JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 419–423
0021-9193/89/010419-05$02.00/0

Vol. 171, No. 1

Hypervariability, a New Phenomenon of Genetic Instability, Related to DNA Amplification in *Streptomyces ambofaciens*

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Received 6 August 1988/Accepted 9 September 1988

The wild-type strain *Streptomyces ambofaciens* DSM 40697 exhibits a high degree of genetic instability. Pigment-defective colonies were observed in the progeny of wild-type colonies at a frequency of about 0.01. While only 13% of these pigment-defective colonies gave rise to homogeneous progeny exhibiting the mutant parental phenotype, 87% of the mutant colonies gave rise to heterogeneous progeny without a preponderant phenotype. This new phenomenon of instability was called hypervariability. In addition, 21% of the mutant strains arising in hypervariable progeny contained highly reiterated DNA sequences, while amplified DNA sequences could be detected in neither stable pigment-defective mutant clones nor in wild-type clones. These results indicate a frequent association between genetic instability and hypervariability and a frequent association between hypervariability and amplification of DNA sequences.

Phenotypic instabilities are commonly observed in *Streptomyces* species. These instabilities are frequently related to such differentiation steps as aerial mycelium formation, sporulation, pigment synthesis, and antibiotic production or resistance. A dramatic increase in this spontaneous mutability can be obtained by several treatments (26). Genomic instabilities are associated with these phenotypic instabilities. Thus, molecular analysis of mutant progeny often reveals genomic rearrangements such as large deletions including genes directly involved in the following phenomena: chloramphenicol sensitivity in *S. coelicolor* A3(2) and *S. lividans* 66 (1, 7), streptomycin sensitivity in *S. glaucescens* (11), melanin formation in *S. reticuli* (25), *S. glaucescens* (12), and several *Streptomyces* species (26), and A-factor biosynthesis in *S. bikiniensis* (14). However, in some cases of reversible phenotypes, it was suggested that the genes involved were affected by transposable elements (8, 9).

Highly amplified DNA sequences (ADS) have been detected within the DNA isolated from variants of many *Streptomyces* species arising either spontaneously (1, 3, 25) or during vegetative growth in the presence of ethidium bromide (10, 19, 24, 25), formation and regeneration of protoplasts (6), or interspecific protoplast fusion (23). In some cases, ADS were associated with particular phenotypes, such as sequential loss of resistance to chloramphenicol and arginine synthesis (2) and loss of resistance to tetracycline and nitrogen assimilation (5). Some others were selected on the basis of high-level antibiotic resistance (7, 15, 17, 21) or overproduction of enzyme inhibitors (K. P. Koller, 6th Int. Symp. Biol. Actinomycetes 1985, p. 177–183). Moreover, ADS formation is frequently associated with large deletions (5, 25).

In *S. ambofaciens*, ADS have already been described (4, 26). We describe here a basic genetic instability preferentially affecting colony pigmentation in spiramycin-producing (20) *Streptomyces ambofaciens* DSM 40697 (16) and a new aspect of genetic instability, called hypervariability, generating numerous mutant phenotypes from the pigment-defective colonies. The molecular analysis of the mutant strains arising from these two steps revealed the relatively frequent occurrence of ADS in the progeny of hypervariable strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type (WT) *Streptomyces ambofaciens* DSM 40697 was used in this work. Representative mutant strains isolated in the course of this study are listed in Table 1. Most of the media used in the culture conditions were described previously (13). *S. ambofaciens* strains were grown at 37°C on plates of Hickey-Tresner (HT) medium (22) for maintenance, sporulation, and mutant isolation. Auxotrophic mutants were detected by replica-plating the WT cultures on minimal medium and further characterized on minimal medium supplemented with combinations of growth requirements. For large-scale isolations of genomic DNA, the *S. ambofaciens* cultures were grown aerobically at 37°C for 48 h in YEME liquid medium supplemented with glycine (0.25%). Small-scale isolations of genomic DNA were performed with mycelium grown at 37°C in HT liquid medium for 24 h.

DNA extraction and restriction endonuclease analyses. The mycelium was harvested by centrifugation, resuspended in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) and mixed with lysozyme (Boehringer Mannheim) (2 mg/ml). Protoplasts were formed by incubation of the mixture at 30°C for 30 min and lysed with sodium dodecyl sulfate (1%) in the presence of proteinase K (50 μg/ml). DNA was purified by two phenol-chloroform extractions followed by one chloroform extraction. The aqueous phase was treated with RNase A (Sigma) (5 mg/ml) for 1 h at 37°C. DNA was precipitated with sodium acetate (0.3 M, pH 5.2) and isopropanol at −20°C. After centrifugation, the pellet was vacuum dried and dissolved in TE buffer. For the small-scale isolations, DNA was purified by the Geneclean process (Bio 101 Inc.). For the large-scale isolations, the DNA was purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients, using a vTi 65.2 rotor (Beckman) at 65,000 rpm for 6 h (400,000 × *g*). Restriction enzymes were purchased from Boehringer Mannheim and used as recommended by the supplier. The restriction fragments were

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TABLE 1. *S. ambofaciens* mutant strains

Strain	Pigmentation[a]	Aerial mycelium[a]	ADS size (kb)
NSA80	−	+	5.2
NSA70	−	+	5.5
NSA190	−	−	7
NSA50	−	+	8
NSA150	−	+	8.2
NSA120	+	+	14.5
NSA130	−	+	16.5
NSA160	−	−	18.5
NSA180	−	+	21.5
NSA100	−	+	21.5
NSA6	−	+	24.8
NSA60	−	+	29
NSA140	−	+	33
NSA170	−	−	35.5
NSA90	+	+	55

[a] −, Mutant phenotype; +, WT character.

electrophoresed on 0.8% agarose gels in TAE buffer (13). Bacteriophage lambda DNA digested by *Hin*dIII was used as a size standard.

RESULTS

Basic genetic instability. Spores of the original strain *S. ambofaciens* DSM 40697 plated on HT agar at 37°C gave rise to colonies exhibiting a high spontaneous mutability of the brown pigmentation seen in the WT colonies. This basic instability is schematized with the relevant frequencies in Fig. 1.A. Platings of spores isolated from 54 independent WT clones showing the typical pigmented powdery appearance (Fig. 2.A.1) were analyzed. Pigment-defective colonies were observed at a high frequency (0.97 ± 0.17%; 27,786 colonies examined). The experimentally determined distribution of these frequencies in the 54 WT clones was significantly different from a random distribution (Poisson's law; $P \leq 0.01$), suggesting heterogeneity of the sampling. This heterogeneity can be explained by undetected mutations arising during the growth of the WT colony harvested to realize the plating. The earlier this mutation event occurs, the higher will be the frequency of occurrence of pigment-defective colonies in a subsequent plating. Two types of pigment-defective colonies were distinguished in the platings: the first type was a homogeneous pigment-negative colony (Fig. 2.A.2); the second one was a colony showing a glossy ring with a grey center (Fig. 2.A.3). The pigmentation of this center followed a gradient ranging from slight to intense. Moreover, 7.8 ± 1.4% of the colonies showed one or several pigment-defective sectors (Fig. 2.A.4), and 7.9 ± 1.3% harbored one or several pigment-defective papillae (Fig. 2.A.5). The distribution of the number of colonies harboring either sectors or papillae could be in accordance with Poisson's law ($P \leq 0.55$ and $P \leq 0.20$, respectively). In addition, sectors and papillae were randomly distributed on an otherwise WT colony ($P \leq 0.50$). A plausible explanation for the occurrence of pigment-defective sectors is to consider a mutation event occurring during the radial growth phase (i.e., the development of the vegetative mycelium). If the mutational event occurs later, during the vertical growth of the aerial mycelium or during sporogenesis, a papilla appears. This basic instability accounts for approximately 17% of the progeny being pigment defective, sectored, and papilla-harboring colonies and was observed at every round of sporulation from a WT colony (Fig. 1.B). The WT platings

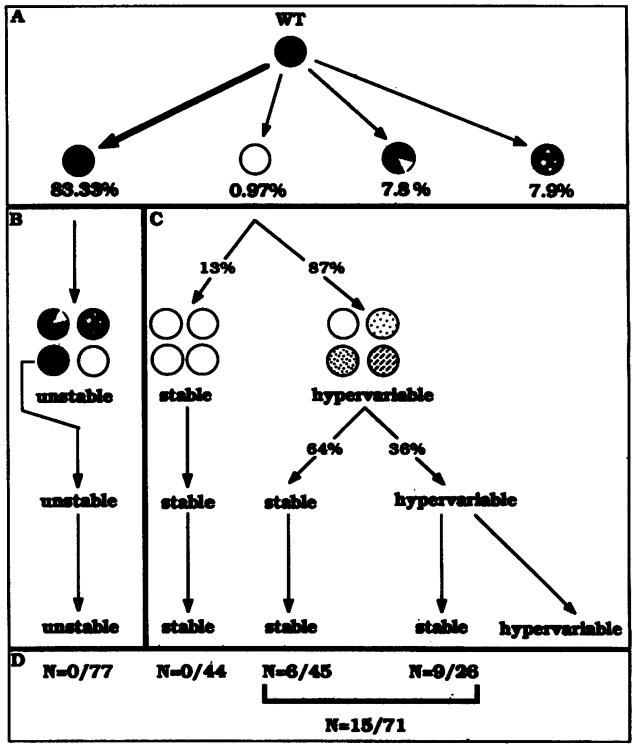

FIG. 1. Flow diagram of genetic instability. Each arrow represents a round of sporulation on HT solid medium. (A) Frequencies of the different mutant phenotypes observed from the WT strain (the WT strain is symbolized by grey circles; open circles represent both pigment-defective and -negative colonies). (B) Successive subclonings of the WT colonies. (C) Successive subclonings of the variant colonies. (D) Molecular analysis of clones isolated from WT, stable, and hypervariable lineages. N = number of clones containing highly amplified DNA sequences/number of clones analyzed.

were investigated for the presence of auxotrophic mutant strains. Two mutant strains were detected among 37,519 colonies tested: one was a His^- mutant with a WT pigmentation phenotype, and the other was an Arg^- mutant with a pigment-negative phenotype. Thus, auxotrophic mutants appeared at a frequency of 5×10^{-5} from the WT strain. Therefore, the genetic instability seems to preferentially affect pigmentation and differentiation processes.

Hypervariability. Studies on the stability of the pigment-defective colonies gave the following results (Fig. 1.C). Spores of 165 independently isolated colonies were plated and the phenotypes were observed. Two types of progeny were distinguished. On the one hand, homogeneous pigment-defective offspring were observed in 21 cases (13%), which gave rise to stable lineages of homogeneous offspring through at least three rounds of sporulation, with more than 300 colonies examined in each plating. On the other hand, heterogeneous offspring were observed in 144 cases (87%). All the pigment-defective colonies with a grey center (Fig. 2.A.3) gave rise to heterogeneous progeny, while pigment-negative colonies of the type shown in Fig. 2.A.2 generated either homogeneous or heterogeneous progeny. These heterogeneous offspring were characterized by the absence of any preponderant phenotype. The number of phenotypes in the progeny derived from the spores of a single colony ranged from 2 to 8, most often 4 or 5. This heterogeneous progeny was called hypervariable, and examples are shown in Figure 2.B (a general view is presented in Fig. 2.B.1 and

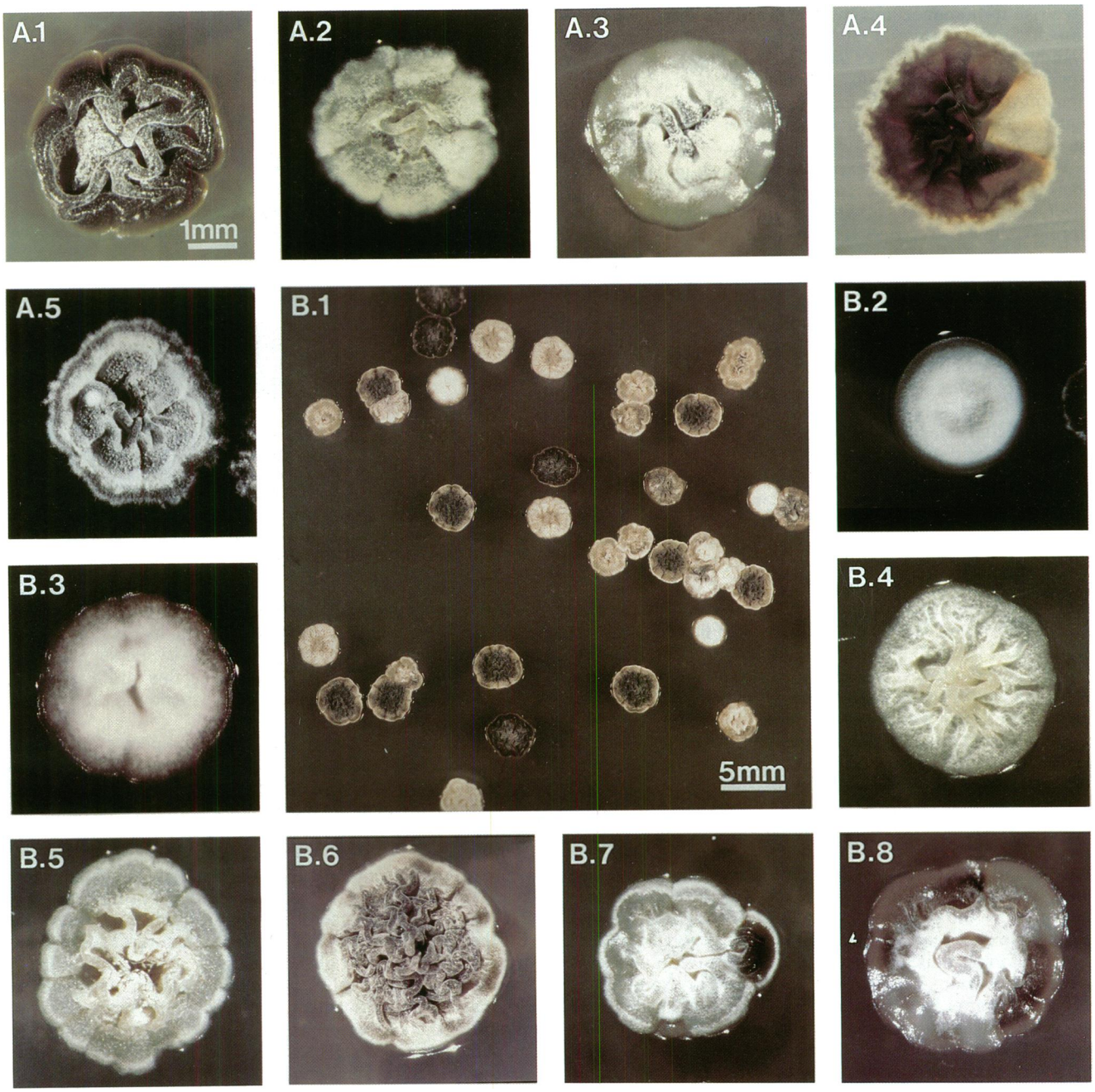

FIG. 2. Illustrations of genetic instability (A) and hypervariability (B) in *S. ambofaciens*. (A.1) WT colony. (A.2) Homogeneous pigment-negative colony. (A.3) Colony with a glossy ring with a grey center. (A.4) WT colony harboring a pigment-defective sector with diascopic lighting. (A.5) Colony harboring a pigment-defective papilla. Papillae appear during sporogenesis so that the appearance of the colony is different from the colony shown in A.1. (B.1) Hypervariable progeny. Close-up views of the different phenotypes are shown in the following pictures. (B.2) Pigment-negative colony with an unwrinkled surface and circular perimeter. (B.3) Pigment-negative colony with a wavy surface and perimeter. (B.4) Mutant colony affected with respect to both pigmentation and aerial mycelium production. (B.5) Same mutant characters as in B.4 with peripheral growing waves. (B.6) Grey wrinkled mutant colony with a wavy ring. (B.7) Pigment-defective colony with two different sectors, defective for aerial mycelium production and WT-like pigmentation. (B.8) Patchwork colony affected with respect to morphology, pigmentation, and aerial mycelium production.

details are presented in Fig. 2.B.2 to 2.B.6). In addition, two hypervariable progeny arising from two independently isolated pigment-defective colonies were different according to the number and the nature of the phenotypes. Among the hypervariable progeny, two examples of mutant colonies with a mosaic phenotype were observed (Fig. 2.B.7 and 2.B.8). The first one harbored two phenotypically different sectors which could correspond to the reversion of the mutant character, illustrating that instability also affects mutant colonies. The second one exhibited a heterogeneous phenotype whose progeny were systematically hypervariable. Colonies exhibiting similar directly observable heterogeneity were present in numerous hypervariable progeny. At the next round of sporulation, 64% of all these mutant strains

gave rise to homogeneous mutant progeny, with the exception of those colonies that harbored sectors, while 36%, whatever their phenotype, gave rise to hypervariable progeny again. Thus, three types of lineages were defined: stable pigment-defective lineages and hypervariable lineages which segregated either stable mutant strains or continuously hypervariable strains.

Association between hypervariability and amplification phenomena. Previous studies allowed us to detect ADS in several mutant strains of *S. ambofaciens* DSM 40697 (4). Here we show by restriction analysis of total DNA of clones isolated from WT, stable pigment-defective, and hypervariable colonies that ADS was detected only in some of the colony types described above (Fig. 1.D). The DNA of 77 WT colonies isolated after three rounds of sporulation and 44 colonies belonging to 44 different stable mutant progeny as well as 71 subcloned colonies randomly selected from 71 different hypervariable progeny were analyzed; 45 colonies of these latter 71 colonies had a mutant phenotype that stabilized after one round of sporulation and 26 were isolated after two rounds of sporulation showing hypervariability, but only the mutant strains with a stabilized phenotype were included in the analysis. All these mutant strains can be considered independent in regard to the initial event of genetic instability yielding the pigment-defective mutants. Highly amplified DNA was detected in 15 of 71 (21%) mutant strains derived from hypervariable progeny, while none was detected in either stable pigment-defective strains (0 of 44) or WT colonies (0 of 77). The 15 of 71 is significantly different from 0 of 44 ($P \leq 10^{-3}$) and 0 of 77 ($P \leq 10^{-4}$). In addition, 9 of 26 and 6 of 45 (Fig. 1.D) are significantly different values ($P = 0.028$). These results clearly indicated that DNA amplification was frequently associated with hypervariability. Moreover, the association between DNA amplification and hypervariability appeared to be stronger when a second hypervariable event had occurred.

The background patterns of total DNA revealed no detectable difference compared with the WT DNA. The sizes of the 15 ADS ranged from 5 to 55 kilobases (kb). Five representative examples of restriction patterns in amplified DNA are shown in Fig. 3. The extent of the amplification measured by the relative fluorescence of the intense bands on agarose gels reached 50% of the total genomic DNA in some mutant strains. An analysis of the restriction patterns suggested overlaps between several ADS. For example, the restriction patterns of strain NSA120 showed six intense bands that were also detectable in the pattern of strain NSA90 (Fig. 3, lanes 6 and 7). In addition, the same 21.5-kb ADS was recovered twice from two independent strains, NSA180 and NSA100 (Fig. 3, lane 4).

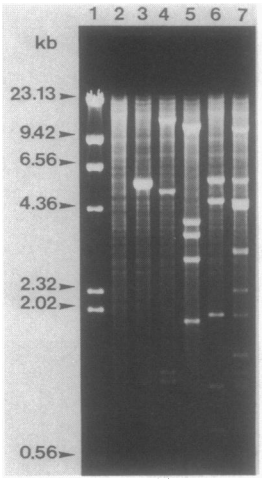

FIG. 3. Restriction patterns of total DNA from the WT and mutant strains of *S. ambofaciens*. DNA was digested with *Bam*HI and electrophoresed on an 0.8% agarose gel at 1 V/cm for 16 h. Lane 1, 0.5 μg of lambda DNA digested with *Hin*dIII; lane 2, WT DNA; lane 3, NSA70 DNA; lane 4, NSA100 DNA; lane 5, NSA6 DNA; lane 6, NSA120 DNA; lane 7, NSA90 DNA. Sizes of ADS, calculated from single and double digestions, are, respectively, 5.5 kb, 21.5 kb, 24.8 kb, 14.5 kb, and 55 kb.

DISCUSSION

In *Streptomyces ambofaciens* DSM 40697, two levels of genetic instability were observed: first, a basic genetic instability revealed by the occurrence of about 1% pigment-defective colonies in the progeny of WT colonies, and second, hypervariability characterized by the absence of a preponderant phenotype in the progeny of 87% of these pigment-defective colonies at the next plating. In addition, analysis of the progeny of more than 1,500 WT colonies did not allow us to observe hypervariability. Thus, hypervariability is closely related to the basic genetic instability. Therefore, hypervariability appeared to result from a succession of two stages: a mutational event, which consisted of the basic genetic instability, and a strong increase in mutability during the development of a pigment-defective colony.

A possible analogous phenomenon has been reported for *Candida albicans* (27). In this case, the WT strain spontaneously exhibits a switching system between seven phenotypes at high frequency (10^{-4}). When this strain is treated with low doses of UV light, this frequency is dramatically increased and reaches 10^{-2}.

In the same way, in the *Streptomyces* species, treatments such as growth on ethidium bromide-containing medium, protoplasting and regenerating, cold conservation, and culture in rich medium have been shown to increase mutability (26). These treatments are far from classic mutagenic conditions.

In *S. ambofaciens*, the high spontaneous mutability could result from a stress, to take up the concept developed by McClintock in the case of genetic instability in maize (18). Further investigations will define the nature of the primary mutational event and of the stress.

The molecular analysis of clones isolated from mutant strains allowed us to detect 15 ADS. In a previous work, we reported the characterization of at least two families of amplifiable units of DNA (AUD) on the WT genome of *S. ambofaciens* (4). The comparison of the restriction patterns of the 15 ADS with the previously mapped ADS allows us to subgroup three ADS (ADS60, ADS130, and ADS140) in the AUD6 family. Further investigations will reveal whether the other ADS belong to other families and whether these families are far from each other on the genome. These facts support the notion of an amplifiable DNA region demonstrated in *S. glaucescens* (10).

In addition, the ADS were detected in 21% of the colonies only from hypervariable progeny. Thus, hypervariability and DNA amplification are closely related. These statistical features lead one to question the relationships between hypervariability and DNA amplification. Two main hypotheses can be drawn from these results.

On one hand, amplification of DNA sequences could be the inducer of numerous phenotypes observed in hypervariable progeny: rearrangements associated with the ADS, such as deletions, would be responsible for the mutant

phenotypes. These rearrangements could take place at multiple loci of a large region to produce such a variability. In this case, the fact that ADS can be detected in only 21% of mutants could be explained two ways. Amplifications could happen progressively through cell divisions and could be detected on agarose gels only after several rounds of sporulation. The case of faint intensified bands in the restriction patterns corresponding to lower-copy-number amplified DNA stretches has been reported (5). The relationships can also be explained by the loss of the ADS either by deletion or by segregation through growth cycles. In this way, unstable ADS have been reported for many *Streptomyces* species (5, 10).

On the other hand, amplification could be induced through a two-step mechanism according to the models currently developed. Thus, Dyson and Schrempf (5) suggest that the rate-limiting step to amplification in *S. lividans* is the formation of a duplicated form of the AUD by an initial recombination event. Further amplification might be generated in a single step by a replication mechanism, as postulated by Young and Cullum based on a rolling-circle model (28). In *S. ambofaciens*, the two steps of the amplification mechanism could be associated with the stages of the induction of hypervariability: the primary mutational event and the subsequent genetic instability, then genomic rearrangements such as amplifications induced by the increased mutability.

ACKNOWLEDGMENTS

This study was supported by grants from C.N.R.S., from Ministère de la Recherche et de l'Enseignement Supérieur, and from Université Nancy I.

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