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Genetic Instability and Genome Structure in *Streptomyces*

Genetička nestabilnost i struktura genoma u vrsta roda *Streptomyces*

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Summary

Streptomyces lividans *66 is used as a model strain to study the mechanisms of genetic instability in* ***Streptomyces****. The instability involves deletion of up to about 1 Mb of the 8 Mb genome and often amplification of neighbouring sequences. Recent work showed that the chromosome of* ***S. lividans*** *is linear and the deletogenic regions were localized to the two ends of the chromosome. A closer study of deletion mutants showed that some deletions had only one end of the chromosome and that one end was replaced by a tandem amplification of chromosomal sequences with deletion of the ca. 300 kb of DNA between the end and the amplification. In contrast deletions affecting the other end resulted in loss of both chromosomal ends and circularization of the chromosome. The reasons for this asymmetry between the chromosome ends will be discussed. Artificial duplications in the chromosome in an amplifiable region can stimulate amplification both of the duplicated sequence and of another amplifiable region about 7 Mb away on the linear chromosome.*

Sažetak

Soj ***Streptomyces lividans*** *66 upotrebljava se kao modelni soj za studij mehanizama genetičke nestabilnosti u vrsta roda* ***Streptomyces****. Genetička nestabilnost uključuje delecije od približno 1 Mb u 8 Mb genomu, a često i amplifikaciju susjednih sekvencija. Nedavno su istraživanja pokazala da je kromosom soja* ***S. lividans*** *66 linearan, te da su regije podložne deleciji smještene uz oba kraja kromosoma. Pomnije studije delecijskih mutanata pokazale su da neke delecije imaju samo jedan nepromijenjeni kraj kromosoma, a da je drugi zamijenjen s tandemskom amplifikacijom kromosomskih sekvencija s delecijom od približno 300 kb DNA između kraja i amplifikacije. Nasuprot tome, ako su delecije obuhvatile drugi kraj, dolazi do gubitka oba kromosomska kraja i cirkularizacije kromosoma. Raspravljat će se o uzrocima te asimetrije između krajeva kromosoma. Umjetno udvostručenje dijela DNA u kromosomu, unutar regije podložne amplifikaciji, može potaknuti amplifikaciju kako podvostručenog dijela DNA tako i dijela DNA podložnog amplifikaciji, udaljenog oko 7 Mb na linearnom kromosomu.*

Introduction

Genetic instability occurs in many and perhaps all *Streptomyces* species *(1)* and was already recognised as early as 1913 *(2)*. It can contribute to strain degeneration of commercially important species *(3)* and lead to lower product yields. The study of genetic instability also gives important insights into genome structure and DNA rearrangements in *Streptomyces* that are of interest for applications.

Unstable genes can be defined as genes that give rise to spontaneous mutants at a frequency of 0.1 % per spore or higher *(1)*. It is found that only certain genes in any species are unstable, with other genes (e.g. typical auxotrophic marker genes) having spontaneous mutation frequencies comparable to those in other bacteria. Sometimes the frequency of mutations can be raised by treatment with agents such as ultraviolet light or ethidium bromide. In most of the cases studied, the instability is associated with the occurence of large deletions of 800 kb *(4)*, 1 Mb *(5)* or over 2 Mb *(6)*, i.e. 10 % or more of the 8 Mb chromosome. In many cases, the deletions are accompanied by DNA amplifications. These consist of tandem repeats of sequences at one border of a deletion and often comprise more than 100 copies. The amplified units vary in size between about 2 kb and 100 kb *(7)*.

In this paper we shall mainly discuss the model species *S. lividans* 66. This species has an unstable chloramphenicol resistance that gives rise to sensitive mutants at a frequency of about 1 % per spore. These mutants are highly unstable themselves and produce arginine auxotrophs at a frequency of about 25 % per spore *(8)*. The arginine auxotrophs have amplified a 5.7 kb fragment to

over one hundred tandem copies and there is a large deletion adjacent to the amplification *(9)*. A second deletion and amplification system was also found which gave rise to a variety of amplifications of sequences within a chromosomal region accompanied by deletion of sequences to one side of the amplifications *(10)*.

A puzzling observation was that it was often not possible to detect a junction fragment to find the second end of the deletion when one end was defined by the amplification. One explanation for this was provided by the discovery that the chromosome of *S. lividans* 66 (and probably other *Streptomyces* species) is linear *(11)*. The deletogenic regions are at both ends of the chromosome *(12)*. In some cases, chloramphenicol-sensitive mutants have deleted both ends of the chromosome and formed a circular chromosome structure which seems to be stable *(5)*. In other cases, one end of the chromosome has been deleted, but the other end seems to be intact *(10)*. All the sequences distal to the DNA amplification have been lost, so the amplification forms the new chromosome end (see Fig. 1). The native chromosome ends seem to have a covalently bound protein that probably acts as a primer *(11)* – this is necessary as the 5' end could not be replicated from an Okazaki fragment. The amplification in the deletion strain is probably a dynamic structure that acts as a sort of »telomere« to prevent the deletion lengthening into essential genes further from the chromosome end.

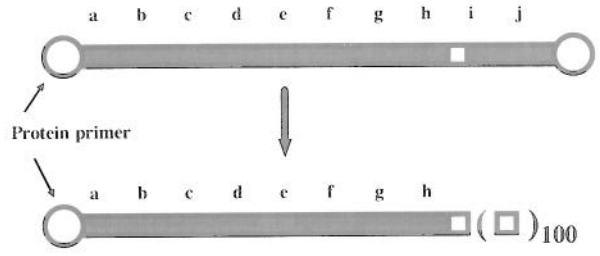

Fig. 1. Tandem amplification is accompanied by the loss of all sequences distal to the amplification. This results in replacement of the protein primer with the tandem repeat

Slika 1. Amplifikacija dijela DNA u jednom nizu popraćena je gubitkom dijela DNA udaljenog od amplifikacije. To dovodi do zamjene proteinske klice s ponavljanjem u jednom nizu

If this sort of genetic instability causes problems in production strains there are several possible approaches to improving the strains:

(i) It may be possible to move genes away from the chromosome ends – there are several integrative vectors available *(13)*. This may not solve the problem if the deletions affect genes controlling a variety of desirable properties of the strain rather than just genes directly involved in product synthesis.

(ii) If a selectable gene is moved distal to genes needed for production, deletions reaching the production genes will also remove the selectable gene leading to loss of the non-producing mutants *(14)*. The selectable gene could be a resistance gene or a gene involved in central metabolism. The problem of strain degeneration lies in the capacity of low-producing mutants to overgrow the production strain rather than in the rate of generation of mutants alone *(15)*.

(iii) It is possible to generate circular chromosomes *(5,11)*, which may be more stable than linear chromosomes. However, there are some data showing that coupled amplifications and deletions can still occur in a strain with a circular chromosome (Sutter & Cullum, in preparation).

(iv) It may be possible to reduce instability by removing potential amplifiable sequences. This ought to reduce the survival of deletion derivatives.

However, it will be necessary to test these approaches on production strains before their feasibility in a production process can be assessed. It is also difficult to estimate how important strain degeneration is in loss of yield in large fermenters.

Results and Discussion

The existence of reproducible DNA amplification suggested that it might be possible to coamplify cloned genes. This was tested in *S. lividans* 66 *(16)* and it was shown that integration of sequences in an amplifiable region allowed efficient coamplification of cloned genes. The clones appeared to be stable so this appproach seems promising to achieve high copy number stable expression. However, the dynamic nature of the amplifications (see Fig. 1.) raises the question of whether instabilities in the cloned sequences could occur. We decided to investigate this initially for spontaneous amplifications.

The strain U101 carries an amplification of 30 kb *(5,10)* and all sequences distal to the amplification are lost (i.e. a structure as in Fig. 1.). Restriction analysis of the amplification and the region in the parent strain indicated that there were no extensive repeated elements flanking the amplifiable sequence. Thus, the expectation was that conserved sequences outside the amplification would not be affected by the DNA rearrangements. This is best seen in Fig. 2, where such sequences are indicated by **a**.

A **d** hybridisation probe showed no hybridisation to the amplified strain (U101) and a **b** or **c** probe showed the expected hybridisation to new amplified fragments. An **ab** fragment was cloned from the parent strain and compared to a cloned **bc** fragment from U101. This allowed sequencing of the junction (Fig. 3.).

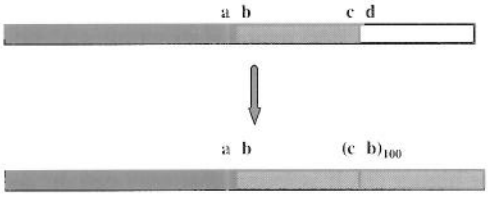

Fig. 2. Generation of amplification showing fate of sequences flanking the junctions of the new duplication

Slika 2. Nastajanje amplifikacije što pokazuje sudbinu dijelova DNA koji omeđuju sjecište novog udvostručavanja

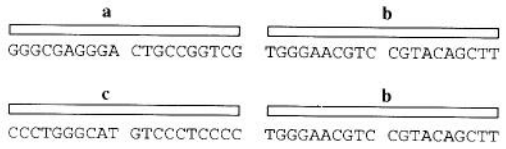

Fig. 3. Amplification junction sequence
Slika 3. Sjecište amplificiranih dijelova DNA

The **a** probe hybridised to a 1.4 kb *Bam*HI fragment both in the parent strain (ZX7) and in U101 (data not shown). However, in U101 there was an unexpected extra band of 5.6 kb. The **ab** region from the parent strain was cloned as a 5.1 kb *Eco*RI-*Kpn*I fragment (see map in reference *10)*. It was possible to clone a 4.9 kb *Eco*RI-*Kpn*I fragment from U101 that hybridised with an **a** probe. DNA sequencing showed that this fragment contained a **bc** junction with an identical sequence to that shown in Fig. 3. However, the *Eco*RI end of the fragment differed in sequence to **a** (including the presence of a *Kpn*I site only 6 bp away from the *Eco*RI site). It is likely that the fragment has arisen as a result of a secondary rearrangement, but further analysis of its structure is needed to clarify this idea.

The presence of a linear chromosome also has consequences for taxonomy and screening. The chromosomes of *Streptomyces* strains are about 8 Mb in size *(12)*. This is nearly twice the size of species such as *Escherichia coli* or *Bacillus subtilis*. However, the known metabolic functions, sporulation and antibiotic production can not account for this large difference in size. Experiments were carried out in which random cosmids were hybridised with *S. lividans* 66 and other related strains belonging to the species group »*S. griseoruber*« defined by numerical taxonomy *(17)*. This showed (Fendrich & Cullum, in preparation) that about half the cosmids were conserved, whereas about half showed little or no hybridisation with one or more members of the species group. This supports the idea that about 4 Mb of sequence is needed for general conserved functions, but raises the question of the role of the other 4 Mb of variable sequence. It is likely that most of this sequence is coding sequence, because any sequencing of *Streptomyces* DNA to date has revealed a tight packing of open reading frames without gaps in between. The existence of variable regions, which have a fairly random distribution in the species group could be explained by genetic exchange i.e. horizontal evolution. Most *Streptomyces* strains possess conjugation systems making transfer of chromosomal material easy to explain. The presence of mismatch repair systems may prevent homologous recombination as observed in *E. coli-Salmonella* crosses *(18)*. However, linear chromosomes might be more susceptible to heterologous recombination than circular chromosomes. It is plausible that the gross gene order is conserved between strains as the position in the chromosome is likely to affect expression of genes (e.g. genes near the replication origin have a higher copy number in fast growing cultures). Circular chromosomes must undergo a double crossing over to generate recombinants, whereas for linear chromosomes a single crossing over event suffices. The crossing over events must be in approximately the same positions in the two chromosomes to prevent generation of large deletions and duplications. A non-homologous recombination event (e.g. transposon-driven) could also result in crossing over. As the crossing over events are likely to be rare, linear chromosomes might be expected to be much more prone to them than circular chromosomes and non-homologous recombinations might result in transfer of variable sequences between strains. Such a recombination is illustrated in Fig. 4.

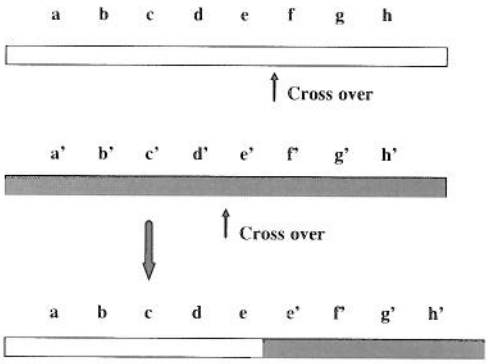

Fig. 4. Generation of recombinants by a single non-homologous cross over
Slika 4. Nastajanje rekombinanata uključuje jednostruki nehomologni »crossingover«

As well as generating diversity in the variable part of the genome, this sort of genetic exchange might create problems in taxonomy. If DNA sequences are used to construct taxonomic trees, the trees might turn out to be different depending in which part of the chromosome the sequences are located. The construction of ordered cosmid gene banks will allow the location of the variable sequences in the chromosome to be studied. It will also allow the selection of genes for taxonomic sequencing experiments. The variable sequences might be of interest as taxonomic markers and it is conceivable that a set of hybridisation probes could be constructed corresponding to classical taxonomic markers. Careful selection of probes might help in the identification of unusual *Streptomyces* for use in screening programmes.

Genetic instability can lead to changes in phenotype of a strain. It can be a serious problem to identify a particular strain. This creates difficulty in studies on biological safety, when it is important to identify strains after release in soil. It can also be a problem in commercial contexts when disputes about strain ownership occur. It was shown in *S. lividans* 66 that a region near one end of the chromosome is very rich in repeated sequences *(5)*. It is possible that such sequences could be used as a sort of »genetic fingerprint« to identify strains. Work to test this possibility is under way.

In this paper, we have shown that genetic instability impinges on many aspects of *Streptomyces* biology that are also important in applications. It is likely that some of these potential applications will be introduced into industrial practice in the next few years.

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