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 crossover 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.
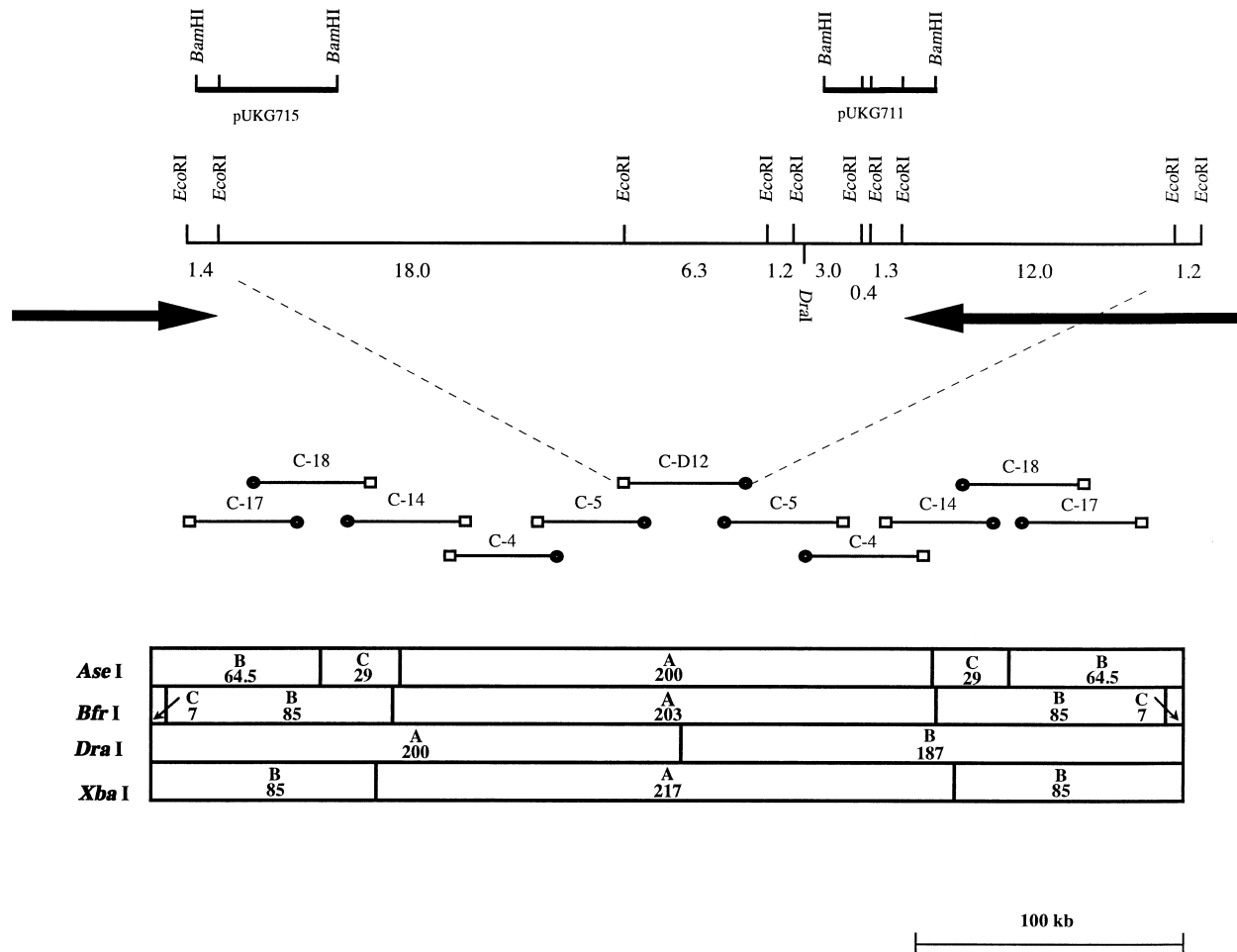
pPZG101

Fig. 1. Restriction map of pPZG101. The cosmid clones are shown orientated relative to the T3 (□) and T7 (●) ends. The map of C-D12 shows the insert flanked by the *Eco*RI sites of the vector. The ends of the inverted repeats (→, ←) and the positions of the cloned *Bam*HI fragments in pUKG711 and pUKG715 are indicated.

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
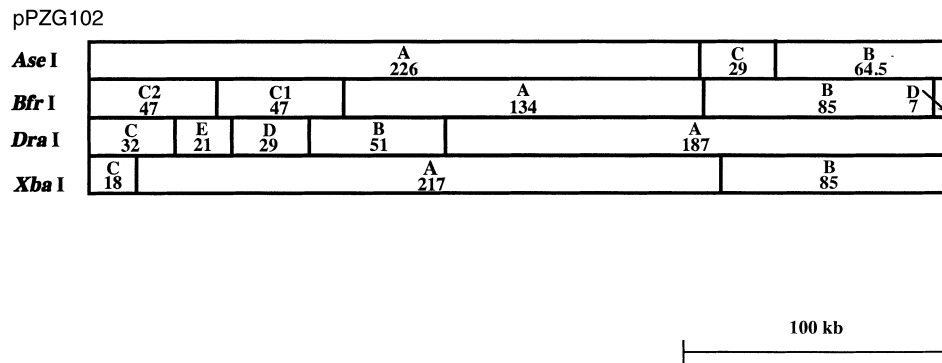
Fig. 2. Restriction map of pPZG102.

is a double band, there are 10 *Eco*RI fragments (i.e. eight *Eco*RI 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 *Eco*RI, *Eco*RI–*Bam*HI, *Dra*I and *Eco*RI–*Dra*I. This allowed assignment of most of the *Eco*RI fragments to one or other of the two insert-derived *Dra*I fragments. However, the 12 kb and 1.4 kb fragments showed cross-hybridization with each other and with both *Dra*I fragments; in addition, these fragments cross-hybridized with the cosmid C-5. The plasmid pUKG711 (see below), which contains a 4.6 kb *Bam*HI fragment of C-D12, hybridized with both the 1.4 kb and the 12 kb *Eco*RI fragments. In addition to the 4.6 kb *Bam*HI fragment, it also hybridized to a 6.3 kb *Bam*HI 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 *Eco*RI fragments of C-D12 (see Fig. 1). The remaining *Eco*RI fragments (the 1.2 kb double band and the 6.3 kb fragment) were ordered using the 1.2 kb *Eco*RI double band extracted from the gel as a hybridization probe against Southern blots of partial *Eco*RI digests of C-D12 and *Dra*I-restricted *Eco*RI 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 *Bam*HI–*Eco*RI fragment of pUKG715 and a 1.6 kb *Eco*RI–*Bam*HI 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 *Ase*I had suggested that pPZG102 (the presumed ancestor of pPZG101) has one end identical with those of pPZG101 (Gravius *et al.*, 1994) and an *Ase*I map of pPZG102 was proposed (Fig. 2). Slices containing pPZG102 DNA were cut from a PFGE gel and digested with the restriction enzymes

*Ase*I, *Bfr*I, *Dra*I and *Xba*I, which gave rise to three, five, five and three fragments respectively (assuming that the intense *Bfr*I-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 *Xba*I digest showed fragments of 85 kb (identical in size to the end fragment of pPZG101), 217 kb and 18 kb. A partial *Xba*I digest gave rise to fragments of sizes corresponding to *Xba*I-A+B (302 kb) and *Xba*I-A+C (235 kb), but not *Xba*I-B+C (103 kb), which yields the map order shown in Fig. 2. Partial digests with *Ase*I also confirmed the previous map.

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

The restriction maps (Figs 1 and 2) of the right-hand ends of pPZG101 and pPZG102 are identical up to the *Dra*I 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 *Bfr*I-D fragment is not present at the left-hand end of pPZG102.

Plasmid integration junction in MV25

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 *Ase*I digests of total DNA. In the parent strain *S. rimosus* R6-501 it hybridized to the *Ase*I-A fragment of pPZG101 and to the chromosomal *Ase*I-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, *Cla*I, *Eco*RI, *Hin*dIII 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 cosmid 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 *Ase*I 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 *Ase*I-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
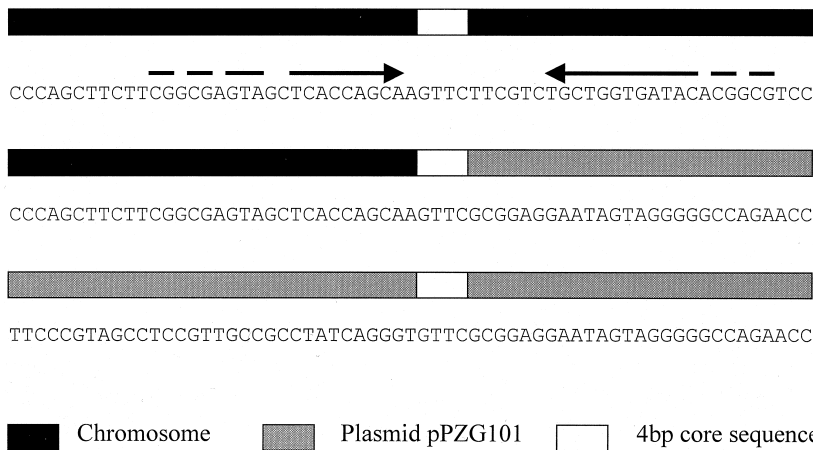
Fig. 3. Sequence of the integration junction in MV25. The middle line shows the sequence in MV25 and the other two lines the parental sequences in R6-501. The arrows show the imperfect inverted repeat in the chromosome.

CCCAGCTTCTTCGGCGAGTAGCTCACCAGCAAGTTCTTCGTCTGCTGGTGATACACGGCGTCC

CCCAGCTTCTTCGGCGAGTAGCTCACCAGCAAGTTCGCGGAGGAATAGTAGGGGGCCAGAACC

TTCCCGTAGCCTCCGTTGCCGCCTATCAGGGTGTTCGCGGAGGAATAGTAGGGGGCCAGAACC

■ Chromosome   ▨ Plasmid pPZG101   ▢ 4bp core sequence

fragments. However, both these *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 *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 *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 *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 μg ml^{-1} oxy-tetracycline, which suggested they might have deleted the cluster. DNA was prepared from the four MV25W mutants, digested with *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 *Bam*HI junction fragment from MV25 was used as a hybridization probe against a Southern blot of *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 *Xba*I site near the recombination event helps its location in the chromosome as *Xba*I is a rarely cutting enzyme. Cosmid C-189, which carries the cross-over point in the parent strain, contains two *Xba*I sites and hybridizes to three *Xba*I fragments of 20, 25 and
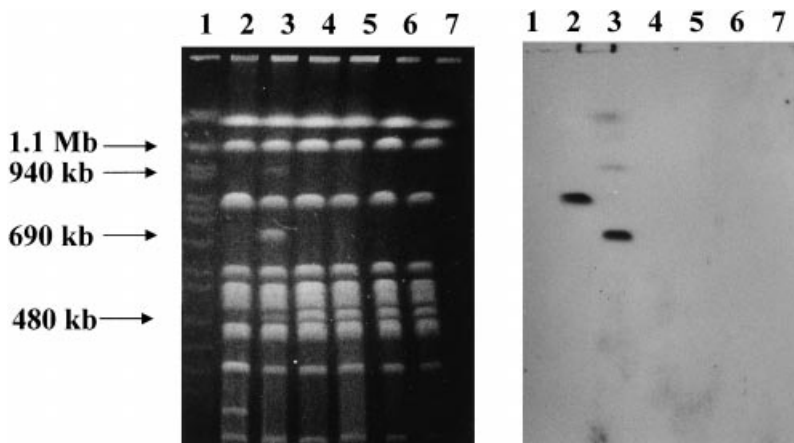
Fig. 4. *Ase*I digestions of MV25 and derivatives.
A. Lane 1, chromosomes of *Saccharomyces cerevisiae*; lane 2, parental strain *S. rimosus* R6–501; lane 3, MV25; lanes 4–7, MV25W1–4. The pulse programme was, 6.0 V cm^{-1}; 15 h with a ramping of 35–60 s followed by 14 h with a ramping of 50–100 s.
B. Southern blot of the gel from Fig. 4A hybridized with cosmid C-25, which carries most of the OTC cluster (S. Pandza *et al.*, unpublished).
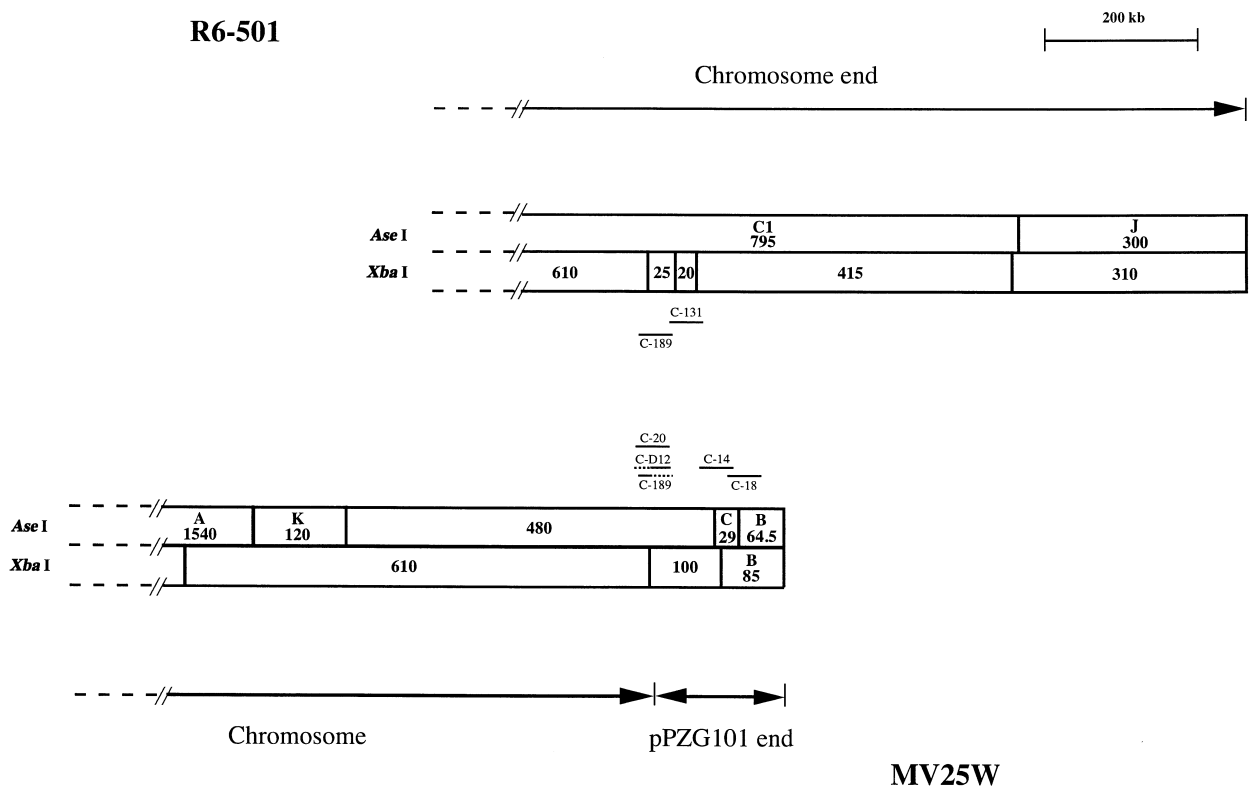
Fig. 5. Aligned restriction maps of the chromosome ends in R6-501 and MV25W strains. The positions of the chromosomal cosmids (C-189, C-131), plasmid cosmids (C-14, C-18, C-D12) and the junction cosmid (C-20) are indicated.

610 kb of *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 *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-A 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). In the *Bfr*I
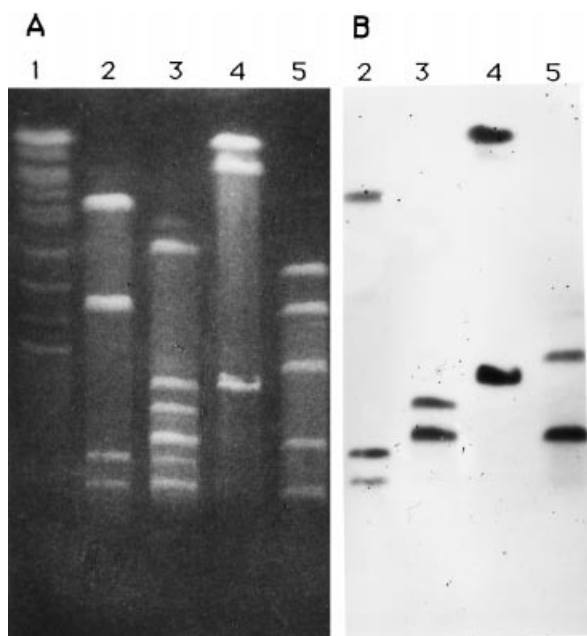
Fig. 6. Restriction analysis of pPZG103 from strain MV17.
A. PFGE gel of DNA from pPZG103 digested with the enzymes
*Ase*I (lane 2), *Bfr*I (lane 3), *Dra*I (lane 4) and *Xba*I (lane 5).
Lane 1 is marker DNA (*S. cerevisiae* chromosomes). The pulse
programme was 6 h at 6.5 V cm^{-1} with a ramping of 5–30 s
followed by 8 h at 5.5 V cm^{-1} with a ramping of 15–20 s.
B. Southern transfer of gel shown in A hybridized with digoxigenin-
labelled DNA of pPZG101.

digests (Fig. 6A, lane 3), the *Bfr*I-B fragment of 180 kb is
also indistinguishable in size from the terminal *Bfr*I frag-
ment 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 chromo-
some the OTC cluster lies on the 795 kb *Ase*I-C1 frag-
ment 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 term-
inal 300 kb fragment to the 795 kb (OTC cluster) fragment,
in addition to the *Xba*I site linking the terminal 310 kb frag-
ment 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 *Xba*I digests
of pPZG103 the *Ase*I-A and B fragments and the *Xba*I-A
and B fragments hybridized (data not shown). The hybrid-
ization results in Fig. 6B, lane 5, suggest that the *Xba*I-C
fragment of pPZG103 lies adjacent to the *Xba*I-D frag-
ment; this was confirmed by a double digestion with *Dra*I
and *Xba*I, which showed that the *Dra*I site lies within the
*Xba*I-C fragment. The two smallest fragments (*Xba*I-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 *Xba*I fragment in the chromosome.
This establishes the *Xba*I 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 propa-
gated via mycelial fragments that are polynucleate and
this makes quantification of stability difficult. We have pre-
viously 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 pheno-
type. 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 neigh-
bouring 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 chromo-
somes, 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 exam-
ined the effect of selection in liquid medium by recon-
struction 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

pPZG103

200 kb

Chromosome end   pPZG101 end

Ase I	B 300	A 666	D 29	C 64.5

Dra I	A 873	B 187

Xba I	B 310	A 415	F 20	E 25	C 205	D 85

C-A4

Fig. 7. Restriction map of pPZG103. The position of chromosomal cosmid C-A4 is shown.

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 *Ase*I 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, *Bam*HI 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 *Bam*HI 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 *Bam*HI 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 *Bam*HI 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

*Bam*HI 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 *Xba*I 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 *Ase*I 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 *Ase*I 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 shown 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 is to be expected that the white derivatives will be counterselected in the colonies used to inoculate the liquid medium. It was hoped to identify a subpopulation of MV25 carrying only one copy of the OTC cluster (i.e. the 690 kb *Ase*I fragment in Fig. 8D) as less pigmented colonies; parallel experiments to identify derivatives of MV17 that had lost pPZG103 showed that pigmentation differences could be reliably identified. However, all potential lightbrown colonies of MV25 gave rise to mixed populations on restreaking. One possible explanation is that the copy number of the OTC cluster is dynamic and can both increase and decrease.

It was shown that three other mutants with integrated plasmid sequences had different plasmid and chromosome integration sites to those in MV25. Re-examination of the *Ase*I digestion data of Gravius *et al*. (1994) in the light of newer data on the plasmid and chromosome structure (this paper and Pandza *et al*., 1997) shows that the DNA rearrangements in these strains are more complicated

than in MV25. Thus, strain 615 does not have an *Ase*I fragment corresponding to either of the end fragments of pPZG102 and also lacks the terminal chromosomal *Ase*I-J fragments. Thus, 615 may well have a circular chromosome. The other two strains (612 and 613) still have at least one free end of pPZG101 but have lost the *Ase*I-B fragment. However, other *Ase*I fragments between *Ase*I-B and the chromosome end are still present. As the three strains have been heavily mutagenized it is also possible that multiple DNA rearrangements have occurred.

A restriction map was established for the plasmid prime pPZG103 that carries the OTC cluster. It was shown that one end was derived from pPZG101 and the other end was derived from a chromosome end (Fig. 7). The crossover site has not been characterized in detail, but the results are consistent with a simple single cross-over as suggested previously (Gravius *et al*., 1994; Hranueli *et al*., 1994; 1995). The chromosome of *S. rimosus* MV17 shows no changes in restriction pattern compared with the parent R6-501 (Gravius *et al*., 1994), so that there 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
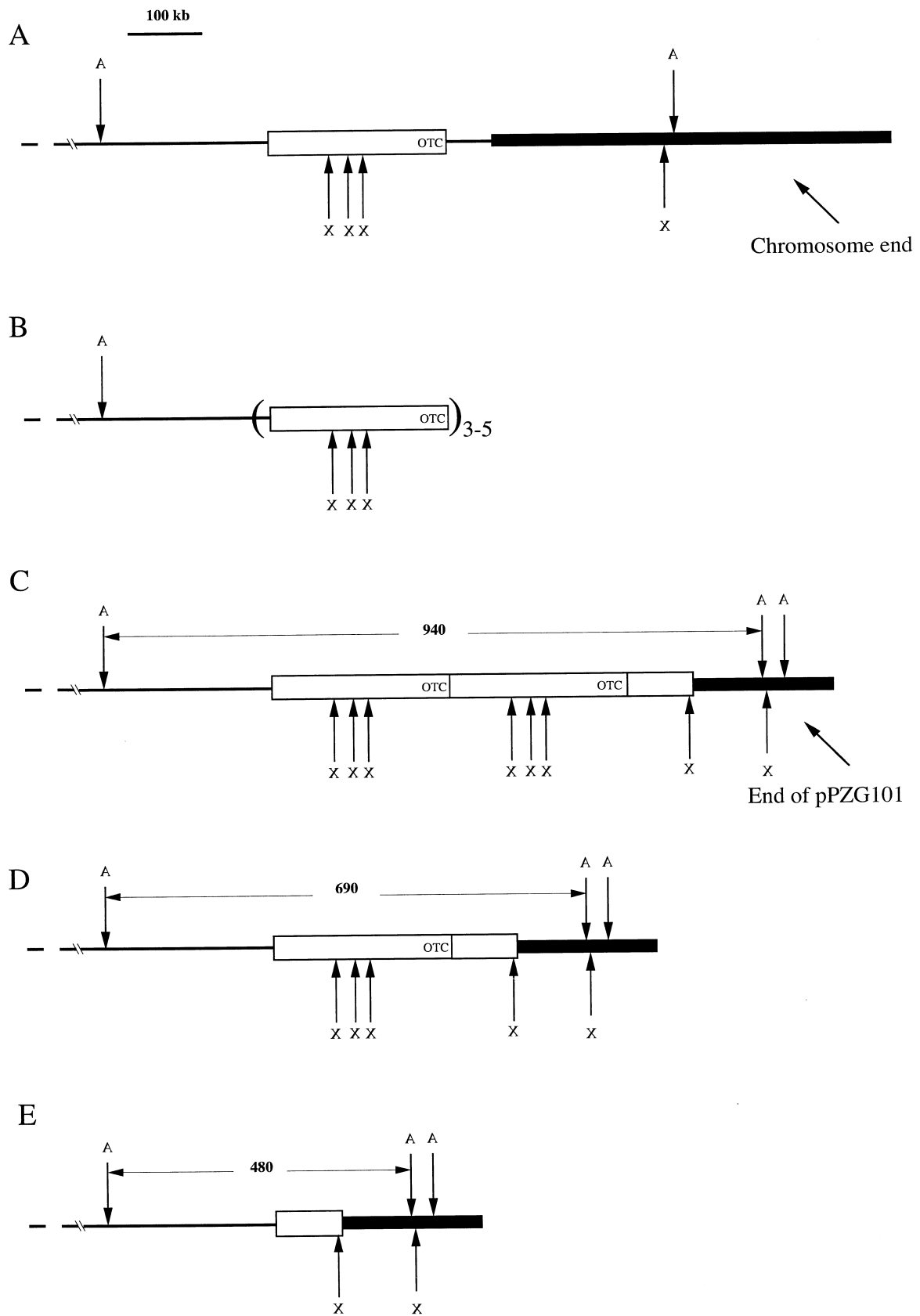
(Gravius *et al.*, 1994). R6-65 is a spontaneous variant of the wild-type strain that was selected for higher antibiotic production. Strains 593, 612, 613 and 615 were obtained by mutagenizing R6-65. Strain 500 was derived by a series of selections of spontaneous variants with higher production starting 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 $0.5\times$ 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 cosmid 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 pUC18 using the host strain JM109 (Yanisch-Perron *et al.*, 1985). 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 *Bam*HI fragment of cosmid C-20 carrying the plasmid integration junction in strain MV25 was cloned into the *Bam*HI site of pUC18 to give pUKG701. pUKG701 DNA was digested with *Eco*RI and religated giving pUKG702, which carries a 1.3 kb *Eco*RI–*Bam*HI insert. pUKG702 DNA was digested with *Xba*I and religated to give pUKG703 carrying a 0.4 kb *Eco*RI–*Xba*I insert. The 4.6 kb *Bam*HI fragment carrying the integration junction in pPZG101 was subcloned from cosmid C-D12 into pUC18 to give pUKG711. The 0.4 kb *Eco*RI fragment of pUKG711 was subcloned into pUC18 to give pUKG712. The 6.3 kb *Bam*HI fragment of cosmid C-D12, which cross-hybridizes with pUKG712, was cloned into pUC18 to give pUKG715. The 5.3 kb *Bam*HI–*Eco*RI fragment of cosmid 189 carrying the integration junction in the chromosome was cloned into pUC18 to give pUKG721. This

was digested with *Xba*I and religated to give pUKG722 carrying a 4.4 kb *Eco*RI–*Xba*I insert. pUKG722 was digested with *Pst*I and religated to give pUKG723 carrying a 0.06 kb *Pst*I–*Xba*I insert.

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Fig. 8. Interpretation of the chromosome structures in MV25 cultures. The restriction sites for *Ase*I (A) and *Xba*I (X) are shown as well as the position of the OTC cluster.
A. The chromosome end region (*Ase*I-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 MV25 W derivatives.

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