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NOTES

Genetic Instability and Associated Genome Plasticity in *Streptomyces ambofaciens*: Pulsed-Field Gel Electrophoresis Evidence for Large DNA Alterations in a Limited Genomic Region

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Using pulsed-field gel electrophoresis (PFGE) analysis, the amplifiable units of DNA (AUD) loci AUD6 and AUD90 of *Streptomyces ambofaciens* DSM40697 could be mapped in the wild-type genome within two adjacent *AseI* restriction fragments estimated to be about 75 and 850 kb. In addition, the genetic instability and formation of very large deletions were strictly correlated. Their sizes were estimated to range from 250 to more than 2,000 kb. These deletions affected the DNA region overlapping both amplifiable loci. PFGE also allowed us to localize the amplified DNA sequences and to establish their structure: amplification takes place at the AUD locus as a tandem array of the wild-type AUD sequence.

The *Streptomyces* genome exhibits a high degree of genome plasticity, consisting of large deletions and amplified DNA sequences (ADS) associated with instability of many characteristics (1, 11, 13). In *Streptomyces ambofaciens* DSM40697 (9), two levels of genetic instability were characterized: (i) a basic genetic instability similar to that reported for other *Streptomyces* spp. and (ii) hypervariability, closely related to the first phenomenon, generating a phenotypic variability from mutant clones (12). At least two DNA regions undergo amplification in close association with hypervariability (3, 12). In addition, an amplifiable locus was recently characterized as a rearrangement hotspot, mainly for deletions (4). An analogous situation implying successive mutational events associated with changes in DNA structure is well documented in *Streptomyces lividans* (5). Using pulsed-field gel electrophoresis (PFGE), we investigated the localization of the amplifiable units of DNA (AUD) on large restriction fragments generated from the wild-type (WT) genome and the size of the deletions associated with genetic instability. We then studied the structure and the physical localization of the ADS in the mutant genome.

Physical relationship between the AUD regions on the WT genome. Forty-eight ADSs, whose sizes ranged from 5.2 to 105 kb, generated through independent genetic instability events were considered. Three probes were used in hybridization experiments: S1, consisting of the cloned extremities of ADS6 (Fig. 1); S120, consisting of a 1.95-kb *Bam*HI fragment of ADS120 (12) prepared as described by Demuyter et al. (3); and S55, a 2.0-kb *Bam*HI fragment cloned from ADS55 (AUD55: 21 kb) in pBR322 (15). Restriction mapping and hybridization experiments with these probes led to the finding that 50% of the ADSs fell in the AUD90 region, 27% were in the AUD6 region, and 23% were in other regions.

Hybridization experiments using AUD-specific probes

with PFGE patterns of *AseI* digests of the WT DNA (14) were carried out. Two signals were detected with the S1 probe: a strong one corresponding to a 75-kb DNA fragment and a fainter one estimated to be 850 kb. This finding is in agreement with the restriction map of AUD6 (Fig. 1). The relative intensity of the signals could be explained by the fact that the 75-kb fragment contained two homologous stretches (i.e., the right side of the AUD and the internal homologous sequence). On the other hand, the 850-kb fragment contained only the left side of AUD6. Both the S120 and S55 (from an ADS belonging to a region other than AUD90 or AUD6) probes revealed the 850-kb fragment. Faint signals could be detected that corresponded to almost all restriction fragments generated by *AseI*, particularly the 200-kb doublet (14), revealing microhomologies throughout the genome. These faint signals could be detected when a large amount of DNA was blotted. The absence of an *AseI* site within AUD90 was confirmed by classical electrophoresis (data not shown). Thus, we were able to map these two AUD regions onto two adjacent restriction fragments. As the AUD6 region overlapped two *AseI* restriction fragments estimated to be 75 and 850 kb, AUD90 was located on the latter 850-kb fragment. In addition, a third AUD locus, AUD55, was located in the same way within the 850-kb fragment.

Hütter et al. (10) called this type of AUD structure type I, corresponding to multiple overlapping AUDs originating from the same region. *Streptomyces glaucescens* provides a well-documented case of a type I AUD locus with a 100-kb region undergoing ADSs highly heterogeneous in length and complexity (7, 8).

Involvement of large polar deletions in basic genetic instability and hypervariability. Thirty-five percent of the unamplified mutant strains of *S. ambofaciens* were found to contain partial or total deletions in the AUD6 region (4), while 65% harbored a WT AUD6 region. PFGE experiments were carried out to estimate the extent of the deletion and to test the presence of a deletion in unamplified mutants.

For this purpose, 26 mutant strains were isolated from 26

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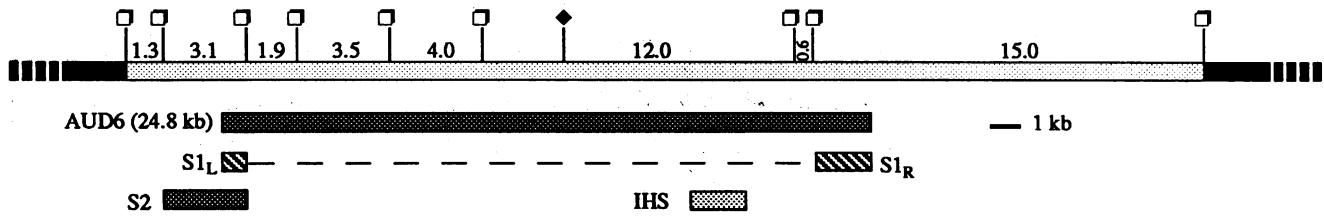


FIG. 1. AUD6 locus. In the restriction map of the AUD6 region in the WT genome, only the *Bam*HI (□) and *Ase*I (◆) restriction sites are shown. The sizes of the *Bam*HI restriction fragments are given in kilobases. *Bam*HI restriction fragments used as probes in hybridization experiments have been cloned: S1 (S1_L and S1_R correspond to the endpoints of the AUD joined in the reiteration fragment); S2, which corresponds to the left flanking sequence of the AUD; and IHS (internal homologous sequence), which represents a sequence, included in the 12-kb *Bam*HI fragment, homologous to S1_R (4).

independent genetic instability events; 14 were derived from the WT strain by the basic genetic instability and exhibited a stable pigment-defective mutant phenotype, and 12 were derived from hypervariability and exhibited various mutant phenotypes (12).

When compared with the WT pattern, several high-molecular-weight DNA fragments were missing (Fig. 2). All 26 mutant strains tested exhibited an altered *Ase*I restriction pattern. The same phenomenon was observed with use of *Dra*I (data not shown). In contrast, 12 independent WT clones were analyzed and found to share the same typical restriction pattern (14). Therefore, each unamplified mutant strain contains a large genomic rearrangement.

In addition, these rearrangements affected the same restriction fragments. The frequency of loss varied from 100% (26 of 26) for the 450-kb fragment to 8% (2 of 26) for the 550-kb fragment. In addition to missing bands, bands of various sizes could be detected in some mutant restriction patterns (17 of 26). They could correspond to joining fragments created by deletions. When no additional fragment could be detected (9 of 26), the joining fragment might

nevertheless exist but could comigrate with one of the preexisting fragments.

The involvement of large deletions was confirmed by hybridization experiments with the S2 (Fig. 1) and S120 probes, which are homologous to the only 850-kb *Ase*I fragment. Since the 850-kb fragment is always missing in the mutant patterns, these probes were expected to reveal joining fragments when the AUD regions were not altered and thus allow us to estimate the extent of the deletions. First, all of the mutant strains deleted in the AUD6 region were shown to lack the 75-kb *Ase*I fragment (Fig. 3, lanes 2 and 7 to 10). The use of S2 allowed us to detect a polymorphism (Fig. 3, lanes 3 to 6 and 12). The hybridizing fragment can be interpreted as a new band consisting of the fusion of the rest of the 850-kb fragment and AUD6. On the other hand, S120 revealed no signal in the mutant strains. This fact demonstrated that the AUD90 region is deleted in all mutant strains so far tested.

Assuming that the DNA rearrangements were the results of a unique deletion event, a speculative *Ase*I restriction map of the unstable region could be drawn (Fig. 4A). We previously demonstrated that the 75- and 850-kb fragments were adjacent. The 450- and 850-kb fragments were always missing. In only two cases could a 850-kb fragment be observed (NSA97H and NSA108H); it did not correspond to the initial 850-kb fragment since the AUD90 region was shown to be deleted in all mutant strains. In addition, the

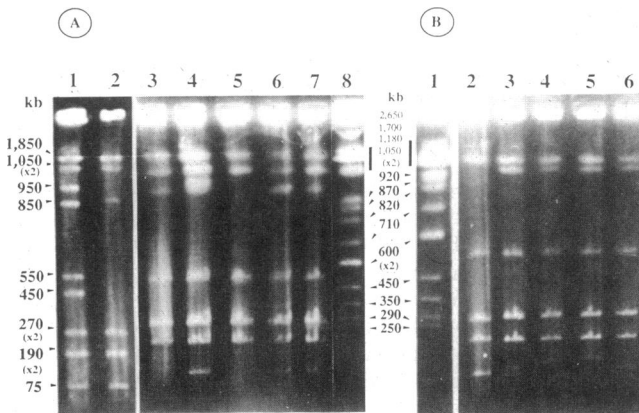


FIG. 2. *Ase*I PFGE restriction patterns of total DNA from the WT and hypervariability-derived strains (A) and from pigment-defective stable strains (B) of *S. ambifaciens*. (A) Lanes: 1, WT DNA; 2, NSA108H; 3, NSA841H; 4, NSA135H; 5, NSA43H; 6, NSA4H; 7, NSA312H; 8, *Saccharomyces cerevisiae* chromosomes used as size standards for lanes 3 to 7. Running conditions were 150 V for 32 h with increased pulse times from 60 to 120 s for lanes 1 and 2; the same conditions were used for lanes 3 to 8 for 30 h. (B) Lanes: 1, *S. cerevisiae* chromosomes used as size standards; 2, NSA833; 3, NSA64; 4, NSA105; 5, NSA49; 6, NSA27. Running conditions were 150 V for 30 h with increased pulse times from 60 to 120 s.

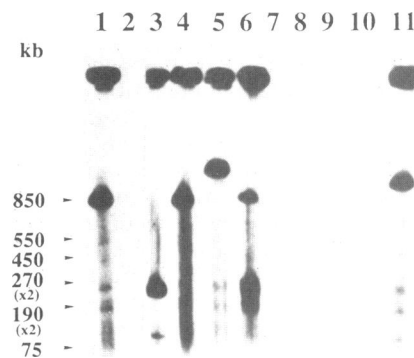


FIG. 3. Hybridization of *Ase*I PFGE restriction patterns of WT and nonamplified mutant strain DNA with the S2 probe. Lanes: 1, WT DNA; 2, NSA43H; 3, NSA4H; 4, NSA97H; 5, NSA850H; 6, NSA108H; 7, NSA28; 8, NSA27; 9, NSA830; 10, NSA49; 11, NSA844. Running conditions were 150 V for 35 h with increased pulse times from 40 to 180 s.

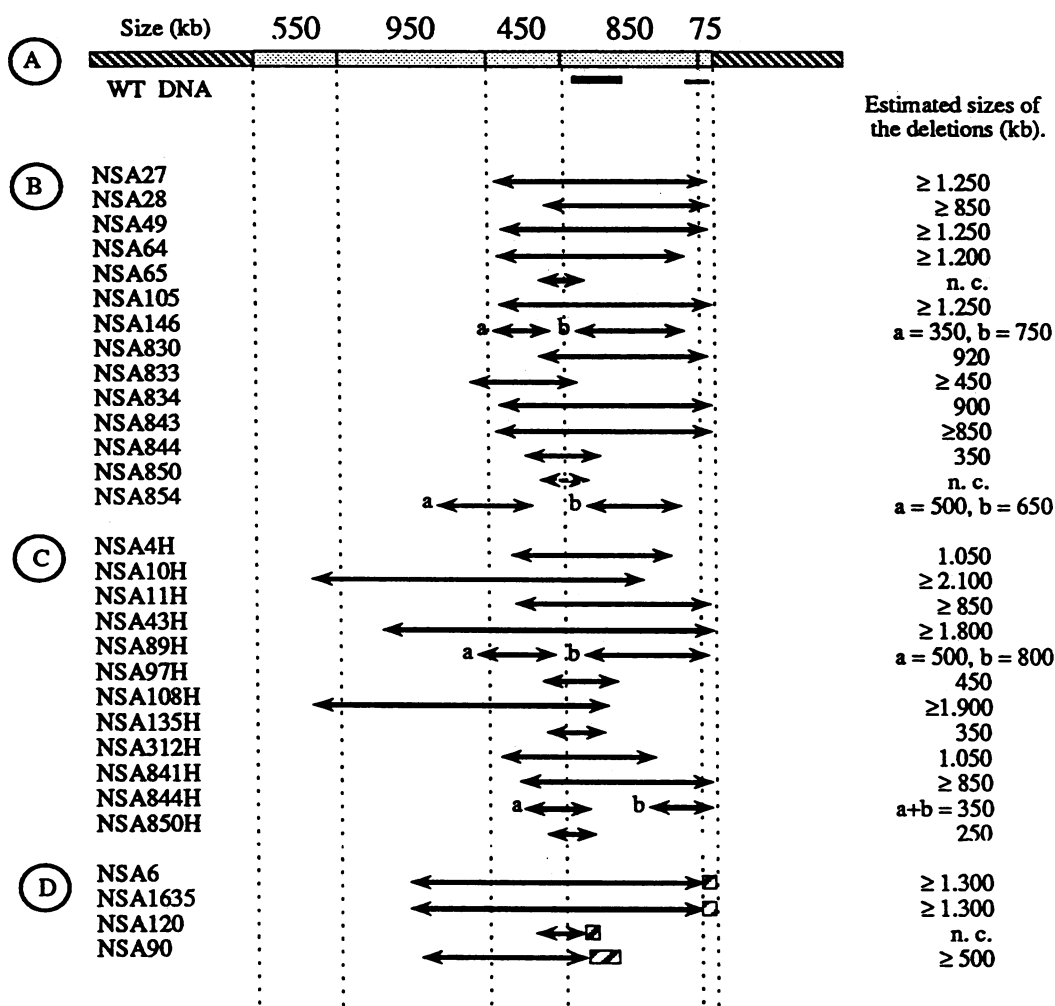


FIG. 4. (A) Speculative *AseI* restriction map of the unstable region in the WT strain; (B) deletions in the pigment-defective stable strains; (C) deletions in the nonamplified mutant strains generated from hypervariability; (D) deletions in amplified strains. Symbols: [diagonal lines], unknown regions on the WT chromosome; [stippled], unstable region; —, AUD6 family; —, AUD90 family; [hatched], amplified DNA; ↔, deletion size estimations using restriction pattern analysis or hybridization experiments with the S2 probe; #, accurate estimation; ≥, minimal estimation; n. c., noncalculable (the lack of additional fragment and hybridization response prevented size estimation).

550-kb fragment was never missing when the 950-kb fragment was present. Thus, we could order these fragments according to this polarity of loss at the opposite side of the 75-kb fragment (Fig. 4A).

Considering only one deletion event, the deletion overlapped the 850-kb fragment and ended in this fragment at the right of the AUD90 region in 50% of cases. In the other 50%, the deletion overlapped the 850-kb fragment and one end lay within or to the right of AUD6. All of the deletion events ended in the 75-kb fragment, since no additional DNA fragment was affected. We were able to estimate the size of the deletion by using the S2 probe in the mutant strains with intact AUD6. When the AUD6 region was altered, no accurate estimation could be made. In some cases, several hybridizing fragments were detected with unequal intensities. These cases could be interpreted as a genomic heterogeneity generated before DNA preparation as described by Demuyter et al. (4). The results are shown in Fig. 4B and C.

The estimated sizes of the deletions range from 250 to more than 2,000 kb, thus including from 3 to 25% of the total genome size, estimated to be 8,000 kb (14). The association

between deletion and genetic instability was frequently reported in *Streptomyces* species (2, 5, 6). In *S. glaucescens*, for example, the double mutant phenotype $Str^- Mel^-$ was ascribed to deletions whose sizes were estimated to range from 270 to over 800 kb (2).

Deletions in ADS-containing strains: structure and localization of ADS. Two ADS6-containing strains were analyzed: NSA6, characterized by a high degree of amplification of AUD6, and NSA1635, isolated in the same progeny of NSA6 but characterized by a low copy number of AUD6. In both cases, the *AseI* restriction pattern revealed the same missing bands as in unamplified mutant strains: the *AseI* fragments of 450, 850, and 950 kb were missing (Fig. 5, lanes 2). Thus, the deletion was estimated at least at 1,300 kb and could reach 2,000 kb since no joining fragment could be detected (Fig. 4D). In contrast, a heavy and smeared DNA band migrating below the 75-kb fragment was detected in NSA6, while the same band was only faint in NSA1635 (Fig. 5, lanes 2). A signal more intense in NSA6 than in NSA1635 (Fig. 5, lanes 3) was revealed with use of S1. This signal could be interpreted as the amplification of a monomer of 24.8 kb.

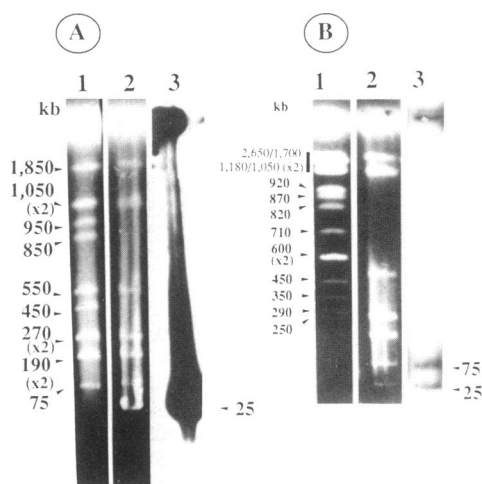


FIG. 5. Hybridization of *AseI* PFGE restriction patterns of ADS6-containing mutant strains with the S1 probe. (A) Hybridization on NSA6 DNA. Lanes: 1, *AseI* restriction pattern of the WT strain used as a reference; 2, NSA6 *AseI* restriction pattern; 3, hybridization pattern of the NSA6 digest with the S1 probe. Running conditions were 150 V for 30 h with increased pulse times from 60 to 160 s. (B) Hybridization on NSA1635 DNA. Lanes: 1, *S. cerevisiae* chromosomes used as size standards; 2, NSA1635 *AseI* restriction pattern; 3, hybridization pattern of the NSA1635 digest with the S1 probe. Running conditions were 150 V for 30 h with increased pulse times from 60 to 120 s.

These observations were consistent with the hypothesis that most of the copies of AUD6 are tandemly reiterated, as already suggested by classical analyses (3). The recent findings of extrachromosomal DNA molecules corresponding to an ADS do not contradict this hypothesis, since the copy number estimated as about 0.6 per genome corresponded to a very small fraction of the amplified DNA (16). In addition, a signal corresponding to the 75-kb fragment was detected in NSA1635. Because of the intense signal in NSA6, it was impossible to detect the presence of this signal in NSA6. However, S1 hybridization on NSA6 DNA revealed the right flanking sequence of AUD6. These findings demonstrated the existence of a large deletion encompassing the AUD90 region, associated with amplification of AUD6.

Two AUDs belonging to the AUD90 locus were studied: AUD120 (15 kb) and AUD90 (55 kb). All of the amplified *Bam*HI fragments generated from ADS120 were present in ADS90 (12), revealing overlapping stretches. These facts suggested that the fragment created by the reiteration of AUD120 was comigrating with one *Bam*HI fragment of ADS90. No *AseI* restriction site could be mapped in either AUD120 or AUD90. *AseI* restriction patterns of NSA120 and NSA90 revealed missing bands: NSA120 lacked the 450- and 850-kb bands (Fig. 6A, lane 3); NSA90 lacked the 450-, 850-, and 950-kb fragments (Fig. 6A, lane 4). No additional fragment could be detected on an ethidium bromide-stained gel. NSA90 exhibited a deletion extending over 500 kb, while NSA120 contained a deletion whose size could not be estimated (Fig. 4D).

To localize the ADSs, PFGE migrations were hybridized with S120. In both strains, a strong signal corresponding to a high-molecular-weight DNA band was detected whose size was estimated to be about 2,000 kb (Fig. 6B). These results were in agreement with the amplification degree of each ADS and the respective size of each AUD. As the 75-kb

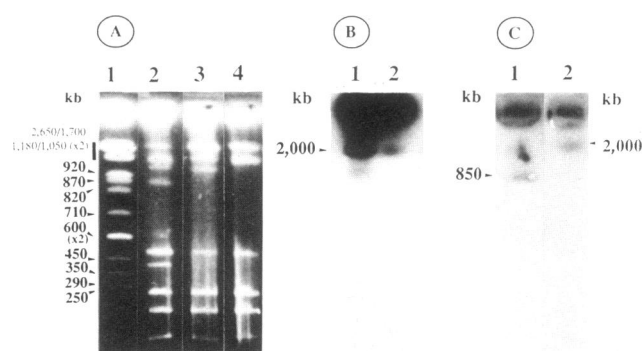


FIG. 6. PFGE analysis of mutant strains containing ADSs deriving from the AUD90 loci and hybridization experiments with AUD-specific probes. (A) PFGE patterns of *AseI* digests of total DNA of WT, NSA120, and NSA90. Lanes: 1, *S. cerevisiae* chromosomes used as size standards; 2, WT DNA; 3, NSA120; 4, NSA90. Running conditions were 150 V for 30 h with increased pulse times from 60 to 120 s. (B) Hybridization of NSA120 (lane 1) and NSA90 (lane 2) *AseI* digests with the S120 probe. (C) Hybridization of WT (lane 1) and NSA120 (lane 2) *AseI* digests with the S2 probe.

fragment was still present, it can be assumed that DNA rearrangements were affecting only the left side of the AUD region. While S2 revealed the original 850-kb fragment in the WT strain (Fig. 6C, lane 1), the probe revealed the 2,000-kb DNA band described above in the mutant strain (Fig. 6C, lane 2). Thus, these results were in accordance with the genomic rearrangements detected in NSA6 and NSA1635: the ADS was mainly detected as a tandem array of AUDs at the original locus. In addition, one end of a large deletion associated with amplification was located in the vicinity of the AUD locus.

These facts, together with the finding that the deletions ended either close to the ADS or even in the proximal copies of the reiterated AUD (4), led to the hypothesis that the loss of the ADS was associated with the generation of various deletions, likely through a mechanism involving an illegitimate recombination event.

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