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Genetic Instability and DNA Amplification in *Streptomyces lividans* 66

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***Streptomyces lividans* 66 exhibits genetic instability, involving sequential loss of resistance to chloramphenicol (Cam^s) and subsequent mutation of *argG*. Associated with this instability is the amplification of a 5.7-kilobase (kb) amplified DNA sequence (ADS). We have characterized a second, independent pathway of genetic instability, involving sequential loss of resistance to tetracycline (Tet^s) followed by mutation in nitrogen assimilation (Ntr). We detected DNA amplification in many of these mutant strains, as well as other reiterations coresident with the 5.7-kb ADS in Cam^s Arg mutants. However, in contrast to the 5.7-kb ADS, none of the novel elements were observed to amplify at high frequency. The mutation of *argG* is due to a deletion, one endpoint of which is defined by the 5.7-kb ADS. This amplification derives from a structure, the tandemly duplicated amplifiable unit of DNA (AUD), present in the wild-type genome. We found that progenitor strains containing just a single-copy AUD failed to reproducibly generate amplification of this element in Cam^s *argG* mutants, and DNA deletion endpoints proximal to the element were found to be unspecific. These results suggest that a duplicated AUD structure is required for high-frequency amplification and that this reiteration can subsequently buffer the extent of deletion formation in the relevant chromosomal region.**

Many *Streptomyces* spp. exhibit an extraordinarily high mutation rate in certain genes involved in both their primary metabolism and their secondary metabolic pathways such as antibiotic synthesis (reviewed in references 14 and 29). The enhancement of this high spontaneous mutability by plasmid-curing agents initially led researchers to propose that these unstable traits might be plasmid encoded (1, 10, 24). Subsequently several such unstable genes were shown to be located on the chromosomes of wild-type strains, while analysis of variant strains indicated that mutant phenotypes were often due to deletions of chromosomal regions containing these genes (11, 16, 21, 28, 35).

A second remarkable phenomenon associated with this genetic instability, which often correlates with deletion of unstable genes, is the formation of very high copy number tandemly amplified DNA sequences (ADS; 8, 9) in the genomes of variant strains of several species (2, 8, 11, 27, 28).

Numerous examples of selected gene amplification have been observed in both procaryotic (6, 18, 26, 34) and eucaryotic systems (reviewed in reference 33). Amplification of drug-resistant genes was detected at low frequency in *Proteus mirabilis* cell populations that had not previously been exposed to antibiotics, suggesting that the subsequent application of a selective regimen for high resistance favored outgrowth of the subpopulation containing the amplified gene (12). DNA amplification in *Streptomyces* spp. occurs in spontaneous segregants of unamplified strains and may result in a 500-fold increase in the copy number of certain sequences which can be subsequently stably inherited. A range of different sequences can be amplified, and to various extents (5 to 30% of the total DNA), in different mutants derived from the same progenitor strain. Two examples of selected gene amplification in *Streptomyces* spp. have been described: amplification of a spectinomycin resistance determinant in *Streptomyces achromogenes* subsp. *rubradiris* (U. Hornemann, C. J. Otto, and G. G. Hoffmann, Abstr. 5th Int. Symp. Genet. Ind. Microorg. 1986, S4-P2, p. 29) and a reiterated sequence carrying an α-amylase inhibitor gene (K. P. Koller and G. J. Riess, Abstr. 5th Int. Symp. Genet. Ind. Microorg. 1986, W1-P15, p. 91). However, as yet no functions have been ascribed to spontaneously derived, unselected amplifications in other species, although it has been shown that those of *S. reticuli* variants do not represent genes for rRNA or tRNA (29). In the absence of obvious selection schemes to obtain such amplifications, the nature of the pressure which favors their generation and subsequent inheritance is still elusive.

Two independent investigations have shown that derivatives of *S. lividans* 66 exhibit the characteristic features of genetic instability and DNA amplification. It was demonstrated that a plasmid-free *S. lividans* strain, TK64, is unstable for both chloramphenicol resistance (Cam^r) and arginine biosynthesis (2), as previously discovered for *S. coelicolor* A3(2) (30). It was shown that a 5.7-kilobase (kb) ADS was present in the genomes of several Cam^s Arg variants of TK64 (2, 3). Another *S. lividans* strain, a Cam^s derivative, M252, was found to segregate morphological and pigmentation variants at high frequency, and the majority of these segregants contained a variety of amplified elements (29).

We chose the wild-type *S. lividans* 66 strain, 1326, as the progenitor strain for our studies, since TK64 might well have already undergone changes in unstable genetic traits as a result of its derivation from the wild type by protoplasting and UV irradiation (15), as shown for a similarly derived strain, TK23, in this paper. The Cam^s phenotype of M252 was a clear indication of at least one difference in unstable characteristics between it and the wild type.

We observed two patterns of genetic instability in morphological-pigmentation variants derived from *S. lividans* 1326. In addition to the previously described Cam^s Arg instabilities, we discovered a second unstable pathway resulting in loss of resistance to tetracycline (Tet^s) and subsequent mutation in nitrogen assimilation (Ntr). We could find a variety of amplified DNA elements in all the strains we

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TABLE 1. Strains of *S. lividans* 66

Strain (source)	Relevant phenotype or genotype (plasmid status)[a]	AUD or ADS status	Mutagenic treatment of progenitor or derivation
1326 and derivatives[b]			
1326 (J.I.I.)[c]	Wild type (SLP2 SLP3)	2 × 6.8-kb AUD	
S52, S84, S93	Cam^s Δ*argG*	5.7-kb ADS	
V23, V112	Cam^s Δ*argG*	5.7-kb ADS	UV
E20, E211, E212	Cam^s Δ*argG*	5.7-kb ADS	Ethidium bromide
Q35, Q421	Cam^s Δ*argG*	5.7-kb ADS	Quinacrine
Q3943, Q3947	Cam^s Δ*argG*	5.7-kb ADS + 12.5-kb ADS	Quinacrine
S11	Cam^s Tet^s Δ*argG*	5.7-kb ADS	
V14	Cam^s Tet^s Δ*argG*	5.7-kb ADS	UV
V111	Cam^s Tet^s Ntr$^-$ Δ*argG*	5.7-kb ADS	UV
E217	Cam^s Tet^s Ntr$^-$ Δ*argG*	5.7-kb ADS	Ethidium bromide
S81, S90, S110, S120	Cam^s Tet^s Ntr$^-$ Δ*argG*	5.7-kb ADS	
Q34, Q40	Cam^s	1 × 6.8-kb AUD	Quinacrine
C68	Cam^s	1 × 6.8-kb AUD	Mitomycin C
S3	Cam^s	2 × 6.8-kb AUD	
Q422, Q432	Cam^s	2 × 6.8-kb AUD	Quinacrine
Q3944	Cam^s Δ*argG*	1 × 6.8-kb AUD	Quinacrine
V27	Cam^s Δ*argG*	1 × 6.8-kb AUD	UV
E203	Cam^s Tet^s Δ*argG*	1 × 6.8-kb AUD	Ethidium bromide
Q36	Cam^s Δ*argG*	ΔLH[d] 6.8-kb AUD	Quinacrine
Q393	Cam^s Δ*argG*	Δ6.8-kb AUD	Quinacrine
TK23 and derivatives	*spc-1* (*)	1 × 6.8-kb AUD	Protoplasting UV (13)
TK23 (J.I.I.)			
910	Cam^s *spc-1* Δ*argG* (*)	Δ6.8-kb AUD + 40-kb ADS	
1331, 1818, 2321	Cam^s *spc-1* Δ*argG* (*)	Δ6.8-kb AUD	
1026	Cam^s Tet^s *spc-1* Δ*argG* (*)	Δ6.8-kb AUD	
1013	Cam^s Tet^s Ntr *spc-1* Δ*argG* (*)	ΔLH 6.8-kb AUD	
617	Cam^s Tet^s Ntr *spc-1* Δ*argG* (*)	18-kb ADS (1 × 6.8-kb AUD)[n]	
1713	Cam^s Tet^s Ntr *spc-1* Δ*argG* (*)	ΔLH 6.8-kb AUD + 18.5-kb ADS (RH[d] 6.8-kb AUD)[n]	
3198 and derivative			
3198 (J.I.I.)	*his-2 leu-2 ura-6 ath-8 str-8* (*)	1 × 6.8-kb AUD	Recombinant (13)
M417	Cam^s *his-2 leu-2 ura-6 ath-8 str-6* Δ*argG* (*)	1 × 6.8-kb AUD	
M7-1 and derivatives			
M7-1	*pro-2 spc-1* (pIJ303)	1 × 6.8-kb AUD	Recombinant (4)
M7-1/A	Cam^s Tet^s Ntr *pro-2 spc-1* Δ*argG* (pIJ303)	1 × 6.8-kb AUD	
M7-1/B	Cam^s Tet^s Ntr *pro-2 spc-1* Δ*argG* (pIJ303)	1 × 6.8-kb AUD + 40-kb ADS	
M7-1/C	Cam^s Tet^s Ntr *pro-2 spc-1* Δ*argG* (pIJ303)	Δ6.8-kb AUD	

[a] (*), Plasmid-free; the plasmid status of 1326 derivatives was not determined.
[b] Variants derived from 1326 sharing a similar clonal origin are denoted by identical characters in the first three positions of their strain number. Other 1326 variants were of independent clonal origin, as were variants derived from all other progenitors.
[c] J.I.I., John Innes Institute, Norwich, England.
[d] LH, Left-hand AUD sequence; RH, right-hand AUD sequence.

examined, but no single element, with the exception of the 5.7-kb ADS, was reiterated at high frequency. It appears necessary for a tandemly duplicated amplifiable unit of DNA (AUD) to be present in the genome of a progenitor for subsequent high-frequency amplification to occur. Moreover, our data suggest that the amplified sequence in mutant genomes may act as a buffer against the extension of deletions into neighboring essential genomic regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The genotypes and phenotypes of *S. lividans* 66 strains and representative variants isolated in the course of this study are described in Table 1. Variants were isolated as spontaneous segregants of the wild type or after mutagenic treatment of wild-type spores, allowing a 10% survival rate. Mutagenesis was by plating spores on complete medium (CM) containing 2.5 μg of ethidium bromide per ml, 200 μg of quinacrine per ml, or 4 μg of mitomycin C per ml, or by irradiation of spores plated on unsupplemented CM with shortwave UV light for 5 min at a distance of 40 cm. CM plates contained (per liter) 3 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of Bacto-Peptone (Difco), 3 g of malt extract (E. Merck AG, Darmstadt, Federal Republic of Germany), 10 g of glucose, and 14 g of agar, with 5 mM $MgCl_2$ added after autoclaving. DNA was isolated

from strains either after growth in liquid CM containing, in addition, between 10 and 34% sucrose and 0.5% glycine, or after cultivation in tryptic soy medium containing (per liter) 10% sucrose, 30 g of Difco tryptic soy broth, 10 g of Difco yeast extract, with 5 mM $MgCl_2$ and 10 mM $CaCl_2$ added after autoclaving. Minimal medium contained, unless otherwise stated, 0.5 g of asparagine per liter as nitrogen source (13).

Antibiotics were used at the following concentrations: chloramphenicol, 8 μg/ml; tetracycline, 25 μg/ml; ampicillin, 50 μg/ml; thiostrepton, 50 μg/ml; spectinomycin, 50 μg/ml; and streptomycin, 10 μg/ml.

Purification and in vitro manipulation of DNA. Total DNA was isolated from *Streptomyces* strains either by a large-scale isolation method (13) or on a small scale by using an adaptation of 2× Kirby mix (13) containing 2% sodium dodecyl sulfate as detergent and 12% EDTA as chelating agent, which was found to prevent excessive enzymatic degradation of the DNA in the preliminary steps of isolation. For both procedures, DNA was spooled after phenol-chloroform extraction and again after RNase treatment and subsequent phenol-chloroform extraction.

Subsequent in vitro manipulations of DNA were by standard methods (20).

DNA hybridization analysis. DNA was transferred to nitrocellulose filters by Southern transfer (32). DNA hybridization probes were made from DNA restriction fragments derived from recombinant bacteriophages or plasmids and isolated by electroelution or by DEAE-membrane capture (Schleicher & Schuell GmbH, Dassel, Federal Republic of Germany). Biotinylation of these DNA fragments was by a standard nick translation reaction (25) for 90 min as recommended for maximal incorporation of biotin-deoxynucleoside triphosphate (Bio-dNTP) analogs (19). Bio-11-dUTP was obtained from Bethesda Research Laboratories, Karlsruhe, Federal Republic of Germany, and Bio-11-dCTP was obtained from Enzo Biochem/Ortho Diagnostic Systems GmbH, Neckargemuend, Federal Republic of Germany. DNA hybridizations were carried out at 42°C in 45% formamide, and filters were stringently washed twice in 0.1% sodium dodecyl sulfate–0.16× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C for 15 min (31). Filters were protein blocked in 3% bovine serum albumin before being incubated with a complex of streptavidin-biotin-alkaline phosphatase (Detek 1-alk; Enzo Biochem) as recommended by the manufacturer. Hybridized probe was visualized by incubation of filters with a substrate for alkaline phosphatase: Nitro Blue Tetrazolium (0.33 mg/ml) plus 5-bromo-4-chloro-3-indolyl phosphate (0.16 mg/ml).

RESULTS

Isolation and initial characterization of *S. lividans* 66 variants. Previous investigators have noted that changes in colony morphology and pigmentation accompanied the loss of unstable phenotypes both in *S. lividans* (2, 29) and in other *Streptomyces* spp. (17, 22, 24, 28, 36). We therefore used these criteria to isolate 120 variant strains derived from the wild-type *S. lividans* 66 strain 1326. Morphologically different clones occurred as isolated single colonies or could be purified from abnormally growing sectors of an otherwise wild-type colony, at spontaneous frequencies of 2 to 5%, or at higher frequencies (between 5 and 10%) after exposure to UV radiation, ethidium bromide, quinacrine, or mitomycin C, treatments which have been demonstrated to enhance genetic instability in *Streptomyces* spp. (5, 11, 17). The majority of isolated variant colonies no longer sporulated or produced aerial mycelia; however, the mycelial colony size, consistency, texture, and pigmentation varied among individual variants.

Subsequent attempts to purify these variant strains, paying precise attention to their relevant morphological and pigmentation characteristics, revealed, in most cases, a high frequency of further segregation. For each example the clonal origin of variant and subvariant strains was noted. The stepwise changes in colony morphology and pigmentation were accompanied by a loss of resistance to certain antibiotics and by auxotrophic mutations. Initial segregants were frequently sensitive to chloramphenicol, tetracycline, or, to a lesser extent, ampicillin. Instability of these and other antibiotic resistances was originally described for *S. coelicolor* A3(2) (7); resistance to erythromycin, kasugamycin, lincomycin, nalidixic acid, puromycin, rifampin, or vancomycin was stably inherited in the *S. lividans* variants we examined. Further segregation of Cam^s variants, at a spontaneous frequency of 10 to 20%, was correlated with mutation in arginine biosynthesis, as previously observed in segregants of TK64 (2). In addition to finding the previously described instabilities, we discovered another class of auxotrophic strains, derived from Tet^s clones at a frequency of 5 to 10%, which grew well on minimal medium containing 20 mM glutamate or 20 mM NH_4Cl as nitrogen source in place of asparagine. The defect in nitrogen assimilation (Ntr) in these strains is currently under further investigation. These two patterns of instability were observed to occur independently in some strains and simultaneously in others.

We also tested sporulating colonies of wild-type appearance for auxotrophy and antibiotic sensitivity by replica plating spores onto appropriate media. No Cam^s, Tet^s, or auxotrophic clones could be detected among this group (0 of 500 tested). This suggests a relationship between the gross changes in the appearance of colonies, used as parameters for variant isolation, and the origin of the auxotrophies and antibiotic sensitivities found in many of these strains.

Total DNA was isolated from each variant and subsequently restricted with *SalI* before gel electrophoresis to screen for the presence of amplified DNA sequences in the genomes of variant strains. This revealed in the majority (94%) of Cam^s Arg strains a common pattern of six high-copy-number DNA fragments derived from amplified DNA sequences. Cleavage with *Bam*HI or *Bgl*II gave just one 5.7-kb amplified band, suggesting that this sequence is similar to the 5.7-kb ADS found in Cam^s Arg variants of TK64 (2, 3). In addition, other lower-copy-number amplified DNA bands were frequently observed in strains containing the 5.7-kb ADS. These extra bands were of comparable stoichiometric amounts and suggested low-level reiterations of other elements greater than 20 kb. However, after subsequent cultivation, further examination showed that these large amplified sequences were often absent from the genomes of progeny populations. This suggests that either reduction in copy number or deletion of certain classes of amplified elements can occur. Hybridization, with the 5.7-kb ADS as probe, revealed no detectable cross-homology between the 5.7-kb ADS and other, coresident amplified DNA species (data not shown). Several subvariants of identical clonal origin contained both the 5.7-kb ADS and a high-copy-number 12.5-kb ADS, which also shared no substantial cross-homology (see Fig. 3). A minority of Cam^s Arg strains (5 of 80) contained no reiterated DNA species. Both Cam^s Tet^s Arg Ntr mutants, which contained the 5.7-kb ADS, and other Tet^s Ntr mutants often contained additional amplified

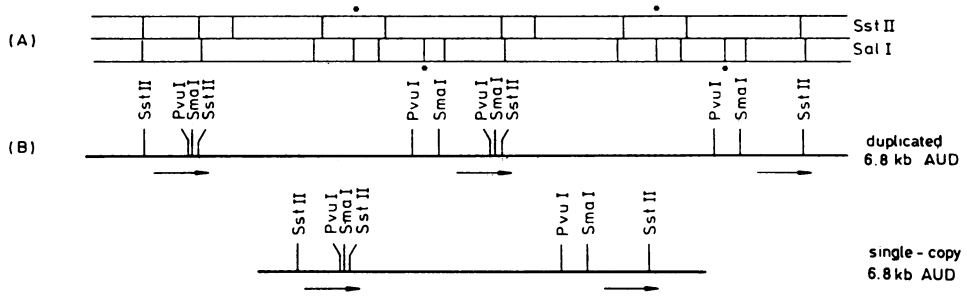

FIG. 1. Restriction map of the *S. lividans* 66 AUD and arrangement of restriction polymorphisms in the duplicated and single-copy AUDs. (A) Restriction map for *Sst*II and *Sal*I in the *S. lividans* 66 AUD. The position of the *Sst*II site missing in comparison with published data (3) is indicated by an asterisk, as is the additional *Sal*I site. (B) Restriction site polymorphisms in the direct repeats for *Pvu*I, *Sst*II, and *Sma*I. The arrows denote the approximate published positions of the direct repeats in the AUD (3). Beneath is the arrangement of the polymorphisms in the single-copy AUD. The sites for *Pvu*I, *Sst*II, and *Sma*I is the adjoining sequences, which allow determination of the repeat structures, are also indicated.

elements. Comparison of the different amplified restriction fragment patterns in these strains showed that no single novel reiteration occurred reproducibly. None of them shared detectable homology with the 5.7-kb ADS (data not shown).

Amplified DNA sequences were discovered in 68% of all variant strains analyzed. However, with the exception of the 5.7-kb amplification, no two identical elements were found to be reiterated and stably inherited in different strains. Storage and recultivation of 5.7-kb ADS-containing strains and subsequent reisolation of chromosomal DNA after several months revealed that this element was stably inherited at high copy number. Interestingly, variants containing the 5.7-kb ADS exhibited greater long-term viability than did many variants lacking an ADS. The variant strains further analyzed in this paper are described in Table 1.

Comparison of AUD and ADS structures in different strains. The accompanying paper describes the characterization of the 44-kb chromosomal region of *S. lividans* 66 containing *argG* and the duplicated 6.8-kb AUD, from which the 5.7-kb ADS is generated, by analysis of relevant recombinant phages from a gene library (4). The structure of the AUD has been described previously, although we noted some differences, notably the absence of an *Sst*II site and the presence of an additional *Sal*I site (Fig. 1A), which may simply reflect errors in the original restriction mapping (3). The complete duplicated AUD structure comprises two copies of an internal sequence, bordered by direct repeats of approximately 1.0 kb. Restriction polymorphisms for enzymes *Pvu*I, *Sst*II, and *Sma*I exist in the three direct repeats in the AUD structure (3).

We found that the structure of this 6.8-kb AUD is variable among *S. lividans* strains. Genomic DNA from strain TK23 (15) and two derivatives, 3198 (15) and M7-1 (4), was cleaved

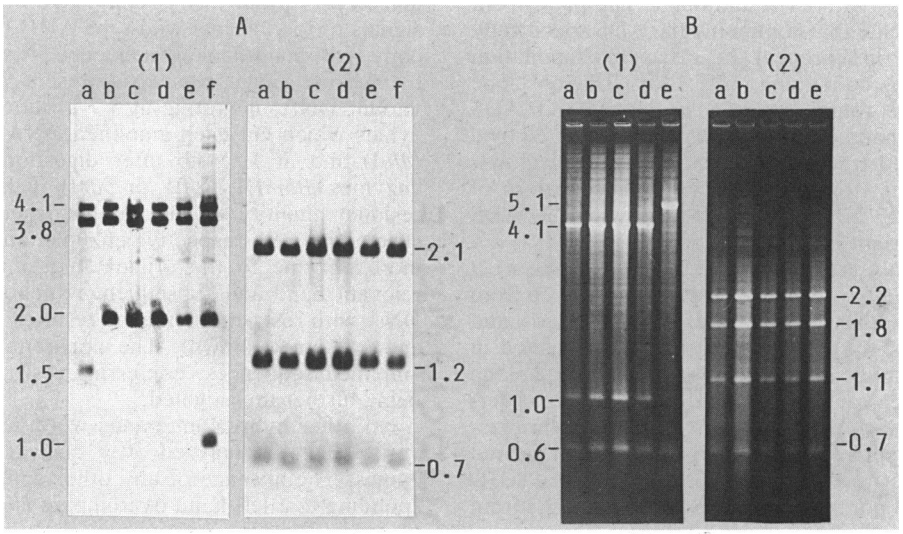

FIG. 2. Comparison of the arrangement of restriction polymorphisms of the AUD and ADS of different *S. lividans* strains. (A) Southern hybridization of the 1.1-kb *Sal*I probe against *Pvu*I digests (panel 1) and *Sst*II digests (panel 2) of chromosomal DNA of strains M7-1/A (Cam^s Tet^s Ntr Arg) (lanes a), derived from the single-copy 6.8-kb AUD strain M7-1, Q3944, (Cam^s Arg) (lanes b), V27 (Cam^s Arg) (lanes c), Q40 (Cam^s) (lanes d), all of which are nonamplified variants of strain 1326, TK23 (lanes e), and *S. lividans* 1326 (lanes f). The sizes, in kilobases, of hybridizing bands are indicated. Each mutant is characterized by the absence of the 1.0-kb *Pvu*I band but a wild-type *Sst*II pattern. The 1.5-kb *Pvu*I band in M7-1/A is a consequence of an adjacent deletion, resulting in loss of the 2.0-kb band (Fig. 4). (B) *Pvu*I (panel 1) and *Sst*II (panel 2) cleavages of chromosomal DNA from strains containing the 5.7-kb ADS: V23 (lanes a), E20 (lanes b), S81 (lanes c), S90 (lanes d), and S110 (lanes e). The sizes, in kilobases, of amplified bands are indicated. The pattern of amplified bands in each strain is explained in the text.

with *Bam*HI and hybridized with a 5.7-kb amplified sequence probe. In contrast to the wild-type pattern of three hybridizing bands, these strains lacked one hybridization signal, namely that migrating at 5.7 kb (data not shown). This result indicates that these strains contain a single copy of the 6.8-kb AUD. Hybridization of the 1.1-kb *Sal*I probe, specific for the direct repeats of the AUD, against *Pvu*I and *Sst*II digests of these DNAs showed that the 1.0-kb *Pvu*I fragment was absent but the 1.2- and 2.1-kb *Sst*II fragments were retained (Fig. 2A). This implied that a copy of the AUD containing the internal direct repeat was deleted; the structure of the single-copy AUD is shown in Fig. 1B.

The 6.8-kb AUD of six stable prototrophic Cam^s strains was investigated. Three strains contained a complete two-copy AUD, similar to the wild type, and a single-copy AUD was present in the other three, as was also reported to be present in one Cam^s mutant of TK64 (3). Examination of the restriction polymorphisms in the remaining direct repeats of the single-copy AUD of each of the three strains indicated, as in the example of TK23, a deletion of the central repeat (Fig. 3A).

Hybridization of the 5.7-kb ADS probe against DNA from the five atypical Cam^s Arg strains derived from *S. lividans* 1326, which did not contain amplifications, also demonstrated an instability of the AUD structure (Fig. 2A and 4). Of the five strains, two (V27 and Q3944) contained a single-copy AUD similar to that of TK23, one (E203) contained the greater part of a single-copy AUD and showed evidence of an adjacent rearrangement, one (Q36) had only a fraction of the AUD remaining, and the fifth strain (Q393, of the same clonal origin as Q3944) showed no hybridization signal, indicating a deletion of the entire AUD. Examination of the arrangement of polymorphic restriction sites in the remaining two direct repeats in the strains containing a single-copy AUD suggested that in each case, the internal direct repeat was missing (Fig. 2A).

We also investigated the structure of the 5.7-kb ADS by appropriate cleavages with *Pvu*I and *Sst*II. The patterns of amplified bands indicate that commonly the ADS specifically contains the internal direct repeat (Fig. 2B). On examination of 20 different strains containing the 5.7-kb ADS, only one exception to this was found. Strain S110 has a 5.7-kb ADS containing the right-hand direct repeat as demonstrated by a 5.1-kb *Pvu*I-amplified fragment, instead of the typical 4.1- and 1.0-kb fragments, together with a standard pattern of *Sst*II fragments (Fig. 2B). The homogeneity of the ADS DNA in any one variant is discussed below.

Investigation of DNA rearrangements adjacent to the AUD or ADS. Investigation of four Cam^s Arg strains derived from TK64 previously demonstrated that in each, the left-hand flanking sequence of the 6.8-kb AUD had been deleted in conjunction with amplification, while the right-hand flanking sequence was not rearranged (3). We confirmed this observation by using derivatives of *S. lividans* 1326. A hybridization probe consisting of a 3.8-kb *Apa*I-*Bgl*II fragment located immediately adjacent to the left end of the wild-type AUD structure (Fig. 4) did not hybridize detectably to DNAs from Cam^s Arg strains containing the 5.7-kb ADS (six strains tested; data not shown), indicating that the deletion of the *argG* gene (4) extends up to at least the left-hand direct repeat of the AUD.

The extent of the deletions in the mutants containing the 5.7-kb amplification was further investigated by hybridization of the 1.1-kb *Sal*I probe, representing the direct repeats of the AUD, against DNAs from the wild type and variants cleaved with a number of different restriction enzymes. The

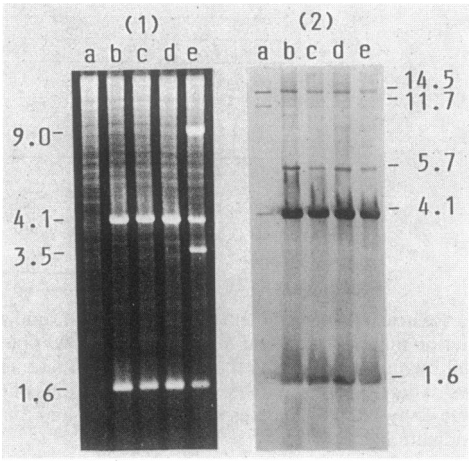

FIG. 3. Analysis of flanking sequences to the 6.8-kb AUD and their arrangement in mutants containing the 5.7-kb ADS. (Panel 1) Chromosomal DNAs from *S. lividans* strains 1326 (lane a), V23 (lane b), S11 (lane c), Q421 (lane d), and Q3947 (lane e) were digested with *Pst*I. Sizes, in kilobases, of the amplified bands are indicated. In addition to the 4.1- and 1.6-kb amplified bands arising from the 5.7-kb ADS, strain Q3947 contains additional 9.0- and 3.5-kb bands stemming from a 12.5-kb ADS. (Panel 2) Hybridization of the 1.1-kb *Sal*I probe against these DNAs. Sizes, in kilobases, of the hybridizing bands are indicated. The patterns of hybridization are explained in the text. A weak homology exists between the probe and the 3.5-kb amplified band of strain Q3947.

results obtained in each analysis with different enzymes were similar. Figure 3 demonstrates the pattern obtained in the hybridization against *Pst*I-cleaved chromosomal DNAs. Against wild-type DNA, hybridization signals equivalent to right-hand and left-hand overlapping flanking sequences of the AUD and the internal, duplicated AUD copy were detected. Variant DNAs contained amplified hybridization signals of the internal wild-type AUD bands and the single-copy right-hand flanking sequence. A second, low-intensity hybridizing signal was also present in each cleavage of variant DNA, migrating at 5.7 kb after digestion with enzymes which cut each amplified DNA copy twice (*Pst*I or *Mlu*I) and at 11.5 kb after digestion with single-cutting enzymes (*Bam*HI, *Bgl*II, or *Sac*I). This band represented a residual quantity of the amplified sequence not fully restricted by each respective enzyme, and neither addition of more enzyme to the original digest nor isolation of the relevant band and subsequent reincubation of the purified DNA with restriction enzyme resulted in any further cleavage (data not shown). The apparent modification to the amplified sequences, conferring resistance to cleavage, is being further investigated.

No other hybridizing bands were detected in any of the variant DNAs analyzed after cleavage with different enzymes. The absence of any other detectable signal, corresponding to a left-hand overlapping flanking sequence, suggested either that deletions in these mutants extended to within the ADS, removing the left-hand direct repeat homologous to the hybridization probe used, or that DNA isolated from any one variant represents a population of molecules which contain variable novel joints at the ADS. Using probes specific to the internal region of the ADS, we have detected hybridization signals which might correspond to the novel joints arising through deletions extending into the ADS in one strain; however, no comparable hybridization signals

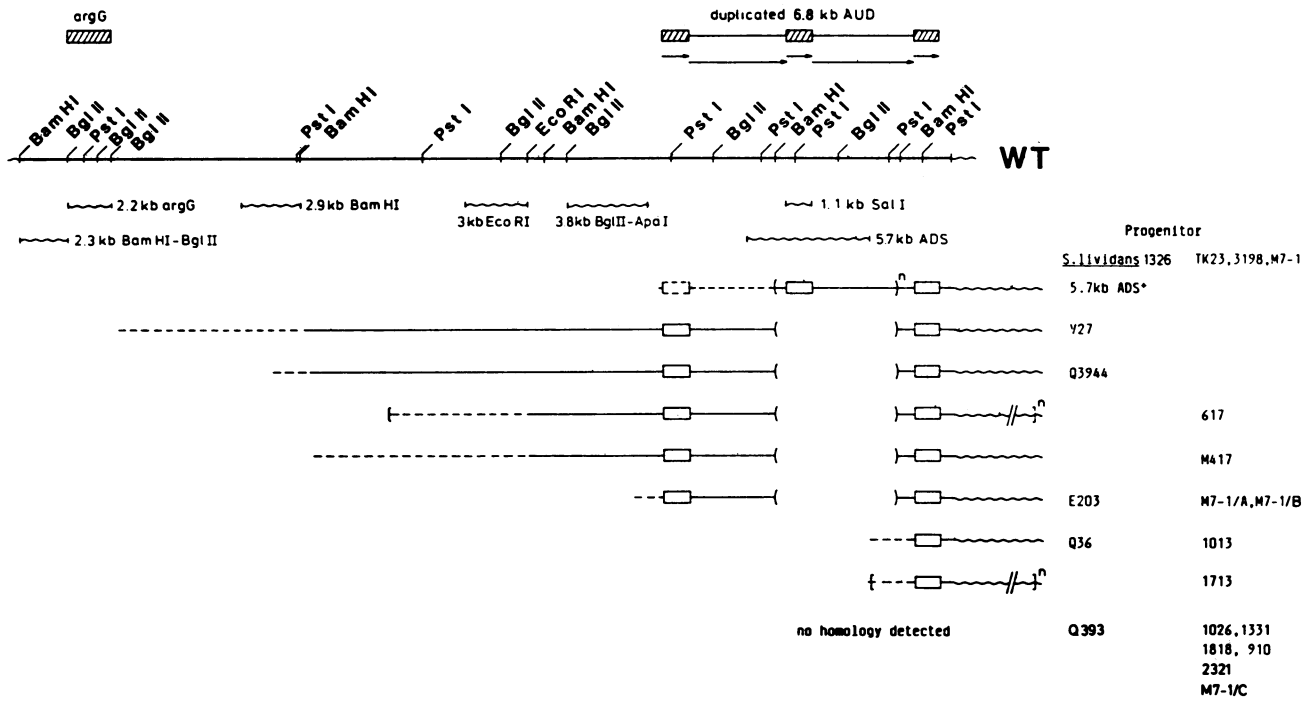

FIG. 4. Rearrangements present in the 44-kb *argG*-AUD chromosomal region in different *S. lividans* strains. The restriction map of the wild-type (WT) arrangement of sequences in this region and the source of different hybridization probes (∿∿∿) are indicated. AUD proximal deletion endpoints were determined by hybridization of the relevant probes against different chromosomal DNA cleavages, thereby determining the endpoints to within particular restriction fragments. Symbols: ——, minimal extent of sequences retained in mutants; -----, possible maximal extent. The left-hand direct repeat is drawn in as dashed lines in 5.7-kb ADS-containing strains because the precise deletion endpoints present in these strains have not been determined. Where applicable, the absence of an AUD copy is denoted. The adjoining list indicates the progenitor strain from which mutants were derived.

were observed in other strains (data not shown), which would support the second explanation. In each case the right-hand flanking sequence was detected unrearranged.

Rearrangements of the AUD proximal sequences in the five atypical Cam^s Arg strains not containing the amplified 5.7-kb element were investigated. Hybridization probes were prepared from several DNA fragments isolated from the 25-kb sequence located between *argG* and the 6.8-kb AUD (Fig. 4) and represented on recombinant phages (4). The extent of the remaining wild-type sequences in the mutant strains was determined by hybridization of these probes to appropriately digested chromosomal DNAs (Fig. 4). An array of different, nonspecific deletion endpoints within or extending beyond this region were found in these strains.

Analysis of rearrangements in variants isolated from TK23, 3198, and M7-1. The lack of amplification and variety of deletion endpoints in the five atypical Cam^s Arg strains described could be correlated, in three of the examples, with the deletion of an AUD copy including the internal direct repeat. That a fourth strain (Q393) should share clonal origin with one of these three (Q3944) and contain an atypical deletion suggested that the single-copy AUD structure might be a feature of a common progenitor strain and that the structure of the AUD in such a prototrophic progenitor strain could influence the nature of the rearrangements, either deletion formation or amplification, which occur in derivatives in which *argG* has been deleted.

To examine this possibility further, we analyzed variants isolated from the prototrophic, plasmid-free strain TK23 and from two marked auxotrophic derivatives, 3198 and M7-1, each containing a single-copy 6.8-kb AUD structure (see above). The three strains exhibited a comparable profile of genetic instability to that of *S. lividans* 1326 for segregation of morphological-pigmentation variants with either Cam^s or Tet^s phenotype or both. Tet^s mutants segregated to Ntr at a frequency of 5 to 10%, and Cam^s mutants segregated to Arg, but only at a frequency of 1 to 5%.

Chromosomal DNAs isolated from 12 Arg mutants of different clonal origin were probed with the 1.1-kb *Sal*I probe. The absence of hybridizing bands in six strains indicated deletion of the entire 6.8-kb AUD region. One such strain (910) contained a novel, approximately 40-kb ADS containing no detectable homology with the 5.7-kb element. The other six mutants examined contained a variety of rearrangements within or adjacent to the 6.8-kb AUD structure, as revealed by hybridization with the 1.1-kb *Sal*I probe and other probes from the 25-kb *argG*-AUD intervening sequence (Fig. 2 and 4). Of particular interest were two Arg mutants of TK23: 617 and 1713. These strains contained amplification of an 18-kb and an 18.5-kb ADS, respectively, both sequences containing homology with the 1.1-kb *Sal*I probe. The 18-kb ADS extends from a point in the left-hand flanking sequence of the 6.8-kb AUD, to include the entire single-copy AUD structure, up to an as yet unmapped position in the right-hand flanking sequence. In strain 1713 a deletion extends into the internal sequence of the 6.8-kb AUD, and the resident 18.5-kb ADS contains the remaining 6.8-kb AUD sequences, including the right-hand direct repeat, plus sequences of the right-hand flanking region which have not yet been fully characterized.

None of the Arg variants derived from progenitors con-

taining a single-copy AUD structure were found to amplify the 5.7-kb element as a discrete unit, and AUD proximal deletion endpoints were found to be unspecific.

DISCUSSION

Genetic instability in several *Streptomyces* spp. have been shown to arise by deletion of particular genes (11, 16, 21, 28, 35). We identified, in addition to a previously described example of genetic instability, a second, independent pathway of instability in *S. lividans* 66. The accompanying paper demonstrates deletion of the *argG* gene which results from the first pathway (4), and deletion is also associated with the Ntr phenotype of mutants derived from the second pathway (P. Dyson and T. Kumar, unpublished results). We observed, coupled to instability, the generation and stable inheritance of amplified DNA sequences in 68% of the variant genomes. A 5.7-kb ADS was present in the majority of Cam^s Arg mutants, as previously described for similar mutants of *S. lividans* TK64 (2). In addition, we found a variety of reiterations either alone or coresident with the 5.7-kb amplified element, in the variant strains we examined. However, with the exception of the 5.7-kb ADS, no two discrete amplified elements the same were commonly found to be generated and stably inherited in any two different variants.

Amplification of the 5.7-kb element arises from a tandemly duplicated AUD structure resident in the wild-type chromosome. In contrast to the situation in the wild type, a single-copy AUD, consisting of two short direct repeats flanking an internal sequence, is present in several *S. lividans* 1326 derivatives, indicating that the nature of the AUD structure is fairly dynamic. Moreover, the arrangement of polymorphic restriction sites within the direct repeat sequences of the AUD suggests that the conversion of single-copy to duplicated status, and vice versa, may operate via unequal crossing-over and intramolecular deletion-recombination, respectively, between the directly repeated sequences.

We have demonstrated for the first time that strains containing a single-copy AUD cannot generate at high frequency the amplification typically found in Cam^s Arg variants derived from the wild type. The duplicated AUD of the wild type might therefore be an intermediate in DNA amplification, analogous to another system of gene amplification in procaryotes. The rate-limiting step to the amplification of drug-resistant determinants in *P. mirabilis* is the initial conversion of a single-copy element to a tandem-duplicated form (21). The latter structure is spontaneously amplified in a proportion of all cells, and the application of a selection pressure for antibiotic resistance allows the outgrowth of the subpopulation containing reiterations of the resistance genes.

The frequency of amplification of any other DNA element in the *S. lividans* genome may therefore be dependent on the copy number, single or duplicated, of the relevant AUD in an immediate progenitor. As we observed in TK23 derivatives, amplification occurs for other single-copy sequences derived from the same chromosomal region as the 6.8-kb AUD when the latter is also present at single copy. This suggests that a similar probability exists for amplification of any discrete single-copy element located in this part of the genome. The irreproducibility of the other amplifications we observed in different variants, for instance in Tet^s Ntr mutants, suggests that these elements may also exist as a single copy in the wild-type genome, and we have demonstrated this for one example (Dyson and Kumar, unpublished results).

We showed that the amplified sequence arising from the duplicated 6.8-kb AUD structure of the wild type consists of a circularly permuted 5.7-kb element, usually containing the internal direct repeat of the AUD. In one exception the right-hand direct repeat was found to be amplified. That any one variant is homogeneous for the specific repeat sequence contained within its ADS may be a consequence of the mechanism of amplification. The site of an initial recombination event at the start of amplification could determine the nature of the first ADS copy and of each subsequent copy. This, in turn, suggests that amplification might be generated by a replication mechanism rather than by additional recombination events.

Amplification of the 5.7-kb ADS was previously shown to be associated with a deletion of one of the flanking sequences of the wild type AUD in derivatives of TK64 (3). We confirmed this observation with strains derived from *S. lividans* 1326 and have shown in addition that the deletion removes at least 30 kb of DNA, including *argG* (4). The ADS proximal deletion endpoint is defined by the amplified sequence itself, and, in agreement with published data (3), we could find no example associated with amplification of rearrangement of the other flanking sequence.

In contrast, we found that a characteristic of Cam^s Arg mutants derived from progenitors containing a single-copy 6.8-kb AUD and hence not containing the 5.7-kb ADS is that deletion endpoints proximal to the AUD are unspecific. In different strains, deletion endpoints were identified in the *argG*-AUD intervening region, within the AUD structure itself, or, for the majority of cases, in the right-hand flanking sequence of the AUD, which had been consequently deleted. We cannot discount at this stage the possibility of another, as yet unidentified, mutation present in the single-copy AUD progenitor strains, which could account for the atypical rearrangements generated in variant genomes. However, our data strongly suggest that the structure of the AUD in a prototrophic strain governs the nature of the rearrangements, either deletion formation or amplification, which occur in derivatives in which *argG* has been deleted.

We speculate that spontaneous amplification is generated from the duplicated AUD structure of wild-type *S. lividans* 66. When subsequent deletions in the same chromosomal arc occur, then, owing to the physical length of reiteration, they terminate within the amplified sequence itself. Progenitor strains such as TK23, in which spontaneous amplification of the 5.7-kb sequence in much rarer, requiring a rate-limiting duplication step, give rise to deletions in the same chromosomal arc whose endpoints are consequently not buffered in the same way and which usually extend further. Alternatively, other amplifications can be generated at low frequency from single-copy elements derived from the same chromosomal region as the 6.8-kb AUD, and these reiterations in turn serve to maintain the integrity of the right-hand unique sequences. In the absence of a highly reproducible amplified sequence buffer to further deletions, the lower frequency of Arg mutants generated from such a progenitor may be a consequence of lethal deletion events which extend so far into the right-hand flanking sequence of the AUD that essential genetic information is lost.

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