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Strain Degeneration in Industrial Streptomycetes

Degeneracija soja u industrijskih streptomiceta

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Summary

Strain degeneration is wide-spread in industrial Streptomycetes strains and describes a variety of spontaneous changes that result in deleterious phenotypes especially for antibiotic yield. Genetic instability (i.e. the occurrence of spontaneous mutations with a high frequency) is common in ***Streptomyces*** *strains and has been studied in several model systems, where a pattern of large-scale DNA deletions and amplifications has been established. However, little work has been performed with high-yielding industrial strains to define the relationship between strain degeneration and genetic instability. Some* ***S. rimosus*** *R6 strains that have been selected for high oxytetracycline production show genetic instability. Two classes of mutants were defined with reduced or no antibiotic production. The Class II mutants carried large deletions including the oxytetracycline gene cluster, which resulted in the loss of both the production and resistance genes. Although this proves that the genes lie in an unstable chromosomal region, the fact that the mutants are sensitive to oxytetracycline makes them unimportant for strain degeneration. In contrast, the commonest low-producing mutants (Class I) remained resistant to the antibiotic and outgrew the parent strain in competition experiments. This suggests that the Class I mutants, which did not show any detectable DNA rearrangements, are responsible for strain degeneration. A hypothesis to explain Class I mutants will be discussed.*

Sažetak

Degeneracija soja uvelike je rasprostranjena pojava u industrijskih streptomiceta, a očituje se nizom spontanih promjena što dovode do pojave štetnih fenotipova, posebno za prinos antibiotika. Genetička je nestabilnost (tj. pojavljivanje spontanih mutacija s velikom učestalošću) česta u vrsta roda ***Streptomyces*** *i proučavana je u nekoliko modelnih sustava. U njih su utvrđene delecije i amplifikacije velikih dijelova DNA. Visokoproizvodnim industrijskim sojevima posvetilo se premalo truda da bi se utvrdio međusobni odnos između degeneracije soja i njegove genetičke nestabilnosti. Neki izolati soja* ***S. rimosus*** *R6, koji su bili selekcionirani na visok prinos oksitetraciklina (OTC), pokazuju genetičku nestabilnost. Okarakterizirane su dvije skupine mutanata sa smanjenom ili potpuno izgubljenom antibiotičkom aktivnošću. Druga skupina mutanata sadržava velike delecije, uključujući i nakupinu OTC-gena. Te delecije dovode do gubitka proizvodnje i otpornosti soja. Iako to dokazuje da su OTC-geni smješteni u nestabilnom kromosomskom području, činjenica da su mutanti II. skupine osjetljivi prema OTC-u čini ih nevažnim za degeneraciju soja. Nasuprot tome, najčešća skupina niskoproizvodnih mutanata (I. skupina) ostaje otporna prema antibiotiku pa stanice mutanata I. skupine u usporednim pokusima mogu prerasti stanice roditeljskog soja. To pokazuje da je I. skupina mutanata, u kojih za sada nisu primijećene nikakve vidljive pregradnje DNA, odgovorna za degeneraciju soja. U ovom će se radu raspravljati o pretpostavkama kojima se može objasniti pojavljivanje mutanata I. skupine.*

Introduction

Strain degeneration is wide-spread in industrial *Streptomyces* strains and describes a variety of spontaneous changes that result in deleterious phenotypes especially for antibiotic yield. It is often accompanied by changes in sporulation, pigmentation and colony morphology. In industrial practice, the effects of strain degeneration are controlled by careful strain maintenance and inocula preparation. However, it is not clear how great the loss of potential yield is due to accumulation of variants during fermentation.

Genetic instability (i.e. the occurrence of spontaneous mutations with a high frequency) is common in *Strepto-*

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myces and has been studied in several model systems *(1,2)*. In most strains that have been studied at a molecular level, genetic instability is associated with large deletions in the chromosome. Pulsed-field gel electrophoresis showed that a *Streptomyces* chromosome consists of about 8 Mb linear DNA *(3-6)*, and that deletions may be up to 2 Mb in size *(7)*. In many cases, sequences flanking the deletions undergo high level DNA amplification giving rise to several hundred tandem copies of a chromosomal sequence. The amplified DNA may account for more than 50 % of the total DNA in the strain *(1,2)*, that is up to 4 Mb.

Genetic instability is also often characterized by changes in sporulation, pigmentation and colony morphology. This makes it interesting to investigate the relationship between genetic instability, that has mainly been studied in model laboratory strains, and strain degeneration, which occurs in high-producing industrial strains. Strain degeneration depends on two critical parameters: (1) mutation frequency and (2) selection effects. A high mutation frequency alone (i.e. genetic instability) is not sufficient to create strain degeneration problems, if the mutants are counter-selected in normal conditions. A good example of this is in the oxytetracycline (OTC) producer *S. rimosus* R6 *(8)*. A high-producing strain showed genetic instability (Class II), which resulted from deletion of the region containing the OTC-cluster. However, this also results in the deletion of the resistance genes present in the cluster. The sensitive variants produced are killed by the OTC produced by the parental strain so that no accumulation of this class of variants is possible. Most *Streptomyces* products of industrial interest are secondary metabolites and production strains have been subjected to multiple rounds of selection to divert the metabolic activities of the cells towards synthesis of the secondary metabolites. This means that mutations that hinder production will often result in higher growth rates, i.e. overgrowth by low-producing variants is a constant danger. Antibiotic synthesis pathways usually involve large clusters of genes [sometimes over 50 kb in size *(9)*]. This means that the spontaneous mutation frequency to non-production may be quite high even in the absence of special mutation mechanisms (e.g. if a typical 1 kb gene has a mutation frequency of 10^{-6}, then a 50 kb pathway would be expected to yield mutants at a frequency of $5 \cdot 10^{-4}$). In the presence of strong selection pressures against high production such mutation frequencies might well be enough to account for strain degeneration.

Although strain degeneration has been reported in the literature many years ago (e.g. *10*), little work has been performed with high-yielding industrial strains to define the relationship between strain degeneration and genetic instability. The first industrial strain in which genetic instability has been studied in detail at the molecular level was the tylosin-producer *S. fradiae*. Some variants in *S. fradiae* showed high copy number tandem repeats of an amplifiable unit of DNA *(11,12)*. Further studies revealed that the mutants contained deletions involving tylosin production and resistance genes, and that the cluster of tylosin biosynthetic genes is interrupted by a structurally unstable segment containing four repeated sequences *(13,14)*. The influence of genetic instability on tylosin production has not been discussed and is probably not a source of strain degeneration in this industrially important species.

Results and Discussion

Our studies have concentrated on *S. rimosus*, the OTC producer, which is an industrially important species where strains have been highly bred for yield improvement. Derivatives of the strain *S. rimosus* R6 are used by the company PLIVA for commercial OTC production. During the strain selection programme to optimize its fermentative/metabolic capacity, derivatives have been isolated that showed considerable increase in OTC production (30–40 % increase on plates) and resistance (Class III mutants, *8*). It was shown that they had increased the copy number of the OTC-cluster. However, the high production was frequently lost indicating the presence of classical strain degeneration in these strains. The strain degeneration was first recognized, because of the extreme morphological instability of the strains. Thus, in this case, genetic instability is a cause of strain degeneration. The study of genome stability was, therefore, initiated in order to understand, and subsequently eliminate, this genetic instability.

Table 1. The frequency of appearance of morphological mutants of *Streptomyces rimosus* R6 during growth of a culture in complete liquid medium

Tablica 1. Učestalost pojavljivanja morfoloških mutanata soja *Streptomyces rimosus* R6 tijekom rasta mikrobne kulture u kompletnom hranjivom mediju

Frequency / % Učestalost / %	Time of growth / h Vrijeme rasta / h		
	6	24	48
Mycelial morphology Morfologija micelija	Small pellets and long hyphae Male nakupine i duge hife	Long hyphae and some mycelial fragments Duge hife i nešto fragmenata micelija	Only mycelial fragments Sami fragmeni micelija
Experiment 1 Pokus 1	1.2	1.6	2.5
Experiment 2 Pokus 2	3.2	2.4	1.9
Mean value Srednja vrijednost	2.2	2.0	2.2

Spontaneous morphological variants arose that were altered in sporulation, pigmentation, colony morphology and OTC production *(8)*. The majority of mutants (Class I) had retained normal levels of OTC resistance and it was shown that low-producing Class I mutants could overgrow the parental high-producing strain in competition experiments. Thus Class I mutants represent an important source of strain degeneration. They can also be classified as genetic instability, because spores give rise to mutants at an extremely high frequency (about 80 % of spores plated). However, when the strain is grown as mycelial fragments it is more stable and gives rise to mutants at frequencies of 1–3 % per plating unit. This observation is very important for strain maintenance. Two possible explanations for this difference are: (1) the sporulation-germination cycle promotes mutation or (2) the mycelial fragments are multi-nucleate and the morphology results from complementation. If the second explanation were true, the frequency of mutants would be expected to rise during growth of a culture, because the mycelium fragments to much smaller units at later growth stages *(15)*. Table 1 shows that there is little difference between the frequencies at different times despite fragmentation.

No changes were detected in Class I mutants using pulsed-field gel electrophoresis. Non-pigmented low-producing Class I mutants give rise to pigmented derivatives at a frequency of about 1 %. These pigmented »revertants« had undergone a DNA rearrangement that resulted in the amplification of a DNA fragment containing the OTC-cluster to 30–50 copies *(8)*. One explanation for these results would be that the Class I variants carry a mutation in a control gene that results in poor expression of pigment and OTC production genes. Suppression of this mutation could be achieved by amplification of the structural genes as reported for RES1-dependent mutations in *S. lividans (16)*. There is no evidence of true reversion of Class I mutations, but the lack of a suitable selective marker prevents detection of rare events.

The derivatives, which show an overproduction of OTC (about 30–40 % higher production on plates) were isolated as rare morphological variants (these Class III variants account for about 0.001 % of morphological variants). They all show an increase of copy number of the OTC-cluster (2–5 copies), which probably accounts for their overproduction and increased resistance. The majority of Class III variants (6/7) carry duplications of the OTC region in the chromosome probably as tandem repeats. Pulsed-field gel electrophoresis experiments showed that they carried a reproducible DNA change with a decrease in the size of the *Xba*I fragment carrying the OTC-cluster from 415 kb to 200 kb as well as the duplication. In order to understand the nature of these changes better, we also analyzed a second class of DNA rearrangements involving the OTC region; the Class II mutants account for about 1 % of morphological mutants and have deleted the OTC-cluster resulting in a loss of production and resistance. The Class II mutants had lost the 415 kb *Xba*I fragment and no new junction fragment could be detected. A reproducible change could be seen in *Asc*I digests suggesting that the deletions in different isolates had identical end points *(8)*. In order to analyze the rearrangements in more detail about 120 cosmids were isolated that hybridized with the 415 kb *Xba*I fragment. Some of the cosmids carry repeated sequences, which retain copies in the Class II deletion strains. Cosmids that do not show homology to the Class II strains could be ordered in two contigs, one of which contains the OTC-cluster. In Class III strains (overproducers) the OTC contig was retained as expected. However, the other contig had been deleted and one end point of the deletion appeared to be identical in both Class II and Class III mutants. One explanation of these data is that the OTC region is flanked by directly repeated sequences. The reproducible deletions would be formed by homologous recombination. A simple model is shown in Fig. 1.

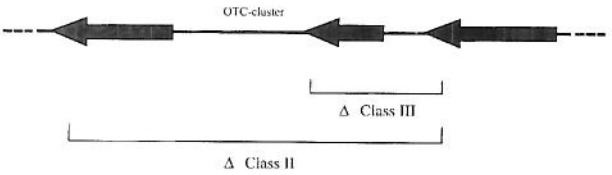

Fig. 1. A simple model to explain deletions in Class II and Class III mutants

Slika 1. Jednostavan model kojim se mogu objasniti delecije u II. i III. skupini mutanata

Recombination between the duplicated copies of the overproducers could result in reduction of copy number and reversion to the parental production level. A better understanding of the structure of the OTC region and its repeated sequences, might help to reduce this problem.

One Class III mutant had arisen by a different mechanism. The parental strain carries a linear plasmid (pPZG101) of 387 kb, whereas the atypical Class III mutant MV17 has a linear plasmid of about 1 Mb in size (pPZG103), which also carries the OTC region. The chromosomal copy of the region is retained, and the plasmid has a copy number of 3–4 *(17)*. As MV17 does not carry tandem repeats of the OTC region, it should not suffer from copy number reduction by homologous recombination. However, when MV17 is plated out on agar medium morphologically atypical colonies are seen. MV17 produces more brown pigment than the normal high-producing strains, whereas the variant colonies are less pigmented or white and are defective in sporulation. These colonies have only low or undetectable OTC production and the high frequency of their appearance prevents commercial use of MV17 as well. One such white variant was analyzed by pulsed-field gel electrophoresis, and it was shown that its plasmid is indistinguishable in size from the pPZG103 in MV17. When genes from the OTC-cluster *(18)* were used as hybridization probes against a Southern blotting of the gel, it could be seen that the plasmid in the white variant hybridized showing that the OTC-cluster was still present *(19)*. Thus the strain degeneration in MV17 is probably also due to Class I mutants.

It seems clear that an increased copy number of the OTC region results in higher production. It is likely that

any problems arising from structural instability of the copy number (recombination between tandem repeats in the chromosome or plasmid loss) can be overcome. If necessary, it should be possible to move copies of the OTC-cluster to more stable chromosomal sites. However, it is likely that Class I mutants will remain a major problem. We are therefore following two strategies to deal with Class I mutants. One strategy is to understand the mechanism of the mutation and to isolate and stabilize the postulated control gene(s). It is likely that this will also yield information of general interest for other strains. It is hoped that this will also provide DNA probes that can be used to measure the proportion of Class I during fermentation. This would allow the development of fermentation conditions to minimize strain degeneration. The second strategy is to isolate promoters whose expression is reduced in Class I mutants. Such a promoter could be coupled to a selectable marker gene (e.g. an antibiotic resistance gene) to allow direct selection against Class I mutants.

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