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Analysis of a genetically unstable region in *Streptomyces lividans* 66–TK64

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Abstract. Genetic and molecular analyses of an unstable region encompassing the gene loci cml arg and a 5·7 kb amplifiable unit of DNA were done. Spontaneous mutants from $Cm1^R \rightarrow Cml^S$ and the revertants from $Cml^S \rightarrow Cml^R$ were analysed for mutations at arg locus and amplification of amplifiable unit of DNA. Twenty-one revertants were analysed. Two of these had large-scale amplification and one of these was also Arg^-. Nine of the revertants which were Arg^+ had low-level or intermediate-level amplification of the 5.7 kb DNA sequence but no deletions of the flanking sequences were detected. Five of the $CmI^{R'}$ revertants, which were also Arg^+, had lost one of the two copies from the doublet of amplifiable unit of DNA. The remaining five revertants did not show any other change. The amplifiable unit of DNA, therefore, not only undergoes amplification but can also suffer specific deletion of one copy. Thus, this region as a whole is characterized by instability and the events appear to take place at more than one locus concomitantly with a high frequency.

Keywords. Genetic analysis; molecular analysis; amplifiable unit of DNA; *Streptomyees lividans.*

1. Introduction

Streptomycin lividans 66-TK64, which is normally chloramphenicol-resistant (CmI^R), mutates to chloramphenicol sensitivity (Cml^S) with a high spontaneous frequency of half a per cent or more (Altenbuchner and Cullum 1984; Neelima and Notani 1990). A high frequency of mutations at a linked arg locus ($Arg^+ \rightarrow Arg^-$) has been observed in Cml^S mutant spores (Altenbuchner and Cullum 1984; Betzler *et al* 1987). Such double mutants are usually accompanied also by the amplification of a 5·7 kb chromosomal DNA sequence in this region (Altenbuchner and Cullum 1984). In wild type strain, the 5·7 kb DNA sequence is present was. a duplication of an internal sequence each flanked by direct repeats and is termed as amplifiable unit of DNA (AUD). Large-scale amplification of AUD to 200 or more copies has been explained to arise from recombinational event(s) between the direct repeats in the DNA replication fork leading to the generation of a rolling circle mode of replication and a concatemer (Young and Cullum 1987). The final resolution and fixation of the amplified DNA is presumed to occur by additional recombinational event(s) explaining also the occurrence of deletions on the left side of AUD (Altenbuchner and Cullum 1985; Young and Cullum 1987). To define further the relationship amongst events at cml and arg loci and AUD, a new screening system was planned and experiments devised. Revertants ($Cm1^R \rightarrow Cml^S \rightarrow Cml^R$) at cml locus were analysed for the allele present at arg locus as also for the amplification of 5·7 kb DNA sequence. We report here the results of our experiments.

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Abbreviation used: AUD, Amplifiable unit of DNA.

2. Materials and methods

2.1 *Bacterial strains*

Streptomyces lividans 66-TK64 was provided by Prof. David Hopwood, John Innes Institute, Norwich, UK. Cml^S mutants were obtained by replica plating isolated colonies from R2YE medium onto minimal medium plates with or without 8 µg/ml of chloramphenicol (Hopwood *et al* 1985). Chloramphenicol-resistant revertants were obtained by plating dense spore suspension of Cml^S mutants on minimal medium containing 8μg/ml of chloramphenicol. Arg^- mutants were isolated by replica plating isolated colonies of Cml^S or $Cm1^{R'}$ mutants from R5 medium onto minimal medium plates with or without arginine (37 g/ml).

2.2 *Chromosomal DNA isolation*

Cells were grown in 5 ml Yeast extract malt extract medium and harvested (Hopwood *et al* 1985). Mycelia pellets were suspended in 400 μl of TE buffer and 100 µ1 of 20 mg/ml lysozyme solution containing 50 *µg/ml* RNase. After incubation at 37°C for 1 h, cells were lysed with 250 p1 of 2% SDS. The lysate was extracted three times with 15 p1 of neutral phenol and 250 p1 of neutral phenol+ chloroform. Last extraction was done with chloroform : isoamylalcohol (24:1). DNA was precipitated with one-tenth volume of 3 M Na-acetate and equal volume of isopropanol. Spooled DNA was washed with 70% ethanol twice and dissolved in 50 µ1 of TE buffer.

2.3 *Restriction digestion*

This was done according to manufacturers' (New England Biolabs, USA; Boehringer–Mannheim, Germany) instructions. Digestion reactions were carried out overnight to ensure complete digestion. Southern transfer of DNA and hybridization were done as described by Dharmalingam (1986), except that hybridizations were done in plastic boxes containing 15 ml of hybridization mixture containing 3×10^7 cpm of ^{32}P-labelled DNA probe. The AUD clone used for hybridization was provided by Dr K Dharmalingam, Madurai Kamaraj University, Madurai.

3. Results

3.1 *Frequencies of forward and reverse mutations at cml locus*

Spontaneous $Cm1^R \rightarrow Cml^S$ mutation frequencies were estimated at 0·3–0·5%. $Cml^S \rightarrow Cm1^{R'}$ back mutation frequency was much lower and was estimated at 10^{-3}–10^{-4}%, Thus, revertants arose with a frequency that was three to four orders of magnitude lower, suggesting that $Cm1^R \rightarrow Cml^S$ are not simple (single site) mutations.

Out of 21 $Cml^{R'}$ revertants that were analysed, one was also Arg^-. The remaining 20 $Cm1^{R'}$ revertants were all Arg^+. Only $Cml^{R'}$ Arg^- showed delayed sporulation. The rest of the revertants, $cmlr_{R'}$ Arg_+, sporulated normally (table 1).

Table 1. Phenotypic and molecular characterization of Cml^S mutants and $Cml^{R'}$ revertants.

Strain Wild type/mutant	Phenotype	Amplification/ deletion of AUD	Arg^- yield (%)	Sporulation
1. *S. lividans* 66-TK64	Cml^R Arg^+	No amplification/ deletion (AUD doublet)		Normal (2–3 days)
2. SC^S (spontaneous mutant)	Cml^S Arg^+	No amplification/ deletion (AUD doublet)	1–4	Normal
3 a. NH1 (spontaneous revertant)	$Cml^{R'}$ Arg^+	High level amplification	25	Normal
b. NH2 (spontaneous revertant)	$Cml^{R'}$ Arg^-	High level amplification	100	Delayed (2–3 weeks)
4. NL1 to NL10 (spontaneous revertants)	$Cml^{R'}$ Arg^+	Intermediate/low level amplification	1–4	Normal
5. NDl to ND4 (spontaneous revertants)	$Cml^{R'}$ Arg^+	Deletion of one copy from AUD doublet	10–12	Normal
6. NNl to NN5 (spontaneous revertants)	$Cml^{R'}$ Arg^+	No amplification/ deletion (AUD doublet)	1–2	Normal

CmI^R, Chloramphenicol resistance; CmI^S, chtoramphenicol sensitivity; $CmI^{R'}$, reversion to CmI^R; Arg^+, argininc prototrophy; Arg-, arginine auxotropby; AUD, amplifiable unit of DNA.

3.2 *Amplification of 5.7 kb DNA sequences in $Cml^{R'}$ revertants*

Amplification or deletion in the AUD region was examined in Cml^S and $CmI^{R'}$ revertants (figures 1 and 2), Total DNA from wild type and $Cml^{R'}$ strains was isolated and cut with BamHI or Xhol, electrophoresed, blotted and hybridized with ^{32}P–labelled AUD probe. High level amplification of 5·7 kb DNA sequence was observed in only two of the Cml revertants (figures 1 and 2, samples 6 and 24) one of which was also Arg^-. The other yielded Are mutants with a frequency of 25% (table 1). Six $Cm1^{R_1}$ Arg^+ isolates were chosen from the 75% Arg^+ population and their total DNA was analysed (figure 3). All of these showed highly amplified 5·7 kb DNA sequence and generated 22-30% Are mutants. Due to the extreme instability at arg locus, it could not be ascertained whether amplified DNA sequence arose from the $Cm1_R$' Arg_+ population or from the Arg^- fraction. However, in another Cml^R revertant (strain NL1) in which Are fraction was only 1%, it had moderately high amplification of 5·7 kb AUD (figures 1 and 2 sample. 15).

Nine out of twenty-one revertants showed a low-level amplification (figures 1 and 2, samples 3, 7, 8, 9, 12, 15, 18, 22, 23) which could be seen only by Southern hybridization with the labelled AUD probe. High level amplification is detectable by cutting DNA with BamHI and electrophoresis. Arg^- fraction arose from these with a frequency of 0·8–4% (table 1).

In all the amplified variants, left and right side AUD-flanking sequences were

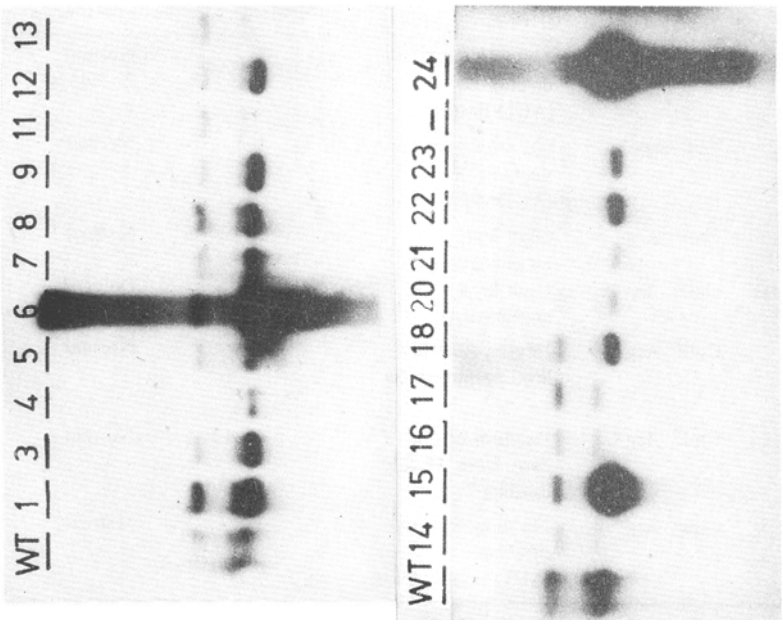

Figure 1. Total DNA of Cml^R 66-TK64 strain and twenty-one chloramphenicol resistant revertants was digested with BamHI and electrophoresed through 0·7% agarose gel. Phage lambda DNA digested with HindIII was used as a molecular weight marker. Samples 4 and 14 to 24 contain ≃0·5 μg of DNA. remaining samples contain ≃1·5 μg of DNA.

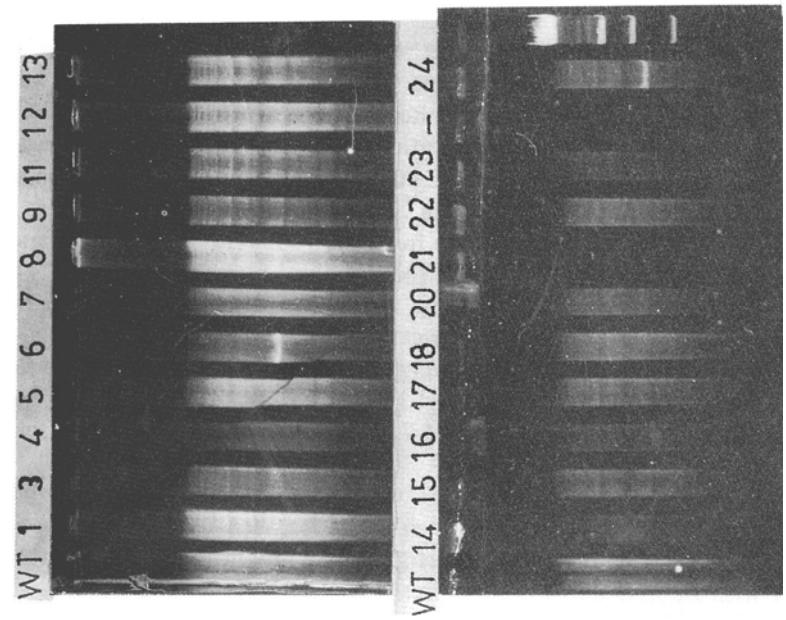

Figure 2. Autoradiogram obtained following Southern transfer of electrophoresed DNA in figure 1 onto nitrocellulose filter and hybridization with ^{32}P-labelled AUD probe.

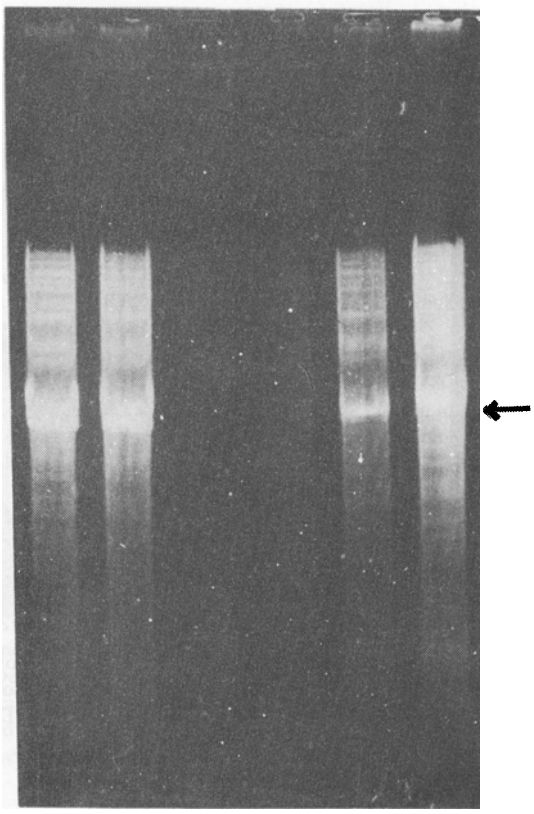

Figure 3. Total DNA of six $Cml^{R'}$ Arg^{+} isolates of NH1 digested with BamHI and electrophoresed through 0·7% agarose gel.

found to be intact. This was confirmed by using Xhol digests of total DNA and then hybridizing with a labelled AUD probe (figures 4 and 5). Xhol digestion yields ~15 kb and 22 kb junction fragments well separated from the amplified 5·7 kb fragment.

Five of the revertants showed deletion of one copy from the AUD doublet (figures 1 and 2 samples 11, 13, 14, 16, 17). Arg^{-} mutants arose from these at a frequency of 10 12% (table 1). Neither DNA amplification nor further deletions were observed in their Arg^{-} variants (data not shown).

The remaining $Cml^{R'}$ revertants were similar to the wild type and Cmls mutants containing AUD doublet without any amplification or deletions (figures 1 and 2, samples 1, 4, 5, 20, 21).

4. Discussion

Study of an unstable region in *S. lividans* comprising of cml and arg gene loci and a 5·7 kb DNA sequence has enabled us to make the following observations: (i) Cml^{R} →Cmls mutation frequency is very much higher, by three to four orders of magnitude, than the reverse mutation frequency of Cmls→$Cml^{R'}$ and are

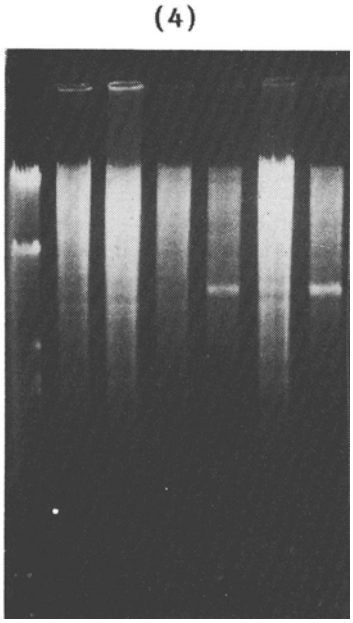

Figure 4. Profiles of DNA of Cin1^{R} 66-TK64 strain, spontaneous chloramphenicol sensitive mutant, and chloramphenicol resistant revertants NN1, NH2, NL1 and NH1, lanes 2 to 7 respectively, digested with Xhol and electrophoresed through 0·7% agarose gel. Bacteriophage T7 DNA digested with Sau 3A1 was used as a molecular weight marker (lane I). Approximately 2µg of DNA was used, except in lanes 5 and 7 which had 1μg of DNA.

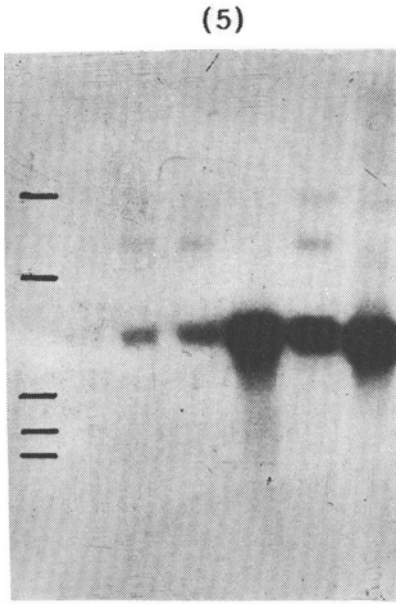

Figure 5. Autoradiogram obtained following Southern transfer of DNA in figure 4 onto nitrocellulose filter and hybridization with ^{32}P-labelled AUD probe

comparable to those observed earlier (Altenbuchner and Cullum 1984; Freeman *et al* 1977). Generally, the reversion frequencies are only about an order of magnitude lower. This gives credence to the notion that most of the forward mutations are not simple point mutations and therefore do not revert easily, (ii) Even amongst the revertants (Cm1$^{R'}$) which primarily were selected for changes at cml locus, there are concomitant changes at arg locus (~4% cases), the amplification of AUD (about 1/3rd of the cases) and deletion of one copy from the doublet of AUD (about 1/5th of the cases), (iii) AUD amplification in some of the present cases seems to have occurred at a lower level. Also, no deletions were associated with it. This is surprising because if amplification was effected through a rolling circle (Young and Cullum 1987), it would mean that it came to a stop short of generating 200-copy AUD equivalent DNA and without producing any of the expected deletions. An alternative possibility in these cases may be that an 'onion-skin' type of amplification is involved here (Stark and Wahl 1984; Stark *et al* 1989). In such a case, deletions may not be expected. In either case, negative regulation of AUD amplification is a possibility.

The basis of instability producing concomitant changes in the cml-arg AUD

region is unknown. The concomitant changes in AUD (amplification/deletion) in $Cm1^{R'}$ revertants are much more frequent than those at arg locus. This suggests the possibility that either AUD or a putative regulatory locus of amplification may be closer to cml locus than to arg.

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