S. rimosus* R6 will allow experiments to see if pPZG101 also acts as a sex factor.

Four of the mutants had plasmid sequences integrated

into the chromosome. Restriction analysis suggested strongly that, in at least three cases, there was still a free plasmid end present. In MV25 (Fig. 3, track 4) two presumed *AseI* junction bands could be seen, but for 612 and 615 (Fig. 4, tracks 7 and 8) only one junction band was present. If the chromosome of *S. rimosus* R6 were linear as in *S. lividans* 66 (Lin *et al.*, 1993) then integration might be explained by exchanging one or both chromosome ends for a plasmid end. Experiments are in progress to construct a restriction map of the chromosome to test this hypothesis.

In one strain (MV17), that overproduces OTC, there is an extra copy of sequences from the OTC region on a prime plasmid (pPZG103) of about 1 Mb that is derived from pPZG101. It is likely that overproduction of the antibiotic is caused by the increased copy number of OTC genes, as other overproducing strains carry reiterations of the OTC region in the chromosome (Gravius *et al.*, 1993). The band intensities suggested that the copy number of the prime plasmid was greater than that of the chromosome. The data would be consistent with a copy number of 3–4, similar to that reported for SCP1 in *S. coelicolor* A3(2) (Kinashi *et al.*, 1991). The data presented here support the idea that formation of linear plasmids carrying chromosomal genes could play an important role in transferring antibiotic biosynthesis genes between different *Streptomyces* as suggested by Kinashi *et al.* (1992). Antibiotic biosynthesis pathways seem to occur as single clusters, which probably carry all the genes necessary for expression in a heterologous host, as demonstrated for actinorhodin biosynthesis by Malpartida & Hopwood (1984).

A linear plasmid could acquire chromosomal sequences by integration into the chromosome near to genes and subsequent imprecise excision as in the case of the circular F plasmid in *E. coli* (Hayes, 1968). However, for genes close to a chromosome end of a linear chromosome, a second possibility exists. If integration occurred by a single crossing-over, it would generate a new plasmid containing one plasmid end and one chromosome end. More detailed analysis will show if this model is relevant for the formation of pPZG103.

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