Chromosomal Circularization in *Streptomyces griseus* by Nonhomologous Recombination of Deletion Ends

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*Streptomyces* linear chromosomes frequently cause deletions at both ends spontaneously or by various mutagenic treatments, and concomitantly display dynamic structural changes such as circularization and arm replacement. We have cloned and sequenced the fusion junctions of circularized chromosomes in two deletion mutants of *Streptomyces griseus*. No homology and a 1-bp overlap were found between the deletion ends of the mutant chromosomes. Taking this together with previous results, we concluded that chromosomal circularization in *Streptomyces* occurs by nonhomologous recombination between deletion ends.

Key words: *Streptomyces*; chromosomal circularization; chromosomal deletion; genome fluidity; nonhomologous recombination

Filamentous soil bacteria, *Streptomyces*, are unusual in carrying an 8–9 Mb linear chromosome:1–7) the genome project for *Streptomyces coelicolor* A3(2), a model strain for *Streptomyces* genetics, has been recently completed.8) *Streptomyces* species are also known to frequently have linear plasmids, which range from 12 to 1700 kb in size.9) These *Streptomyces* linear replicons have principally the same structural features; terminal inverted repeats (TIRs) are present at both ends and the 5’ ends are linked covalently to a terminal protein. *Streptomyces* linear chromosomes easily undergo terminal deletions spontaneously or by various mutagenic treatments.10,11) The sizes of deletions sometimes reach up to 2 Mb.12) The deleted chromosomes subsequently show several types of rearrangements; circularization, arm replacement, and amplification. The last phenomenon may be an intermediate state to reach a final stable state.

Chromosomal circularization in *Streptomyces* was first indicated by detection of a macrorestriction fusion fragment in deletion mutants of *Streptomyces lividans*1,13) and *Streptomyces ambofaciens*.4) It was finally confirmed in *Streptomyces griseus* by cloning and sequencing of the fusion junctions of the circularized chromosomes.14) No homology and a 6-bp microhomology were detected between the right and left deletion ends of the mutant chromosomes. This result suggested that chromosomal circularization occurred by nonhomologous recombination between both deletion ends. Qin and Cohen15) reported that nonhomologous recombination also generated circular plasmids from telomere-damaged linear plasmids in *S. lividans*. To reveal if nonhomologous recombination is general in circularization of *Streptomyces* linear replicons, we analyzed two additional circularized chromosomes of *S. griseus* mutants.

Materials and Methods

Bacterial strains, cosmids, plasmids, and media. *S. griseus* strain 2247, used for mutation, was described previously.2) The cosmid library was previously constructed for strain 22472) and ordered at both chromosomal end regions.16) *E. coli* XL1-Blue and pUC19 were used for cloning and sequencing of DNA fragments. Glucose-meat extract-peptone (GMP) medium contains 1.0% glucose, 0.4% peptone, 0.2% yeast extract, 0.2% NaCl, and 0.025% MgSO4 7H2O (pH 7.0). MB plates contain 1.0% mannitol, 0.1% yeast extract, 0.2% Polypepton (Wako Chemicals, Osaka), 0.1% meat extract, and 1.5% agar (pH 7.0).

Mutation of *S. griseus* 2247. A spore suspension of strain 2247 in distilled water was UV-irradiated at a distance of 60 cm under a germicidal lamp (15W) for 0.5–5 min with constant agitation. Portions were taken out every 30 sec, kept in the dark for 2 hr to prevent photoreactivation, and spread and grown on MB plates at 28°C for several days. The spore sus-
pension, which was irradiated for 2.5–3.5 min, gave a survival of 0.1–1% and used for isolation of deletion mutants.

**DNA isolation and pulsed-field gel electrophoresis.** *S. griseus* strains were reciprocally cultured at 28°C in a 500-ml Sakaguchi flask containing 100 ml of GMP medium and total DNAs were isolated as described by Suwa et al.\(^{17}\) Gel samples for PFGE were prepared by the mycelium method as described previously,\(^{2,18}\) digested in gels, and separated by contour-clamped homogeneous electric fields (CHEF).\(^{19}\) CHEF was done in 0.5 x TBE buffer using 1.0% agarose at 15°C. *E. coli* strains were cultured at 37°C in LB medium, and cosmids and plasmid DNAs were extracted as described by Sambrook *et al.*\(^{20}\)

**Southern hybridization.** DNA fragments were separated by CHEF or conventional agarose gel electrophoresis and transferred to nylon membrane filters by the capillary method. Hybridization was done using the DIG system (Roche Diagnostics GmbH, Mannheim, Germany) overnight at 70°C in standard buffer according to the supplier’s protocol. After hybridization, washing was done twice for 5 min each in 2 x wash solution at room temperature, and then twice for 15 min each in 0.1 x wash solution at 70°C.

**DNA sequencing.** Nucleotide sequencing was done by the dideoxy termination method using a dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and the ABI-373S sequencing system (PE Biosystems, Foster City, CA). Genetyx-Mac 10.2 (Software Development, Tokyo) and FramePlot 2.3.2\(^{21}\) were used for analysis of sequence data.

**Results**

**Chromosomal deletion in mutants, No. 9 and No. 83**

Surviving colonies of *S. griseus* after UV irradiation were picked up randomly. Their total DNAs were digested with *Bam*HI, separated by conventional agarose gel electrophoresis, and analyzed by Southern hybridization using pSGE1 as a probe, which contains the 2.7-kb end *Sal*I fragment of the 2247 chromosome.\(^{22}\) Among 60 colonies tested, two colonies, No. 9 and No. 83, showed no hybridizing signals, which indicated that both chromosomal ends were deleted in these mutants. Colonies of mutant No. 9 have a bald appearance on solid MB medium, while mutant No. 83 sporulates as the parent strain 2247 does. Both mutants grow normally in solid and liquid cultures.

To measure the sizes of deletions, the ordered terminal cosmids of the 2247 chromosome\(^{23}\) were hybridized to the *Bam*HI digest of total DNAs of the two mutants. As shown in Figs. 1A and 1B, mutant No. 9 showed fewer signals compared with strain 2247, when probed by the right end cosmids 6E12 and cosmids F1B6, which is more than 500 kb from the left end. These results showed that the right and left deletion endpoints in mutant No. 9 are located on the cosmids 6E12 and F1B6, and their deletion sizes are 30 and 550 kb, respectively (Fig. 2). By the same analysis shown in Figs. 1C and 1D, the right and left deletion endpoints in mutant No. 83 were on cosmids 6C8 and F2D2, and their deletion sizes were 130 and 170 kb, respectively (Fig. 2).

**Chromosomal circularization in mutants, No. 9 and No. 83**

To see widely the structural changes that occurred at the chromosomal ends of the two deletion mutants, Southern hybridization analysis was done using macrorestriction fragments of strains, 2247, No. 9, and No. 83 (Fig. 3). When the right end cosmid 6E12 was used as a probe, the *Spe*I digest of strain 2247 showed the 280-kb right end fragment as well as the 520-kb left end fragment, due to the presence of TIRs at both ends, while the *Spe*I digest of mutant No. 9 gave a new 450-kb fragment. When the left deletion end cosmids F1B6 was used a probe, strain 2247 gave a 350-kb fragment, while mutant No. 9 gave the same 450-kb fragment, as detected by cosmids 6E12. These results indicated that in mutant No. 9, the right and left deletion ends of the chromosome were combined to generate the 450-kb fusion *Spe*I fragment; namely, the linear chromosome was circularized.

![Fig. 1. Southern Hybridization Analysis of the Deletion Sizes in Mutants No. 9 and No. 83.](image-url)
Fig. 2. Restriction and Cosmid Maps at Both Chromosomal Ends of S. griseus 2247 and the Deleted Regions in Mutants No. 9 and No. 83. The deleted regions in mutants No. 9 and No. 83 as well as the previously analyzed mutants 404–23 and N2 are indicated by dashed lines. All of the four mutants were found to contain a circular chromosome. Af, AfII; As, AseI; Sp, SpeI; Ss, SspI; L, linear; C, circular.

Fig. 3. Southern Hybridization Analysis of Chromosomal Circularization in Mutants No. 9 and No. 83. Gel samples of strain 2247 and mutants No. 9 and No. 83 were digested with SpeI or AseI, separated by CHEF electrophoresis, and hybridized with the deletion endpoint cosmids; 6E12 (A), F1B6 (B), 6C8 (C), and F2D2 (D). CHEF was done at 150 V with 24-s pulses for 30–35 h.

Similarly, cosmids 6C8 and F2D2, which contain the right and left deletion endpoints of the No. 83 chromosome, hybridized to AseI fragment E (550 kb) and AseI fragment F1 (480 kb) of the 2247 chromosome, respectively, and hybridized to the same 800-kb fusion fragment of the No. 83 chromosome (Figs. 3C and 3D). Therefore, circularization occurred in this mutant, too.

**Restriction and hybridization analysis of fusion junctions**

To analyze the chromosomal circularization more precisely, we tried to clone the fusion junctions from the two mutants and corresponding right and left deletion endpoints from strain 2247. In the case of mutant No. 9, an 8.1-kb fusion fragment was clearly seen in its BamHI digest, when probed by the right end cosmid 6E12 (Fig. 1A). When probed by the left deletion end cosmid F1B6, the 8.1-kb fusion frag-
The fusion fragment was not detected (Fig. 1B), due to their short homology. Therefore, the No. 9 DNA was digested with another enzyme, *Sac*I, and probed by F1B6, which revealed a fusion fragment at 3.8 kb (Fig. 1B). The two fusion fragments were cloned and plasmid p2F9 was obtained from the former. When the *Bam*HI digest of the 2247 DNA was probed by p2F9, two positive signals were detected at 13.6 and 1.0 kb (Fig. 4A), which correspond to the right and left endpoints, respectively. These fragments were also subcloned from cosmids, 6E12 and F1B6, and put through restriction and hybridization analysis. Comparison of the three restriction maps showed that the fusion junction is on a shaded 0.5-kb *Afl*II-*Bam*HI fragment (Fig. 4B).

In the case of mutant No. 83, the fusion fragment was not easily identified among many hybridizing signals in Figs. 1C and 1D. However, precise restriction and hybridization analysis finally located the fusion junction on a 3.6-kb *Bam*HI fragment of the No. 83 DNA, the right deletion endpoint on a 2.7-kb *Bam*HI fragment of cosmid 6C8, and the left deletion endpoint on a 3.3-kb *Bam*HI fragment of cosmid F2D2. These fragments were cloned from the No. 83 DNA or subcloned from cosmids 6C8 and F2D2, respectively. As shown in Figs. 5A and 5B, the right (Probe A, 2.7 kb) and left (Probe B, 3.3 kb) deletion end fragments hybridized to the same 3.6-kb fusion fragment. Restriction maps were constructed for these three clones and compared, which located the fusion junction on a central 0.8-kb *Apa*I-*Pvu*I fragment (Fig. 5C).

**Nucleotide sequences of fusion junctions in mutants, No. 9 and No. 83**

The nucleotide sequences around the fusion junctions in mutants No. 9 and No. 83 and those around their corresponding regions in strain 2247 were determined. By sequence comparison, the fusion junction in mutant No. 9 was located at nt 291–292 in Fig. 6. No homology was found between the right and left deletion end sequences, and no amplification was detected around the junction. No initiation and stop codons were found in the determined sequences, but two ORFs were identified based on the unique codon usage of the high GC *Streptomyces* DNAs.\(^{23}\) Homology search for databases found that the ORF at the right deletion end codes a hypothetical protein and the ORF at the left deletion end may code glycosyl hydrolase (pfam02837). The two ORFs, which have opposite directions, were completely destroyed by deletion and circularization in mutant No. 9.

The nucleotide sequence around the fusion junction in mutant No. 83 is compared with those around the right and left deletion endpoints (Fig. 7). In this case, a G residue is present at both the right and left deletion ends, which form a fusion junction at nt 222. No amplification was found near the fusion junction. Two ORFs were identified at the right and left deletion ends, both of which are coded on the complementary strand. Homology search revealed that the ORF at the right deletion end codes a hypothetical protein, and the ORF at the left deletion end code phosphate acyltransferase (smart00563). Both ORFs were completely destroyed by deletion.
Fig. 5. Location of the Fusion Junction in Mutant No. 83 by Restriction and Hybridization Analysis.
(A, B) The BamHI digest of the 2247 DNA showed the same junction fragment (3.6 kb), when probed by the right (Probe A, 2.7 kb) and left (Probe B, 3.3 kb) deletion end probes. (C) The fusion junction in mutant No. 83 was located on a 0.8-kb Apal-PvuI fragment. All sites are shown for Apal (Ap), BamHI (B), BglII (Bg), PvuI (Pv), and SalI (Sa). For SacII (SII) and SmaI (Sm), only the sites flanking the determined sequences in Fig. 7 are shown.

Fig. 6. Nucleotide Sequences of the Fusion Junction (J) in Mutant No. 9 and Corresponding Right (R) and Left (L) Deletion Ends in Strain 2247.
The fusion junction is located at nt 291–292. Putative two ORFs, which have opposite directions, are shown in the one-letter code.
Fig. 7. Nucleotide Sequences of the Fusion Junction (J) in Mutant No. 83 and Corresponding Right (R) and Left (L) Deletion Ends in Strain 2247.

The fusion junction is composed of a G residue at nt 222. Putative two ORFs, both of which are coded on the complementary strand, and circularization, and a possible fused ORF in mutant No. 83 has a frame shift and therefore cannot encode a functional protein.

Discussion

In this study, we have elaborated chromosomal circularization in two *S. griseus* deletion mutants, No. 9 and No. 83, by restriction and hybridization analysis and finally by nucleotide sequencing. No homology was detected between the right and left deletion endpoints in mutant No. 9 and only a 1-bp overlap was found in mutant No. 83. In addition, no amplification was detected around the fusion junctions in both mutants. We previously reported chromosomal circularization in *S. griseus* mutants, 404–23 and N2.14) In these cases, no homology and a 6-bp microhomology were found between the right and left deletion ends. In any of the four cases, no hotspot was detected as a deletion endpoint. From these results, we concluded that chromosomal circularization occurs by nonhomologous recombination and the sequences at both deletion ends play no significant roles in circularization. It should be noted that all of the four circularized mutants grow normally in spite of their chromosomal arm deletions, up to 550 kb in mutant No. 9.

Chromosomal circularization was also indicated in *S. lividans*1,13) and *S. ambofaciens*4) by detection of a fused macrorestriction fragment. In addition, Qin and Cohen15) reported the circularization of artificial pSLA2 plasmids in *S. lividans* by nonhomologous recombination of deletion ends. Therefore, circularization may be a common strategy of *Streptomyces* linear replicons, when both of the two telomeres were deleted.

In addition to circularization, *Streptomyces* chromosomes display several structural changes to survive from the loss of the extreme ends that may be essential for terminal replication.24,25) Fischer et al.26) reported that homologous recombination of two sigma factor-like ORFs caused chromosomal arm replacement in *S. ambofaciens*, which generated unusually long TIRs in the mutants. We also found that in *S. griseus* mutant MM9, homologous recombination between two similar lipoprotein-like ORFs caused chromosomal arm replacement and generated 450-kb TIRs in place of the original 24-kb TIRs.27) If recombinational DNA repair occurs between the intact and deleted TIR regions on the same chromosome, intact TIRs could be reproduced. We proposed a hypothesis that this type of repair may occur frequently in *Streptomyces* and TIRs may guarantee homologous sequences for recombination at both chromosomal ends.27) Only when recombination occurred outside the TIRs can we recognize it as chromosomal arm replacement. Furthermore, recombination between the ends of a linear chromosome and a linear plasmid was observed, which caused chromosomal end exchange in *Streptomyces rimosus*28) and repair of a damaged telomere in *S. lividans*.15) Qin and Cohen29) reported another strategy of
Streptomyces, in which duplication of the right half of pSLA2 generated symmetric linear plasmids, when the left telomere was damaged. Nucleotide sequencing of the centers of symmetry revealed that small palindromic sequences were originally present in pSLA2. We previously isolated a similar symmetric plasmid pSLA2-L1 from S. rochei. All of these rearrangements may be strategies of Streptomyces to recover an extreme end when one of the two telomeres was deleted.

As described above, Streptomyces linear chromosomes and plasmids display dynamic structural changes to survive from lethal deletions; circularization, arm replacement, repair of intact TIRs, and formation of symmetric linear plasmids. The mechanisms underlined in these rearrangements are different; nonhomologous recombination for circularization, homologous recombination for arm replacement and repair of TIRs, and misreplication at a small palindrome sequence for formation of a symmetric linear plasmid. These dynamic structural changes of Streptomyces linear replicons are quite interesting in respect of the evolution of genome structures. Most of these deletion mutants grow normally. Therefore, terminal regions carry dispensable genes and are deletable except for the extreme ends, and could accept various foreign DNAs. This genome fluidity of Streptomyces may give it an extraordinary ability to produce many secondary metabolites.

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References


