Recombination between the linear plasmid pPZG101 and the linear chromosome of *Streptomyces rimosus* can lead to exchange of ends

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**Summary**

The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* R6 can integrate into the chromosome or form a prime plasmid carrying the oxytetracycline biosynthesis cluster. The integration of plasmid pPZG101 into the linear chromosome of *S. rimosus* R6-501 in mutant MV25 was shown to be due to a single cross-over at a 4 bp common sequence. pPZG101 had integrated into a 250 kb DNA sequence that was reiterated at a low level. This sequence includes the oxytetracycline biosynthesis cluster, so that homologous recombination generated a mixed population carrying different copy numbers of the region. The 1 Mb linear plasmid pPZG103 in mutant MV17 had also arisen from a cross-over between pPZG101 and the chromosome, so that one end of pPZG103 consists of c. 850 kb of chromosomal sequence including the oxytetracycline biosynthesis cluster. The plasmid pPZG101 was shown to consist of a unique central region of about 30 kb flanked by terminal inverted repeats of about 180 kb. Analysis of a presumed ancestor plasmid pPZG102 suggested that the long terminal repeats had arisen by a recombination event during the strain development programme.

**Introduction**

Linear plasmids are common in *Streptomyces* species (Hirochika *et al*., 1984; Chardon-Loriaux *et al*., 1986; Kinashi and Shimaji, 1987; Keen *et al*., 1988; Rathos *et al*., 1989; Chen *et al*., 1993). The chromosome of most or all species is also linear and about 8 Mb in size (Lin *et al*., 1993; Lezhava *et al*., 1995; Leblond *et al*., 1996), although both stable (Redenbach *et al*., 1993) and unstable (Fischer *et al*., 1997; Volff *et al*., 1997) circular derivatives can also be produced. In *Escherichia coli* K-12, the F plasmid can integrate into the chromosome to form Hfr strains and chromosomal sequences can be transferred to the F plasmid to form F′ plasmids (see Broda, 1979). In *S. coelicolor* A3(2), genetic evidence showed that a plasmid SCP1 could integrate into the chromosome to form high-frequency transfer derivatives (Vivian and Hopwood, 1973) and could pick up chromosomal genes to form SCP1′ plasmids (Hopwood and Wright, 1976). It is now known that SCP1 is linear (Kinashi and Shimaji-Murayama, 1991) like the chromosome of *S. coelicolor* A3(2) (Lin *et al*., 1993). The structure of one integration event that involves deletion of part of SCP1 including loss of free plasmid ends is known (Hanafusa and Kinashi, 1992).

In *S. rimosus* R6-501, there is a linear plasmid pPZG101 of 387 kb length whose ends are long inverted repeats (Gravius *et al*., 1994). Mutants were found in which plasmid sequences were integrated into the chromosome, and restriction mapping suggested that a free plasmid end was still present. Recent work (Pandza *et al*., 1997) has shown that the chromosome is a linear molecule of 8 Mb in size, similar to the chromosome in other *Streptomyces* strains. It was suggested that integration could occur because of replacement of a chromosome end with a plasmid end (Gravius *et al*., 1994; Hranueli *et al*., 1994; 1995). In another mutant, the linear plasmid had increased in size from 387 kb to about 1 Mb and had formed a plasmid prime carrying the oxytetracycline (OTC) biosynthesis genes. The demonstration that the OTC cluster was only about 600 kb from the chromosome end (Pandza *et al*., 1997) suggested that the prime plasmid could have originated by replacement of a plasmid end by a chromosome end. pPZG101 has long inverted repeats (at least 95 kb; Gravius *et al*., 1994), whereas some strains from earlier in the selection process carried a smaller plasmid pPZG102 of about 310 kb, which did not carry the extremely long inverted repeats. This suggested that the long inverted repeats had originated by recombination between the ends.

In this paper, we characterize the plasmid integration event into the chromosome of one mutant (MV25) and the structure of the plasmid prime carrying OTC genes (pPZG103). We also map pPZG102.
Results

Establishment of an ordered cosmid bank for pPZG101

The three *Ase* I fragments of pPZG101 (Gravius et al., 1994; Fig. 1) were eluted from gels, labelled with digoxigenin and used for colony hybridization against a cosmid library of *S. rimosus* R6-501 that had previously been constructed (Rausch et al., 1993). Fifteen positive clones were isolated, and six clones were selected for further analysis. One clone C-D12 carried a *Dra* I site (there is a unique *Dra* I site near the centre of pPZG101; Fig. 1) and clones C-14 and C-18 carried *Ase* I sites. Ordering of the cosmids was carried out by cross-hybridization experiments. Total DNA of each cosmid was labelled with digoxigenin and hybridized with Southern blottings of *Eco* RI–*Bam* HI double digests of the cosmids. In addition, *T3* and *T7* RNA polymerases were used to produce hybridization probes from the ends of each insert. Both the *T3* and *T7* probes of cosmid C-D12 hybridized to cosmid C-5, which suggested that both terminal inverted repeats might extend into C-D12. This idea was tested by hybridizing the cosmids to *Dra* I digests of total DNA of *S. rimosus*. In each case, both *Dra* I fragments of pPZG101 hybridized strongly, as would be expected if the inverted repeats extended into C-D12. The most distal cosmid (C-17) lies 10–20 kb from the ends and does not contain the *Bfr* I site, which lies about 7 kb from the ends (Gravius et al., 1994). The cosmids were used as hybridization probes against Southern blots of *Eco* RI digests of total DNA from *S. rimosus* R6-501. Comparison of the results with *Eco* RI digests of the cosmid DNA did not reveal any unexplained bands, which suggested that the *Eco* RI maps of both inverted repeats are identical.

Digestion of C-D12 DNA with *Eco* RI showed the presence of nine bands (data not shown). As the 1.2 kb band
is a double band, there are 10 EcoRI fragments (i.e. eight EcoRI sites derived from pPZG101 and the two vector sites flanking the cloning site). Insert fragments were isolated, labelled and hybridized to Southern blots of digests of the cosmid DNA restricted with EcoRI, EcoRI–BamHI, DraI and EcoRI–DraI. This allowed assignment of most of the EcoRI fragments to one or other of the two insert-derived DraI fragments. However, the 12 kb and 1.4 kb fragments showed cross-hybridization with each other and with both DraI fragments; in addition, these fragments cross-hybridized with the cosmid C-5. The plasmid pUKG711 (see below), which contains a 4.6 kb BamHI fragment of C-D12, hybridized with both the 1.4 kb and the 12 kb EcoRI fragments. In addition to the 4.6 kb BamHI fragment, it also hybridized to a 6.3 kb BamHI fragment of C-D12, which was cloned into pUC18 to give pUKG715. Hybridization experiments using pUKG711 and pUKG715 as probes allowed the ordering of most of the EcoRI fragments to one or other of the two insert-derived DraI fragments. However, the 12 kb and 1.4 kb fragments showed cross-hybridization with each other and with both DraI fragments; in addition, these fragments cross-hybridized with the cosmid C-5. The plasmid pUKG711 (see below), which contains a 4.6 kb BamHI fragment of C-D12, hybridized with both the 1.4 kb and the 12 kb EcoRI fragments. In addition to the 4.6 kb BamHI fragment, it also hybridized to a 6.3 kb BamHI fragment of C-D12, which was cloned into pUC18 to give pUKG715. Hybridization experiments using pUKG711 and pUKG715 as probes allowed the ordering of most of the EcoRI fragments of C-D12 (see Fig. 1). The remaining EcoRI fragments (the 1.2 kb double band and the 6.3 kb fragment) were ordered using the 1.2 kb EcoRI double band extracted from the gel as a hybridization probe against Southern blots of partial EcoRI digests of C-D12 and DraI-restricted EcoRI partial digests. This gave the map in Fig. 1. Cross-hybridization experiments between pUKG711 and pUKG715 located the ends of the terminal inverted repeats to a 1.3 kb BamHI–EcoRI fragment of pUKG715 and a 1.6 kb EcoRI–BamHI fragment of pUKG711 (Fig. 1). Thus, the unique region at the centre of the plasmid is only 30–32 kb in size so that the inverted repeats are about 180 kb in size.

Restriction map of pPZG102

Previous work based on digestion with AseI had suggested that pPZG102 (the presumed ancestor of pPZG101) has one end identical with those of pPZG101 (Gravius et al., 1994) and an AseI map of pPZG102 was proposed (Fig. 2). Slices containing pPZG102 DNA were cut from a PFGE gel and digested with the restriction enzymes AseI, BfrI, DraI and XbaI, which gave rise to three, five, five and three fragments respectively (assuming that the intense BfrI-C band is a double band). Parallel digestions of pPZG101 were also carried out (data not shown). In each case the sum of the fragment sizes of pPZG102 DNA was 320 kb. The XbaI digest showed fragments of 85 kb (identical in size to the end fragment of pPZG101), 217 kb and 18 kb. A partial XbaI digest gave rise to fragments of sizes corresponding to XbaI-A+B (302 kb) and XbaI-A+C (235 kb), but not XbaI-B+C (103 kb), which yields the map order shown in Fig. 2. Partial digests with AseI also confirmed the previous map.

The map for BfrI and DraI was established by conducting double digests. In BfrI–XbaI double digests (data not shown), one of the 47 kb BfrI-C fragments was cut to give fragments of 18 kb (i.e. the XbaI-C fragment) and 29 kb. This located the BfrI-C2 fragment to the left hand end of the plasmid (Fig. 2). The BfrI–XbaI double digest also showed that the 85 kb XbaI-B fragment had been replaced by a slightly smaller fragment. This shows that the 7 kb BfrI-D fragment must lie at the right-hand plasmid end. The reduction in size of the 85 kb BfrI-B fragment shows that it must be adjacent to the BfrI-D fragment. Similarly, DraI–XbaI double digests confirmed the location of the 187 kb DraI-A fragment to one plasmid end and showed that the 32 kb DraI-C fragment lies at the other plasmid end (Fig. 2). A BfrI–DraI double digest allowed ordering of the rest of the fragments. The appearance of a 95 kb fragment showed that the BfrI-A fragment must be adjacent to the BfrI-B fragment because if the order of the BfrI-A and BfrI-C1 fragments in Fig. 2 were reversed only smaller fragments would be present. The order of the DraI fragments in Fig. 2 is the only one consistent with the BfrI–DraI double digestion.

The restriction maps (Figs 1 and 2) of the right-hand ends of pPZG101 and pPZG102 are identical up to the DraI site (i.e. at least 187 kb of identity). However, any perfect inverted repeat at the end of pPZG102 must be much shorter than in pPZG101. In particular, the 7 kb BfrI-D fragment is not present at the left-hand end of pPZG102.
Although part of pPZG101 had integrated into the chromosome in *S. rimosus* strain MV25, at least one free plasmid end was still present (Gravius et al., 1994). A cosmid gene bank of MV25 containing 1728 clones was constructed in sCos-1, and 41 clones hybridizing to digoxigenin-labelled DNA of pPZG101 were isolated by colony hybridization. The 41 cosmids were screened for potential junction clones that also contained non-plasmid sequences. Double digests of cosmid DNA with *Bam*HI and *Eco*RI were separated by agarose gel electrophoresis and subjected to Southern blotting. The filters were hybridized with labelled pPZG101 DNA to detect plasmid sequences. Two cosmids contained insert fragments that did not hybridize. One of these cosmids (C-20) was investigated further. It was used as a hybridization probe against *Asel* digests of total DNA. In the parent strain *S. rimosus* R6-501 it hybridized to the *Asel*-C fragment of pPZG101 and to the chromosomal *Asel*-C band. In MV25 there were two hybridizing bands of about 690 kb and 480 kb that had previously been identified as potential junction fragments (Gravius et al., 1994). Total DNA of R6-501 and MV25 was digested with *Bam*HI, *Clal*, *Eco*RI, *Hind*III and *Sal*I and Southern blottings hybridized with labelled DNA of cosmid C-20. This showed differences between the two strains. In the *Bam*HI digest, 6.3 kb and 4.6 kb fragments of *S. rimosus* R6-501 were missing and there was a new fragment of 4.4 kb in MV25. The same enzymes were used to digest DNA of cosmid C-20 and Southern blots were hybridized with labelled DNA of pPZG101 and labelled total DNA of strain *S. rimosus* R6-593, which has lost pPZG101 completely (Gravius et al., 1994) and is thus a probe for chromosomal DNA. The 4.4 kb *Bam*HI fragment hybridized with both probes supporting the idea that it is a junction fragment. It was eluted from a preparative gel and cloned into pUC18 to produce pUKG701. The insert in pUKG701 was restriction mapped and proved to contain two sites for *Eco*RI and one site for *Xba*I. To localize the junction more precisely, pUKG701 DNA was doubly digested with *Bam*HI and *Eco*RI and the three insert fragments used as hybridization probes against *Bam*HI-digested total DNA of *S. rimosus* strains R6-501 and MV25. This localized the junction to a 1.3 kb *Eco*RI-*Bam*HI fragment carrying the *Xba*I site. The previous hybridization results (Gravius et al., 1994) would require the *Xba*I site to be from the chromosome. This located the junction to a 0.4 kb *Eco*RI-*Xba*I fragment that was obtained in pUKG703 by deletion of restriction fragments of pUKG701 (see Experimental procedures).

Once the junction fragment had been isolated, it was necessary to obtain the corresponding clones from pPZG101 and the chromosome. It was found that cosmid C-D12, which carries the *Dra*I site of pPZG101 (see Fig. 1), shows considerable cross-hybridization with the junction cosmids C-20. When pUKG701 was used as a probe against restriction digests of C-D12 DNA there was strong hybridization to a 4.6 kb *Bam*HI fragment, which was subcloned into pUC18 to give plasmid pUKG711 (there was also weak hybridization to the 6.3 kb *Bam*HI fragment, which carries the other end of the terminal inverted repeat; see Fig. 1). Restriction mapping of pUKG711 and comparison with the map of pUKG701 suggested that the junction lay on a 0.4 kb *Eco*RI fragment, which was subcloned into pUC18 to give plasmid pUKG712. Further analysis of C-20 suggested that the chromosomal sequences lay at the T3 promoter end of the insert. A T3 polymerase RNA probe was used to isolate 2 cosmid clones from the *S. rimosus* R6-501 cosmid bank. One of the clones C-189 was analysed in more detail. Hybridization experiments with a pUKG701 probe localized the junction to a 4.5 kb *Bam*HI-*Eco*RI fragment that was cloned into pUC18 to give plasmid pUKG721. The junction was located to a 94 bp *Xba*I-*Pst*I fragment that is present in plasmid pUKG723 (see Experimental procedures).

The junction inserts in pUKG703, pUKG712 and pUKG723 were small enough to sequence the whole insert from both strands and the sequences of these three fragments have been deposited in the EMBL Nucleotide Sequence Database (accession numbers X99898, X99899 and X99900). Comparison of the sequences shows that the recombination event occurred at a 4 bp sequence present both in the chromosome of *S. rimosus* R6-501 and in the linear plasmid pPZG101 (Fig. 3). There is no further obvious homology between the chromosome and plasmid sequences. The cross-over position in the chromosome is flanked by an imperfect inverted repeat (16/20 bases; Fig. 3). The sequences were also compared with the EMBL database, but no significant matches were found.

### Structure of the plasmid integration event in MV25

Although two *Asel* junction bands could be seen in MV25, there were only single junction bands for *Bfr*I and *Xba*I (Gravius et al., 1994). It was suggested that both ends of the chromosome might have been replaced by plasmid ends, but this is ruled out by the continuing presence in MV25 of the *Asel*-J fragment, which is the terminal fragment of the chromosome (Pandza et al., 1997). In the work to characterize the junction sequence for integration (see above), there were no data supporting the presence of a second integration junction. A further puzzle was provided by the hybridization data of *S. rimosus* R6-501 and MV25 with the junction cosmid C-20. As noted above, *Bam*HI fragments of 6.3 kb and 4.6 kb were replaced by a new fragment of 4.4 kb. Our original interpretation of these data was that the two missing fragments in MV25 corresponded to the chromosomal and plasmid junction

fragments. However, both these \textit{Bam} HI fragments are derived from pPZG101 and correspond to the fragments cloned in pUKG711 and pUKG715 (Fig. 1), whereas the chromosomal junction fragment of 8.5 kb is still present in MV25.

It was noticed that the relative intensity of the two \textit{Ase} I junction fragments varied in different DNA preparations, which suggested the presence of a mixed population. In some preparations a further fragment of 940 kb could be seen (Fig. 4A, lane 3). Southern blots showed that the 690 kb and 940 kb fragments hybridized with the OTC cluster, whereas the 480 kb fragment did not hybridize (Fig. 4B, lane 3). The hybridization also revealed a third new hybridizing fragment in MV25, larger than the 1.1 Mb \textit{Ase} I-B fragment. Four colonies of MV25 were used to inoculate liquid cultures. When mycelial fragments were spread on agar medium, a small number of pale colonies (<1%) were found that resembled those of class II mutants that had deleted the whole OTC region (Gravius \textit{et al.}, 1993). One such colony was chosen from each of the four independent cultures (MV25W1–4) and used for further analysis. All four proved to be sensitive to 10 \(\mu\)g ml\(^{-1}\) oxytetracycline, which suggested they might have deleted the cluster. DNA was prepared from the four MV25W mutants, digested with \textit{Ase} I and separated using PFGE. The digestion patterns were identical in each case and differed from that of MV25 only in that the 690 kb and 940 kb junction bands were missing, but the 480 kb band was still present (Fig. 4A, lanes 4–7). The four derivatives showed no hybridization with an OTC probe (Fig. 4B, lanes 4–7). The clone pUKG701 carrying the 4.4 kb \textit{Bam} HI junction fragment from MV25 was used as a hybridization probe against a Southern blot of \textit{Bam} HI digests of DNA from the parent strain (R6-501), MV25 and a MV25W mutant (data not shown). This showed that the 8.5 kb chromosomal fragment has disappeared in MV25W but is still present in MV25.

The presence of the \textit{Xba} I site near the recombination event helps its location in the chromosome as \textit{Xba} I is a rarely cutting enzyme. Cosmid C-189, which carries the cross-over point in the parent strain, contains two \textit{Xba} I sites and hybridizes to three \textit{Xba} I fragments of 20, 25 and 1998 Blackwell Science Ltd, \textit{Molecular Microbiology}, 28, 1165–1176

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**Fig. 3.** Sequence of the integration junction in MV25. The middle line shows the sequence in MV25 and the other two lines the parental sequences in R6-501. The arrows show the imperfect inverted repeat in the chromosome.

**Fig. 4.** \textit{Ase} I digestions of MV25 and derivatives. A. Lane 1, chromosomes of \textit{Saccharomyces cerevisiae}; lane 2, parental strain \textit{S. rimosus} R6–501; lane 3, MV25; lanes 4–7, MV25W1–4. The pulse programme was, 6.0 V cm\(^{-1}\); 15 h with a ramping of 35–60 s followed by 14 h with a ramping of 50–100 s. B. Southern blot of the gel from Fig. 4A hybridized with cosmid C-25, which carries most of the OTC cluster (S. Pandza \textit{et al.}, unpublished).
610 kb of \textit{S. rimosus} R6-501, whereas pUKG722, which carries the chromosomal junction sequence of MV25, only hybridizes to the 25 kb fragment. This locates the integration point to the 25 kb fragment about 45 bp from the Xba I site. During analysis of the OTC region, cosmids were isolated that hybridized with the 415 kb Xba I fragment that carries the OTC cluster (Gravius, 1994). One of these cosmids (C-131) contains two Xba I sites and hybridizes with chromosomal DNA fragments of 415, 25 and 20 kb and also cross-hybridizes with C-189. These linking clones establish the Xba I map in the region of plasmid integration and other work had shown that the 415 kb fragment is adjacent to the 310 kb terminal Xba I fragment (Pandza et al., 1997).

Integration of a pPZG101 end into the chromosome by a single cross-over would yield the observed Ase I junction fragment of about 480 kb and the Xba I fragment of 100 kb (see Fig. 5). This results in deletion of the OTC cluster, which lies distal to the integration point. The origin of the larger junction fragments of 690, 940 and $>1100$ kb will be considered in the Discussion.

**Structure of pPZG103**

The plasmid prime pPZG103 is derived from pPZG101 and carries chromosomal sequences including the OTC cluster (Gravius et al., 1994). Slices containing DNA from pPZG103 were cut from PFGE gels of undigested total DNA of \textit{S. rimosus} strain MV17 and digested with the enzymes Ase I, Bfr I, Dra I and Xba I (Fig. 6A). After PFGE, Southern blots were hybridized against DNA of pPZG101 as a probe (Fig. 6B). With all four enzymes hybridizing fragments were seen identical in size to the end fragments of pPZG101 (i.e. the Ase I-B and -C, the Bfr I-B, the Dra I-B and the Xba I-B fragment of pPZG101; in Fig. 6B, lane 3, the 7 kb Bfr I-C fragment has run out of the gel but was seen in gels with a pulse programme designed to resolve the small fragments). In each case there is a single additional hybridizing fragment that is not present in pPZG101. There is only weak hybridization to the 873 kb Dra I-A fragment (the strongly hybridizing 1 Mb fragment in Fig. 6B, lane 4, is undigested plasmid). These data show that one end of pPZG101 up to the Dra I site is present in pPZG103. The fact that the Ase I-B fragment of pPZG103 hybridizes with pPZG101 (Fig. 6B, lane 2) suggests the order of Ase I fragments shown in the map (Fig. 7). This would mean that the 300 kb Ase I-B fragment is at the end of pPZG103. This fragment is indistinguishable in size from the chromosomal Ase I-J fragment that lies at the end of the chromosome (Pandza et al., 1997).

**Fig. 5.** Aligned restriction maps of the chromosome ends in R6-501 and MV25W strains. The positions of the chromosomal cosmids (C-189, C-131), plasmid cosmids (C-14, C-18, C-D12) and the junction cosmid (C-20) are indicated.
digests (Fig. 6A, lane 3), the Bfr I-B fragment of 180 kb is also indistinguishable in size from the terminal Bfr I fragment of the chromosome. Moreover, previous work showed that the OTC cluster lies on the Ase I-A fragment of pPZG103 (Gravius et al., 1994), whereas in the chromosome the OTC cluster lies on the 795 kb Ase I-C1 fragment that lies adjacent to the Ase I-J end fragment (Pandza et al., 1997). This suggests that pPZG103 could carry one end of the chromosome and one end of pPZG101.

The cosmid C-A4 carries the Ase I site linking the terminal 300 kb fragment to the 795 kb (OTC cluster) fragment, in addition to the XbaI site linking the terminal 310 kb fragment with the 415 kb fragment carrying the OTC cluster (Pandza et al., 1997). When DNA from C-A4 was used as a hybridization probe against Ase I and XbaI digests of pPZG103 the Ase I-A and B fragments and the XbaI-A and B fragments hybridized (data not shown). The hybridization results in Fig. 6B, lane 5, suggest that the XbaI-C fragment of pPZG103 lies adjacent to the XbaI-D fragment; this was confirmed by a double digestion with Dral and XbaI, which showed that the Dral site lies within the XbaI-C fragment. The two smallest fragments (XbaI-E and F are not resolved from one another in Fig. 6A, lane 5) are indistinguishable in size from the two fragments proximal to the 415 kb XbaI fragment in the chromosome. This establishes the XbaI map in Fig. 7.

Stability of mutants

The strain carrying the plasmid prime pPZG103 (MV17) and the strain with the integrated plasmid (MV25) are both class III strains (Gravius et al., 1993) that are deeply pigmented and sporulate poorly. They have to be propagated via mycelial fragments that are polynucleate and this makes quantification of stability difficult. We have previously shown that MV17 gives rise to class I mutants, which produce little OTC and are pale, but that these mutants do not show any change in the size of pPZG103 (Hranueli et al., 1994). During propagation of MV17, we noticed that lighter brown colonies that resembled the parent strain R6-501 occurred at a frequency of about 0.1–1%. The exact frequency is difficult to determine because pigment from neighbouring colonies can mask the phenotype. On restreaking, the light-brown colonies retained the phenotype. Three independent light-brown derivatives of MV17 were selected for further study. They gave rise to class I, II and III mutants at similar frequencies to the parent strain R6-501, although the class III strains (e.g. MV17) are more unstable. DNA was prepared for PFGE analysis and it was found that all three mutants had lost the plasmid pPZG103.

Comparison of Ase I digests of MV25 and MV25W (Fig. 4A, lanes 3 and 4) showed that the 480 kb, 690 kb and 940 kb fragments of MV25 were less intense than neighbouring fragments in the gel, whereas the 480 kb fragment of MV25W is of comparable intensity. This suggested that MV25 consisted of a mixed population. Comparison of the 480 kb fragments of MV25 and MV25W by scanning the gel photograph suggested that the 480 kb fragment population accounted for about 15% of the MV25 chromosomes, although the relative intensities of the bands varied between different MV25 preparations. As described above, it was possible to isolate the 480 kb subpopulation as white colonies (the MV25W mutants). When white colonies were restreaked on agar, all of the resulting colonies were white, i.e. this phenotype was stable. The proportion of white colonies varied between different MV25 cultures from under 1% to over 2.5%. We tried to isolate light-brown colonies from MV25 in a similar fashion to MV17 above. It was possible to identify potential light-brown derivatives, but on restreaking they gave rise to colonies showing a similar range of variation to the original MV25 strain. MV25 colonies produce OTC on agar and this is expected to select against the sensitive white population. We examined the effect of selection in liquid medium by reconstruction experiments. Two cultures were inoculated with a mixture of MV25 and MV25W and the proportion of white colonies monitored over 84 h of growth. There was
a small increase in the proportion of white colonies in the cultures (from 33% to 38% and from 47% to 59% respectively) that showed there was no selection against white derivatives in the liquid cultures.

**Other plasmid integration events**

Three further strains carrying integrated plasmid (612, 613 and 615) were characterized previously (Gravius et al., 1994). They had been extensively mutagenized and are OTC non-producers. The strains are pale in colour, grow poorly and are morphologically stable. However, this may only reflect the fact that they have lost most of the morphological characters used. Although the AseI digests had suggested that they were different from MV25, it was still possible that the same chromosome or plasmid integration site had been used. To check this, BamHI digests of the three strains were used for Southern blotting experiments using plasmid pUKG701 (carries the junction fragment of MV25) as a probe (data not shown). In R6-501 there are two hybridizing BamHI fragments from the plasmid pPZG101 of 4.6 kb and 6.3 kb (corresponding to the inserts in pUKG711 and pUKG715 in Fig. 1). As the three strains 612, 613 and 615 are derived from R6-65 (carries pPZG102, which lacks the long terminal inverted repeats), they do not have the 6.3 kb fragment. All three showed the 4.6 kb BamHI fragment, whereas in MV25 this fragment is replaced by the 4.4 kb junction fragment. The chromosomal integration site of MV25 corresponds to a 8.5 kb BamHI fragment that is replaced by the 4.4 kb junction fragment in MV25W. Strain 612 lacks this fragment and there is no extra fragment present. Thus, in this strain the MV25 integration site in the chromosome has been deleted. In strains 613 and 615, the 8.5 kb BamHI fragment is present. These data show that the plasmid and chromosomal integration site(s) in 612, 613 and 615 is/are different from those in MV25.

**Discussion**

The plasmid pPZG101 has long, inverted repeats of about 180 kb and a short, central, unique region of about 30 kb. Comparison with the probable ancestor plasmid pPZG102 (Figs 1 and 2) suggests that the long, inverted repeats may have been generated by recombination between two copies of the plasmid in opposite orientation. This could be similar to the recombination between two different linear plasmids observed in *Rhodococcus* (Kalkus et al., 1993).

The integration site in *S. rimosus* MV25 was characterized. The DNA sequences did not show extensive homology and recombination across short repeat sequences has been observed in many other systems (e.g. Nakano et al., 1984). The integration occurred at a location proximal to the OTC cluster. MV25 showed a mixed population and part of the population that can be isolated as MV25W derivatives had lost the OTC cluster, as would be expected if a single cross-over had replaced the chromosome end with a plasmid end (Fig. 5). However, part of the population still carries the OTC cluster and the cluster occurs on a 200 kb XbaI fragment instead of the parental 415 kb fragment. The same rearrangement occurs in most class III mutants that have reiterated a 250 kb DNA region carrying the OTC cluster and deleted distal sequences (Gravius et al., 1993; S. Pandza et al., unpublished). It is probable that this leads to the replacement of the chromosome end by an amplified DNA sequence (Fig. 8A and B), as suggested for amplifications observed in *S. lividans* (Rauland et al., 1995). The fact that this sequence is much larger (250 kb...
compared with less than 30 kb in *S. lividans*) may explain the lower copy number (3–50 copies compared with 50–100 copies in *S. lividans*; Gravius *et al.*, 1993). Figure 8C shows that integration of pPZG101 into such an amplified region would give rise to an *Asel* fragment of about 940 kb in size as observed in part of the MV25 population, and that this fragment would carry two copies of the OTC cluster. A larger fragment of 1200 kb would be produced if there were three copies of the AUD instead of two. Thus, the observed fragment of more than 1100 kb (Fig. 4B) is explained by the presence of three or more copies of the AUD (the fragment is larger than the resolution limit for the PFGE programme used). The *Asel* junction fragments of 690 and 480 kb in the other subpopulations could be derived by cross-overs between the c. 100 kb-long homologous regions flanking the OTC cluster (Fig. 8D and E). This explains the fact that only single *Bfr* I and *Xba*I junction fragments were seen with MV25 (Gravius *et al.*, 1993) as there are sites for these enzymes in the repeats flanking the OTC region. This model also explains why the 8.5 kb *Bam* HI fragment that carries the chromosomal integration site is still present in the MV25 population but not in the MV25W subpopulation.

Scanning of gel photographs suggested that the MV25W subpopulation accounts for about 15% of the chromosomes in MV25 cultures. However, the number of white colonies is usually only about 1%. This can be explained by the fact that multinucleate mycelium fragments were plated. It is likely that only fragments that do not contain any copies of the OTC cluster will yield white colonies as these colonies do not show any brown progeny on restreaking. Reconstruction experiments showed that the white derivatives were not selected against in liquid medium. However, in contrast to liquid medium, considerable OTC production takes place on the agar medium and it seems to be no barrier to maintaining extra copies of chromosome ends. This is similar to the case of *S. lividans* 66, in which the chromosome ends are identical to those of the linear plasmid SLP2 (Lin *et al.*, 1993). The existence of such plasmid primes might be important for the evolution of *Streptomyces* as they would allow transfer of considerable segments of chromosomes between strains. It might mean that genes near the chromosome ends would be more mobile.

This paper shows that there is considerable lability of the linear ends of plasmids and chromosome in *S. rimosus* with exchange of ends between plasmids and the chromosome being possible. This would allow rapid evolution of end sequences as exchange resulting in replacement of one end could then be followed by a recombination replacing the second end analogous to the process resulting in the formation of pPZG101 from pPZG102. The existence of chromosomes with two different ends was somewhat unexpected as the circularity of genetic maps in various *Streptomyces* species including *S. rimosus* (Pigac and Alačević, 1979) suggests that the two ends interact strongly. However, it is still possible that only a short stretch of identical sequence is necessary for replication of the ends; the linear plasmids pSLA2 from *S. rochei* (Hirochika *et al.*, 1984) and SLP2 from *S. lividans* 66 (Chen *et al.*, 1993) have identical 12 bp end sequences, but the ends do not show any further homology.

**Experimental procedures**

**Bacterial strains and growth conditions**

The *S. rimosus* R6 strains used were 65 (Pigac and Alačević, 1979), 501, MV17, MV25 (Gravius *et al.*, 1993) and 593...
(Gravius et al., 1994). R6-65 is a spontaneous variant of the wild-type strain that was selected for higher antibiotic production. Strains 593, 612, 613 and 615 were obtained by mutagenizing R6-65. Strain 500 was derived by a series of selections of spontaneous variants with higher production starting with strain 65. Strain 500 had lost the lysogenic phage RP2 and strain 501 was obtained by relysogenizing. MV17 and MV25 are spontaneous class III mutants derived from strains 501 and 500 respectively. Media and growth conditions were as described in Gravius et al. (1993).

**DNA methods**

DNA preparation methods, restriction digests, agarose gel electrophoresis (standard and PFGE), Southern transfers, labelling of DNA with digoxigenin, hybridization and detection were as described in Gravius et al. (1993), except that PFGE was carried out using a Bio-Rad CHEF DRIII apparatus with 1% agarose in 0.5 x TBE buffer at 14°C with a separation angle of 120°. Isolation of linear plasmids from agarose gels and restriction digestion were as described in Gravius et al. (1994). Construction of the cosmids gene bank of MV25 was as described in Rausch et al. (1993) using the vector sCos-1 (Evans et al., 1989). Labelling of the ends of the cosmid inserts using T3 or T7 polymerase was as described in Redenbach et al. (1996). Subclones of cosmids were made in the vector HUSAR (Evans et al., 1989). DNA sequencing was carried out on an Applied Biosystems 373A fluorescent sequencer using a cycle sequencing kit according to the manufacturer’s instructions. Sequence comparisons were carried out using the FASTA program on the HUSAR system of DKFZ (Heidelberg, Germany).

**Construction of plasmid subclones**

The 4.4 kb BamHI fragment of cosmid C-20 carrying the plasmid integration junction in strain MV25 was cloned into the BamHI site of pUC18 to give pUKG701. pUKG701 DNA was digested with EcoRI and religated giving pUKG702, which carries a 1.3 kb EcoRI–BamHI insert. pUKG702 DNA was digested with XbaI and religated to give pUKG703 carrying a 0.4 kb EcoRI–XbaI insert. The 4.6 kb BamHI fragment carrying the integration junction in pUKG701 was subcloned from cosmid C-D12 into pUC18 to give pUKG711. The 0.4 kb EcoRI fragment of pUKG711 was subcloned into the vector pUC18 to give pUKG712. The 6.3 kb BamHI fragment of cosmid C-D12, which cross-hybridizes with pUKG712, was cloned into pUC18 to give pUKG715. The 5.3 kb BamHI–EcoRI fragment of cosmid 189 carrying the integration junction in the chromosome was cloned into pUC18 to give pUKG721. This was digested with XbaI and religated to give pUKG722 carrying a 4.4 kb EcoRI–XbaI insert. pUKG722 was digested with PstI and religated to give pUKG723 carrying a 0.06 kb PstI–XbaI insert.

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**References**


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**Fig. 8.** Interpretation of the chromosome structures in MV25 cultures. The restriction sites for AseI (A) and XbaI (X) are shown as well as the position of the OTC cluster.

A. The chromosome end region (AseI-J and C1 fragments) in the parent strain R6-501.

B. The rearrangement in a class III mutant with loss of the chromosome end and tandem amplification of the region containing the OTC cluster.

C. Integration of the end of pPZG101 to leave two tandem copies of the OTC region — this generates the 940 kb junction fragment in MV25.

D. Loss of one copy of the OTC region by homologous recombination to generate the 690 kb junction fragment in MV25.

E. Loss of all OTC copies to generate the 480 kb junction fragment in MV25 and the MV25W derivatives.


