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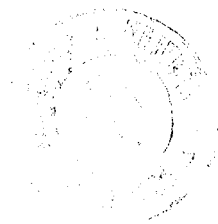

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en
Génétique Moléculaire

par

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HYPERVARIABILITE GENETIQUE CHEZ *STREPTOMYCES AMBOFACIENS* : PLASTICITE D'UN ARC GENOMIQUE.

Soutenue le 2 Juillet 1990 devant la commision d'examen

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SOMMAIRE

AVANT-PROPOS

Les résultats expérimentaux sont présentés sous forme d'un résumé. Les résultats obtenus au cours de cette thèse ont été exposés dans les publications et les propositions d'articles suivantes:

1. **Characterization of two families of spontaneously amplifiable units of DNA in *Streptomyces ambofaciens*.**
   Demuyter P., Leblond P., Decaris B. and J-M. Simonet.
   The Journal of General Microbiology (1988) **134**: 2001-2007.

2. **Genetic instability and hypervariability correlated with DNA amplification in *Streptomyces ambofaciens*.**
   Demuyter P., Leblond P., Moutier M., Simonet J-M. and B. Decaris.
   Abstr. 208th Meeting of the Genetical Society of Great Britain
   Heredity (1988) **61**:315.

3. **Hypervariability, a new phenomenon of genetic instability, related to DNA amplification in *Streptomyces ambofaciens*.**
   Leblond P., Demuyter P., Moutier L., Laakel M., Decaris B. and J-M. Simonet.
   Journal of Bacteriology (1989) **171**:419-423

4. **Genetic instability and hypervariability in *Streptomyces ambofaciens*: towards an understanding of a mechanism of genome plasticity.**
   Leblond P., Demuyter P., Simonet J-M. and B. Decaris.
   Molecular Microbiology (1990) in press.

5. **Genome plasticity associated with genetic instability and hypervariability in *Streptomyces ambofaciens*.**
   Simonet J-M., Decaris B., Leblond P. and P. Demuyter.
   Journal of Cellular Biochemistry (1990) Abstr. UCLA Symposium on Molecular and Cellular Biology, CC417, p. 129.

6. **Pulsed-field gel analysis of the genome of *Streptomyces ambofaciens* strains.**
   Leblond P., Francou F.X., Simonet J-M. and B. Decaris.
   Article soumis à *FEMS Microbiology Letters*.

7. **A chromosomal region is a hotspot for multiple rearrangements associated with genetic instability in *Streptomyces ambofaciens* DSM40697.**
   Demuyter P., Schneider D., Leblond P., Simonet J-M. and B. Decaris.
   Proposition d'article pour *Molecular and General Genetics*.

8. **Genetic instability and associated genomic plasticity in *Streptomyces ambofaciens*: PFGE evidences for large DNA alterations in a limited genomic region.**
   Leblond P., Demuyter P., Simonet J-M. et B. Decaris.
   Proposition d'article pour *Molecular and General Genetics*.

ABREVIATIONS ET DEFINITIONS

ADS, Amplified DNA Sequence
AUD, Amplifiable Unit of DNA
PFGE, Pulsed-Field Gel Electrophoresis
Microhomologie, homologie partielle de courte étendue.
Arc génomique, grande région du génome
RAL, Replication Activating Locus

INTRODUCTION

Les *Streptomyces*[1] constituent un modèle d'étude original des phénomènes d'instabilité génétique. En effet, ces phénomènes semblent ubiquitaires dans les nombreuses espèces de *Streptomyces*. L'instabilité se manifeste par l'apparition spontanée à haute fréquence (supérieure à 10^{-3}) de mutants à partir de la souche sauvage. De nombreux caractères phénotypiques, appartenant principalement au métabolisme secondaire et à la différentiation, sont affectés: formation du mycélium aérien, production de spores, résistance et/ou production d'antibiotiques, production d'enzymes extracellulaires dont la tyrosinase impliquée dans la pigmentation des colonies bactériennes de plusieurs espèces de *Streptomyces* (tableau 1). Un petit nombre seulement de caractères de prototrophie se sont révélés instables. Ces mutants apparaissent suivant deux processus différents: soit directement à partir de la souche sauvage sous forme d'un double mutant auxotrophe-déficient pour la formation de mycélium aérien (Redshaw *et al.*, 1979; Coyne *et al.*, 1984), soit à haute fréquence à partir de mutants sensibles à un antibiotique, produits par un premier événement d'instabilité (Altenbuchner et Cullum, 1984; Dyson et Schrempf, 1987).

De nombreux traitements comme l'exposition à un rayonnement ultraviolet, la culture en présence d'agents intercalants de l'ADN (bromure d'éthidium, acridine orange, acriflavine...), la conservation au froid, la culture à "haute température", les chocs nutritionnels, une protoplastisation suivie de régénération augmentent considérablement les fréquences spontanées de mutants (tableau 1). Certains de ces traitements sont classiquement utilisés dans des expériences de mutagénèse. Cependant, les conditions d'expérience utilisées sur les *Streptomyces* sont très éloignées de la mutagénèse classique. De plus, les traitements comme la conservation des spores au froid, la culture à "haute température" ou les chocs nutritionnels produisent des mutants à haute fréquence et ne sont pas considérés comme des agents mutagènes.

L'analyse moléculaire des phénomènes d'instabilité a révélé de fréquents remaniements génomiques associés aux phénotypes mutants (pour revue, Hütter et Eckhardt, 1988; Leblond *et al.*, 1990). Ces remaniements sont de deux natures: délétions et amplifications de séquences d'ADN (ADS)(tableau 2).

Lorsque le gène instable est connu, il s'avère compris dans une délétion de grande taille (souvent plusieurs dizaines de kilobases). C'est le cas pour le gène de la phosphotransférase conférant la résistance à la streptomycine chez *Streptomyces glaucescens* (Hintermann *et al.*, 1984 et 1985) ou encore le gène de la tyrosinase intervenant dans la secrétion de la mélanine chez *S. glaucescens* et *Streptomyces reticuli* (tableau 2)(Birch *et al.*, 1989; Schrempf, 1985). La taille de ces délétions a été estimée à environ 250 kb pour chaque caractère muté chez *Streptomyces lividans* et *Streptomyces coelicolor* A3(2) (Cullum *et al.*, 1988) et jusqu'à environ 800 kb chez *S. glaucescens* (Birch *et al.*, 1989).

En plus de ces délétions, de nombreux clones mutés contiennent des ADS à des degrés d'amplification divers (tableau 2). Les gènes mutés ont été, dans tous les cas étudiés, localisés dans la région adjacente au locus contenant les régions amplifiables: gène de l'arginosuccinate synthétase chez *S. lividans* (Betzler *et al.*, 1987), gènes de résistance et de production de la tylosine chez *Streptomyces fradiae* (pour revue, Baltz et Seno, 1988) ou encore gènes de la tyrosinase et de la phosphotransférase chez *S.*

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[1] *Les Streptomyces sont des bactéries à coloration gram+, filamenteuses, immobiles et sporulantes appartenant au groupe des Actinomycètes.*

Tableau 1: Caractères instables chez les *Streptomyces*.

Espèce de *Streptomyces*	Phénotypes	Fréquences		Références
		Spontanées	Induites	
S. acrimycini IPV1610	Cam^S	1%	U.V. 4%	Wright et Hopwood, 1977
	Ery^S	0,1-0,6%	U.V. 0,7-5,2%	Freeman et Hopwood, 1978
S. alboniger ATCC12461	Amy^-Arg^-	0,3%	AF. 2% B.E.T. 20%	Redshaw *et al.*,1979
S. bikiniensis ISP5235	Str^S	0,45-1,8%	U.V. 3,6-5,3%	Kirby et Lewis, 1981
S. bikiniensis ATCC11062	Mel^-	<0,5%	AF. <0,5% B.E.T. <0,5%	Shaw et Piwowarski, 1977
	$Str^{-(*)}$	nd	B.E.T. 2-4% AF 4-16%	
S. cattleya NRRL8057	Amy^- (Amy^-Arg^-: 56%, $Amy^-Arg^-Met^-$: 11%, Amy^-Met^-: 5%, Amy^-Aux: 11%)	0,5%	U.V. nd	Usdin *et al.*, 1985 Coyne *et al.*, 1984
S. coelicolor A3(2)	Cam^S	0,5%	U.V. >5%	Freeman *et al.*, 1977
S. glaucescens ETH22794	Str^S	0,3% 0,2-1,4%	U.V. 2,7-11% B.E.T. >90%	Freeman et Hopwood, 1978 Crameri *et al.*, 1983
	Mel^-	<1%	C.B.T. 13% B.E.T. >17%	Suter *et al.*, 1978
S. lividans 66 TK64	Cam^S	1%	nd	Altenbuchner et Cullum, 1984
S. lividans 66 1326	Cam^S Tet^S	2-5% 2-5%	5-10% 5-10%	Dyson et Schrempf, 1987
S. griseus CUB609	Str^S	0,2%	U.V. 1,8-10%	Freeman et Hopwood, 1978
S. griseus NCIB8506	Str^S	0,5-1,6%	U.V. 0,5-1,6%	Kirby et Lewis, 1981
S. scabies PA10	Amy^- (Amy^-Arg^-: 27%)	0,01%	AF. 6% B.E.T. 20%	Redshaw *et al.*, 1979
S. scabies A26	Mel^-	0,2%	AC.2-60%	Gregory et Huang, 1964
S. species M-506	Mel^-	5%	C.H.T.>60% AC. 20%	El-Kersh et Vezina, 1978
S. violaceus-ruber ATCC3355	Amy^- (Amy^-Arg^-: 39%)	$<10^{-3}$	AF. 2% B.E. 20%	Redshaw *et al.*, 1979

*: Les cas d'instabilité décrits affectant la production d'antibiotiques sont trés nombreux; pour revue Hütter et Eckhardt (1988).

Abréviations: AC., acridine orange; AF., acriflavine; Arg^-, déficience de la biosynthèse de l'arginine; Aux, cas d'auxotrophie complexe; Amy^-, déficience de la production de mycélium aérien; Cam^S, sensibilité au chloramphénicol; C.B.T., conservation des spores à basse température; B.E.T., bromure d'éthidium; Ery^S, sensibilité à l'érythromycine; C.H.T., croissance à haute température; Mel^-, déficience de la biosynthèse de la mélanine; Met^-, auxotrophie pour la méthionine; nd, non déterminé; Str^S, sensibilité à la streptomycine; Tet^S, sensibilité à la tétracycline; U.V., rayonnement ultraviolet.

Tableau 2: Amplifications d'ADN chez les *Streptomyces*.

Espèce de *Streptomyces*	Taille ADS (kb)	Phénotype mutant	Traitement	Délétion	Références
S. achromogenes subsp. *rubradiris*	8	Spc^{HR}	Spontané et sélection	détectée	Hornemann *et al.*, 1987 Hornemann *et al.*, 1989
S. ambofaciens DSM40697	5,2 to 105	Hypervariable	Spontané	détectée	Demuyter *et al.*, 1988 Leblond *et al.*, 1989
S. antibioticus	détectée	Mel^-	Spontané	*mel*	Schrempf, 1985
		Surproduction d'oléandomycine	Sélection	nd	Orlova et Danilenko, 1983
S. azureus	détectée	Mel^-	B.E.T./Spontané	*mel*	Schrempf, 1985
S. coelicolor A3(2)	30	Cam^{HR}	Spontané and sélection	nd	Flett et Cullum, 1987
S. fradiae	10.5	Spc^R Tyl^S	Spontané	nd	Fishman et Hershberger, 1983
		Tyl^S	Protoplastisation	nd	
S. glaucescens	2,9 à 35	Mel^- Str^S	B.E.T.	*melC/sph*	Hasegawa *et al.*, 1985 Ono *et al.*, 1982
S. griseus	6,7	Kan^{HR}	Protoplastisation and sélection	nd	Ishikawa *et al.*, 1988
S. jumonjinensis et *S. lipmanii*	6	Tun^R	Fusion interspécifique de protoplastes	nd	Robinson *et al.*, 1981
S. lividans 66	5,75	Cam^S Arg^-	Spontané	détectée	Altenbuchner et Cullum 1984 et 1985
	5,75 à 40	Cam^S Arg^-/Tet^S Ntr^-	U.V. Quinacrine	*argG*	Dyson et Schrempf, 1987
		Cam^S Arg^- Tet^S Ntr^-	B.E.T. Mitomycine C		
S. scabies	détectée	Mel^-	B.E.T./Spontané	*mel*	Schrempf, 1985
S. reticuli	20 à 50	Mel^-	B.E.T./Spontané	*mel*	Schrempf, 1985
S. rimosus	10,3	Kan^{HR}	Spontané and sélection	nd	Potekhin et Danilenko, 1985
S. tendae	37	Surproduction de l'inhibiteur de l'α-amylase	NTG/AC	nd	Koller, 1985

Abréviations identiques au tableau 1 sauf: ADS, Amplified DNA Sequence (séquence d'ADN amplifiée); *argG*, gène structural de l'arginosuccinate synthétase; Cam^{HR}, hyperrésistance au chloramphénicol; Kan^{HR}, hyperrésistance à la kanamycine; *mel*, gène structural de la tyrosinase; NTG, N-méthyl-N'-nitro-N-nitrosoguanidine; Ntr^-, déficience de l'assimilation de l'azote minéral; Spc^R, résistance à la spectinomycine; Spc^{HR}, hyperrésistance à la spectinomycine; *sph*, gène de l'hydroxystreptomycine phosphotransférase; Tun^R, résistance à la tunicamycine; Tyl^S, sensibilité à la tylosine.

glaucescens (Birch *et al.*, 1989). L'ADS peut représenter jusqu'à 50 % de l'ADN total de la bactérie. La pression de sélection conduisant à l'amplification reste inconnue, si toutefois elle existe. Cependant, la sélection pour la surproduction d'un antibiotique (Orlova et Danilenko, 1983), d'un inhibiteur d'enzyme (Koller, 1985) ou pour la résistance à un antibiotique (Hornemann *et al.*, 1987; Flett et Cullum, 1987) a permis la mise en évidence de l'amplification et de la surexpression de plusieurs gènes. L'analyse de la structure fine des séquences amplifiables (AUD) a révélé deux types de structures (en accord avec la nomenclature proposée par Hütter *et al.*, 1988): le type I est caractérisé par une grande hétérogénéité en taille des AUD provenant d'une grande région chromosomique unique. Le type II est caractérisé par une grande reproductibilité de l'amplification d'une AUD unique (Altenbuchner et Cullum, 1985; Fishman *et al.*, 1985; Hornemann *et al.*, 1987; Dyson et Schrempf, 1987).

La plasticité du génome des *Streptomyces* pourrait être le reflet des caractéristiques d'organisation et de la composition de leur génome.

Les estimations de la taille du génome sont contradictoires. En effet, suivant les techniques utilisées (chromatographie haute pression, cinétique de renaturation...), les valeurs oscillent entre 4,9 x 10^3 et 10,5 x 10^3 kb (Begnini *et al.*, 1975, Gladek et Zakrewska, 1984; Genthner *et al.*, 1985). Récemment, la technique d'électrophorèse en champs pulsés (PFGE) a fourni un nouveau moyen d'investigation de la taille du génome. Le chromosome de *S. coelicolor* A3(2) a été estimé à 5,8 x 10^3 kb (Hopwood *et al.*, 1990, Simonet *et al.*, 1990). La technique PFGE a permis d'estimer la taille du génome de quatre isolats de *Streptomyces ambofaciens* entre 6,5 x 10^3 kb et 8,2 x 10^3 kb suivant les souches (Leblond *et al.*, article soumis). Le génome est donc de 1,3 à 1,8 fois plus important que celui de *Escherichia coli* [estimé par la même technique à 4,6 x 10^3 kb (Smith *et al.*, 1987)].

L'organisation du chromosome bactérien semble elle aussi originale: la cartographie génétique montre l'existence de deux secteurs symétriques "silencieux" sur une carte génétique circulaire de *S. coelicolor* A3(2)(Chater et Hopwood, 1984). Ce fait était interprété comme un point chaud de recombinaison. Récemment, la cartographie physique du chromosome a confirmé que le génome était bien circulaire mais aussi qu'il comportait deux arcs chromosomiques quasi vide de marqueurs (Hopwood *et al.*, 1990).

De plus, l'ADN de *Streptomyces* montre un très fort pourcentage de bases G et C, 70 à 74 % suivant les espèces (pour revue, Hütter et Eckhardt, 1988). Enfin, la complexité du génome est caractérisée par la présence de 4 à 11 % de séquences d'ADN répétées (qui formeraient plusieurs classes en fonction du pourcentage en G+C)(Usdin *et al.*, 1984 et 1988) et 2 % d'ADN "foldback" (Antonov *et al.*, 1978).

De nombreux éléments génétiques extrachromosomiques ont été caractérisés: les plasmides circulaires (pour revue, Hopwood *et al.*, 1986) ou linéaires (pour revue, Kinashi et Shimaji, 1987) sont de tailles très variées: de 4 à 200 kb pour les plasmides circulaires caractérisés, jusqu'à 520 kb pour les plasmides linéaires (Kinashi *et al.*, 1987). Certains plasmides sont impliqués dans les phénomènes de fertilité et de létalité zygotique et sont susceptibles de porter des fonctions de régulation ou des gènes impliqués dans la production d'antibiotiques. Ce dernier cas a été démontré pour le plasmide SCP1 de *S. coelicolor* A3(2) et le gène structural de la méthylénomycine bien avant la caractérisation physique du plasmide (Wright et Hopwood, 1976). Plusieurs de ces éléments rentrent en interaction avec le chromosome par intégration-excision à des sites spécifiques. Chez *S. ambofaciens* ATCC23877 en particulier, pSAM2 (11,1

kb)(Pernodet *et al.*, 1981) code pour une intégrase similaire à celle des phages tempérés et est capable de s'intégrer dans le génome de nombreuses espèces de *Streptomyces* (Boccard *et al.*, 1989).

De même, de nombreux bactériophages ont été caractérisés (pour revue, Chater, 1986). Chez *S. ambofaciens* ATCC23877, pSAM1 (80 kb) tout d'abord décrit comme circulaire (Ikeda *et al.*, 1982; Pernodet *et al.*, 1984) a été décelé sous forme concatémérique linéaire (Leblond *et al.*, article soumis) simultanément à une activité lytique. Ces faits suggèrent que pSAM1 sous forme circulaire serait un actino-prophage comme dans le cas de ΦSF1 chez *S. fradiae* (Chung, 1982). Des séquences d'insertions et des éléments transposables ont été caractérisés chez les *Streptomyces* (pour revue Chater *et al.*, 1988) qui pourraient participer à la fluidité du génome soit par mutation insertionnelle ou encore en participant à la cointégration d'éléments extrachromosomiques de grande taille avec le chromosome. Ainsi, IS*446* serait impliqué dans l'intégration du plasmide SCP1 chez *S. coelicolor* A3(2) (Chater *et al.*, 1988).

Cette profusion d'éléments génétiques extrachromosomiques ainsi que les caractéristiques propres du chromosome font des *Streptomyces* un modèle d'étude attractif de la fluidité du génome associé ou non à l'interaction de molécules extrachromosomiques.

L'association étroite entre les phénomènes d'instabilité génétique et de plasticité du génome chez les *Streptomyces* pose le problème des relations de cause à effet pouvant exister entre ces phénomènes.

La haute mutabilité spontanée est-elle la conséquence directe des remaniements du génome? Dans ce cas, le problème pourrait trouver sa solution dans l'étude de la structure des régions impliquées dans la plasticité génomique (régions délétées et séquences amplifiables). Dans ce cas, les déterminants de la plasticité génomique seraient directement dépendants des caractéristiques de l'ADN et du génome des *Streptomyces*.

L'instabilité génétique et la plasticité génomique sont-elles les conséquences d'un événement mutationnel primaire source d'une cascade d'événements moléculaire? Dans ce cas, l'étude de la séquence des événements mutationnels, mutation/délétion/amplification pourrait permettre de cerner et d'identifier la mutation initiale.

RESULTATS

I. PHENOMENOLOGIE DE L'INSTABILITE GENETIQUE CHEZ *STREPTOMYCES AMBOFACIENS.*

Les résultats résumés dans ce chapitre ont été exposés dans les publications 1, 2 et 3.

A. - Instabilité génétique de base -

Chez *Streptomyces ambofaciens*, souche DSM40697, l'instabilité génétique est révélée par une mutabilité spontanée importante du caractère pigmentation: ainsi, la souche sauvage produit spontanément 17% de colonies soit entièrement dépigmentées (0,97 ± 0,17%; 27.786 colonies examinées), soit présentant au moins un secteur mutant (7,8 ± 1,4%) ou une papille mutante (7,9 ± 1,3%)(illustrations des différents phénotypes dans l'article 2 en annexe et figure 1, cadre A). Cette instabilité est observée à chaque sous-clonage d'une colonie de phénotype sauvage (figure 1, cadre B).

Ces fréquences sont le produit de l'analyse de 54 clones sauvages isolés et sous clonés indépendamment. La distribution des colonies dépigmentées dans la descendance des 54 clones est différente d'une distribution aléatoire. Cette hétérogénéité de la population testée peut être expliquée par un effet clonal de la mutation: des papilles mutantes indétectables par simple observation ont pu se développer à la surface des colonies sauvages entraînant l'apparition de clones dépigmentés dans la descendance. Plus la mutation intervient précocement dans le développement, plus le nombre de colonies mutantes sera important à l'étalement suivant. Les distributions des colonies portant soit des secteurs soit des papilles pourraient être en accord avec une distribution aléatoire.

Les différents aspects de ces mutations pourraient traduire le moment où interviendrait la mutation dans le cycle de différentiation de la bactérie. En effet, si la mutation intervient lors de la phase de croissance radiale, alors un secteur angulaire mutant peut apparaître. De même, si la mutation intervient au cours de la croissance aérienne ou de la sporulation alors une papille peut apparaître. Cette instabilité affecte également la sporulation, la production de mycélium aérien et la production d'antibiotiques.

En revanche, la fréquence de mutants d'auxotrophie est inférieure à 5×10^{-5}. En effet, seulement deux mutants d'auxotrophie (His^- et Arg^-) ont été détectés parmi 37.519 colonies testées (M. Laakel, 1986, DEA). L'instabilité génétique affecte donc préférentiellement des caractères du métabolisme secondaire et de la différentiation.

B. - Hypervariabilité -

L'étude de la stabilité des mutants dépigmentés a permis de caractériser un nouveau phénomène d'instabilité génétique que nous avons appelé hypervariabilité (figure 1, cadre C). Les colonies dépigmentées présentent un centre allant de totalement dépigmenté à légèrement pigmenté. Or, seuls 13% de ces colonies mutantes produisent des lignées stables mutantes de phénotype parental. La stabilité de ces souches a été vérifiée sur au moins 3 cycles de sporulation. Par contre, 87% produisent des lignées hypervariables, phénotypiquement hétérogènes, sans phénotype majoritaire. Le nombre

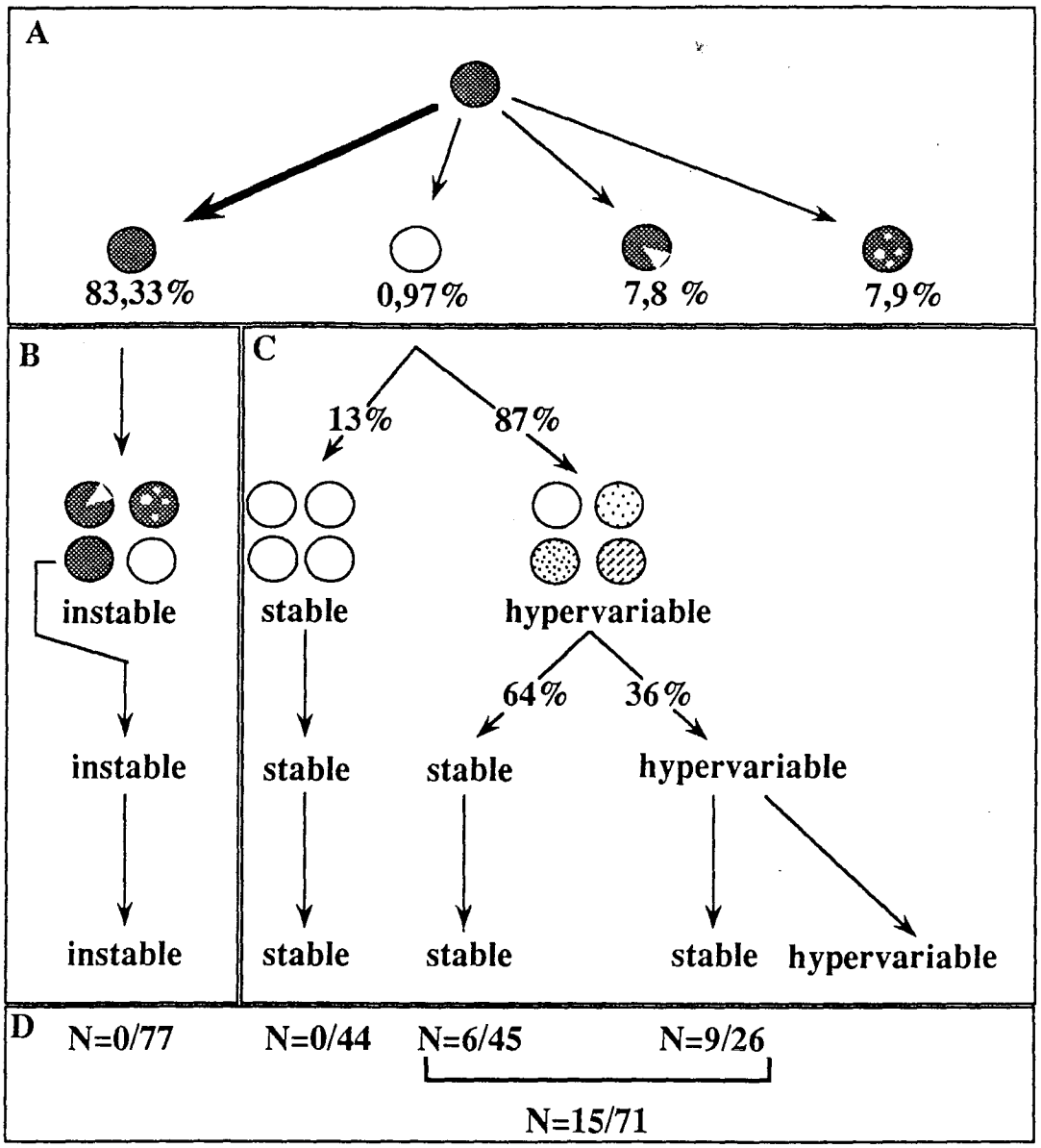

Figure 1: Diagramme de l'instabilité génétique chez *Streptomyces ambofaciens* DSM40697. (A) Fréquences des différents phénotypes mutants observés à partir de la souche sauvage. (B) Sous clonages successifs de colonies de type sauvage; (C) Sous clonages successifs des variants; (D) Analyse moléculaire des colonies isolées à partir des trois lignées WT, stables et hypervariables; N = nombre de clones contenant une ADS rapporté au nombre de clones analysés.

Colonie de phénotype sauvage,
Colonie mutante dépigmentée,
Colonie mutante à secteur dépigmenté,
Colonie mutante à papilles dépigmentées,
Colonies de phénotypes variés issues de l'hypervariabilité.

de phénotypes différents dans une même descendance hypervariable varie de 2 à 8 avec une moyenne de 4 ou 5. L'hypervariabilité se maintient dans la descendance de 36% des clones issus d'un premier événement d'hypervariabilité tandis que 64% donne une descendance mutante homogène de type stabilisé. Le phénomène d'hypervariabilité se maintenant dans une partie des descendances, le terme de lignée hypervariable se justifie. *A posteriori*, nous avons observé que les lignées mutantes phénotypiquement stables dérivaient des colonies totalement dépigmentées. Ainsi, trois types de lignées mutantes ont été isolés à partir de la souche sauvage: stable, stabilisé et hypervariable.

L'hypervariabilité est étroitement liée à l'instabilité génétique de base puisque l'hétérogénéité phénotypique n'est observée que dans la descendance de colonies de phénotype mutant. Aucun cas d'hétérogénéité n'a été observé dans la descendance de plus de 1.500 colonies de phénotype sauvage.

II. L'AMPLIFICATION D'ADN EST FREQUEMMENT ASSOCIEE A L'HYPERVARIABILITE.

Les résultats résumés dans ce chapitre ont été exposés dans la publication 2 et 3.

Lors d'études préliminaires du génome des lignées mutantes, nous avions détecté des séquences d'ADN hautement réitérées. D'autre part, l'amplification a souvent été associée à l'instabilité génétique (cf. tableau 2, introduction). Nous avons donc testé l'hypothèse d'une corrélation entre phénotype mutant et amplification d'ADN chez *S. ambofaciens*.

L'analyse de restriction de l'ADN total de clones provenant des trois types de lignées mutantes ainsi que de la souche sauvage a été réalisée (figure 1, cadre D). Ainsi, 77 clones sauvages (isolés et sous clonés de façon indépendante), de 44 mutants phénotypiquement stables (provenant de 44 événements d'instabilité différents) et de 71 colonies tirées au hasard dans 71 descendances hypervariables indépendantes ont été analysés. Seuls les clones phénotypiquement stables provenant de l'hypervariabilité ont pu être analysés. Cependant, parmi ceux-ci, 45 ont été isolés après un événement d'hypervariabilité unique et 26 après deux événements successifs.

Des ADS ont été détectées dans 21% (15/71) des clones issus de l'hypervariabilité alors qu'aucun cas d'amplification n'a été détecté ni dans les clones mutants phénotypiquement stables (0/44) ni dans les clones sauvages (0/77)(figure 1, cadre D). De plus, l'amplification semble plus fréquente dans les clones ayant subi plusieurs événements d'hypervariabilité (9/26 pour deux événements contre 6/45 pour un événement unique; ces fréquences sont statisquement différentes).

Ces résultats montrent une stricte corrélation entre les phénomènes d'hypervariabilité et d'amplification.

III. CARACTERISATION DE LOCI AMPLIFIABLES SUR LE GENOME SAUVAGE.

Les résultats résumés dans ce chapitre ont été exposés dans la publication 1.

Au total, 48 événements d'amplification indépendants, décelés dans l'ADN total de clones isolés dans 48 descendances hypervariables différentes ont été considérés. Ces ADS montrent une grande hétérogénéité en taille et en degré d'amplification. Ainsi, la taille des ADS varie de 5,2 à 105 kb et l'estimation du degré d'amplification peut atteindre jusqu'à 50% de l'ADN génomique total.

Dans un premier temps, quatre ADS dont la taille varie de 5,8 à 24,8 kb ont été cartographiées par analyse de restriction. La comparaison des cartes de restriction suggérant des chevauchements, des expériences d'hybridations croisées nous ont permis de caractériser deux familles d'AUD. Ainsi, l'AUD6 (24,8 kb) contient entièrement les AUD101 (17 kb) et AUD102 (5,8 kb), ces deux dernières se chevauchant sur plus d'un kilobase (figure 2.A). En revanche, aucune homologie n'a été détectée entre ces trois ADS et la quatrième, l'ADS103 (AUD103: 12,8 kb). Nous avons donc défini la notion de famille d'AUD. La famille AUD6 regroupe les ADS6, ADS101 et ADS102, la famille AUD103 contient l'ADS103.

La région de l'AUD6, semblant particulièrement instable, a été caractérisée dans le génome sauvage par hybridation (figure 2.B). De cette façon, nous avons montré que l'ADS était constituée de répétitions en tandem de l'AUD. L'AUD ne subirait pas de remaniement décelable avant amplification. Ceci a également été démontré dans 3 autres cas (AUD101, AUD102, AUD55). La réitération de l'AUD6 crée un nouveau fragment de restriction pour une enzyme donnée qui est homologue des deux séquences flanquantes de l'AUD6 dans le génome sauvage. Le fragment de réitération *Bam*HI de 2,9 kb a été cloné et constitue la sonde S1 (figure 2.B).

De plus, ces expériences de cartographie de l'ADN sauvage nous ont permis de montrer que l'AUD est présente sous forme simple copie. D'autre part, il n'existe pas de longues séquences répétées aux extrémités de l'AUD, ce qui confirmait les données obtenues par cartographie des ADS. Cependant, la sonde S1 révèle une séquence homologue d'environ 1 kb à l'intérieur de l'AUD; cette séquence est en fait homologue de la partie droite de l'AUD (figure 2.B).

Les 48 ADS (dont les ADS6, ADS101, ADS102 et ADS103) ont été hybridées avec la sonde S1 de façon à détecter les ADS qui appartiendraient à la famille AUD6. Dix d'entre elles dont la taille varie entre 5,8 et 35 kb présentent une homologie de séquence avec l'AUD6. Toutes les autres souches amplifiées présentent un profil WT en ce qui concerne la région AUD6. Les profils de restriction de 24 ADS (dont la taille varie entre 10 et 67 kb) n'appartenant pas à la famille AUD6 révélent de grandes similarités et ont donc été regroupées. Cette répartition a été confirmée pour 17 d'entre elles par hybridation avec une sonde constituée d'un fragment *Bam*HI (S120) appartenant à l'AUD120. L'ensemble de ces ADS a été regroupé dans la famille de l'AUD90. Les 13 ADS restantes (taille variant de 5,2 à 105 kb) dont l'ADS103 n'ont pu être localisées dans l'une ou l'autre des familles précitées. Notamment, l'AUD55 (ADS55, 21 kb) a été cartographiée suivant le même procédé que l'AUD6 et ne révèle aucune homologie avec les deux régions amplifiables déjà définies.

En conclusion, 2 régions distinctes ont été caractérisées qui sont le siège de 73% des événements d'amplification.

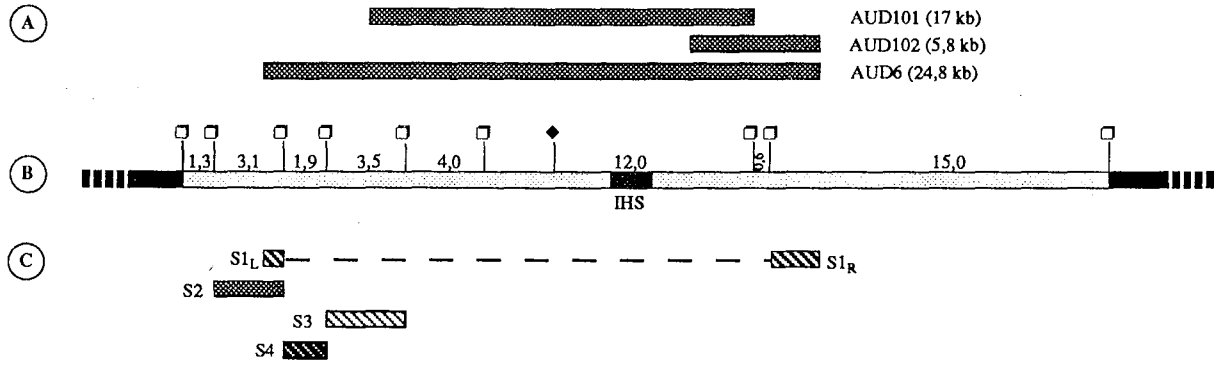

Figure 2: Locus AUD6.
(A) Localisation relative des ADS6, ADS101 et ADS102 dans la région de l'AUD6. (B) Carte de restriction de la région de l'AUD6 dans le génome sauvage. Seuls les sites *Bam*HI (❐) et *Ase*I (◆) ont été figurés. La taille des fragments est donnée en kilobases, la séquence homologue (IHS) à une partie de la séquence flanquante droite de l'AUD6 (S1R) est localisée par un cadre hachuré.
(C) Fragments de restriction *Bam*HI utilisés comme sondes dans les expériences d'hybridation, S1 (S1L+S1R, séquence créée par la réitération de l'AUD) et S2 sont des fragments clonés, S3 et S4 sont préparés directement à partir de l'ADN total de la souche amplifiée NSA6.

IV. LOCALISATION DES LOCI AMPLIFIABLES SUR LE GENOME SAUVAGE.

Les résultats résumés dans ce chapitre ont été exposés dans les publications 5, 6 et 8.

La technique de PFGE a été utilisée afin de localiser sur le chromosome les deux loci amplifiables. Cela a nécessité au préalable l'adaptation de la technique aux caractéristiques de *Streptomyces*. L'utilisation de cette technique a permis la caractérisation du génome sauvage par l'analyse de son contenu extrachromosomique et des profils de restriction produits par les enzymes à sites de coupure rares.

A. - Etude du génome sauvage de *S. ambofaciens* par électrophorèse en champs pulsés (PFGE) -

La technique de préparation d'un ADN total intact (de très haut poids moléculaire) a été développée sur *S. ambofaciens* et a permis de caractériser le génome par des profils de restriction *Ase*I et *Dra*I. La souche sauvage présente de façon reproductible le même profil caractéristique (12 clones indépendants analysés). Ce sont deux enzymes à sites de coupure rares compte tenu de leur séquence de reconnaissance uniquement composée de base A et T et du pourcentage élevé en base G et C (73%) du génome de *S. ambofaciens*. L'analyse du contenu extrachromosomique ne révéla aucune molécule libre dans la souche DSM40697.

Trois autres isolats géographiques de *S. ambofaciens* (ATCC15154, ETH9427 et ETH11317) ont été caractérisés par la même technique. La souche ATCC15154 contient une molécule linéaire de 80 kb qui présente une homologie avec le plasmide circulaire pSAM1. Cette molécule a été également détectée sous forme concatémérique linéaire. Cette molécule pourrait correspondre au génome du phage détecté dans cette souche. La réplication de cette molécule serait thermo-sensible. De même dans les souches ETH, une série de molécules compatible avec la concaténérisation d'un monomère de 45 kb a été détectée. Cependant, cette molécule ne présente pas d'homologie avec pSAM1.

Les profils de restriction par *Ase*I et *Dra*I de ces quatre isolats ont été comparés: la souche DSM40697 présente une grande similarité de profil avec les souches ETH (qui partagent le même profil avec les deux enzymes), mais diffère totalement de la souche ATCC15154.

Cette technique a permis d'estimer la taille totale de l'ADN génomique en additionnant la taille des fragments de restriction estimée par rapport à des marqueurs de taille de haut poids moléculaire: 8.000 kb pour la souche DSM40697, 8.200 kb pour les ETH et 6.500 pour la souche ATCC15154.

Le profil *Ase*I de la souche DSM40697 est caractérisé par 12 fragments de restriction dont la taille varie entre 75 et 1.850 kb; *Dra*I génère 10 fragments de 210 à 2.500 kb. Plusieurs fragments pourraient être dupliqués: les fragments *Ase*I de 1.050, 270 et 190 kb; le fragment *Dra*I de 310 kb.

B. - Localisation des loci amplifiables -

Les sondes d'ADN correspondant à 3 AUD ont été utilisées dans des expériences d'hybridation sur des fragments de restriction *Ase*I de la souche sauvage: la sonde S1 correspond aux extrémités clonées de l'ADS6 (figure 2.C); la sonde S120 correspond à un fragment amplifié commun à de nombreuses ADS de la famille AUD90, la sonde S55 est constituée d'un fragment *Bam*HI de 2,0 kb cloné correspondant à un fragment d'une AUD n'appartenant pas à ces deux familles d'AUD.

Les résultats ont permis de montrer que l'AUD6 chevauche deux fragments de restriction *Ase*I de 75 et 850 kb; la présence de ce site à l'intérieur de l'AUD a été confirmée sur la souche amplifiée par analyse de restriction et par hybridation sur l'ADN sauvage non amplifié. D'autre part, la sonde S120 comme la sonde S55 révèlent le même fragment *Ase*I de 850 kb. L'absence de site *Ase*I dans ces AUD a été confirmée par analyse classique. Les trois loci amplifiables sont localisés sur un arc chromosomique représentant au moins 10% du génome.

V. L'INSTABILITE GENETIQUE EST STRICTEMENT CORRELE A LA PLASTICITE DU GENOME.

Les résultats résumés dans ce chapitre ont été exposés dans les publications 5, 7 et 8.

A. - L'AUD6 subit des délétions fréquentes dans les processus d'instabilité et d'amplification -

1. - Polarité des délétions de l'AUD dans les mutants non amplifiés -

Un échantillonage des souches mutantes phénotypiquement stables et de souches issues de l'hypervariabilité provenant d'événements d'instabilité génétique indépendants a été analysé par hybridation pour connaître la structure de la région de l'AUD6 dans leur génome.

67 souches mutantes non amplifiées ont ainsi été analysées dont 30 phénotypiquement stables et 37 issues de l'hypervariabilité. 27/67 présentent un profil d'hybridation différent de la souche sauvage, à savoir différent du triple signal correspondant aux deux séquences flanquantes de l'AUD6 (3,1 et 15 kb avec *Bam*HI) et à la séquence homologue interne à l'AUD (compris dans la fragment de 12 kb *Bam*HI)(figure 2.B). Ces modifications correspondent à une délétion partielle ou totale de l'AUD6. Les différents types de délétions ont été appelés $\Delta 1$ à $\Delta 6$ (figure 3). Ces remaniements affectent indifféremment les souches phénotypiquement stables (16/30) et les souches issues de l'hypervariabilité (11/37).

Ces délétions révèlent une polarité de l'événement dans la région de l'AUD6. Ainsi, l'AUD6 serait dans 23% des cas la borne droite (figure 3) de délétion.

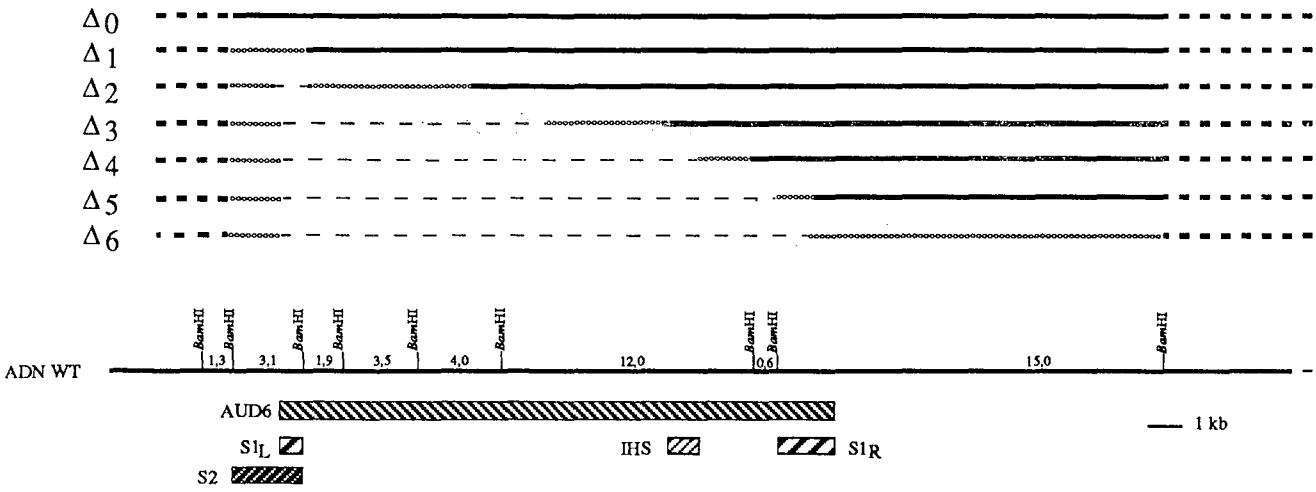

Figure 3: Délétions partielles ou totales dans la région de l'AUD6 dans les souches mutantes non amplifiées de *S. ambofaciens*.

- AUD6,
- Sonde S1,
- Sonde S2,
- Séquence homologue à $S1_R$,
- Séquences délétées (démonstration par hybridation),
- Séquences supposées délétées,
- Séquences inconnues,
- Séquences non délétées,
- Δ_0 Région AUD6 non délétée,
- Δ_x Délétion partielle ou totale de la région de l'AUD6.

2. - Les souches phénotypiquement stables sont le siège d'une hypervariabilité génomique dans la région de l'AUD6 -

Deux colonies appartenant à une lignée stable de phénotype dépigmenté issues de l'instabilité génétique de base ont été sous-clonées. Les descendants présentent une hétérogénéité génotypique dans la région de l'AUD6.

3. - Des délétions sont associées à l'amplification dans la région de l'AUD6 -

Les amplifications ADS6 (AUD6: 24,8 kb), ADS101 (AUD101: 17 kb) et ADS102 (AUD102: 5,8 kb) ont été analysées pour rechercher d'éventuels événements de délétion associée au phénomène d'amplification (figure 2.A). Pour cela, plusieurs sondes correspondant à différentes régions de l'AUD6 ont été utilisées (figure 2.C). Dans les ADS, la séquence S2 est délétée. Dans les ADS6 et ADS101, les sondes permettent de détecter des fragments de restriction créés par un événement de délétion qui pénètre dans au moins la première copie de l'AUD dans l'ADS. Dans la souche NSA102, une délétion a été décelée dans la proximité de l'ADS dont l'extrémité n'a pu être précisément localisée.

L'amplification dans la région de l'AUD6 est donc étroitement corrélée à une délétion localisée du même côté de l'AUD que dans les clones non amplifiés ce qui renforce la notion de polarité des événements. Ces résultats indiquent également qu'une étape de duplication au moins de l'AUD doit intervenir avant la délétion.

En revanche, 29/29 clones contenant une ADS n'appartenant pas à la famille AUD6 présentent un profil d'hybridation sauvage pour S1. L'amplification dans une région distincte n'implique donc pas de remaniement de l'AUD6. Cette fréquence est significativement différente de la fréquence de remaniement dans les clones non amplifiés.

4. - Instabilité des amplifications: cas de l'ADS6 -

Dans le cas de l'ADS6, la stabilité de l'amplification a été étudiée sur plusieurs cycles de sporulation: dès le second cycle de sporulation après la détection de l'ADS, un certain nombre de clones (en moyenne 35%) ne présentent plus l'ADS mais des délétions de type Δ (partielles ou totales de l'AUD6) analogues à celles décelées dans les clones non amplifiés. Ce phénomène est confirmé dans la descendance des clones restant amplifiés après le premier cycle de sporulation. Ces résultats révèlent l'instabilité des amplifications, et en particulier de l'ADS6.

B. - Mise en évidence de grandes délétions associées à l'instabilité -

Un échantillonage de souches issues des phénomènes d'instabilité a été analysé par la technique PFGE. Pour cela, la technique a été adaptée aux souches mutantes en faisant varier les conditions de lyse. Chaque souche a été caractérisée par un profil de restriction *Ase*I et comparée à la souche sauvage de référence.

Les profils obtenus par l'analyse de 14 souches mutantes stables et 12 issues de l'hypervariabilité ont été analysées. Toutes présentent un profil différent de la souche de référence: certains grands fragments sont systématiquement absents, d'autres disparaissent à des fréquences variées. Certaines souches présentent des nouveaux fragments de restriction. Les deux fragments de 850 kb et 75 kb qui contiennent les loci amplifiables sont perdus respectivement dans 92% et 45% des cas. Il n'y a pas de différence significative entre les deux populations et le tableau 3 représente l'ensemble des résultats.

Afin de connaître la nature de ces remaniements, des expériences d'hybridation ont été réalisées à l'aide des sondes spécifiques des AUD6 et AUD90 (S2 et S120). Dans tous les clones testés, la sonde S120 ne révèle aucun fragment de restriction; la délétion de cette séquence a été confirmée sur plusieurs mutants par hybridation sur des profils *Hin*dIII en PFGE et *Bam*HI en électrophorèse classique. Dans les mutants de type Δ0 (figure 3), S2 hybride sur des fragments de restriction *Ase*I de taille variable. Les mutants de type Δ1 à Δ6 (figure 3) n'hybrident pas avec S2 ce qui confirme les résultats acquis par analyse classique. Ces résultats sont compatibles avec l'existence de grandes délétions. La taille de ces délétions peut être estimée lorsqu'un fragment nouveau est détecté avec S2. En outre, dans certains cas, l'apparition de plusieurs nouveaux fragments est interprétée comme l'intervention de plusieurs délétions.

Si l'on considère que le remaniement observé dans les souches mutantes est la résultante d'un événement moléculaire unique, il est possible de dresser une carte spéculative des fragments *Ase*I (figure 4.A). La figure 4 (parties B et C) représente les interprétations en termes de délétions des remaniements détectés dans les souches stables et hypervariables. Ainsi, comme dans le cas de l'analyse classique de l'instabilité génomique de la région de l'AUD6, une polarité de l'événement est observée: par exemple, le fragment de 550 kb n'est jamais absent si celui de 950 kb n'est l'est pas.

La taille des délétions varie entre 250 et plus de 2.000 kb, c'est à dire affectant jusqu'à 25% du génome. Ces délétions sont strictement corrélées à l'instabilité génétique de base et à l'hypervariabilité.

Quatre souches amplifiées ont été analysées en PFGE. Deux présentent une amplification dans la région de l'AUD90 (ADS90, 55 kb et ADS120, 15 kb); l'AUD90 est chevauchante sur toute l'AUD120. Deux présentent l'AUD6 à des degrés d'amplification différents (ADS6 et ADS1635 provenant de la même descendance).

Ces quatre souches présentent un profil de restriction *Ase*I modifié par rapport à la souche sauvage. La région affectée par les délétions est la même que dans les mutants non amplifiés; le nombre de fragments absents traduisant la taille de la délétion varie suivant les souches. Par contre, les deux souches présentant l'ADS6 présentent les mêmes remaniements. La figure 4.D schématise ces interprétations.

Aucune bande additionnelle n'est détectée pour les souches amplifiées dans la région de l'AUD90. Pour les souches amplifiées dans la région de l'AUD6, la même bande de 25 kb est observée plus intense dans l'ADS6 que dans l'ADS1635. Ces résultats sont cohérents avec la localisation respective des sites *Ase*I dans ces AUD et avec la réitération en tandem de l'AUD.

Profil PFGE *Ase*I WT (kb)	Fréquence de perte des fragments
kb	
1850	0%
1050	0%
950	23%
850	92%
550	8%
450	100%
270	0%
190	0%
75	45%

Tableau 3: Fréquence de perte des fragments *Ase*I dans les souches mutantes de *S. ambofaciens*. Les fragments de 1.050, 270 et 190 kb présentent une intensité double et sont représentés en double épaisseur.

C - Structure et localisation de l'ADS -

Pour les ASD6 et ADS1635, le fragment additionnel de 25 kb correspond effectivement à l'ADS6; la séquence flanquante gauche contenue dans le fragment de 850 kb est délétée alors que la séquence droite est intacte.

Pour les ADS120 et ADS90, l'amplification est détectée (avec S120) comme un fragment de haut poids moléculaire qui comigre avec le fragment de 1.850 kb. La sonde S2, qui est homologue avec la séquence flanquante de droite de l'AUD90 (fragment *Ase*I de 850 kb), révèle le même fragment de restriction. L'ADS est donc présente sous forme d'une répétition en tandem au locus de l'AUD sauvage. Aucune sonde n'est disponible pour détecter la séquence flanquante de gauche créée par la délétion associée.

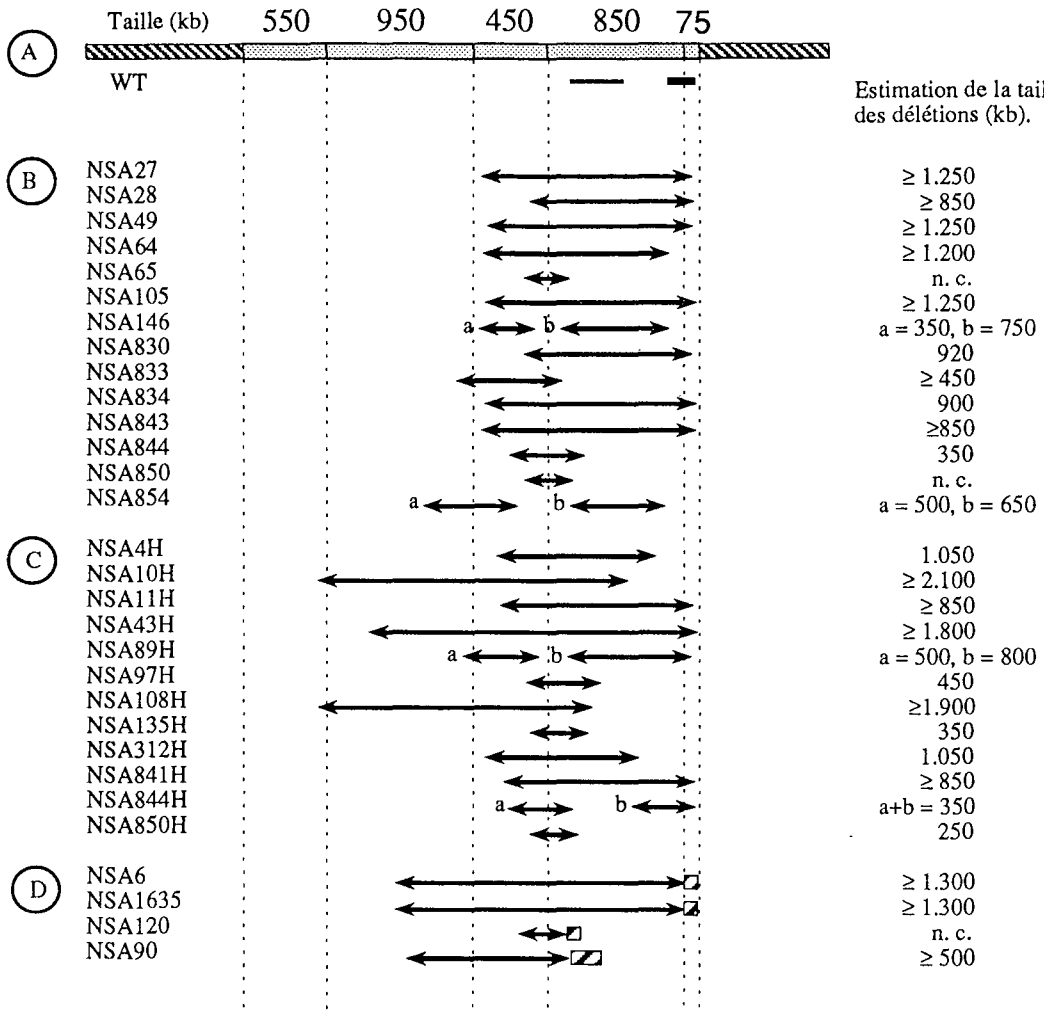

Figure 4: (**A**) Carte de restriction spéculative des fragments *Ase*I de la région génomique instable dans la souche sauvage. (**B**) Délétions dans les souches mutantes stables. (**C**) Délétions dans les souches non amplifiées issues de l'hypervariabilité. (**D**) Délétions dans les souches amplifiées.

Régions inconnues du chromosome,
Région instable,
Famille AUD6,
Famille AUD90,
Unité amplifiée,
Estimation de la taille des délétions par hybridation avec S2 ou par analyse de restriction; #, estimation relativement précise; ≥, estimation minimale; n. c., estimation non calculable par absence de fragment additionnel.

DISCUSSION

Les thèmes abordés dans cette discussion ont été en partie évoqués dans la revue publiée dans Molecular Microbiology (publication 4).

I. - NOTION DE REGIONS AMPLIFIABLES -

La cartographie de restriction et les expériences d'hybridations croisées ont permis de regrouper ces séquences amplifiées en familles. Deux grandes familles ont ainsi été caractérisées: la famille de l'AUD90 [dont dérive l'ADS90 (55 kb)] et qui regroupe plus de 50% des ADS détectées; la famille de l'AUD6 [dont dérive l'ADS6 (24,8 kb)] qui regroupe 23 % des ADS; enfin, 27% des ADS n'ont pu être affectées dans ces familles mais cependant ne semblent pas former de groupe. Deux d'entre elles, ADS55 (21 kb) et ADS430 (40 kb), présentent une homologie de séquence. Bien que les 2 familles regroupent plus de 73% des ADS, il n'est pas exclu que les 27% restants soient dispersés sur le génome ou sur un arc génomique.

Des sondes spécifiques utilisées sur des profils de restriction en PFGE de la souche sauvage ont permis de localiser ces familles les unes par rapport aux autres sur le chromosome sauvage. Ainsi, l'AUD6 chevauche deux fragments *Ase*I de 75 et 850 kb; les familles de l'AUD90 et l'ADS55 sont localisées sur ce dernier fragment de restriction. Les régions amplifiables à l'origine de plus de 75% des ADS sont donc situées dans le même arc chromosomique.

Une localisation plus précise de l'AUD90 sur le fragment *Ase*I de 850 kb est possible. En effet, plusieurs clones mutants sont affectés par des délétions dont la taille, relativement petite, a pu être estimée aux environs de 250 à 450 kb (NSA844, NSA97H, NSA850H, voir figure 4 résultats chapitre IV). Ces délétions chevauchent les fragments de 450 et 850 kb. Par ailleurs, la région de l'AUD90 est vraisemblablement délétée dans ces clones. Ces faits suggèrent que l'AUD90 serait localisée à l'extrémité gauche (figure 4) du fragment de 850 kb (dans les 400 kb flanquants). Les régions amplifiables AUD6 et AUD90 seraient donc séparées par plus de 400 kb.

Chez les autres *Streptomyces*, deux types principaux de structure de régions amplifiables ont été décrites: le type I et le type II (en accord avec la nomenclature proposée par Hütter *et al.*, 1988).

Le plus fréquemment décrit est le type II qui est caractérisé par une structure symétrique comprenant deux séquences identiques de 4 à 5 kb flanquées de trois répétitions directes de 0,7 à 1,7 kb. Cette structure a été décrite pour la première fois chez *S. lividans* 66 TK64 par Altenbuchner et Cullum (1985). La forme dupliquée est interprétée comme le résultat d'un crossing over inégal survenu lors du passage de la fourche de réplication entre les deux brins d'ADN néo-répliqués dans une souche contenant initialement une seule copie de l'AUD. Dyson et Schrempf (1987) proposèrent que cette structure dupliquée serait l'étape initiale du processus d'amplification. Cette hypothèse est appuyée par la description d'amplifications à faible nombre de copies détectées dans une souche de *S. lividans* 66 (TK23) ne présentant qu'un seul exemplaire de l'AUD. Cependant, plusieurs autres AUD de type II avec une structure non dupliquée ont été décrites chez *S. fradiae* (Fishman et Hershberger, 1985) et *Streptomyces achromogenes* subsp. *rubradiris* (Hornemann *et al.*, 1987) et ont été détectées amplifiées.

Le type I a été principalement décrit chez *S. glaucescens* (Hasegawa *et al.*, 1985; Häusler *et al.*, 1989) bien qu'observé aussi chez *S. reticuli* (Schrempf, 1983), *Streptomyces scabies* (Schrempf, 1985) et *S. coelicolor* (Flett *et al.*, 1987). Ce type d'amplification est caractérisé par la non reproductibilité de l'amplification des séquences. Ces AUD ne présentent pas de structure commune et notamment pas de séquences répétées de grande taille aux extrémités.

De plus, des ADS de tailles variées et à faible nombre de copies ont été détectées chez un *Streptomyces* où ont été caractérisées les AUD de type II; ces ADS pourraient correspondre à l'amplification de structures de type I moins favorablement amplifiées et donc décelées plus rarement (Dyson et Schrempf, 1987).

Chez *S. glaucescens*, l'analyse d'une banque cosmidique du génome sauvage a permis de montrer que toutes les ADS détectées sauf une (11 ADS cartographiées) provenaient d'un locus unique couvrant environ 100 kb. De plus, le séquençage des extrémités de quatre ADS a révélé, pour trois d'entre elles, des courtes répétitions imparfaites de 16 à 27 paires de base, appelées microhomologies (Häusler *et al.*, 1989). Ces répétitions sont spécifiques de chaque ADS considérée.

Chez *S. ambofaciens*, les séquences d'ADN amplifiées montrent une grande hétérogénéité en taille, de 5,2 à 105 kb, et en degré d'amplification (l'estimation atteint 50% de l'ADN génomique total dans certains clones). Malgré cette hétérogénéité, certaines ADS sont retrouvées plusieurs fois dans des événements d'amplification indépendants sur la base des profils de restriction: les ADS 102 (5,8 kb), ADS34 (67 kb), ADS239 (44 kb) et ADS17 (5,2 kb) sont retrouvées 2 fois; l'ADS24 est retrouvées 3 fois; enfin, les ADS 20 (18 kb) et ADS100 (22 kb) sont retrouvées 4 fois. La région amplifiable est composée de plusieurs loci amplifiables (dont au moins deux sont à l'origine de plus de 75 % des ADS détectées); l'ensemble de cette région couvre de l'ordre de 600 kb. Les ADS sont très hétérogénes à partir de chaque locus amplifiable. De plus, l'analyse de restriction des AUD ne révéle aucune longue répétition à leurs extrémités (5 cas étudiés). Cette situation répond donc à la structure de type I.

Cependant, la détection de loci amplifiables distincts, regroupant 50% et 25% des ADS et séparés par plus de 400 kb, suggère l'existence de signaux définissant chaque locus. Cette hypothèse est renforcée par l'existence d'une structure spécifique de ces régions. En effet, au cours de l'étude de la structure de l'AUD6, nous avons décelé une séquence interne à l'AUD homologue (IHS, figure 2) à l'une des extrémités. D'autre part, un fragment de restriction *Bam*HI de 1,9 kb contenu dans l'ADS6 révéle la présence de séquences homologues dispersées dans l'ADS6 (D. Schneider, com. pers.). Une situation analogue a également été révélée dans l'ADN de la souche NSA90 (résultats non publiés). En outre, ces séquences ne montrent pas d'homologie entre elles. D'autre part, nous ne pouvons pas exclure la possibilité de microhomologies aux extrémités des ADS comme chez *S. glaucescens* malgré l'absence d'homologie décelable par hybridation entre $S1_R$ et $S1_L$ chez *S. ambofaciens* (résultats figure 2).

Ces homologies qui sont spécifiques de chaque locus amplifiable pourraient définir des signaux et donc participer au processus d'amplification.

Nous sommes donc en présence d'une situation intermédiaire entre type I et type II où des loci distincts du génome sont susceptibles d'être amplifiés (régions amplifiables de

type I), chaque locus amplifiable pouvant être caractérisé par la répétition d'une séquence spécifique. Ces loci seraient eux mêmes contenus dans un arc génomique instable.

Cependant, la détection de ces "loci chauds" pour l'amplification pourrait traduire la plus grande stabilité de certaines ADS et donc leur observation et leur étude plus fréquente. En outre, cette spécificité de région pourrait résulter de la contre-sélection des événements d'amplification dans une autre région du génome: l'amplification et les remaniements associés devenant non viables dans un locus contenant des gènes essentiels.

II.- STRUCTURE ET LOCALISATION DE L'ADN AMPLIFIE CHEZ *STREPTOMYCES AMBOFACIENS* -

Chez *S. ambofaciens*, les ADS ont été détectées sous forme de réitération en tandem de l'AUD au locus sauvage: tout d'abord, un fragment homologue de chaque extrémité de l'AUD a été caractérisé comme le fragment-joint créé par la réitération; d'autre part, les profils de restriction en PFGE de souches mutantes amplifiées (4 cas étudiés) hybridés avec des sondes spécifiques d'ADS ont montré qu'un seul fragment de restriction compatible avec la réitération en tandem de l'AUD était présent. Dans le cas où l'enzyme utilisée coupe à l'intérieur de l'AUD (cas de l'ADS6), l'ADS est décelée sous forme d'un fragment très intense dont la taille correspond à l'AUD. D'autre part, lorsque l'enzyme utilisée coupe à l'extérieur de l'AUD (cas de l'ADS90 et ADS120), le fragment de restriction portant l'ADS contient aussi une séquence flanquante de l'AUD sauvage. Ces faits démontrent que l'amplification intervient sur le chromosome au locus de l'AUD. Chez *S. glaucescens*, la situation semble être équivalente (Häusler *et al.*, 1989).

III.- STRUCTURES PREAMPLIFIABLES -

Chez *S. ambofaciens*, la cartographie de 4 ADS et leur comparaison à chaque AUD sauvage respective ne révèle aucune différence détectable au niveau de la structure interne de l'AUD. Sauf dans le cas de l'ADS7 (22 kb) où l'AUD a subi une délétion interne avant amplification, l'ADS est constituée de la réitération en tandem de l'AUD sauvage. Les seules séquences créées au moment de l'amplification sont la séquence-joint (issue de la réitération) et la séquence flanquante gauche subissant une délétion (5 cas sur 5).

Plusieurs auteurs ont proposé que l'amplification soit précédée par un remaniement de l'AUD qui lui confère son caractère amplifiable. Ces remaniements sont de plusieurs types.

Dyson et Schrempf (1987) proposèrent que, chez *S. lividans* l'AUD, subît une duplication préalable au cours des réplications précédant l'amplification par un événement de recombinaison. Hütter et Eckhardt (1988) proposèrent deux modèles pour la création d'un état amplifiable: soit une délétion entre deux séquences répétées éloignées l'une de l'autre sur le génome sauvage créant ainsi une structure à répétitions finales (figure 5.A),

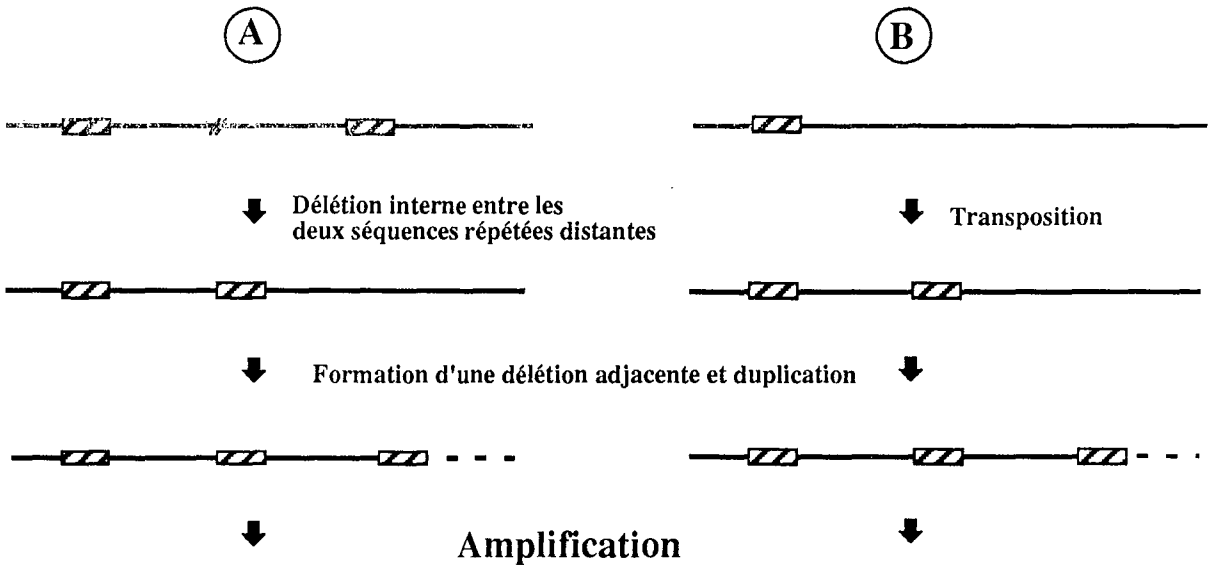

Figure 5: Modèles de formation d'une unité amplifiable selon Hütter et Eckhardt (1988).
(**A**) Création de l'AUD par délétion entre deux séquences répétées distantes.
(**B**) Création de l'AUD par transposition duplicative d'une séquence.
Symboles:
—— ADN génomique,
—//— Grande distance sur le génome,
▨ Séquence frontière de l'AUD,
- - - Séquence délétée.

soit une transposition d'une séquence à un locus proche du locus initial (figure 5.B). Ces hypothèses impliquent qu'une distance optimale entre les deux répétitions soit obtenue pour permettre l'amplification.

Chez *S. ambofaciens*, nous avons montré que la potentialité à amplifier une séquence était strictement associée à l'hypervariabilité. En effet, aucun cas d'amplification n'a été détecté directement dans un clone sauvage ni dans un mutant phénotypiquement stable. Il pourrait donc exister un état amplifiable créé lors de l'hypervariabilité dont la nature reste inconnue.

IV.- DELETIONS -

Chez les *Streptomyces*, l'instabilité génomique a été admise comme source de la variabilité phénotypique. L'amplification, premier phénomène mis en évidence, puis la formation de grandes délétions ont été tenues pour responsables de la perte des caractères instables (pour revue, Hütter et Eckhardt, 1988; Leblond *et al.*, 1990).

Chez *S. ambofaciens*, de nombreuses délétions ont été détectées dans une AUD (AUD6); cette région semble constituer une région "borne" de la grande région chromosomique subissant les délétions à moins qu'un gène essentiel soit situé au delà et qu'une délétion plus importante ne soit létale. La technique de PFGE a permis d'établir une stricte corrélation (30 cas étudiés) entre délétions et instabilité génétique, ce qui avait été postulé jusqu'alors. Les délétions ont été décelées dans toutes les lignées mutantes: phénotypiquement stables, issues de l'hypervariabilité, amplifiées ou non. Chez les autres espèces de *Streptomyces*, les délétions sont localisées soit immédiatement adjacentes à l'ADS soit dans la proximité de celle-ci (Dyson et Schrempf, 1987; Häusler *et al.*, 1989; Betzler *et al.*, 1987, Hornemann *et al.*, 1989). Chez *S. ambofaciens*, la taille des délétions est estimée de 250 à plus de 2.000 kb et est analogue à celles détectées chez *S. glaucescens* (Birch *et al.*, 1989). Dans les autres espèces de *Streptomyces*, les délétions sont détectées mais leur taille maximale n'a pu être encore estimée.

Chez *S. ambofaciens*, les délétions détectées dans les clones amplifiés incluent au moins la première copie de l'AUD. De plus, les clones mutants issus de l'instabilité génétique (phénotypiquement stables) comme les clones dérivant de l'hypervariabilité montrent une hypervariabilité génotypique. Enfin, les clones ayant subi une délétion sont susceptibles de subir à nouveau des délétions.

V. - VERS UNE EXPLICATION DE LA PLASTICITE GENOMIQUE: LES DELETIONS MULTIPLES ET SUCCESSIVES -

Deux hypothèses sur la succession des événements moléculaires constituant la plasticité génomique peuvent être émises.

La première hypothèse implique l'intervention de délétions successives, responsables de la variabilité phénotypique. Dans les lignées hypervariables, l'amplification serait possible grâce à la création de structures préamplifiées par des délétions différentes de celles affectant les clones phénotypiquement stables. Les délétions seraient l'origine et le principale mode de perte des ADS.

Dans la deuxième hypothèse, les phénomènes de délétion et d'amplification sont indépendants. Dans ce cas, l'amplification ne dépendrait pas d'une délétion préalable et les délétions affectant les clones mutants auraient les mêmes caractéristiques. La détection d'amplifications dans les seules lignées hypervariables traduirait un effet de sélection. En fait, les lignées mutantes seraient définies par les fréquences respectives de ces événements.

Dans la première hypothèse, les délétions créent la potentialité d'amplifier une AUD. La figure 6.A représente la structure de la région instable dans le génome sauvage. Cette structure est simplifiée puisqu'elle ne mentionne qu'une seule famille d'AUD et un seul RAL (Replication Activating Locus). La potentialité à amplifier une séquence dépend de la séquence délétée au cours du processus d'amplification (fonction de contrôle de la réplication) et/ou encore de la séquence fusionnée ou créée par la fusion des séquences résiduelles. Cette séquence pourrait contenir une séquence consensus capable de promouvoir l'initiation de la réplication. Dans ce cas, le facteur déterminant serait la distance séparant le site d'initiation de l'AUD après délétion. D'autre part, la structure formée par la délétion et la fusion à l'AUD pourrait avoir des fonctions d'origine de réplication. Ainsi, Palzkill et Newton (1988) ont décrit une origine de réplication plasmidique de levure (ARS, Autonomously Replicating Sequence) comme une région contenant de 9 à 30 répétitions directes ou inversées de 9 à 11 pb. Ainsi, l'AUD est caractérisée par la répétition de séquences homologues entre elles à différents degrés et d'autres séquences homologues ont été détectées sur le génome sans être localisées dans l'AUD. La fusion de plusieurs répétitions pourrait créer une structure analogue à une ARS et donc réinitier la réplication au locus de l'AUD. Chez *S. glaucescens*, quatre répétitions de 38 pb en orientation directe ou inversée ont été détectées à l'extrémité d'une AUD qui pourrait avoir ce rôle (A. Häusler, 1989, thèse). L'existence de plusieurs de ces loci serait démontrée puisque les délétions décelées dans les clones amplifiés sont de tailles très différentes.

La figure 6.B représente les remaniements génomiques dans les clones phénotypiquement stables; des grandes délétions de taille variable (une seule délétion est représentée) atteignent la région de l'AUD6 dans 50% des cas. La stabilité du phénotype dépigmenté pourrait être due à la perte du gène structural ou d'un élément de contrôle indispensable au caractère de pigmentation. Une hypothèse permettant d'expliquer qu'aucun cas d'amplification n'ait été détecté dans ces lignées pourrait être la délétion systématique du RAL ou un mauvais positionnement.

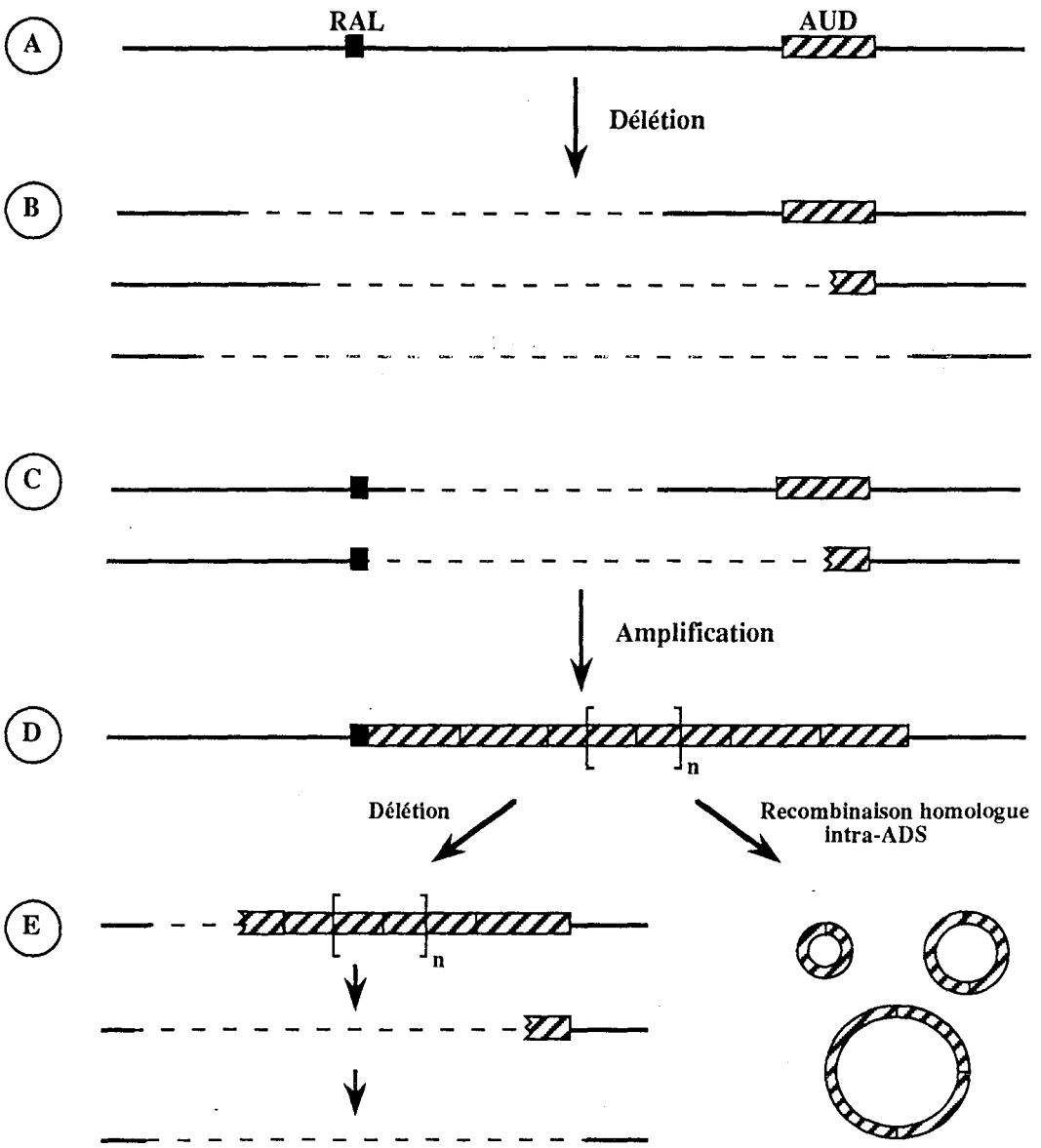

Figure 6: Modèle de la plasticité génomique chez *S. ambofaciens*.
(**A**) Structure du génome en amont d'une AUD dans la souche sauvage. (**B**) Délétions dans l'arc génomique instable affectant les clones phénotypiquement stables. (**C**) Délétions affectant les clones issus de l'hypervariabilité. (**D**) Amplification en tandem au locus sauvage de l'AUD. (**E**) Perte de l'ADS par délétion (recombinaison homologue ou hétérologue) ou par recombinaison entre copies réitérées de l'ADS (recombinaison homologue ou hétérologue entre séquences partiellement homologues).
Symboles:
— ADN génomique,
■ RAL, Replication Activating Locus,
▨ AUD, Amplifiable Unit of DNA,
- - - Séquence délétée,
Formes circulaires oligomériques de l'ADS.

La figure 6.C permet d'expliquer les remaniements qui mènent à la variabilité génotypique et phénotypique et à l'amplification dans les clones générés par l'hypervariabilité. Des délétions de tailles variées atteignent la région instable, sans affecter systématiquement le RAL. Dans certains cas, le RAL est fusionné à l'AUD de façon convenable pour promouvoir l'amplification. Les délétions affecteraient des régions de contrôle ou structurales des gènes régissant la morphologie et le métabolisme secondaire. Il en résulterait la variabilité phénotypique.

Ces délétions pourraient avoir lieu par recombinaison soit entre séquences partiellement ou totalement homologues soit par l'intermédiaire de recombinases site-spécifiques. De même dans les clones amplifiés, les délétions entraînant la perte partielle ou totale de l'ADS (sans retour à la séquence sauvage au locus AUD) pourraient avoir lieu entre des séquences situées dans l'ADS et des séquences externes à l'ADS non encore délétées par les événements précédents. D'ailleurs, l'ADS pourrait constituer une zône hautement recombinogène et donc présenter une instabilité accrue du génome. Ainsi, les séquences minisatellites hypervariables des cellules humaines sont le siège d'une recombinaison homologue intense: en particulier, des séquences "chi-like" ont été détectées dans ces réitérations qui pourraient être la source de leur amplification par crossing-over inégal mais aussi de leur variabilité (pour revue, Jarman et Wells, 1989). La perte de l'amplification pourrait se produire aussi par recombinaison homologue entre les copies réitérées de l'AUD et expliquer la détection de molécules extrachromosomiques oligomères de l'AUD dans les souches amplifiées (figure 6.D)(Simonet *et al.*, en préparation).

Dans la deuxième hypothèse, toute AUD aurait la potentialité de s'amplifier sans délétion préalable. Délétions et amplifications affecteraient tous les clones mutants issus de l'instabilité génétique mais à des fréquences variées. Ainsi, l'intervention de délétions à une fréquence plus élevée dans les lignées mutantes phénotypiquement stables ne permettrait pas de détecter le stade amplifié. La fréquence plus élevée de délétion dans ces clones serait associée au phénotype mutant stable. De plus, l'association délétion-amplification établie dans les clones hypervariables amplifiés serait le résultat d'un fréquence plus élevée des événements de délétions par rapport à ceux d'amplifications. De fait, le stade amplifié non délété serait fugace et donc non encore décelé.

VI.- MODELES D'AMPLIFICATION -

Les ADS détectées chez les *Streptomyces* montrent une très grande hétérogénéité en ce qui concerne la taille et le degré d'amplification, tout en présentant une grande homogénéité pour ce qui est de la séquence réitérée. Chez *S. lividans*, des ADS hétérogènes, c'est à dire présentant des remaniements dans quelques copies de la réitération ont été mentionnées (Dyson et Schrempf, 1987). Cependant, cette hétérogénéité pourrait résulter de remaniements subséquents à l'amplification.

L'amplification peut résulter de crossing over inégaux multiples entre séquences néo-répliquées. Cependant, les degrés d'amplification atteints chez les *Streptomyces* impliqueraient de très nombreux événements de recombinaison, ce qui est improbable. De

plus, compte tenu des séquences répétées dispersées dans les loci amplifiables, des événements de recombinaison favoriseraient l'introduction d'une hétérogénéité dans les copies réitérées.

L'intervention d'un processus de réplication fidèle semble plus probable puisqu'il peut en une seule étape, générer un haut degré d'amplification. Ainsi, chez *S. ambofaciens*, certaines souches mutantes présentent un degré d'amplification qui atteint 50% de l'ADN génomique total. De même, chez les cellules de mammifères, l'amplification impliquerait la réplication d'une structure amplifiable résultant d'un remaniement initial de l'ADN sauvage.

Les mécanismes proposés dans le domaine des *Streptomyces* s'appliquent aux AUD de type II. Ces modèles diffèrent par leur première étape: Dyson et Schrempf (1987) proposèrent que l'étape limitante du processus soit la duplication préalable de l'AUD (présente en copie unique dans la plupart des souches sauvages de *Streptomyces*) par un mécanisme de crossing-over inégal entre les chromatides nouvellement répliquées. Puis, un mécanisme de type cercle tournant chromosomique serait responsable de l'amplification. Young et Cullum (1987) proposèrent que l'étape limitante soit l'emprisonnement d'une fourche de réplication sur le chromosome par un événement de recombinaison entre une répétition de l'AUD néo-répliquée et une répétition homologue non encore répliquée. Ce modèle permettait d'expliquer l'apparition de délétions uniquement d'un côté de l'amplification: des événements de recombinaison hétérologues permettant la réassimilation de la fourche de réplication sur le chromosome.

Chez les cellules de mammifères en culture, l'amplification est généralement constituée de répétition en tandem d'une structure "tête-bêche" d'une séquence créée dans les premières étapes de l'amplification. Un des modèles proposés repose sur la réinitiation multiple au locus de l'AUD et la recombinaison homologue entre les chromatides néo-répliquées. Ce modèle appelé "pelure d'oignon" ("onion skin") a été initialement proposé pour l'amplification des gènes d'ARN ribosomaux dans les oocytes de Batraciens et le chorion des Drosophiles (Stark et Wahl, 1984; Schimke, 1984). Cependant , il n'explique pas la mise en place de la structure en répétition inversée détectée dans la plupart des cas.

Hyrien *et al.* (1987 et 1988) proposèrent un modèle (modèle "spirale") où l'amplification est réalisée sur le chromosome au locus de la séquence amplifiable. La mise en place de la structure réplicative inversée est due à une erreur réplicationnelle: glissement de la machinerie réplicationnelle par un mécanisme de recombinaison "copy choice" (Streisinger, 1966; Brunier *et al.*, 1988) sur une séquence riche en base A/T (séquence *Alu*-like). Les séquences *Alu*-like sont connues pour induire de telles aberrations de la réplication (Glickman et Ripley, 1984). Ensuite, deux fourches de réplication sont emprisonnées sur le chromosome créant la structure en spirale.

Ce modèle tend à expliquer les différentes localisations de l'ADN amplifié dans les cellules de mammifères en culture et chez les eucaryotes inférieurs. En effet, de nombreux cas d'amplification de gènes de résistance à des drogues ont été décrits aussi bien dans des lignées de cellules cancéreuses de mammifères que chez *Leishmania* (pour revue, Stark et Wahl, 1984). Chez ces organismes, l'ADN amplifié a pu être localisé, soit intégré de façon linéaire sur le chromosome au locus originel ou à un autre locus (HSR, Homogeneous Staining Region), soit sous forme circulaire libre (DM, Double Minute).

Ainsi, l'excision par recombinaison homologue d'une molécule contenant la structure réplicative "spirale" pourrait générer les formes circulaires libres (DM) avec possibilité de réintégration à de multiples loci. Les formes circulaires non réplicatives constitueraient des intermédiaires de perte de l'ADS. Chez *Leishmania*, la stabilisation de la résistance à la drogue utilisée avait préalablement été attribuée à l'intégration sur le chromosome de la forme DM de l'ADS (Beverley *et al.*, 1984). Cependant, Garvey et Santi (1986) ont récemment montré par analyse en PFGE que l'amplification était stable sous forme d'une grande molécule circulaire et instable sous forme de chromosomes DM. La situation pourrait donc différer quant à la localisation de l'amplification chez *Leishmania* et les lignées cancéreuses des cellules de mammifères.

Récemment, chez *S. ambofaciens*, des formes circulaires oligomériques de l'AUD ont été détectées en gradient de chlorure de césium dans l'ADN total de souches amplifiées (Simonet *et al.*, en préparation). Le rôle de ces structures est actuellement recherché: forme intermédiaire du processus d'amplification ou perte de l'ADS par recombinaison homologue.

Des formes circulaires libres pourraient être des intermédiaires dans le mécanisme d'amplification. Ainsi, Gruss et Ehrlich (1989) ont montré que la formation de concatémères plasmidiques (HMW, High Molecular Weight) d'une insertion était liée au mode de réplication du plasmide, et en particulier à la réplication sous forme simple brin impliquée dans la réplication du type cercle tournant.

L'amplification, phénomène ubiquitaire, pourrait malgré les différences importantes de structure des séquences amplifiables entre les organismes étudiés, dépendre des mêmes mécanismes moléculaires. D'ailleurs, des réarrangements multiples (inversions, translocations, délétions et aberrations chromosomiques) ont été détectés associés à l'amplification. De plus, il semble maintenant démontré que l'amplification chez les mammifères est étroitement associée à la formation de délétions (Stark, 1989).

VII. - MUTATIONS PRIMAIRES -

La détection d'événements multiples et successifs dans les lignées mutantes suggère que l'instabilité génomique et en particulier les délétions ne sont pas l'origine de l'instabilité génétique mais la conséquence d'une mutation primaire. Ainsi, dans les cellules de mammifères en culture, Tlsty *et al.* (1989) ont montré que l'amplification d'un gène de résistance à une drogue (PALA pour N-phosphonoacéthyl-L-aspartate) se produisait au hasard et spontanément dans la population cellulaire et que la probabilité de réamplification d'une cellule (cellule tumorale) portant déjà une amplification est plus importante que dans une cellule normale (saine). De plus, les mutations de type amplification et grande délétion dans ces lignées cellulaires sont plus fréquentes que les mutations ponctuelles suggèrant une mutation primaire affectant la cellule cancéreuse (Tlsty *et al.*, 1989). En accord avec ces résultats, Rice *et al.* (1989) ont montré que la fréquence de lignées cellulaires résistantes à deux drogues était 10 à 100 fois supérieure à la fréquence d'une lignée résistante à une seule drogue. Ces faits supportent l'hypothèse d'une mutation primaire qui augmenterait la fréquence spontanée de remaniements ou induirait les remaniements génomiques.

A. - Recombinaison homologue -

La plasticité génomique pourrait être le résultat de la recombinaison homologue entre séquences répétées. Cependant, la recombinaison homologue *recA*-dépendante nécessite des séquences parfaitement homologues sur au moins 16 paires de base minimum chez *E. coli* et de 20 à 100 pb en général chez les autres procaryotes (pour revue, Radding, 1988). Ainsi, chez *Streptomyces*, les longues séquences répétées directes (entre 0,7 et 2,2 kb suivant les espèces) cartographiées dans les AUD de type II sont susceptibles de constituer un bon substrat pour une recombinaison homologue. Chez *S. ambofaciens*, les multiples homologies détectées dans les différentes familles d'AUD pourraient, suivant leur nature (homologie totale ou partielle), promouvoir une recombinaison homologue ou illégitime. Des mutantations de suppression de recombinaison pourraient induire des événements de recombinaison illégitime (entre séquences non entièrement homologues) au locus de ces structures répétées. Ainsi, Rayssiguier *et al.* (1989) ont montré que la recombinaison interspécifique entre *E. coli* et *Salmonella typhimurium* est déréprimée chez des mutants déficients dans le système de réparation des misappariements. De telles structures pourraient subir l'amplification suivant des modèles de type Young et Cullum (1987) ou Dyson et Schrempf (1987) aussi bien qu'être le substrat de délétions. Chez *Bacillus subtilis*, des séquences amplifiées stables peuvent être induites sur le chromosome par l'intermédiaire de séquences répétées de 2 à 4 kb (Jannière *et al.*, 1985).

Au contraire, les microhomologies détectées aux extrémités des AUD chez *S. glaucescens* (Häusler *et al.*, 1989) ou les courtes séquences répétées impliquées dans les "points chauds" de délétions chez *E. coli* (Whoriskey *et al.*, 1987) ne peuvent promouvoir la recombinaison homologue.

B. - Erreurs réplicationnelles -

Une mutation affectant la stabilité du complexe réplisome-ADN pourrait induire des erreurs réplicationnelles à des sites préférentiels. Ainsi, Glickman et Ripley (1984) ont montré que la polymérase se comporte de façon aberrante lorsqu'elle réplique des séquences comportant des palindromes ou pouvant former des structures en "épingle à cheveux". La fréquence de ces erreurs pourrait être augmentée sous des conditions de croissance modifiant le rythme de réplication (traitement avec des intercalants de la double hélice, germination ou reprise de croissance après conservation au froid). Sur ces résultats, Hyrien *et al.* (1988) proposèrent le modèle d'amplification "spirale" où la première étape est un glissement de la fourche de réplication sur une séquence *Alu*-like susceptible de former des palindromes. Chez les *Streptomyces*, la présence de multiples palindromes pourrait résulter du fort taux en base G et C (72%) du génome.

Brunier *et al.* (1989) ont étudié la recombinaison entre séquences répétées de petite taille (9 pb) chez *E. coli*. Les auteurs montrent que des délétions peuvent intervenir entre ces séquences par un mécanisme de "copy choice", c'est à dire par la formation d'un hybride partiel sur un seul brin d'ADN par l'intermédiaire des séquences répétées. La fourche de réplication glisse alors sur une telle structure et provoque une délétion détectable sur les doubles brins d'ADN néo-répliqués. De tels événements seraient à l'origine de l'excision propre de certains transposons (Brunier *et al.*, 1988).

C. - Enzymes de la topologie de l'ADN: les topoisomérases -

Les topoisomérases induisent des coupures simple ou double brins sur le génome à des sites multiples et dispersés. Ces enzymes qui sont impliquées dans la maintenance de la superhélicité de l'ADN pourraient être impliquées dans les mécanismes de la plasticité du génome.

Les topoisomérases de type I induisent des coupures simple brin et restent attachées à l'extrémité d'un brin. Les sites de reconnaissance de la topoisomérase I du foie de rat consistent en une séquence consensus de quatre nucléotides ($5'$A/T-G/C-A/T-$T3'$)(Champoux et Bullock, 1988). La fréquence de coupure varie fortement en fonction de la composition de ces sites. Ces enzymes pourraient être impliquées dans l'excision du virus SV40 dans les cellules de rat: l'analyse des séquences aux sites d'excision révèle, en effet, une corrélation avec la présence de sites potentiels de coupure d'une topoisomérase. De même, ces enzymes pourraient être impliquées dans les remaniements observés dans les séquences codantes des immunoglobulines (Krawinkel *et al.*, 1986).

Les topoisomérases de type II, telle que la gyrase, induisent des coupures double brin. Miura-Masuda et Ikeda (1990) ont montré que la gyrase pouvait être à l'origine de délétions dans des souches de *E. coli recA*. Lors de l'analyse des sites de coupure, une séquence de 6 pb qui serait commune au site de reconnaissance de la gyrase (Lockshon et Morris, 1985) a été détectée. De plus, l'acide oxolinique, inhibiteur de l'activité de superenroulement de la gyrase mais pas de l'activité de coupure ni de la reconnaissance de sites, augmente la fréquence de délétions renforçant l'hypothèse de l'intervention de cette enzyme.

En outre, des séquences répétées dispersées ont été décelées sur le génome de *E. coli* et *S. typhimurium* (Stern *et al.*, 1984; Gilson *et al.*, 1987). Ces séquences appelées PU (Palindromic Unit)(Gilson *et al.*, 1987) ou REP (Repetitive Extragenic Palindromic sequence)(Stern *et al.*, 1984) sont extragéniques. Elles sont composées de répétitions inversées de 20 à 40 nucléotides qui ont la potentialité de former des structures en "épingles à cheveux" et qui peuvent être groupées sur le génome. Le nombre de ces loci est estimé de 100 à 200 par génome (Stern *et al.*, 1984). Ces structures pourraient être impliquées dans la régulation de l'expression des cistrons qu'elles encadrent (Dahl *et al.*, 1989). Récemment, l'implication de ces séquences dans les remaniements génomiques a été fortement suggérée (Yang et Ames, 1988; Miura-Masuda et Ikeda, 1990; Shyamala *et al.*, 1990). En effet, ces séquences sont reconnues par une protéine, qui d'abord inconnue (Gilson *et al.*, 1986) s'est révélée être une gyrase (Yang et Ames, 1988). Ensuite, l'analyse de séquences impliquées dans des événements de duplication chez *S. typhimurium* révèle des séquences REP (Shyamala *et al.*, 1990). Par ailleurs, de nombreux autres cas de délétions impliquant des séquences répétées de très petite taille ont été décrits (Farabaugh *et al.*, 1978; Albertini *et al.*, 1982; Jones *et al.*, 1982; Schaaper *et al.*, 1986) qui pourraient résulter d'événements moléculaires analogues. L'échange de brins d'ADN entre complexes topoisomérase-ADN permet de générer des délétions comme d'installer un système de réplication du type cercle tournant sur un brin. La séquence amplifiable correspond alors au segment compris entre les deux sites de reconnaissance de l'enzyme. Dans ce modèle, l'amplification ne nécessite pas de délétion préalable ce qui rejoint l'hypothèse où délétion et amplification sont des événements indépendants.

Par ailleurs, Michel et Ehrlich (1985) ont décrit l'intervention du produit du gène II du phage M13 dans l'induction de délétions. Ce gène est impliqué dans l'introduction d'une coupure simple brin à l'origine de réplication du réplicon. Dans ce cas, l'initiation de la réplication serait à l'origine des délétions tout comme elle serait impliquée dans le mécanisme d'amplification.

D. - Recombinases site-spécifiques -

Plusieurs cas de recombinases site-spécifiques associées à la formation de délétions ont été décrits chez les procaryotes. Ces enzymes peuvent être impliquées dans des remaniements programmés dans les processus de différenciation. Comme nous l'avons mentionnée plus haut, l'induction de l'opéron responsable de la fixation de l'azote chez *Anabaena* résulte de la délétion de segments de 11 et 55 kb. Une recombinase (produit du gène *xisA*) a été caractérisée et serait responsable de la délétion de 11 kb (Lammers *et al.*, 1986).

Chez les *Streptomyces*, nous avons peu de données sur les séquences impliquées dans les remaniements. Cependant, chez *S. glaucescens*, le séquençage des sites de délétions révèle une homologie de séquence avec les sites d'attachement des plasmides intégratifs (pour revue, Boccard *et al.*, 1989). Ainsi, le site d'attachement de pIJ408 (Hopwood *et al.*, 1984) présente une séquence consensus dont 5 pb ($^{5'}$TCGAA$^{3'}$) sont retrouvées dans 2 cas étudiés sur 3, dans le troisième cas l'homologie n'est que partielle. Par ailleurs, des bactériophages ont été mis en évidence dans trois souches de *S. ambofaciens* sur les quatre à notre disposition. L'intégrase d'un plasmide intégratif ou d'un bactériophage pourrait donc reconnaître ces sites et induire une recombinaison illégitime.

Les remaniements pourraient être également la conséquence directe d'un événement spécifique d'excision d'un grand réplicon (plasmide linéaire ou bactériophage). Ainsi, chez *S. fradiae* (Stonesifer *et al.*, 1986) et *S. achromogenes* subsp. *rubradiris* (Hornemann *et al.*, 1987), certains marqueurs, dont les séquences amplifiables, localisées sur le chromosome sont transférés à très haute fréquence par rapport à d'autres lors d'expériences de croisement. Cette région pourrait alors s'exciser comme cela a été observé chez *S. coelicolor* A3(2) (Bibb *et al.*, 1981) ou encore être mobilisée par un facteur de fertilité.

VIII.- SIGNIFICATION BIOLOGIQUE DE LA PLASTICITE GENOMIQUE -

La fréquence des événements de remaniement du génome chez les *Streptomyces* pose la question de leur signification biologique.

Plusieurs aspects du phénomène d'instabilité génétique semblent relever d'un processus aléatoire. Ainsi, les remaniements génomiques n'affectent qu'une partie de la population comme dans le cas de la cancérisation chez les cellules de mammifères ou lors de l'apparition de cellules résistantes à certaines drogues chez *Leishmania* (pour revue, Stark, 1989). De plus, la résultante de l'instabilité génétique semble le plus souvent caractérisée par une diminution des performances métaboliques des mutants par rapport à la référence sauvage. L'instabilité revêt donc un aspect délétère. Ainsi, un phénomène aléatoire peut donc apparaître comme programmé et systématique si sa fréquence est très

élevée. Chez *Podospora*, la sénescence est un phénomène aléatoire qui affecte la totalité de la population. L'arrêt de croissance est corrélé à des délétions et des amplifications affectant le génome mitochondrial (Vierny-Jamet, 1988).

Par ailleurs, certains aspects de l'instabilité génétique ne semblent pas être aléatoires. En effet, les fonctions du métabolisme secondaire semblent être préferentiellement affectées par l'instabilité génétique. D'autre part, la plasticité génomique semble spécifique d'une région. Si les remaniements génomiques sont à la source des phénotypes mutants (comme cela est démontré chez plusieurs *Streptomyces*; pour revue, Leblond *et al.*, 1990), l'arc génomique pourrait ne pas contenir de gènes essentiels, dont la délétion entraînerait la létalité. Des arcs chromosomiques vides de marqueurs génétiques connus ont d'ailleurs été récemment décelés sur une carte physique du chromosome de *S. coelicolor* A3(2)(Hopwood *et al.* ,1990). Ainsi, les remaniements pourraient être aléatoires à l'intérieur de cette région et pourraient avoir comme finalité la création d'une variabilité phénotypique et non pas un rôle dans la différentiation du microorganisme comme chez les Ciliés (pour revue, Yao, 1989) ou chez *B. subtilis* (Stragier *et al.*, 1989). Les délétions pourraient créer des cadres d'expression nouveaux par la fusion de promoteurs à des séquences codantes préexistantes (expression augmentée ou diminuée), ou créer de nouveaux gènes par fusion de séquences préexistantes. L'amplification peut permettre la surexpression des gènes portés par l'ADN amplifiable. Chez *S. ambofaciens*, plusieurs mutants présentant des phénotypes avantageux par rapport aux caractères sauvages ont été isolés: hypersporulation ou encore vitesse de croissance supérieure (résultats non publiés). De même dans le cas de l'opéron *lacI* de *E. coli*, l'intervention de délétions produit des mutants aux capacités métaboliques variées (Whoriskey *et al.*, 1987).

Par ailleurs, il existe, dans d'autres systèmes biologiques, des phénomènes qui n'affectent pas toute la population et qui sont programmés. Ainsi, chez *Anabaena*, la différentiation de cellules végétatives en hétérocystes fixateurs d'azote atmosphérique est effective dans environ 10% des cellules et est étroitement associée à des délétions site-spécifiques à l'intérieur de l'opéron nif (pour revue, Haselkorn, 1989). Chez *Candida albicans*, une variabilité phénotypique importante est générée à partir de la souche sauvage à une fréquence comparable à l'instabilité génétique des *Streptomyces* (Slutsky *et al.*, 1985). Récemment, l'analyse du caryotype des souches mutantes a révélé une réorganisation importante du génome incluant délétions, translocations et ploïdies partielles (Suzuki *et al.*, 1989; Rustchenko-Bulgac *et al.*, 1990). De même, les phénomènes de variation de phase chez les Trypanosomes et *Borrelia* ou la génération de la multiplicité des gènes des immunoglobulines dans les lymphocytes génèrent la variabilité au sein d'une population (Borst et Greaves, 1987). Tous ces organismes sont confrontés à un environnement variable (organismes supérieurs pour *Candida*, le sol pour les *Streptomyces*...) Ces remaniements pourraient permettre de générer une variabilité génotypique et phénotypique permettant une meilleure colonisation de leur environnement et une meilleure dissémination de l'espèce.

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ANNEXES

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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 $\mu g\ ml^{-1}$). 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 *Hin*dIII 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 ^{32}P-labelled by nick-translation (Rigby *et al.*, 1977) using [^{32}P]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%, polyvinylpyrolidone 0·1%, denatured salmon sperm DNA 200 $\mu g\ ml^{-1}$, 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 ^{32}P-labelled probe (7×10^7 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.

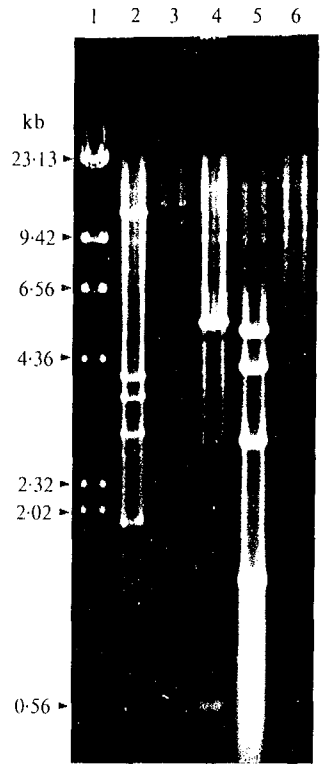

Fig. 1. Restriction patterns of total DNA from the WT and mutant strains of *S. ambofaciens*. DNA was digested with *Bam*HI and electrophoresed on 0·6% agarose gel at 1 V cm^{-1} for 18 h. Tracks: 1, 0·5 μg of λ DNA digested with *Hin*dIII as size marker; 2, 1·5 μg of NSA6 DNA; 3, 0·75 μg of NSA101 DNA; 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 $4·9 \times 10^3$ 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

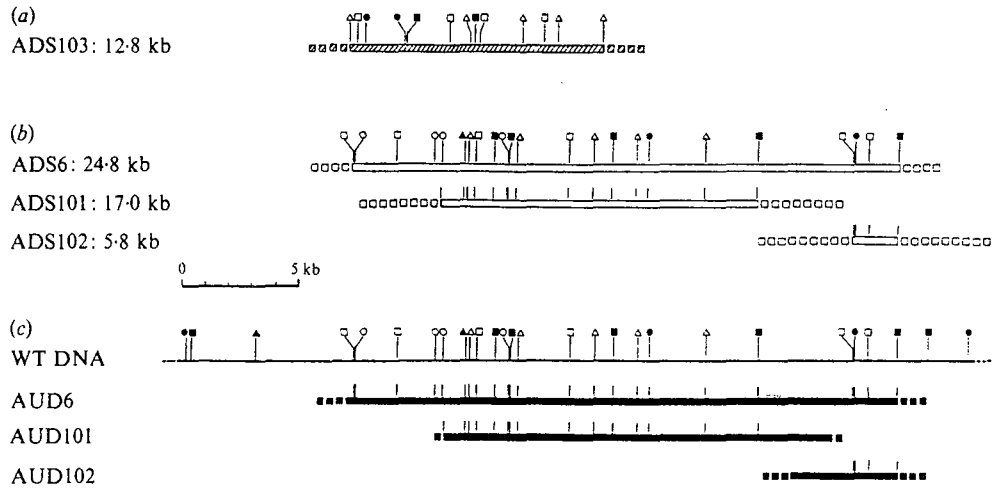

Fig. 2. Restriction maps of the two families of ADS in mutant strains of *S. ambofaciens*. (*a*) ADS in NSA103 ('ADS103 family'). (*b*) ADS in NSA6, NSA101, NSA102 ('ADS6 family'). (*c*) Amplifiable units of DNA (AUD) and identification of flanking sequences based on hybridization experiments. Symbols 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. 3*a*, track 2). The hybridization pattern (Fig. 3*b*, 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. 3*b*, 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. 3*b*, tracks 4, 5, 6 and 7). All typical reiterated DNA stretches of ADS101 and ADS102 were detected as heavy bands (Fig. 3*b*, tracks 4 and 5). Under the same hybridization conditions, no intense signals were detected with *Bam*HI digests of NSA103 and WT DNA (Fig. 3*b*, 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. 2*c*). These results proved that AUD6 is present as a single copy: most particularly, *Xho*I digestion generated only two flanking

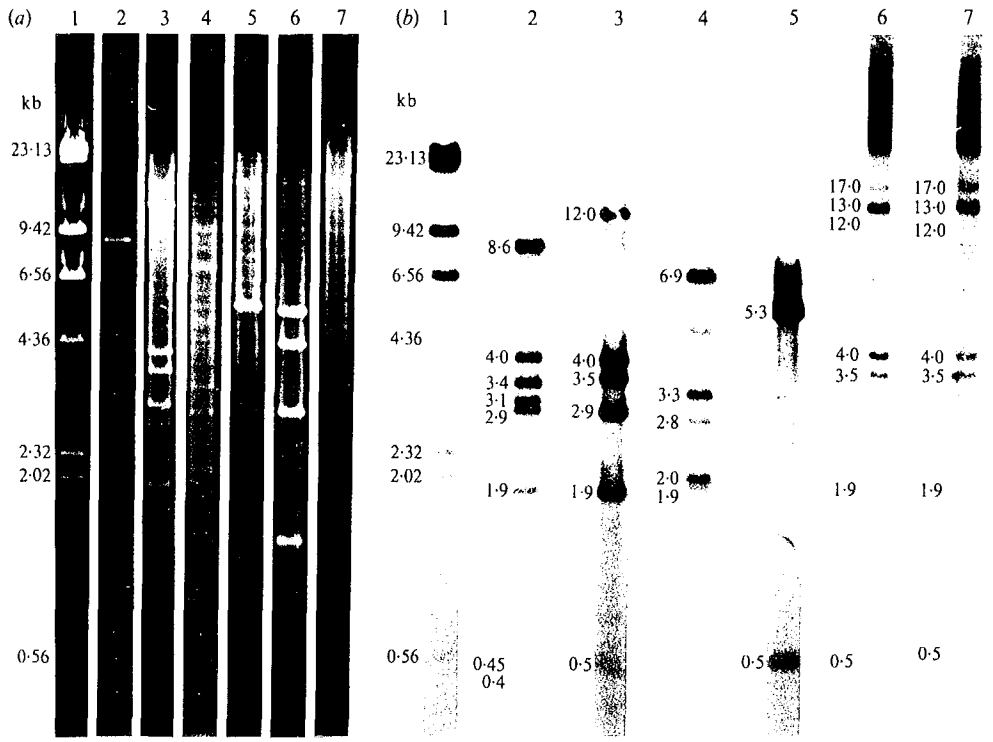

Fig. 3. Hybridization of ^{32}P-labelled ADS6 DNA with digests of DNA from mutant and WT strains. (*a*) Agarose gel separation of total DNA prepared from the WT and from mutant strains with amplified DNA. (*b*) Southern blot hybridization. Tracks: 1, *Hin*dIII-digested λ DNA; 2, eluted ADS6 DNA triple 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 & Schrempf, 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

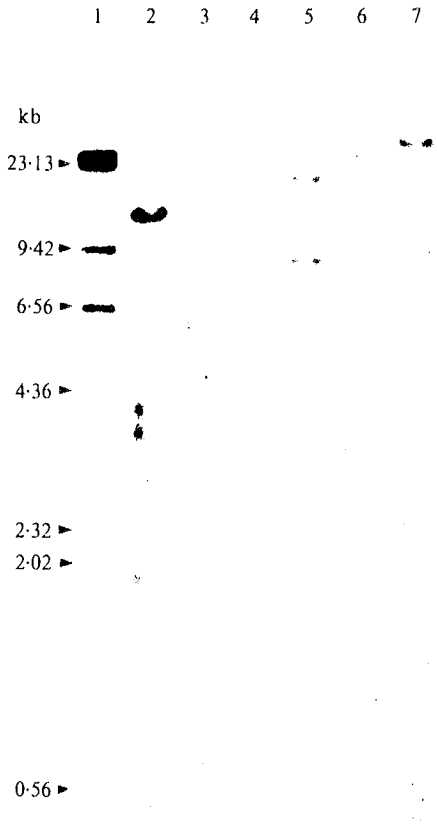

Fig. 4. Hybridization of ^{32}P-labelled ADS6 DNA with total WT DNA digested with a range of restriction enzymes. Tracks: 1, *Hin*dIII-digested λ DNA; 2, *Bam*HI; 3, *Bcl*I; 4, *Bgl*II; 5, *Pst*I; 6, *Pvu*II; 7, *Xho*I.

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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GENETIC INSTABILITY AND HYPERVARIABILITY CORRELATED WITH DNA AMPLIFICATION IN *STREPTOMYCES AMBOFACIENS*.

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The wild type (WT) strain *Streptomyces ambofaciens* DSM 40697 exhibits a high degree of genetic instability. A frequency of about 1% of pigment-defective colonies was observed in the progeny of WT colonies. In contrast, auxotrophic mutants appeared at the frequency of 5×10^{-5}. Therefore, this genetic instability seemed to preferentially affect characters involved in pigmentation and differenciation processes.

Out of 165 pigment-defective colonies independently isolated, only 21% gave rise to an homogeneous progeny exhibiting the parental phenotype. 87% of the mutant colonies gave rise to an heterogeneous progeny. Such a progeny was characterized by the absence of a preponderant phenotype and the number of phenotypes ranged from 2 to 8, most often 4 or 5. This new phenomenon of high mutability was called "hypervariability".

Molecular analysis of the total DNA of 71 independent clones arising in hypervariable progenies revealed highly amplified DNA in 21% of the clones, while no amplification was detected neither in 44 independent stable pigment-defective nor in 77 independent WT colonies. These results indicated that the hypervariability and amplification phenomena were correlated. The size of the amplified DNA sequences (ADS) ranged from 5 kb to 52 kb and the extent of the amplification could reach 50% of the total genomic DNA. The restriction patterns analysis suggested overlaps between several ADS. This fact is in accordance with the notion of amplifiable regions in the WT genome.

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

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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

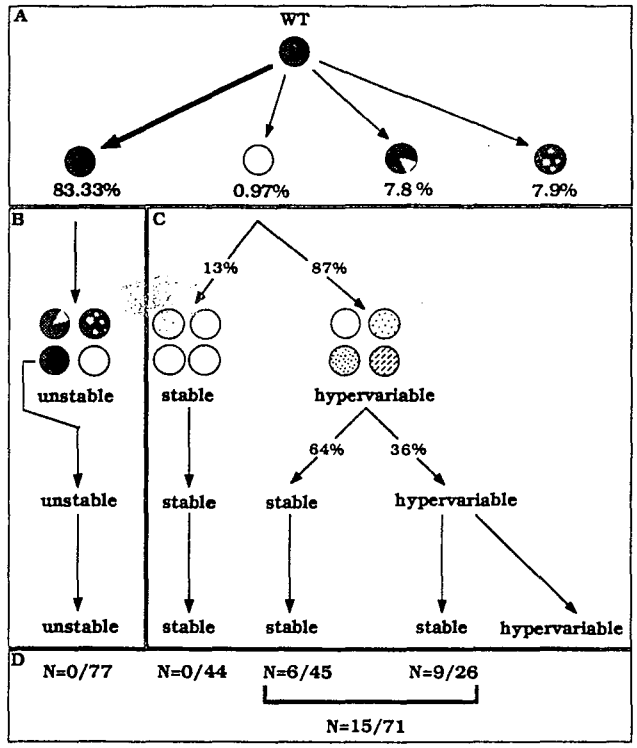

FIG. 1. Flow diagram of genetic instability. Each arrow represents a round of sporulation on HT solid medium. (A) Frequencies of the different mutant phenotypes observed from the WT strain (the WT strain is symbolized by grey circles; open circles represent both pigment-defective and -negative colonies). (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

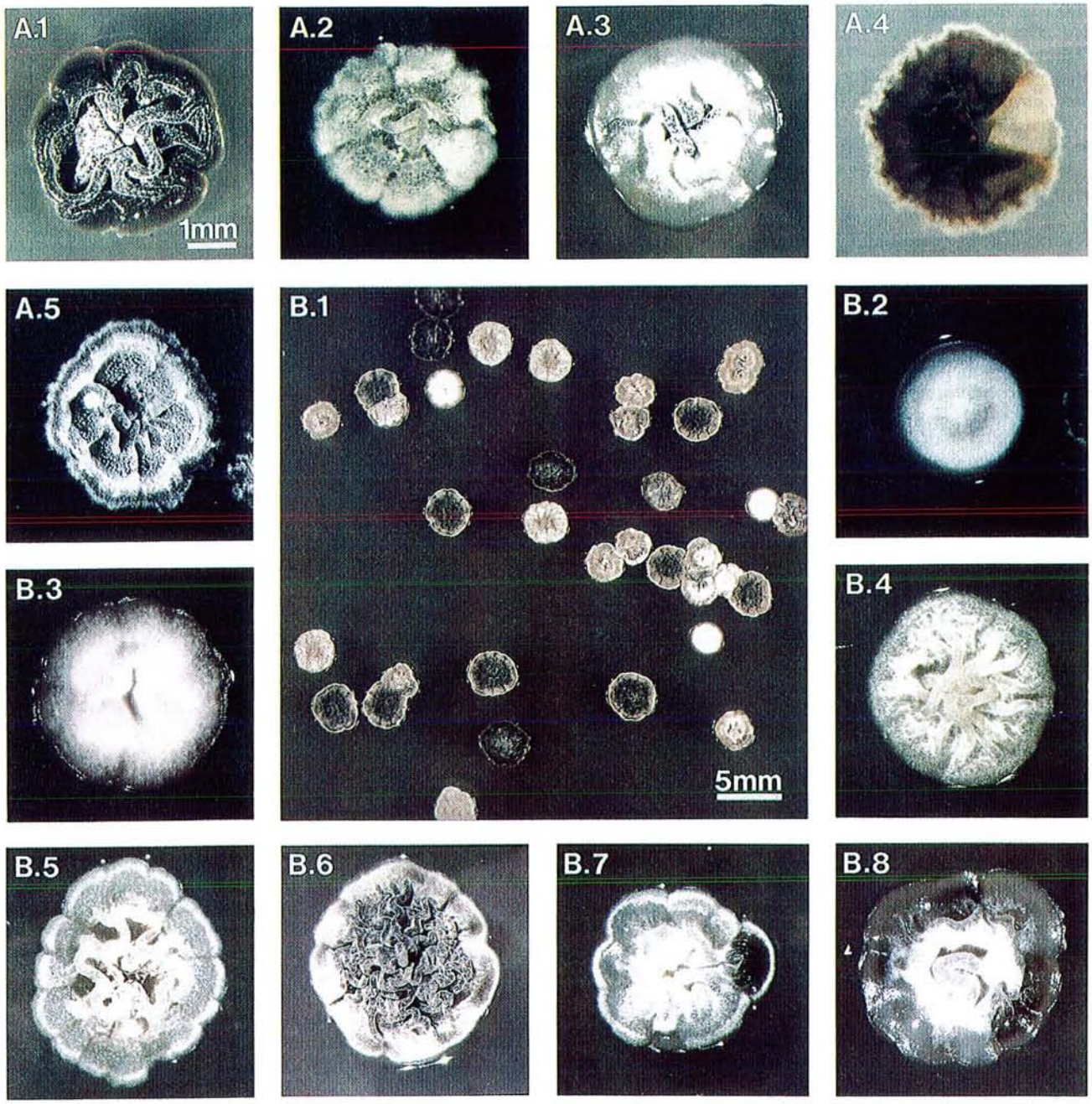

FIG. 2. Illustrations of genetic instability (A) and hypervariability (B) in *S. ambofaciens*. (A.1) WT colony. (A.2) Homogeneous pigment-negative colony. (A.3) Colony with a glossy ring with a grey center. (A.4) WT colony harboring a pigment-defective sector with diascopic lighting. (A.5) Colony harboring 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).

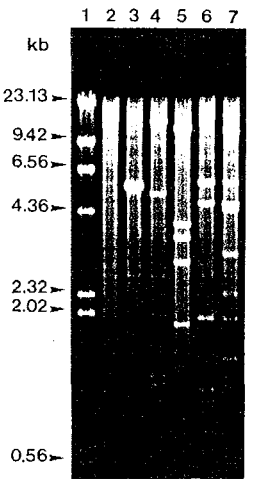

FIG. 3. Restriction patterns of total DNA from the WT and mutant strains of *S. ambofaciens*. DNA was digested with *Bam*HI and electrophoresed on an 0.8% agarose gel at 1 V/cm for 16 h. Lane 1, 0.5 μg of lambda DNA digested with *Hin*dIII; lane 2, WT DNA; lane 3, NSA70 DNA; lane 4, NSA100 DNA; lane 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.

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Genetic instability and hypervariability in *Streptomyces ambofaciens*: towards an understanding of a mechanism of genome plasticity

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Summary

Many *Streptomyces* species exhibit a very high degree of genetic instability which is usually manifested as genomic rearrangements such as large deletions. In *Streptomyces ambofaciens* DSM40697, two levels of genetic instability were previously described: (i) a basic genetic instability similar to that reported for other strains, and (ii) hypervariability, a phenomenon that we believe to be a new aspect of instability closely associated with DNA amplification. A large DNA region undergoes deletions, amplifications and large genomic changes strictly associated with both aspects of genetic instability. The genetic and molecular analyses of the different aspects of genetic instability allow us to propose that they result from a cascade of molecular events and to investigate the relationships between genetic instability phenomena and genome fluidity.

Genetic instability in *Streptomyces* species

Several phenotypic traits are repeatedly reported to be unstable in *Streptomyces* species (Hütter and Eckhardt, 1988). The characters affected include aerial mycelium formation, spore production, antibiotic resistance and/or production, and the formation of extracellular enzymes such as tyrosinase (involved in pigmentation). Some of these characters have been shown to be controlled by pleiotropic regulatory genes such as the unstable *afsA* gene, which affects streptomycin production, streptomycin resistance and spore production. The number of prototrophic characters that are reportly unstable (leading to auxotrophy) is more limited. Moreover, the metabolic steps affected by the instability are common to all mutants of the same character (e.g. tyrosinase is always absent from non-pigmented (Mel^-) mutants and arginosuccinate synthetase is always absent from Arg^- mutants). Thus, specific mutable genes seem to be reproducibly affected, indicating that genetic instability occurs at 'hot spots'.

In *Streptomyces ambofaciens* DSM40697, a basic genetic instability affects the pigmentation character and produces pigment-defective colonies at a frequency of $0.97 \pm 0.17 \times 10^{-2}$ (Leblond et al., 1989). Auxotrophic mutants were obtained from the same wild-type clones at a frequency close to the classical mutation rate (5×10^{-5}). The pigmentation process is expressed late in growth, indicating that, as in other *Streptomyces* species, the basic instability preferentially affects characters that are developmentally regulated.

Treatments used to induce genetic instability in *Streptomyces* strains are by no means always classical mutagenic conditions. For example, cold storage, growth at high temperature or nutritional shifts generate mutant strains at high frequencies and yet are not normally considered to be mutagenic. Several hypotheses might explain the induced increase in mutability. For example, the stability of the replisome-DNA complex might be affected under growth conditions that modify the replication rhythm (particularly treatment with intercalating dyes, spore germination or the resumption of growth, after cold storage), resulting in errors in DNA replication. Such aberrant behaviour could occur at palindromic or short repeated sequences, which are frequent in the G+C-rich (72%) genome of *Streptomyces* (Usdin and Kirby, 1988). Alternatively indirect effects on DNA replication could trigger DNA damage and consequently an SOS-like response although there is, as yet, no evidence for such a response in *Streptomyces*. The resulting increase in recombination could cause deletions to occur between repeated sequences (Usdin and Kirby, 1988). Whatever the mutational mechanism, there are many reports of

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intercalating dyes inducing deletions either in chromosomal (Hintermann *et al.*, 1984; Schrempf, 1985) or plasmid DNA (Schrempf, 1985; Crameri *et al.*, 1986).

Plasmid loss has been proposed as a possib le explanation for unstable characters in *Streptomyces*. Indeed, the strongest pieces of genetic evidence supporting this hypothesis were the high frequencies of loss of only certain characters, which was consistent with the loss by segregation of extrachromosomal DNA. Moreover, typical plasmid-curing agents such as intercalating dyes or u.v. light as well as protoplast regeneration efficiently increase the spontaneous loss of the characters, although they also generate mutations, especially via deletions.

It has been established that particular characters such as tylosin production, amplifiable DNA or resistance to several antibiotic resistances in *Streptomyces fradiae* or *Streptomyces achromogenes* subspecies *rubradiris* can be conjugally transferred at high frequencies relative to presumed chromosomal markers (Stonesifer *et al.*, 1986; Hornemann *et al.*, 1987), suggesting the existence of autonomous or chromosomally integrated plasmids. Such unstable elements have not yet been physically characterized using classical methods for isolating plasmid DNA, which suggests that they are either too large to be purified without breakage or are excised from the chromosome only under particular conditions. Moreover, some characters cannot be localized unambiguously relative to known chromosomal markers by recombination mapping. For example, while a locus for the A-factor was chromosomally defined in *Streptomyces coelicolor* A3(2), attempts to map it on the chromosome in *S. griseus* were unsuccessful (Hara *et al.*, 1983), and the chloramphenicol-resistance functions could not be mapped to a unique chromosomal site in *S. coelicolor* A3(2) (Freeman *et al.*, 1977). There are indeed numerous reports of the detection of plasmid DNA in Streptomyces (reviewed by Hütter and Eckhardt, 1988), and pulsed-field gel electrophoresis has been used recently to detect giant linear plasmids in several antibiotic-producing *Streptomyces* (Kinashi *et al.*, 1987). The first demonstration that genes required for antibiotic synthesis could be located on a plasmid was the report by Wright and Hopwood (1976), who showed that the genes for methylenomycin were located on plasmid SCP1 from *S. coelicolor* A3(2). Kinashi *et al.* (1987) ascribed the methylenomycin-resistance locus to a family of linear plasmids ranging from 410–590 kb.

To our knowledge, no clear correlation between plasmid presence and unstable characters has been reported. Moreover, in the well-documented case of *Streptomyces glaucescens*, Crameri *et al.* (1984) showed that although the unstable genes for streptomycin resistance and the melanin loci were physically linked, the streptomycin genes could be unambiguously mapped whereas a melanin gene (*melC*) could not (Birch *et al.*, 1989). However, this result was proposed to be the result of interference with the recombination frequencies caused by additional mutations occurring concomitantly with the *melC* mutation rather than by the extrachromosomal inheritance of the melanin trait.

High rates of spontaneous mutation could also be explained by the integration-excision cycle of a transposon-like sequence, as reported in eukaryotic systems. The reports of reversible instability systems (Freeman *et al.*, 1977; Kirby and Lewis, 1981; Potekhin and Danilenko, 1985; Flett and Cullum, 1987) would be consistent with this hypothesis. However, no correlation between the presence of a transposon and unstable characters has been demonstrated. In addition, Flett and Cullum (1987) showed that the frequent reversion from chloramphenicol sensitivity to resistance in *S. coelicolor* A3(2) was probably due to a second-site suppressor mutation.

Genetic instability in *S. ambofaciens* strains

In our laboratory, four *S. ambofaciens* isolates from different localities have been investigated for unstable characters: *S. ambofaciens* DSM40697, ATCC23877, ETH9427 and ETH11317. A basic genetic instability affecting the pigmentation character in *S. ambofaciens* DSM40697 was characterized (Leblond *et al.*, 1989). Thus, the wild-type strain spontaneously produces not only pigment-defective colonies (frequency, $0.97 \pm 0.17 \times 10^{-2}$) but also colonies with either pigment-defective sectors ($7.8 \pm 1.4 \times 10^{-2}$) or pigment-defective papillae ($7.9 \pm 1.3 \times 10^{-2}$), each of which probably represents different aspects of the same mutational event occurring at different stages during colony development. Thus, a sector will appear if a mutation occurs during the radial development of the vegetative mycelium, whereas a papilla will appear if a mutation occurs during the vertical growth of the aerial mycelium, and totally non-pigmented colonies could result from a mutation occurring during sporogenesis and present in the spore DNA. This hypothesis is supported by the distribution of pigment-defective colonies after plating of spores of 54 independent wild-type clones, which was significantly different from a random distribution and revealed a clonal effect of the mutational event. The earlier this mutation occurs, the higher the frequency of pigment-defective colonies after plating out.

Both pigmentation and aerial mycelium production are unstable in *S. ambofaciens* ATCC23877, ETH9427 and ETH11317 (J.-N. Volff, unpublished data). The frequencies of fully pigment-defective, sectored or papillae-harbouring colonies were not significantly different between the four isolates when observed at the same time of growth. However, *S. ambofaciens* ATCC23877, ETH9427 and ETH11317 produced colonies without aerial mycelia at a

frequency of about 0.01, whereas only colonies with aerial mycelia were produced by *S. ambofaciens* DSM40697. In addition, *S. ambofaciens* ATCC23877 spontaneously generated a new type of mutant colony with a fully pigmented mycelia (vegetative and aerial) whereas only the aerial mycelium was pigmented in a wild-type colony. These mutants occurred at a frequency of $2.7 \pm 0.6 \times 10^{-2}$. These frequencies did not vary significantly when colonies were grown at 30°C or 37°C, except for sectored colonies for which the frequency increased from $0.28 \pm 0.13 \times 10^{-2}$ at 30°C to $7.8 \pm 1.4 \times 10^{-2}$ at 37°C.

Information on the mechanism of the genetic instability was provided by the distribution of pigment-defective papillae on colonies raised from the wild-type strains. Whereas in *S. ambofaciens* DSM40697, ETH9427 and ETH11317 this distribution could be consistent with a random distribution, *S. ambofaciens* ATCC23877 produced some colonies with significantly more papillae than others. This heterogeneity reveals an instability which is manifested at the next generation, since the experimental distributions of the progeny of colonies chosen at the extremities of the distribution were significantly different and remained heterogeneous. These facts suggest the existence of an initial genetic step that switches on instability.

Hypervariability

Studies on the stability of the pigment-defective colonies arising from wild-type *S. ambofaciens* (DSM40697) allowed us to characterize a new aspect of genetic instability called hypervariability (Leblond *et al.*, 1989). This new phenomenon was defined as the spontaneous occurrence of a phenotypic heterogeneity from a pigment-defective colony generated by the basic genetic instability. 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 2–8, most often 4 or 5. This heterogeneity was observed in the progeny of 87% of pigment-defective colonies. No heterogeneous progeny were observed after plating more than 1500 wild-type clones, indicating that hypervariability is observed after the basic genetic instability event has occurred.

Similar heterogeneity can be generated in this and other *Streptomyces* species in other ways, e.g. when wild-type spores are plated on complete solid medium (Hickey-Tresner medium) containing low doses of ethidium bromide. When protoplasts are regenerated from a wild-type strain, phenotypic variability is observed among the surviving colonies. Analogous features have been observed by others in different strains after growth in rich liquid medium (D. Schneider, unpublished data).

These observations allow us to propose a hypothetical pathway leading to hypervariable lineages in which hypervariability observed in the progeny of a pigment-defective colony could result from the genomic heterogeneity of this colony. This hypothesis allows us to introduce the notion of 'genomic hypervariability' triggered during the development of a pigment-defective colony and affecting the pigmentation genes so that all the genotypically heterogeneous colonies exhibit the original pigment-defective phenotype.

Genomic hypervariability could result from numerous different mutations triggered by the primary mutational event that destabilized the genome. This instability would be manifested as a dramatic increase in mutability at the next round of sporulation, generating genomic heterogeneity in a pigment-defective colony. Hypervariability in Streptomycetes might be compared to the phenotypic variability observed in maize, with multiple mutations leading to genomic heterogeneity in pigment-defective colonies being induced through a sequential mechanism analogous to the 'breakage-fusion-bridge cycle' in chromosomes of plant cells (McClintock, 1951).

Hypervariability and heterogeneity observed after non-classical mutagenic treatments share numerous similar characteristics and could be generated via the same mutational pathway. Although the induced hypervariability seems to be produced via multiple independent mutational events, as in a mutagenic process, it could be explained by the cascade hypothesis if, for example, the ethidium bromide triggers a unique mutation that causes numerous mutations to be spontaneously generated, leading to hypervariability. Thus, the induced hypervariability could be useful as a model system for studying spontaneous hypervariability. Further work is required to refine this model.

Genome plasticity

Molecular analyses of mutant strains arising from spontaneous or induced instability often reveal DNA rearrangements such as deletions, amplifications or both (reviewed by Hütter and Eckhardt, 1988). Amplifications or amplified DNA sequences (ADSs) derived by high reiteration of amplifiable units of DNA (AUDs; nomenclature according to Fishman and Hershberger, 1983). These phenomena seem to be ubiquitous in *Streptomyces*, but the selective pressure leading to high copy-number amplifications generally remains unknown. Indeed, except for amplification of selected genes for antibiotic resistance, the phenotypes conferred by the ADSs are not yet specified. In addition, the frequencies at which ADSs occur in highly selected genes could be quite different to those of spontaneous ADSs resulting from general genetic instability, and the two types of ADSs

could arise from structurally different AUDs. This point must be considered when analysing the structure of induced ADSs produced under highly selective conditions as models for ADSs in general.

In some cases, a correlation has been established between the presence of an ADS and a particular phenotype. For example, all melanin-negative mutants (Mel^-) of *Streptomyces scabies* (Schrempf, 1985) and 94% of the double chloramphenicol-sensitive arginine auxotroph (Cam^S Arg^-) mutants of *Streptomyces lividans* 66 (Altenbuchner and Cullum, 1985; Dyson and Schrempf, 1987) were shown to contain ADSs. In the latter case, this double phenotype was associated with the amplification of a 6.8 kb AUD (Altenbuchner and Cullum, 1984). Similarly, all ADS-containing strains of *S. glaucescens* were streptomycin-sensitive (Str^S) and Mel^- (Hasegawa *et al.*, 1985). In *S. ambofaciens* DSM40697, the molecular analysis of clones independently isolated from stable, hypervariable and wild-type colonies also reveals a correlation between hypervariability and DNA amplification. ADSs were detected in 21% of strains derived from hypervariable progeny, whereas none were detected in either stable pigment-defective strains or wild-type colonies (Leblond *et al.*, 1989). Although the majority of ADS-containing mutant strains were pigment-defective, they were also often affected in numerous other morphological traits. Analogous results were obtained in studies of a mutant clone of *S. lividans* 66 (named M252); 30% of the progeny of this strain was morphologically heterogeneous and 70% of these variant clones contained ADSs (Schrempf, 1985).

Studies on the mechanism of amplification in *S. ambofaciens* are simplified by the fact that ADSs can be reproducibly generated from hypervariable lineages at high frequency without the need to apply exogenous selective pressure. Thus, many different ADSs can be structurally analysed. In addition, stable pigment-defective and hypervariability-derived strains that do not contain any ADSs could be useful tools for trapping a possible pre-amplifiable state.

The size of the ADSs in *S. ambofaciens* ranges from 5.2–55 kb and the degree of amplification is quite variable between strains. Restriction analysis and cross-hybridization experiments allowed us to detect overlapping stretches between some of ADSs and thus to characterize at least two families of AUDs (Demuyter *et al.*, 1988). This notion of a large genomic region likely to undergo amplifications or deletions was already suggested by Hasegawa *et al.* (1985) for *S. glaucescens*. On the other hand, when the structural gene or DNA sequence corresponding to the unstable character was cloned, the mutant trait was ascribed to large deletions including this sequence. For example in *S. lividans* 66, the double mutant phenotype Cam^S Arg^- probably results from the deletion of the sequence conferring chloramphenicol resistance and the arginosuccinate synthetase structural gene (Flett and Cullum, 1987; Dyson and Schrempf, 1987). The size of these deletions was estimated, by hybridization of the total DNA of the mutant strain against a cosmid bank prepared from a wild-type strain, to be about 250 kb each (Cullum *et al.*, 1988). In the same way, the double mutant phenotype Str^S Mel^- in *S. glaucescens* was ascribed to deletion of the phosphotransferase structural gene and *melC*, the tyrosinase structural gene (Hintermann *et al.*, 1984; 1985). Recently, the sizes of these deletions were shown to range from 270 kb to over 800 kb by cosmid bank analyses (Birch *et al.*, 1989).

In *S. ambofaciens* DSM40697, the genomic structure in the AUD6 region was investigated in mutant strains isolated from stable and hypervariable lineages. For this purpose, restriction digests of the total DNA of the mutant strains were hybridized with the cloned end points of ADS6. These hybridization patterns revealed a high frequency (about 35%) of DNA rearrangements such as duplications or, more frequently, deletions in the close

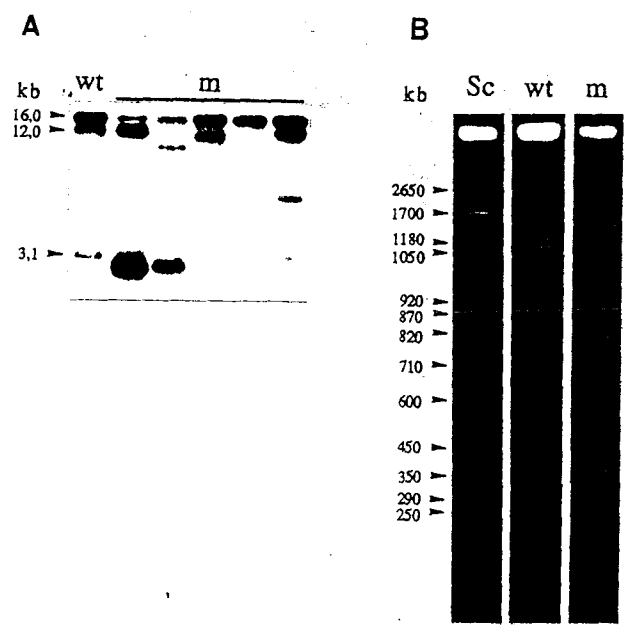

Fig. 1. Hybridization and electrophoretic analyses of total DNA of wild-type (WT) and mutant strains in *S. ambofaciens*.
A. Southern blots of *Bam*HI digests of total DNA from the WT and mutant (m) strains probed with the cloned extremities of ADS6. The hybridization pattern of AUD6 in the WT strain is characterized by the two flanking sequences (16 kb and 3.1 kb) and a sequence homologous to only one extremity within a 12 kb restriction fragment of the AUD.
B. Pulsed-field electrophoresis analysis of *Ase*I digests of total DNA from WT and a mutant strain of *S. ambofaciens*.
S.c., *Saccharomyces cerevisiae* chromosomes used as size standard. The *Ase*I WT pattern is characterized by nine detectable restriction fragments ranging from about 75 kb to 1850 kb. The mutant pattern (m) shows several missing fragments (totalling more than 2000 kb) and a new additional one (estimated to be about 150 kb).

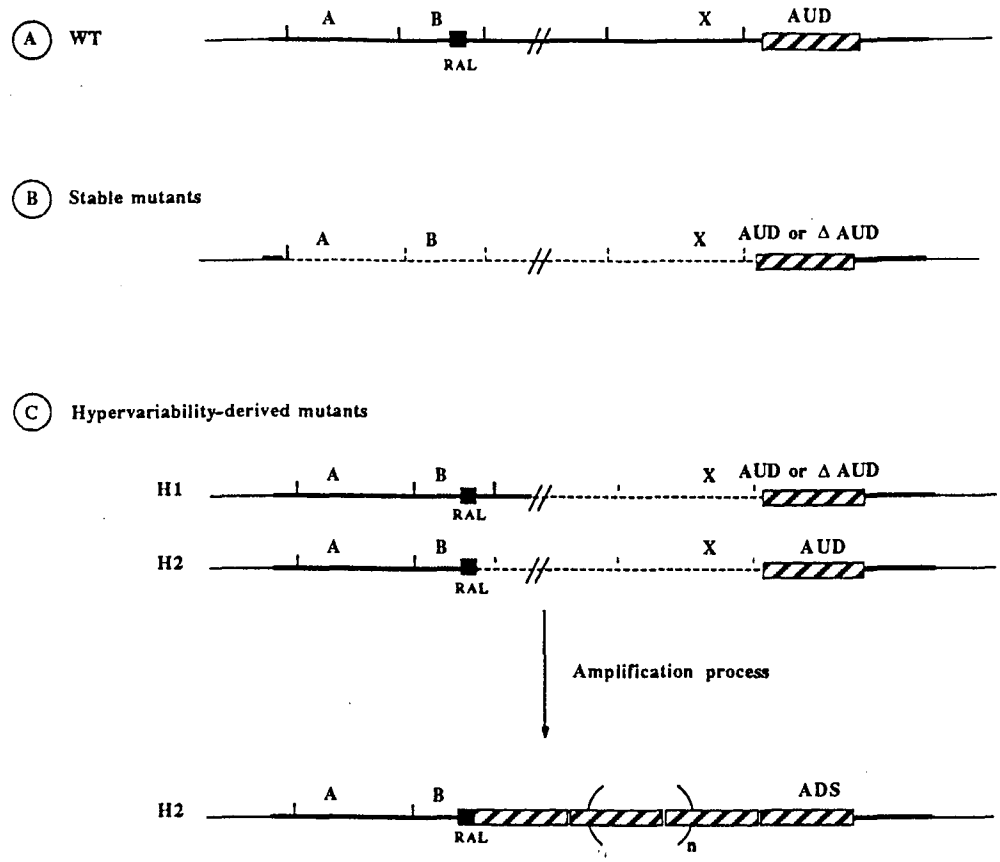

Fig. 3. Hypothetic genomic structure upstream from an AUD region in wild-type (WT), stable and hypervariable strains. A to X represent unstable character structural or regulatory genes in the WT genome. The dotted lines represent deleted areas. The hatched boxes represent an AUD either entirely or partially deleted. The black box represents a replication activating locus (RAL).

proximity of AUD6 in the hypervariable strains as well as stable mutant strains (Fig. 1A) whereas none could be detected in wild-type clones. AUD6 was either partially or totally deleted. In addition, these deletions affected only one flanking sequence of the AUD. Thus, the AUD6 region constitutes a 'hot spot' for genomic rearrangements (P. Demuyter *et al.*, in preparation). In addition, *Ase*I and *Dra*I restriction patterns of the same mutant strains were carried out using pulsed-field electrophoresis (PFE). Stable as well as hypervariability-derived strains (amplified or not) reveal large genomic changes relative to the wild-type strain. All wild-type clones shared the same characteristic restriction pattern. An example of a mutant pattern (Fig. 1B) shows that several large bands are missing while only one new band appears, suggesting that a very large deletion has occurred (exceeding 2000kb). However, important structural changes such as the circularization of large DNA stretches or DNA modification at the restriction sites could explain the absence of several restriction fragments in PFE since large circular molecules are known to be unable to penetrate the gel and large linear fragments (more than several thousand kilobases) could remain unresolved. These structural changes associated with genetic instability phenomena could affect more than 20% of the genome (estimated to about 8000kb by restriction analysis) (Leblond *et al.*, in preparation).

In earlier studies, deletions were reported to be associated with amplifications (see Hütter and Eckhardt, 1988). These deletions were detected either immediately adjacent or in the proximity of ADSs (Hasegawa *et al.*, 1985; Dyson and Schrempf, 1987; Häusler *et al.*, 1989). Hence, deletions are a common feature of the amplification mechanism and are responsible for the mutant characters if the genes corresponding to the unstable traits are located in the proximity of the AUD. This situation has been well documented in *S. lividans* 66 (Dyson and Schrempf, 1987), *S. glaucescens* (Birch *et al.*, 1989) and S. fradiae (for review, see Baltz and Seno, 1988).

Figure 2 illustrates the structures of the two main types of AUD found in *Streptomyces*. The first (type II in the nomenclature proposed by Hütter *et al.*, 1988) was characterized in *S. lividans* 66 TK 64 and comprises a symmetrical structure including two large identical

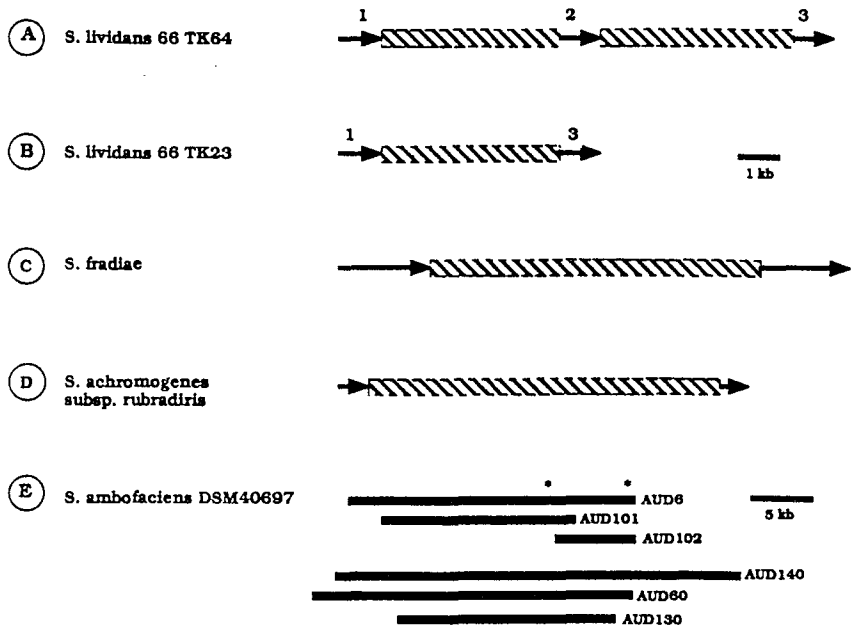

Fig. 2. Main structures of the amplifiable unit of DNA (AUD) in *Streptomyces species*.
A. Duplicated structure in *S. lividans* 66 TK64 (type II AUD) (Altenbuchner and Cullum, 1985). Sequence 2 shows restriction-site polymorphism suggesting an unequal recombination event between sequences 1 and 3 during a replication cycle.
B. Unduplicated form of the AUD of *S. lividans* 66 TK64 found in a closely related strain *S. lividans* 66 TK23 (Dyson and Schrempf, 1987).
C. Unduplicated form of the AUD of *S. fradiae* (Fishman *et al.*, 1985).
D. Unduplicated form of the AUD of *S. achromogenes* subspecies *rubradiris* (Hornemann *et al.*, 1987).
E. Amplifiable region in *S. ambofaciens* DSM40697 (type I AUD).
These AUDs were shown to belong to the same amplifiable region since overlapping sequences between them were characterized by restriction analyses and cross-hybridization experiments (Demuyter *et al.*, 1988; Leblond *et al.*, 1989). Asterisks indicate the repeated sequence homologous to the cloned extremities of the AUD6.

sequences (4–5 kb; hatched in Figure) flanked by three short direct repeats (0.7–1.7 kb; bars in Fig. 2A) (Altenbuchner and Cullum, 1985). This duplicated structure was assumed to be formed from a single AUD by unequal recombination and was further assumed to play the role of a rate-limiting step in the high amplification process. This hypothesis was supported by the report of a lower frequency of ADSs in the related strain *S. lividans* 66 TK23, in which a single copy of the central sequence flanked by two direct repeats was detected (Fig. 2B) (Dyson and Schrempf, 1987), although in *Streptomyces achromogenes* subspecies *rubradiris* (Fig. 2D) (Hornemann *et al.*, 1987) and *S. fradiae* (Fig. 2C) (Fishman *et al.*, 1985) the AUD is composed only of a central sequence flanked by two long direct repeats, but can nevertheless trigger amplification.

The second type of structure (type I according to Hütter *et al.*, 1988) was mainly detected in both *S. glaucescens* and *S. ambofaciens* (Fig. 2E) (Hasegawa *et al.*, 1985; Demuyter *et al.*, 1988). On the basis of overlapping sequences, the ADSs in both species could be grouped into families covering large DNA regions. In addition, sequencing of the end points of three *S. glaucescens* AUDs revealed short, imperfect tandem repeats called 'microhomologies' (Häusler *et al.*, 1989). In *S. ambofaciens*, hybridization experiments with the cloned extremities of ADS6 allowed us to detect repeated sequences within AUD6 (Fig. 2E). However, we do not know if this is a common characteristic of AUDs in *S. ambofaciens* and so its role in deletion formation and amplification is hypothetical. In addition, we cannot exclude the possible existence of short repeats at the end points of the ADS, as in *S. glaucescens*, that are undetectable by hybridization. It may be that long terminal repeats provide a favourable structure for reproducible amplification while short repeats (at the extremities or inside the AUD) could trigger amplification more randomly at multiple loci within a given region.

In spite of these structural differences, amplification could occur by a common mechanism based on an over-replication process. Such a model, involving rolling circle-like replication, has been postulated by Young and Cullum (1987). The first step of this model is a recombination event between long repeated sequences, producing a rolling circle in the chromosome. However, other events such as template switching could be responsible for triggering over-replication of a particular sequence (Hyrien *et al.*, 1988).

Towards an understanding of the molecular mechanism for genetic instability in *S. ambofaciens*

The high level of genomic instability associated with the generation of a variety of phenotypes raises questions about the biological significance and the mechanism of genetic instability. The phenomenon could reflect a general property of the *Streptomyces* genome related to its high G+C content or its general organization. Several organisms showing differentiation or a complex development cycle simultaneously exhibit large genomic rearrangements such as site-specific recombinations and deletions to create genes or expression frames, as in

Bacillus subtilis (Stragier *et al.*, 1989) or *Anabaena* species (Golden *et al.*, 1988), or large deletions and gene amplifications in a specific genomic region associated with, for example, differentiation in ciliated protozoans (Blackburn and Karrer, 1986).

In *S. ambofaciens* DSM40697, mutant phenotypes arising from both basic instability and consequent hypervariability were associated with DNA rearrangements. About 35% of the mutants carried either deletions or duplications in the AUD6 region, demonstrating that it constitutes a hot spot for DNA rearrangements. Moreover, PFE analyses revealed a strict correlation between instability (basic instability and hypervariability) and large genomic changes, and amplified DNA was detected in mutant strains generated only after a hypervariability event, suggesting the existence of a pre-amplifiable state present only in hypervariability-derived strains. Thus, genomic instability is probably the source of mutant phenotypes generated by genetic instability. In particular, amplification could occur after a primary DNA rearrangement event and thus appear to be a consequence of this event.

We would now like to present a model which could explain the genomic instability in *S. ambofaciens* (Fig. 3). The model embodies many of the ideas presented previously by others, together with refinements that we have introduced. In the wild-type genome (Fig. 3A), the structural genes corresponding to unstable characters (A to X) are grouped in a large region extending over several hundred kilobases that contain several dispersed AUDs. We observed that 13% of the pigment-defective colonies spontaneously generated from the wild-type strain gave rise to stable pigment-negative strains, suggesting that pigmentation determinants have been deleted, possibly together with a variable number of flanking genes (Fig. 3B).

Deletions and large genomic rearrangements have also been detected in amplified and non-amplified mutants of hypervariability-derived strains. Thus, mutations in the hypervariability-derived strains could be deletions too (Fig. 3C). For example, a small initial deletion could be enlarged during the development of a pigment-defective colony, generating a population of mutant genomes. Variations in the size of the second deletion would cause different determinants to be affected and would explain the phenotypic heterogeneity. Spores of a given colony containing variable genomes would then give rise to the two types of progeny: (i) stable mutant strains (64%), and (ii) hypervariable progeny in which further deletions could occur (36%).

Hütter and Eckhardt (1988) postulate that AUDs could be created prior to amplification either by an internal deletion between two DNA repeats or by transposition of one flanking sequence. In *S. ambofaciens*, this hypothesis can be largely discounted since we have demonstrated in the case of the AUD6 that the ADS was composed of tandem reiterations of the wild-type AUD. Thus, the potential for an AUD to be amplified is probably not due to a primary rearrangement of the AUD sequence itself. Moreover, taking into account the facts that no ADSs could be detected in a large sampling of wild-type clones and that all mutants exhibit large genomic rearrangements, it seems plausible that amplification is a consequence of a primary DNA rearrangement, i.e. the mere existence of an entire AUD in the wild-type genome cannot trigger amplification efficiently, so the primary rearrangement events are more likely to provide the amplifiable state.

To take up a notion mentioned by Häusler *et al.* (1989), the potential of an AUD to be amplified would result from the nature of the joining fragment after deletion. In our model, a replication activating locus (RAL) is joined to the AUD (Fig. 3C). To explain why only 21% of the hypervariability-derived strains contain ADSs, we propose that strains without ADSs could result from deletions of RAL during the cascade of deletion events or by formation of joining fragments where RAL is not correctly positioned relatively to the AUD to trigger amplification (Fig. 3C). Several RAL sequences would be dispersed in this large DNA region and thus would trigger amplification of many potential AUDs, generating the many phenotypes known to be presented by ADS-containing strains. RAL could be analogous to the amplification enhancer at the autosomal chorion gene of *Drosophila* (Delidakis and Kafatos, 1989). Furthermore, this model implies that all strains with amplified DNA segments have also suffered large deletions, as suggested for *S. ambofaciens* and numerous other *Streptomyces* species.

The close association between genetic instability and genomic plasticity leads us to consider the nature of the relationships between these phenomena. First, the phenotypic variability indicative of genetic instability could be the direct consequence of the DNA rearrangements which are the origin of genome plasticity. Second, genetic instability and genome plasticity could be the consequences of a primary mutational event, the basic genetic instability and hypervariability being the phenotypic expression of a cascade of DNA rearrangements. The possibility that the primary mutation could affect recombination suppressors is raised by the observation made by Rayssiguier *et al.* (1989) that recombination between *Escherichia coli* and *Salmonella typhimurium* occurs more efficiently when the strains are deficient in mismatch DNA repair. In addition, a *Streptomyces cattleya* mutant strain deficient in a DNA-repair system was reported to exhibit enhanced genetic instability (Hromic and Kirby, 1989).

Relaxed recombination in these hypothetical mutants could occur more favourably at the frequently repeated sequences detected in the *Streptomyces* genome (Usdin

and Kirby, 1988). Moreover, the finding of imperfect palindromic sequences at the extremities of AUDs and at the ends of a deletion in *S. glaucescens* (Häusler *et al.*, 1989; Birch *et al.*, 1989) supports the hypothesis that short repeated sequences are required for genome plasticity of *Streptomyces*. These imperfect palindromic sequences could be analogous to the PU sequences (Palindromic Unit) reported in *E. coli* and related species (Gilson *et al.*, 1987). In addition, Brunier *et al.* (1989) have shown that while recombination by a breakage-reunion mechanism is efficient on repeated sequences longer than 18 bp, copy choice recombination is highly efficient when repeated sequences as small as 9 bp are used as substrates. Thus, the high G+C content of the *Streptomyces* genome could provide many targets (repeated sequences or PU-like sequences) for such a recombinational mechanism.

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GENOME PLASTICITY ASSOCIATED WITH GENETIC INSTABILITY AND HYPERVARIABILITY IN *STREPTOMYCES AMBOFACIENS*.

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Streptomyces ambofaciens DSM40697 exhibits a high degree of genetic instability revealed by the occurence of about 1 % of pigment-defective colonies from the WT strain. While only 13 % of these mutant colonies show a homogeneous mutant progeny, 87 % give rise to hypervariable progeny.

In the strain DSM40697, the molecular analysis of the total DNA isolated from WT, stable and hypervariable lineages reveals DNA amplifications related to phenotypic hypervariability. Two families of amplifiable unit of DNA, the AUD6 and AUD103 families have been characterized.

In order to detect other rearrangements of the AUD6 region in various mutant strains, hybridization experiments were carried out using the cloned fragment generated by the tandem reiteration of the AUD as a probe. Hybridization patterns reveal no rearrangement in the WT strains, and a high frequency of large deletions overlapping the AUD6 region in the mutant strains isolated not only from the hypervariable lineages, but also from the stable ones. Therefore, deletion events are closely related to the genetic instability. This fact suggest a mechanism of genetic instability through a cascade of mutational events.

A few patterns show one duplication of a sequence homologous to the probe. Similar analysis of subclones of the AUD6 amplified strain reveal a loss of the amplification associated with a rearrangement of the AUD in 30 % of the strains. Thus, the AUD6 region is an unstable region undergoing duplication, amplification or deletion. Hence, this region could be a hot spot for rearrangements involved in the mechanism of genome plasticity.

Pulsed field gel electrophoresis was used in order to detect DNA rearrangements at the level of the whole genome. Analysis of the large restriction fragments generated by the enzymes *Ase*I and *Dra*I indicates a minimal size for the WT genome of some 6,500 kbp. The restriction patterns of independant stable pigment-defective strains as well as independant hypervariable strains reveal missing bands compared to the WT. Some of the missing bands are common to all the mutants and add up more than 2,000 kbp (i.e., about 30 % of the estimated genome). This was not observed if *Hin*dIII was used instead of *Ase*I or *Dra*I. This fact suggests perhaps that the missing bands are not deleted but have undergone structural changes.

PULSED-FIELD GEL ELECTROPHORESIS ANALYSIS OF THE GENOME OF *STREPTOMYCES AMBOFACIENS* STRAINS.

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Key Words: Macrorestriction pattern analysis, *Streptomyces ambofaciens*, Pulsed-field gel electrophoresis, Concatemeric linear molecules, Genome size, Intraspecific polymorphism.

SUMMARY

The genome of four *Streptomyces ambofaciens* strains of organisms from different geographical locations (ATCC15154, DSM40697, ETH9247 and ETH11317) was analysed by pulsed-field gel electrophoresis (PFGE). The PFGE technique has allowed the study of the extrachromosomal content of these strains and the characterization of their genomic DNA by restriction analyses.

Electrophoretic migration of undigested DNA allowed us to detect a 80 kb-length linear molecule with concatemeric forms in *S. ambofaciens* ATCC15154. These extrachromosomal molecules were shown to be homologous to the circular plasmid pSAM1 (80 kb) suggesting that pSAM1 could exist not only in circular form but also in linear form. In the same way, a 45 kb-length linear molecule was detected in *S. ambofaciens* ETH9427 and ETH11317. In contrast, no extrachromosomal DNA could be detected in *S. ambofaciens* DSM40697.

Restriction patterns using the rare-cutting enzymes *Ase*I and *Dra*I were derived. The analysis of the macrorestriction patterns indicated a close relationship between the DSM- and ETH- strains. Indeed, three types of restriction patterns were distinguished: while *S. ambofaciens* ETH9427 and ETH11317 were characterized by the same pattern and share more than 75% of comigrating fragments with the strain DSM40697, *S. ambofaciens* ATCC15154 exhibited a restriction pattern different from the other three. The total genome sizes of *S. ambofaciens* ATCC15154, DSM40697, ETH9427 and ETH11317 were estimated to be about 6,500 kb, 8,000 kb, 8,200 kb and 8,200 kb respectively.

INTRODUCTION

Pulsed-field gel electrophoresis (PFGE) provides a new approach to study the size and the organization of bacterial genomes. *Escherichia coli* genome was the first report of genome size estimation and physical mapping of the entire genome using PFGE [1]. Data concerning the size of the chromosome of *Streptomyces* species are differ. Measurement of reassociation kinetics and separation of single-strand and double-stranded by high-pressure liquid chromatography (HPLC) led to estimations ranging from 4.9 x 10^3 kb to 10.5 x 10^3 kb [2, 3, 4]. Recently, the PFGE technique allowed to estimate the genome size of *Streptomyces coelicolor* A3(2) to be about 5.8 x 10^3 kb [5]. In addition, PFGE studies of*Streptomyces ambofaciens* were briefly reported [6].

The aim of this study was to investigate the extrachromosomal content of *S. ambofaciens*, to estimate the genome size and to compare the restriction patterns of the four strains assumed to belong to the same species. For this purpose, *Ase*I and *Dra*I (which are rare-cutting enzymes for the high G+C content of *Streptomyces* DNA) digests of preparations of high molecular weight DNA were highly resolved into characteristic restriction patterns using PFGE.

MATERIALS and METHODS

Bacterial strains. The four wild type (WT) strains of *S. ambofaciens* used in this work are listed in table 1.

Plasmid DNA preparation. Plasmid DNA (pSAM1) was extracted from *S. ambofaciens* ATCC15154 by an alkaline lysis method [10] and further purified by a cesium chloride-ethidium bromide gradient.

DNA preparation, restriction analysis and pulsed-field gel electrophoresis (PFGE) conditions. S. ambofaciens mycelia were grown in Hickey-Tresner liquid medium [11] supplemented with glycine (0.25%) at 30°C for 8 to 24 hours, or at 37°C for 8 hours. Mycelia were washed in ES solution (10.3% Sucrose 0.25 M EDTA), resuspended in ES solution containing lysozyme (Boehringer Mannheim)(20 mg/ml) and finally embedded in 1.5% low melting point agarose [LMPA (Appligène)] dissolved in ES solution to an end concentration of 0.5% LMPA. The mixture was dispensed in a plug former and incubated for 10 mn at 0°C. Then, the agarose plugs were incubated in a solution of 0.5 M EDTA, 1% N-lauroylsarcosine, 5 mg/ml Pronase E (Sigma) for 12 h at 50°C. After washing the plugs for 3 h in 10 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) with continuous shaking, 1 mM phenylmethanesulphonylfluoride [PMSF (Boehringer Mannheim)] was added to inhibit proteinases. Plugs are stored at 4°C in 0.5 M EDTA.
For restriction analysis, slices of the agarose plugs containing the high molecular weight genomic DNA were washed with the appropriate restriction buffer (according to [12]) at room temperature and incubated at 37°C with 50 units of the restriction enzyme [*Ase*I (Biolabs) or *Dra*I (Boehringer Mannheim)].
Chromosomes of *Saccharomyces cerevisiae* prepared as in [13] were used as high molecular weight standards [14]. Concatemers of bacteriophage lambda DNA provided a convenient size marker up to 1,000 kb (Bio-Rad, USA). Electrophoreses were performed in 1% agarose gels in 0.5x TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) at 14°C in a CHEF (Contour Clamped Homogeneous Electric Field, Biorad, USA) system [15]. Start and end pulse times were usually adapted to maximize resolution of DNA fragments depending on the molecular weights. After electrophoresis, gels were stained with ethidium bromide (E.B.).

Radioactive labelling of DNA. Southern blotting and hybridization. pSAM1 plasmid DNA was labelled with ^{32}P (Amersham) according to [16] using the Multiprime DNA labelling system kit (Amersham) enabling high specific activity (4×10^9 dpm/µg). For hybridization experiments, Southern blots were performed using the capillary transfer method with the Vacugene system (LKB) on Hybond-N membranes (Amersham). Because of the large size of DNA fragments to be transferred, the DNA was depurinated with 0.25 HCl for 30 mn before denaturation (in 1.5 M NaCl, 0.5 M NaOH for 1 hour), neutralized (in 1.5 M Tris-HCl, 1.5 M NaCl for 30 mn) and transfered in 20x SSC (1x SSC= 0.15 M-sodium chloride, 0.015 M-sodium citrate) for 90 mn. Prehybridization, hybridization and washing conditions were as previously described [17].

Densitometric analysis. Densitometric readings of the PFGE restriction patterns were performed using an Ultrascan densitometer (LKB, Sweden). Peak areas were measured by integrative calculation.

RESULTS

Extrachromosomal DNA molecules

In order to detect extrachromosomal DNA molecules, undigested samples of total DNA of the four strains were electrophoresed under migration conditions which resolved linear fragments ranging from 20 kb to 3,000 kb. Thus, a typical bacterial chromosome would be able to enter the gel taking account of its presumably large size and circular form.

While undigested DNA of *S. ambofaciens* DSM40697 grown at 30°C and 37°C (strain B) did not contain extrachromosomal bands (less than 1,000 kb) on a E.B.-stained gel (figure 1.α., lane B; figure 1.β., lane B), DNA preparations of *S. ambofaciens* ATCC15154 (strain A), ETH9427 (strain C) and ETH11317 (strain D) exhibited extrachromosomal DNA molecules dependant on the growth temperature (figure 1.α. and 1.β., lanes A, C, and D, respectively). In strain A, an extrachromosomal molecule estimated to be about 80 kb-length relative to a ladder of concatemers of lambda DNA was detected after growth in liquid cultures performed at 37°C for 8 hours (figure 1.β., lane A) while no extrachromosomal DNA could be detected at 30°C (figure 1.α., lane A). Strains C and D exhibited the same pattern of extrachromosomal DNA bands consisting of at least seven detectable bands after growth at 30°C for 8 hours (figure 1.α., lanes C and D). The intensity of these bands decreased in those bands corresponding to the higher molecular weight. The size of the four more intense bands were measured relative to a ladder of lambda concatemers under optimized migration conditions: these were respectively 45 kb, 90 kb, 135 kb and 180 kb. When the same strains were grown either at 30°C for 24 hours (data not shown) or at 37°C for 8 hours (figure 1.β., lanes C and D), a single band comigrating with the shorter one of the ladder previously reported was detected. In addition, these molecules were assumed to be linear since their positions on the gel relatively to the linear size standards did not significantly change under different switching conditions. These facts were consistent with the concatemerization of a 45 kb-length monomer. This ladder of concatemers could correspond to the transient step of replication either of a linear plasmid or of bacteriophage DNA. Both hypotheses are suitable since the presence of giant linear plasmids (reviewed in [18]) was reported in several antibiotic-producing *Streptomyces* species as well as *Streptomyces* phages [19].

In addition, several high molecular weight bands (at least three) were detected in strains C and D DNA after growth at 37°C (fig. 1.β., lanes C and D). Using migration conditions which resolved preferentially high molecular weight fragments (long pulse times), no discrete band could be detected (data not shown). Thus, these bands could be due to degradated total DNA remaining unresolved under short switching pulse times.

In strain A, the presence of a 80 kb-length molecule could be accounted for by the linearization of the circular plasmid pSAM1. To test this hypothesis, a Southern blot corresponding to the agarose gel of figure 1 was hybridized with ^{32}P-labelled pSAM1 DNA extracted as cccDNA from strain A. An intense hybridizing band heavy signal corresponding to the 80 kb molecule detected on the E.B.-stained gel was observed (figure 2.β., lane A). In addition, several faint signals corresponding to DNA bands estimated to be 165 kb, 240 kb, 330 kb, 425 kb and 500 kb were revealed. These DNA bands were not detectable by ethidium bromide staining(figure 1.β., lane A). Taking account into size estimation errors, these results are consistent with the concatemerization of a 80 kb-length monomer highly homologous to the plasmid pSAM1. Thus, pSAM1

could be present in the strain A in linear and circular forms. No signal could be detected in the same strain after growth at 30°C (figure 2.α., lane A).

In strains B, no signal could be detected on the gel when probing native DNA after growth at 30°C or 37°C (figure 2.α. and 2.β., lane A). In strains C and D, no hybridization signal could be detected corresponding either to the ladder of linear molecules observed at 30°C (figure 2.α., lanes C and D) or to the unique DNA band observed at 37°C (figure 2.β., lanes C and D). So, these molecules did not show any homology with pSAM1 DNA.

In all cases, a strong signal was observed corresponding to the wells. Taking into account the large size of the probe and the repeated sequences dispersed all over the genome as demonstrated by Usdin [20], these signals seemed to be non specific. Hence in strains C and D, a faint signal revealing the high molecular weight smeared DNA could correspond to low homology between pSAM1 and total DNA of *S. ambofaciens* genome. In addition, Pernodet *et al.* [8] showed that pSAM1 was not present in strain B supporting this hypothesis. So, the existence of an integrated form of pSAM1 in the four *S. ambofaciens* strains could be excluded.

Restriction analysis of genomic DNA

Restriction pattern comparison

Considering the high G+C content of the streptomycetes, about 74% [21], restriction enzymes with recognition sequences that contain only A and T nucleotides such as *Dra*I (TTTAAA), *Ase*I (ATTAAT) or *Ssp*I (AATATT) were expected to produce a few bands which could be resolved into a characteristic pattern. While *Dra*I and *Ase*I were found to be convenient, *Ssp*I reproducibly produced partial digests and thus, was not extensively used in further experiments.

Chromosomal DNA of each strain was digested as described above and electrophoresed under migration conditions convenient to resolve each restriction fragment: pulse times were ramped from 5 s to 40 s for 24 h at 180 V to resolve the shortener fragments, the high molecular weight fragments (up to 1,000 kb) were resolved using 100 s to 250 s ramped pulse times for 50 h at 150 V. The size was estimated relative to the linear *Saccharomyces cerevisiae* chromosomes for the high molecular weight fragments and to a ladder of lambda DNA concatemers between approximatively 50 kb to 1,000 kb. The use of these molecular weight standards could have lead to the underestimation (5-10%) of the *Streptomyces* DNA fragments since Maniloff [22] reported that G+C-rich DNA fragments migrate more quickly than A+T-rich DNA fragments of the same size.

Restriction patterns of the four *S. ambofaciens* strains using *Ase*I and *Dra*I are respectively shown in the figures 3.α. and 4.α. Figures 3.β. and 4.β. constitute a schematic interpretation including the estimated size of each *Ase*I and *Dra*I restriction fragment.

The genome of strains A, B, C and D was respectively cleaved by *Ase*I into 14, 12, 12 and 12 fragments ranging from 39 kb to 1,850 kb and by *Dra*I into 10, 10, 12 and 12 fragments ranging from 30 kb to 2,500 kb. Each strain reproducibly displayed a unique restriction pattern for a given enzyme. Indeed, 12 independently isolated and subcloned strain B WT clones were analyzed and shared the same characteristic restriction pattern. Using either restriction enzyme, three types of pattern could be distinguished among the four strains. While A and B were unique, Strains C and D had the same restriction pattern.

The restriction patterns of strains B and C/D showed a high degree of similarity since 7 of the 12 *Ase*I fragments and 8 of the 10 *Dra*I fragments of strain B patterns were found in C and D digests.

Strain A exhibited numerous differences with the three other strains either with *Ase*I or with *Dra*I. The number of comigrating DNA stretches between strain A and the three others is quite low; the only 1,850 kb and 270 kb *Ase*I fragments and the 2,500 kb, 710 kb, 230 kb and 210 kb *Dra*I fragments were common between these strains. Thus, while strains C and D and strain B seemed to be closely related, strain A seemed to be phylogenetically divergent.

In all cases, a strong intensity corresponding to the wells was observed on E.B.-stained gel after enzyme cleavage. In addition, whatever the DNA probe used in hybridization experiments, a strong signal corresponded to the wells (data not shown). These results suggested that a large amount of total DNA remained trapped in the slots, perhaps due to lysis products which complex the DNA and prevent its entry into the matrix gel.

Genome size estimation

The first aim of this study was to estimate the size of the genome of *S. ambofaciens*. The molecular size of the chromosomal DNA was determined by adding up the size of all the restriction fragments taking account of their relative intensity. In order to confirm direct observation that some DNA fragments were multiplets, densitometric readings of each *Ase*I and *Dra*I restriction pattern were performed. For this purpose, the estimated copy number of each restriction fragment was calculated taking account into the area of the peak on the densitometric lines and the size ratios. The figure 5 illustrates this method with the compilation of 11 densitometric readings of *Ase*I restriction patterns generated from strain B genome. The relative intensity of the restriction fragments on E.B.-stained gel was decreased in those fragments corresponding to the higher molecular weights. In addition, 3 DNA fragments of 190 kb, 270 kb and 1,050 kb exhibited a significantly higher intensity. To demonstrate that these restriction fragments were multiplets, densitometric readings of *Saccharomyces cerevisiae* electrophoretic karyotypes were used as reference (figure 5.B). Hence, the compilation of 5 karyotypes also revealed a decreased intensity considering the higher molecular weight chromosomes. A change of incline could be observed around 1,000 kb as in *S. ambofaciens* analysis. In addition, the slopes of the curves, before and after the change of incline, were similar in both analyses. These facts could be explained either by statistical degradation during DNA preparation or by trapping and smearing of high molecular weight DNA fragments using large field intensities [23]. Moreover, in *S. cerevisiae* analysis, the chromosomes V and VIII, comigrating as an unique DNA band [14], were revealed as a doublet using our analytic method. So, these DNA bands (190 kb, 270 kb, 1,050 kb) were demonstrated to be in non-molar ratios. Taking account into the total genome size of *S. ambofaciens* determined using *Dra*I (table 2), these fragments were interpreted as doublets taking account into their relative intensity ranging from 1.5 (1,050 kb) to 2 (190 kb). The same method was applied to confirm that several other *Ase*I and *Dra*I fragments were doublets in the *S. ambofaciens* patterns: strain A, *Ase*I-50 kb and *Ase*I-600 kb; strain B, *Dra*I-310 kb; strains C and D, *Ase*I-1,050 kb and *Ase*I-550 kb. These DNA fragments were counted as double in the genome size calculations. The only *Ase*I-600 kb fragment in strain A could be resolved as two discrete bands suggesting the other intense bands might be duplicated and not just comigrating fragments.

In strains C and D, the 30 kb *Ase*I fragment could be identified as part of the extrachromosomal molecule previously described; partially digested genomic DNA provided a ladder of bands ending with this 30 kb band (data not shown). Thus, the extrachromosomal molecule contained an *Ase*I restriction site. In strain A, no *Ase*I DNA fragment could correspond to the linear extrachromosomal molecule since DNA preparation was performed after growth at 30°C where no extrachromosomal molecule was observed (figure 1.α., lane A).

The estimated genome sizes of the four strains with *Ase*I and *Dra*I are summarized in table 2. In spite of possible inaccuracies in estimating sizes of especially large DNA fragments, total genome size calculations were similar using two different restriction enzymes. In addition, these values were consistent with the size of the chromosome of *S. coelicolor* A3(2) estimated to about 5,800 kb by PFGE analysis [5]. However, the total size of the chromosome could be undervalued: first, because of the underestimation of each restriction fragment relatively to the size standards [22], and secondly, because of the possible loss of short restriction fragments that could be generated from A+T-rich regions of the chromosome. In addition, we cannot exclude the existence of large circular molecules that could be trapped in the well.

DISCUSSION

PFGE analyses of the extrachromosomal content of *S. ambofaciens* strains allowed us to demonstrate the presence of linear extrachromosomal DNA with concatemeric forms in three of the four strains studied. So, PFGE analysis of undigested genomic DNA revealed itself as a convenient way to study the presence and possibly the replication process of the linear DNA molecules often reported in *Streptomyces* genomes [reviewed in 18]. This method could extend the investigation methods for strain characterization since plasmids as well as phage genome are considered as part of the bacterial genome. Indeed, these DNA molecules could not be detected through any other analytic method taking account of their size and linear structure.

Indeed, at least two linear extrachromosomal molecules have been detected since the extrachromosomal molecules present in strains C, D DNAs are different from the molecule detected in strains A DNA, as shown by the hybridization with pSAM1 DNA and size determination. Concatemeric forms could have been found in both cases. These oligomeric could correspond to replication intermediates via a rolling circle-like mechanism. In addition, the fact that the detectable extrachromosomal content varied at different growth temperatures suggested that the replication process was temperature-dependent.

In strain A, the linear molecule was estimated about 80 kb and was shown to be highly homologous to pSAM1 DNA. Thus, this molecule could correspond to a linear form of pSAM1 which could exist not only in circular form but also in linear form. In this hypothesis, pSAM1 could be analogous to the actinophage ϕSF1 of *Streptomyces fradiae* (84 kb)[24] detected as circular prophage state in ϕSF1-resistant strains and in its linear, infectious state in phage-sensitive strains. However, the only finding suggesting a rolling circle-like replication process is that ϕSF1 is circularly permuted and terminally redundant. In our knowledge, PFGE was not applied to the *S. fradiae* genome, and thus no ϕSF1-concatemers could be detected. The presence of phage particules in the four *S. ambofaciens* strains is currently investigated.

Restriction analyses and subsequent PFGE resolution of *S. ambofaciens* genomic DNA has yielded interesting results concerning the relationship between the different *S. ambofaciens* isolates. Thus, while high-cleavage-frequency restriction enzymes as *Bam*HI or *Pvu*II and subsequent classical electrophoretic separation did not allow the detection of any difference between strain B and strains C and D (data not shown), macrorestriction PFGE analyses allowed the differentiation of these strains. In the same way, while only several differences could be detected between strain A and the three other strains by classical restriction analysis, the PFGE restriction pattern revealed a quite different genomic structure for strain A. The related genomic structure of strains C and D and strain B suggested that these strains were more phylogenetically related with each other than with strain A.

The use of low-cleavage-frequency restriction enzymes and subsequent PFGE analysis can yield important information. Whereas large DNA rearrangements (such as inversions or translocations) as well as point mutations at restriction sites remain undetected in the complex pattern generated by a high-cleavage-frequency restriction enzyme since these alterations affect only two fragments, such DNA rearrangements could be easily observed in low number fragments PFGE pattern.

The genome sizes corroborate the data obtained from the analysis of the macrorestriction patterns: strains B, C and D seem to be closely related and could have derived from each other by large genomic rearrangements. In contrast, strain A exhibited different traits in its macrorestriction pattern as well as its genome size. Thus, strain A could have earlier diverged from a common *S. ambofaciens* ancestor.

However, we can not rule out the possibility that *S. ambofaciens* genome consists of several large molecules (linear or circular) as recently reported in *Rhodobacter sphaeroides* [25]. In particularly, strains B, C and D exhibited intense DNA fragments that could correspond to duplicated regions constituting an additional molecule. However, Hopwood *et al.* [5] reported the construction using PFGE of a unambiguous circular physical map of *Streptomyces coelicolor* A3(2) consistent with the genetic map.

To our knowledge, *S. ambofaciens* is the largest bacterial chromosome estimated and reported using restriction analysis and PFGE resolution [25, 26, 27, 28]. Macrorestriction pattern analysis offers a way to study the genetic instability often reported in the *Streptomyces* genus at the genome level [28, 29]. Thus, this method of analysis of the genomic structure allows the large genomic rearrangements, mainly amplifications and deletions, associated with hypervariability and genetic instability in *S. ambofaciens* DSM40697 (strain B)[17, 29, 30] to be followed. In addition, this approach could offer a method of studying the physical linkage between resistance and production genes in the spiramycin-producer *Streptomyces ambofaciens*.

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Table 1: Strains of *Streptomyces ambofaciens*.

Stock designation	Strain	Source	Plasmid content pSAM1	pSAM2
A	*S. ambofaciens* ATCC15154	Derived from ATCC23877 [7] after UV irradiation	+	integrated form [8]
B	*S. ambofaciens* DSM40697	[9]	-	- [8]
C	*S. ambofaciens* ETH9427	[9]	no published data	
D	(*)*S. ambofaciens* ETH11317	[9]	no published data	

ATCC: American Type Culture Collection,
DSM: Deutsche Sammlung von Mikroorganismen,
ETH: Eidgenosiche Technischen Hochschule (Zürich),
+/- Detection of plasmid DNA,
(*) *S. ambofaciens* ETH11317 was also called *S. aureofaciens* [9].

Table 2: Estimations of the genome sizes of the *S. ambofaciens* strains (A, B, C and D) with *Ase*I and *Dra*I.

Strain	A	B	C	D
***Ase*I**	**6,594**	**7,745**	**8,075**	**8,075**
***Dra*I**	**6,485**	**7,840**	**8,340**	**8,340**

Figure 1: PFGE analysis of undigested DNA of *S. ambofaciens* strains after growth at 30°C for 8 hours (α) and at 37°C for 8 hours (β). Lane λ: concatemers of lambda DNA used as size standard; lane A: strain A; lane B: strain B; lane C: strain C; lane D: strain D. Running conditions were 180 V for 24 hours with ramped pulse times from 60 s to 120 s.

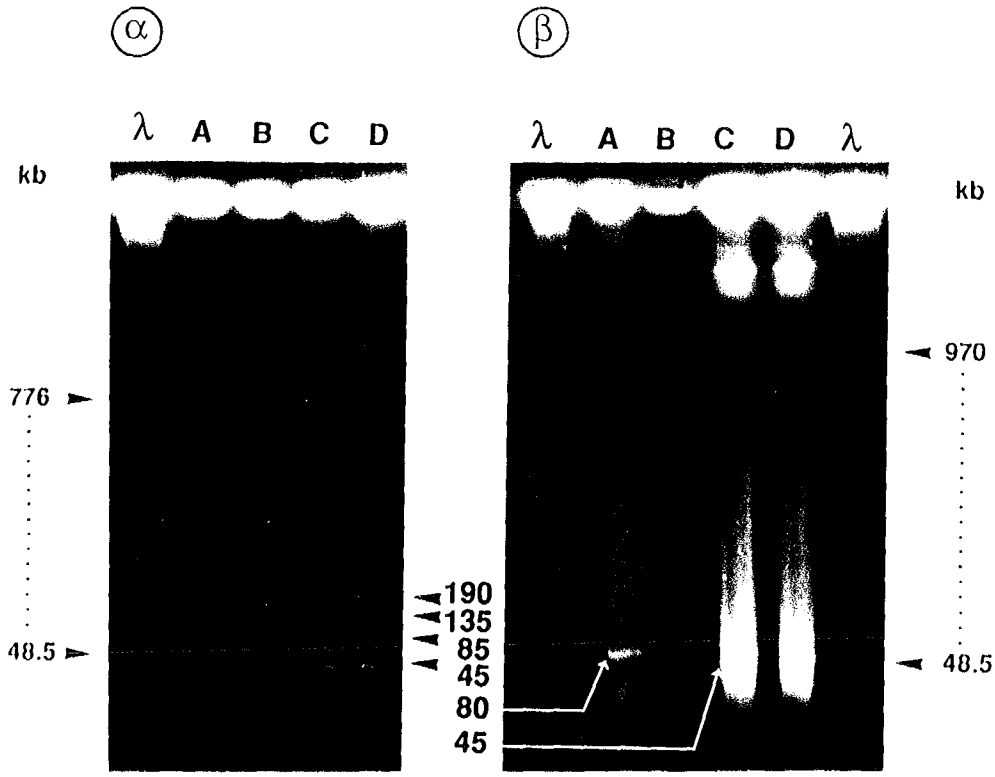

α
β
λ A B C D
λ A B C D λ
kb
776
48.5
190
135
85
45
80
45
kb
970
48.5

Figure 2: Hybridization of ^{32}P-labelled pSAM1 DNA with PFGE patterns of undigested DNA of *S. ambofaciens* strains.
(α) After growth at 30°C for 8 hours, (β) after growth at 37°C for 8 hours.
Lane λ: hybridization of ^{32}P-labelled λ DNA with concatemers of λ DNA, lane A: strain A; lane B: strain B; lane C: strain C; lane D: strain D.
Autoradiograph exposure time: 36 h.

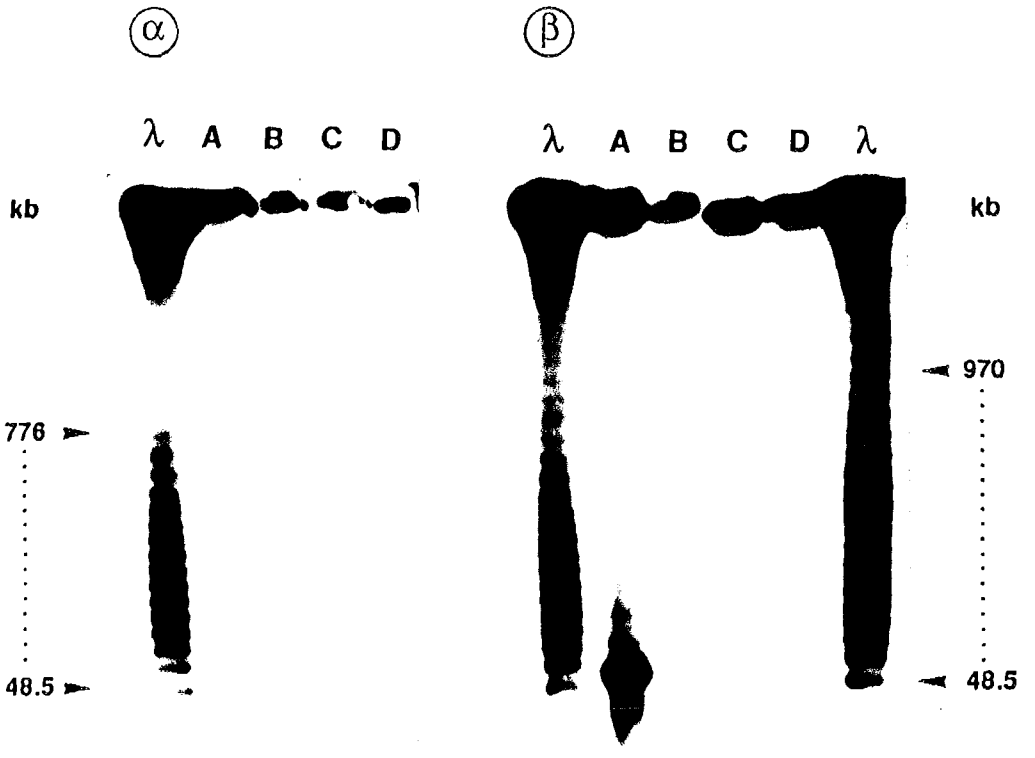

α
β
λ A B C D
λ A B C D λ
kb
kb
776
48.5
970
48.5

Figure 3: PFGE macrorestriction pattern analyses of *Ase*I digests of genomic DNA of *S. ambofaciens* strains.

(α) *Ase*I-PFGE restriction patterns. Lane S. c.: chromosomes of *Saccharomyces cerevisiae* used as size standards; lane A: strain A; lane B: strain B; lane C: strain C; lane D: strain D. Running conditions were 150 V for 35 hours with ramped pulse times from 60 s to 180 s.

(β) Schematic interpretation of (**A**). The estimated size and the stoechiometry of each restriction fragment are mentioned relative to the *Saccharomyces cerevisiae* chromosomes.

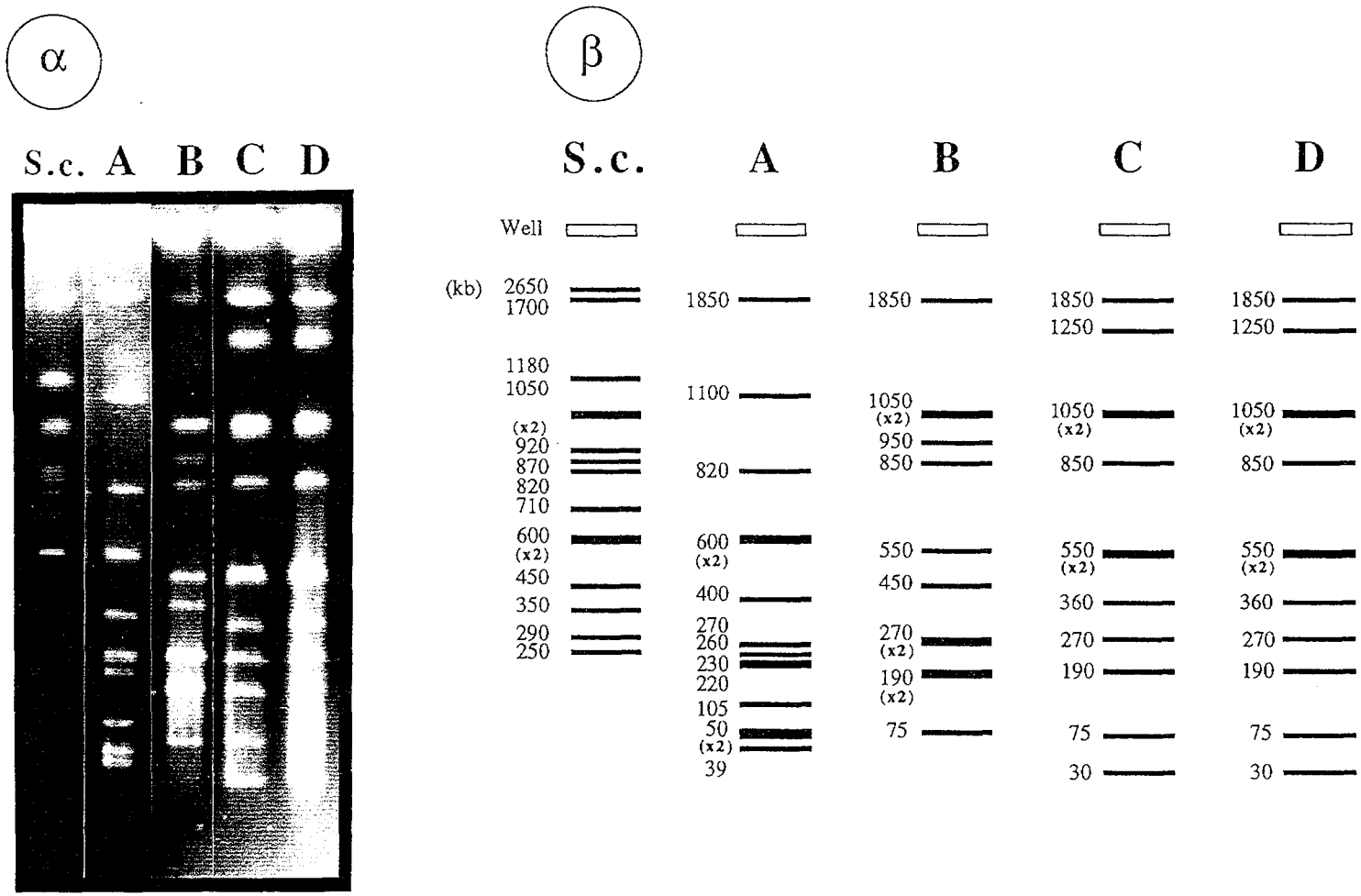

α
S.c. A B C D
β
S.c.
A
B
C
D
Well
(kb)
2650
1700
1180
1050
(x2)
920
870
820
710
600
(x2)
450
350
290
250
1850
1100
820
600
(x2)
400
270
260
230
220
105
50
(x2)
39
1850
1050
(x2)
950
850
550
450
270
(x2)
190
(x2)
75
1850
1250
1050
(x2)
850
550
(x2)
360
270
190
75
30
1850
1250
1050
(x2)
850
550
(x2)
360
270
190
75
30

Figure 3

Figure 4: PFGE macrorestriction pattern analyses of *Dra*I digests of genomic DNA of *S. ambofaciens* strains.

(α) *Dra*I-PFGE restriction patterns. Lane λ: concatemers of λ DNA used as size standards; lane A: strain A; lane B: strain B; lane C: strain C; lane D: strain D. Running conditions were as in figure 3.α.

(β) Schematic interpretation of (α). The estimated size and the stoechiometry of each restriction fragment are mentioned relative to the *Saccharomyces cerevisiae* chromosomes and concatemers of λ DNA.

α


S.c. λ A B C D

β

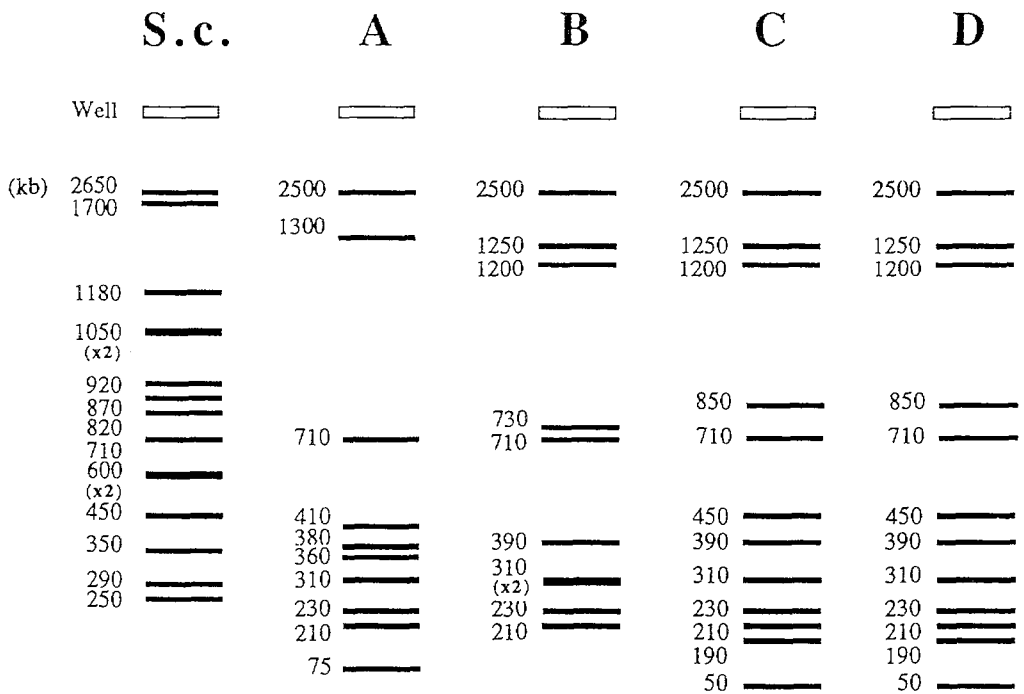

S.c.
A
B
C
D
Well
(kb)
2650
1700
1180
1050
(x2)
920
870
820
710
600
(x2)
450
350
290
250
2500
1300
710
410
380
360
310
230
210
75
2500
1250
1200
730
710
390
310
(x2)
230
210
2500
1250
1200
850
710
450
390
310
230
210
190
50
2500
1250
1200
850
710
450
390
310
230
210
190
50

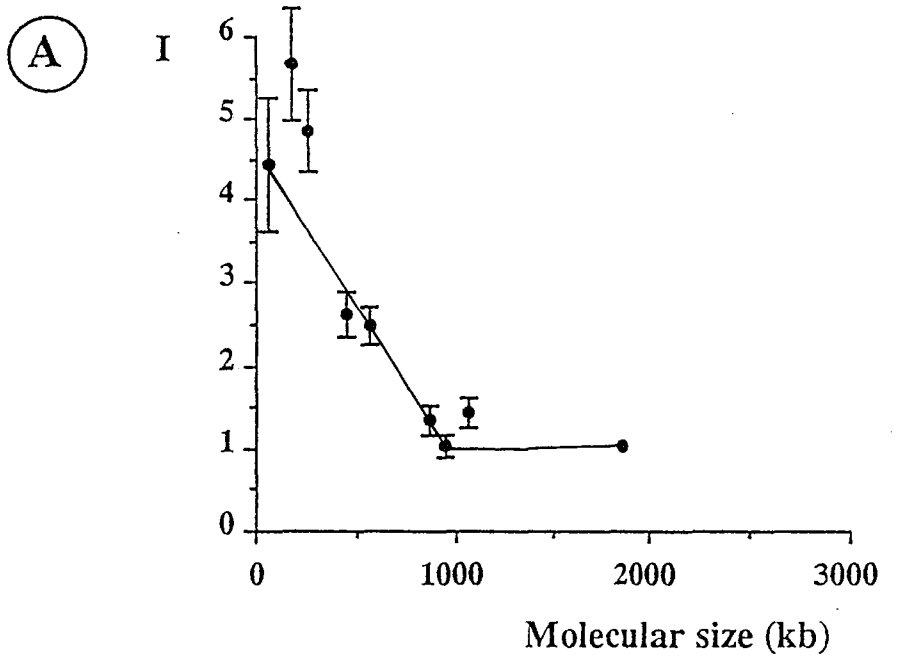
Figure 4

Figure 5: Relative intensity on E.B.-stained gel of the *Ase*I restriction fragments of *S. ambofaciens* strain B (A) and of *Saccharomyces cerevisiae* chromosomes (B).
The relative intensity (I) is expressed relatively to the 1,850 kb fragment (reference) for *S. ambofaciens* and to the 2,650 kb chromosome for *S. cerevisiae* taking into account the size ratios (SR) of the DNA bands:

$$I = \frac{\text{Relative area of band X}}{\text{Relative area of the reference}} \times SR$$

$$SR = \frac{\text{Size of band X}}{\text{Size of the reference}}$$

The relative area of the peaks on densitometric readings are calculated by integration.


A
I
6
5
4
3
2
1
0
0
1000
2000
3000
Molecular size (kb)

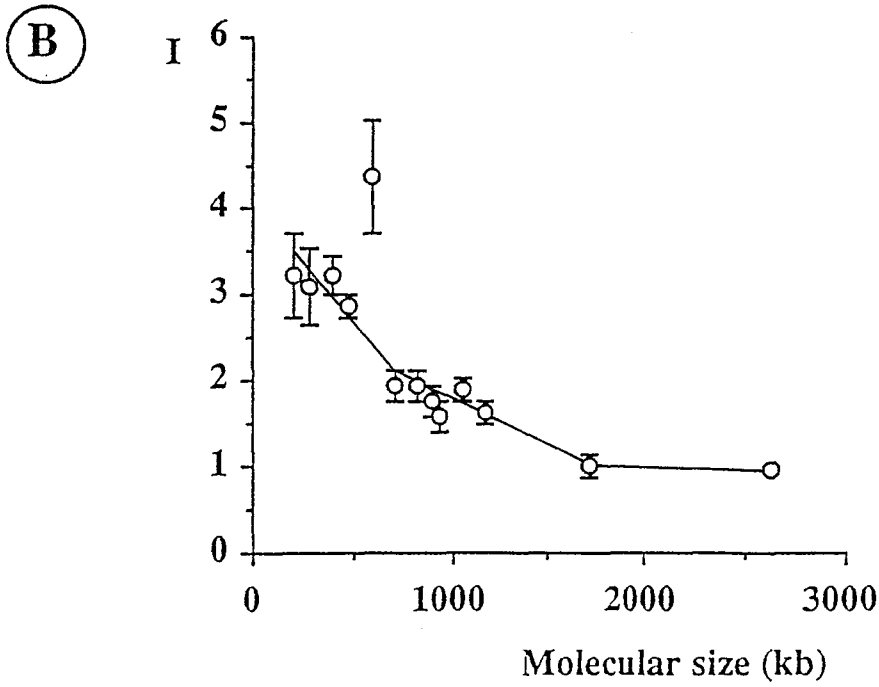

B
I
6
5
4
3
2
1
0
0
1000
2000
3000
Molecular size (kb)

Figure 5

GENETIC INSTABILITY AND ASSOCIATED GENOME PLASTICITY IN *STREPTOMYCES AMBOFACIENS*: PFGE EVIDENCE FOR LARGE DNA ALTERATIONS IN A LIMITED GENOMIC REGION

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Keywords: Genetic instability, Hypervariability, Amplification, Deletion, PFGE.
Abbreviations: AUD, Amplifiable Unit of DNA; ADS, Amplified DNA Sequence; WT, Wild-Type; PFGE, Pulsed-Field Gel Electrophoresis; EB, Ethidium Bromide.

SUMMARY

Streptomyces ambofaciens exhibits a high degree of genomic instability (amplifications and deletions) closely related to genetic instability. Amplified DNA sequences (ADS), arising only in the progeny of hypervariability-derived strains, are demonstrated to originate mainly from two amplifiable loci. Pulsed-field gel electrophoresis (PFGE) analysis of the wild type (WT) genome allowed us to localize the amplifiable loci within two adjacent *AseI* restriction fragments estimated to be about 75 kb and 850 kb. In addition, the formation of large deletions and genetic instability were strictly correlated since deletions were detected in all mutant strains isolated from the WT strain. Phenotypically stable as well as hypervariability-derived mutant strains (amplified or not) harbored large deletions whose sizes were estimated to range from 250 kb to more than 2,000 kb. These deletions affected the DNA region that includes the amplifiable loci. Thus, a large chromosomic region corresponding to about 25% of the total genome size was shown to undergo amplifications and large deletions. PFGE also allowed us to localize the ADS and to establish their structure: amplification takes place at the AUD locus as a tandem array of the WT AUD sequence.

INTRODUCTION

DNA rearrangements can be associated with differentiation and genetic instability processes in micro-organisms. Indeed, developmentally-controlled site-specific recombinations and deletions create a transcriptional factor gene in *Bacillus subtilis* (Stragier et al., 1989) and result in the formation of a complete nitrogen fixation open reading frame in *Anabaena* species (Golden et al., 1988). In the same way, programmed chromosome breakage and deletion are involved in ciliate macronucleus differentiation (Yao, 1989).

In addition, high frequency DNA rearrangements were associated with the occurrence of phenotypic variability affecting colony morphology and symbiotic properties in *Rhizobium* (Flores et al., 1988) and in colony morphology and virulence in the pathogenic yeast *Candida albicans* (Slutsky et al., 1985; Suzuki et al., 1989). In the same way, the *Streptomyces* genome exhibits a high degree of genome plasticity consisting in large deletions and amplified DNA sequences (ADS) associated with instability of many characters (Hütter and Eckhardt, 1988; Leblond et al., 1990). No differentiation or developmental significance could be assigned to these phenomena, but could rather be the consequence of incidental rearrangements.

In *Streptomyces ambofaciens*, two levels of genetic instability were characterized; a basic genetic instability similar to that reported for other *Streptomyces*, and hypervariability, closely related to the first phenomenon, generating a phenotypic variability from mutant clones (Leblond et al., 1989). Both aspects were associated with genome plasticity: at least two DNA regions undergo amplification in close association with hypervariability (Demuyter et al., 1988; Leblond et al., 1989), furthermore, Demuyter et al. (1990) reported that one of these amplifiable regions constitutes a hotspot for successive deletion events.

Here, we investigate the localization of the amplifiable units of DNA (AUD) in the WT genome and the ADS in the mutant genome using pulsed-field gel electrophoresis (PFGE). Then, we study the localization and the size of the deletions associated with genetic instability.

MATERIALS and METHODS

Bacterial strains and plasmid. Streptomyces ambofaciens DSM40697 (Hütter, 1967) was used in this work as the wild type (WT). Mutant strains are described in the Results section. *Escherichia coli* HB101 (Boyer et Roulland-Dussoix, 1969) and the plasmid pBR322 (Bolivar, 1978) were the host and vector for cloning.

Media and culture conditions. Streptomyces strains (WT and mutants) were grown at 37°C on plates of Hickey Tresner medium (Hopwood et al., 1985) for mutant isolation and spore production. Large scale DNA isolation was performed on mycelium grown for 48 h at 37°C in YEME liquid medium (Hopwood et al., 1985) supplemented with glycine (0.25%). Small scale DNA isolations were performed after growth for 24 h at 37°C in HT liquid medium supplemented with glycine (0.25%).
E. coli strains were grown at 37°C in LB liquid medium. Transformant strains were tested on LB plates supplemented with ampicillin (50 µg/ml) or tetracycline (25 µg/ml).

Isolation of total DNA from S. ambofaciens strains and restriction analysis. DNA extraction and purification were carried out according to Demuyter et al. (1988). Restriction enzymes *Bam*HI, *Hin*dIII, *Dra*I were purchased from Boehringer Mannheim (FRG); *Ase*I from New England Biolabs (Beverly, Mass., USA) and used according to the manufacters' recommandations. DNA fragments were electrophoresed on 0.8% agarose gels according to Sambrook et al. (1989). Bacteriophage λ DNA digested with *Hin*dIII was used as size standard (Daniels et al., 1983).

DNA probes. S120 consisted in 1.95 kb *Bam*HI fragment of ADS120 purified as described in Demuyter et al. (1988) from total DNA of NSA120 mutant strain (see table 1). S1 corresponded to the extremities of AUD6 cloned in pBluescriptKS as described in Demuyter et al. (1990). S55 consisted in a 2.0 kb *Bam*HI fragment of ADS55 cloned in pBR322 (this work).

Cloning of DNA fragment. Plasmid pBR322 DNA was isolated according to Sambrook et al. (1989). Purified total DNA from strain NSA55 (containing ADS55) was digested to completion with *Bam*HI. After ethidium bromide-staining, the appropriate band was isolated and purified from agarose by the Geneclean process (Bio 101, USA). Extracted DNA fragments were coprecipitated with *Bam*HI-cut pBR322 (ratio 3/1) using isopropanol. The DNA mixture was dissolved in ligation buffer and ligated with T4 DNA ligase (Boehringer Mannheim, FRG) according to the suppliers before *E. coli* strain transformation. Colonies with an ampicillin resistant phenotype but tetracycline sensitive phenotype were presumed to carry pBR322 with inserts. These chimeric plasmids were further analyzed using the boiling method (Sambrook et al., 1989).

Pulsed-field gel electrophoresis analysis (PFGE). S. ambofaciens mycelia were grown for 8 hours in Hickey Tresner liquid medium supplemented with glycine (0.25%) at 37°C. Mycelia were washed in ES solution (10.3% Sucrose 0.25 M EDTA), resuspended in ES solution containing lysozyme (Boehringer Mannheim, FRG)(20 mg/ml) and finally embedded in 1.5% low melting point agarose [LMPA (Appligène,

Strasbourg, France)] dissolved in ES solution to a final concentration of 0.5% LMPA. The mixture was dispensed in a plug former and incubated for 10 mn to 30 mn at 0°C depending on the strain. Then, the agarose plugs were incubated in a solution of 0.5 M EDTA, 1% N-lauroylsarcosine, 5 mg/ml Pronase E (Sigma, USA) for 12 h at 50°C. After washing the plugs for 3 h in 10 ml TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) with continuous shaking, 1 mM phenylmethanesulphonylfluoride [PMSF (Boehringer Mannheim, FRG)] was added to inhibit proteinases. Plugs were stored at 4°C in 0.5 M EDTA.
For restriction analysis, slices of the agarose plugs containing the high molecular weight genomic DNA were washed with the appropriate restriction buffer according to Sambrook et al. (1989) at room temperature and incubated at 37°C with 50 units of the restriction enzyme [*Ase*I (Biolabs) or *Dra*I (Boehringer Mannheim)].
Chromosomes of *Saccharomyces cerevisiae* (strain 288C) prepared as in Bellis et al. (1987) were used as high molecular weight standards (Frutos et al., 1989). Concatemers of bacteriophage λ DNA provided a convenient size marker up to 1,000 kb (Bio-Rad, USA). Electrophoreses were performed in 1% agarose gels in 0.5x TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) at 14°C in a CHEF (Contour Clamped Homogeneous Electric Field, Biorad, USA) system (Chu et al., 1986). Initial and final pulse times were usually adapted to maximize resolution of DNA fragments depending on the molecular weights. Gels were stained with ethidium bromide (EB).

Southern analysis.The DNA probe was ^{32}P-labeled using the Multiprime DNA labeling system kit (Amersham, UK). The labeling could be carried out either with purified DNA or directly in the agarose matrix. λ DNA was ^{32}P-labeled by nick-translation (Rigby et al., 1977) using a nick-translation kit (Amersham, UK).
For hybridization experiments, Southern (Southern, 1975) blots were carried out using the capillary transfer method with the Vacugene system (LKB) onto Hybond-N membranes (Amersham, UK). The DNA was depurinated with 0.25 HCl for 30 mn before denaturation (in 1.5 M NaCl, 0.5 M NaOH for 1 hour), neutralized (in 1.5 M Tris-HCl, 1.5 M NaCl for 30 mn) and transfered in 20x SSC (1x SSC = 0.15 M-sodium chloride, 0.015 M-sodium citrate) for 90 mn. Prehybridization, hybridization and washing conditions were as previously described in Demuyter et al. (1988).

RESULTS

Physical relationship between the AUD regions on the wild-type genome.

In *S. ambofaciens* DSM40697, the occurrence of highly amplified DNA (ADS) was correlated with spontaneous hypervariability; indeed, 21% of the mutant strains arising in phenotypically heterogeneous progeny contained ADS, while ADS could not be detected, either in stable pigment-defective mutant clones, or in WT clones (Leblond et al., 1989). Each studied ADS-containing strain was generated through independent genetic instability events, i.e., isolated from hypervariable progeny raised from different pigment-defective colonies (generated from the WT strain). According to this protocol, 48 ADSs were considered. Their size ranged from 5.2 kb to 105 kb (table 1). Several mutant strains could be isolated containing ADS presumed to araise from the same AUD on the basis of their restriction patterns (the amplification degree differed). The cloned fragment corresponding to the extremities of the ADS6, previously mapped by Demuyter et al. (1988), constituting the S1 probe (figure 1) was used against Southern blots of *Bam*HI digests of the ADS-containing strains (data not shown). 11 ADSs (sizes ranging from 5.8 kb to 35 kb) could unambiguously be assigned to the AUD6 family. All other ADS-containing strains exhibited a WT hybridization pattern using S1 against *Bam*HI digests (Demuyter et al., 1990). Moreover, 24 ADSs (sizes ranging from 10 kb to 67 kb) constituted the AUD90 family according the high similarity of their *Bam*HI restriction patterns. In addition, hybridization experiments using a 1.95 kb-*Bam*HI fragment as a probe (S120) common to 17 ADSs assumed to belong to this family allowed us to confirm the distribution of these ADSs (data not shown). However, 13 ADSs could be assigned neither in AUD6 region nor in AUD90 region: either because of the lack of hybridization response with S1 or S120, or because of the lack of restriction pattern similarity. For example ADS55 (AUD55, 21 kb) and ADS450 (AUD450, 32 kb) were shown to be homologous and presumed to belong to other AUD regions.

In conclusion, 73% (35 of 48) of the ADSs could be grouped in two independant AUD loci: 50% (24 of 48) in the AUD90 region, 27% (13 of 48) in the AUD6 region.

In order to investigate the distance between the AUD loci, PFGE analyses of the WT DNA and subsequent hybridization experiments with AUD-specific probes were carried out. First, preliminary PFGE studies of the WT strain allowed us to characterize the WT genome with *Ase*I and *Dra*I restriction patterns (Leblond et al., submitted). For the following experiments, only *Ase*I was extensively used since the WT DNA was restricted to 12 well-resolved restriction fragments. First, the Southern blot of *Ase*I digest of the WT DNA was hybridized with ^{32}P-labeled S1 probe. Two unequal strong signals corresponding to DNA fragments estimated to be about 75 kb and 850 kb relative to the size standards were detected (figure 2.A). This fact suggested that AUD6 overlapped two *Ase*I restriction fragments. This *Ase*I site was mapped relative to *Bam*HI restriction sites by direct restriction analysis in NSA6 and confirmed by hybridization with S1 in the WT strain (figure 1). The relative intensity of the signal could be explained *a posteriori* since the 75 kb fragment contained two homologous stretches (the right side of the AUD and the internal homologous sequence mentioned

in Demuyter et al., 1990) while the 850 kb fragment contained only the left side of the AUD6. Both S120 and S55 probes (a 2 kb *Bam*HI fragment from ADS55 cloned in pBR322) revealed a heavy signal corresponding to the 850 kb fragment (figure 2.B). Faint signals could be detected correponding to almost all restriction fragments generated by *Ase*I revealing microhomologies all over the genome. These faint signals could be detected when a large amount of DNA were blotted. The absence of *Ase*I site within the AUD90 was confirmed by classical electrophoresis (data not shown).

Therefore, at least three DNA regions (AUD6, AUD90 and AUD55) likely to undergo amplifications and encompassing at least 77% of amplification were localized in a large chromosomal region corresponding to about 10% of the WT genome.

Unamplified mutant strains having undergone large deletions

1. Correlation between PFGE restriction pattern modifications and mutation

Classical restriction analysis revealed that 35% of the unamplified mutant strains of *S. ambofaciens* contained partial or total deletions in the AUD6 region (Demuyter et al., 1990) 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 deletion in all unamplified mutants.

For this purpose, 26 mutant strains were independently isolated following the same protocol used for ADS-containing strains: 14 were derived from the WT strain by the basic genetic instability and exhibited a stable pigment-defective mutant phenotype; 12 were derived from hypervariability and exhibited various mutant phenotypes (Leblond et al., 1989). No ADS could be detected in these strains either on agarose gel or using S1 as a probe in hybridization experiments.

Restriction patterns were realized for each mutant strain and compared to the WT: several high molecular weight DNA fragments were missing. Figure 3 shows examples of mutant restriction patterns. Table 2 summarizes the results of all the sampling. These patterns were obtained reproductibly and were not due to partial digestion. All 26 mutant strains tested, exhibited an altered *Ase*I restriction pattern. The same phenomenon was observed using *Dra*I (data not shown). In contrast, 12 independently isolated and subcloned WT clones were analysed and shared the same typical restriction pattern (Leblond et al., submitted). So, a strict correlation could be established between unamplified mutant strains and large genomic rearrangements.

In addition, the same restriction fragments were always found to be missing. The frequency of loss decreased from 100% (26 out of 26) for the 450 kb fragment to 8% (2 out of 26) for the 550 kb fragment (table 2). With this sample, the frequencies of loss for each fragment in phenotypically stable lineages and hypervariability-derived strains were not significantly different. Thus, large rearrangements occured in both kinds of mutant lineage.

In addition to missing bands, bands of various sizes could be detected in some mutant restriction patterns (17 of 26, table 2). The new fragment could correspond to joining fragments created by a deletion event. In some cases, several additional fragments have been detected that could correspond to the joining fragments of multiple deletion events. Then, when no additional fragment could be detected (9 out of 26), the joining fragment could comigrate with one of the pre-existing fragments, could remain unresolved if high molecular weight or could leave the gel if low molecular weight.

2. Evidence that large deletions affect the AUD loci

We subsequently carried out hybridization experiments with AUD-specific probes in order to estimate the extent of the deletions by identification of the joining fragment. The second approach was performed using probes S120 or S2 (figure 1). The use of this probe was justified because this sequence was homologous to the only 850 kb *Ase*I fragment and thus, gave a simple hybridization pattern (only one main signal).

All the mutant strains characterized using classical analysis (Demuyter et al., 1990) as deleted in the AUD6 region were shown to lack in the 75 kb *Ase*I fragment (including the right side of the AUD6 region).

Thus, in mutant strains with intact AUD6, we expected to detect the rest of the 850 kb fragment after deletion. In the same way, S120 would have allowed us to detect the DNA fragment generated by the deletion in the mutant strain with partially or totally deleted AUD6 taking into account the relative location of the AUD6 and AUD90 regions.

As expected, S2 revealed a hybridization polymorphism among the mutant strains with intact AUD6, while no signal could be detected in all strains affected in the AUD6 region (figure 4). Conversely, using the same Southern blot after removing S2, S120 did not reveal any signal whatever the mutant strain.

In table 3 are listed the hybridization responses to S2 and the sizes of the *Ase*I hybridizing fragments in PFGE for each strain. A hybridizing fragment corresponding to a junction was detected in 10 cases out of 19 strains tested. In these cases, assuming that only one deletion event is required to explain the DNA rearrangement, the size of the deletion could be estimated.

Thus, mutant strains are affected by large deletions including the AUD90 region and reaching the AUD6 region in about 35% of cases. The left-side of the deletion could lie in 550 kb, 950 kb or 850 kb *Ase*I fragments assuming that all the missing fragments were affected by a deletion.

Deletions in ADS-containing strains. Structure and localization of ADS.

In order to investigate the structure, the localization of the ADS and the associated DNA rearrangements, PFGE analyses were carried out on mutant strains containing ADS originating from the AUD6 region as well as from the AUD90 region.

Classical analyses of ADSs in the AUD6 region allowed to conclude that most of the copies of the AUD were tandemly reiterated since only two main hybridization signals corresponding to the flanking sequences of the ADS could be detected in these clones (Demuyter et al., 1988; 1990). In addition, an *Ase*I restriction site was unambiguously mapped in the AUD6 region using PFGE and hybridization with S1. Thus, ADS6 was expected to appear as a 24.8 kb *Ase*I fragment in a PFGE analysis.

Two ADS6-containing strains were analysed: NSA6 previously 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 (detectable only by hybridization)(Demuyter et al., 1990).

In both cases, the *Ase*I restriction pattern revealed the same missing bands as in unamplified mutant strains: the *Ase*I fragments of 450 kb, 850 kb and 950 kb were missing. In contrast, a heavy and smeared DNA band migrating below the 75 kb fragment was detected in NSA6 while this same band was only faint in NSA1635 (figures 5.A and 5.B respectively, lane 2). Using Labeled S1 against Southern blots of *Ase*I digests, both strains revealed a strong signal corresponding to the previously described band. The signal was more intense in NSA6 than in NSA1635 (figure 5.A and 5.B respectively, lane 3). In addition, a signal correponding 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 Southern blots of *Bam*HI digests of NSA6 revealed the right flanking sequence of AUD6.

These facts were consistent with the existence of a large deletion encompassing the AUD90 region, associated with amplification of AUD6. S2 and S120 did not reveal any hybridizing fragment on PFGE restriction patterns, demonstrating the presence of a large deletion whose size was at least 1,300 kb.

Two mutant strains containing an ADS deriving from the AUD90 locus were investigated for the structure of the ADS and the correlation between amplification and large deletion. AUD120 (15 kb) and AUD90 (55 kb) were shown to overlap: all the amplified fragments generated from ADS120 were present in ADS90 using *Bam*HI restriction analysis (Leblond et al., 1989). These facts suggested that the fragment created by the reiteration of AUD120 was co-migrating with one *Bam*HI fragment of ADS90. No *Ase*I restriction site could be mapped in either AUD120 or AUD90 using classical analysis, supporting hybridization experiments, where S120 revealed the only 850 kb *Ase*I fragment in PFGE (figure 2.B).

*Ase*I restriction patterns of NSA120 and NSA90 revealed missing bands: NSA120 lacked the 450 kb and 850 kb bands (figure 6.A, lane 3; table 2); NSA90 lacked the 450 kb, 850 kb and 950 kb fragments (figure 6.A, lane 4; table 2). No additional fragment could be detected on EB-stained gel. In order to localize the ADSs, PFGE migrations were hybridized with S120 (which corresponded to an amplified *Bam*HI fragment). In both strains, a strong signal corresponding to a high molecular weight DNA band was detected on the gel. The size was estimated to be about 2,000 kb despite the low resolution in the these molecular sizes (figure 6.B). These results were in agreement with the amplification degree of each ADS and the respective size of each AUD. Assuming that DNA rearrangements were affecting only the left side of the AUD region (the 75 kb fragment was not missing), we attempted to localize the right flanking sequence using S2 against the NSA120 *Ase*I restriction pattern. While S2 evidenced the original 850 kb fragment in the WT strain (figure 6.C, lane 1), the probe revealed the 2,000 kb DNA band described above in the mutant strain (Figure 6.C, 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 loci. In addition, a large deletion closely associated with amplification was shown to terminate in the vicinity of the AUD locus.

DISCUSSION

Localisation of the characterized AUD families in a large chromosomal region

In *S. ambofaciens*, ADS-containing strains arose only from hypervariability at the frequency of 0.21 (Leblond et al., 1989). Previously, we reported the mapping of four ADSs belonging to two different AUD loci (Demuyter et al., 1988). Here, we studied the AUD loci considering 48 independent amplification events. These ADSs showed a high degree of heterogeneity in size and amplification extent. However, amplification of the same AUD (on the basis of the restriction pattern) could be observed in independent clones. On the basis of restriction analyses and cross-hybridization experiments, 77% of the ADSs were shown to originate from only two AUD regions called AUD90 and AUD6 families.

Using AUD90 and AUD6 specific probes and PFGE *Ase*I-restriction pattern of the WT strain, we were able to map these two AUD regions onto two adjacent restriction fragments. Indeed, as the AUD6 region overlapped two *Ase*I restriction fragments estimated to be 75 kb and 850 kb, AUD90 region 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. We concluded that the AUD regions in *S. ambofaciens* extend over 200 kb (totalling the largest ADS of each AUD group, table 1). According to Hütter et al. (1988), this type of AUD structure was called type I, corresponding to multiple overlapping AUDs originating from the same region. *S. glaucescens* provides a well-documented case of type I AUD locus with a 100 kb region undergoing ADSs highly heterogeneous in length and complexity (Hasegawa et al., 1985; Häusler et al., 1989). The type I was defined in contrast to highly reproductible ADSs generated from a single AUD sequence (type II). This AUD was characterized by a symmetrical structure including two or three large identical sequences (Altenbuchner and Cullum, 1985; Fishman et al., 1985; Dyson and Schrempf, 1987, Hornemann et al., 1987). In the case of *S. ambofaciens*, at least two separated AUD loci were characterized from which ADSs are generated. Each region could be limited either by consensus-like sequences or the amplification process itself: amplification could involve a particular molecular event (deletion or duplication) that could be AUD specific. Hence, amplification in one of the AUD regions could be non random. In addition, within each AUD region, several ADSs could be reproducibly observed (table 1). Thus, these AUDs could be analogous to type II AUD amplification. Therefore, the notions of AUD of type I/II are not exclusive. Indeed, particular sequences or structures could induce the amplification process; for example microhomologies (Häusler et al., 1989) dispersed all along the AUDs as detected in AUD6 (Demuyter et al., 1990) could define an AUD and could be involved in the amplification process. Besides, unstable type I-like ADSs were found in addition to reproductibly obtained type II ADSs (Dyson and Schrempf, 1987).

The involvement of large polar deletions both in basic genetic instability and hypervariability

PFGE analyses revealed important alterations of restriction pattern in all mutant strains of *S. ambofaciens*. Indeed, 5 large restriction fragments (sizes ranging from 75 kb to 950 kb) were always found to be missing with different frequencies (table 3). The frequencies of loss varied from 8% to 100%. Thus, assuming that the observed DNA rearrangements were the results of a unique molecular event, a speculative *Ase*I restriction map of the unstable region could be drawn (figure 7.A). We previously demonstrated that the 75 kb and 850 kb fragments were adjacent. The 450 kb and 850 kb fragments were always missing. In only two cases, a 850 kb fragment could be observed (NSA97H and NSA108H) but 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 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 (figure 7.A). This speculative map does not foresee of the nature of the DNA rearrangement either.

Hybridization experiments allowed us to conclude that the observed DNA alterations corresponded to large deletions. Using AUD-specific probes (S1 for AUD6, S120 for AUD90), the AUD90 region was shown to be deleted in all mutant strains tested while the 75 kb fragment was affected only in 50% of cases. These latter data were consistent with classical restriction analysis results. 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 ended within or to the right of the AUD6. All the deletion events ended in the 75 kb fragment since no additional DNA fragment was affected.

Using S2 probe (homologous to the left-flanking sequence of AUD6) on *Ase*I-digest Southern blots, we could estimate the size of the deletion by detection of the DNA fragment generated by the deletion. This estimation could be only realized in mutant strain with intact AUD6.

The sizes of the deletion may range from 250 kb to more than 2,000 kb (figure 7.B and 7.C), thus including from 3% to 25% of the total genome size, recently estimated using PFGE (Leblond et al., submitted). In some cases, several hybridizing fragments were detected with unequal intensities. These cases could be analysed as a genomic heterogeneity generated before DNA preparation. Such genomic heterogeneity was extensively described in Demuyter et al., (1990). In mutant strains with altered AUD6 region, the size of the deletion could not be estimated accurately. However, in three cases (NSA89H, NSA854 and NSA146), two possible joining fragments were detected either on agarose gel or by hybridization with S1 and could be explained by the involvement of two deletion events. In these cases, at least two successive deletion events could have occurred. Such successive deletions were detected using classical restriction analysis (Demuyter et al., 1990).

Deletion as the primary molecular event of the amplification process

Large deletions were also detected in ADS-containing strains. The same DNA region was affected and the size of the deletions were varied. In NSA6 (ADS6) and NSA1635 (ADS6), the deletion was estimated to be larger than 1,300 kb and could reach 2,000 kb since no joining fragment could be detected (figure 7.D). In the NSA90 region, two strains were studied: NSA90 (ADS90) and NSA120 (ADS24). NSA90 exhibited a deletion extending over 500 kb while NSA120 contained a deletion which could not be estimated (figure 7.D). The location of the ADS was investigated using amplified fragment as a probe: when *Ase*I cut within the AUD (AUD6), the ADS was detected as "monomers" of AUD size; when *Ase*I do not cut within the AUD (AUD120), the ADS was detected as a large DNA stretch consistent with the amplification degree. Thus, we concluded that large deletions are associated with the amplification process and that at least most of the ADS is present as a tandem array at the chromosomal AUD locus.

Large deletions have often been reported in *Streptomyces* species associated with genetic instability (for review Hütter and Eckhardt, 1988; Leblond et al., in press). In *S. glaucescens*, the double mutant phenotype Str^S Mel^- was due to deletion of the corresponding structural genes with deletion whose size ranged from 270 kb to over 800 kb (Birch et al., 1989). The association between deletion and amplification was frequently reported in *Streptomyces* (Flett and Cullum, 1987; Dyson and Schrempf, 1987; Birch et al., 1989; Cullum et al., 1990).

In *S. ambofaciens*, a precise analysis of the deletion endpoints accompanying three ADSs occurring at the AUD6 locus (ADS6, ADS101, ADS102) showed that deletion were ending close to the ADS if not in the first copies of the reiterated AUD (in the case of NSA6 and NSA101). In contrast, all ADSs originated from the AUD90 and others exhibited a WT AUD6 region. In addition, the study of the stability of ADS6 leads to the conclusion that the loss of the ADS was associated with the generation of various deletions of the AUD6 (Demuyter et al., 1990).

All these data strongly suggest that ADSs could result from one or several primary deletion events and is lost through additional deletion events. This correlation between deletion and amplification could be clarified if both phenomena were generated from the same basic molecular mechanism. Indeed, microhomologies (Häusler et al., 1989) or palindromic sequences reported in *Streptomyces* (Usdin et al., 1988) could provide a suitable substrate for illegitimate recombination as demonstrated by Brunier et al. (1989) on a plasmidic model for the breakage-reunion model. This mechanism could trigger deletion formation by template switching as well as production of a rolling circle-like structure on the chromosome at the passage of the replication fork. Then, over-replication could occur as postulated by Young and Cullum (1987).

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Table 1: *S. ambofaciens* ADS-containing mutant strains. Strains are grouped considering the overlapping of the AUDs.

GROUP I	GROUP II	Others
NSA6 (AUD6, 24.8 kb)	NSA11 (AUD11, 27 kb)	NSA3 (AUD3, 30 kb)
NSA9 (AUD9, 24.5 kb)	NSA20 (AUD20, 18 kb)	NSA17 (AUD17, 5.2 kb)
NSA14 (AUD14, 20 kb)	NSA24 (AUD24, 15 kb)	NSA18 (AUD18, 60 kb)
NSA60 (AUD60, 29 kb)	NSA34 (AUD34, 67 kb)	NSA25 (AUD25, 5.5 kb)
NSA101 (AUD101, 17 kb)	NSA44 (AUD20, 18 kb)	NSA46 (AUD17, 5.2 kb)
NSA102 (AUD102, 5.8 kb)	NSA68 (AUD68, 49 kb)	NSA54 (AUD54, 19 kb)
NSA130 (AUD130, 16.5 kb)	NSA83 (AUD100, 22 kb)	NSA55 (AUD55, 21 kb)
NSA140 (AUD140, 35 kb)	NSA90 (AUD90, 55 kb)	NSA72 (AUD72, 7 kb)
NSA150 (AUD150, 33 kb)	NSA93 (AUD93, 25 kb)	NSA96 (AUD96, 8 kb)
NSA160 (AUD160, 11.5 kb)	NSA100 (AUD100, 22 kb)	NSA103 (AUD103, 12.5 kb)
NSA420 (AUD102, 5.8 kb)	NSA131 (AUD34, 67 kb)	NSA124 (AUD124, 7.5 kb)
	NSA157 (AUD24, 15 kb)	NSA300 (AUD300, 105 kb)
	NSA171 (AUD171, 14kb)	NSA450 (AUD450, 32 kb)
	NSA179 (AUD179, 10 kb)	
	NSA180 (AUD100, 22 kb)	
	NSA207 (AUD68, 49 kb)	
	NSA213 (AUD213, 50 kb)	
	NSA239 (AUD239, 44 kb)	
	NSA253 (AUD24, 15kb)	
	NSA259 (AUD20, 18 kb)	
	NSA265 (AUD265, 47 kb)	
	NSA270 (AUD213, 50 kb)	
	NSA272 (AUD20, 18 kb)	
	NSA430 (AUD100, 22 kb)	

Table 2: Analysis of the *Ase*I PFGE restriction pattern of the mutant strains of *S. ambofaciens*. The frequencies of loss of the restriction fragments are given considering the only non amplified mutant strains (14 phenotypically stable and 12 hypervariability-derived strains). *NSA1635 derived from the same hypervariable progeny as NSA6. *Ase*I doublets fragments are noticed by x2.

	*Ase*I fragments (kb)	1,850	1,050 (x2)	270 (x2)	190 (x2)	550	950	450	850	75	Additional fragments
Pigment-defective stable strains	NSA27	+	+	+	+	+	+	-	-	-	100 kb
	NSA28	+	+	+	+	+	+	-	-	-	undetected
	NSA49	+	+	+	+	+	+	-	-	-	faint band 100 kb
	NSA64	+	+	+	+	+	+	-	-	+	90 kb
	NSA65	+	+	+	+	+	+	-	-	+	undetected
	NSA105	+	+	+	+	+	+	-	-	-	100 kb
	NSA146	+	+	+	+	+	+	-	-	-	50 and 100 kb
	NSA830	+	+	+	+	+	+	-	-	-	475 kb
	NSA833	+	+	+	+	+	-	-	-	+	undetected
	NSA834	+	+	+	+	+	+	-	-	-	475 kb
	NSA843	+	+	+	+	+	+	-	-	-	undetected
	NSA844	+	+	+	+	+	+	-	-	+	950 kb
	NSA850	+	+	+	+	+	+	-	-	+	undetected
	NSA854	+	+	+	+	+	-	-	-	+	900 kb
Hypervariability-derived strains	Non amplified mutants										
	NSA4H	+	+	+	+	+	+	-	-	+	undetected
	NSA10H	+	+	+	+	-	-	-	-	+	650 kb
	NSA11H	+	+	+	+	+	+	-	-	-	undetected
	NSA43H	+	+	+	+	+	-	-	-	-	faint band 100 kb
	NSA89H	+	+	+	+	+	-	-	-	+	900 and 50 kb
	NSA97H	+	+	+	+	+	+	-	+	+	undetected
	NSA108H	+	+	+	+	-	-	-	+	+	faint band 850 kb
	NSA135H	+	+	+	+	+	+	-	-	+	undetected
	NSA312H	+	+	+	+	+	+	-	-	+	250 kb
	NSA841H	+	+	+	+	+	+	-	-	-	undetected
	NSA844H	+	+	+	+	+	+	-	-	-	950 kb
	NSA850H	+	+	+	+	+	+	-	-	+	1,050 kb
	Amplified mutants										
	NSA120	+	+	+	+	+	+	-	-	+	undetected
	NSA90	+	+	+	+	+	-	-	-	+	undetected
	NSA6	+	+	+	+	+	-	-	-	+	25 kb
	NSA1635*	+	+	+	+	+	-	-	-	+	undetected
Frequency of loss		0%	0%	0%	0%	8%	23%	100%	92%	45%	

Table 3: Hybridization analysis of *Ase*I PFGE restriction patterns of mutant strains of *S. ambofaciens* with S2 probe: +, detection of a hybridization response to S2 probing; -, no hybridization response to S2; when a signal is observed, the estimated size of the corresponding DNA fragment is given in kilobases, the large letters correspond to heavy signals and small letters correspond to faint signals; nd, not determined.

	Strain	Hybridization	Size
Pigment-defective stable strains	NSA27	-	-
	NSA28	-	-
	NSA49	-	-
	NSA64	+	75/90
	NSA65	+	nd
	NSA105	-	-
	NSA146	-	-
	NSA830	-	nd
	NSA833	+	nd
	NSA834	-	nd
	NSA843	-	-
	NSA844	+	950
	NSA850	+	nd
	NSA854	+	190/25
Hypervariability-derived strains	NSA4H	+	270/80/60
	NSA10H	+	nd
	NSA11H	-	20/80/90
	NSA43H	-	-
	NSA89H	-	-
	NSA97H	+	850
	NSA108H	+	850/270/190
	NSA135H	+	950
	NSA312H	+	250
	NSA841H	+	nd
	NSA844H	-	-
	NSA850H	+	1050
	NSA120	+	>1,800
	NSA90	+	nd
	NSA6	-	25
	NSA1635	-	25

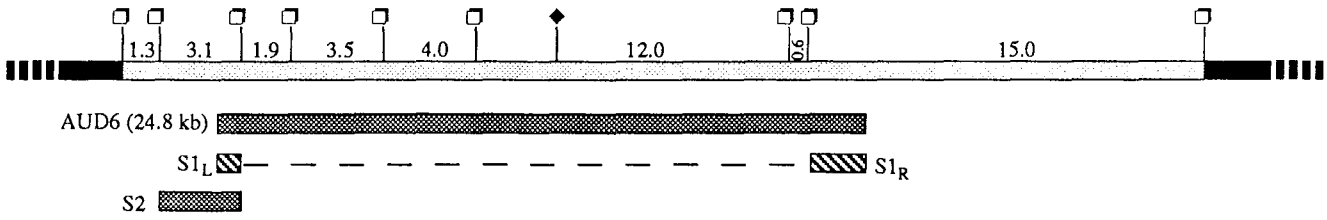
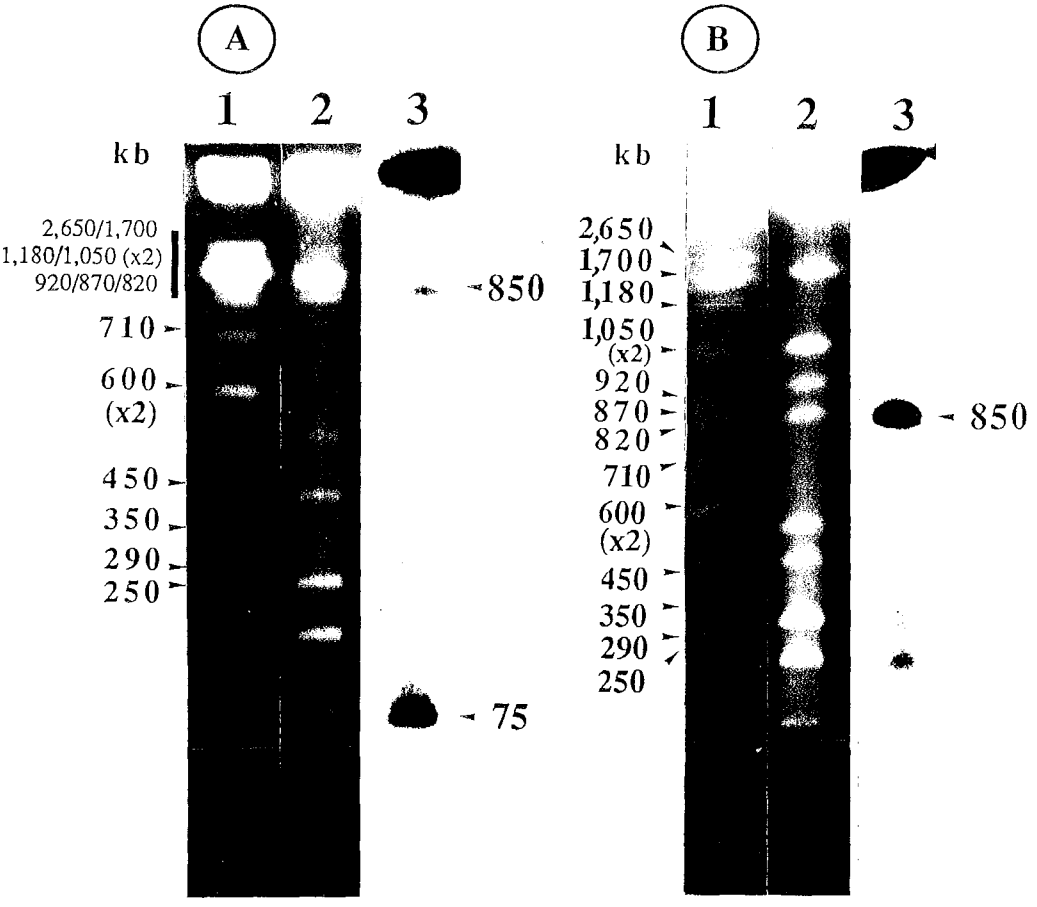


Figure 1: AUD6 locus. Restriction map of the AUD6 region in the WT genome: the only *Bam*HI (❐) and *Ase*I (◆) restriction sites are mentioned. The sizes of the *Bam*HI restriction fragments are given in kilobases. *Bam*HI restriction fragments used as probes in hybridization experiments have been cloned: S1 ($S1_L$ and $S1_R$, correspond to the end point of the AUD joined in the reiteration fragment), S2 correponds to the left flanking sequence of the AUD.

Figure 2: Hybridization patterns of AUD-specific probes on *Ase*I PFGE restriction patterns of *S. ambofaciens* WT DNA.
(**A**) Lane 1: *Saccharomyces cerevisiae* (strain 288C) chromosomes used as size standard; lane 2: WT DNA, lane 3: hybridization pattern with S1 probe. PFGE running conditions were 150 V for 30 hours with increased pulse times from 60 s to 100 s.
(**B**) Lane 1: *S. cerevisiae* chromosomes; lane 2: WT DNA; lane 3: hybridization pattern with S120 probe. Running conditions were the same as in (a) except for pulse times increased from 60 s to 160 s.


A
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2
3
kb
2,650/1,700
1,180/1,050 (x2)
920/870/820
710
600
(x2)
450
350
290
250
850
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B
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kb
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1,700
1,180
1,050
(x2)
920
870
820
710
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(x2)
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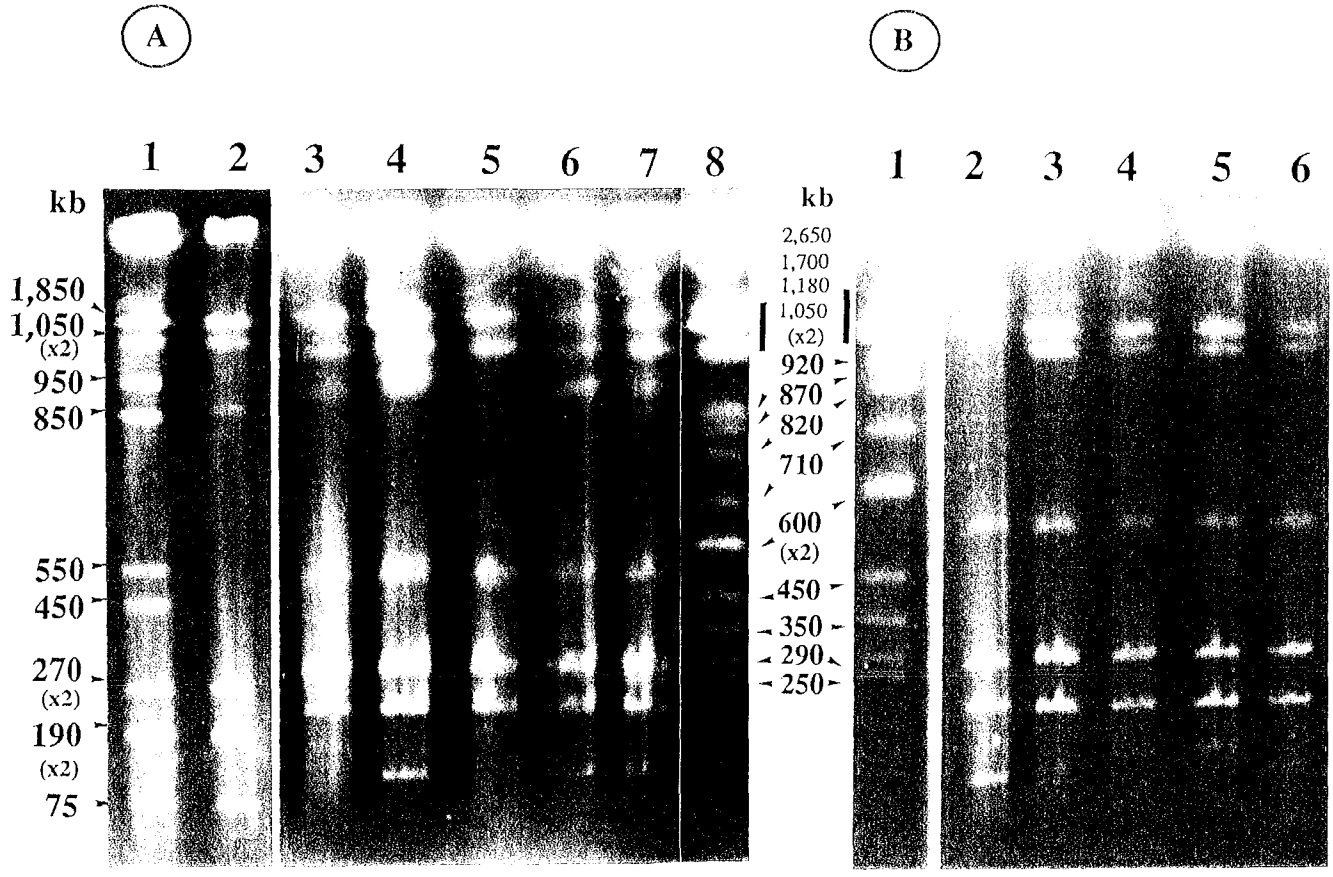
Figure 3: *Ase*I PFGE restriction patterns of total DNA from the WT, hypervariability-derived strains (A) and pigment-defective stable strains (B) of *S. ambofaciens*.
(A) Lane 1: WT DNA; lane 2: NSA108H; lane 3: NSA841H; lane 4: NSA135H; lane 5: NSA43H; lane 6: NSA4H; lane 7: NSA312H; lane 8: *S. cerevisiae* chromosomes used as size standard for lanes 3 to 7. Running conditions were 150 V for 32 hours with increased pulse times from 60 s to 120 s for lanes 1 and 2; the same conditions were applied on lanes 3 to 8 for 30 hours.
(B) Lane 1: *S. cerevisiae* chromosomes used as size standard; lane 2: NSA833; lane 3: NSA64; lane 4: NSA105; lane 5: NSA49; lane 6: NSA27. Running conditions were 150 V for 30 hours with increased pulse times from 60 s to 120 s.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(x2)
920
870
820
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(x2)
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290
250
B
1 2 3 4 5 6

Figure 4: Hybridization of *Ase*I PFGE restriction patterns of WT and non amplified mutant strains DNA with S2 probe.
Lane 1: WT DNA; lane 2: NSA43H; lane 3: NSA4H; lane 4: NSA97H; lane 5: NSA850H; lane 6: NSA108H; lane 7: NSA28; lane 8: NSA27; lane 9: NSA830; lane 10: NSA49; lane 11: NSA844. Running conditions were 150 V for 35 hours with increased pulse times from 40 s to 180 s. The size of the hybridizing fragments are reported in table 3.

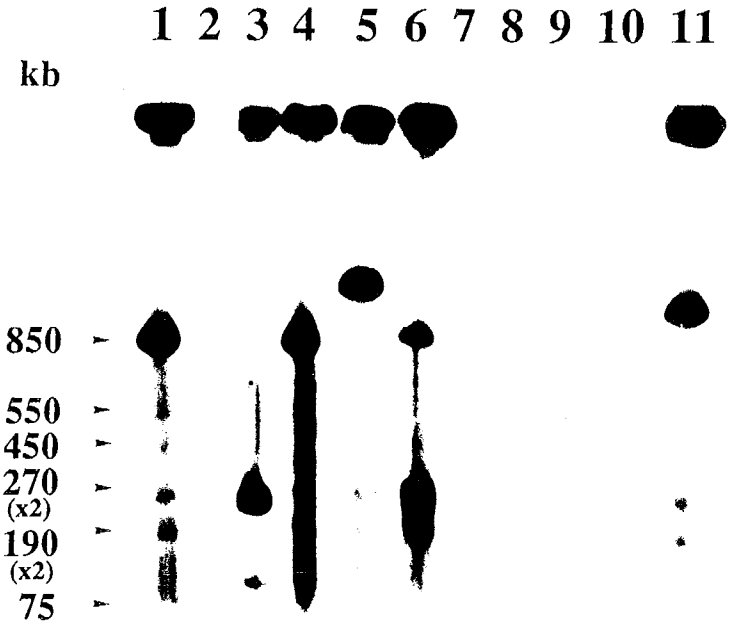
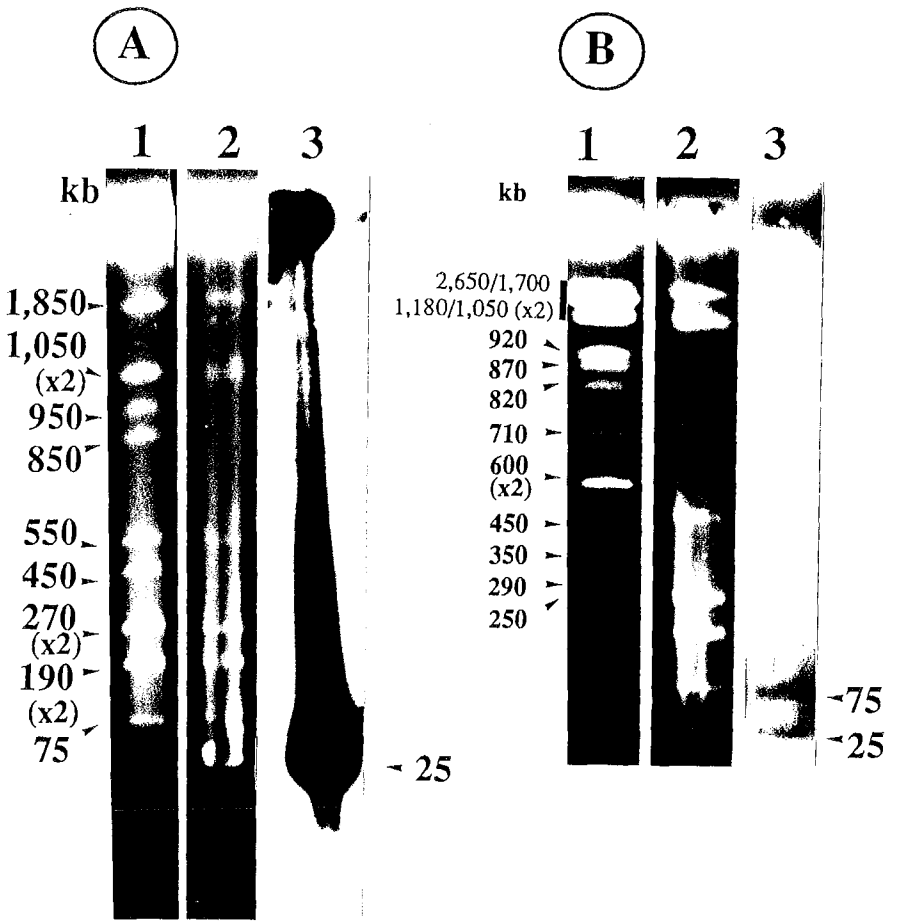

1 2 3 4 5 6 7 8 9 10 11
kb
850
550
450
270
(x2)
190
(x2)
75

Figure 5: Hybridization of *Ase*I PFGE restriction patterns of ADS6-containing mutant strains with S1 probe.
(A) Hybridization on NSA6 DNA. Lane 1: *Ase*I restriction pattern of the WT strain used as reference; lane 2: NSA6 *Ase*I restriction pattern; lane 3: hybridization pattern of NSA6 digest with S1 probe. Running conditions were 150 V for 30 hours with increased pulse times from 60 s to 160 s.
(B) Hybridization on NSA1635 DNA. Lane 1: *S. cerevisiae* chromosomes used as size standard; lane 2: NSA1635 *Ase*I restriction pattern; lane 3: hybridization pattern of NSA1635 digest with S1 probe. Running conditions were 150 V for 30 hours with increased pulse times from 60 s to 120 s.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1,180/1,050 (x2)
920
870
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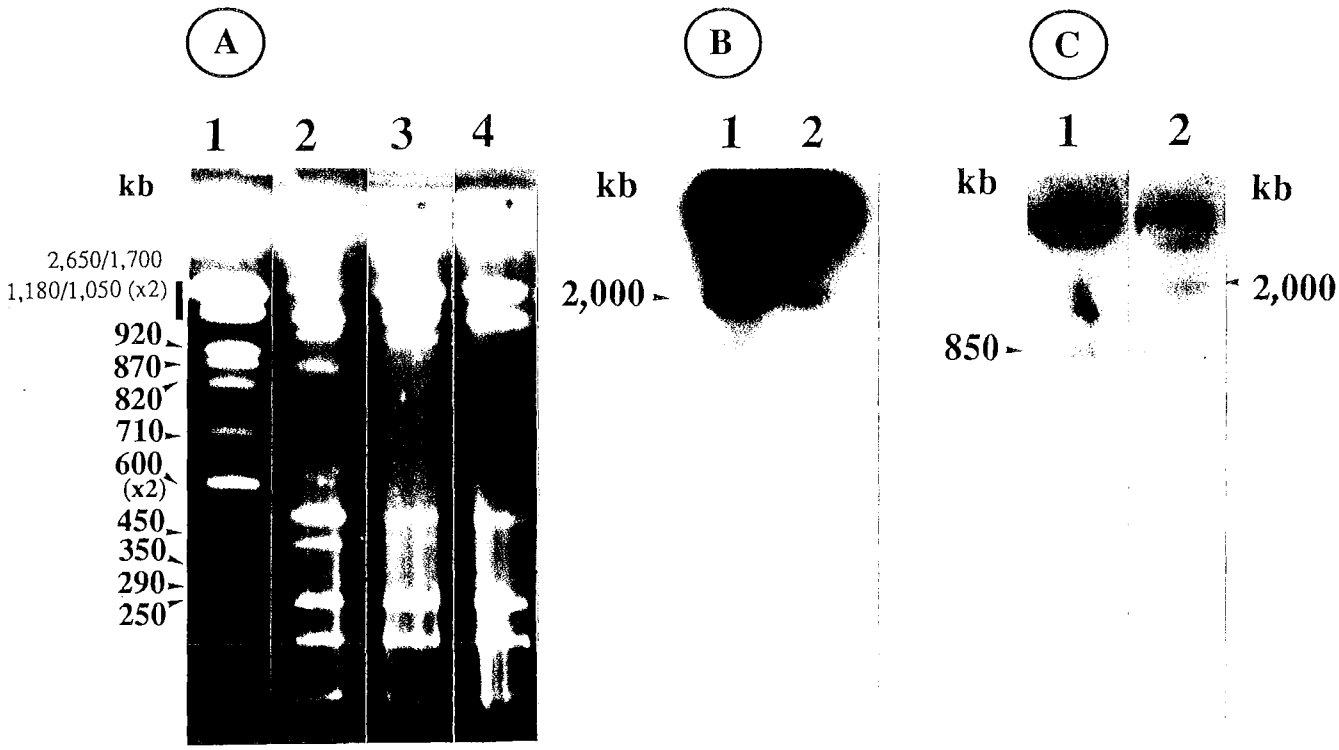
600
(x2)
450
350
290
250
75
25

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


A
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kb
2,650/1,700
1,180/1,050 (x2)
920
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450
350
290
250
B
1
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kb
2,000
C
1
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kb
kb
2,000
850

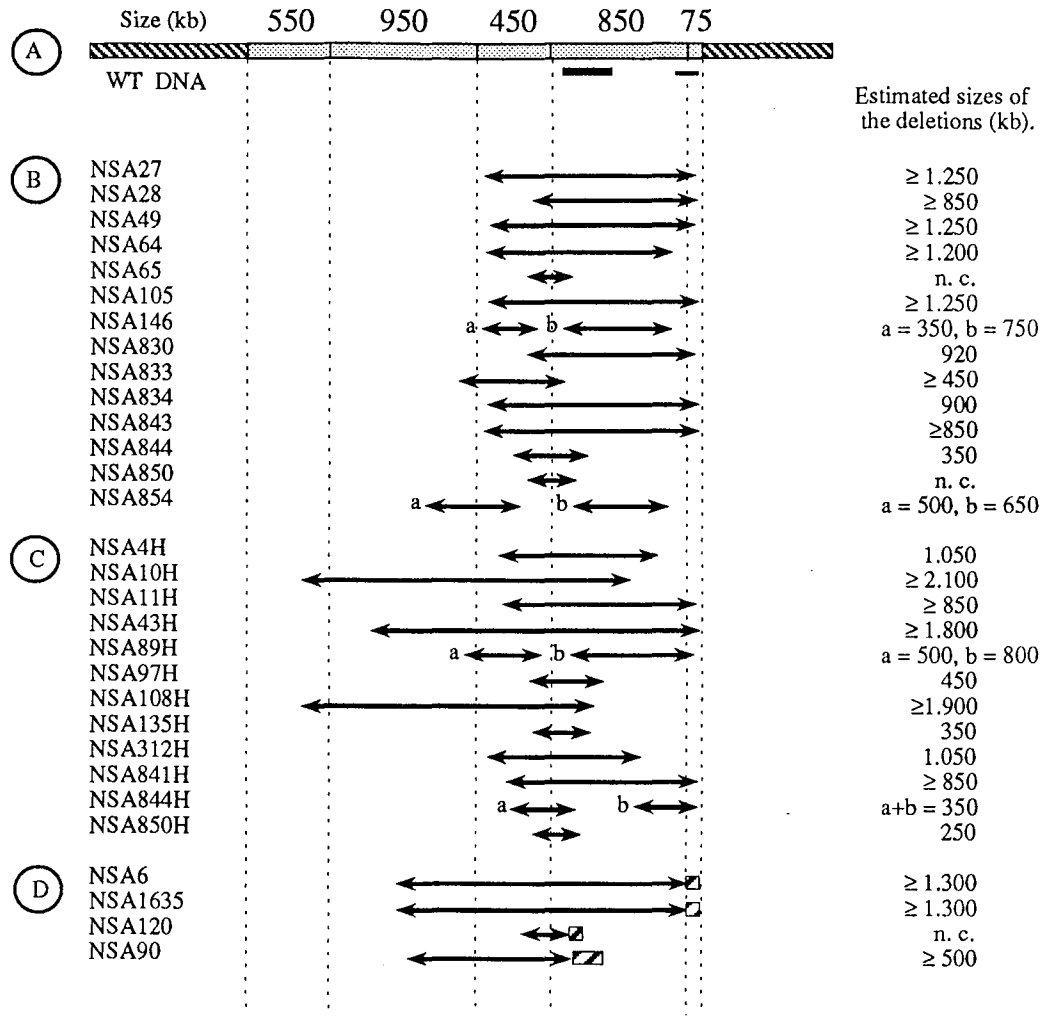

Figure 7 : (**A**) Speculative *Ase*I restriction map of the unstable region in the WT strain. (**B**) Deletions in the pigment-defective stable strains. (**C**) Deletions in the non amplified mutant strains raised from hypervariability. (**D**) Deletions in amplified strains.

- Unknown regions on the WT chromosome,
- Unstable region,
- AUD6 family,
- AUD90 family,
- Symbolizes amplified DNA,
- Deletion size estimations using restriction pattern analysis or hybridization experiments with S2 probe; #, accurate estimation; ≥, minimal estimation; n. c., non calculable, the lack of additional fragment and hybridization response prevent us from size estimation.

A CHROMOSOMAL REGION AS A HOTSPOT FOR MULTIPLE REARRANGEMENTS ASSOCIATED WITH GENETIC INSTABILITY IN *STREPTOMYCES AMBOFACIENS* DSM40697.

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Key words: Genetic instability- Hotspot of rearrangements- *Streptomyces ambofaciens*- AUD structure- Genomic heterogeneity.

SUMMARY

Genetic instability in *Streptomyces ambofaciens* DSM40697 involves genomic rearrangements such as amplifications and deletions of particular DNA sequences. The various amplifications were located in at least two amplifiable regions, one of which called AUD6 (for Amplifiable Unit of DNA) was revealed to be a rearrangement hotspot. Indeed, 30% of the mutant strains studied present rearrangements (amplifications or deletions or both) in the AUD6 locus. The AUD6 contains a sequence presenting reiterations which are specific to the AUD. Moreover, one of the endpoints of the AUD6 shows homology with an internal sequence. In the amplified strains, deletions occured at one side of the ADS (for Amplified DNA Sequence) and removed a part of the proximal copy of this ADS. Thus, this process involves multiple rearrangements as does the loss of an amplification.

INTRODUCTION

Streptomycetes often exhibit a high degree of genetic instability and many characters have been reported to be unstable (for reviews, see Hütter and Eckhardt, 1988; Leblond et al., 1990a). In *S. ambofaciens* DSM40697, a basic genetic instability produces pigment-defective (Pig^-) colonies at the frequency of about 1%. A second aspect of this genetic instability, called hypervariability, has been characterized: a pigment-defective colony generated by the basic genetic instability leads to a phenotypically heterogeneous progeny (Leblond et al., 1989).

Genetic instability could be due to plasmid loss (Usdin et al., 1985) or chromosomal rearrangements by formation of large deletions including the unstable gene (for review, see Hütter and Eckhardt, 1988).

Highly amplified DNA sequences (ADS) have been detected within the DNA isolated from mutants of many *Streptomyces* species (for review, see Cullum et al., 1986). These amplifications appeared either spontaneously (Schrempf, 1983; Altenbuchner and Cullum, 1984, 1985) or after various treatments (Robinson et al., 1981; Hasegawa et al., 1985). In *S. ambofaciens* DSM40697, several ADSs which were isolated in hypervariability-derived strains, have already been described (Demuyter et al., 1988; Leblond et al., 1989).

The amplification formation is, moreover, frequently associated with large deletions. These deletions were detected either immediately adjacent or in close proximity to the ADS (Hasegawa et al., 1985; Birch et al., 1989). Altenbuchner and Cullum (1984, 1985) showed that in *Streptomyces lividans*, the chloramphenicol sensitive mutant strains were extremely unstable: in their progeny, spontaneous arginine auxotrophs (Arg^-) appeared at a frequency of about 25%. The Arg^- strains presented the amplification of a particular 5.7 kb chromosomal fragment and a deletion at one side of the amplifiable region. In several *Streptomyces*, the extent and the localization of chromosomal deletions are quite different. In *Streptomyces glaucescens*, the deletions were found to extend to over 800 kb without affecting the growth (Birch et al., 1989). In *Streptomyces coelicolor*, silent chromosomal arcs exist where very few markers were localized (Chater and Hopwood, 1984). These arcs could then correspond to large stretches of "dispensable" DNA. In bacteria other than *Streptomyces* (for example, *Escherichia coli* and *Bacillus subtilis*), it was shown that the frequency of deletion formation on plasmids was increased by the presence of palindromic sequences or direct repeats (DasGupta et al., 1987; Peeters et al., 1988). The same phenomenon could exist at the chromosome level so that the deletion formation could then be explained by intramolecular recombination.

In *S. ambofaciens* DSM40697, two amplifiable regions have been characterized. One of these regions, the AUD6 (for Amplifiable Unit of DNA) locus, is presented in this work. The lineages of different mutant strains were analyzed for the rearrangements found in the AUD6 locus. This region seems to be a rearrangement hotspot. These rearrangements could proceed through several steps.

MATERIALS and METHODS

Bacterial strains and plasmid. S. ambofaciens DSM40697 (Hütter, 1967) was used in this work as the wild type strain (WT). Amplified mutant strains spontaneously isolated from the WT are listed in the table 1.
Escherichia coli JM109 (Yanisch-Perron et al., 1985) and the plasmid pBluescriptKS (Short et al., 1988) were the host and vector for cloning.

Media and culture conditions. The media used in the culture conditions were described previously (Hopwood et al., 1985). *Streptomyces* strains were grown at 37°C on plates of Hickey Tresner (HT) medium (Hopwood et al., 1985) for mutant isolation and spore production. Large scale DNA isolation was carried out after growth for 48 hours at 37°C in YEME liquid medium (Hopwood et al., 1985) supplemented with glycine (0.25%). Small scale DNA isolations were performed after growth for 24 hours at 37°C in HT liquid medium supplemented with glycine (0.25%).

E. coli strains were grown at 37°C in LB liquid medium (Maniatis et al., 1982).

Isolation of total DNA and restriction analysis. DNA extraction and purification were performed according to Demuyter et al.(1988). Restriction enzymes were purchased from Boerhinger Mannheim (FRG) and used according to the manufacter's recommandations. DNA fragments were electrophoresed on 0.8% agarose gels according to Maniatis et al. (1982). Bacteriophage lambda DNA digested with *Hin*dIII was used as size standard (Daniels et al., 1983).

Cloning of DNA fragment. The 2.9 kb *Bam*HI joint-segment generated by tandem reiteration of the AUD6 was isolated from the total DNA of the NSA6. This fragment was cloned and used as the probe S1 (figure 1). The left part of S1 in the WT AUD6 was called $S1_L$ and the right part $S1_R$. The 3.1 kb *Bam*HI left flanking fragment of the AUD6 was isolated from the total DNA of the NSA60. This fragment was cloned and used as the probe S2. Plasmid pBluescriptKS DNA was used as cloning vector. Purified total DNA from strains NSA6 and NSA60 (containing ADS6 and ADS60, respectively) was digested to completion with *Bam*HI and electrophoresed. After ethidium bromide-staining, the appropriate bands were isolated and purified from agarose by the Geneclean process (Bio 101, USA). Extracted DNA fragments were co-precipitated with *Bam*HI linearized pBluescriptKS (ratio 5/1). The DNA was dissolved in ligation buffer and ligated using T4 DNA ligase (Boerhinger Mannheim, FRG). Transformation was carried out as described by Maniatis et al. (1982). White colonies were presumed to carry pBluescriptKS with inserts. These chimeric plasmids were further analyzed.

The probes S3 and S4, which correspond to the 3.5 kb and 1.9 kb *Bam*HI fragments of the AUD6 respectively, were recovered by elution from the total DNA of the NSA6 strain. To verify that these two probes were not contamined with same size fragments, similar controls as in Demuyter et al. (1988) were done.

^{32}P-labeling of DNA. Southern blotting and hybridization. DNA probes were ^{32}P-labeled (Amersham, UK) according to Feinberg and Vogelstein (1983) using the Multiprime DNA labeling system kit (Amersham, UK) or using a nick-translation kit (Amersham, UK). The labeling could be carried out either with purified DNA or directly in the agarose matrix. Lambda DNA was ^{32}P-labeled by nick-translation (Rigby et al., 1977).

For hybridization experiments, Southern blottings were carried out using the capillary transfer method with the Vacugene system (LKB, Sweden) (Southern, 1975) onto Hybond-N membranes (Amersham, UK). The DNA was depurinated with 0.25M HCl for 30 minutes before denaturation (in 1.5M NaCl, 0.5M NaOH for 1 hour), neutralization (in 1.5M Tris-HCl, 1.5M NaCl for 30 minutes) and vaccuum-transfered with 20x SSC (1x SSC= 0.15M sodium chloride, 0.015 M sodium citrate) for 90 minutes. Prehybridization, hybridization and washing conditions were previously described in Demuyter et al. (1988).

Estimation of the ADSs copy number. The densitometer used was a LKB UltroScan XL (Sweden). The apparent copy number of the ADSs was estimated by measuring the relative peak area, from hybridization patterns with the S1 probe, of the 15 kb *Bam*HI fragment (which is the right flanking sequence of the AUD6 and which is, thus, present as a single copy) and of the 2.9 kb *Bam*HI fragment (which is the segment generated by the reiteration). These relative areas are then corrected with respect to the size of homology of S1 in the 15 kb fragment (2.1 kb) and in the 2.9 kb fragment (2.9 kb) to give the estimated copy number of the amplification. This copy number is only apparent because the mutant strains could present a genomic heterogeneity.

RESULTS

Structure of the AUD6 region

Restriction patterns of amplified DNA extracted from mutant strains isolated from hypervariable lineages allowed us to map eight new overlapping amplifiable sequences with respect to the three AUDs previously described by Demuyter et al. (1988) (figure 1). Two of these AUDs, the AUD102 and the AUD420, were indistinguishable by the methods used. Appart for this case, all the sequences seemed to be different in length. A twelfth amplification (ADS7) was recovered but not independently of the others since it derived from the ADS6 containing strain. Nothing in the map revealed the presence of long direct repeats. This was confirmed by hybridization for the ADS6. This family of amplifications allowed us to define the AUD6 region.

In order to detect rearrangements other than amplifications of the AUD6 locus in various mutant strains isolated from stable and hypervariable lineages, hybridization experiments were carried out with the S1 probe (figure 1).

Southern blots of *Bam*HI digests of total DNA from WT and NSA6 strains were hybridized with the S1 probe. The hybridization pattern of the WT DNA (figure 2A lane 1) revealed three bands: 15 kb, 12 kb, and 3.1 kb. According to the restriction map of the AUD6 region, the 15 kb and 3.1 kb fragments were the flanking sequences of the AUD6. S1 was used on 31 restriction patterns of independently isolated WT colonies derived from DSM40697 and the same pattern was always recovered. Since the AUD6 was detected as a single copy in the WT genome, the fact that S1 hybridized with the 12 kb *Bam*HI fragment demonstrated the presence of a sequence homologous to the probe in this fragment. Hybridization of the S1 probe with various digests of the WT DNA confirmed the existence of this internal homologous sequence (IHS) of less than 1 kb localized within the 12 kb *Bam*HI fragment (figure 1). To determine whether the IHS was homologous to $S1_L$ or to $S1_R$, S2 was used as a probe. *Bam*HI and *Xho*I WT hybridization patterns (figure 2B lanes 11 and 13) revealed only a 3.1 kb *Bam*HI band and a 8.6 kb *Xho*I band. No signal corresponding to the IHS was detected demonstrating that it was homologous to the only right extremity of the AUD6.

Deletions in the AUD6 region

Genomic rearrangements in the AUD6 region were investigated in 96 mutant strains independently isolated from different mutational events. 30 mutant strains derived from phenotypically stable lineages (A strains). The 66 other strains were isolated from hypervariable lineages (Leblond et al., 1989) and presented a stabilized phenotype. 37 of these 66 strains were not amplified (B strains) and 29 presented various amplifications (C strains). Mutant clones were analyzed after two successive rounds of subcloning.

Southern blots of *Bam*HI digests of total DNA from mutant clones were probed with the S1 probe. The majority of the mutant strains (40/30+37) were unaltered in the AUD6 region (figure 2A lane 3). Six different hybridization patterns were revealed in 16/30 A strains and in 11/37 B strains (figure 3). A seventh potential pattern may exist (çalled Δ5) but this kind of rearrangement was not observed in the sample of strains studied here. Some or all of the bands of the characteristic WT pattern (15 kb, 12 kb, 3.1 kb) were missing in altered patterns (figure 2A). These differences can be explained by partial (Δ1 to Δ5) or complete (Δ6) deletions of the AUD6. A partial deletion of the AUD6 generated a fragment which can hybridize with S1 according to the localization of the

right endpoint of the deletion (figure 2A lanes 4 and 6). This endpoint can also be located between the 3.1 kb and 12 kb length fragments (Δ2) or inside the 12 kb length fragment between the IHS and the 0.6 kb sequences (Δ4) (figure 2A lanes 5 and 7).

The extent of the deletions was polarized to the right. For example, no strain was deleted for $S1_R$ without deletion of the 12 kb *Bam*HI fragment. These observations could result from a selection of essential genes which were located to the right of the AUD, in spite of the existence of deletions of the entire AUD (Δ6). Nevertheless, the deletions could be initiated through particular structures or sequences lying within the left region of the AUD. The frequencies of rearrangements were not significantly different in the stable clones and in the non amplified hypervariable clones (figure 3).

Hybridization patterns of 29 different strains carrying amplifications not originating from the AUD6 region revealed no rearrangement in the AUD6 locus.

In the ADS6 DNA, two faint bands of 15 kb and 3.1 kb and two heavy bands of 12 kb and 2.9 kb were the expected signals in a *Bam*HI hybridization pattern (figure 1). However, the 3.1 kb fragment of the left flanking sequence of the ADS6 was absent or masked by the heavy spot of the 2.9 kb reiterated fragment (figure 2A lane 2). The flanking sequence was investigated in NSA6 using S2 as a probe (figure 1) and hybridized with Southern blots of *Bam*HI and *Xho*I digests of total DNA from NSA6 strain (figure 2B lanes 10 and 12). The expected band corresponding to the same flanking sequence, which is a 8.6 kb *Xho*I fragment, disappeared in the *Xho*I pattern of the NSA6 strain. Moreover, no band other than the 25 kb fragment, which corresponded to the ADS, was present. The same fact appeared for the *Bam*HI pattern of the NSA6 strain, which presented only the 2.9 kb reiterated fragment. The amplification ADS6 was associated with a deletion which could be localized at the left side of the AUD and which could remove a part of the proximal copy of the ADS, since no joint-fragment was after deletion detected on the hybridization patterns.

A deletion could also take place at the left side of the ADS101 and ADS102 since no signal was obtained with Southern blots of *Bam*HI and *Xho*I digests of total DNA from the NSA101 and NSA102 strains probed with S2 (data not shown).

In order to specify the right endpoints of the deletions associated with the amplification in the three strains NSA6, NSA101 and NSA102, the four probes S1, S2, S3 and S4 were used.

Hybridizations were carried out using S4 and the total DNA of NSA6 digested with *Bam*HI and *Pvu*II (figure 1). In the WT strain, S4 hybridized with five *Bam*HI fragments (figure 4 lane 1) whose sizes corresponded to those of the AUD6 region. Similar results were obtained with *Pvu*II (figure 4 lane 3). Thus, S4 showed several homologous sequences in the AUD6. Since no other band was present, these homologies were not dispersed on the genome but were specific to this locus. This probe is a useful tool for studying the rearrangements in the amplified strains. The different intensities of the signals in figure 4 could be explained if the homologies of S4 were not perfect in all the fragments.

The *Bam*HI and *Pvu*II hybridization patterns of the total DNA of NSA6 probed with S4 confirmed the homologies found in the WT (figure 4 lanes 2 and 4). The *Bam*HI pattern revealed an additional 2.9 kb band. Since this fragment cannot correspond to S1 (because S1 did not hybridize with S4), this fragment could be a joint-fragment generated by the deletion. The *Pvu*II pattern showed a 6.1 kb fragment which could constitute the joint-fragment in question. These results suggested that the deletion, at the left side of the ADS6, removed a part of the proximal copy of the ADS and that the right endpoint of this deletion was localized in the 1.9 kb *Bam*HI fragment.

The same experiments were carried out with NSA101 and NSA102 using S3 and S4 as probes (data not shown). This led to the conclusion that in NSA101, a deletion occured at the left side of the ADS101 and removed part of the proximal copy of the ADS. Moreover, hybridizations with S3 confirmed the homologies found with S4 (data not shown). Hybridizations with the NSA102 strain showed that S3 and S4 were deleted in this strain (data not shown) and since this ADS was shorter than the other two, it was impossible to investigate whether the deletion removed a part of the proximal copy of the ADS102.

The fact that the proximal copy of an ADS was partially deleted implies that a deletion event occured after the amplification process (or at least after a first step of duplication to preserve one intact copy of the AUD). This process could remove several copies of the ADS. The deletion events presented the same polarity in the amplified strains as in the unamplified strains.

Instability of the ADS6 amplification

In order to test whether an ADS was lost by successive steps of deletion, the stability of the amplification was studied in several mutant clones derived from the NSA6 lineage by hybridization experiments with S1. Four successive rounds of sporulation were carried out from the amplified progenitor NSA6 and the different studied clones were randomly isolated as single colonies in this lineage for the first two rounds of sporulation. Only colonies which conserved the ADS were subcloned for the third round of sporulation and only the colonies with a very low copy number were subcloned for the fourth round of sporulation.

The NSA6 amplified progenitor led, after the first round of sporulation, to 5/5 studied clones which conserved the ADS but with an apparent reduced copy number (figure 5). After the second round of sporulation, 12 randomly chosen colonies in the progeny of the NSA6 progenitor were studied: 4 of them presented the ADS, 5 showed a loss of the entire ADS associated with a partial deletion of the AUD6 (one with a Δ2 and four with a Δ3 rearrangement) and 3 had also lost the ADS but with a complete deletion of the AUD (Δ6). One colony (NSA631) presenting an ADS including about 29 copies was subcloned and the eight clones which were studied in the progeny had conserved the ADS. The apparent copy number ranged from 1.3 to 47 (figure 5). Thus, the copy number of the ADS was heterogeneous. The apparent copy number of 1.3 could be explained by a genomic heterogeneity of the corresponding colony containing two genomes: one which was amplified and one which did not present the ADS.

Another colony (NSA641) which had conserved the ADS with a copy number of 18 (figure 5) was subcloned and 14 colonies were obtained after the third round of sporulation. 5/14 clones studied presented the loss of the ADS associated with a partial deletion of the AUD (three Δ2 and two Δ3). 2/14 presented a modified ADS (which included about ten copies) in which an internal deletion occured removing a part of the 12 kb fragment. This AUD was called AUD7 (figure 1). This fact implied that this deletion occured before the amplification process. 5/14 showed the typical ADS6 pattern (with a copy number ranging from 19 to 98) and 2/14 studied clones ($NSA641_1$ and $NSA641_2$) presented the amplified pattern but the apparent copy number of these two ADSs was determined to be reduced to only 0.4 and 0.3 copy, respectively (figure 5). This fact could be interpreted as a transitory step of loss of the ADS. These two strains were then subcloned and in all the clones tested (58), the ADS6 was lost in association with a partial or complete deletion of the AUD6.

Thus, the ADS6 was an unstable amplification and when this amplification was lost, the AUD6 was never intact and Δ2 seemed to be the most frequent deletion observed. There was also Δ3 and Δ6 clones. In a few cases (3), a modified amplification was recovered in the progeny of the ADS6 containing strain.

Moreover, the apparent copy number of the amplification could increase or decrease at each round of sporulation but the sample studied was too small to test statistical significance.

AUD6 locus rearrangements in the stable Pig⁻ mutants

Spores of two Pig⁻ (pigment-defective) colonies (PIG1⁻ and PIG2⁻), independently isolated in the progeny of two WT colonies, were plated on HT medium at the rate of about 100 spores/plate (five plates), incubated at 37°C and observed after growth for five days. All the mutant clones exhibited the parental Pig⁻ phenotype. These platings were classified as homogeneous stable progeny because no other phenotype was detected in the sample of colonies observed in the case of PIG1⁻ and PIG2⁻.

Genomic rearrangements at the AUD6 locus were investigated in a sample of 32 and 31 mutant colonies isolated from the progenies of PIG1⁻ and PIG2⁻, respectively. Hybridization patterns showed a very high degree of heterogeneity. Partial or complete deletions at the AUD locus were detected at high frequency (20/32 and 31/31, PIG1⁻ and PIG2⁻, respectively). Among the colonies presenting rearrangements several were more frequent than others. In the progeny of PIG1⁻, one clone presented a Δ2 rearrangement, 15 a Δ3 one, 2 a Δ5 one and 2 a Δ6 one. In the progeny of PIG2⁻, 11 clones showed a Δ3 rearrangement, 5 a Δ4 one and 15 a Δ6 one.

DISCUSSION

The AUD6 locus as a hotspot for DNA rearrangements

A high degree of genomic instability mainly affecting two chromosomal regions (AUD90 and AUD6) was revealed in mutant strains derived from independent genetic instability events. This work reports the rearrangements detected at the AUD6 locus. The structure found for the AUD6 family (12 AUDs in this family) is very similar to the AUD locus observed in *S. glaucescens* (Häusler et al., 1989). Indeed, heterogeneity in size and amplification degree was observed in this family of ADS. According to the nomenclature proposed by Hütter et al. (1988), the AUD6 family of *S. ambofaciens* can be ranged as type I amplifications.

DNA amplifications were previously reported in 21% of hypervariability derived strains (Leblond et al., 1989). Among 48 ADS containing strains isolated from independent hypervariable lineages, 11 (23%) were mapped in the AUD6 locus. Deletions accompanying ADS were found. These deletions occured at one side of the ADSs and removed in two cases a part of the proximal copy of the ADS. In addition, pedigree analyses of the amplified strain NSA6 revealed a loss of the amplification associated with a partial or complete deletion of the AUD6. The study of the apparent copy number revealed a high heterogeneity in the progeny of the parental NSA6 strain.

On the other hand, taking into account the three types of strains (A strains: phenotypically stable strains; B strains: unamplified hypervariable strains; C strains: ADS containing strains), about 30% of the mutant strains presented rearrangements in the AUD6 region (16/30 A strains, 11/37 B strains and 0/29 C strains). Moreover, the detected deletions presented a different right endpoint in the various studied mutant strains. 20% of these harbored deletions ending in the AUD6 suggesting that this locus constitutes a rearrangement hotspot in *S. ambofaciens* DSM40697. Indeed, these deletions presented an endpoint included in a 25 kb DNA region (AUD6) consisting in about 0.3 % of the whole genome (estimated to about 8,000 kb by Leblond et al., submitted).

Therefore, a close relationship between the structural instability of this locus and the genetic instability was established.

The ability of the AUD6 locus to undergo sequential rearrangements

Several observations suggested a multi-step process in the rearrangements of the AUD6 locus rather than a single step mechanism. In the case of NSA6 and NSA101, a deletion event occured after the amplification process. Thus, the loss of the amplification ADS6 also suggested a multi-step process. One mutant clone (NSA7) isolated from the lineage of the amplified strain NSA6 exhibited a deletion in the AUD6 tandemly reiterated. These observations were consistent with a rearrangement within the AUD before the amplification process.

DNA pattern analyses of progenies of two pigment-negative colonies revealed a high degree of genomic heterogeneity. A genomic hypervariability could be triggered during the development of a Pig^- colony affecting the pigmentation genes: the genotypically heterogeneous colonies exhibited the Pig^- phenotype. In one progeny, no WT AUD6 locus was recovered. On the basis of the existence of a single genome in a spore, the genomic hypervariability could result from several mutational events which occured during the growth of the colony. This clonal effect suggests that these mutations

could occur at different stages during the colony development. Thus, the basic genetic instability could consist in a high frequency (1%) of a primary mutational event inducing a genomic instability preferentially directed through specific loci such as the AUD6 locus.

Genome plasticity in the AUD6 locus

Previously, a diagram has been reported to explain the rearrangements into the upstream region of the amplifiable loci in the mutant strains (Leblond et al., 1990a). The principles of the model are applied here to the AUD6 locus to account for the high degree of plasticity of this locus in the phenotypically stable and hypervariable mutant clones.

In *S. ambofaciens* DSM40697, Leblond et al. (1990b) have shown, using PFGE, that all the rearrangements detected were located in a chromosomal arc and that the deletions were from 250 up to 2,000 kb in size. The two amplifiable regions were located in the chromosomal arc which presented the genome plasticity. In *S. lividans*, Cullum et al. (1988) have shown that the minimum size of the deletion associated with the amplification in the chloramphenicol sensitive and Arg$^-$ mutants was of about 250 kb. In *S. glaucescens*, the deletion sizes ranged from 250 to 800 kb (Birch et al., 1989).

The AUD6 contained a sequence homologous to several DNA fragments belonging to the AUD. These homologies were specific to the AUD6 and were recovered in another *S. ambofaciens* strain: the ATCC strain (Dary A., com. pers.). The AUD90 showed also such homologies but were different of those of the AUD6. Moreover, the right endpoint of the AUD6 presented an homology with an internal sequence (IHS). Such a structure was reported for the biosynthetic genes of erythromycin in *Saccharopolyspora erythraea* (Katz et al., 1990). The different deletions observed in the AUD6 could occur by recombination between two of these homologies. Thus, ADSs could be lost through two mechanisms. First, homologous recombination could occur between reiterated copies of the AUD. Secondly, recombination (homologous or illegitimate) events, analogous to those generating polar deletions in unamplified mutant strains and assumed to trigger amplifications (Leblond et al., 1990a), could occur between a sequence external to the ADS and a sequence belonging to the amplification.

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Table 1: Amplified mutant strains used in this work.
For each strain are presented the origin of the strain, the type of amplification and the deletions observed. Δ2 represents in this table a deletion which removes a part of the proximal copy of the corresponding ADS without affecting the 12 kb *Bam*HI fragment (figure 2). For the NSA102, + reveals the presence of a deletion at the left side of the ADS but we were not able to localize precisely the right endpoint of this deletion.
nd: not determined.

Strain	Origin	Type of amplification	Deletion
NSA6	Hypervariable lineage	ADS6	Δ2
NSA60	Hypervariable lineage	ADS60	nd
NSA101	Hypervariable lineage	ADS101	Δ2
NSA102	Hypervariable lineage	ADS102	+
NSA631	Derived from NSA6	ADS6	nd
NSA641	Derived from NSA6	ASD6	nd
$NSA641_1$	Derived from NSA641	ADS6	nd
$NSA641_2$	Derived from NSA641	ADS6	nd

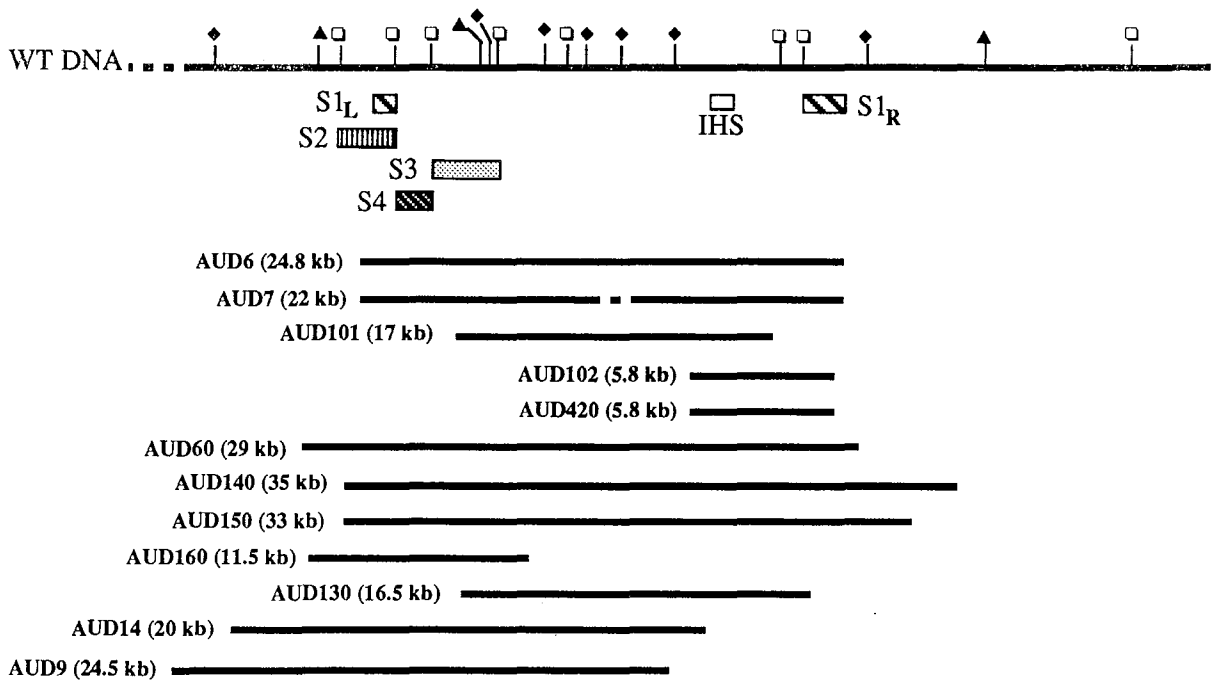
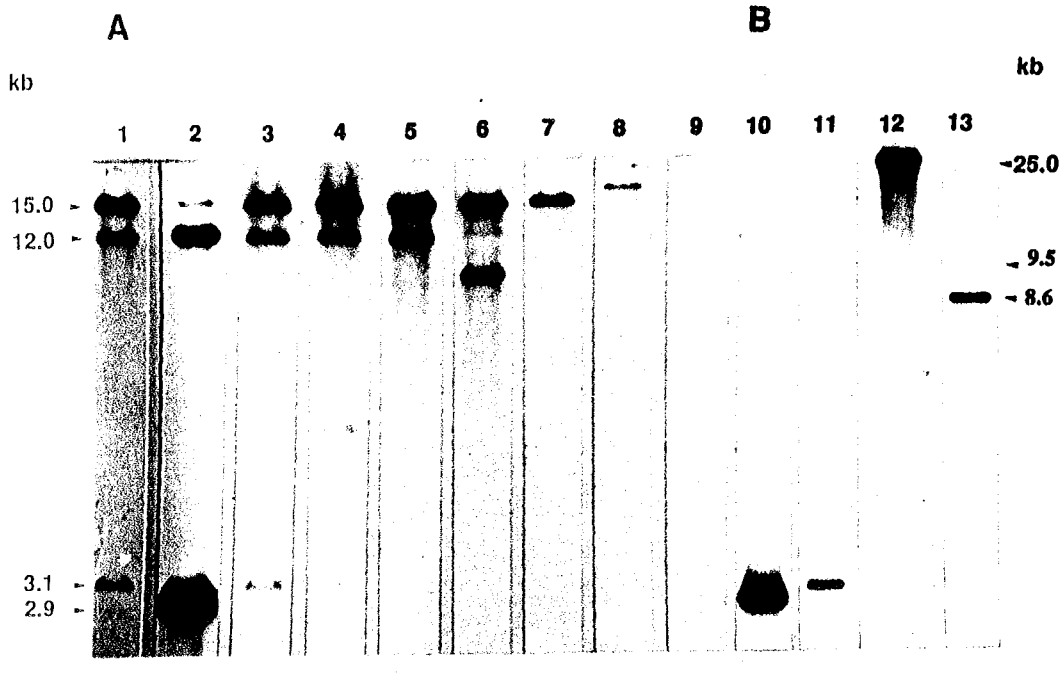
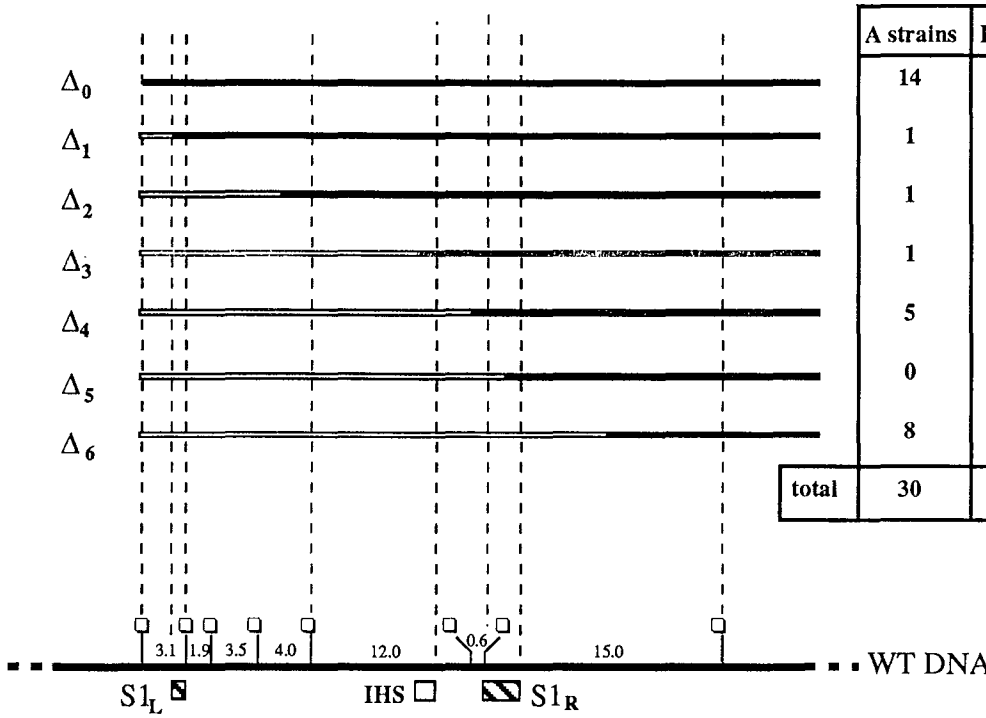


Figure 1: Map of the AUD6 locus of *S. ambofaciens* DSM40697. The eleven independent AUDs belonging to the AUD6 family are showed. The AUD7 presents an internal deletion of the WT AUD (symbolized by dotted lines) and derives from the NSA6 strain. The following restriction enzymes were used: *Bam*HI (❑), *Pvu*II (◆) and *Xho*I (▲). The following symbols are used: S1, S2, IHS, S3 and S4.

Figure 2:
(**A**) Hybridization patterns of the total DNAs of the WT strain and of various mutant strains which were digested with *Bam*HI. The probe used was the cloned endpoints of the ADS6 (S1). Lane 1: WT pattern. Lane 2: pattern of the amplified NSA6 strain. Lane 3: pattern of the strains without detectable rearrangement in the AUD6 region (Δ0). Lanes 4 to 8: patterns which correspond to partial deletions of the AUD6 (Δ1 to Δ5). Lane 9: pattern which corresponds to a total deletion of the AUD6 (Δ6).
(**B**) *Bam*HI (lanes 10 and 11) and *Xho*I (lanes 12 and 13) hybridization patterns of the total DNAs of the WT strain (lanes 11 and 13) and of the NSA6 strain (lanes 10 and 12). The probe used was S2 which was cloned.
The differences of intensity between a single copy signal (fragment of 15 kb) in the NSA6 strain and a single copy signal (15 kb, 12 kb and 3.1 kb fragments) in the WT and the mutant strains were due to a greater amount of DNA and to a longer exposure of the filters for the WT and mutant strains.

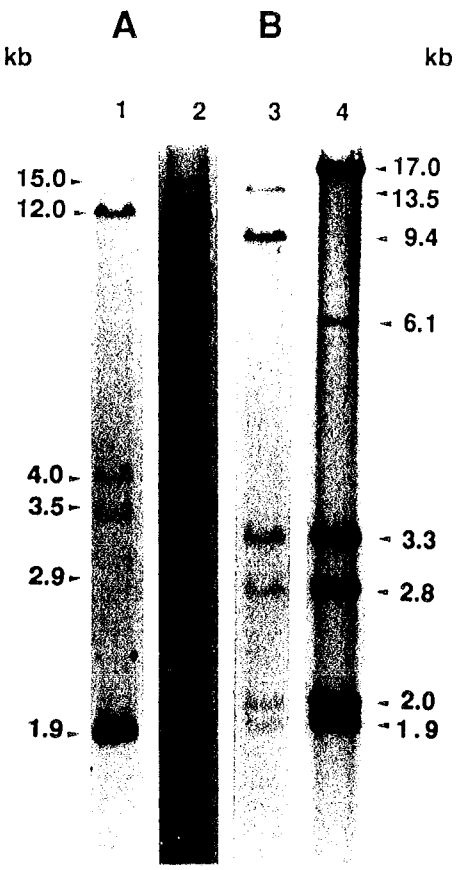

A
B
kb
kb
1
2
3
4
5
6
7
8
9
10
11
12
13
15.0
12.0
3.1
2.9
25.0
9.5
8.6



	A strains	B strains	C strains
	14	26	29
	1	1	0
	1	2	0
	1	0	0
	5	3	0
	0	0	0
	8	5	0
total	30	37	29

Figure 3: Rearrangements in the AUD6 region. Δ1 to Δ6 are the different observed deletions. The black lines over the WT map represent the sequences which are not deleted. The white boxes are the sequences which are deleted in the different mutant strains. The right endpoint of the deletion is localized in each case in a *Bam*HI fragment. For Δ0, no deletion is detectable. The sequences left to the 3.1 kb *Bam*HI fragment are not known to be deleted and are supposed to be deleted in the cases of Δ1 to Δ6 but the extent of the deletions is unknown. The *Bam*HI restriction sites (❑) are shown. S1▨, IHS:□.The number of clones presenting rearrangements in the AUD6 region for different mutant strains (strains A, B and C) are shown at the right of this figure.

Figure 4:
Hybridization patterns using the S4 probe, which is the 1.9 kb *Bam*HI fragment of the ADS6. The total DNAs of the WT strain (lanes 1 and 3) and of the NSA6 strain (lanes 2 and 4) were digested with *Bam*HI (**A**) and *Pvu*II (**B**).
The *Bam*HI patterns revealed a strong hybridization with a 1.9 kb fragment (which corresponds to the probe) and with four other fragments whose sizes corresponded to those of the AUD6 region. The same fact was recovered with the *Pvu*II patterns.


A
B
kb
kb
1
2
3
4
15.0
12.0
4.0
3.5
2.9
1.9
17.0
13.5
9.4
6.1
3.3
2.8
2.0
1.9

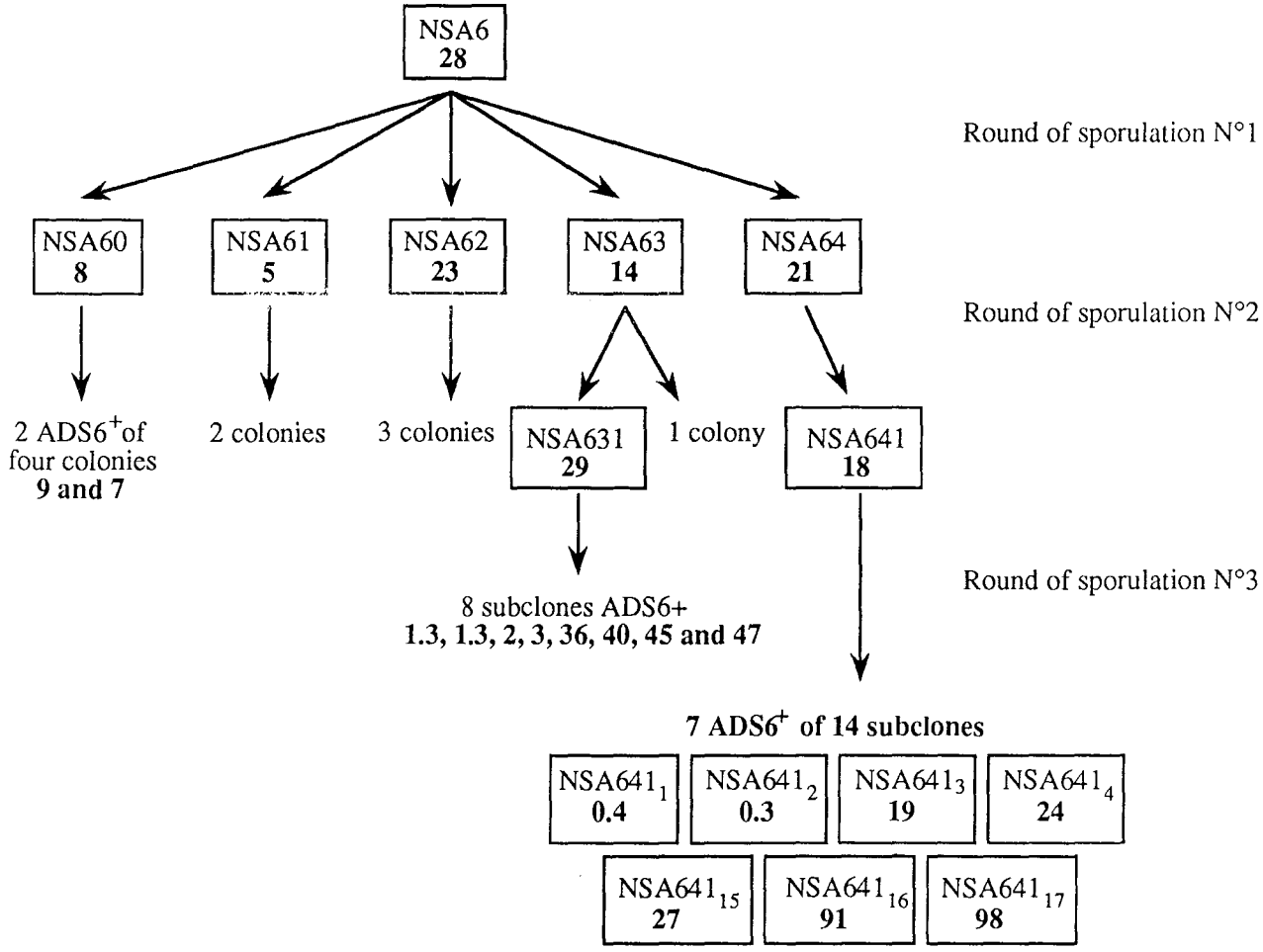


Figure 5: Pedigree analysis of mutant clones in the lineage of the NSA6 strain. All the mutant strains were analyzed by their *Bam*HI hybridization pattern using S1 as a probe. At each round of sporulation, the number of clones studied and the number of clones presenting an amplified pattern for the ADS6 (ADS6+) are shown. The apparent copy number of the ADS6 in the different amplified strains is indicated in bold characters.

"Genetic hypervariability in *Streptomyces ambofaciens*: plasticity of a genomic arc."

The wild-type (WT) strain *Streptomyces ambofaciens* DSM40697 exhibits a high degree of genetic instability. Pigment-defective colonies were spontaneously generated from the wild-type colonies at the frequency of 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 aspect of genetic instability was called hypervariability. In contrast, auxotrophic mutants were obtained from the same WT strain at the frequency of 5×10^{-5} suggesting that genetic instability preferentially affects characters that are developmentally-regulated.

Amplified DNA sequences (ADS) were detected in 21% of strains derived from hypervariable progeny, whereas none were detected in either stable pigment-defective or wild-type colonies. So, both hypervariability and DNA amplification were associated. The size of the AUDs (Amplifiable Unit of DNA) ranged from 5.2 kb to 105 kb and the degree of amplification is quite variable between strains. The analysis of 48 independently isolated ADSs allowed to characterize at least two non-overlapping AUD loci (AUD6 and AUD90) generating more than 73% of the amplification events. No group could be formed with the remaining AUDs. More than 75% of the AUDs could be mapped, using PFGE analysis of the WT genome, on two adjacent large DNA fragments corresponding to about 10% of the genome.

The structure of the AUD6 loci was investigated in the WT genome using classical analysis: the AUD was present as a single copy and no large repeated sequences could be found at the extremities.

The study of the genomic structure of the AUD6 locus in mutant strains led to the conclusion that deletions were intimely associated to genetic instability. Hence, 35% of non amplified strains isolated from stable and hypervariable lineages revealed partial or total deletion of the AUD6. The deletion process revealed a polarity. A similar genomic heterogeneity was found in the progeny of two stable mutant strains. Thus, the AUD6 locus constituted a "hotspot" for genomic rearrangements (amplification and multiple deletions). Moreover, successives deletions were detected in the progeny of some mutant strains and seemed to be implied in the deamplification process.

PFGE analysis of the same mutant strains (amplified or not) revealed also a polar process involving large deletions. Their size ranged from 250 kb to more than 2,000 kb. These deletions affected a large chromosomal arc including the AUD loci. The chromosomal location and the tandem array structure of the ADS was demonstrated in one amplified mutant strain using PFGE.

The close association between genetic instability and giant deletions suggests the dominating part of deletions in the mecanism of genome plasticity and the possible involvement of a primary mutational event triggering these deletions.