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Characterization of Two Families of Spontaneously Amplifiable Units of DNA in *Streptomyces ambofaciens*

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Four highly amplified DNA sequences (ADS) ranging from 5·8 to 24·8 kb were found in spontaneous mutant strains of *Streptomyces ambofaciens* DSM 40697. Restriction patterns of total DNA were hybridized with purified ADS6 (24·8 kb) as a probe to detect the amplifiable regions in the wild-type (WT) genome. The results suggested that the amplifiable unit of DNA (AUD) was present as a single copy in the WT genome. Moreover, similarities suggested by the restriction maps of three of the ADS were confirmed by hybridization experiments. The fourth ADS did not hybridize with the three others. Therefore, two families of DNA sequences are potentially amplifiable in the *S. ambofaciens* genome.

INTRODUCTION

Streptomycetes often exhibit a high degree of genetic instability. Many characters have been reported to be unstable, for example antibiotic resistance (Freeman *et al.*, 1977; Hütter *et al.*, 1981; Altenbüchner & Cullum, 1984), pigment synthesis (Suter *et al.*, 1978; Schrempf, 1983), and aerial mycelium formation (Usdin *et al.*, 1985). The frequency of mutant colonies after treatments such as UV light exposure (Freeman & Hopwood, 1978), cold storage (Suter *et al.*, 1978) or growth in the presence of ethidium bromide or acridine orange (Suter *et al.*, 1978; Hütter *et al.*, 1981) reaches 10% or higher. Genetic instability could be due to plasmid loss (Hara *et al.*, 1983; Usdin *et al.*, 1985) or to chromosomal instability by formation of large deletions including the unstable gene (Hintermann *et al.*, 1984, 1985; Usdin *et al.*, 1985; Flett & Cullum, 1987). In the latter case, no reversion could be observed. In addition, highly reiterated DNA sequences are commonly observed in *Streptomyces* DNA associated with deletions (Ono *et al.*, 1982; Altenbüchner & Cullum, 1985; Hasegawa *et al.*, 1985; Dyson & Schrempf, 1987). Moreover, highly reiterated stretches have been detected after ethidium bromide treatments (Schrempf, 1982, 1983; Ono *et al.*, 1982; Hasegawa *et al.*, 1985), interspecific protoplast fusion (Robinson *et al.*, 1981), selection for overexpression of the spectinomycin-resistance gene (Hornemann *et al.*, 1987), protoplasting and regenerating (Fishman & Hershberger, 1983), or spontaneously (Altenbüchner & Cullum, 1984; Baltz & Stonesifer, 1985).

The work reported here concerns *Streptomyces ambofaciens* DSM 40697, a spiramycin producer. Preliminary observations showed that this strain exhibits a high frequency (0·9%) of spontaneous pigment-negative mutant colonies. Highly reiterated DNA sequences were detected and characterized in spontaneous mutant strains of *S. ambofaciens*. These investigations led to the identification of two DNA regions in the *S. ambofaciens* genome which are susceptible of a high degree of amplification.

Abbreviations: ADS, amplified DNA sequence(s); AUD, amplifiable unit of DNA; WT, wild-type.

METHODS

Strains. The wild-type (WT) *Streptomyces ambofaciens* DSM 40697 strain was used. Mutant strains isolated from it are described in Results.

Media and culture conditions. *Streptomyces* strains were grown at 37 °C on plates of Hickey Tresner medium (Hopwood *et al.*, 1982). Before DNA extraction, the *Streptomyces* cultures were grown aerobically at 37 °C in YEME liquid medium supplemented with glycine (0.25%) for 48 h (Hopwood *et al.*, 1982).

DNA extraction. The mycelium was harvested by centrifugation, resuspended in TE buffer (Tris/HCl 10 mM, EDTA 1 mM, pH 8.0) and mixed with lysozyme (Boehringer Mannheim) (2 mg ml⁻¹). Protoplasts were formed by incubation of the mixture at 30 °C for 30 min and lysed with sodium dodecyl sulphate (SDS) (1%) in the presence of proteinase K (50 µg ml⁻¹). DNA was purified by two phenol/chloroform extractions followed by one chloroform extraction. The aqueous phase was treated with ribonuclease A (Sigma) (5 mg ml⁻¹) for 1 h at 37 °C. DNA was precipitated with sodium acetate (0.3 M, pH 5.2) and 2-propanol at -20 °C. After centrifugation, the pellet was vacuum-dried and dissolved in TE buffer. DNA was purified by equilibrium density centrifugation in CsCl/ethidium bromide gradients, using a vTi 65.2 rotor (Beckman) at 65000 r.p.m. for 6 h (400000 g).

Restriction endonuclease digestion analysis. Restriction enzymes were purchased from Appligène or Boehringer Mannheim and used as recommended by the suppliers. DNA fragments were electrophoresed on 0.6% agarose gels according to Maniatis *et al.* (1982). Bacteriophage λ DNA digested by *Hind*III was used as size standard.

Isolation of DNA fragments. Restriction fragments to be used for the nick-translation step were purified from agarose gels by electro-elution followed by chromatography on commercially available columns (Schleicher and Schüll) or by the GeneClean process (Bio 101).

Nick-translation, prehybridization, hybridization and autoradiography. DNA fragments were ³²P-labelled by nick-translation (Rigby *et al.*, 1977) using [³²P]dCTP and a nick-translation kit (Amersham). For hybridization experiments, DNA fragments were electrophoresed on agarose gels, and blotted on Hybond-N membranes (Amersham) according to Southern (1975). DNA fragments were covalently linked to the membrane by exposure to UV light for 5 min.

Prehybridization and hybridization solutions consisted of formamide 50% (v/v), Ficoll 0.1%, bovine serum albumin 0.1%, polyvinylpyrrolidone 0.1%, denatured salmon sperm DNA 200 µg ml⁻¹, 6× SSC (1× SSC = 0.15 M-sodium chloride, 0.015 M-sodium citrate), 0.5% SDS. After blotting, the nylon membrane was incubated for 4 h at 42 °C in a sealed plastic bag containing the prehybridization mixture. The hybridization mixture, consisting of prehybridization solution containing ³²P-labelled probe (7 × 10⁷ d.p.m. µg⁻¹), was added and the bag was further incubated overnight at 42 °C. The nylon membrane was then removed, washed with 2× SSC, 0.1% SDS at room temperature for 1 h (one change of washing solution), then washed with 0.1× SSC, 0.1% SDS at 50 °C for 2 h (with three changes of washing solution) and finally rinsed twice with 0.1× SSC, 0.1% SDS and four times with 0.1× SSC. Autoradiographies were performed using Hyperfilm-MP (Amersham) with an intensifying screen. Films were exposed at room temperature for 6 h to 3 d.

RESULTS

Isolation of variant strains

Genetic instability is revealed by the occurrence of spontaneous pigment-negative mutant colonies at high frequency. With the WT *S. ambofaciens* DSM 40697, 0.9% of colonies were pigment-negative and 7.4% contained pigment-negative sectors. Mutant strain NSA101 was subcloned from a pigment-negative homogeneous clone (sector-free). Some pigment-negative colonies are affected by a reverse instability and give rise either to homogeneous WT colonies or to revertant sectors on mutant colonies. Mutant strain NSA103 was isolated from such a revertant sector.

A novel phenomenon arising from pigment-negative colonies and affecting numerous morphological characters, especially pigmentation, was the occurrence of progeny without any preponderant phenotype. The mutant strains exhibit highly variable pigmentation ranging from WT to pigment-negative phenotype. Mutant strains NSA6 and NSA102 were subcloned from such a progeny.

Characterization of amplified DNA

Strains NSA6, NSA101, NSA102 and NSA103 were characterized and compared to the WT by restriction analysis of total DNA (Fig. 1). This revealed intense bands which represent highly amplified DNA. The background pattern showed no detectable difference from the WT DNA, showing that the mutant strains are derived from *S. ambofaciens* DSM 40697.

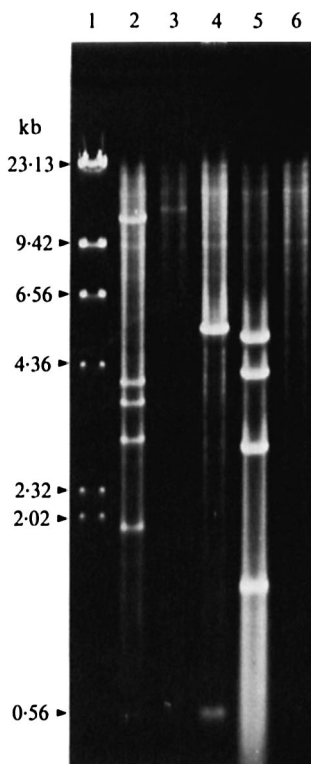


Fig. 1. Restriction patterns of total DNA from the WT and mutant strains of *S. ambofaciens*. DNA was digested with *Bam*HI and electrophoresed on 0.6% agarose gel at 1 V cm^{-1} for 18 h. Tracks: 1, 0.5 μg of λ DNA digested with *Hind*III as size marker; 2, 1.5 μg of NSA6 DNA; 3, 0.75 μg of NSA101 DNA; 4, 1 μg of NSA102 DNA; 5, 1 μg of NSA103 DNA; 6, 1 μg of WT DNA.

Undigested DNA of the mutant strains showed no detectable extrachromosomal bands on agarose gels (data not shown). In addition, no covalently closed circular DNA was observed in equilibrium density centrifugation on CsCl gradients, so the amplifications were not present in this form.

In each strain, the total size of the amplified DNA deduced by digestion with different enzymes was about the same. Hence the amplified fragments are contiguously organized in amplified DNA sequences (ADS). The sizes calculated from compilation of the single and double digestions, and the restriction maps of the four ADS, are shown in Fig. 2(a, b). ADS6, ADS101 and ADS102 show high similarities, while ADS103 is very different from the three others. All the known cleavage sites of ADS101 and ADS102 are recovered in ADS6. It was not possible to deduce from the restriction maps whether ADS101 and ADS102 overlapped. The restriction maps indicate that there are two families of ADS represented: the first family includes ADS6, ADS101 and ADS102, the second family is represented by ADS103.

Assuming a genome size of $4.9 \times 10^3 \text{ kb}$ (Genthner *et al.*, 1985), and according to the amount of DNA in the slots, the relative fluorescence of the intense bands in the agarose gel permitted an estimate of the proportion of amplified DNA in each mutant strain (NSA6, 20%; NSA101, 5–10%; NSA102, 20%; NSA103, 50%) and of the number of copies of the ADS (ADS6, 50 copies; ADS101, 20 copies; ADS102 and ADS103, 200 copies).

Hybridization between reiterated sequences

Strain NSA6, containing the largest ADS (24.8 kb) was chosen to provide the DNA probe. DNA of strain NSA6 was digested with *Xho*I plus *Pst*I to generate three predominant fragments

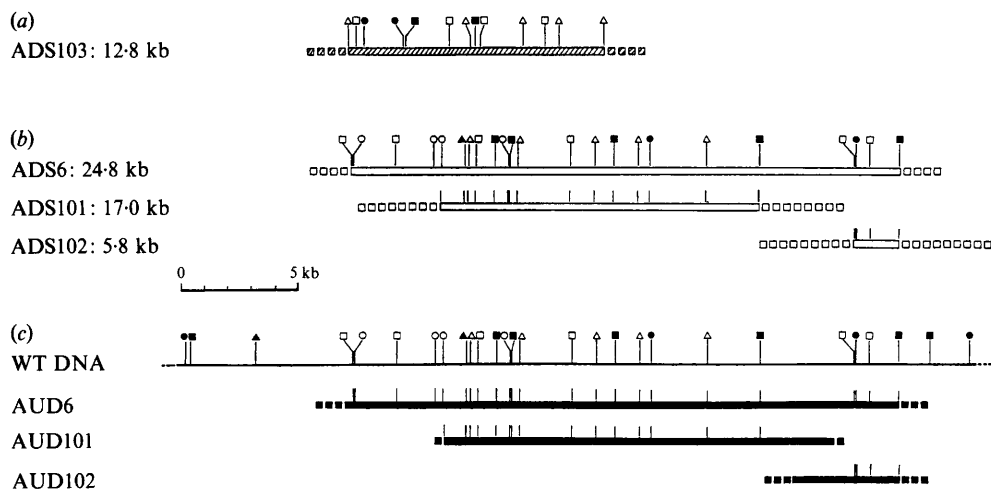


Fig. 2. Restriction maps of the two families of ADS in mutant strains of *S. ambifaciens*. (a) ADS in NSA103 ('ADS103 family'). (b) ADS in NSA6, NSA101, NSA102 ('ADS6 family'). (c) Amplifiable units of DNA (AUD) and identification of flanking sequences based on hybridization experiments. Symbols used for cleavage sites: □, *Bam*HI; ■, *Bcl*I; ○, *Bgl*II; ●, *Pst*I; △, *Pvu*II; ▲, *Xho*I. The dotted lines represent unknown areas.

(7.8, 8.4 and 8.6 kb). After electrophoresis, these fragments were eluted, purified and labelled by nick-translation. However, it was essential to control the presence of DNA of the same size which could comigrate with eluted DNA. These fragments cleaved again with *Bam*HI were therefore used as target in a hybridization test (Fig. 3a, track 2). The hybridization pattern (Fig. 3b, track 2) is characteristic of ADS6, including the faint bands generated by partial digestion. The background due to the presence of contaminating DNA allows an estimation of the quality of the probe: it was suitable to detect a 1.3 kb single homologous sequence in the WT DNA digested with *Bcl*I as shown in further results (Fig. 4, track 3). In the hybridization test performed on DNA of NSA6, all typical DNA stretches are observed (Fig. 3b, track 3).

Southern blots of NSA101, NSA102, NSA103 and WT DNA digested with *Pvu*II or *Bam*HI were hybridized with the ADS6 probe (Fig. 3b, tracks 4, 5, 6 and 7). All typical reiterated DNA stretches of ADS101 and ADS102 were detected as heavy bands (Fig. 3b, tracks 4 and 5). Under the same hybridization conditions, no intense signals were detected with *Bam*HI digests of NSA103 and WT DNA (Fig. 3b, tracks 6 and 7). Longer exposure of these latter autoradiographs revealed typical ADS6 sequences, with the exception that one fragment (2.9 kb) was absent and two additional fragments (13 and 17 kb) were found. This experiment shows that a DNA sequence homologous with ADS6 is present in DNA of both the WT and NSA103. This homologous DNA sequence corresponds to the amplifiable unit of DNA (AUD6). In addition, the two bands of 13 and 17 kb correspond to hybridization with flanking sequences of the AUD. The 2.9 kb fragment is generated by tandem reiteration of the AUD.

The fact that DNA of NSA103 and the WT shows the same hybridization pattern with the ADS6 probe suggests that the DNA of NSA103 can be considered as identical to the WT DNA with respect to ADS6.

Southern blots of NSA101 DNA digested with various restriction enzymes were probed with a *Bcl*I digest of ADS102 (5.8 kb fragment) (data not shown). The results confirmed an overlap between ADS101 and ADS102.

Restriction map of AUD region

Southern blots of various digests of WT DNA were hybridized with the ADS6 probe to allow the restriction mapping of AUD6 and its flanking DNA (Fig. 2c). These results proved that AUD6 is present as a single copy: most particularly, *Xho*I digestion generated only two flanking

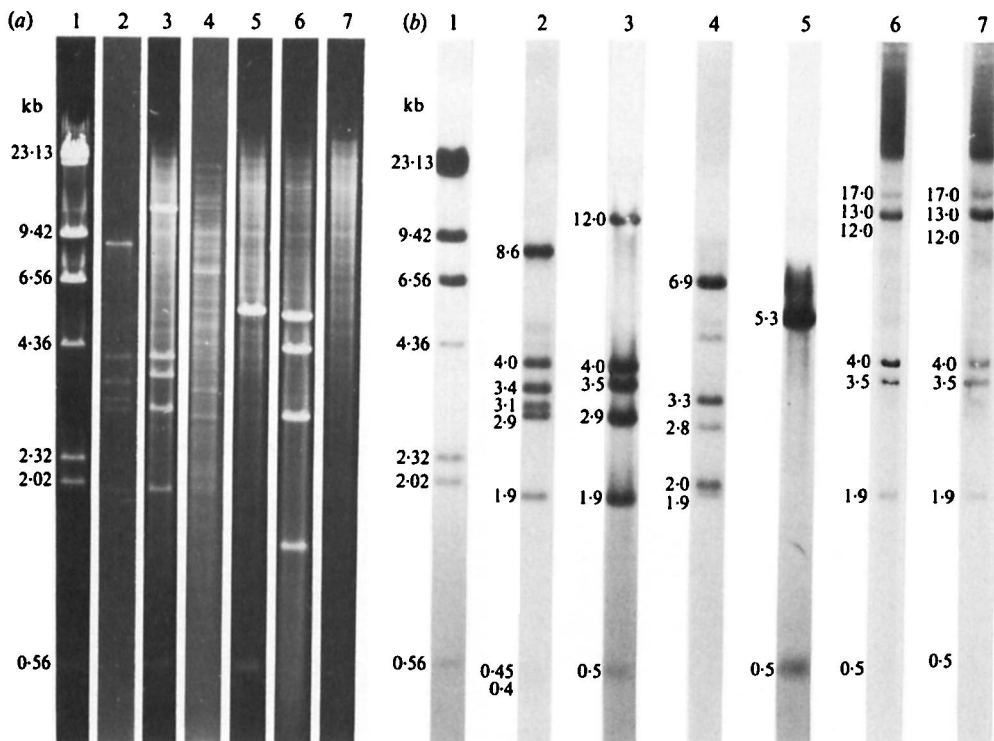


Fig. 3. Hybridization of ^{32}P -labelled ADS6 DNA with digests of DNA from mutant and WT strains. (a) Agarose gel separation of total DNA prepared from the WT and from mutant strains with amplified DNA. (b) Southern blot hybridization. Tracks: 1, *Hind*III-digested λ DNA; 2, eluted ADS6 DNA triple digested with *Xho*I/*Pst*I/*Bam*HI; 3, NSA6 total DNA digested with *Bam*HI; 4, NSA101 total DNA digested with *Pvu*II; 5, NSA102 total DNA digested with *Bam*HI; 6, NSA103 total DNA digested with *Bam*HI; 7, WT DNA digested with *Bam*HI. Restriction enzyme digestions, agarose gel electrophoresis, nick-translation and membrane hybridization were done as described in Methods (electrophoretic conditions: 1 V cm^{-1} for 18 h, 0.6% agarose). Autoradiograph exposure times: tracks 1, 3 and 5 for 4 h, tracks 2 and 4 for 7 h, tracks 6 and 7 for 60 h.

sequences (Fig. 4, track 7). This confirmed that the AUD is not duplicated in the WT genome.

Accordingly, two 'families' of AUD are characterized in the WT DNA: the 'AUD6 family', which includes AUD6, AUD101, AUD102, and the 'AUD103 family', represented by the ADS103.

DISCUSSION

This work reports the identification of at least two 'families' of amplifiable units of DNA in *S. ambofaciens* DSM 40697. Analogous results related to overlapping reiterated sequences were reported for the *Streptomyces glaucescens* genome after mutagenic treatments (Hasegawa *et al.*, 1985). However, possible rearrangements associated with DNA amplification have not yet been investigated in our *S. ambofaciens* mutant strains.

The accuracy of the AUD restriction map does not reveal any long direct repeats as in other *Streptomyces* species (Altenbüchner & Cullum, 1985; Fishman *et al.*, 1985; Hornemann *et al.*, 1987). A model based on homologous recombination between direct repeats of the AUD during the replication has been proposed for DNA amplification in *Streptomyces lividans* 66 (Young & Cullum, 1987). However, this model seems unlikely in *S. ambofaciens*, since although a duplicated, pre-amplified AUD was detected in the WT strain *S. lividans* 66 (Dyson & Schrepf, 1987), AUD6 is present as only a single copy in the WT *S. ambofaciens* genome.

The genomic location of the AUD and ADS is not yet known. A location as stable

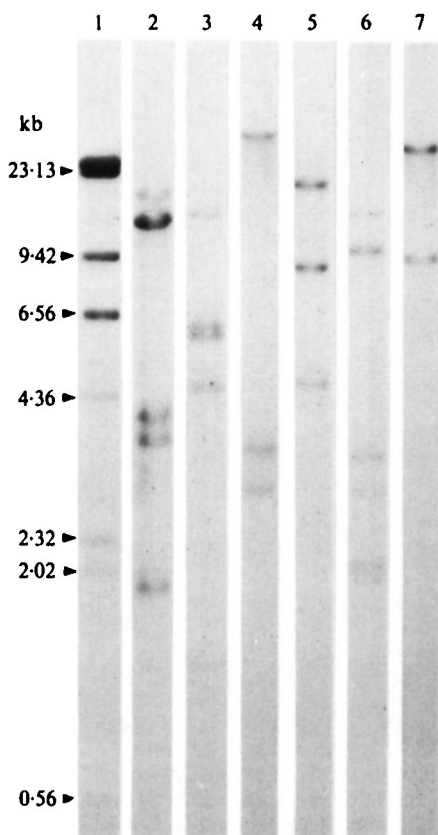


Fig. 4. Hybridization of ^{32}P -labelled ADS6 DNA with total WT DNA digested with a range of restriction enzymes. Tracks: 1, *Hind*III-digested λ DNA; 2, *Bam*HI; 3, *Bcl*I; 4, *Bgl*II; 5, *Pst*I; 6, *Pvu*II; 7, *Xho*I.

extrachromosomal DNA undetectable by classical methods remains possible. Stable amplified DNA in drug-resistant *Leishmania* has been reported as large extrachromosomal circles (Garvey & Santi, 1986), and giant plasmids (410–560 kb) have recently been characterized in *Streptomyces coelicolor* A3(2) (Kinashi *et al.*, 1987).

Additional information about the structure and location of the AUD and ADS are essential for an understanding of the phenomenon of amplification and its relationships with genetic instability.

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