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Occurrence of Deletions, Associated with Genetic Instability in *Streptomyces ambofaciens*, Is Independent of the Linearity of the Chromosomal DNA

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The chromosomal structures of mutant strains of *Streptomyces ambofaciens* which have arisen from genetic instability were investigated by using pulsed-field gel electrophoresis and probing with sequences cloned from the unstable region which maps near the ends of the linear chromosomal DNA. The chromosomal structures of seven mutant strains harboring large deletions were classified into three types. (i) Deletions internal to one chromosomal arm were characterized in two of the mutant strains. In these strains, a linear chromosomal structure was retained, as were parts of the terminal inverted repeats sequences (TIRs) and the proteins bound to them. (ii) Four of the mutants presented a deletion including all sequences from the TIRs. A junction fragment homologous to sequences originating from internal region of both arms was characterized. Consequently, the chromosomal DNA of these strains was deduced to be circularized. Furthermore, chromosomal stability was assessed in the progeny of these circular DNA mutants. Additional deletion events were detected in 11 mutants among the 13 strains isolated, demonstrating that circular chromosomes do not correspond to a stabilization of the chromosome structure and that the occurrence of deletion could be independent of the presence of chromosomal ends. (iii) A mutant with DNA amplification was shown to have a linear chromosome with a deletion of all sequences between the amplified region and the end of the chromosome. The other chromosomal arm remained unaffected by deletion and associated with protein.

All *Streptomyces* species studied so far have been found to be subject to a particularly high degree of genetic instability (mutation frequencies higher than 10^{-3}). This phenomenon was shown to be closely related to chromosomal rearrangements such as large-scale deletions, up to 2 Mb, and DNA sequence amplifications in a particular chromosomal area called the unstable region (1, 3, 8, 9, 15, 20, 23, 25).

Another striking feature of this genus is the linearity of the chromosomal DNA first demonstrated in *Streptomyces lividans* 66 (18). The ends consist of terminal inverted repeats (TIRs) covalently bound to proteins. This structure was also described for *S. griseus*, *S. rimosus*, and *S. coelicolor* A3.2 (6, 17, 22). In all four wild-type (WT) isolates of *S. ambofaciens*, the chromosomal DNA is a linear molecule of about 8,000 kb (13). In *S. ambofaciens* DSM40697, the chromosome ends are flanked by TIRs of 210 kb, covalently bound to proteins. The unstable region was localized on the physical map and corresponds to both ends of the chromosome (13). This situation was similar to that described for *S. lividans* 66 and *S. coelicolor* A3(2) (12, 14, 18).

In *S. ambofaciens*, genetic instability leads to chromosomal rearrangements such as the amplification of particular DNA sequences and large polar deletions. The two adjacent fragments *Ase*I-G and *Ase*I-E, constituting one end of the linear chromosome (Fig. 1), were always missing in the mutant strains tested so far. The *Ase*I J fragment (on the same chromosomal arm) and the *Ase*I D and F fragments (on the other arm [Fig. 1]) were missing only when G and E were deleted. In addition, the *Ase*I F fragment was missing only when D was deleted. These findings defined the so-called polarity of loss of the unstable fragments (15). Most amplifications were located in two amplifiable regions called AUD90 (AUD stands for amplifiable unit of DNA) and AUD6 (2, 16) (Fig. 1); the latter was described as a hotspot for rearrangements (7).

The localization of the unstable region at both ends of the chromosome raised the question about the relationship between chromosomal rearrangements and linearity. Here we report the genomic structures of mutant strains of *S. ambofaciens*, using a combined approach of pulsed-field gel electrophoresis (PFGE) and probing with both *Ase*I linking clones from chromosome mapping (13) and ordered cosmids available from the growing encyclopedia of clones covering the unstable region. The results showed that the formation of deletion was not directly related to chromosomal DNA linearity. The resulting structure of the chromosomal DNA can be either linear or circular, both structures being viable. In addition, mutants with circular chromosome were subject to genetic instability with a frequency even higher than that characterized for the wild-type strain. We showed that this instability was also related to chromosomal deletions.

MATERIALS AND METHODS

Bacterial strains, culture conditions, reagents, and enzymes. *S. ambofaciens* strains were grown at 30°C in yeast extract-malt extract liquid medium for 36 to 48 h (10). Mutants were isolated on Hickey-Tresner (HT) medium at 30°C (19). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (24). Restriction enzymes and molecular biology reagents were purchased from New England Biolabs (Beverly, Mass.) and Boehringer Mannheim (Mannheim, Germany).

DNA extraction. Total DNA from *S. ambofaciens* was extracted as described previously (13). Cosmid DNA was extracted from *E. coli* by the alkaline lysis method (24).

Preparation of DNA for PFGE analysis. DNA was prepared by the method of Redenbach et al. (21). For restriction analysis, slices of the agarose plugs con-

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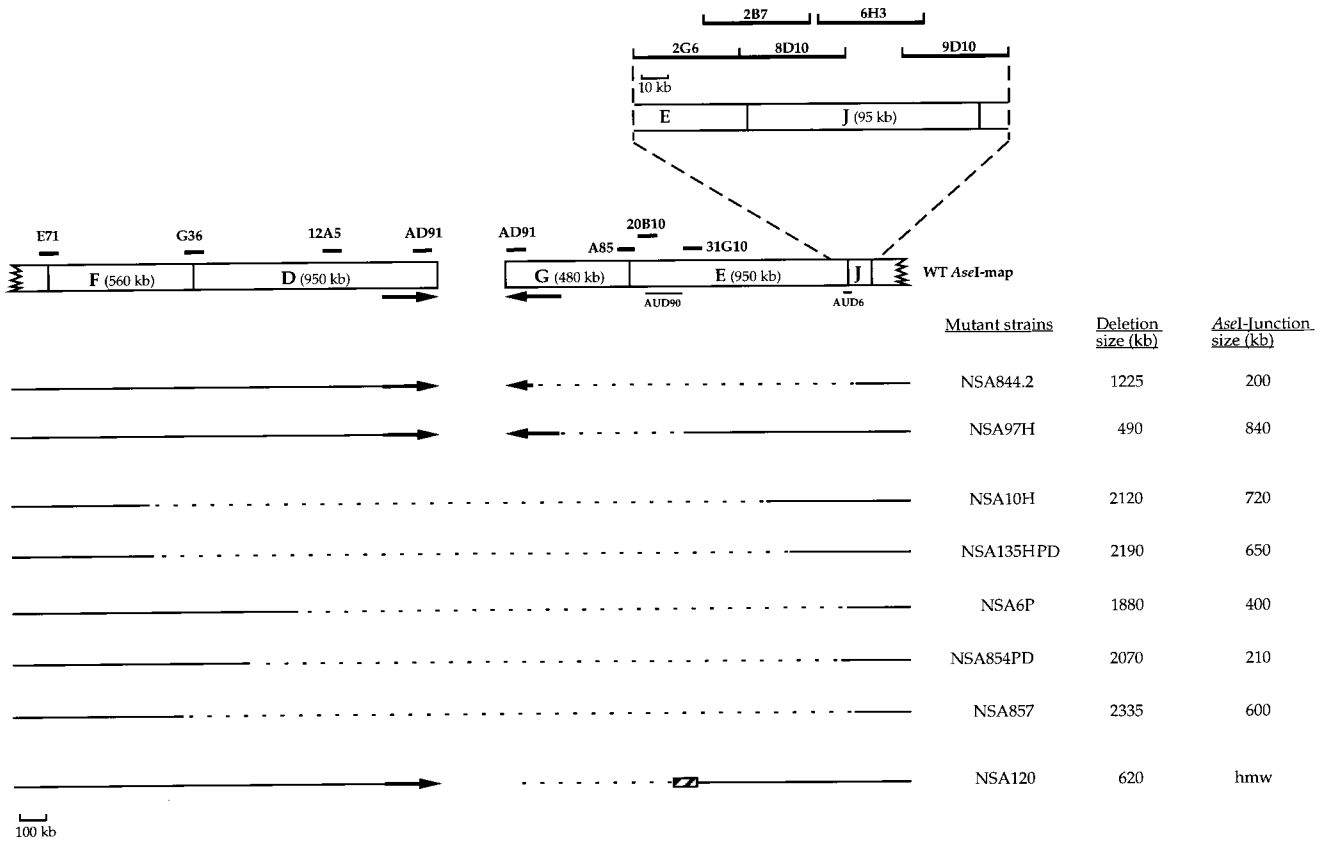

FIG. 1. Extents of the deletions in *S. ambofaciens* mutant strains. The *Ase*I restriction map of the unstable region of the WT chromosome and the detailed overlapping of cosmids surrounding the *Ase*I-J fragment are shown at the top. The size of the *Ase*I-J fragment was revised from the PFGE estimation of 75 kb to 95 kb by measuring the corresponding parts of the inserts of the three overlapping cosmids, 8D10, 6H3, and 9D10. Cosmids used to study the DNA rearrangements are indicated as black bars. The black arrows represent the WT sequences of the TIRs. The deleted regions are shown as dotted lines (thin lines represent undeleted DNA). The hatched box symbolizes DNA amplification. hmw, high-molecular-weight fragment (larger than 2,000 kb).

taining the DNA were incubated (in the buffer recommended by the supplier, New England Biolabs or Boehringer Mannheim) with 20 U of restriction enzyme at 37°C for 3 to 12 h. PFGE runs were performed in a CHEF system (Bio-Rad, Hercules, Calif.) (5). In all experiments, 1% agarose gels were run, using an electrophoresis buffer of 0.5× TBE (50 mM Tris-borate buffer [pH 8.0], 0.1 mM EDTA). Pulse times were optimized depending on the sizes of the DNA fragments to be separated. Lambda concatemeric DNA and *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as size standards. The proteinase-untreated samples of DNA were prepared as described by Lin et al. (18).

DNA labeling and hybridization. Cosmid and chromosomal DNAs were labeled using a nonradioactive digoxigenin (DIG) labeling kit (Boehringer Mannheim). DNA fragments were transferred to nylon membranes (Hybond-N [Amersham, Little Chalfont, England] or Nytran-N [Schleicher & Schuell, Dassel, Germany]) by the method of Smith and Summers (26) and cross-linked by exposure to UV light for 10 min. Hybridizations with DIG-labeled probes were carried out as specified by the manufacturer at 68°C. Detection of DIG-labeled nucleotides was by either chemiluminescent or a colorimetric reaction. To clone *Ase*I-J as overlapping cosmids, the fragment was recovered from a low-melting-point agarose gel and directly labeled after denaturation for 10 min at 100°C. The labeling mixture was kept at 37°C to avoid solidification of the agarose.

RESULTS

Origin of the mutant strains of *S. ambofaciens*. The strains used in this work (Table 1) arose either from basic genetic instability (i.e., isolated in the progeny of the WT strain on the basis of their stable pigment-defective phenotype) or from hypervariability (i.e., isolated in a pigment-defective mutant progeny characterized by the absence of any preponderant phenotype) (15, 16). After several years of spore conservation at −20°C, some strains (NSA844.2, NSA135HPD, NSA6P, and NSA854PD) have undergone additional rearrangements compared to the previous analyses (Table 1).

Internal deletion in one chromosomal arm. Strain NSA844.2 harbors a linear chromosomal DNA with a deletion internal to the right chromosomal arm (according to the map orientation in Fig. 1). A new *Ase*I fragment of 200 kb (Fig. 2A) was shown to correspond to the new chromosome end by hybridizing both cosmids AD91 (flanking the DNA extremities) and 6H3 (inside the *Ase*I J fragment) (Fig. 2B). In addition, the intensity of this

TABLE 1. *S. ambofaciens* strains used in this work

Strain(s)	Origin	Reference or source
NSA844.2	NSA844	15
NSA97H	NSA97H	16
NSA10H	NSA10H	16
NSA135HPD	NSA135H	15
NSA6P	NSA6	15
NSA854PD	NSA854	15
NSA120	NSA120	15
NSA136.1H, NSA136.2H, NSA137.1H, NSA137.2H, NSA138.1H	NSA135HPD	This study
NSA8.1, NSA9.1, NSA9.2, NSA9.3	NSA6P	This study
NSA857, NSA858.1, NSA859.1, NSA859.3	NSA854PD	This study

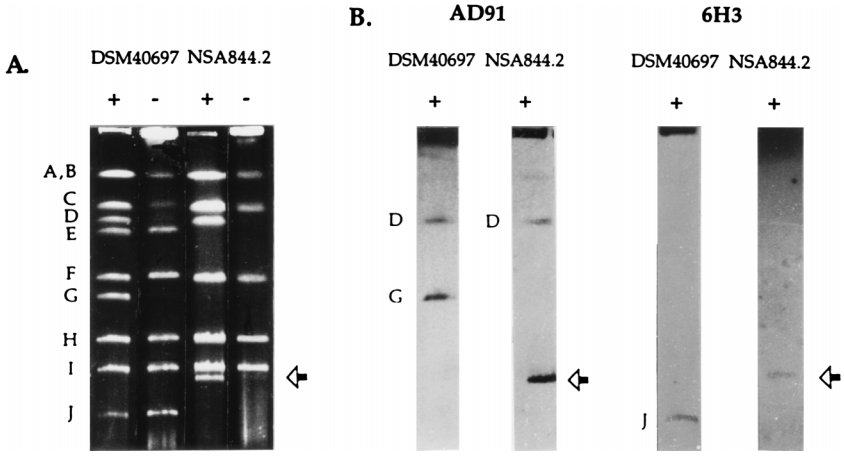

FIG. 2. (A) *Ase*I restriction patterns of genomic DNA of *S. ambofaciens* strains. Running conditions were as follows: 1% agarose gel; 200 V for 24 h with a ramped pulse time from 20 to 130 s. Fragments are lettered according to the chromosome map (13). + and − represent pronase-treated and pronase-untreated samples of DNA, respectively. The open arrow indicates the junction fragment. The higher intensity of *Ase*I C, H, and I fragments is explained by their proximity to the replication origin (13). The large fragments in the pronase-untreated lanes are slightly degraded. (B) Southern blots of the pronase-treated lanes probed with labeled AD91 and 6H3 cosmid DNA. In the lane showing NSA844.2 hybridized with AD91, the faint signal detected at the top of the gel (i.e., PFGE resolution front, where fragments cannot be separated on the basis of molecular weight) is probably due to partial digests caused by the high concentration of DNA.

fragment was found to be fainter in the proteinase-untreated sample of DNA, as was the *Ase*I D fragment, showing that proteins were covalently bound to both of them (Fig. 2A) (13, 18). The extent of deletion was estimated as 1,225 kb by adding the sizes of the missing fragments (*Ase*I-G [480 kb], E [850 kb], and J [95 kb]), subtracting the 200 kb of the new fragment.

The deletion was shown to include parts of the right TIR by hybridization of labeled A50 cosmid DNA (Fig. 3B) onto a *Bam*HI pattern of total DNA from strain NSA844.2. The *Bam*HI fragment of 2.8 kb, including the end of the right TIR, was entirely deleted, while on the other arm, the *Bam*HI fragment of 7 kb was still detected (Fig. 3B). Since all sequences of the TIR are present on the left arm, the whole set of cosmid covering this region gave a WT hybridization pattern. However, cosmid AD105 detected an extra *Bam*HI fragment of 6.5 kb interpreted as the left deletion terminus. The right deletion terminus was identified in cosmid 2B7 as a novel junction-containing *Bam*HI fragment of 10 kb. It was surprising to find that the right and left *Bam*HI junction fragments were of different sizes, 10 and 6.5 kb, respectively. This finding indicated that the rearrangement in strain NSA844.2 was not a simple recombination event between two points, which would have generated a single fusion fragment. In addition, sequences present in the left junction-containing *Bam*HI fragment of 6.5 kb appeared to result from the duplication of a unique sequence cloned in cosmid 6H3, in the WT strain, thereby generating a nontandem duplication at the junction point.

Similarly, both deletion termini have been mapped internally to the right chromosomal arm of strain NSA97H. In this strain, the new chromosomal extremity consists of a new *Ase*I fragment of 840 kb resulting from the deletion of a 490-kb region overlapping fragments *Ase*I-G and -E (Fig. 1). The left and right deletion termini were mapped in cosmids A50 (inside the TIR) and 31G10, respectively, generating at the junction a single *Bam*HI fragment of 3 kb. Thus, in strain NSA97H, the rearrangement may correspond to a recombination event between two points that leads to the loss of the intervening 490 kb of DNA.

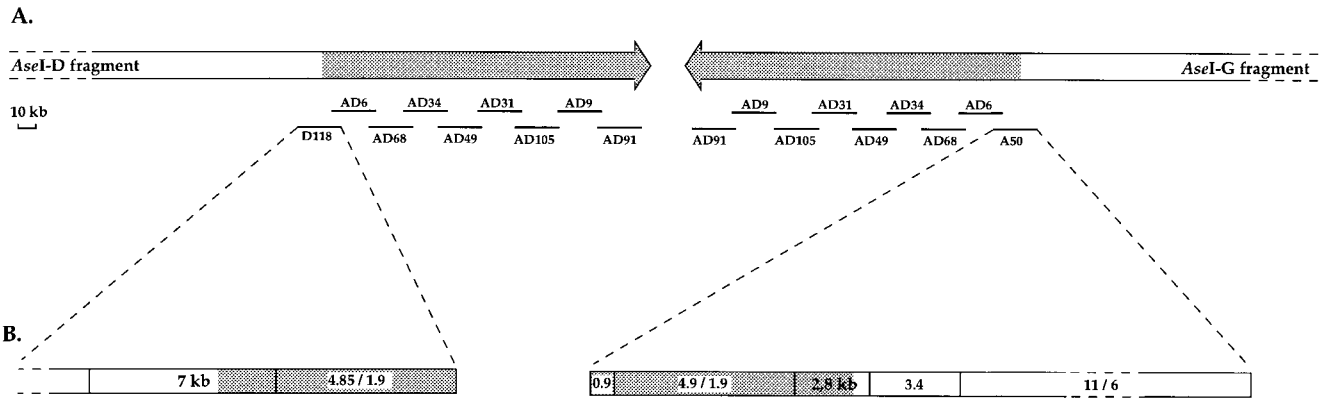

FIG. 3. TIRs at the chromosomal ends. (A) The TIRs are symbolized by the grey arrows at the extremities of the terminal *Ase*I restriction fragments. Nine overlapping cosmids were used to clone this region. (B) *Bam*HI restriction map of the region overlapping the beginning of the TIRs (grey zone) on both chromosomal arms. The fragment sizes are indicated in kilobases.

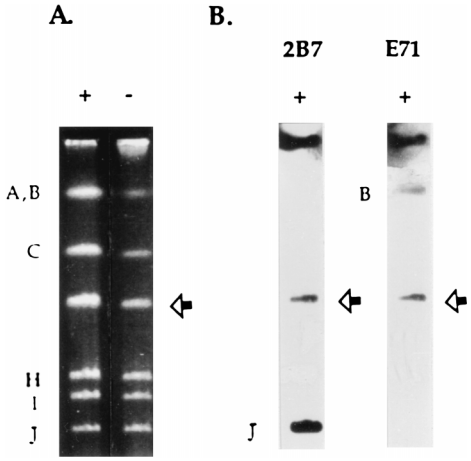

FIG. 4. (A) *Ase*I restriction patterns of genomic DNA from strain NSA10H. Running conditions were as follows: 1% agarose gel; 200 V for 24 h with a ramped pulse time from 40 to 150 s. Fragments are lettered according to the chromosome map (13). DNA samples were pronase-treated (+) or not pronase untreated (−). The open arrow indicates the new 720-kb fragment which was not covalently bound to a protein. (B) Southern blots of the pronase-treated lane probed with labeled 2B7 and E71 cosmid DNA (Fig. 1).

Deletion and chromosome circularization. Four mutant strains (NSA10H, NSA135HPD, NSA6P, and NSA854PD) were shown to have deletions containing all the sequences of cosmids A85, 12A5, and AD91, indicating the loss of both chromosomal ends (Fig. 1). In each case, several *Ase*I fragments originating from both chromosomal arms were missing and a new *Ase*I fragment was characterized. Its electrophoretic migration was not impaired in proteinase-untreated DNA samples; therefore, it was not bound to a protein. For example, in NSA10H, a 720-kb *Ase*I fragment was shown to be homologous to both cosmids 2B7 and E71, originating from the right and the left chromosomal arms, respectively (Fig. 4). Thus, it represents the *Ase*I junction fragment resulting in circularization of the chromosomal DNA. The extend of the deletion was estimated to 2,120 kb (Fig. 1). Similar results were obtained for NSA135HPD, NSA6P, and NSA854PD (summarized on Fig. 1).

Instability of circular chromosomes. Phenotypic stability was surveyed in the progeny of three mutants harboring a circularized chromosome: NSA135HPD (pigment-defective aerial mycelium resulting in light grey colonies), NSA6P (pigment-negative mycelium resulting in white colonies), and NSA854PD (light grey colonies). Spores recovered from an isolated colony were plated on Hickey-Tresner medium. The frequencies of appearance of colonies exhibiting a phenotype different from that of the parental strain (i.e., affected in aerial mycelium formation, pigment synthesis, or colony size) were 5.7×10^{-2} for NSA135HPD (63 of 1,099 colonies), 7.1×10^{-2} for NSA6P (54 of 763 colonies), and 2.3×10^{-2} for NSA854PD (28 of 1,213 colonies). Under the same conditions, the WT strain exhibited 3.5×10^{-3} pigment-defective or -negative colonies (5 of 1,418 colonies), frequency consistent with that reported previously (16).

From the progeny of each strain, mutants were subcloned and tested for the presence of additional DNA rearrangements. The results of hybridization of cosmids E71, G36, 2G6, 8D10, and 6H3 (Fig. 1) onto *Bam*HI patterns of total DNA are summarized in Table 2.

In the progeny of strain NSA135HPD, extra rearrangements could not be tested on the left side of the parental deletion since no useful DNA probe was available left of G36, which was already deleted in the parental strain NSA135HPD (Fig. 1). Sequences homologous to cosmid E71, localized left of G36, were never found to be deleted. However, on the right side, three of the five mutants exhibited extra deletions. For strains NSA136.1H and NSA137.1H, the presence of 2G6 does not exclude the possibility of an additional deletion in the remaining *Ase*I-E sequences. In the progeny of NSA6P, all four mutants showed additional deleted areas on the left side, while only one, NSA8.1, was found to also have a deleted area on the right side. In the offspring of NSA854PD, three of the four strains tested had further deletions on the left side and two had further deletions on the right side. For NSA857, the circularity of the chromosome was checked by PFGE. Thus, an *Ase*I junction fragment of about 600 kb resulted from a 2,315-kb deletion, compared to a 2,070-kb deletion in the parental strain NSA854PD.

In total, 11 of 13 mutants analyzed were shown to have a deletion larger than that of their progenitor.

Amplification associated with the loss of one end of the chromosome. NSA120 was previously characterized by a high degree of amplification of a 15-kb region belonging to the locus AUD90 (15). The *Ase*I pattern of this strain lacked both G and E fragments (Fig. 1). The other chromosomal end consisting of *Ase*I-D was not deleted, and when proteinase treatment was omitted, its migration in a PFGE gel was impaired by protein bound to its extremity. Consequently, its chromosomal DNA was thought to be linear. Using a DNA probe overlapping the AUD90 locus (cosmid 20B10 [Fig. 1]), we showed that the deletion was directly adjacent to the DNA amplification. All sequences tested between the amplification and the TIR gave no hybridization signal. Finally, cosmid AD91 DNA revealed only the *Ase*I D fragment (not shown), suggesting that all sequences from the other chromosomal end were deleted. The amplified DNA was detected as a high-molecular-weight *Ase*I fragment (larger than 2,000 kb) (15). Consequently, the chromosomal end might be replaced on this arm by the reiterated DNA sequence.

DISCUSSION

The extreme plasticity of the *S. ambofaciens* genome was confirmed in this study of the chromosomal structure of deletion mutant strains.

TABLE 2. Localization of the deletion termini in 3 circular DNA deleted mutants and in their offspring[a]

Parental strain	Left terminus	Right terminus	Offspring	Left terminus	Right terminus
NSA135HPD	*Ase*I-F	*Ase*I-E	NSA136.1H	*Ase*I-F (ND)	*Ase*I-E (ND)
			NSA136.2H	*Ase*I-F (ND)	8D10 (+)
			NSA137.1H	*Ase*I-F (ND)	*Ase*I-E (ND)
			NSA137.2H	*Ase*I-F (ND)	8D10 (+)
			NSA138.1H	*Ase*I-F (ND)	2G6 (+)
NSA6P	*Ase*I-D	2G6	NSA8.1	*Ase*I-F (+)	8D10 (+)
			NSA9.1	*Ase*I-F (+)	2G6 (−)
			NSA9.2	*Ase*I-F (+)	2G6 (−)
			NSA9.3	*Ase*I-F (+)	2G6 (−)
NSA854PD	*Ase*I-D	2G6	NSA857	*Ase*I-F (+)	8D10 (+)
			NSA858.1	*Ase*I-D (ND)	2G6 (+)
			NSA859.1	*Ase*I-F (+)	2G6 (−)
			NSA859.3	G36 (+)	2G6 (−)

[a] Left and right refer to the map orientations in Fig. 1, (+) and (−) indicate the presence and absence, respectively, of additional deletion, (ND) (not determined) indicates the lack of suitable probe in the region under consideration.

In two of the mutants, the localization of both deletions within the same chromosomal arm leaves a linear DNA structure ending in TIRs which are associated with proteins. In both cases, the left-hand deletion termini mapped within the TIR, resulting in shortening of the TIRs from 210 kb in the WT strain to about 100 and 200 kb in NSA844.2 and NSA97H, respectively. The TIRs are highly variable in length between different species of *Streptomyces*, from 24 kb in *S. griseus* (17) to 550 kb in *S. rimosus* (6); here we show evidence for the first time of heterogeneity in TIR length between strains of the same species. The cause of this variation appears to have been not the loss of the end of one chromosome but rather a recombination events between two points within the same chromosomal arm. An example of this type of rearrangement is the deletion of 32.6 kb of DNA, previously identified in strain GLA600 of *S. glaucescens* in a region cloned using contiguous cosmids (3). The chromosome structure of this species was not described by the authors; however, the rearrangement appears to map approximately 150 kb away from an unclonable gap in the unstable region. This deletion event is analogous to the loss of internal DNA of strain NSA97H. We have also shown that a duplication of a previously unique sequence was associated with the deletion junction in strain NSA844.2. Birch et al. (3) have described this type of duplication in three of the five cases that they investigated. The rearrangement described for *S. glaucescens* GLA24 with a complex junction was consistent with the deletion that we characterized for strain NSA844.2.

Four of the mutants presented a circular chromosome with deletion encompassing the entire TIRs on both chromosomal arms. In each case, the deletion was approximately 2,000 kb. Circular chromosomes have been identified by Redenbach et al. in *S. lividans* (21). In addition, the chromosome of this species was also artificially circularized by a double-crossover event on both chromosomal arms (18). These circular chromosomes were shown to be highly unstable when selection pressure was removed (4). In *S. ambofaciens*, the phenotypic instability, characterized in the progeny of three spontaneous circular DNA mutants, was significantly higher than in the wild-type strain. Among the 13 clones isolated, each of 11 showed a larger deletion than the progenitor. From changes in chromosomal structure, this increased level of genetic instability might be caused by the difficulties of circular chromosomes to terminate the replication and to partition.

In strain NSA120, the amplification might act as the new extremity, since the deletion encompassed all sequences between AUD90 and the end of the chromosome. Similar structures have been found in *S. lividans* 66 (20) and *S. achromogenes* subsp. *rubradiris* (11). We showed that the chromosome of this amplified strain is linear and that there are no TIRs left, since the two extremities are different in sequence. It is still not known if the structure of this extremity consists of a replicating DNA loop, as suggested by Young and Cullum's model (27), or by a free end composed of tandem repeats.

In conclusion, chromosome circularization in *S. ambofaciens* does not prevent the loss of DNA caused by genetic instability. This finding indicates that the presence of DNA ends does not promote chromosomal deletions but rather that the resulting chromosomal structure of the mutants may be a consequence of the localization of the deletion termini. For example, if they are on the same chromosomal arm, the structure will be linear, leaving the ends intact, as seen in strains NSA844.2 and NSA97H, whereas if they are on both arms, then the chromosome will be circularized, with the loss of both ends. According to this hypothesis, the loss of all sequences from one chromosome end, associated with amplification, as described for strains NSA120 could be the consequence of the mechanism leading to creation of these DNA repetitions.

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