UDC 579.873.7:579.254.22
ISSN 1330-9862
(FTB-1342)

original scientific paper

Persistence of the Chromosome End Regions at Low Copy Number in Mutant Strains of *Streptomyces rimosus* and *Streptomyces lividans*

Dalia Denapaite[1a], *Andrea Paravić Radičević*[1,2], *Branka Čajavec*[1,3b], *Iain Hunter*[3], *Daslav Hranueli*[2c] *and John Cullum*[1*]

[1]LB Genetik, University of Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany

[2]PLIVA Inc., Research Division, Prilaz baruna Filipovića 25, HR-10 000 Zagreb, Croatia

[3]University of Strathclyde, Department of Pharmaceutical Sciences, 204 George Street, Glasgow G1 1XW, UK

Received: June 11, 2004
Accepted: November 22, 2004

Summary

Streptomycetes are important antibiotic producing bacteria that often exhibit genetic instability. One or both ends of the linear *Streptomyces* chromosome are lost spontaneously, resulting in viable mutant strains sometimes lacking hundreds of genes. We examined some strains of *Streptomyces rimosus* and *Streptomyces lividans*, which had been classified as »deletion mutants« and appeared to have lost chromosome end sequences. We discovered that the »deleted« sequences were still present in vegetative mycelium at a very low copy number so that they were normally not detected. The copy number in *S. rimosus* was estimated as $0.1–1.0 \cdot 10^{-3}$/chromosome. *Streptomyces* spores contain the disappearing chromosome end sequences at a higher copy number than the vegetative mycelium, promoting their inheritance via spore preparations. This, in effect, represents a separation between germ line and deleted vegetative genomes, which has not been recognised before in *Streptomyces*, and has practical implications both for strain preservation and genetic studies.

Key words: linear chromosome, linear plasmid, deleted DNA, reversion, *Streptomyces*

Introduction

Streptomyces species grow as a branched substrate mycelium with multinucleoid compartments (*1*). When conditions become less favourable they produce aerial mycelium that divides up into haploid spores that are resistant to desiccation. Many species show genetic instability affecting the ends of the linear chromosome (*2–5*). In *S. rimosus*, spontaneous mutants were isolated (*6*) that showed an altered colony morphology as well as changes in the production of and resistance to the antibiotic oxytetracycline (OTC). They were classified on

*Corresponding author; Phone: ++49 631 20 54 062; Fax: ++49 631 20 54 090; E-mail: cullum@rhrk.uni-kl.de

Present addresses:

[a] LB Mikrobiologie, University of Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany

[b] Innovationskolleg Theoretische Biologie, Humboldt-Universität zu Berlin, Invalidenstraße 43, D-10115 Berlin, Germany

[c] Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, HR-10 000 Zagreb, Croatia

the basis of their OTC resistance. Class I mutants showed unchanged resistance levels. They were very heterogeneous in their colony morphology and antibiotic production and no large scale DNA changes could be detected. Class III mutants showed increased resistance and production, which was correlated with an increased copy number of the OTC cluster. Class II mutants were sensitive to OTC and did not appear to produce any OTC. They sporulated poorly. Restriction analysis using pulsed-field gel electrophoresis suggested that the Class II mutants carried identical large deletions. This deleted region in *S. rimosus* includes the OTC biosynthesis cluster, which lies near one chromosome end, as well as the chromosome end (*4*). It was therefore surprising when more sensitive experiments showed that the Class II mutants still produce small amounts of OTC (about 0.1 μg/mL compared to 10 mg/mL in the parent strain). Further experiments showed that the Class II mutants gave rise to apparent revertants at low frequency that produced parental amounts of OTC (*7*). Southern hybridisation indicated that these strains had regained the OTC cluster and it appeared unlikely that their appearance was due to contamination with the parental strain. In this paper, we examine the genome structure of these strains using pulsed-field gel electophoresis to rule out contamination as an explanation.

The 50-kb linear plasmid SLP2 of *S. lividans* 66 has one end identical to the chromosome end (*8*). Some mutants that had circularised the chromosome, resulting in deletion of terminal sequences, had lost SLP2, and therefore SLP2 could not be reintroduced into such strains (*9*). It was suggested that such strains had deleted genes needed for replication of the linear chromosome and plasmid ends and later work identified the terminal protein gene (*tpg*) in this region (*10*). One of the strains, 27AA, has a circular chromosome and has deleted about 270 kb from the left hand chromosome end and about 30 kb from the right hand end (*11*,*12*). Strain 27AA still sporulates and it was chosen as a host strain for experiments to try to identify genes involved in terminal replication by complementation. During this work apparent revertants of the deletion mutation were isolated, which cannot be explained by contamination.

The reappearance of deleted sequences could be explained if they were present at low copy number in the mycelium. This was tested for *S. rimosus* using real time PCR and for *S. lividans* by increasing the detection time for Southern blots. In *S. lividans* it was also shown that the sequences were enriched in spores.

Materials and Methods

Strains, plasmids and culture conditions

27AA is a spontaneous chloramphenicol-sensitive mutant of the wild type *S. lividans* 66 strain 1326 (*13*), which has also lost linear plasmid SLP2 (*11*). The following *S. rimosus* strains were used: R6-500, MV9, MV15 (*6*), nine brown OTC-producing pseudorevertants MV9-R1, -R2, -R3, -R4, -R5, -R6, -R7 and MV15-R1, -R3 (*7*) and M4018 (*14*). The media and growth conditions for *S. lividans* strains (*15*) and *S. rimosus* strains (*16*,*17*) were as described previously. The cosmids carrying *S. lividans* sequences (26, 54, 54/3, 68, 69, 69/6, 85, 85/3 and 90) were described earlier (*18*). Cosmid 26 lies in the *Ase*I-H_1 right hand terminal fragment of the chromosome. All the other cosmids come from the left hand terminal fragment (*Ase*I-A). Cosmid 90 lies proximal to the deletion in strain 27AA, whereas all the other cosmids lie within the deletion. Cosmid pPZG25 (*7*) carries most of the OTC cluster of *S. rimosus* R6. Eight cosmid clones (C-11, C-13, C-16, C-21, C-25, C-61, C-136 and C-189) from the deletion region in Class II mutants (*4*) were used.

Plasmid pOJ436 (*19*) was introduced into strain 27AA using protoplast transformation with selection for apramycin resistance (50 μg/mL) to give strain 27AA::pOJ436. Cosmid clone DNA was introduced into 27AA::pOJ436 using protoplast transformation with selection for kanamycin (5 μg/mL). From 30 to 150 transformants/μg of cosmid DNA were obtained (control transformations with plasmid pIJ702 (*20*) gave more than 10^6 transformants/μg of DNA). This yielded small poorly growing colonies. After restreaking three times on kanamycin-containing minimal medium colonies of normal size were obtained. For further analysis 6–8 colonies per transformation were used.

General DNA techniques

Standard general methods were used for DNA manipulation in *Streptomyces* (*21*) and in *E. coli* (*22*). DNA was isolated from *Streptomyces* spores using the method of Kutchma *et al*. (*23*), except that no pretreatment with acetone was performed. Spores were harvested after the 10-day growth on Soya-Mannitol agar medium and filtered through cotton wool (*23*). For DNA extraction, 50 mg (wet weight) of spores were used.

Southern transfers used transfer in an ammonium acetate buffer (*24*) to a nylon membrane (Amersham). PFGE gels were first subjected to depurination with HCl. Probe DNA was labelled by random priming (*25*) using the conditions recommended by the manufacturers of a non-radioactive digoxigenin labelling kit (Boehringer Mannheim). Hybridisation and detection with colour reaction were done according to the manufacturer's instructions.

PFGE of *S. lividans* (*18*) and DNA preparation for PFGE of *S. rimosus* (*6*) were carried out as described previously. A BioRad CHEF DR-III apparatus was used for electrophoresis.

Quantitative real time PCR

PCR reactions were carried out using a LightCycler machine (Roche). Two primer pairs were synthesised to amplify the *recA* and *otcC* genes of *S. rimosus*:

*recA*fwd	5′–GGCACTGACCGCGAGAAG–3′
*recA*rev	5′–GCAGCGCCTGGCTCATCAG-3′
*otcC*fwd	5′-GGCAACCCACTGATGATCC-3′
*otcC*rev	5′-GATCACCGGGAACATGTGC-3′

The primers were used in PCR reactions at concentrations of 3, 3, 2 and 1.5 μM, respectively. Reactions were carried out as recommended by the manufacturer using the LightCycler-DNA Master SYBR Green 1 kit (Roche) with herring sperm carrier DNA. The PCR program used was: denaturation: 95 °C for 30 s (1 cycle);

amplification: 95 °C for 0 s, 54 °C for 5 s, 72 °C for 10 s, 87 °C for 0 s (45 cycles); melting curve analysis: 95 °C for 0 s, 70 °C for 15 s, 95 °C for 0 s (1 cycle).

Relative concentration of gene sequences was calculated from the number of cycles needed for the appearance of a signal and the exponential rate of the increase of the signal.

Results

Characterisation of apparent revertants from Class II mutants of S. rimosus

Class II mutants are pale in colour and produce extremely low levels of oxytetracycline (*6*,*7*). Hybridisation experiments showed that there were large chromosomal deletions including the *otc* biosynthesis cluster. Nine brown oxytetracycline-producing Class IV mutants (previously called »pseudorevertants«) were isolated from the Class II mutants MV9 and MV15 (*7*). Five had colony morphology indistinguishable from that of the parental type, whereas the other four showed much stronger sporulation. Hybridisation experiments showed that the Class IV mutants had regained the *otc* cluster, but contamination with the parental strain seemed very unlikely. Therefore, we examined the *Xba*I and *Ase*I restriction patterns of the mutants.

Fig. 1A shows *Xba*I digests of DNA from the Class II mutant MV9, its parent R6-500 and five Class IV derivatives of MV9. In comparison with R6-500 (track 1), MV9 (track 2) has lost the band of 415 kb (which carries the OTC cluster) and the band of 610 kb, and has gained a new band of 930 kb. Two Class IV mutants with parental colony morphology MV9-R3 (track 4) and MV9--R7 (track 7) show patterns indistinguishable from that of R6-500. However, the three strongly sporulating Class IV mutants MV9-R1 (track 3), MV9-R4 (track 5) and MV9-R5 (track 6) show patterns identical to each other, but differing from those of R6-500 and M9. In comparison with MV9 they have lost the 930 kb band and gained a new band of 560 kb. When Southern blots were hybridised with cosmid pPZG25 that contains most of the OTC cluster (*7*), three strongly sporulating strains showed hybridising bands of 200 kb (Fig. 1B). Two Class IV mutants with parental colony morphology showed hybridising bands of 415 kb like that of the R6-500 parent. *Ase*I digests of the strains were also examined (data not shown). When Southern blots were hybridised with the OTC-cluster cosmid pPZG25, all five Class IV mutants showed a single hybridising band of the same size as the *Ase*I-C_1 band in R6-500 (795 kb). Two Class IV mutants with parental colony morphology showed *Ase*I patterns identical to that of R6-500. The other three mutants showed new *Ase*I bands of 350 kb and 710 kb, which were not present in either R6-500 or MV9. These mutants also showed loss of the 550 kb *Ase*I-E band. The chromosome of *S. rimosus* has long terminal repeats of about 550 kb, which include the *Ase*I-J fragment (*4*). The ends of the inverted repeats lie in the *Ase*I-E fragment and the OTC-containing fragment *Ase*I-C_1. *Xba*I and *Ase*I digests were also carried out with the other Class IV mutants and showed similar results.

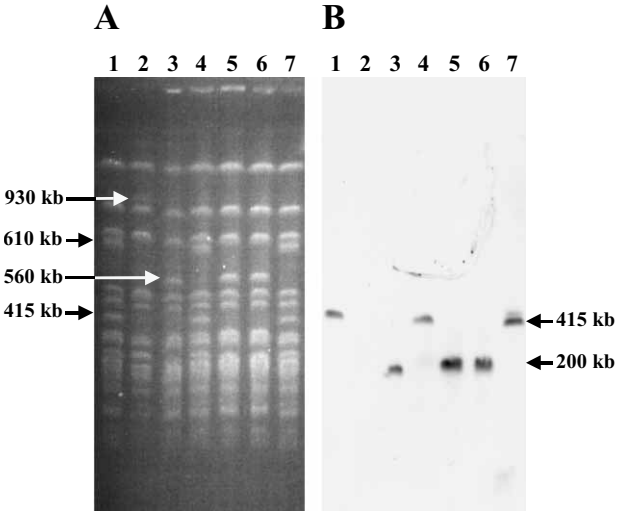

Fig. 1. PFGE of *Xba*I digests of *S. rimosus* strains. **(A)** track 1: parental strain *S. rimosus* R6-500; track 2: MV9; track 3: MV9R-1; track 4: MV9R-3; track 5: MV9R-4; track 6: MV9R-5; track 7: MV9R-7. The gel was run at 6 V/cm with the program: 16 h with ramping of 40–60 s, followed by 20 h with ramping of 60–130 s. **(B)** Southern blot of the gel hybridised with cosmid pPZG25

These experiments showed that the Class IV mutants can be divided into two classes. Class IVA mutants (*i.e.* MV9-R3 and -R7) are indistinguishable from R6-500 in colony morphology and PFGE patterns. However, Class IV B mutants (*i.e.* MV9-R1, -R4 and -R5) differ from R6-500 in PFGE pattern and are also different from every other *S. rimosus* strain that we have examined. Thus, the occurrence of Class IV B mutants can not be explained by contamination.

Detection of the deleted sequences at low copy number in S. rimosus

As the Class II mutants, which have deleted the OTC cluster, still produce very small amounts of OTC (*7*) and give rise to Class IV mutants, which recover the OTC cluster, we suspected that the deleted sequences might still be present at low copy number in the mycelium. Therefore, we repeated hybridisation experiments to confirm the presence of deletions in the Class II mutant MV9 of *S. rimosus*. Eight cosmids were used and the loss of sequences was confirmed. Experiments were also carried out with longer detection of the digoxigenin marker (20-hour detection with an alkaline phosphatase kit). In one experiment weak signals with a pattern like that of R6-500 were seen with cosmid C-21, but this was not reproducible (data not shown).

As the deleted sequences might still be present at a very low copy number, we tried to detect them using PCR. A primer pair that amplified a 500 bp fragment of the oxytetracycline biosynthesis cluster gene *otcC* gene was used. In preliminary experiments DNA of MV9 was used and a 500 bp band appeared after 40 cycles of PCR. It was decided to use a real time PCR method to estimate the copy number. Two primer pairs were designed to amplify a 0.5 kb internal fragment of the *otcC* gene and, as a control, a similar sized internal fragment of the *recA* gene, which is on the opposite chromosome

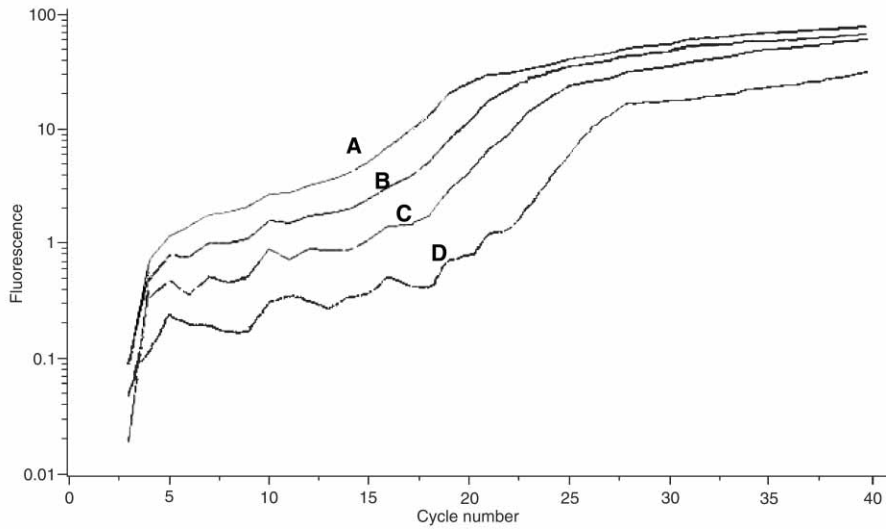

Fig. 2. Real time PCR with *otcC* primers. Fluorescence was plotted against the number of cycles. The results are shown for the following four DNA samples: (**A**) M4018 undiluted, (**B**) M4018 10-fold diluted, (**C**) M4018 100-fold diluted and (**D**) MV9 undiluted

arm to the *otc* cluster (*4*). These primers were used with DNA of *S. rimosus* strain M4018, which does not carry a terminal deletion. Both primer pairs produced PCR products that were detected by fluorescence signals. As expected, a detectable signal first appeared after a number of cycles and the signal increased exponentially until a saturating value was reached, as can be seen for the *otcC* primers (Fig. 2, curve A). Comparable signals were also obtained for the *recA* primers. When 10-fold and 100-fold dilutions of the M4018 DNA were used, more cycles were needed until a signal was observed, but the signal still showed an exponential region before saturation (Fig. 2, curves B and C). The number of cycles until the first appearance of the signal was in agreement with the displacement expected for the dilutions used, thus establishing a standard. DNA from the Class II strains MV7 and MV9 was also used. In each case, the *recA* primers gave comparable signals to M4018. The *otcC* primers also gave signals that showed a region of exponential increase followed by saturation, as can be seen for MV9 DNA (Fig. 2, curve D). These signals appeared in the PCR reactions about 10 cycles later than the *recA* signals. This allowed us to calculate that the copy number of *otcC* in MV7 and MV9 was 1000–5000 lower than that of *recA*. The copy number in MV7 appeared to be somewhat lower than in MV9. Experiments were also carried out to see if the copy number changed during serial subculture in liquid medium. A culture of MV9 was grown for 2 days and then diluted 100-fold into fresh medium. A second subculture was undertaken after another 2 days. Mycelium samples were taken after each 2-day growth period and used for DNA preparation. All 3 samples showed similar results for *otcC* copy number to that presented in Fig. 2.

Apparent reversion of a deletion mutant of S. lividans 66

S. lividans 66 shows a high frequency of spontaneous mutants that have undergone deletion of the chromosome ends to produce circular chromosomes (*18*). Such mutants have often lost genes needed for the maintenance of the linear plasmid SLP2 (*12*). We were interested in trying to identify such genes by complementing them with sequences from cosmid clones. Most of the deletion strains are defective in sporulation, but the strain we used, 27AA, still sporulated (*12*). We wanted to introduce cosmid sequences by homologous recombination analogously to the method used in *S. coelicolor* A3(2) (*9*). However, as there is no homology between the cosmid clones from the deletion region and the chromosome of the strain 27AA, it was necessary to introduce homologous sequences into the chromosome. The integrative plasmid pOJ436 (*19*) has some common sequences with the SuperCos1 cosmid vector (*26*), which we hoped would be enough to allow homologous recombination.

pOJ436 was introduced into the strain 27AA and its integration site was confirmed by PFGE. DNA from 7 cosmids of the deletion region (54, 54/3, 68, 69, 69/6, 85 and 85/3) as well as cosmid 90, which lies proximal to the deletion, was used to transform protoplasts of strain 27AA::pOJ436 with selection for the kanamycin resistance gene of SuperCos1. Colonies from each of the 8 transformation experiments were selected and used to prepare spore suspensions. DNA was isolated from each of the 8 transformants digested with *Bam*HI and used for Southern blottings. The Southern blottings were hybridised with probes prepared from each of the 8 cosmids. Fig. 3 shows the results of the blottings using one of the deletion cosmids (85/3) as a probe. As expected, the *S. lividans* 66 wild type strain 1326 (track 3) gives a series of bands, as does another strain without the deletion (track 6). There are no bands with the deletion strain 27AA (track 4). There is a single band in the strain carrying pOJ436 (track 5), because of the homology with the SuperCos1 vector of cosmid 85/3. The results for the 7 transformants with cosmids from the deletion region were surprising. As expected, each cosmid gave signals with its own transformant. However, in each case, the other 6 deletion cosmids (as well as cosmid 90 from outside the deletion) also gave signals of comparable strength

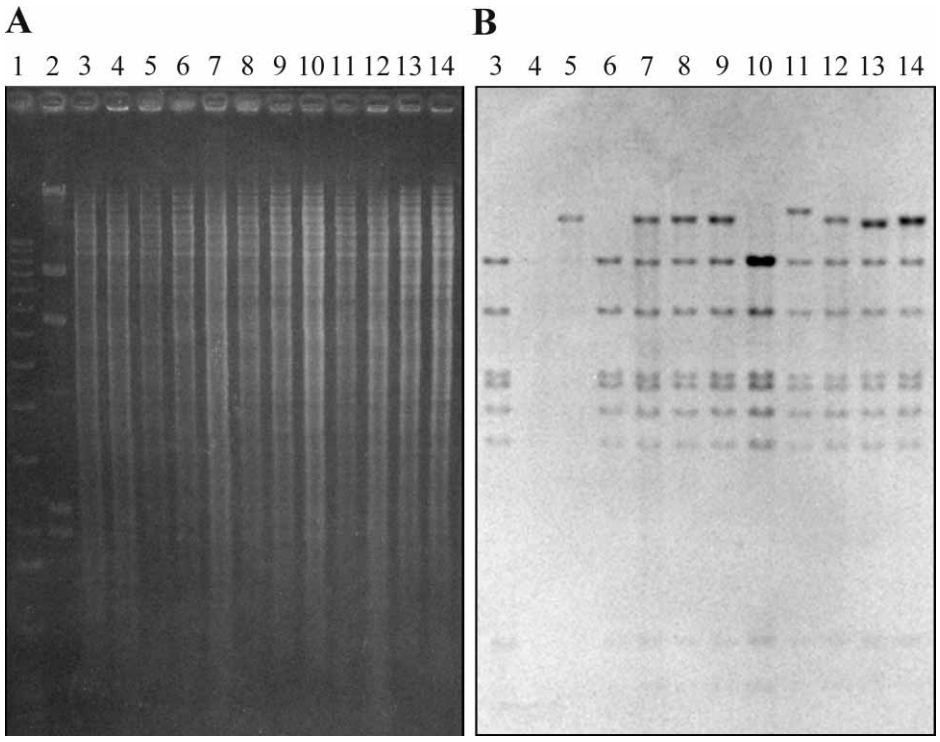

Fig. 3. (**A**) *Bam*HI digests of total DNA of *S. lividans* strains. Track 1: marker 1 kb DNA ladder (Fermentas); track 2: marker λ *Hin*dIII; track 3: wild-type strain 1326; track 4: strain 27AA; track 5: strain 27AA::pOJ436; track 6: strain WU10; tracks 7–14: transformants of 27AA with cosmids 90 (track 7), 85 (track 8), 85/3 (track 9), 69 (track 10), 69/6 (track 11), 54/3 (track 12), 54 (track 13), 68 (track 14). **(B)** Southern blot of the gel hybridised with digoxigenin-labelled DNA of cosmid 85/3

i.e. the deleted sequences seemed to have reappeared. As we were not working with any other strains carrying pOJ436 in the laboratory, contamination did not seem to be an explanation for these results.

The signal corresponding to the integrative plasmid pOJ436 is unaltered in most cases suggesting that integration has not occurred by homologous recombination between pOJ436 and the cosmid clone. Blocks for PFGE analysis were also prepared and used for *Ase*I digests. Southern blots were hybridised with probes prepared from cosmid 26 that comes from the middle of the *Ase*I-H_1 fragment, which is the right hand terminal fragment of the chromosome (*27*). In strain 27AA, which has a circular chromosome, fusion of the *Ase*I-A and *Ase*I-H_1 fragment generates a large *Ase*I-A´ fragment that hybridises with cosmid 26. In the 7 transformants with the deletion plasmids, there is hybridisation to a fragment of the same size as *Ase*I-H_1 (data not shown), suggesting that chromosome linearity has been restored.

The »deleted« sequences of S. lividans are still present at low copy number

The apparent reversion of the deletion mutation in strain 27AA suggested that the »deleted« sequences might still be present at low copy number in the mycelium, as was found for *S. rimosus* in the experiments reported above. When Southern transfers of total DNA from strain 27AA digested with *Bam*HI were detected using an alkaline phosphatase colour detection system, no signals with the 7 deletion cosmids were seen after 20 min, whereas there were strong signals with the parent strain 1326. However, after 20-hour detection, weak signals were reproducibly observed. Cosmid 90, which is not in the deletion region, gave comparable signals in both 1326 and 27AA. Fig. 4 shows that, using cosmid 69/6 as a probe, the restriction pattern of 27AA is indistinguishable from that of the parent strain 1326. Similar results were obtained with the other 6 deletion cosmids. Thus, terminal regions seem to be present at low copy number.

If the deletions had arisen as a single crossover, then the deleted sequences should be present as a linear molecule of about 300 kb in size. Repeated attempts to detect this molecule by hybridisation of cosmid probes to Southern blots of pulsed-field gel electrophoresis (PFGE) gels of undigested DNA were unsuccessful. When total DNA of *S. lividans* is restricted with *Dra*I, the left hand chromosome end is carried on the small 30 kb *Dra*I-VII terminal fragment (*27*). DNA from 27AA and the parent strain 1326 was digested with *Dra*I. The *Dra*I-VII band can be seen in Fig. 5A (track 3) and it shows hybridisation with a probe that carries sequences from the chromosome terminal region (Fig. 5B, track 3). There is also a hybridising band of 50 kb due to the linear plasmid SLP2, which has homology to the chromosome end (*8*), and is also present in undigested DNA (Fig. 5B, track 4). When normal detection conditions (20 min alkaline phosphatase reaction) were used, no hybridising band was seen with DNA from 27AA (Fig. 5B, track 2). However, when the detection reaction was continued for 20 h, a faint band of 30 kb could also be seen with the DNA from 27AA (Fig. 5C, track 2). This suggests that the deleted region is indeed present as a linear molecule.

Enrichment of the end region in spores

The initially used 27AA culture had been propagated from a single spore isolate, which suggested that there was stable retention of the low copy number terminal regions. In order to test the stability, 3 independent single spore isolates of 27AA were used to establish cultures and total DNA was prepared. In addition, 3 single spore isolates of 27AA::pOJ436 were used. Southern blots of *Bam*HI digests of 6 DNA preparations were hybridised with probes from 6 deletion cosmids (85, 85/3, 69/6, 69, 54/3 and 54). In each case, after 20-hour detection, faint bands of the same size as those in the wild type strain 1326 were present (data not shown). Thus, the low copy number ends are retained both through the protoplasting-regeneration steps used to transform 27AA with pOJ436 and through single spore isolates. This might be explained if these sequences were preferentially segregated into the spores.

Spores were harvested from well-sporulated plates of 27AA and 1326. It was noticed that the yield of spores was somewhat lower for 27AA (30–60 mg/plate) than for 1326 (60–100 mg/plate). DNA was prepared from spores and mycelial cultures of the two strains and Southern blots of *Bam*HI digests hybridised with the 6 cosmids. As seen earlier, the signals with mycelial DNA were much weaker with 27AA than with 1326 (Fig. 6A, tracks 1 and 2). However, the signals from the spore DNA were comparable in the two cases (Fig. 6B, tracks 1 and 2).

Strain 27AA often segregates red poorly-sporulating colonies. One such colony was picked and showed the same phenotype stably on restreaking. DNA was made from a mycelial culture from this colony and »spore« DNA was also made from material scraped off the upper surface of the cultures grown on plates. The mycelial DNA did not show any hybridisation with the cosmids, whereas weak bands were present in »spore« DNA (data not shown).

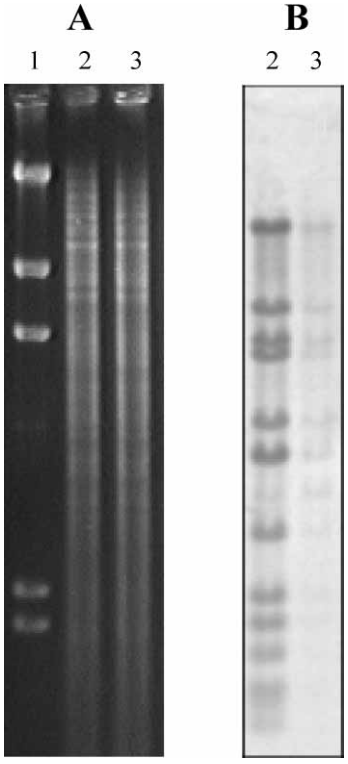

Fig. 4. (A) *Bam*HI digests of total DNA from *S. lividans* 66 strains. Track 1: marker – λ *Hin*dIII; track 2: 1326; track 3: 27AA. **(B)** Southern blot of the gel hybridised with digoxigenin-labelled DNA of cosmid 69/6. Detection was carried out for 20 h

Discussion

In both *S. rimosus* and *S. lividans* 66 there appeared to be reversion of »deletion mutants«. In *S. rimosus*, Class II mutants that had »deleted« the OTC region appeared to give rise to Class IV mutants which had regained

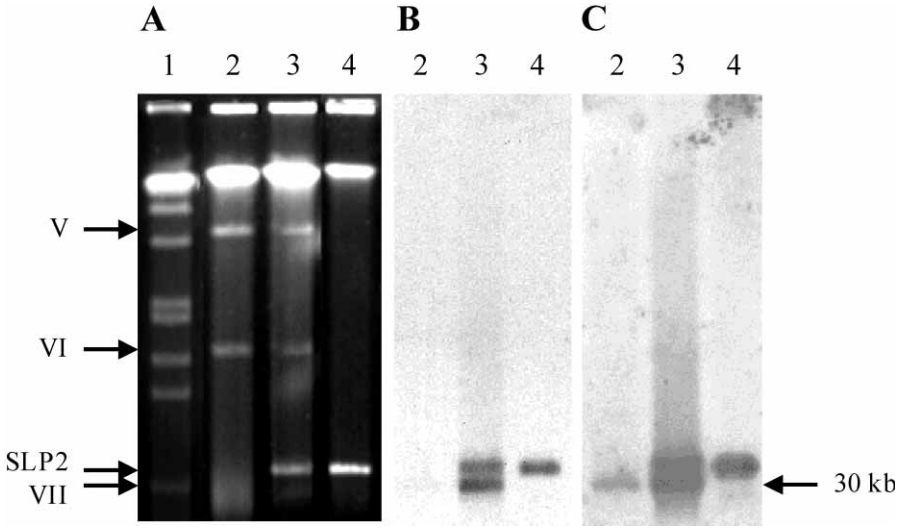

Fig. 5. PFGE analysis of *S. lividans* strains. **(A)** Track 1: marker – *Ase*I digest of total DNA of *S. coelicolor* A3(2) strain M145; track 2: 27AA digested with *Dra*I; track 3: 1326 digested with *Dra*I; track 4: 1326 undigested. Running conditions: 6V/cm for 24 h with ramping of 5–30 s. **(B)** Southern blot of the gel hybridised with cosmid 68. Detection 20 min. **(C)** As (B) with detection 20 h. The positions of the *Dra*I–V, –VI and –VII bands and the SLP2 band are indicated

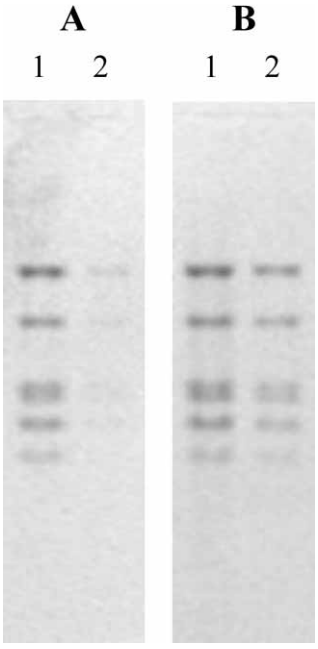

Fig. 6. Southern blots of the DNA isolated from spores and mycelium of *S. lividans* strains. Track 1: 1326, track 2: 27AA. Total DNA was digested with *Bam*HI and the cosmid 54/3 was used as a probe, 20-hour detection was used. **(A)** DNA isolated from mycelium. **(B)** DNA isolated from spores

the region. The Class IVA mutants were indistinguishable from the parent strain R6-500 so that contamination, although unlikely, could not be ruled out as an explanation. However, the Class IV B mutants differed in PFGE pattern from all other *S. rimosus* strains that had been worked with in the laboratory so that contamination was not a possible explanation. Real time PCR detected a fragment of the *otc*C gene at a copy number of $0.2–1.0 \cdot 10^{-3}$ per chromosome in the two Class II »deletion« mutants examined. The experiments reported here (Fig. 1) used a better PFGE program than the one used earlier (*6*). This allowed the detection of the 610 kb *Xba*I fragment and the 930 kb fragment. The observed fragments would be explained (Fig. 7) if Class II mutants in *S. rimosus* resulted from a fusion of two copies of the chromosome in inverted orientation (*28*). Such a structure has been found in mutants of *S. ambofaciens* (*29*). The recombination event should also generate a linear molecule of about 1.85 Mb in size carrying two copies of the OTC cluster (Fig. 7). The reintegration of the OTC region to produce Class IV B mutants seems to be a complex event, which also affects the *Ase*I-E fragment that lies near the opposite end of the chromosome. In *S. lividans* 66, apparent reversion of »deletion mutants« occurred when a selection for integration of cosmid clones was carried out. The revertant strains carried the integrative plasmid pOJ436, which had not been introduced

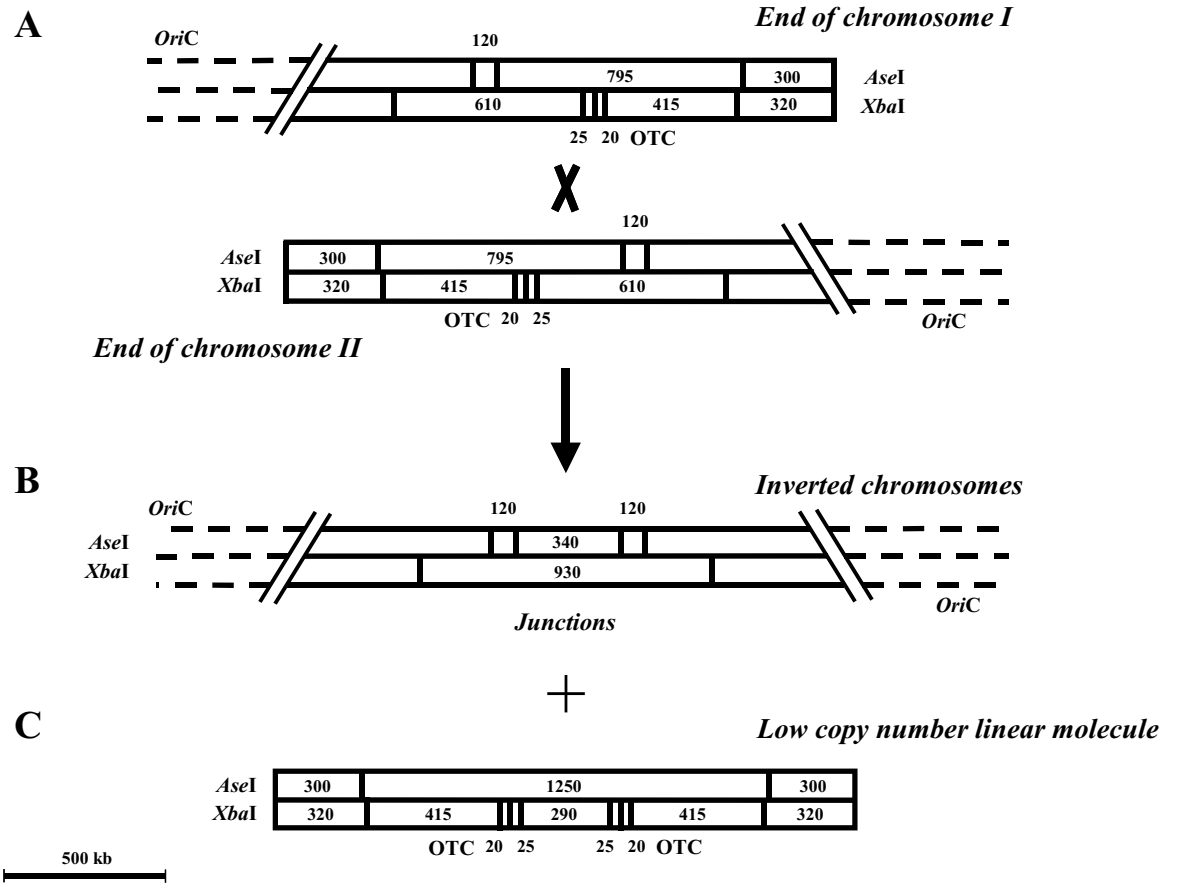

Fig. 7. A model to explain the structure of Class II deletion mutants in *S. rimosus*. Two copies of the chromosome undergo a recombination in inverse orientation **(A)**, which generates a double chromosome with two origins of replication **(B)** and a linear molecule that carries two copies of the OTC gene-cluster but no known origin of replication **(C)**

into other strains in the laboratory. This ruled out contamination as an explanation. Southern blotting showed that the »deleted« sequences were still present at low copy number in the »deletion« strain. If *S. lividans* strain 27AA had arisen by a single crossover to circularise the chromosome, the recombination event should also produce a linear molecule of about 300 kb in size carrying the deleted regions and both chromosome ends. We did not succeed in detecting such a molecule, but it is known that detection of weak bands in Southerns of PFGE is very inefficient (*30*). However, a 30 kb terminal *Dra*I fragment was detected (Fig. 5), strongly suggesting the presence of a linear molecule. Thus, in both *S. lividans* 66 and *S. rimosus*, linear molecules without any known internal replication origin would be generated if the chromosomal deletions had arisen by reciprocal recombination events. It is possible that the mechanisms suggested for replication of the linear ends (*31,32*) could occasionally result in replication of the whole molecule like in *Bacillus* phage ϕ29 (*33*). This replication would probably be inefficient, accounting for the low copy number.

Low copy number sequences were propagated stably in cultures of both *S. rimosus* and *S. lividans*. In the case of *S. rimosus*, the material was scraped from the surface of the colonies as the Class II strains do not sporulate. For *S. lividans*, standard spore preparation methods were used, which included filtering through cotton wool (*21*). Although this procedure has proven very successful over the years for propagating pure cultures and carrying out crosses, it can not be ruled out that some multiple nucleoid mycelium is transferred. The low copy number sequences in *S. lividans* also persisted through the protoplasting and regeneration steps used to transform with plasmid pOJ436. Protoplasting is also thought to yield units carrying a single chromosome. However, even if the spore isolation and protoplasting steps sometimes give rise to multiple chromosome units, it seems very unlikely that the number of chromosomes present is high enough to allow stable inheritance of the low copy number sequences by random segregation. This suggests that there must be a selection for the retention of sequences.

There seems to be a correlation between the presence of the terminal regions and sporulation. Previously it has been reported that *S. lividans* strains that have circularised the chromosome and lost the terminal sequences sporulate poorly (*18*). The sporulating strain 27AA has retained the terminal region at low copy number in the mycelium, whereas derivatives of 27AA that sporulate poorly do not contain detectable copies of the terminal regions in the mycelium. The terminal sequences are enriched in spores of 27AA. *S. rimosus* Class II mutants sporulate very poorly, whereas the Class IV derivatives that have regained at least a part of the deleted region at normal chromosomal copy number sporulate much better. These results might be explained if there was a sporulation gene close to one chromosome end. Other possible explanations would be the preferential segregation of chromosome ends into spores or the over-replication of the low copy number sequences in aerial mycelium. As *Streptomyces* strains are usually propagated via spores or by scraping material from the colony surface, this would result in retention of the sequences despite their inefficient replication. As *S. lividans* and *S. rimosus* are among the most distantly related strains in the genus *Streptomyces* (*34*), it seems likely that these phenomena are general.

Acknowledgments

This work was supported by the grant HRV 01/003 (to J.C. and D.H.) from the International Bureau of the BMBF, Federal Republic of Germany and the Ministry of Science and Technology, Republic of Croatia, by the Wellcome Trust grant 051550/Z/97 (to I.S.H. and D.H.), and by the grant 058008 (to D.H.) from the Ministry of Science, Education and Sports, Republic of Croatia.

References

1. K. Flärdh, K.C. Findlay, K.F. Chater, Association of early sporulation genes with suggested developmental decision points in *Streptomyces coelicolor* A3(2), *Microbiology*, *145* (1999) 2229–2243.
2. C.W. Chen, C.H. Huang, H.H. Lee, H.H. Tsai, R. Kirby, Once the circle has been broken: Dynamics and evolution of *Streptomyces* chromosomes, *Trends Genet*. *18* (2002) 522–529.
3. G. Fischer, T. Wenner, B. Decaris, P. Leblond, Chromosomal arm replacement generates a high level of intraspecific polymorphism in the terminal inverted repeats of the linear chromosomal DNA of *Streptomyces ambofaciens*, *Proc. Natl. Acad. Sci. USA*, *95* (1998) 14296–14301.
4. K. Pandza, G. Pfalzer, J. Cullum, D. Hranueli, Physical mapping shows that the unstable oxytetracycline gene cluster of *Streptomyces rimosus* lies close to one end of the linear chromosome, *Microbiology*, *143* (1997) 1493–1501.
5. J.N. Volff, J. Altenbuchner, Genetic instability of the *Streptomyces* chromosome, *Mol. Microbiol*. *27* (1998) 239–246.
6. B. Gravius, T. Bezmalinović, D. Hranueli, J. Cullum, Genetic instability and strain degeneration in *Streptomyces rimosus*, *Appl. Environ. Microbiol*. *59* (1993) 2220–2228.
7. D. Hranueli, N. Perić, H. Petković, G. Biuković, Z. Toman, J. Pigac, B. Borovička, A. Bago, I. Crnolatac, T. Maršić, L. Zhou, S. Matošić, P.G. Waterman, J. Cullum, I.S. Hunter, Novel hybrid polyketide compounds produced by genetic engineering of the oxytetracycline biosynthetic pathway, *Food Technol. Biotechnol*. *37* (1999) 117–125.
8. Y.-S. Lin, H.M. Kieser, D.A. Hopwood, C.W. Chen, The chromosomal DNA of *Streptomyces lividans* 66 is linear, *Mol. Microbiol. 10* (1993) 923–933.
9. M. Redenbach, H.M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, D.A. Hopwood, A set of ordered cosmids and a detailed genetic and physical map of the 8 Mb *Streptomyces coelicolor* A3(2) chromosome, *Mol. Microbiol*. *21* (1996) 77–96.
10. K. Bao, S.N. Cohen, Terminal proteins essential for the replication of linear plasmids and chromosomes in *Streptomyces*, *Genes Dev*. *15* (2001) 1518–1527.
11. A. Arnold, *Ph. D. Thesis*, University of Kaiserslautern, Germany (1994).
12. M. Redenbach, A. Arnold, U. Rauland, J.A. Cullum, Structural instability of the *Streptomyces lividans* 66 chromosome and related effects, *Actinomycetologica*, *8* (1994) 97–102.
13. N.D. Lomovskaya, N.M. Mkrtumian, N.L. Gostimskaya, V.N. Danilenko, Characterization of temperate actinophage ϕC31 isolated from *Streptomyces coelicolor* A3(2), *J. Virol*. *9* (1972) 258–262.

14. M.J. Butler, E.J. Friend, I.S. Hunter, F.S. Kaczmarek, D.A. Sugden, M. Warren, Molecular cloning of resistance gene and architecture of a linked gene cluster involved in the biosynthesis of tetracycline by *Streptomyces rimosus*, *Mol. Gen. Genet*. *215* (1989) 231–238.

15. J. Altenbuchner, J. Cullum, DNA amplification and an unstable arginine gene in *Streptomyces lividans* 66, *Mol. Gen. Genet*. *195* (1984) 134–138.

16. D. Hranueli J. Pigac, M. Vešligaj, Characterization and persistence of actinophage RP2 isolated from *Streptomyces rimosus* ATCC 10970, *J. Gen. Microbiol*. *114* (1979) 295–303.

17. M. Vešligaj, M. Filipović, J. Pigac, D. Hranueli, Isolation of *Streptomyces rimosus* mutants with reduced actinophage susceptibility, *Appl. Environ. Microbiol. 41* (1981) 986–991.

18. M. Redenbach, F. Flett, W. Piendl, I. Glocker, U. Rauland, O. Wafzig, R. Kliem, P. Leblond, J. Cullum, The *Streptomyces lividans* chromosome contains a 1 Mb deletogenic region flanked by two amplifiable regions, *Mol. Gen. Genet. 241* (1993) 255–262.

19. M. Bierman, R. Logan, K. O'Brien, E.T. Seno, R.N. Rao, B.E. Schoner, Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp., *Gene, 116* (1992) 43–49.

20. E. Katz, C.J. Thompson, D.A. Hopwood, Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*, *J. Gen. Microbiol. 129* (1983) 2703–2714.

21. T. Kieser, M.J. Bibb, M.J. Buttner, D.A. Hopwood: *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, UK (2000).

22. J. Sambrook, E.F. Fritsch, T. Maniatis: *Molecular Cloning: A Laboratory Manual* (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

23. A.J. Kutchma, M.A. Roberts, D.B. Knaebel, D.L. Crawford, Small-scale isolation of genomic DNA from *Streptomyces* mycelia or spores, *Biotechniques, 24* (1998) 452–456.

24. G.E. Smith, M.D. Summers, The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper, *Anal. Biochem. 109* (1980) 123–129.

25. A.P. Feinberg, B. Vogelstein, A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity, *Anal. Biochem. 132* (1983) 6–13.

26. G.A. Evans, K. Lewis, B.E. Rothenberg, High efficiency vectors for cosmid microcloning and genomic analysis, *Gene, 79* (1989) 9–20.

27. P. Leblond, M. Redenbach, M.J. Cullum, Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2), *J. Bacteriol. 175* (1993) 3422–3429.

28. J. Cullum, D. Denapaite, L.-I. Horvat, T. Maršić, A. Paravić, D. Hranueli, I. Hunter: Genetic Instability and the Biotechnology of *Streptomyces*. In: *Current Studies of Biotechnology, Vol. 1 – Biomedicine*, Z. Kniewald, I. Friganović, J. Kniewald, S. Novak, W. Schönfeld, V. Silobrčić, R. Spaventi, B. Šimić, B. Zamola (Eds.), Croatian Society of Biotechnology, Zagreb (2000) pp. 7–13.

29. T. Wenner, V. Roth, G. Fischer, C. Fourrier, B. Aigle, B. Decaris, P. Leblond, End-to-end fusion of linear deleted chromosomes initiates a cycle of genome instability in *Streptomyces ambofaciens*, *Mol. Microbiol. 50* (2003) 411–425.

30. T.J. Leach, R.L. Glaser, Quantitative hybridisation to genomic DNA fractionated by pulsed-field gel electrophoresis, *Nucleic Acids Res. 26* (1998) 4787–4789.

31. C.-H. Huang, Y.-S. Lin, Y.-L. Yang, S.-W. Huang, C.W. Chen, The telomeres of *Streptomyces* chromosomes contain conserved palindromic sequences with potential to form complex secondary structures, *Mol. Microbiol. 28* (1998) 905–926.

32. Z. Qin, S.N. Cohen, Replication at the telomeres of the *Streptomyces* linear plasmid pSLA2, *Mol. Microbiol. 28* (1998) 893–903.

33. M. Salas, Protein-priming of DNA replication, *Ann. Rev. Biochem. 60* (1991) 39–71.

34. S.T. Williams, M. Goodfellow, E.M. Wellington, J.C. Vickers, G. Alderson, P.H. Sneath, M.J. Sackin, A.M. Mortimer, A probability matrix for identification of some *Streptomycetes*, *J. Gen. Microbiol. 129* (1983) 1815–1830.

Stalna prisutnost krajeva kromosoma u malom broju kopija u mutanata sojeva bakterija *Streptomyces rimosus* i *Streptomyces lividans*

Sažetak

Vrste roda *Streptomyces*, proizvođači antibiotika, pripadaju bakterijama u kojih je vrlo učestala genska nestabilnost. Jedan ili oba kraja linearnoga kromosoma streptomiceta često se spontano izgube delecijom, pri čemu nastaju mutanti kojima katkada nedostaje stotine gena. Iscrpno su analizirani neki sojevi vrsta *S. rimosus* i *S. lividans* što su bili razvrstani među »delecijske mutante«. Ti su sojevi izgubili sekvencije DNA s krajeva kromosoma. Otkriveno je da se vrlo mali broj izgubljenih sekvencija DNA još uvijek nalazi u vegetativnom miceliju. Taj mali broj kopija sprječava da se njihova prisutnost jednostavno uoči. Broj je kopija sekvencija DNA s jednoga kraja kromosoma u bakterije *S. rimosus* procijenjen na $0{,}1–1{,}0 \cdot 10^{-3}$ po kromosomu. Spore streptomiceta sadržavaju sekvencije DNA s krajeva kromosoma u većem broju kopija od broja kopija istih sekvencija u vegetativnom miceliju. Prema tome, te se sekvencije DNA nasljeđuju procesom sporulacije. U vrsta roda *Streptomyces* ta biološka pojava upućuje na razlike između genoma spora (koje omogućavaju klijanje) i genoma vegetativnih stanica. Stalna prisutnost krajeva kromosoma u malom broju kopija u streptomiceta do sada nije opisana i može imati praktične posljedice kako za čuvanje bakterijskih sojeva, tako i za njihova genetička istraživanja.