Genetic instability associated with insertion of IS6100 into one end of the *Streptomyces lividans* chromosome

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Analysis of 548 recombinant strains of *Streptomyces lividans* carrying chromosomal insertions of IS6100 revealed that six mutants contained DNA amplifications. The amplifications differed in size but included IS6100 sequences. Hybridization with representative cosmid clones containing sequences from the unstable regions of the chromosome indicated that, in each mutant, DNA rearrangements affected just one of the chromosome ends. The amplifications were derived either from a region immediately proximal to the terminal inverted repeat (TIR) or further distal, from a previously characterized type I amplifiable unit of DNA. There was no evidence for extensive deletions accompanying the amplifications and chromosome linearity was maintained with, at least in five mutants, clear evidence for no loss of either TIR. The nature of the rearrangements provides evidence that insertions affecting the integrity of a chromosome end can contribute to genetic instability in *Streptomyces*.

Keywords: *Streptomyces lividans*, insertion sequence, IS6100, genetic instability

INTRODUCTION

The linear *Streptomyces* chromosome can exhibit high-frequency genetic instability, undergoing spontaneous deletions at rates that can be higher than 0.1 % of spores (reviewed by Volff & Altenbuchner, 1998). These deletions often affect the chromosome ends, including the terminal inverted repeats (TIRs), the 5' ends of which are covalently bound by a terminal protein believed to be essential for replication of terminal sequences (Lin *et al.*, 1993). In the absence of this complex, viability can be maintained by chromosomal circularization. Moreover, tandem amplification of specific chromosomal sequences is frequently associated with the deletions.

These phenomena have been investigated in some detail in *Streptomyces lividans*. In this species, the extremities of the chromosome consist of 30 kb TIRs, which contain

two transposable elements: Tn4811 and IS1372 (Lin *et al.*, 1993; Fischer *et al.*, 1996). A physical genetic map of a 1 Mb region spanning both chromosome ends has been assembled (Redenbach *et al.*, 1993). The regions immediately adjacent to the TIRs lack any housekeeping genes; indeed, in the related *Streptomyces coelicolor* A3(2), with the exception of *argG*, no essential genes (defined as genes needed for growth in laboratory conditions) are located within 1.2 Mb of either end (Redenbach *et al.*, 1996). Sequential deletions in one chromosomal arm can remove a TIR, a chloramphenicol-resistance marker and *argG*. The majority of these mutants contain a highly amplified 5.7 kb sequence which defines, or is close to, the deletion end point (Altenbuchner & Cullum, 1984; Betzler *et al.*, 1987). This sequence originates from a type II amplifiable unit of DNA (AUD_{5.7}) in progenitor strains; type II AUDs give rise to amplified sequences of identical structure in independently isolated mutants and consist of an internal sequence flanked by direct repeats of at least 0.8 kb (Volff & Altenbuchner, 1998). Instability affecting the other chromosomal arm has been shown to cause large deletions, including the TIR, often ending at amplified sequences of different sizes in independently isolated mutants (Rauland *et al.*, 1995). These arise from type I AUDs, which are characteristically heterogeneous

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Abbreviations: AUD, amplifiable unit of DNA; TIR, terminal inverted repeat.

in their size and end points, but are derived from overlapping chromosomal regions.

The evolution of this genetic organization, with the absence of essential genes in unstable regions and AUD structures that can amplify, possibly to protect against further deletion, implies that genetic instability may not be an unusual fate for a streptomycete, even when living in its natural environment. This instability may be associated with chromosomal linearity, because circularized derivatives lack functions to ensure their stable propagation. Models to account for frequent formation of terminal deletions propose that collapsed replication forks may be repaired by homologous recombination between chromosomes in the multi-nucleoid mycelium (Volff & Altenbuchner, 1998). The frequency of these events is likely to be stimulated by genotoxic agents which are well known to increase levels of spontaneous instability. Alternatively, replicative transposition of a transposable element normally resident in an unstable region could promote deletion formation (Chen, 1996; Volff & Altenbuchner, 1998). Although there is no evidence to date for transposition of an indigenous element causing rearrangements of unstable regions, in this study we characterize genetic rearrangements associated with transposition of the heterologous IS6100 into one chromosome end of *S. lividans*. IS6100, originating from *Mycobacterium fortuitum*, belongs to the IS6 family of transposable elements and generates cointegrates as the end product of its replicative transposition mechanism (Martin *et al.*, 1990; Smith & Dyson, 1995).

METHODS

Growth conditions, strains and transposon mutagenesis. IS6100 was introduced into *S. lividans* strain 66 (Hopwood *et al.*, 1985) on the temperature-sensitive delivery vector pUCS30 (Smith & Dyson, 1995) by PEG-mediated transformation of protoplasts and selection on regeneration media as described previously (Smith & Dyson, 1995). Individual insertion mutants were obtained from outgrowing sectors of colonies grown at the non-permissive temperature for replication of pUCS30, as has been described previously (Smith & Dyson, 1995), in the absence of thiostrepton-induction of transposition. Purified transposition mutants were used to inoculate a tryptic-soy-containing broth (Dyson & Schrepf, 1987), and grown, without antibiotic selection, at 40 °C prior to DNA isolation. Antibiotic resistance of transposition mutants was tested on GHM medium (containing l⁻¹: 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g CaCO₃, 12 g agar) supplemented with either chloramphenicol (8 µg ml⁻¹) or tetracycline (25 µg ml⁻¹). Prototrophy was tested for by growth on minimal medium (Hopwood *et al.*, 1985). *Escherichia coli* strain F⁻ Z⁻ ΔM15 (Rüther *et al.*, 1981) was employed as a host to maintain the other plasmids and cosmids used in this study. These plasmids were introduced by a standard calcium-chloride-mediated transformation procedure (Sambrook *et al.*, 1989). The plasmids and cosmids used as hybridization probes were: pUCS19 (Smith & Dyson, 1995), containing IS6100; pJD201 (Betzler *et al.*, 1987), containing an AUD_{5,7} repeat sequence; pSL100 (Betzler *et al.*, 1987), containing *argG*; and cosmids containing either *S. lividans* telomeric sequences: 68/7, 11/9, 26, 66 and A7

(Redenbach *et al.*, 1993) or *S. coelicolor* A3(2) DNA: F37 and F42 (Redenbach *et al.*, 1996).

DNA manipulations. *S. lividans* chromosomal DNA was isolated as described previously (Dyson & Schrepf, 1987). Plasmid and cosmid DNA was isolated from *E. coli* by alkaline lysis (Sambrook *et al.*, 1989). Restriction enzymes were from either Life Technologies or New England Biolabs, and were used as recommended by the suppliers. Restriction fragments required for probe synthesis were purified by electrophoresis in 0.8% agarose gels prepared in TAE buffer (Tris/acetate/EDTA; Sambrook *et al.*, 1989) and subsequently isolated from sodium-iodide-dissolved gel slices by binding to glassmilk using a GeneClean II kit, following the manufacturer's instructions (Bio 101).

Restricted chromosomal DNA was electrophoresed in 0.8% agarose gels run in HEPES buffer (Ray *et al.*, 1992) to avoid Tris-mediated DNA cleavage. The extent of amplification was estimated by densitometric analysis of restricted amplified DNA fragments in each gel lane after ethidium bromide staining of the gel. A Quantity One (Bio-Rad) software package was used for the analysis. The sizes of individual amplified restriction fragments were calculated by comparison with a λ HindIII size marker. The DNA was transferred to a Hybond-N nylon membrane (Amersham) using a vacuum-blotting system (Stratagene). An IS6100-specific probe was prepared by gel purification of a 0.9 kb *Sma*I fragment of pUCS19. An internal pUCS30 probe was prepared by gel purification of a 2.3 kb HindIII–EcoRI pUCS30 fragment that spans a unique *Xho*I site. Probes were labelled with digoxigenin-11-dUTP by random priming using a kit from Boehringer Mannheim. Hybridization conditions and subsequent detection by colour reaction with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate were according to the manufacturer's instructions (Boehringer Mannheim).

Inserts for PFGE were prepared from 4-d-old cultures grown in tryptic soy medium, as described previously (Leblond *et al.*, 1993). Undigested or *Afl*II-digested DNA was separated by PFGE in 1% agarose gels in a contour-clamped homogeneous electric field system (Chu *et al.*, 1986) supplied by Bio-Rad. The running buffer was 0.5 × TBE (Sambrook *et al.*, 1989) supplemented with 50 µM thiourea. Pulse times were optimized with respect to the length of DNA to be separated.

RESULTS AND DISCUSSION

Isolation and characterization of IS6100 transposon mutants containing amplified DNA sequences

Analysis of DNA isolated from 548 individual transposon mutants showed that six clones (i.e. 1%) contained amplified DNA fragments, and although some of these six shared amplified *Bam*HI restriction fragments of similar molecular mass, no two possessed identical profiles of amplified fragments. Southern hybridization with an IS6100 probe revealed that in each case at least one of the amplified fragments contained the insertion sequence (Fig. 1). Amplification of intervening sequences between two directly repeated copies of an insertion sequence has been observed in other bacteria (Peterson & Rownd, 1985). If this had occurred here, concatameric amplification of the cointegrate of pUCS30 would have resulted in a single amplified *Bam*HI restriction fragment of 15.4 kb. However, assuming the amplified restriction

Fig. 1. Amplification of IS6100 sequences in transposon mutants of *S. lividans*. *Bam*HI-digested genomic DNA from wild-type, and mutants 2E, 3N, 19C, 18I, 9A and 5B (lanes 1–7, respectively) was separated by gel electrophoresis (a). A Southern blot of this DNA was hybridized with an IS6100 probe (b). The positions of size markers (λ HindIII) are indicated.

Table 1. Characteristics of amplification mutants

Mutant	Amplified DNA		Colony phenotype*
	Size (kb)	Copy no.	
2E	85	4	Amy [−] Pig [−]
3N	65	15	Amy ⁺ Pig ⁺
5B	55	7	Amy [−] Pig [−]
9A	80	5	Amy ⁺ Pig ⁺
18I	270	5	Amy ⁺ Pig ⁺
19C	280	5	Amy ⁺ Pig ⁺

* Amy, aerial mycelial formation; Pig, production of pigmented antibiotics.

fragments in each mutant were derived from single DNA amplifications, the total sizes of the amplified units were estimated to vary from 55 to 280 kb in the different mutants (Table 1). A 15.4 kb fragment comigrating with linearized pUCS30 and hybridizing with the IS6100 probe could not be detected in any of the six strains (results not shown). The extent of amplification was estimated to vary between 4- and 15-fold in the different mutants (Table 1).

Hybridization with a probe specific for non-IS6100 sequences of pUCS30 also indicated unusual arrangements of the integrated sequences in these amplifications. Hybridization of this probe to *Bam*HI-digested chromosomal DNA from non-amplified transposon mutants carrying a 'normal' cointegrate reveals a single band varying in size in different isolates. However, this probe hybridized to three amplified restriction fragments of strain 9A, two amplified restriction fragments from mutants 19C, 3E and 18I, one amplified fragment from

mutant 3N (the hybridizing fragments from each mutant were of different molecular mass), and not at all to DNA from strain 5B.

Examination of the morphological and pigmentation phenotypes of the mutants when grown on complete medium with glucose as the carbon source revealed that four mutants sporulated normally and exhibited normal pigmentation, whereas two mutants were deficient in both aerial mycelium formation and pigmentation (Table 1). We also examined the phenotypes with regard to typical unstable traits in *S. lividans*. All six mutants were resistant to chloramphenicol and tetracycline, and were prototrophic. Southern hybridization with AUD₅₋₇ and *argG* probes against *Bam*HI-restricted DNA from the wild-type and the six mutants did not detect any rearrangements associated with this chromosomal end (data not shown). The absence of rearrangements was confirmed by hybridizing a cosmid 11/9 probe, containing approximately 12 kb of TIR sequence (see Fig. 2), which detected a similar 900 kb *A*/III PFGE fragment from this chromosomal end in wild-type and in all six mutants (data not shown).

Rearrangements affect one chromosome end

The extent of the rearrangements in each mutant was analysed by hybridizing cosmid probes encompassing the other chromosome end against *Bam*HI-digested wild-type and mutant DNA separated by conventional electrophoresis and *A*/III-digested DNA separated by PFGE. These analyses showed that there were no extensive deletions in any of the mutants, and that the amplifications were derived from either of two regions (Fig. 2).

The end point of one amplifiable region was proximal to the innermost end of the TIR. Mutants 2E, 3N, 5B and 9A contained different-sized overlapping amplified sequences from this region, all detected by hybridization with cosmid 26. The rearrangements in strain 2E were confirmed by PFGE and hybridization with the cosmid 66 probe, indicating an absence of both the 22 and 300 kb *A*/III fragments, and the presence of a novel fragment of approximately 680 kb. The other cosmid probes detected similar fragments in both wild-type and 2E, including the 80 kb *A*/III fragment proximal to the TIR. In contrast, PFGE analysis of mutant 3N revealed that the rearrangements extended into the TIR-proximal 80 kb *A*/III fragment, which was absent, as were the 22 kb and 300 kb *A*/III fragments; these were replaced with fragments of approximately 510 kb and >1.2 Mb. Hybridization with cosmid probe 66 against *Bam*HI-digested DNA revealed that the majority of wild-type fragments were retained in mutant 3N, indicating the limited extent of any rearrangements flanking the amplified DNA. This was confirmed by hybridization with the other cosmid probes, thus providing no evidence for other rearrangements. The TIR-proximal 80 kb *A*/III fragment of mutant 5B was found to be retained, but rearrangements affected the 22 kb and 300 kb *A*/III fragments, which were replaced by a 450 kb

Fig. 2. Extent of rearrangements in amplified mutants. (a) A map of the wild-type chromosome ends indicating the sizes (kb) of the *Af*III fragments, and the positions of the AUD_{5.7}, AUD type I, *argG* and the TIRs (from Redenbach *et al.*, 1993). The locations of the cloned *S. lividans* sequences present in the cosmids used as hybridization probes are indicated by solid bars under the *Af*III restriction map; the approximate equivalent locations of the *S. coelicolor* DNA in cosmids F37 and F42 are represented by dashed bars. (b) Approximate positions of the amplified DNA in each mutant are represented by solid boxes, including IS6100 sequences (triangles) whose precise locations in the various amplified structures were not determined. Dashed lines flanking the amplified DNA represent the furthest possible extent of any adjacent rearrangements.

Fig. 3. Retention of the TIR proximal to the amplifications. (a) A *Xho*I restriction map of the TIRs and the immediate flanking region contained in cosmid 68/7, the extent of which is denoted beneath the map. The sizes (kb) of *Xho*I restriction fragments are indicated (from Redenbach *et al.*, 1993; Volff & Altenbuchner, 1997). (b) Hybridization with a cosmid 68/7 probe against *Xho*I-digested chromosomal DNA from wild-type and mutants 2E, 3N, 9A, 5B, 19C and 18I (lanes 2–8, respectively). The sizes (kb) of the TIR-specific fragments detected and their copy number are indicated to the right of the blot. An amplified 9.6 kb *Xho*I fragment is evident in mutant 9A. A λ *Hind*III size marker is in lane 1.

fragment. Hybridization with cosmid probe 66 against *Bam*HI-digested 5B DNA revealed a novel 19 kb amplified DNA fragment, a novel single-copy 12 kb fragment and two unrearranged single-copy fragments, indicating the limit to the extent of rearrangements in this mutant. In the overlapping amplifications contained in these four mutants, the end point of the 80 kb amplification of mutant 9A mapped closest to the TIR, since it was detected by probes of both cosmids 11/9 and 68/7 (Fig. 3). This amplification overlaps the junction between the TIR and flanking unique sequence DNA, which, from sequence analysis, contains numerous long

direct repeats (Volff & Altenbuchner, 1997). The other amplification end point was concluded to lie in the region spanned by cosmid 66, which detected novel amplified and single-copy fragments of 25 kb and 13 kb, respectively, in addition to several wild-type single-copy fragments. This was confirmed by the loss of the 80, 22 and 300 kb *Af*III fragments with the appearance of at least one novel *Af*III fragment of 410 kb. None of the four TIR proximal amplifications contained an internal *Af*III site, despite originating from a chromosomal region represented by cosmids 26 and 66, in which *Af*III sites are present. This is evidence for internal deletions

associated with the rearrangements in these amplified mutants.

Strains 19C and 18I contained larger amplifications, of 280 and 270 kb, respectively. These overlapped a type I AUD region more distal to the TIR. The presence of this AUD had previously been described in association with rearrangements in 2-deoxygalactose-resistant mutants of *S. lividans* strain U001 (Rauland *et al.*, 1995). The amplifications in mutants 19C and 18I were both detected with cosmid probe A7 containing these AUD type I sequences (Fig. 2); hybridization against *Afl*III digests showed that the 300 kb fragment of wild-type was absent, whereas 280 kb and 270 kb unit length amplified *Afl*III fragments were present in strains 19C and 18I, respectively. To investigate further the extent of the rearrangements in these two mutants, two cosmids from the *S. coelicolor* genome library were used as probes: cosmid F37, containing sequences adjacent but TIR-distal to a homologous AUD region, and cosmid F42, containing a region some 120 kb further inside the chromosome (Redenbach *et al.*, 1996). Whereas the cosmid F37 probe hybridized to amplified DNA fragments in both mutants, including a common 23 kb *Bam*HI fragment of mutant 18I identified by the cosmid A7 probe, the hybridization patterns of the mutants revealed with the F42 probe were similar to the wild-type. Moreover, for both mutants, the other *S. lividans* TIR proximal cosmid probes detected similar hybridizing patterns to the wild-type, suggesting retention of the chromosome end. Mutants 19C and 18I therefore differ from most 2-deoxygalactose mutants derived from strain U001, in which amplifications define end points of large deletions that remove the chromosome end (Rauland *et al.*, 1995). Interestingly, the formation of these particular rearrangements required the prior integration of plasmid pMT664, carrying a copy of IS466, into the chromosome of the progenitor U001. However, as the site of integration was 1.75 Mb distal from the TIR, and sequences homologous to pMT664 were not found associated with the rearrangements, the role of the integrated plasmid and insertion sequence in provoking the instability is unclear.

Linear topology of the mutant chromosomes

The retention of at least one unrearranged chromosome end suggested that the mutant chromosomes would be linear. This was confirmed by comparing the migration of the chromosome in PFGE samples prepared with or without proteinase K treatment. Chromosomal or linear plasmid DNA with bound terminal proteins does not enter a gel during PFGE, whereas the DNA in samples in which these proteins are digested migrates according to its molecular mass (Lin *et al.*, 1993). This analysis revealed that, just as in the wild-type, the mutant chromosomes were linear and, moreover, each mutant strain retained the 50 kb linear plasmid SLP2 (data not shown). Most *S. lividans* mutants containing amplified DNA have been found to contain circularized chromosomes (Volff & Altenbuchner, 1998). Where chromosomal linearity was maintained only one TIR was

retained. Due to the duplication of TIR sequences, the question of whether both TIRs are present is complicated. To investigate this further in the IS6100-amplified mutants, we looked for the presence of a unique 4.5 kb *Xho*I fragment which overlaps the innermost portion of the TIR in question (Redenbach *et al.*, 1993; Volff & Altenbuchner, 1997). *Xho*I chromosomal digests were probed with cosmid 68/7, which contains a region overlapping the TIR together with adjacent unique sequences. This revealed the 4.5 kb fragment in strains 2E, 3N, 5B, 18I and 19C (Fig. 3). Both this fragment, and the adjacent 7.7 kb fragment from the unique region, were not evident in strain 9A, confirming rearrangements associated with amplification of the TIR flanking sequence. The remaining TIR-internal *Xho*I fragments could be attributed to the TIR of the unrearranged chromosome end. Although the strength of the hybridization signals was consistent with these fragments being duplicated, and hence both TIRs being present, this requires confirmation.

Concluding remarks

Transposition of IS6100 into one end of the *S. lividans* chromosome stimulated rearrangements in this region, resulting in low-level amplification of the insertion sequence and adjacent chromosomal DNA. In contrast to commonly described rearrangements affecting the *S. lividans* chromosome ends, the amplifications were not accompanied by extensive deletions affecting terminal sequences and chromosomal linearity was maintained. Linearity was also maintained in some *S. ambofaciens* mutants carrying deletions affecting one chromosome end (Fischer *et al.*, 1997). The expected cointegrate structures resulting from simple intermolecular transposition of IS6100 were not present, indicating that in the derivation of the mutants additional recombination events, leading to amplification and affecting both the inserted DNA and sequences internal to the TIR, are likely to have occurred. The results suggest that insertions affecting the integrity of a chromosomal end can provoke recombination, and indicate that transposition can contribute to the high-frequency genetic instability of streptomycetes. Due to the lack of essential genes at the ends, the region may act as a 'sink' for the accumulation of transposable elements, and these could subsequently act to destabilize the chromosome.

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