Doctoral Thesis

Structural and evolutional analysis of *Streptomyces* linear replicons

（放線菌線状レプリコンの構造および進化に関する解析）

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2. 公表論文（Articles）

(1). The *tap-tpg* gene pair on the linear plasmid functions to maintain a linear topology of the chromosome in *Streptomyces rochei*.
Yosi Nindita, Zhisheng Cao, Yingjie Yang, Kenji Arakawa, Yuh Shiwa, Hirofumi Yoshikawa, Michihira Tagami, Alexander Lezhava and Haruyasu Kinashi.
Molecular Microbiology, **95**(5): 846-858 (2015).

(2). Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2).
Yosi Nindita, Tomoya Nishikawa, Kenji Arakawa, Guojun Wang, Kozi Ochi, Zhongjun Qin and Haruyasu Kinashi.
FEMS Microbiology Letters, **347**(2): 149–155 (2013).

3. 参考論文（Thesis Supplements）

pSLA2-M of *Streptomyces rochei* is a composite linear plasmid characterized by self-defense genes and homology with pSLA2-L.
Yingjie Yang, Toru Kurokawa, Yoshifumi Takahama, Yosi Nindita, Susumu Mochizuki, Kenji Arakawa, Satoru Endo and Haruyasu Kinashi.
Bioscience, Biotechnology, and Biochemistry, **75**(6): 1147-1153 (2011).

主　論　文
(Main Thesis)

Contents

Chapter 1
Introduction

The saprophytic filamentous soil bacteria *Streptomyces* is Gram-positive bacteria with a high G+C content. This genus of bacteria is well characterized by three distinct properties; complex morphological differentiation, the ability to produce secondary metabolites, and the possession of a linear chromosome. The life cycle of *Streptomyces*, as illustrated in Figure 1, starts as a spore. On solid media, the spore develops as vegetative mycelium. After several days, vegetative mycelium produces branches extending into the air, known as aerial mycelium. The aerial hyphae undergo septation creating compartments that contain single nucleoid. Finally, these compartments differentiate to create spore chain.

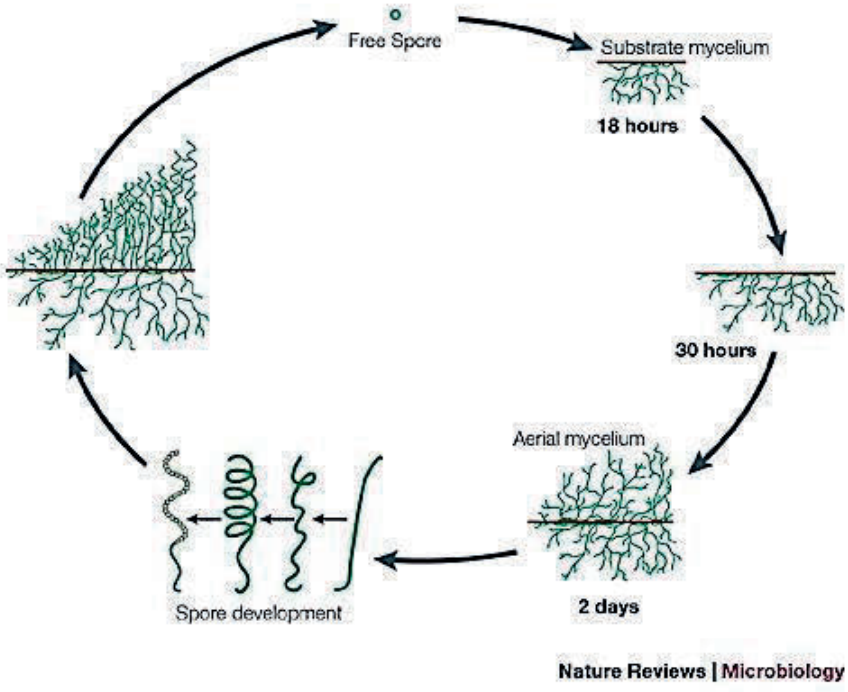

Figure 1. Life cycle of *Streptomyces coelicolor*

Streptomyces species are known for their ability to produce a wide variety of

medically and agriculturally important secondary metabolites. The antibiotic biosynthetic genes in *Streptomyces* form a gene cluster and are usually located on the chromosome. However, it is known that in several cases, a large linear plasmid is involved in antibiotic production. For examples, SCP1 in *S. coelicolor* A3(2) for methylenomycin production (Kirby *et al.*, 1975; Kirby and Hopwood, 1977; Kinashi *et al.*, 1987; Kinashi and Shimaji-Murayama, 1991), pPZG103 in *S. rimosus* MV17 for oxytetracycline (Gravius *et al.*, 1994), and pSLA2-L in *S. rochei* 7434AN4 for lankamycin and lankacidin (Kinashi *et al.*, 1994; Kinashi *et al.*, 1998; Suwa *et al.*, 2000).

Pulsed-field gel electrophoresis (PFGE) analysis revealed that *Streptomyces* carries a linear chromosome with a size of around 8-9 Mb (Leblond *et al.*, 1991; Kieser *et al.*, 1992; Leblond *et al.*, 1993; Lin *et al.*, 1993; Lezhava *et al.*, 1995). The linearity of *Streptomyces* chromosome was first proved for *S. lividans* (Lin *et al.*, 1993), and the complete linear genome sequence of the model species *S. coelicolor* A3(2) was determined in 2002 (Bentley *et al.*). Apart from the chromosome, *Streptomyces* also carries a linear plasmid(s) (Kinashi and Shimaji, 1987; Kinashi *et al.*, 1987; Sakaguchi, 1990; Hinnebusch and Tilly, 1993; Netolitzky *et al.*, 1995). *Streptomyces* linear chromosomes and plasmids have the common structural components; terminal inverted repeat (TIRs) present at both ends, a terminal protein linked covalently to the 5'-end (Volff and Altenbuchner, 1998; Bao and Cohen, 2001), and an origin of replication (*oriC*) located in the center of the replicons (Calcutt and Schmidt, 1992; Zakrzewska-Czerwinska and Schrempf, 1992), where the bidirectional replication is initiated and proceeds to the telomeres (Musialowski *et al.*, 1994). The telomere sequence of several *Streptomyces* chromosomes and linear plasmids have been isolated and sequenced (Huang *et al.*, 1998). Most of these terminal region share conserved palindrome DNA

sequences with various ranges of size.

The linear chromosomes of *Streptomyces* are very unstable and undergo terminal deletions spontaneously (Leblond and Decaris, 1994; Volff and Altenbuchner, 1998). The sizes of deletions are various in each case, up to 2.3 Mb in *S. ambofaciens* (Fischer *et al.*, 1997). Terminal deletions are generally followed by DNA rearrangements such as amplification, arm replacement, and circularization (as occurred in *Streptomyces griseus,* illustrated in Figure 2).

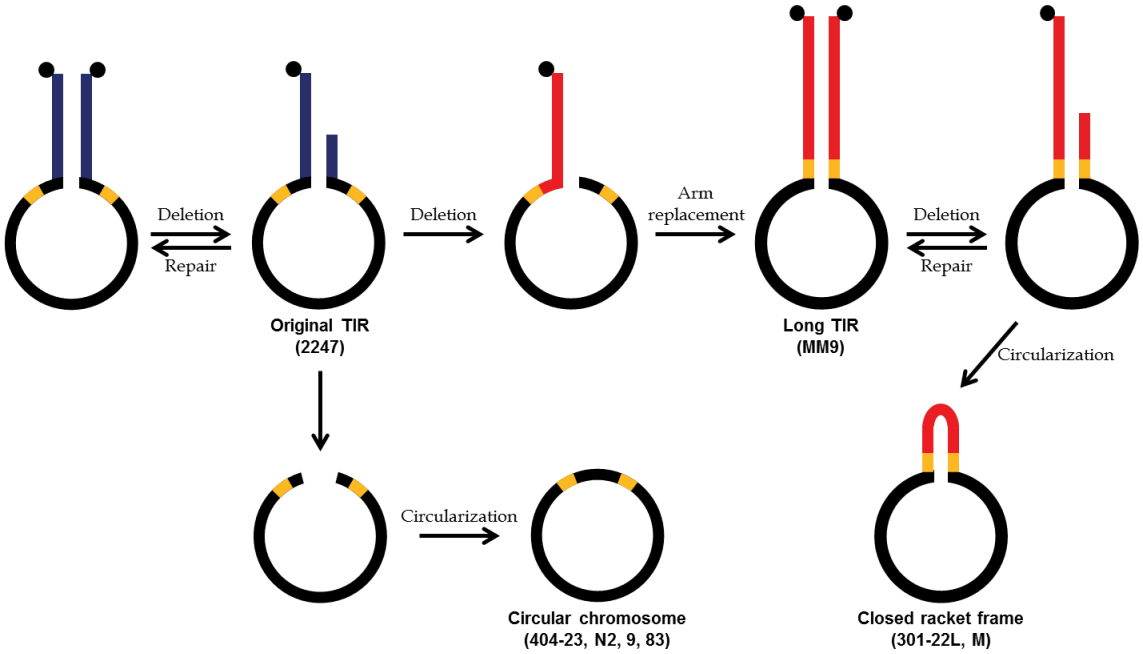

Figure 2. Chromosomal rearrangement in *Streptomyces griseus*

Usually, the telomeres and parts of the flanking chromosomal regions are deleted and the deletion ends are joined to form a circular chromosome (Kameoka *et al.*, 1999; Inoue *et al.*, 2003; Kinashi, 2008). The wild type chromosome can also be circularized artificially by targeted recombination (Lin *et al.*, 1993; Volff *et al.*, 1997). Chromosomal circularization was indicated by detection of a macrorestriction fragment in the deletion mutants of *S. lividans* (Lin *et al.*, 1993; Redenbach *et al.*, 1993) and *S. ambofaciens* (Leblond *et al.*, 1996). It was finally confirmed in *S. griseus* by cloning

and sequencing of the fusion junctions of the circularized chromosomes (Kameoka *et al.*, 1999; Inoue *et al.*, 2003). No sequence homology was found between the left and right deletion ends in two mutants, and only 1-bp and 6-bp homology was found in other mutants. Accordingly, it was proposed that nonhomologous recombination between the left and right deletion ends caused chromosomal circularization (Inoue *et al.*, 2003). Microhomology was also detected at the fusion points of the circularized chromosomes of *S. avermitilis* mutants (Chen *et al.*, 2010).

In this thesis, I analyzed additional cases of chromosomal deletion in *Streptomyces* species. Chapter 2 in this thesis contains the basic methods and materials used in the experiment. Chapter 3 contains the study on the chromosomal deletion in *Streptomyces rochei* 7434AN4 concomitant to the loss of its linear plasmids. Chapter 4 consists of the analysis of the telomere deletion in *Streptomyces coelicolor* strain No. 4. Chapter 5 is the general conclusion of two main topics in this thesis.

In the first topic (Chapter 3), I examined the functional genes essential to maintain the linearity of the chromosome in *S. rochei* 7434AN4. This strain carries three linear plasmids, pSLA2-L, pSLA2-M, and pSLA2-S. All of the plasmidless mutants obtained from this strain suffered from terminal deletions, followed by chromosomal circularization. This fact suggests that these linear plasmids were essential to keep the chromosomal telomere in this strain. Further analyses revealed that the chromosome of *S. rochei* 7434AN4 does not carry an intact *tap-tpg* gene pair, which is important for end-patching in linear replicons. In this study, I showed by complementation and curing experiments that the *tap-tpg* gene pair of pSLA2-M function in maintaining the chromosomal telomere of *S. rochei* 7434AN4.

In the second topic of this thesis (Chapter 4), I examined chromosomal circularization in *S. coelicolor* A3(2). Strain No. 4 used in this study is an *eshA* mutant

of wild-type *S. coelicolor* A3(2) strain 1147. The loss of *eshA*, which is located at 131 kb from the right end of the chromosome, indicated that strain No. 4 underwent deletion beyond this locus. Southern hybridization and sequence analysis detected the telomere deletion in both left and right ends of the chromosome, with the sizes of 237 kb and 851 kb, respectively. Further analyses also revealed the fusion junction of the circularized chromosome in this mutant.

Chapter 2
Basic materials and methods

2.1. Bacterial cultures

Escherichia coli XL1-Blue was grown in Luria-Bertani (LB) media (Table 1) at 37°C. *Streptomyces* strains were grown at 28°C in YM media (Table 2). For preparation of protoplasts, *Streptomyces* strains were grown in YEME media (Table 3). Protoplasts were regenerated in R1M media (Table 4) and overlaid with SNA media (Table 6) containing antibiotic for selection of *Streptomyces* transformants.

Table 1. Luria Bertani media

NaCl	1%
Yeast extract	0.5%
Polypeptone	1%
Agar (when necessary)	2%
	Adjust pH to 7.0

Table 2. YM media

Malt extract	1%
Yeast extract	0.4%
Glucose	0.4%
Agar (when necessary)	2%
	Adjust pH to 7.3

Table 3. YEME media

Malt extract	0.3%
Yeast extract	0.3%
Polypeptone	0.5%
Glucose	1%
Sucrose	34%
	Adjust pH to 7.2
After autoclaving, add:	
$MgCl_2 \cdot H_2O$	5 mM
Glycine	0.5%

When necessary, appropriate antibiotics were added in the following concentration: ampicillin (50 μg/ml), kanamycin (50 μg/ml), and thiostrepton (50 μg/ml in solid media and 2 μg/ml in liquid media).

Table 4. R1M media

K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	4.07 g
Casamino acids	0.1 g
L-asparagine	2 g
Yeast extract	8 g
Polypeptone	5 g
Glucose	10 g
Sucrose	103 g
Trace element solution	2 ml
	Fill up to 800 ml
Agar	20 g
After autoclaving, add:	
5.73% TES buffer (pH 7.2)	100 ml
7.37% $CaCl_2 \cdot 2H_2O$	100 ml
0.5% KH_2PO_4	10 ml

Table 5. Trace element solution

$ZnCl_2$	0.004%
$FeCl_3 \cdot 6H_2O$	0.02%
$CuCl_2 \cdot 2H_2O$	0.001%
$MnCl_2 \cdot 4H_2O$	0.001%
$Na_2B_4O_7 \cdot 10H_2O$	0.001%
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.001%

Table 6. SNA media

Nutrient broth	0.8%
Agar	0.7%

2.2. Basic DNA manipulation in *E. coli*

2.2.1. Preparation of *E. coli* competent cells

Single colony of *E. coli* XL1-Blue was incubated overnight in 5 ml of LB media at 37°C. One milliliter of the pre-culture was inoculated in 100 ml LB media, and the

culture was incubated at 18°C. When the OD_{600} reached between 0.4-0.6, culture vessel was transferred to an ice-water bath for 10 minutes. Cells were harvested by centrifugation and washed with ice-cold Inoue Transformation buffer (TB, Table 7). The pellet was resuspended in 17 ml of TB by gently swirling and 1.2 ml of DMSO was added to the suspension. The suspension was dispensed into 100 µl aliquots and stored in −80°C.

Table 7. Inoue transformation buffer

$MnCl_2 \cdot 4H_2O$	55 mM
$CaCl_2 \cdot 2H_2O$	15 mM
KCl	250 mM
PIPES (0.5 M, pH 6.7)	10 mM

2.2.2. *E. coli* transformation

An appropriate amount of DNA (up to 10 µl) was added to 100 µl of competent cells and was mixed by swirling gently. The mixture tube was stored on ice-water bath for 30 minutes, and was then transferred in 42°C water bath for 90 seconds with gentle swirling. The tube was rapidly placed on ice-water bath for 1-2 minutes. 900 µl of fresh LB media was added to the tube. The mixture was incubated at 37°C for 1 hour. Appropriate volume of transformed competent cells was spread onto LB solid media containing suitable antibiotics. The plates were incubated in 37°C for 12-16 hours.

2.2.3. Isolation of plasmid DNA from *E. coli*

Plasmid DNA isolation was performed using alkaline-lysis method. The cell pellet from 1.5 ml of overnight culture was resuspended with 100 µl of solution I (Table 8). The suspension was treated with 200 µl of solution II (Table 9) and the resultant was neutralized with 150 µl of solution III (Table 10). The mixture was mixed with 450 µl of phenol:chloroform=1:1 (v/v) and centrifuged. The aqueous upper layer was

transferred to a new tube. Nucleic acids were precipitated from the supernatant by adding 2.5 volumes of ice-cold 99% ethanol, mixed by gentle inversion. The mixture was stored in −80°C for 30 minutes, and centrifuged. The supernatant was removed, and the resulting pellet was washed with 300 µl of ice-cold 70% ethanol. The dried pellet was dissolved in 30 µl of TE buffer (Table 11) and stored in −20°C.

Table 8. Solution I

Glucose	50 mM
Tris-base	25 mM
EDTA	10 mM
	Adjust pH to 8.0

Table 9. Solution II

NaOH	0.2 M
SDS	1%

Table 10. Solution III

Potassium acetate	29.4 g
	Adjust pH to 5.2
	Fill up to 100 ml

Table 11. TE buffer

Tris-Cl (pH 8.0)	100 mM
EDTA (pH 8.0)	10 mM

2.3. Basic manipulation in *Streptomyces*

2.3.1. Preparation of *Streptomyces* protoplasts

Streptomyces strains were grown in YEME media at 28°C for 24 hours. The cells were harvested and washed with 10.3% sucrose solution. The cell was treated with lysozyme (1 mg/ml in P buffer, Table 12) at 37°C for 1 hour. The protoplasts

suspension was passed through a cotton filter to exclude the remaining mycelia, and the filtrate was centrifuged. P buffer was added to resuspend the precipitate, and the suspension was dispensed into 50 µl aliquots in chilled, sterile microtubes. The protoplasts aliquots were stored at −80°C.

Table 12. P buffer

K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	2.02 g
Sucrose	103 g
Trace element solution	2 ml
	Fill up to 800 ml
After autoclaving, add:	
5.73% TES buffer (pH 7.2)	100 ml
3.68% $CaCl_2 \cdot 2H_2O$	100 ml
0.5% KH_2PO_4	10 ml

2.3.2. Transformation of *Streptomyces*

An appropriate amount of DNA (up to 5 µl) was added to 50 µl of protoplast solution. T buffer (Table 13) containing 25% PEG-1000 was added to the mixture. The mixture was immediately spread onto R1M solid media. After 24-32 hours of incubation at 28°C, the plates were overlaid with SNA media containing thiostrepton, and further incubation until the colonies grew out.

Table 13. T buffer

Trace element solution	0.2 ml
2.5% K_2SO_4	1 ml
10.3% Sucrose	25 ml
Distilled water	75 ml
To 9.3 ml solution, add:	
5 M $CaCl_2$	0.2 ml
1 M Tris-Maleic acid buffer (pH 8.0)	0.5 ml

2.3.3. Isolation of *Streptomyces* total DNA

Streptomyces total DNA was prepared by a neutral method as described by Suwa *et*

al., (2000) with a slight modification. The mycelium from 5 ml culture broth was harvested and washed with 10.3% sucrose solution. The mycelia were resuspended in 3 ml lysozyme solution (1 mg/ml in Tris-sucrose-EDTA buffer, Table 14), to which 0.25 ml of 0.5 M EDTA (pH 8.0) was added and were incubated at 37°C for one hour. Actinase E (5 mg/ml in Tris-sodium-EDTA buffer, Table 15) was added to the solution and incubated at 37°C for another one hour. Then the mixture was treated with 0.25 ml of 10% SDS was then shaken for an additional 30 minutes at 37°C. The mixture was then mixed with 0.5 ml of 5 M NaCl, incubated at 37°C for another 30 minutes and left on 4°C overnight. The mixture was centrifuged after vortex vigorously, and the supernatant fluid was precipitated with equal volume of 2-propanol. To purify the DNA, the precipitate was dissolved in TE buffer and extracted with phenol:chloroform 1:1 (v/v). The aqueous layer was precipitated with 3 times volume of 99% chilled ethanol, dissolved in TE buffer, and stored in −20°C.

Table 14. Tris-sucrose-EDTA buffer

Sucrose	0.3 M
Tris-base	25 mM
EDTA	30 mM
	Adjust pH to 7.0

Table 15. Tris-sodium-EDTA buffer

NaCl	50 mM
Tris-base	30 mM
EDTA	5 mM
	Adjust pH to 8.0

2.3.4. Pulsed-field gel electrophoresis (PFGE)

DNA samples for pulsed-field gel electrophoresis (PFGE) were obtained according to the method as described previously (Kinashi *et al.*, 1994; Lezhava *et al.*, 1995) with a slight modification. *Streptomyces* strains were grown in YM medium for 24 hours and

washed twice with 10.3% sucrose by centrifugation. The mycelium was homogenized with Tris-sucrose-EDTA buffer. The cell suspension was mixed with equal volume of 2% low-melting-point agarose (type VII, Sigma), and then was poured into the cast. The agarose plug (14 ml) was treated with 25 ml lysozyme solution (2 mg/ml in Tris-sucrose-EDTA buffer) at 37°C for 5 hours. The lysozyme solution was replaced with 25 ml of Actinase E (5 mg/ml in Tris-sodium-EDTA buffer) and 13 ml of 10% SDS. After overnight incubation at 37°C, the sample was washed with Tris-sucrose-EDTA buffer, and was stored in 0.5 M EDTA in 4°C.

For in-gel digestion, agarose samples were washed twice with TE buffer for 30 min. Actinase E was inactivated by shaking the gels in 100 μM phenylmethylsulfonyl fluoride for one hour. The gels were then washed on TE buffer for 30 min and twice in the reaction buffer for 30 min each. Digestion was carried out in reaction buffer containing 100 μg of bovine serum albumin (BSA) per ml at 37°C overnight. After digestion, the gels were stored at 4°C in 0.5 M EDTA (pH 8.0).

Contour-clamped homogeneous electric field gel electrophoresis, CHEF mapper® XA (Bio-Rad) was used for PFGE analysis. This analysis was carried out in 0.5XTBE (Table 16) at 14°C using 1% agarose with a suitable condition for each run.

Table 16. 0.5×TBE buffer

Tris	44.5 mM
Boric acid	44.5 mM
EDTA	1 mM

2.4. Southern hybridization

2.4.1. Preparation of probe

Southern hybridization was carried out using DIG labeling kit (Roche) according to the manufacture's protocol. A 15 μl of DNA solution was boiled for 10 minutes and

placed on ice immediately. To the denatured DNA, 2 μl of hexanucleotide mixture solution and 2 μl of dNTP labeling mixtures solution were added. After adding 1 μl of Klenow enzyme, the mixture was incubated at 37°C for more than 4 hours. The labeling was stopped by the addition of 2 μl of 0.5 M EDTA, 2.5 μl of 4 M LiCl, and 75 μl of 99% ethanol. The mixture was placed at -80°C for 30 minutes. After centrifugation, the pellet was washed with 70% ethanol, and dissolved with 30 μl of distilled water.

2.4.2. Hybridization

After the gel electrophoresis image stained by Ethidium Bromide (EtBr) was taken, the agarose gel was rinsed with 0.25 M HCl for 10 minutes with gentle agitation, and then continued to soak in alkaline transfer buffer (Table 17) for 15 minutes twice and neutralization buffer (Table 18) for 20 minutes. The DNA was transferred onto the nitrocellulose membrane by upward capillary transfer method for 8-24 hours. After washing with 2XSSC briefly, the membrane was irradiated by UV light to fix the single-stranded DNA. The membrane was then placed in Hybri-bag Hard (Cosmo Bio, Tokyo, Japan) filled with hybridization buffer (Table 20) and was incubated at 70°C. After one hour, the denatured probe was added to the Hybri-bag, and the membrane was further incubated overnight at 70°C. The incubated membrane was rinsed with 2XSSC-0.1% (w/v) SDS for 5 minutes at room temperature twice and with 0.1XSSC-0.1% (w/v) SDS at 70°C for 15 minutes twice.

Table 17. Alkaline transfer buffer

NaOH	20 g
NaCl	87.65 g
Fill up to	1000 ml

Table 18. Neutralization buffer

Tris base	121.1 g
NaCl	87.65 g
Fill up to	1000 ml
	Adjust pH to 8.0

Table 19. 20xSSC

NaCl	175.32 g
Sodium citrate bi-hydrate	88.23 g
Fill up to	1000 ml

Table 20. Hybridization buffer

20xSSC	250 ml
Skim milk	5 g
10% SDS	1 ml
10% N-lauroylsarcosine	10 ml
Fill up to	1000 ml

2.4.3. Detection

The membrane was rinsed in buffer I (Table 21) and soaked in buffer II (0.5% (w/v) skim milk in buffer I) for 30 minutes at room temperature with gentle agitation. After washing with buffer I, the membrane was incubated in buffer I containing anti-digoxygenin-AP Fab fragment (Roche) (2 μl in 10 ml) for 1 hour. The membrane was washed with buffer I for 15 minutes twice, and then soaked in buffer III (Table 22). The DIG-labeled DNA was detected using color development solution (45 μl NBT solution (Table 23) and 35 μl X-phosphate solution (Table 24) in 10 ml buffer III).

Table 21. Buffer I

Tris base	121.1 g
NaCl	87.5 g
Fill up to	1000 ml
	Adjust pH to 7.5

Table 22. Buffer III

Tris base	121.1 g
NaCl	87.65 g
$MgCl_2 \bullet 6H_2O$	10.165 g
Fill up to	1000 ml
	Adjust pH to 9.5

Table 23. NBT solution

Nitrobluetetrazolium salt	75 mg
70% (v/v) dimethylformamide	1 ml

Table 24. X-phosphate solution

5-Bromo-4-chloro-3-indolylphosphate toluinidium salt	50 mg
100% dimethylformamide	1 ml

Chapter 3

The *tap-tpg* gene pair on the linear plasmid functions to maintain a linear topology of the chromosome in *Streptomyces rochei*

3.1. Introduction

Streptomyces rochei 7434AN4 used in this study is a producer of two unrelated polyketide lankamycin (LM) and lankacidin (LC) [Figure 3]. This strain carries three linear plasmids, pSLA2-L (210,614 bp), pSLA2-M (113,464 bp), and pSLA2-S (17,526 bp) (Hayakawa *et al.*, 1979; Kinashi *et al.*, 1994). Several derivative mutants with different plasmid profiles from this strain have been obtained by Kinashi *et al.* (1994), several of the pulsed-field gel electrophoresis of which are as shown in Figure 4. Their complete nucleotide sequences having been determined. Two thirds of the largest linear plasmid pSLA2-L is occupied by secondary metabolism related genes, including the biosynthetic gene clusters for macrolide antibiotics, lankacidin and lankamycin, a cryptic type-II polyketide, and a carotenoid (Mochizuki *et al.*, 2003). pSLA2-M

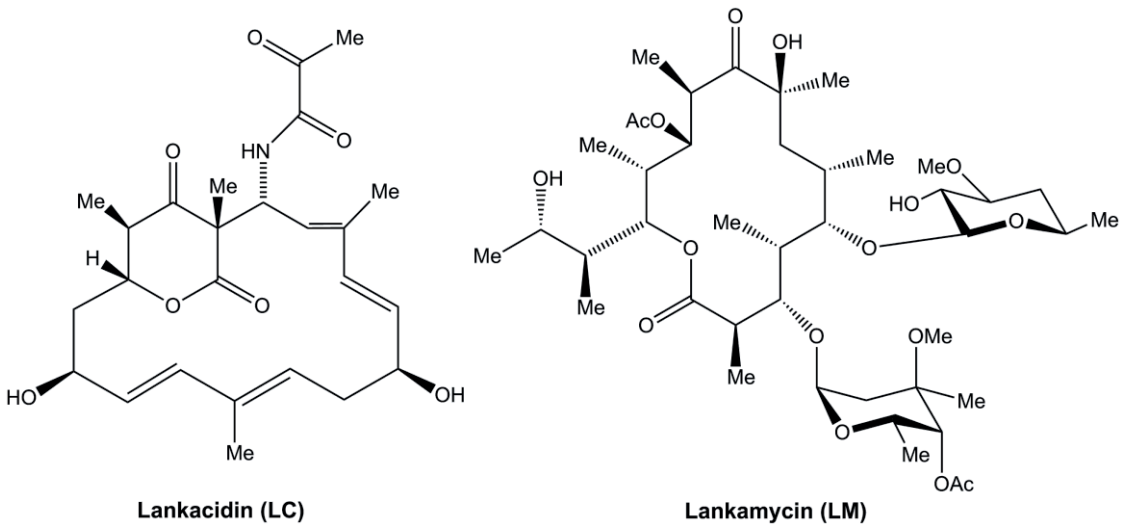

Figure 3. Chemical structures of lankacidin (left) and lankamycin (right) produced by *S. rochei* 7434AN4

contains self-defense genes such as a CRISPR cluster and a *ku70/ku80*-like gene (Yang *et al.*, 2011), which may be involved in immunity against phage infection and repair of double-strand DNA breaks, respectively. Both of pSLA2-L and pSLA2-M contain a *tap-tpg* gene pair, encoding a telomere-associated protein (Tap) and a terminal protein (TP) for end patching. Bidirectional replication of *Streptomyces* linear plasmids and chromosomes from a central origin generates 3'-leading-strand overhangs at the telomeres, which require end patching to produce full-length duplex DNA molecules (Qin and Cohen, 1998). In the latter process, Tap recruits TP to the 3'-end overhang (Bao and Cohen, 2003) and TP functions as a primer for DNA synthesis (Qin and Cohen, 1998; Yang *et al.*, 2006). In contrast to pSLA2-L and pSLA2-M, the smallest linear plasmid pSLA2-S does not contain a *tap-tpg* gene pair or biosynthetic genes (DDBJ AB905438).

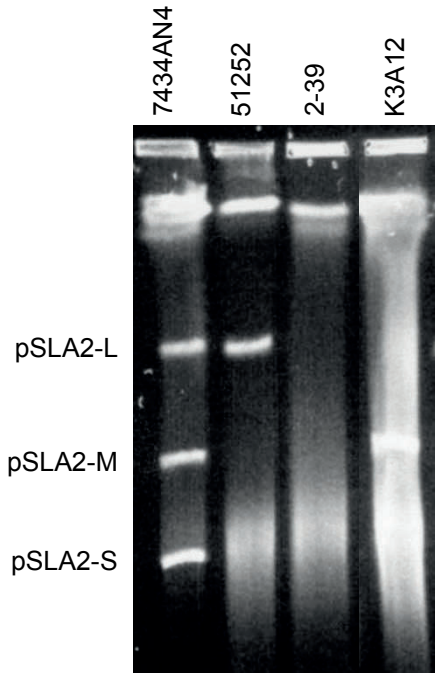

Figure 4. PFGE analysis of *S. rochei* 7434AN4 and its derivative mutants with different plasmid profile.

The genome project of the wild-type strain 7434AN4 has been started in the collaboration with RIKEN and then Tokyo University of Agriculture, and the National Institute of Infectious Diseases. In addition, a plasmidless mutant 2-39 was selected to clone the telomere fragments of the *S. rochei* chromosome, because it carries none of the three linear plasmids (Kinashi *et al.*, 1994), which was expected to avoid confusion in sequence analysis. However, this mutant did not show a hybridizing signal of telomere, suggesting that terminal deletions and circularization of the chromosome have occurred concomitant with a plasmid loss. This idea was confirmed by the finding of a fusion sequence in the 2-39 genome libraries. In addition, the data hitherto obtained from the genome project of strain 7434AN4 indicates that the chromosome itself does not contain *tap-tpg* gene pair. This result led to the idea that the *tap-tpg* of pSLA2-L or pSLA2-M functions to maintain a linear topology of the chromosome in strain 7434AN4. This hypothesis was finally confirmed by complementation and curing experiments of the *tap-tpg* of pSLA2-M in a plasmidless strain of *S. rochei*. The details of these experimental processes are described in my thesis.

3.2. Materials and methods

3.2.1. Bacterial strains, plasmids, and media

Streptomyces strains used in this study were derived from the wild-type strain *S. rochei* 7434AN4 and are listed in Table 25. Plasmid pUC19 was used for cloning in *Escherichia coli* XL-1 Blue and nucleotide sequencing, while the *E. coli-Streptomyces* shuttle vector pRES18 (Ishikawa *et al.*, 1996) was used for DNA manipulation in *Streptomyces*.

Table 25. Bacterial strains and plasmids

Names	Description	Source/ref.
Strains		
S. rochei 7434AN4	Wild-type strain (pSLA2-L, -M, -S)	(Kinashi *et al.*, 1994)
S. rochei 51252	Ultraviolet irradiation of 7434AN4 (pSLA2-L)	(Kinashi *et al.*, 1994)
S. rochei 1-2	Ultraviolet irradiation of 7434AN4 (pSLA2-L, -M)	(Kinashi *et al.*, 1994)
S. rochei K3A12	High temperature culture of 1-2 (pSLA2-M)	(Kinashi *et al.*, 1994)
S. rochei 2-39	Protoplast regeneration of 51252 (no plasmid)	(Kinashi *et al.*, 1994)
S. rochei YN-P7	Protoplast regeneration of 51252 (no plasmid)	This study
S. rochei YN-145	Protoplast regeneration of 51252 (no plasmid)	This study
S. rochei YN-T3	Introduction of pYN15 into 51252 (pSLA2-L, pYN15)	This study
S. rochei YN-C119	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C149	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C220	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C227	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C149-1	Same genotype with YN-C149 (pYN15)	This study
S. rochei YN-C149-2	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-3	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-4	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-5	Same genotype with YN-C149 (pYN15)	This study
S. rochei YN-C149-6	Same genotype with YN-C149 (pYN15)	This study
Plasmids		
pEcoEnd	0.4-kb *Eco*RI end fragment of pSLA2-L in pUC19	(Hiratsu *et al.*, 2000)
pCZ106	4.6-kb *Bam*HI-*Eco*RI end fragment of chromosome in pUC19	This work
pCZ117	2.3-kb fusion fragment of 2-39 in pUC19	This work
pYN15	*tapRM-tpgRM* of pSLA2-M in pRES18	This work
pYN16	*tpgR1* of pSLA2-L in pUC19	This work

3.2.2. PCR, conventional nucleotide sequencing, and analysis of DNA folding

PCR amplification was performed on a 2720 Thermal Cycler (Applied Biosystems) with KOD-plus DNA polymerase (Toyobo). Primer pairs listed in Table 26 were used for PCR amplification.

Table 26.PCR primers

tpgRM-f5	TTGAATTCGCGTTCAGGTTCTGGGTTATAG
tpgRM-r5	TTAGATCTACGTACTCGGAGTTGGTGTTG
2-39-229-f	TTGAATTCAAGTCCAGGCAGTAGAACTGGA
2-39-229-r	TTCTGCAGTTTATCACCGCTTCGGAGTAAG
tpgR1-F4	ATGGATCCAGAGCAGCAATTCGAGGAGCAC
tpgR1-R4	ATGAATTCTGGTCCAGATGGTGGAGGTCAC

Table 27.Accession numbers of the nucleotide sequences

Accession number	Description	Source/ref.
AB088224	pSLA2-L	(Mochizuki *et al.*, 2003)
AB597522	pSLA2-M	(Yang *et al.*, 2011)
AB905437	pSLA2-S	This study
AB905439	Contig 229 of mutant 2-39, containing the fusion junction of the circularized chromosome	This study
AB905441	Telomere of the 7434AN4 chromosome	This study
AB905442	Contig 586 of strain 7434AN4, containing a nucleotide-binding protein gene at the fusion junction of the 2-39 chromosome	This study
AB905443	Contig 634 of strain 7434AN4, containing a *ftsK* gene at the fusion junction of the 2-39 chromosome	This study
AB907705	Contig 95 of strain 7434AN4, containing a truncated *tpg* homolog of chromosome	This study

Nucleotide sequencing was performed by the dideoxy termination method, using BigDye Terminator v3.1/v1.1 Cycle Sequencing Kits (Life Technologies) and a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA). Genetyx-Mac 17.0.2 (Software Development, Tokyo) and FramePlot 4.0beta (Ishikawa and Hotta, 1999) were used for analysis of sequence data. All of the nucleotide sequences determined in this study have been submitted to the DDBJ database, whose accession numbers are listed in Table 27.

Folding of 3'-leading strand overhangs at the telomeres was analyzed using the mfold Web Server (http://bi.biopapyrus.net/app/mfold.html).

3.2.3. Whole shotgun genome sequencing of *S. rochei* strains 7434AN4 and 2-39

The sequencing library of strain 7434AN4 was prepared from 5 μg of total DNA with a GS General Library Prep Kit according to the manufacturer's protocol. Two runs of sequencing were performed using the Genome Sequencer FLX (Roche, Branford, CT) with the GS Sequencing Kit and the GS PicoTiterPlate Kit (70×75). Imaging and signal processing were done using GS FLZ SW v2.3, gsRunProcessor fullProcessing, De novo assembly was performed using Newbler v2.3 (Roche).

The sequencing library of strain 2-39 was prepared from 5 μg of total DNA with a media insert size of 500 bp for a multiplexed paired-end read format according to the Illumina protocols. The final product was validated using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The barcoded library was sequenced as multiplexed paired-end 100 bp reads on a genome analyzer II system (Illumina, San Diego, CA). The quality of sequenced library was assessed by using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and the reads were trimmed to 80 bp. After trimming reads, *de novo* assembly was performed using Velvet (Zerbino and Birney, 2008) with parameters optimized by the VelvetOptimizer (http://www.bioinformatics.net.au/ software.velvetoptimiser.shtml).

3.2.4. Construction of plasmids pCZ106, pCZ117, and pYN16

The 4.6-kb *Bam*HI-*Eco*RI fragment of the chromosome next to the 0.4-kb *Eco*RI telomere fragment was shotgun cloned from 7434AN4 DNA into pUC19 to give plasmid pCZ106. The 2.3-kb fusion fragment of the chromosome was amplified from

the 2-39 DNA using primers 2-39-229-f and 2-39-229-r, digested with *Eco*RI and *Pst*I, and cloned into pUC19 to give plasmid pCZ117. The 0.9-kb fragment covering the *tpgR1* gene on pSLA2-L was amplified from 7434AN4 DNA using primers tpgR1-F4 and tpgR1-R4, digested with *Bam*HI and *Eco*RI, and cloned into pUC19 to give plasmid pYN16.

3.2.5. Complementation and curing experiments

The *tapRM-tpgRM* gene pair of pSLA2-M was amplified by PCR using total DNA of strain K3A12, which carries only pSLA2-M, and primers tpgRM-f5 and tpgRM-r5. The resulting 4.5-kb amplified fragment was digested with *Eco*RI and *Bam*HI and cloned into pRES18 to give the complementation plasmid pYN15. This plasmid was introduced into strain 51252, which carries only pSLA2-L, by PEG-assisted transformation to give strain YN-T3. Protoplast regeneration of strain YN-T3 and its pSLA2-L-less derivative YN-C149 was performed on R1M plates (Zhang *et al.*, 1997) with/without 50 μg/ml of thiostrepton.

3.3. Results

3.3.1. Telomere sequences of the chromosome are identical to the right end sequences of pSLA2-L and pSLA2-M

Although the complete nucleotide sequences of the three linear plasmids, pSLA2-L (Mochizuki *et al*., 2003, AB088224), pSLA2-M (Yang *et al*., 2011, AB597522), and pSLA2–S (AB905437), have been determined, little was known about the chromosomal sequence of *S. rochei* 7434AN4 including the telomere sequences. Concerning to the relationship between the telomere sequences of *Streptomyces* linear chromosomes and coexisting linear plasmids, two different cases have been reported. The telomere sequences of the *Streptomyces lividans* chromosome and the right end sequence of the

linear plasmid SLP2 are identical (Bey *et al.*, 2000). On the other hand, the telomere sequences of *S. coelicolor* A3(2) chromosome (Bentley *et al.*, 2002) and linear plasmid SCP1 (Kinashi *et al.*, 1991; Bentley *et al.*, 2004) were totally different. Consequently, the presence of another gene pair, essential for end patching of SCP1, was expected and a novel type of gene pair, *tac* (telomere-associated protein of SCP1)-*tpc* (terminal protein of SCP1) was identified on SCP1 (Huang *et al.*, 2007).

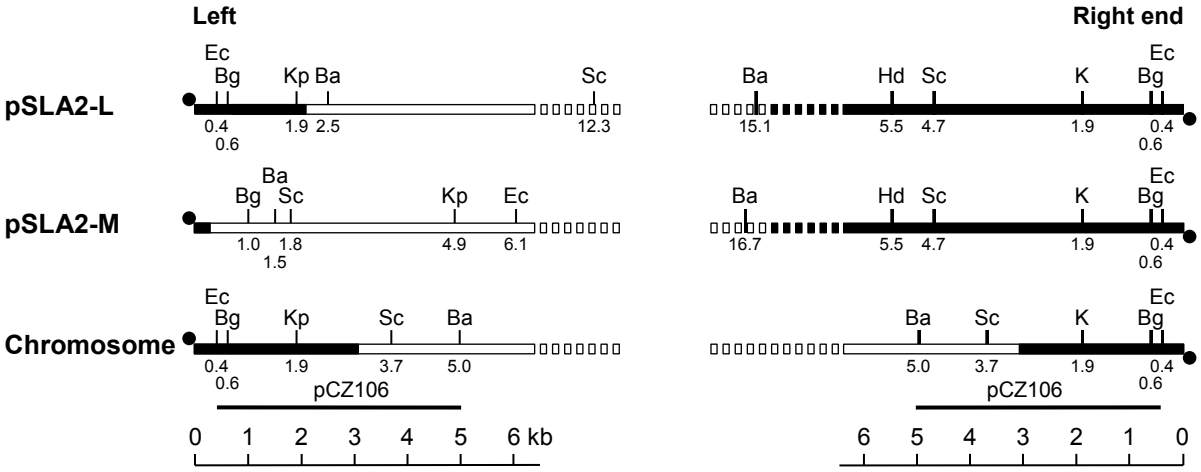

Figure 5. Restriction maps of the left and right ends of chromosome, pSLA2-L, and pSLA2-M

Terminal proteins bound to the 5'-ends are indicated by filled circles. Homologous regions to the right end of pSLA2-L are drawn in thick black lines. Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Hd, *Hin*dIII; Kp, *Kpn*I; Sc, *Sac*I.

In *S. rochei* 7434AN4, the right end sequences of pSLA2-L and pSLA2-M are almost (99.9%) identical up to 14.6 kb from the end (Figure 5). The homology between the left and right end sequences (terminal inverted repeat, TIR) of pSLA2-L is extending to 2.1 kb. The TIR of pSLA2-M is short (352 bp) with a relatively low similarity (321/352, 91.2% identities). Expecting a sequence similarity between the telomeres of the chromosome and pSLA2-L, 7434AN4 DNA was digested with various enzymes and probed by plasmid pEcoEnd containing the 0.4-kb right end fragment of pSLA2-L (Hiratsu *et al.*, 2000). As shown in Figure 6A, when 7434AN4 DNA was

digested with *Bam*HI, four expected signals of the linear plasmids were observed at 16.7 kb (right end of pSLA2-M= M-R), 15.1 kb (right end of pSLA2-L= L-R), 2.5 kb (left end of pSLA2-L= L-L), and 1.5 kb (left end of the pSLA2-M= M-L) (see Table 28 and Figure 5 for the restriction sites that gave a hybridizing signal). In addition, a new hybridizing signal appeared at 5.0 kb (indicated by asterisk). This possible telomere fragment of the chromosome was further analyzed using other restriction enzymes.

Table 28. Expected restriction size (in kb) of both ends (L and R) of chromosome (Ch), pSLA2-L (L) and pSLA2-M (M)

Enzyme	Ch (L & R)	L-L	L-R	M-L	M-R
*Bam*HI	5.0	2.5	15.1	1.5	16.7
*Bgl*II	0.6	0.6	0.6	1.0	0.6
*Eco*RI	0.4	0.4	0.4	6.1	0.4
*Kpn*I	1.9	1.9	1.9	4.9	1.9
*Sac*I	3.7	12.3	4.7	1.8	4.7

When 7434AN4 DNA was digested with *Sac*I, a hybridizing signal of the chromosome appeared at 3.7 kb, in addition to three plasmid signals at 12.3 kb (L-L), 4.7 kb (L-R and M-R), and 1.8 (M-L). On the other hand, when 7434AN4 DNA was digested with *Kpn*I, *Bgl*II, or *Eco*RI, only two plasmid signals were observed, one common signal derived from L-L, L-R, and M-R and another from M-L. In the latter three cases, the telomere signal of the chromosome was overlapped with the common signal. These results indicate that the left and right telomeres of the chromosome have identical restriction sites with L-L, L-R, and M-R at least up to the *Kpn*I site (1.9 kb from the ends), giving the same *Kpn*I (1.9 kb), *Bgl*II (0.6 kb), and *Eco*RI (0.4 kb) fragments.

The left and right telomeres of the chromosome were further analyzed by Southern hybridization of large fragments separated by pulsed-field gel electrophoresis (PFGE). As shown in Figure 6B, *Hin*dIII digest of 7434AN4 gave two telomere signals at 90 and

70 kb in addition to the linear plasmid signals at 159 kb (L-L) and 5.5 kb (L-R and M-R). The M-L fragment (105 kb) of pSLA2-M was not observed here due to its low homology to the 0.4-kb telomere probe. The sizes of the two telomere fragments suggest that the length of the TIR at both ends of the chromosome is shorter than 70 kb.

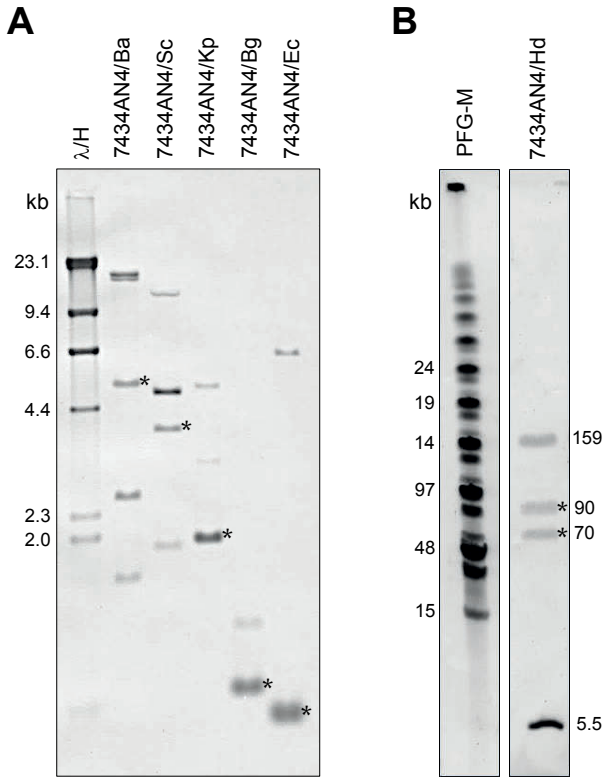

Figure 6. Southern hybridization analyses of end fragments of the chromosome, pSLA2-L, and pSLA2-M separated by conventional agarose gel electrophoresis (A) and pulsed-field gel electrophoresis (B)

Telomere fragments of the chromosome are indicated by asterisk. Plasmid pEcoEnd, containing 0.4 kb right end fragment of pSLA2-L, was used as a probe for hybridization. λ DNA digested with *Hin*dIII (λ/Hd) and MidRange I PFG marker (PFG-M) were used as a size marker. Ba, *Bam*HI; Bg, *Bgl*II; Ec, *Eco*RI; Hd, *Hin*dIII; Kp, *Kpn*I; Sc, *Sac*I.

Since telomere fragments of the chromosome smaller than 1.9 kb are inseparable from those of L-L, L-R, and M-R, an adjacent 4.6-kb *Bam*HI-*Eco*RI fragment, locating at 0.4 kb from the chromosomal end was shotgun cloned (pCZ106) and sequenced (AB905441). Comparison of the sequences of pCZ106 and L-R revealed that their

homology is extending up to 2.7 kb from the *Eco*RI site (2590/2636, 98.3% identities). We speculate that the 0.4-kb extreme end sequences of the left and right telomeres of the chromosome (C-L and C-R), L-L, L-R, and M-R are identical based on the following results. 1) C-R, C-L, L-L, L-R, and M-R gave the same hybridizing signals when digested with *Kpn*I, *Bgl*II, and *Eco*RI (Figure 6A). 2) L-L, L-R, and M-R have been cloned and sequenced, which revealed their identical end sequence (Hiratsu *et al.*, 2000; Mochizuki *et al.*, 2003; Yang *et al.*, 2011). 3) In the on-going genome project of strain 7434AN4, no sequence heterogeneity has been detected in the 0.4-kb extreme end region derived from the chromosome, pSLA2-L, and pSLA2-M. Collectively, we concluded that the left and right telomere sequences of chromosome are identical to each other and are 98.5% (3027/3073) identical to the right end sequence of pSLA2-L and pSLA2-M up to 3.1 kb from the ends (100% identical up to nt 1937).

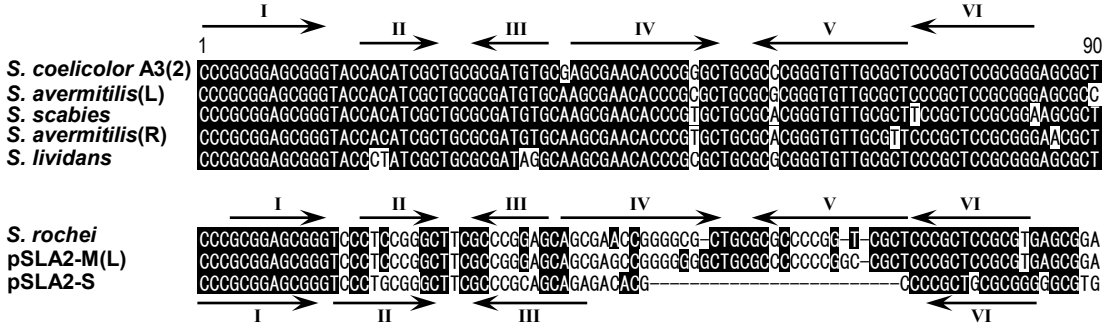

Figure 7. Alignments of the telomere sequences of representative *Streptomyces* species and *S. rochei* linear replicons

S. coelicolor A3(2) (NC_003888), *S. lividans* (AF194023), *S. avermitilis* MA-4680 (NC_003155), *S. scabies* 87.22 (FN554889), *S. rochei* 7434AN4 (this study), pSLA2-L (AB088224), pSLA2-M (AB597522), and pSLA2-S (AB905437). Highly conserved nucleotides in representative telomeres are drawn in white letters. The telomere sequences of *S. avermitilis* are different in the left (L) and right (R) arms. The telomere sequence of *S. rochei* is identical to that of pSLA2-L and to the right end sequence of pSLA2-M, while the left end sequence of pSLA2-M is a little bit different. Three pairs of inverted repeat sequences, I and VI, II and III, and IV and V, are indicated by arrowhead lines.

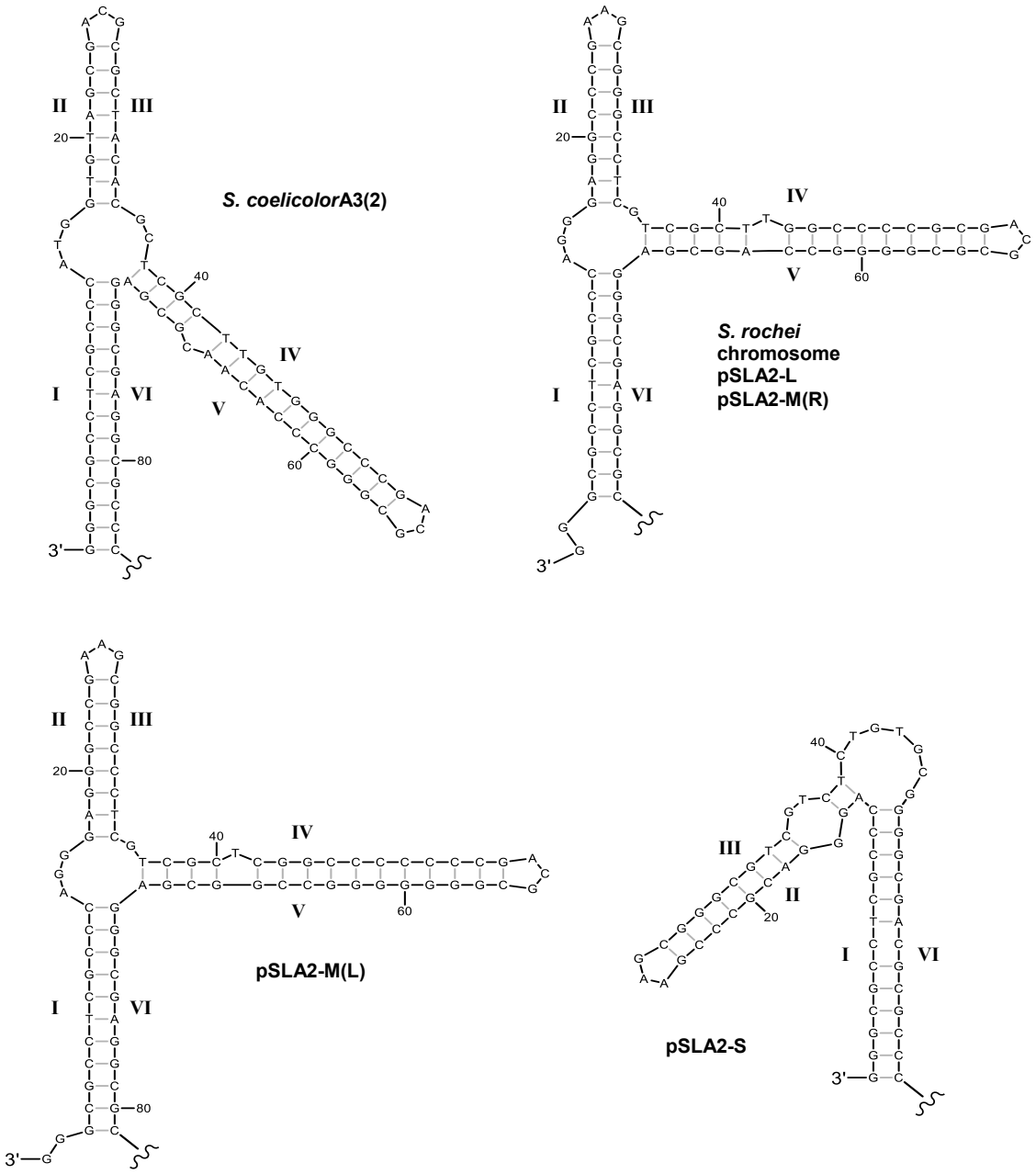

Figure 8. Secondary foldback structures formed by the 3'-single-stranded DNAs

Foldback structure comparison between the chromosome of *S. coelicolor*A3(2), *S. rochei* chromosome [identical to pSLA2-L and pSLA2-M (R)], pSLA2-M (L) and pSLA2-S. Sequences II-III and sequences IV-V form a hairpin loop, while sequences I-VI form a base part.

Streptomyces linear chromosomes and plasmids have a conserved sequence at the extreme ends and their 3'-leading strand overhangs are possible to form a Y-shaped secondary foldback structure (Huang *et al.*, 1998; Qin and Cohen, 1998). This structure may be recognized by Tap and TP to initiate a protein-primed DNA synthesis for end

patching (Bao and Cohen, 2003). The telomere sequence of the *S. rochei* chromosome also shows high similarity to typical telomere sequences (Figure 7) and therefore its 3' single stranded DNA can form a Y-shaped foldback structure (Figure 8), where sequences II and III and sequences IV and V form two hairpin loops and sequences I and VI form a base part.

3.3.2. Chromosome circularization occurred in mutant 2-39 concomitant with a plasmid loss

The plasmidless strain 2-39 has been obtained by protoplast regeneration of strain 51252, which carried only pSLA2-L (Kinashi *et al.*, 1994). Strain 2-39 did not give a telomere signal when probed by the telomere clone pEcoEnd (Figure 9A), indication terminal deletions of the chromosome. *Streptomyces* linear chromosomes frequently suffer deletions at both ends spontaneously or by various mutagenic treatments, leading to chromosomal circularization, arm replacement, and amplification (Volff and Altenbuchner, 1998), among which circularization occurs more frequently.

To know if the chromosomal circularization also occurred in strain 2-39, we searched for a fusion sequence in its genome library and found a candidate contig 229. We considered this as a fusion contig, because it contains on each side two independent sequences of contigs 634 and 586 of strain 7434AN4. Based on the sequence of contig 229, a 2.3-kb fusion fragment was amplified and cloned from 2-39 DNA to give plasmid pCZ117. The nucleotide sequence of pCZ117 was identical to that of contig 229, which indicates that this contig was not an artifact generated during construction of the library (Figure 10). Chromosomal circularization was further analyzed by Southern hybridization using pCZ117 as a probe. As shown in Figure 11A, *Afl*III digest of 51252 DNA gave two hybridizing signals of the deletion end fragment at 4.8 and 1.7 kb, while that of 2-39 DNA gave a signal of the fusion fragment at 4.2 kb. Similar results were

obtained for large fragments separated by PFGE. *Hin*dIII digest of 51252 and 2-39 DNAs showed two deletion end fragments at 500 and 450 kb and one fusion fragment at 650 kb, respectively (Figure 11B).

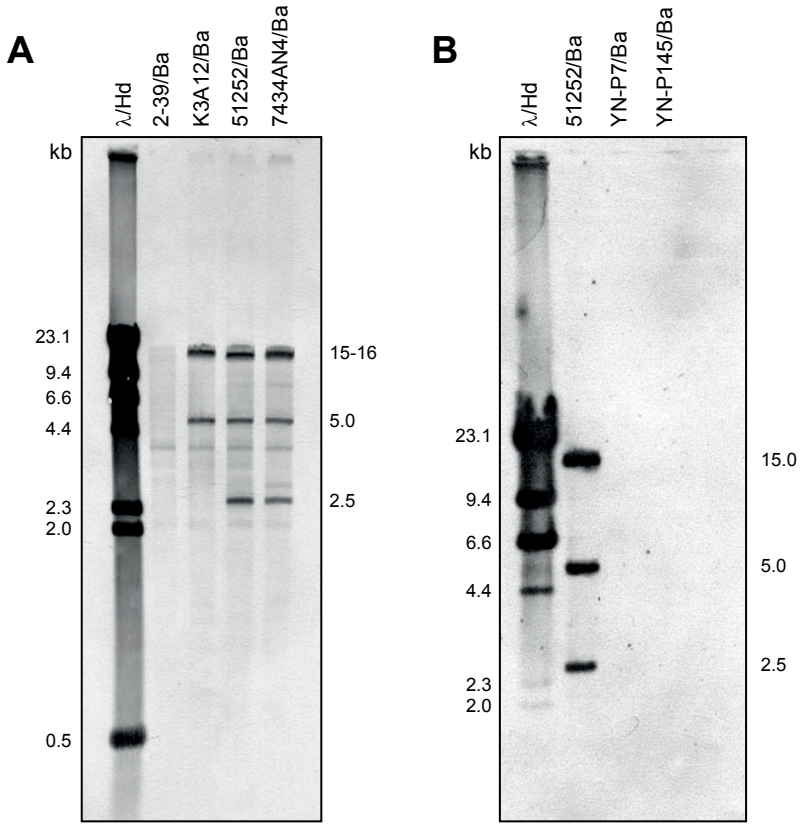

Figure 9. Southern hybridization analyzes of end fragment chromosome in several mutants with different plasmid profile

Plasmid pEcoEnd was used as a probe for hybridization. λ DNA digested with *Hin*dIII (λ/Hd) were used as a size marker. Ba, *Bam*HI.

The fusion sequence of the chromosomal 2-39 (contig 229) and the corresponding deletion end sequences of the 7434AN4 chromosome (contigs 634 and 586) are aligned and compared in Figure 10. Between two deletion ends of the chromosome, a 9-bp microhomology was identified, which is shorter that 20 bp required for homologous recombination in *E. coli* (Watt *et al.*, 1985). At the deletion end in contig 634, a cell division protein (FtsK, 1331 aa) is encoded. On the other hand, at the other deletion end

in contig 586, a putative nucleotide-binding protein (NBP, 423 aa) is encoded. The generated fusion gene encodes for a protein (1162 aa), in which due to frame coincidence, N-terminal 422 aa of the FtsK protein were replaced by N-terminal 253 aa of the nucleotide-binding protein.

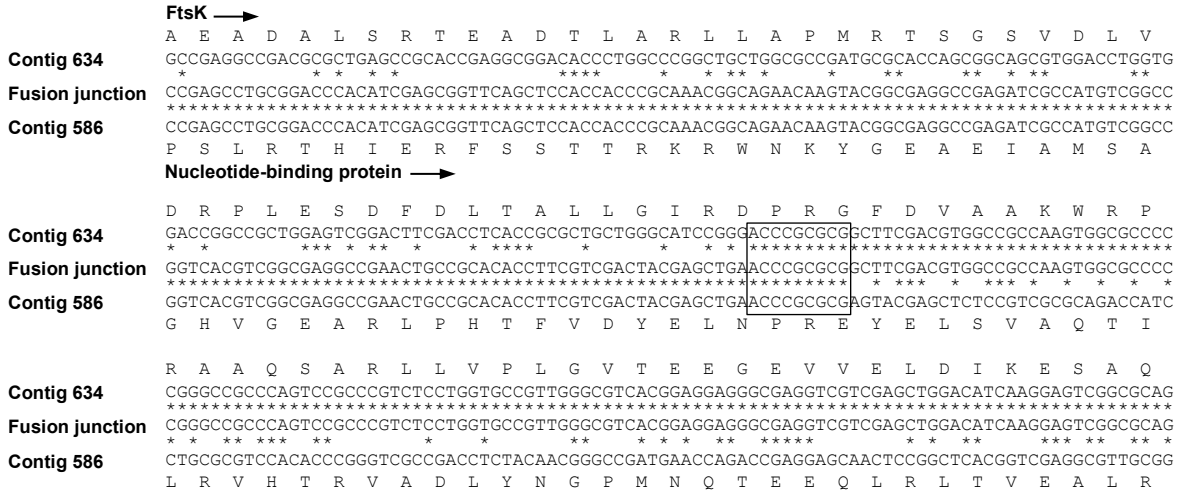

Figure 10. Nucleotide sequence comparison of the deletion ends of the chromosome of strain 7434AN4 and the fusion junction of the plasmidless mutant 2-39

Identical nucleotides are indicated by asterisks, and the 9 bp homologous sequences between the two deletion ends are enclosed by a square. Amino acid sequences of the cell division protein (FtsK) and the nucleotide-binding protein are shown upper and under the nucleotide sequences.

To know whether chromosomal circularization was specific to mutant 2-39 or is general in plasmidless mutant, strain 51252 was subjected to protoplast regeneration to cure pSLA2-L. By using the antibiotic producing ability coded on pSLA2-L as a marker, two non-producers, YN-P7 and YN-P145, were selected from 192 regenerated colonies. When probed by the telomere clone, two colonies did not show the telomere signals of the chromosome or pSLA2-L (Figure 9B). This result indicates that two events, curing of pSLA2-L and terminal deletions of the chromosome, have occurred concomitantly in the newly obtained plasmidless strains, too, which possibly led to chromosomal circularization. To analyze the deletion sizes in mutants, YN-P7 and YN-P145, their

total DNAs were digested with *Afl*III and probed by the fusion plasmid pCZ117. As shown in Figure 11A, mutant YN-P7 showed two intact signals (4.8 kb and 1.7 kb) of both deletion end fragments, while mutant YN-P145 gave only a signal (4.8 kb) of one deletion end fragment. Similar results were obtained for large fragments separated by PFGE. *Hin*dIII digest of YN-P7 DNA showed two hybridizing signals of both deletion end fragments at 500 and 450 kb, while that of YN-P145 showed a signal of one deletion end fragment at 500 kb (Figure 11B). These results indicate that deletion sizes at the left and right chromosomal arms were different from strain to strain, and therefore where were no hot spots for terminal deletion. When grown on solid media, mutant 2-39 did not produce aerial mycelium or spores (bald phenotype). On the other hand, mutants YN-P7 and YN-P145 did not form spores, but produces white mycelium (Figure 12).

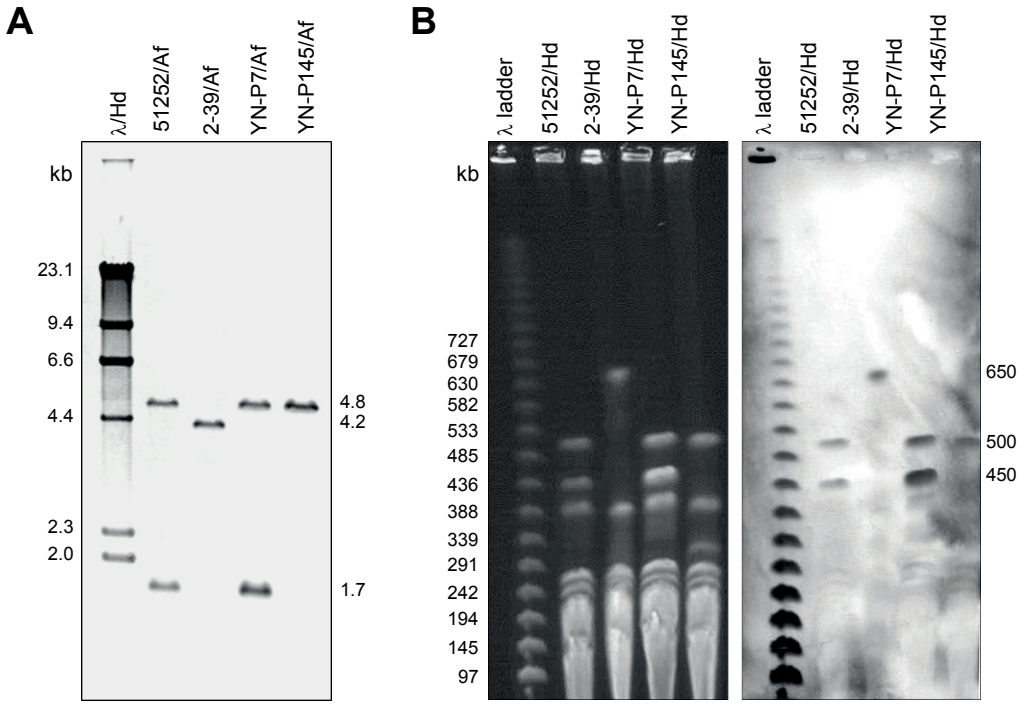

Figure 11. Southern hybridization analysis of chromosomal deletions in three plasmidless mutants

Total DNA of 51252 (parent strain) and three plasmidless mutants 2-35, YN-P7 and YN-P145 were digested with *Afl*III (A) and *Hin*dIII (B), separated by conventional gel electrophoresis and pulsed-field gel electrophoresis, respectively. Fusion clone pCZ117 was used as a probe in Southern hybridization. Af, *Afl*III; Hd, *Hin*dIII.

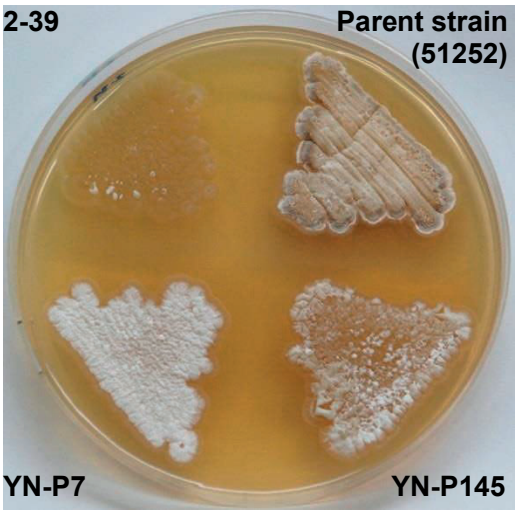

Figure 12. Phenotype of plasmidless mutants of *S. rochei* (2-39, YN-P7 and YN-P145) compared with parent strain 51252

3.3.3. Introduced *tap-tpg* of pSLA2-M functioned to maintain a linear chromosome

As described above, the genome project of the wild-type strain 7434AN4 is in progress. This strain carries all of the three linear plasmids pSLA2-L, pSLA2-M, and pSLA2-S. Consequently, we identified the *tap-tpg* gene pairs of pSLA2-L (*tapR1-tpgR1*) and pSLA2-M (*tapRM-tpgRM*) in the genome library of strain 7434AN4. However, we have not found an additional *tap-tpg* pair of the chromosome at this stage, where a draft assembly with ca. 450-fold genome coverage gave 340 contigs containing over 500-bp nucleotides (8,999 kb in total size). Instead, we identified one gene in contig 95, which has a relatively short sequence homologous to nt 117-221 of *tpgR1* of pSLA2-L (96/105, 91% identities) [Figure 13, AB907705]. We speculate that this gene was generated by at least two recombination events, which resulted in deletion of both 5'- and 3'-termini of the original *tpg* gene. Thus, a *tap* gene, which is always located upstream of *tpg*, was neither found around this truncated *tpg* homolog.

To further analyze the presence/absence of an additional *tpg* gene on the

chromosome, we carried out Southern hybridization experiments using the *tpgR1* clone of pSLA2-L (pYN16) as a probe (Figure 14). When digested with *Fsp*I, 7434AN4 DNA gave two hybridizing signals of *tpgR1* (6.5 kb) and *tpgRM* (3.4 kb), while 51252 DNA gave only a signal of *tpgR1* (6.5 kb). On the other hand, 2-39 DNA did not show any hybridizing signals. The signal of the truncated *tpg*-homolog was neither detected due to its short homology. These results led us to the idea that the chromosome does not contain a *tap-tpg* gene pair and instead pSLA2-L or pSLA2-M functions to maintain a linear chromosomal topology in strain 7434AN4. To support this hypothesis, we carried out following complementation and curing experiments.

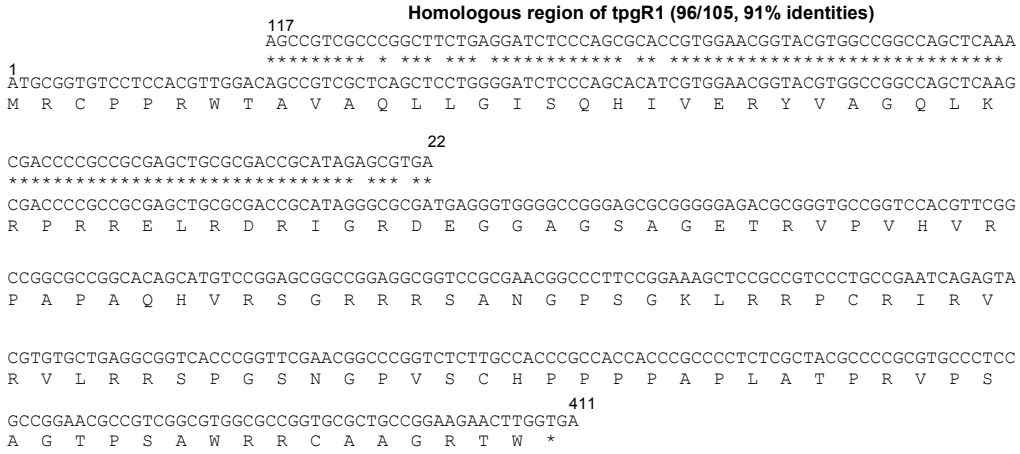

Figure 13. Nucleotide sequence of the truncated *tpgR1* homolog found in contig 95 of strain 7434AN

Nucleotides 117-223 of *tpgR1*, to which this gene shows high homology, are drawn upper the sequence.

The *tapRM-tpgRM* gene pair of pSLA2-M was cloned into the *Streptomyces-E. coli* shuttle vector pRES18 (Ishikawa *et al.*, 1996) to give plasmid pYN15, which was then introduced into strain 51252 carrying only pSLA2-L. The sequence of *tapRM-tpgRM* of pSLA2-M is a little bit different from that of *tapR1-tpgR1* of pSLA2-L, which was expected to avoid recombination between the two gene pairs during experiments. The obtained transformant, YN-T3, was analyzed by Southern hybridization using the

telomere probe. As shown in Figure 15A, strain YN-T3 gave three telomere signals at 12.3 kb (L-L), 4.7 kb (L-R), and 3.7 kb (C-L and C-R), in addition a signal of linearized pYN15 at 10.3 kb (homology of the vector part gave this signal).

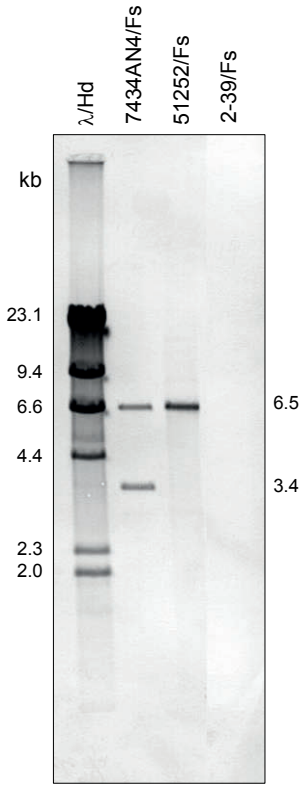

Figure 14. Search for additional *tpg* gene by Southern hybridization

Total DNAs of *S. rochei* 7434AN4, 51252 and 2-39 were digested with *Fsp*I and analyzed by Southern hybridization using the *tpgR1* of pSLA2-L as a probe. The *tpgR1* of pSLA2-L and *tpgRM* of pSLA2-M gave hybridizing signals at 6.5 kb and 3.4 kb, respectively, but no additional *tpg* signals were detected. Fs, *Fsp*I.

Then, strain YN-T3 was subjected to protoplast regeneration to cure pSLA2-L in the presence of thiostrepton, which was added to maintain pYN15. Among 96 regenerated colonies tested, eight colonies still kept pYN15 but lost pSLA2-L. All of the eight pSLA2-L-less strains ($pYN15^{+}$, $pSLA2\text{-}L^{-}$) did not show the signals of pSLA2-L at 12.3 kb (L-L) or 4.7 kb (L-R), but still showed the telomere signal of the chromosome at 3.7 kb and the pYN15 signal at 10.3 kb (Figure 15B, four representative strains are shown here). This result indicates that *tapRM-tpgRM* of pSLA2-M could

maintain a linear chromosome in place of pSLA2-L.

Finally, strain YN-C149, one of the pSLA2-L cured strains carrying only pYN15, was subjected again to protoplast regeneration under no pressure of thiostrepton. A total of 192 regenerated colonies were tested for their thiostrepton sensitivity; 24 colonies were thiostrepton-sensitive and remaining 168 colonies were still thiostrepton-resistant. All of the 24 sensitive colonies and 15 of the resistant colonies were subjected to Southern hybridization analysis. All colonies of the former group showed neither the

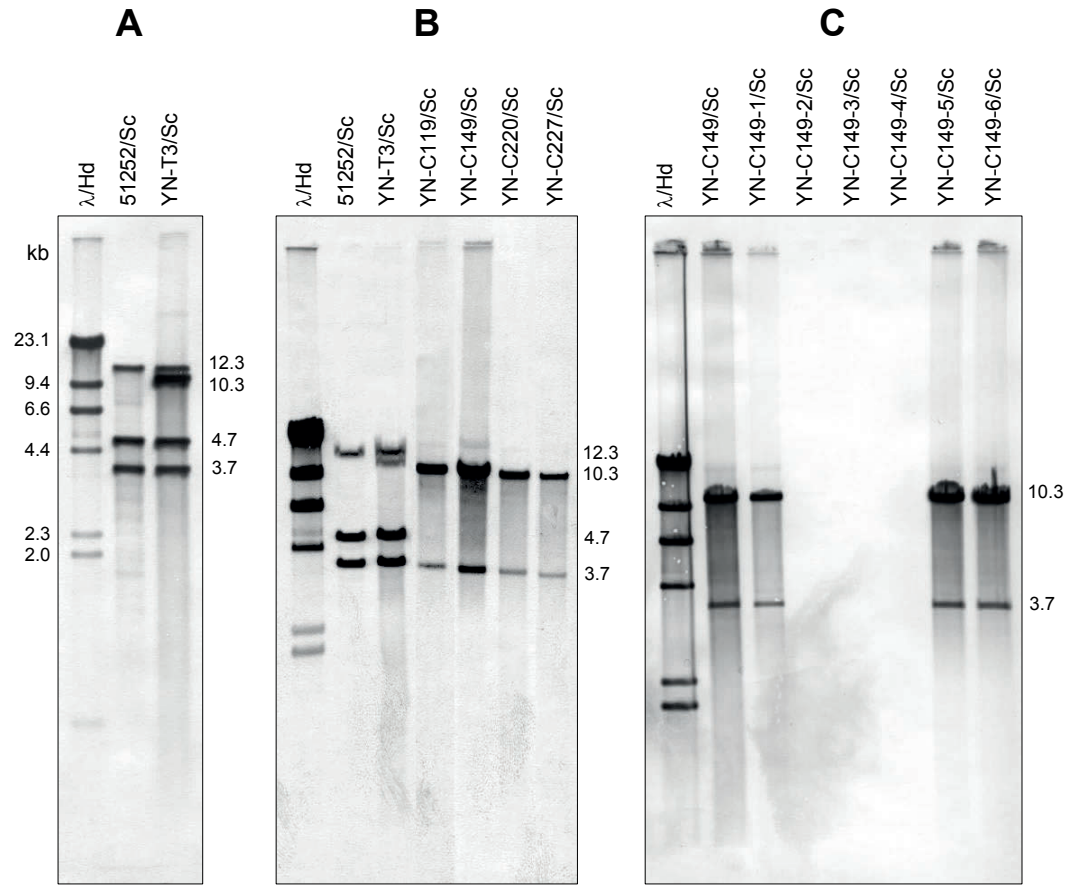

Figure 15. Effects of plasmid pYN15 containing *tapRM-tpgRM* gene pair of pSLA2-M on chromosomal topology of *S. rochei* strains

Total DNAs of various *S. rochei* strains with/without pYN15 were digested with *Sac*I and analyzed by Southern hybridization using pEcoEnd as a probe. (A) Strain YN-T3 obtained by introduction of pYN15 into strain 51252. (B) pSLA2-L cured strains, YN-C119, YN-C149, YN-C220, and YN-C227 obtained by the first protoplast regeneration of strain YN-T3. (C) Strains YN-C149-1~6 obtained by the second protoplast regeneration of strain YN-C149.

pYN15 signal nor the telomere signal, while all colonies of the latter group showed both signal (Figure 15C, six representative strains are shown here). Based on a complete correlation between the presence of the telomere and plasmid pYN15, we concluded that the *tapRM-tpgRM* of pSLA2-M functions to maintain a linear chromosome in strain YN-C149 in place of pSLA2-L. However, the intensity of the telomere signal was weak in most of the pSLA2-L-cured strains compared with the parent strain YN-T3, which suggests a possibility that an additional subsidiary gene(s) or region(s) is necessary for a full maintenance of the linear chromosome.

3.4. Discussion

In this study, the following results were obtained on the sequence and topology of the linear chromosome of *S. rochei* 7434AN4. 1) The left and right telomere sequences of the chromosome are identical to each other and are almost identical to the right end sequences of pSLA2-L and pSLA2-M up to 3.1 kb. 2) The telomere sequence of *S. rochei* is similar to the typical telomere sequences of *Streptomyces* and could form a Y-shaped secondary foldback structure. 3) Chromosomal circularization occurred in mutant 2-39 concomitant with a plasmid loss by terminal deletion followed by nonhomologous recombination of the deleted ends. 4) The *tapRM-tpgRM* gene pair of pSLA2-M functioned to maintain a linear chromosome in plasmidless strains in place of pSLA2-L.

As shown in Figure 8, the identical telomere sequences of the chromosome, pSLA2-L, and pSLA2-M can form a Y-shaped foldback structure similar to that of *S. coelicolor* A3(2). Even the least homologous left end sequence of pSLA2-M shows high similarity in this region and forms a similar Y-shaped structure. On the other hand, the smallest linear plasmid pSLA2-S lacks sequences IV and V, although 14 nucleotides at the extreme end are identical to those of typical *Streptomyces* telomeres (Figure 7).

Sequences I and VI and sequences II and III of pSLA2-S can make a foldback structure with a single hairpin loop (Figure 8). All of the replication experiments of *S. rochei* linear replicons have been carried out using pSLA2-S or its derivative plasmids. These results suggest that the minimal structure essential for end patching is a foldback structure with a single hairpin loop rather than a Y-shaped structure with double loops.

Chromosomal circularization of *Streptomyces* species has been confirmed at a sequence level for *S. griseus* (Kameoka *et al.*, 1999; Inoue *et al.*, 2003), *S. avermitilis* (Chen *et al.*, 2010), and *S. coelicolor* A3(2) (Nindita *et al.*, 2013). In these cases, no homology or only 1-bp to 6-bp homology was identified between the left and right deletion ends. In this study, 9-bp homology between the deletion ends for circularization of the *S. rochei* chromosome was identified, which is still much shorter than the minimum size (20 bp) of homology required for homologous recombination (Watt *et al.*, 1985). This result again supports nonhomologous recombination of deletion ends of *Streptomyces* linear chromosomes proposed previously (Kameoka *et al.*, 1999).

Disruption of the *tpgL* gene on the chromosome of *S. lividans* showed that TP is essential to maintain a linear chromosome as well as the introduced pSLA2 plasmid (pSLA2-S) (Bao and Cohen, 2001). Complementation and curing experiments in this study proved that the *tapRM-tpgRM* of pSLA2-M functioned to maintain a linear chromosome in pSLA2-L-cured strains of *S. rochei*. The *tapR1-tpgR1* of pSLA2-L may function similarly, because their gene products have high sequence similarity to those of *tapRM-tpgRM*; TpgR1 and TpgRM are 98.4% identical (181/184 aa) and TapR1 and TapRM are 75.5% identical (558/739 aa). In addition, strain 51252, which carries only pSLA2-L, stably keeps a linear chromosome as strain K3A12 does, which carries only pSLA2-M. My laboratory previously tried to cure pSLA2-L, pSLA2-M, and pSLA2-S from strain 7434AN4, by protoplast regeneration and other curing methods. All of the

mutants with a possible combination of the three linear plasmids have been obtained except for one that carries only pSLA2-S (Kinashi *et al.*, 1994). Now I realize that this result did not happen by chance; neither the *S. rochei* chromosome nor pSLA2-S supports end patching of itself or another without an intact *tap-tpg* gene pair.

A truncated *tpg* homolog was identified on the *S. rochei* chromosome (Figure 13), which might have been generated by recombination. The truncated *tap-tpg* homologs (*SGR6987-6986*) were also found on the chromosome of *S. griseus* IFO13350 (Suzuki *et al.*, 2008). The latter chromosome contains a novel type of gene pair (*gtpB-gtpA*) for end patching of the atypical telomere sequence. Thus, the deficiency of *tap-tpg* is rescued in different ways in two strains; by introducing linear plasmids, pSLA2-L and pSLA2-M, containing an intact *tap-tpg* pair in *S. rochei* 7434AN4, while by acquiring a novel set of the *gtpB-gtpA* genes and the atypical telomeres possibly from a certain linear plasmid in *S. griseus* IFO13350 (Suzuki *et al.*, 2008). The fact that another atypical telomere sequence of *S. griseus* strain 2247 was totally different from that of strain IFO13350 (Goshi *et al.*, 2002) supports the latter idea.

The number of examples where biosynthetic gene clusters are located on a giant linear plasmid is increasing gradually (Chater and Kinashi, 2007; Kinashi, 2011) since the discovery of SCP1 from *S. coelicolor* A3(2) (Kinashi *et al.*, 1987); SCP1 carries the biosynthetic gene cluster for methylenomycin (Bentley *et al.*, 2004). In addition, the methylenomycin cluster was found as an integrated form into the chromosome of *S. coelicolor* A3(2) (Hanafusa and Kinashi, 1992; Yamasaki *et al.*, 2001) as well as into circular plasmid pSV1 of *S. violaceoruber* (Yamasaki *et al.*, 2003). The gene clusters for the angucycline antibiotics were also identified on linear plasmid pSA3239 of *S. aurofaciens* (Novakova *et al.*, 2013) as well as at both ends of the chromosome of *S. ambofaciens* (Pang *et al.*, 2004). Recombination between a linear plasmid and a

chromosome near the end of the latter causes an exchange, which transferred the oxytetracycline biosynthetic cluster from the *S. rimosus* chromosome to a 387-kb linear plasmid pPZG101 (Pandza *et al.*, 1998). When recombination occurred between SCP1 and the center of the chromosome, two chimeric chromosomes were generated in *S. coelicolor* A3(2) strain 2106 (Yamasaki and Kinashi, 2004). This may be considered as a model of chromosome duplication. In addition, I revealed in this study that linear plasmids, pSLA2-L and pSLA2-M, function even in maintaining a linear topology of the chromosome in *S. rochei* 7434AN4. Thus, the idea that giant linear plasmids have played critical roles in genome evolution and horizontal transfer of secondary metabolism was further supported.

Chapter 4
Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2)

4.1. Introduction

Streptomyces chromosomes are unusually unstable and are often subject to deletion and amplification spontaneously or by various mutagenic treatments (Volff and Altenbuchner, 1998). The size of chromosomal deletions reaches up to 2 Mb in some *Streptomyces* species (Fischer *et al.*, 1997; Chen *et al.*, 2010). Studies of this genetic instability of *Streptomyces* faced great difficulty previously, because *Streptomyces* chromosomes had been considered to be circular for a long time. We now have a correct idea of the linear structure of *Streptomyces* chromosomes and a powerful method, pulse-field gel electrophoresis, for physical analysis of their arrangements. It is known at present that chromosomal deletion occurs from both ends. However, the instability of *Streptomyces* chromosomes has not been clarified well. For example, an amplifiable unit of DNA (AUD) was tandemly amplified several hundred times to form amplified DNA sequence (ADS) in *S. lividans* mutants (Altenbuchner and Cullum, 1985), but the gross structures of the mutants chromosomes have not been clarified.

The most frequent effect of *Streptomyces* chromosomes following terminal deletion is circularization. Chromosome circularization was indicated by detection of a macrorestriction fragment in deletion mutants of *S. lividans* (Lin *et al.*, 1993; Redenbach *et al.*, 1993) and *Streptomyces ambofaciens* (Leblond *et al.*, 1996). It was finally confirmed in *Streptomyces griseus* by cloning and sequencing of the fusion junctions of the circularized chromosomes (Kameoka *et al.*, 1999; Inoue *et al.*, 2003). No sequence homology was found between the left and right deletion ends in two

mutants, and only 1-bp and 6-bp homology was found in other mutants. Accordingly, it was proposed that nonhomologous recombination between the left and right deletion ends caused chromosomal circularization (Inoue *et al.*, 2003). Microhomology was also detected at the fusion points of circularized chromosomes of *Streptomyes avermitilis* mutants (Chen *et al.*, 2010).

Chromosomal arm replacement is another outcome of deleted chromosomes. When one chromosomal arm is deleted and the left and right arms carry a homologous sequence in an inverted orientation, homologous recombination between them causes arm replacement, which recovers a telomere and generates longer terminal inverted repeats (TIRs) at both ends. This phenomenon was first reported for *S. ambofaciens* (Fischer *et al.*, 1998) and was followed by *S. griseus* (Uchida *et al.*, 2003) and *S. coelicolor* A3(2) (Widenbrant *et al.*, 2007). Even the long TIRs formed by arm replacement suffer terminal deletion. When an inverted repeat sequence is present at the deletion end inside the long TIR, it could form a hairpin structure, which invades the opposite TIR strand during replication leading to a circular chromosome with an extremely large palindrome (Uchida *et al.*, 2004). Similar various rearrangements were observed in *Streptomyces* linear plasmids when deletions were introduced at specific locations within telomeres (Qin and Cohen, 2002).

In spite of this extensive analyses, chromosomal circularization has not been proved for the model species *S. coelicolor* A3(2) at a sequence level. In this study, I first report the cloning and sequencing of a fusion junction of a circularized chromosome of *S. coelicolor* A3(2) and show that chromosomal circularization occurred by non-homologous recombination in this species, too. At the end of the study, I discuss on stability and evolution of *Streptomyces* chromosomes.

4.2. Materials and methods

4.2.1. Bacterial strains, plasmids and cosmid libraries.

Streptomyces strains used in this study were derived from the wild-type strain *Streptomyces coelicolor* A3(2) and are listed in Table 29. Plasmid pUC19 was used for cloning in *Escherichia coli* XL-1 Blue and nucleotide sequencing. The cosmid libraries of *S. coelicolor* A3(2) used in this study have been constructed and ordered by Redenbach *et al*. (1996) and Zhou *et al.* (2012).

Table 29. Bacterial strains and plasmids

Names	Description	Source/ref.
Strains		
S. coelicolor 1147	Prototrophic wild type ($SCP1^+$, $SCP2^+$)	Kawamoto *et al.*, 2001
S. coelicolor M145	$SCP1^-$, $SCP2^-$	Bentley *et al.*, 2002
S. coelicolor No. 4	*eshA* null mutant (*eshA::tsr*) of 1147 ($eshA^-$, $argG^-$)	Kawamoto *et al.*, 2001
Cosmids and Plasmids		
8-65	Cosmid library of *S. coelicolor* (nt. 7,790,510-7,825,517)	Zhou *et al.*, 2012
3-14	Cosmid library of *S. coelicolor* (nt. 230,501-274,102)	Zhou *et al.*, 2012
pLUS221	1.3-kb *Bam*HI end fragment of *S. coelicolor* A3(2)	Huang *et al*., 1998
pOPP	Fusion clone of No. 4, 1.7-kb *Bam*HI-*Eco*RI PCRfragment in pUC19 with primer del-L and del-R	This study

4.2.2. DNA manipulation and Southern hybridization

Table 30. GMP medium

Meat extract	0.2%
Yeast extract	0.2%
Polypeptone	0.4%
Glucose	1%
NaCl	0.5%
	Adjust pH to 7.0

S. coelicolor A3(2) wild type and mutant strains were reciprocally grown in liquid GMP medium (Table 30) in Sakaguchi flasks at 28 °C for 3 days. DNA manipulation

for *Streptomyces (*Kieser *et al.*, 2000) and *E. coli* (Sambrook and Russell, 2001) was carried out according to standard procedures.

4.2.3. PCR and nucleotide sequencing

Two primers for PCR amplification, del-L, 5′-CACCGAATTCTGAGCGATGGT CGTCGTGA-3′ (the *Eco*RI site is underlined) and del-R, 5′-ATACGGATCC TTCGCGATCGTCCCGCTGA-3′ (the *Bam*HI site is underlined), were designed based on Southern hybridization analysis of the left and right deletion ends of mutant No 4. PCR was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster city, CA) with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Nucleotide sequencing was performed by the dideoxy termination method, using Big-Dye Terminator v3.1/v1.1 Cycle Sequencing Kits (Life Technologies, Carlsbad, CA) and a 3130xl Genetic Analyzer (Life Technologies).

4.3. Results and discussion

4.3.1. Analysis of chromosomal deletion in mutant No. 4

S. coelicolor A3(2) strain No. 4 used in this study is an *eshA* (named for a defect of extension of sporogenic hyphae; Kwak *et al.*, 2001) mutant obtained by cultivation of the wild-type strain 1147 at a high temperature and shows several defective phenotypes (Kawamoto *et al.*, 2001). The *eshA* gene encodes a nucleotide-binding protein, disruption of which caused a loss of actinorhodin production due to a reduced level of ppGpp (Saito *et al.*, 2006). The *eshA* gene was identified as SCO7699 (nt 8535532-8536947 of the genome sequence) located at 131 kb from the right end (Bentley *et al.*, 2002), suggesting that at least the right chromosomal arm was deleted beyond this locus. To clarify whether only the right telomere was deleted or both telomeres were, Southern hybridization analysis was carried out using labeled pLUS221, which contains the 1.3-

kb end *Bam*HI fragment of the chromosome (Huang *et al.*, 1998). As shown in Figure 16A, the reference strain M145 showed a positive signal at 1.3 kb, whereas no hybridizing signal was observed for mutant No. 4. As the *S. coelicolor* A3(2) chromosome has the 24-kb TIRs at the left and right ends, this result indicates that both telomeres were deleted in mutant No. 4.

To determine the range of deletions in the left and right arms, we used the original cosmid library constructed by Redenbach *et al.* (1996) as probe for hybridization, but could not identify the deletion-end cosmids. Therefore, we used another cosmid library recently constructed by Zhou *et al.* (2012) and finally identified the left deletion-end cosmid, 3-14, and the right deletion-end cosmid, 8-65. As shown in Figure 16B, cosmid 3-14 (covers nt. 230,501-274,102 of the chromosome) and cosmid 8-65 (nt. 7,790,510-7,825,517) gave fewer hybridizing signals for mutant No. 4 compared with strain M145. Referring to the restriction maps of cosmid 3-14 and 8-65, the left and right deletion ends were delimited. It was revealed that the 4.2-kb *Bam*HI fragment of cosmid 3-14 and the 13.6-kb *Bam*HI fragment extending over the right end of cosmid 8-65 were fused to generate a 3.3-kb *Bam*HI fragment (Figure 16B and Figure 17). Similarly, the 3.9-kb *Sph*I fragment of cosmid 3-14 and the 6.2-kb *Sph*I fragment of cosmid 8-65 were fused to generate a 4.2-kb *Sph*I fragment. It should be noted that cosmids 3-14 and 8-65 hybridized to the same 3.3-kb *Bam*HI fragments, and the hybridization intensity of the former was stronger than the latter. This result indicates that the 3-14 sequence in the 3.3-kb *Bam*HI fragment is larger than the 8-65 sequence.

Based on these results, two primers, del-L and del-R, were synthesized and used for PCR amplification, which gave a 1.7-kb amplified fragment. This fragment was digested with *Bam*HI and *Eco*RI and cloned into pUC19 to give plasmid pOPP.

As expected, the pOPP hybridized to both the left and right deletion-end fragments of strain M145 and to the fusion fragment of mutant No. 4 (Figure 16C).

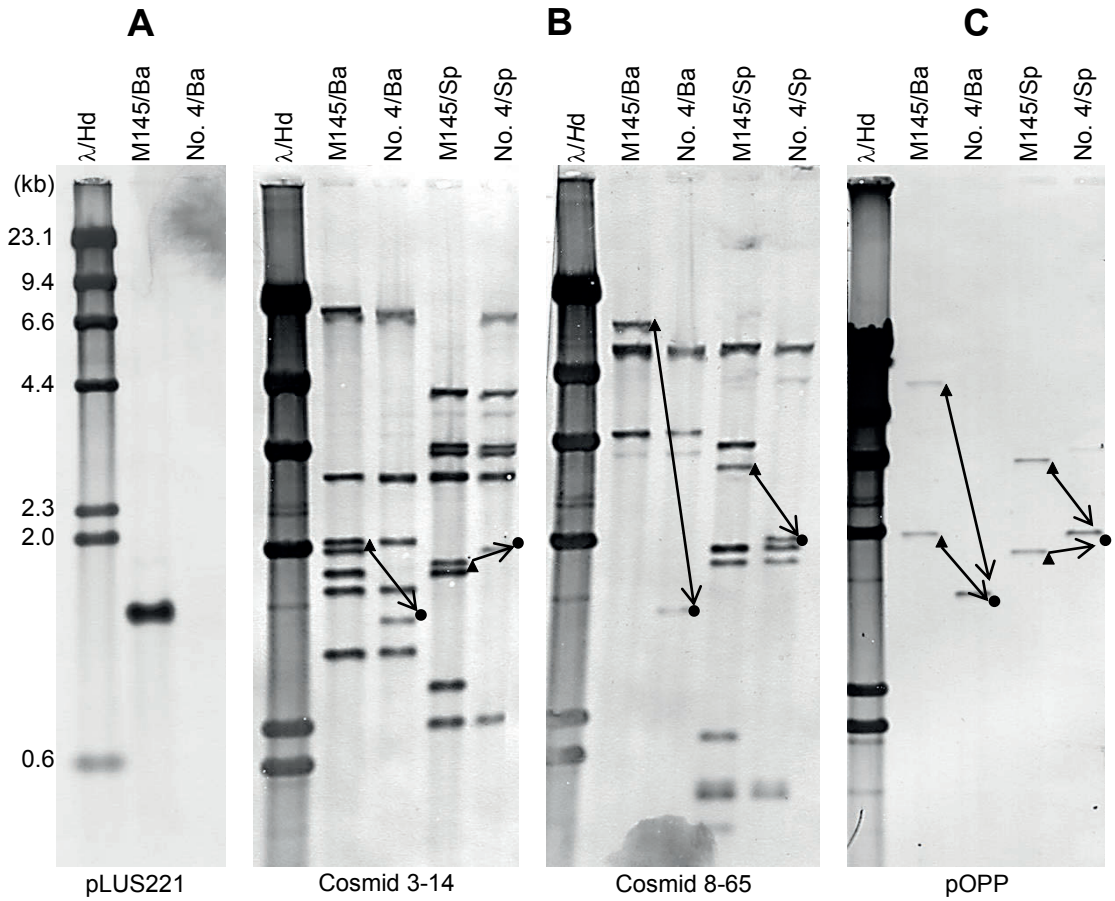

Figure 16. Southern hybridization analyses of chromosomal deletion and circularization

(a) Analysis of telomere deletion using the telomere clone pLUS221. (b) Analysis of the deletion ranges using cosmids 3-14 and 8-64. Fragments at the deletion end and newly appeared fusion fragments are marked with asterisk and connected each other by arrow. (c) Analysis of chromosomal circularization using the fusion clone pOPP. Lambda DNA digested with *Hin*dIII was used as size markers, and their sizes are shown on the left side. The hybridization probes are indicated under each figure. λ, lambda DNA; Ba, *Bam*HI; Hd, *Hin*dIII; Sp, *Sph*I.

4.3.2. Sequence analysis of the fusion junction

Nucleotide sequencing of plasmid pOPP determined the fusion junction of the circularized chromosome of mutant No. 4. The sequences around the left and right deletion ends of strain M145 and the fusion junction of mutant No. 4 are aligned and

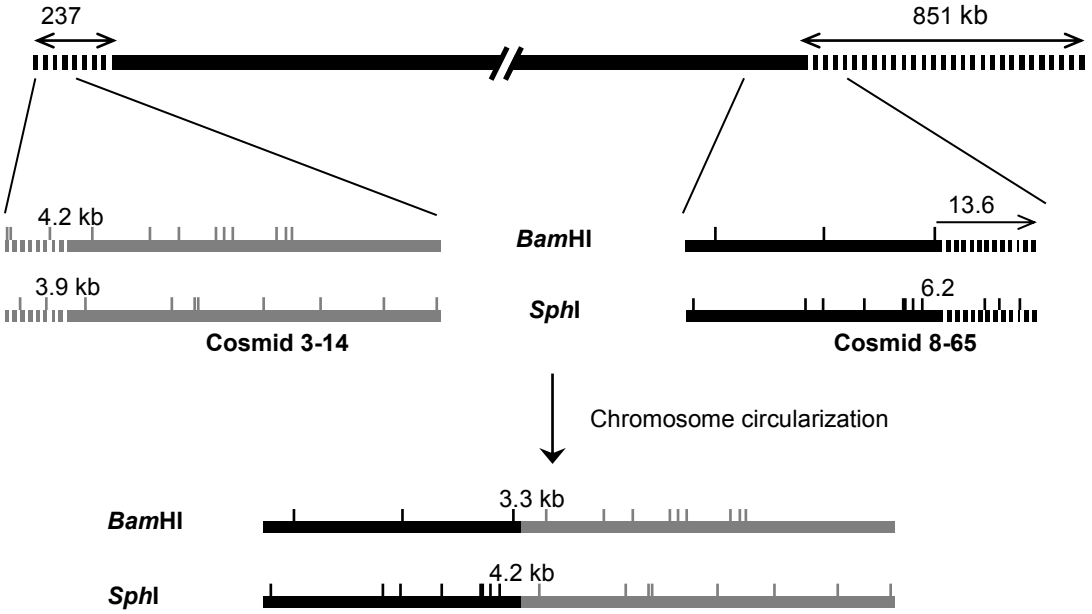

Figure 17. Restriction maps of deletion-end regions in strain 1147 and the fusion region in mutant No. 4 (deletion regions are indicated by broken lines).

compared in Figure 18. Between the left and right deletion ends, a 6-bp microhomology was identified, which is much shorter than the minimum size (20 bp) of homology required for homologous recombination (Watt *et al.*, 1985). This result again supports nonhomologous recombination of two deletion ends, which was proposed for chromosomal circularization of other *Streptomyces* species. At the left deletion end, a putative sporulation control protein, SpoOM (SCO247), is encoded. On the other hand, at the right deletion end, a possible binding-protein-dependent transport protein (SCO7030) is encoded. The generated fusion gene encodes for a protein, in which due to frame coincidence, the N-terminal 16-aa of the SCO247 protein were replaced by the N-terminal 9-aa of SCO7030. As this protein carries most (325 aa/341 aa) of the SCO247 sequence, it may function as the SCO247 protein does in the parent strain 1147.

Mutant No. 4 lost a total of 1088 kb DNA (237 kb from the left end and 851 kb from the right end) during terminal deletion and circularization. Although mutant No. 4 shows several defective phenotypes, it grows normally (Kawamoto *et al.*, 2001). Thus,

many genes located in the deleted terminal regions are not essential for survival. As the *eshA* gene is located at 131 kb from the right end, it is possible that deletion of other genes rather than *eshA* caused some of the defective phenotypes found in mutant No. 4.

Figure 18. Sequence alignments of the left and right deletion ends and the fusion junction

The aa sequences of the SCO0247 and SCO7030 proteins are shown above and below each nucleotide sequence.

4.3.3. Circularized *Streptomyces* chromosomes are stably maintained.

There have been contradictory issues on stability of circularized *Streptomyces* chromosomes. Lin & Chen (1997) and Volff *et al.* (1997) independently constructed artificially circularized chromosomes of *S. lividans* and studied their genetic instability. In both cases, the circularized chromosome showed higher frequencies of deletion and amplification. Thus, they claimed that the circularized chromosomes were more unstable than the parent linear chromosomes. However, it should be noted that in both cases, the deleted sequences in the artificially circularized chromosomes were restricted to the left and right TIR region about 30 kb each. Therefore, deletable genes such as the chloramphenicol resistance gene (*cmlR*) and the arginine biosynthetic gene (*argG*) and an amplifiable sequence such as AUD were still retained. Fischer *et al.* (1997) also prepared circularized chromosomes of *S. ambofaciens* with extremely larger deletions (more than 2 Mb) by mutagenic treatments and reported their genetic instability, too. In this case, it may be possible that the extremely large deletions eliminated regions

important for stable maintenance of the circularized chromosome.

In contrast to the examples described above, my laboratory group and other groups obtained stably maintained circular chromosomes by mutagenic treatments of *S. griseus* (Kameoka *et al.*, 1999; Inoue *et al.*, 2003), *S. coelicolor* A3(2) (this work), and *S. avermitilis* (Chen et al., 2010). In these cases, the sizes of deletions were 480 kb (130 kb at the left and 350 kb at the right end), 580 kb (30 kb + 550 kb), and 300 kb (130 kb + 170 kb) for *S. griseus*, 1088 kb (237 kb + 851 kb) for *S. coelicolor* A3(2), and 1939 kb (1611 kb + 328 kb) for *S. avermitilis*, respectively. Thus, the deletable sizes seem to have some relation to the chromosomal size of each species: 7.8 kb (calculated from *Ase*I fragments) from *S. griseus* strain 2247 (Lezhava *et al.*, 1995), 8668 kb for *S. coelicolor* A3(2) (Bentley *et al.*, 2002), and 9026 kb for *S. avermitili*s (Ikeda *et al.*, 2003). Larger chromosomes may contain larger terminal region dispensable for survival (Kirby, 2011). It was speculated that terminal deletions proceed progressively until to appropriate points, where circularized chromosomes reach a stable state and are stably maintained. Thus, mutant chromosomes with small deletions or amplification may be intermediates in this process, which are finally converted to more stable circular chromosomes.

Linear replicons always have a problem of terminal replication, namely incomplete replication of the 5'-ends. *Streptomyces* linear chromosomes solve this problem as follows. Linear chromosomes are replicated bidirectionally from an internal origin (Musialowski *et al.*, 1994). This leaves single-strand overhangs at the 3'-ends (Chang and Cohen, 1994), which are filled by a novel patching synthesis primed by terminal protein (Qin and Cohen, 1998). It was suggested that *Streptomyces* linear chromosomes were generated by integration of a linear plasmid into a circular chromosome (Volff and Altenbuchner, 2000). If circularized chromosomes are stably maintained, why have

Streptomyces kept a linear chromosome in the evolutionary history. The following advantages could be raised for linear chromosomes. (1) The genome sizes of *Streptomyces* are about two times larger than those of bacteria such as *E. coli* and *Bacillus subtilis*. Therefore, *Streptomyces* linear chromosomes could accommodate many genes that are indispensable for sophisticated morphological differentiation and adaptation to environmental changes of this soil-living genus. The size of circular chromosomes is limited, because large super-twisted circular replicons are difficult to be unwound and resolved to single strands during replication. (2) A single crossover with another linear replicon near the end of chromosome leads to end exchange (Pandza *et al.*, 1998), by which *Streptomyces* linear chromosomes could obtain genes necessary for secondary metabolism and environmental adaptation. In addition, when a single crossover occurs near the center of chromosome, it could give two chimeric chromosomes. This event actually occurred in *S. coelicolor* A3(2) strain 2106 (Yamasaki and Kinashi, 2004), which was considered as a model of chromosomal multiplication.

Structural comparison of *Streptomyces* linear replicons and studies of their genetic instability will give us important hints how circular chromosomes have been converted to linear chromosomes in the evolutionary history (Volff and Altenbuchner, 2000; Chen *et al.*, 2002; Kirby, 2011).

Chapter 5
Conclusions

Terminal deletion frequently occurred in the linear chromosome of *Streptomyces*. *Streptomyces rochei* 7434AN4, which carries three linear plasmids pSLA2-L, pSLA2-M, and pSLA2-S, showed telomere deletion concomitant with a loss of linear plasmids pSLA2-L and pSLA2-M. The chromosomal deletion in three plasmidless mutants: 2-39, YN-P7, and YN-P145, was proved by Southern hybridization analysis of fragments separated by conventional and PFGE gel electrophoreses. The fusion junction in strain 2-39 was identified by comparing the sequence data of this mutant and the wild-type 7434AN4, and a 9-bp homology was identified between the deletion ends. Southern hybridization and sequence analysis of *S. rochei* 7434AN4 revealed that the chromosome carries no intact *tap-tpg* gene pair. Our hypothesis was that the *tap-tpg* gene pair of either pSLA2-L or pSLA2-M compensates the lack of these genes on the chromosome. To prove this hypothesis, the complementation plasmid pYN15 containing *tapRM-tpgRM* from pSLA2-M, was introduced into strain 51252 (carries only pSLA2-L). Curing experiments of pSLA2-L and pYN15 from the mutants showed that the linearity of the chromosome is maintained by *tap-tpg* gene pair from either of the linear plasmids.

Chromosome circularization was also successfully determined in the model species *Streptomyces coelicolor* A3(2). Strain No. 4 is an *eshA* mutant from *S. coelicolor* strain 1147, obtained by cultivation at a high temperature. The lack of *eshA* gene that located at 131 kb from the right end of the chromosome suggests that this strain lost the right arm. Southern hybridization analysis confirmed the loss of both left and right ends of the chromosome. The cosmid library of this strain, constructed by Redenbach *et al.* (1996) and Zhou *et al.* (2012), was used to determine the deletion range in mutant No. 4.

Additional analyses revealed that mutant No. 4 lost 237 kb from the left end and 851 kb from the right end of the chromosome. The fusion fragment was amplified by PCR method, and sequence analysis identified 6-bp of homology between the right and left deletion ends.

Thus, the chromosomal telomere deletion in *S. rochei* and *S. coelicolor* A3(2) analyzed in this study led to chromosome circularization. Short homology observed in the fusion junction (9-bp in *S. rochei* strain 2-39 and 6-bp in *S. coelicolor* strain No. 4) suggests that chromosomal circularization occurred by nonhomologous recombination of two deletion ends.

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References

Altenbuchner, J. and Cullum, J. (1985). "Structure of an amplifiable DNA sequence in *Streptomyces lividans* 66." *Mol. Gen. Genet.* **201**(2): 192-197.

Bao, K. and Cohen, S. N. (2001). "Terminal proteins essential for the replication of linear plasmids and chromosomes in *Streptomyces*." *Genes Dev.* **15**(12): 1518-1527.

Bao, K. and Cohen, S. N. (2003). "Recruitment of terminal protein to the ends of *Streptomyces* linear plasmids and chromosomes by a novel telomere-binding protein essential for linear DNA replication." *Genes Dev.* **17**(6): 774-785.

Bentley, S., Brown, S., Murphy, L., Harris, D., Quail, M., Parkhill, J., Barrell, B., McCormick, J., Santamaria, R. and Losick, R. (2004). "SCP1, a 356,023bp linear plasmid adapted to the ecology and developmental biology of its host, *Streptomyces coelicolor* A3(2)." *Mol. Microbiol.* **51**(6): 1615 - 1628.

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J. and Hopwood, D. A. (2002). "Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)." *Nature*. **417**(6885): 141-147.

Bey, S.-J., Tsou, M.-F., Huang, C.-H., Yang, C.-C. and Chen, C. W. (2000). "The homologous terminal sequence of the *Streptomyces lividans* chromosome and SLP2 plasmid." *Microbiology*. **146**(4): 911-922.

Calcutt, M. J. and Schmidt, F. J. (1992). "Conserved gene arrangement in the origin region of the *Streptomyces coelicolor* chromosome." *J. Bacteriol.* **174**(10): 3220-3226.

Chang, P. C. and Cohen, S. N. (1994). "Bidirectional replication from an internal origin in a linear *Streptomyces* plasmid." *Science*. **265**(5174): 952-954.

Chater, K. F. and Kinashi, H. (2007). *Streptomyces* Linear Plasmids: Their Discovery, Functions, Interactions with Other Replicons, and Evolutionary Significance. Microbial Linear Plasmids. F. Meinhardt and R. Klassen, Springer Berlin Heidelberg. **7:** 1-31.

Chen, C. W., Huang, C.-H., Lee, H.-H., Tsai, H.-H. and Kirby, R. (2002). "Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes." *Trends Genet.* **18**(10): 522-529.

Chen, W., He, F., Zhang, X., Chen, Z., Wen, Y. and Li, J. (2010). "Chromosomal instability in *Streptomyces avermitilis*: major deletion in the central region and stable circularized chromosome." *BMC Microbiology*. **10**(1): 198.

Fischer, G., Decaris, B. and Leblond, P. (1997). "Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA." *J. Bacteriol.* **179**(14): 4553-4558.

Fischer, G., Wenner, T., Decaris, B. and Leblond, P. (1998). "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**(24): 14296-14301.

Goshi, K., Uchida, T., Lezhava, A., Yamasaki, M., Hiratsu, K., Shinkawa, H. and Kinashi, H. (2002). "Cloning and analysis of the telomere and terminal inverted repeat of the linear chromosome of *Streptomyces griseus*." *J. Bacteriol.* **184**(12): 3411-3415.

Gravius, B., Glocker, D., Pigac, J., Pandza, K., Hranueli, D. and Cullum, J. (1994). "The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome." *Microbiology*. **140**(9): 2271-2277.

Hanafusa, T. and Kinashi, H. (1992). "The structure of an integrated copy of the giant linear plasmid SCP1 in the chromosome of *Streptomyces coelicolor* 2612." *Mol. Gen. Genet.* **231**(3): 363-368.

Hayakawa, T., Tanaka, T., Sakaguchi, K., Otake, N. and Yonehara, H. (1979). "A linear plasmid-like DNA in *Streptomyces sp.* producing lankacidin group antibiotics." *J. Gen. Appl. Microbiol.* **25**(4): 255-260.

Hinnebusch, J. and Tilly, K. (1993). "Linear plasmids and chromosomes in bacteria." *Mol. Microbiol.* **10**(5): 917-922.

Hiratsu, K., Mochizuki, S. and Kinashi, H. (2000). "Cloning and analysis of the replication origin and the telomeres of the large linear plasmid pSLA2-L in *Streptomyces rochei*." *Mol. Gen. Genet.* **263**(6): 1015-1021.

Huang, C.-H., Tsai, H.-H., Tsay, Y.-G., Chien, Y.-N., Wang, S.-L., Cheng, M.-Y., Ke, C.-H. and Chen, C. W. (2007). "The telomere system of the *Streptomyces* linear plasmid SCP1 represents a novel class." *Mol. Microbiol.* **63**(6): 1710-1718.

Huang, C. H., Lin, Y. S., Yang, Y. L., Huang, S. W. and Chen, C. W. (1998). "The telomeres of *Streptomyces* chromosomes contain conserved palindromic sequences with potential to form complex secondary structures." *Mol. Microbiol.* **28**(5): 905-916.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M. and Ōmura, S. (2003). "Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*." *Nat. Biotechnol.* **21**(5): 526 - 531.

Inoue, S., Higashiyama, K., Uchida, T., Hiratsu, K. and Kinashi, H. (2003). "Chromosomal circularization in *Streptomyces griseus* by nonhomologous recombination of deletion ends." *Biosci. Biotechnol. Biochem.* **67**(5): 1101-1108.

Ishikawa, J. and Hotta, K. (1999). "FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content." *FEMS Microbiol. Lett.* **174**(2): 251-253.

Ishikawa, J., Niino, Y. and Hotta, K. (1996). "Construction of pRES18 and pRES19, *Streptomyces-Escherichia coli* shuttle vectors carrying multiple cloning sites." *FEMS Microbiol. Lett.* **145**(1): 13-116.

Kameoka, D., Lezhava, A., Zenitani, H., Hiratsu, K., Kawamoto, M., Goshi, K., Inada, K., Shinkawa, H. and Kinashi, H. (1999). "Analysis of fusion junctions of circularized chromosomes in *Streptomyces griseus*." *J. Bacteriol.* **181**(18): 5711-5717.

Kawamoto, S., Watanabe, M., Saito, N., Hesketh, A., Vachalova, K., Matsubara, K. and Ochi, K. (2001). "Molecular and functional analyses of the gene (eshA) encoding the 52-kilodalton protein of *Streptomyces coelicolor* A3(2) required for antibiotic production." *J. Bacteriol.* **183**(20): 6009-6016.

Kieser, H. M., Kieser, T. and Hopwood, D. A. (1992). "A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome." *J. Bacteriol.* **174**(17): 5496-5507.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. and Hopwood, D. A., Eds. (2000).

Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, England.
Kinashi, H. (2008). "Antibiotic production, linear plasmids and linear chromosomes in *Streptomyces*." *Actinomycetologica*. **22**(1): 20-29.
Kinashi, H. (2011). "Giant linear plasmids in *Streptomyces*: a treasure trove of antibiotic biosynthetic clusters." *J. Antibiot.* **64**(1): 19-25.
Kinashi, H., Fujii, S., Hatani, A., Kurokawa, T. and Shinkawa, H. (1998). "Physical Mapping of the Linear Plasmid pSLA2-L and Localization of the eryAI and actI Homologs." *Biosci. Biotechnol. Biochem.* **62**(10): 1892-1897.
Kinashi, H., Mori, E., Hatani, A. and Nimi, O. (1994). "Isolation and characterization of linear plasmids from lankacidin-producing *Streptomyces* species." *J. Antibiot.* **47**(12): 1447-1455.
Kinashi, H. and Shimaji-Murayama, M. (1991). "Physical characterization of SCP1, a giant linear plasmid from *Streptomyces coelicolor*." *J. Bacteriol.* **173**(4): 1523-1529.
Kinashi, H., Shimaji-Murayama, M. and Hanafusa, T. (1991). "Nucleotide sequence analysis of the unusually long terminal inverted repeats of a giant linear plasmid, SCP1." *Plasmid*. **26**(2): 123-130.
Kinashi, H. and Shimaji, M. (1987). "Detection of giant linear plasmids in antibiotic producing strains of *Streptomyces* by the OFAGE technique." *J. Antibiot.* **40**(6): 913-916.
Kinashi, H., Shimaji, M. and Sakai, A. (1987). "Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes." *Nature*. **328**: 454-456.
Kirby, R. (2011). "Chromosome diversity and similarity within the Actinomycetales." *FEMS Microbiol. Lett.* **319**(1): 1-10.
Kirby, R. and Hopwood, D. A. (1977). "Genetic determination of methylenomycin synthesis by the SCP1 plasmid of *Streptomyces coelicolor* A3(2)." *J. Gen. Microbiol.* **98**(1): 239-252.
Kirby, R., Wright, L. F. and Hopwood, D. A. (1975). "Plasmid-determined antibiotic synthesis and resistance in *Streptomyces coelicolor*." *Nature*. **254**(5497): 265-267.
Kwak, J., McCue, L. A., Trczianka, K. and Kendrick, K. E. (2001). "Identification and characterization of a developmentally regulated protein, EshA, required for sporogenic hyphal branches in *Streptomyces griseus*." *J. Bacteriol.* **183**(10): 3004-3015.
Leblond, P. and Decaris, B. (1994). "New insights into the genetic instability of *Streptomyces*." *FEMS Microbiol. Lett.* **123**(3): 225-232.
Leblond, P., Demuyter, P., Simonet, J. M. and Decaris, B. (1991). "Genetic instability and associated genome plasticity in *Streptomyces ambofaciens*: pulsed-field gel electrophoresis evidence for large DNA alterations in a limited genomic region." *J. Bacteriol.* **173**(13): 4229-4233.
Leblond, P., Fischer, G., Francou, F.-X., Berger, F., Guérineau, M. and Decaris, B. (1996). "The unstable region of *Streptomyces ambofaciens* includes 210kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA." *Mol. Microbiol.* **19**(2): 261-271.
Leblond, P., Redenbach, M. and Cullum, J. (1993). "Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2)." *J. Bacteriol.* **175**(11): 3422-3429.
Lezhava, A., Mizukami, T., Kajitani, T., Kameoka, D., Redenbach, M., Shinkawa, H., Nimi, O. and Kinashi, H. (1995). "Physical map of the linear chromosome of *Streptomyces griseus*." *J. Bacteriol.* **177**(22): 6492-6498.

Lin, Y.-S., Kieser, H. M., Hopwood, D. A. and Chen, C. W. (1993). "The chromosomal DNA of *Streptomyces lividans* 66 is linear." *Mol. Microbiol.* **10**(5): 923-933.

Lin, Y. S. and Chen, C. W. (1997). "Instability of artificially circularized chromosomes of *Streptomyces lividans*." *Mol. Microbiol.* **26**(4): 709-719.

Mochizuki, S., Hiratsu, K., Suwa, M., Ishii, T., Sugino, F., Yamada, K. and Kinashi, H. (2003). "The large linear plasmid pSLA2-L of *Streptomyces rochei* has an unusually condensed gene organization for secondary metabolism." *Mol. Microbiol.* **48**(6): 1501-1510.

Musialowski, M. S., Flett, F., Scott, G. B., Hobbs, G., Smith, C. P. and Oliver, S. G. (1994). "Functional evidence that the principal DNA replication origin of the *Streptomyces coelicolor* chromosome is close to the *dnaA-gyrB* region." *J. Bacteriol.* **176**(16): 5123-5125.

Netolitzky, D. J., Wu, X., Jensen, S. E. and Roy, K. L. (1995). "Giant linear plasmids of β-lactam antibiotic producing *Streptomyces*." *FEMS Microbiol. Lett.* **131**(1): 27-34.

Nindita, Y., Nishikawa, T., Arakawa, K., Wang, G., Ochi, K., Qin, Z. and Kinashi, H. (2013). "Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2)." *FEMS Microbiol. Lett.* **347**(2): 149-155.

Novakova, R., Knirschova, R., Farkasovsky, M., Feckova, L., Rehakova, A., Mingyar, E. and Kormanec, J. (2013). "The gene cluster aur1 for the angucycline antibiotic auricin is located on a large linear plasmid pSA3239 in *Streptomyces aureofaciens* CCM 3239." *FEMS Microbiol. Lett.* **342**(2): 130-137.

Pandza, S., Biuković, G., Paravić, A., Dadbin, A., Cullum, J. and Hranueli, D. (1998). "Recombination between the linear plasmid pPZG101 and the linear chromosome of *Streptomyces rimosus* can lead to exchange of ends." *Mol. Microbiol.* **28**(6): 1165-1176.

Pang, X., Aigle, B., Girardet, J.-M., Mangenot, S., Pernodet, J.-L., Decaris, B. and Leblond, P. (2004). "Functional angucycline-like antibiotic gene cluster in the terminal inverted repeats of the *Streptomyces ambofaciens* linear chromosome." *Antimicrob. Agents Chemother.* **48**(2): 575-588.

Qin, Z. and Cohen, S. N. (1998). "Replication at the telomeres of the *Streptomyces* linear plasmid pSLA2." *Mol. Microbiol.* **28**(5): 893-903.

Qin, Z. and Cohen, S. N. (2002). "Survival mechanisms for *Streptomyces* linear replicons after telomere damage." *Mol. Microbiol.* **45**(3): 785-794.

Redenbach, M., Flett, F., Piendl, W., Glocker, I., Rauland, U., Wafzig, O., Kliem, R., Leblond, P. and Cullum, J. (1993). "The *Streptomyces lividans* 66 chromosome contains a 1 MB deletogenic region flanked by two amplifiable regions." *Mol. Gene. Genet.* **241**(3): 255-262.

Redenbach, M., Kieser, H. M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H. and Hopwood, D. A. (1996). "A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome." *Mol. Microbiol.* **21**(1): 77-96.

Saito, N., Xu, J., Hosaka, T., Okamoto, S., Aoki, H., Bibb, M. J. and Ochi, K. (2006). "EshA accentuates ppGpp accumulation and is conditionally required for antibiotic production in *Streptomyces coelicolor* A3(2)." *J. Bacteriol.* **188**(13): 4952-4961.

Sakaguchi, K. (1990). "Invertrons, a class of structurally and functionally related genetic elements that includes linear DNA plasmids, transposable elements, and genomes of adeno-type viruses." *Microbiol. Mol. Biol. Rev.* **54**(1): 66-74.

Sambrook, J. and Russell, D. W. (2001). Molecular Cloning, A laboratory manual. 3.

Cold Spring Harbor Laboratory Press, New York, NY.
Suwa, M., Sugino, H., Sasaoka, A., Mori, E., Fujii, S., Shinkawa, H., Nimi, O. and Kinashi, H. (2000). "Identification of two polyketide synthase gene clusters on the linear plasmid pSLA2-L in *Streptomyces rochei*." *Gene*. **246**(1-2): 123-131.
Suzuki, H., Marushima, K., Ohnishi, Y. and Horinouchi, S. (2008). "A novel pair of terminal protein and telomere-associated protein for replication of the linear chromosome of *Streptomyces griseus* IFO13350." *Biosci. Biotechnol. Biochem.* **72**(11): 2973-2980.
Uchida, T., Ishihara, N., Zenitani, H., Hiratsu, K. and Kinashi, H. (2004). "Circularized chromosome with a large palindromic structure in *Streptomyces griseus* mutants." *J. Bacteriol.* **186**(11): 3313-3320.
Uchida, T., Miyawaki, M. and Kinashi, H. (2003). "Chromosomal arm replacement in *Streptomyces griseus*." *J. Bacteriol.* **185**(3): 1120-1124.
Volff, J. N. and Altenbuchner, J. (1998). "Genetic instability of the *Streptomyces* chromosome." *Mol. Microbiol.* **27**(2): 239-246.
Volff, J. N. and Altenbuchner, J. (2000). "A new beginning with new ends: linearisation of circular chromosomes during bacterial evolution." *FEMS Microbiol. Lett.* **186**(2): 143-150.
Volff, J. N., Viell, P. and Altenbuchner, J. (1997). "Artificial circularization of the chromosome with concomitant deletion of its terminal inverted repeats enhances genetic instability and genome rearrangement in *Streptomyces lividans*." *Mol. Gen. Genet.* **253**(6): 753-760.
Watt, V. M., Ingles, C. J., Urdea, M. S. and Rutter, W. J. (1985). "Homology requirements for recombination in *Escherichia coli*." *Pro. Natl. Acad. Sci.* **82**(14): 4768-4772.
Widenbrant, E. M., Tsai, H.-H., Chen, C. W. and Kao, C. M. (2007). "*Streptomyces coelicolor* undergoes spontaneous chromosomal end replacement." *J. Bacteriol.* **189**(24): 9117-9121.
Yamasaki, M., Ikuto, Y., Ohira, A., Chater, K. and Kinashi, H. (2003). "Limited regions of homology between linear and circular plasmids encoding methylenomycin biosynthesis in two independently isolated streptomycetes." *Microbiology*. **149**(5): 1351-1356.
Yamasaki, M. and Kinashi, H. (2004). "Two chimeric chromosomes of *Streptomyces coelicolor* A3(2) generated by single crossover of the wild-type chromosome and linear plasmid SCP1." *J. Bacteriol.* **186**(19): 6553-6559.
Yamasaki, M., Redenbach, M. and Kinashi, H. (2001). "Integrated structures of the linear plasmid SCP1 in two bidirectional donor strains of *Streptomyces coelicolor* A3(2)." *Mol. Gen. Genet.* **264**(5): 634-642.
Yang, C. C., Chen, Y. H., Tsai, H. H., Huang, C. H., Huang, T. W. and Chen, C. W. (2006). "In Vitro Deoxynucleotidylation of the Terminal Protein of *Streptomyces* Linear Chromosomes." *Applied and Environmental Microbiology*. **72**(12): 7959-7961.
Yang, Y., Kurokawa, T., Takahama, Y., Nindita, Y., Mochizuki, S., Arakawa, K., Endo, S. and Kinashi, H. (2011). "pSLA2-M of *Streptomyces rochei* is a composite linear plasmid characterized by self-defense genes and homology with pSLA2-L." *Biosci. Biotechnol. Biochem.* **75**(6): 1147-1153.
Zakrzewska-Czerwinska, J. and Schrempf, H. (1992). "Characterization of an autonomously replicating region from the *Streptomyces lividans* chromosome." *J. Bacteriol.* **174**(8): 2688-2693.
Zerbino, D. R. and Birney, E. (2008). "Velvet: Algorithms for de novo short read

assembly using de Bruijn graphs." *Genome Res.* **18**(5): 821-829.

Zhang, H., Shinkawa, H., Ishikawa, J., Kinashi, H. and Nimi, O. (1997). "Improvement of transformation system in *Streptomyces* using a modified regeneration medium." *J. Ferment. Bioeng.* **83**(3): 217-221.

Zhou, M., Jing, X., Xie, P., Chen, W., Wang, T., Xia, H. and Qin, Z. (2012). "Sequential deletion of all the polyketide synthase and nonribosomal peptide synthetase biosynthetic gene clusters and a 900-kb subtelomeric sequence of the linear chromosome of *Streptomyces coelicolor*." *FEMS Microbiol. Lett.* **333**(2): 169-179.

公表論文
（Articles）

1. The *tap-tpg* gene pair on the linear plasmid functions to maintain a linear topology of the chromosome in *Streptomyces rochei*.
   Yosi Nindita, Zhisheng Cao, Yingjie Yang, Kenji Arakawa, Yuh Shiwa, Hirofumi Yoshikawa, Michihira Tagami, Alexander Lezhava and Haruyasu Kinashi.
   Molecular Microbiology, **95**(5): 846-858 (2015).

2. Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2).
   Yosi Nindita, Tomoya Nishikawa, Kenji Arakawa, Guojun Wang, Kozi Ochi, Zhongjun Qin and Haruyasu Kinashi.
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The *tap-tpg* gene pair on the linear plasmid functions to maintain a linear topology of the chromosome in *Streptomyces rochei*

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Summary

***Streptomyces rochei* 7434AN4 carries three linear plasmids, pSLA2-L (211 kb), pSLA2-M (113 kb) and pSLA2-S (18 kb), their complete nucleotide sequences having been determined. Restriction and sequencing analysis revealed that the telomere sequences at both ends of the linear chromosome are identical to each other, are 98.5% identical to the right end sequences of pSLA2-L and pSLA2-M up to 3.1 kb from the ends and have homology to those of typical *Streptomyces* species. Mutant 2-39, which lost all the three linear plasmids, was found to carry a circularized chromosome. Sequence comparison of the fusion junction and both deletion ends revealed that chromosomal circularization occurred by terminal deletions followed by nonhomologous recombination. Curing of pSLA2-L from strain 51252, which carries only pSLA2-L, also resulted in terminal deletions in newly obtained mutants. The *tap-tpg* gene pair, which encodes a telomere-associated protein and a terminal protein for end patching, is located on pSLA2-L and pSLA2–M but has not hitherto been found on the chromosome. These results led us to the idea that the *tap-tpg* of pSLA2-L or pSLA2-M functions to maintain a linear chromosome in strain 7434AN4. This hypothesis was finally confirmed by complementation and curing experiments of the *tap-tpg* of pSLA2-M.**

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Introduction

The saprophytic and filamentous soil bacteria, *Streptomyces*, are well known to produce varieties of secondary metabolites including antibiotics, anticancer agents, immunosuppressants, herbicides and enzyme inhibitors. It is also a characteristic feature of this genus to carry an 8–9 Mb linear chromosome in place of a usual circular bacterial chromosome (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). In addition, *Streptomyces* frequently possess multiple linear plasmids with principally same structural features (Hayakawa *et al.*, 1979; Kinashi *et al.*, 1987; Kinashi, 1994). Namely, *Streptomyces* linear chromosomes and plasmids have inverted repeat sequences at the left and right ends and the 5'-ends are covalently bound to a terminal protein (TP). Accumulated data suggest that *Streptomyces* linear plasmids have played critical roles in genome evolution and horizontal transfer of secondary metabolism (Chen *et al.*, 2002; Chater and Kinashi, 2007; Kinashi, 2008; 2011). However, involvement of *Streptomyces* linear plasmids in host primary metabolism has not been reported.

Streptomyces rochei 7434AN4 used in this study carries three linear plasmids, pSLA2-L (210 614 bp), pSLA2-M (113 464 bp) and pSLA2-S (17 526 bp) (Hayakawa *et al.*, 1979; Kinashi *et al.*, 1994), their complete nucleotide sequences having been determined. Two thirds of the largest linear plasmid pSLA2-L is occupied by secondary metabolism-related genes, including the biosynthetic gene clusters for macrolide antibiotics, lankacidin and lankamycin, a cryptic type-II polyketide and a carotenoid (Mochizuki *et al.*, 2003). pSLA2-M contains self-defense genes such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated genes and a *ku70/ku80*-like gene (Yang *et al.*, 2011), which may be involved in immunity against phage infection and repair of double-strand DNA breaks respectively. Both pSLA2-L and pSLA2-M contain a *tap-tpg* gene pair, encoding a telomere-associated protein (Tap) and a TP for end patching. Bidirectional replication of *Streptomyces* linear plasmids and

chromosomes from a central origin generates 3'-leading-strand overhangs at the telomeres, which require end patching to produce full-length duplex DNA molecules (Qin and Cohen, 1998). In the latter process, Tap recruits TP to the 3'-end overhang (Bao and Cohen, 2003) and TP functions as a primer for DNA synthesis (Qin and Cohen, 1998; Yang *et al*., 2006). In contrast to pSLA2-L and pSLA2-M, the smallest linear plasmid pSLA2-S does not contain a *tap-tpg* gene pair or biosynthetic genes (DDBJ AB905438, see Supporting Information Table S1 for the accession numbers of the *S. rochei* sequences described in the text).

We have started the whole genome shotgun sequencing project of the wild-type strain 7434AN4 in collaboration with RIKEN, Tokyo University of Agriculture and the National Institute of Infectious Diseases (NIID). In addition, we tried to clone the telomere fragments of the *S. rochei* chromosome. For this purpose, we chose at first a plasmid-less mutant 2-39, because it carries none of the three linear plasmids (Kinashi *et al*., 1994) and therefore was expected to avoid confusion in sequence analysis. However, to our surprise, this mutant did not show a hybridizing signal of telomere, which suggests that terminal deletions and circularization of the chromosome have occurred concomitant with a plasmid loss. This idea was confirmed by finding of a fusion sequence in the 2-39 genome library. In addition, the data hitherto obtained from the genome project of strain 7434AN4 suggest that the chromosome itself does not contain a *tap-tpg* gene pair. This result led us to the idea that the *tap-tpg* of pSLA2-L or pSLA2-M functions to maintain a linear topology of the chromosome in strain 7434AN4. This hypothesis was finally confirmed by complementation and curing experiments of the *tap-tpg* of pSLA2-M in plasmid-less strains of *S. rochei*. The details of these experimental processes are described in this paper.

Results

Telomere sequences of the chromosome are identical to the right end sequences of pSLA2-L and pSLA2-M

Although the complete nucleotide sequences of the three linear plasmids, pSLA2-L (Mochizuki *et al*., 2003, AB088224), pSLA2-M (Yang *et al*., 2011, AB597522) and pSLA2-S (AB905437), have been determined, little was known about the chromosomal sequence of *S. rochei* 7434AN4 including the telomere sequences. Concerning the relationship between the telomere sequences of *Streptomyces* linear chromosomes and coexisting linear plasmids, two different cases have been reported. The telomere sequences of the *Streptomyces lividans* chromosome and the right end sequence of linear plasmid SLP2 are identical (Huang *et al*., 2003). On the other hand, the telomere sequences of the *Streptomyces coelicolor* A3(2) chromosome (Bentley *et al*., 2002) and linear plasmid SCP1 (Kinashi *et al*., 1991; Bentley *et al*., 2004) were totally different. Consequently, the presence of an additional gene pair, essential for end patching of SCP1, was expected, and a novel type of gene pair, *tac* (telomere-associated protein of SCP1)-*tpc* (terminal protein of SCP1), was identified on SCP1 (Huang *et al*., 2007).

In *S. rochei* 7434AN4, the right end sequences of pSLA2-L and pSLA2-M are almost (99.9%) identical up to 14.6 kb from the ends (Fig. 1A). The homology between the left and right end sequences [terminal inverted repeat (TIR)] of pSLA2-L is extending to 2.1 kb. The TIR of pSLA2-M is short (352 bp) with a relatively low similarity (321/352, 91.2% identities). Expecting a sequence similarity between the telomeres of the chromosome and pSLA2-L, 7434AN4 DNA was digested with various enzymes and probed by plasmid pEcoEnd containing the 0.4 kb right end fragment of pSLA2-L (Hiratsu *et al*., 2000). As shown in Fig. 1B, when 7434AN4 DNA was digested with BamHI, four expected signals of the linear plasmids were observed at 16.7 kb (right end of pSLA2-M = M-R), 15.1 kb (right end of pSLA2-L = L-R), 2.5 kb (left end of pSLA2-L = L-L) and 1.5 kb (left end of pSLA2-M = M-L) (see Fig. 1A for the restriction sites that gave a hybridizing signal). In addition, a new hybridizing signal appeared at 5.0 kb (Fig. 1B, indicated by asterisk). This possible telomere fragment of the chromosome was further analyzed using other restriction enzymes. When 7434AN4 DNA was digested with SacI, a hybridizing signal of the chromosome appeared at 3.7 kb, in addition to three plasmid signals at 12.3 kb (L-L), 4.7 kb (L-R and M-R) and 1.8 kb (M-L). On the other hand, when 7434AN4 DNA was digested with KpnI, BglII or EcoRI, only two plasmid signals were observed, one common signal derived from L-L, L-R and M-R and another from M-L. In the latter three cases, the telomere signal of the chromosome was overlapped with the common signal. These results indicate that the left and right telomeres of the chromosome have identical restriction sites with L-L, L-R and M-R at least up to the KpnI site (1.9 kb from the ends), giving the same KpnI (1.9 kb), BglII (0.6 kb) and EcoRI (0.4 kb) fragments.

The left and right telomeres of the chromosome were further analyzed by Southern hybridization of large fragments separated by pulsed-field gel electrophoresis (PFGE). As shown in Fig. 1C, HindIII digest of 7434AN4 DNA gave two telomere signals at 90 and 70 kb in addition to the linear plasmid signals at 159 kb (L-L) and 5.5 kb (L-R and M-R). The M-L fragment (105 kb) of pSLA2-M was not observed here because of its low homology to the 0.4 kb telomere probe. The sizes of the two telomere fragments suggest that the length of the TIR at both ends of the chromosome is shorter than 70 kb.

Because telomere fragments of the chromosome smaller than 1.9 kb are inseparable from those of L-L, L-R

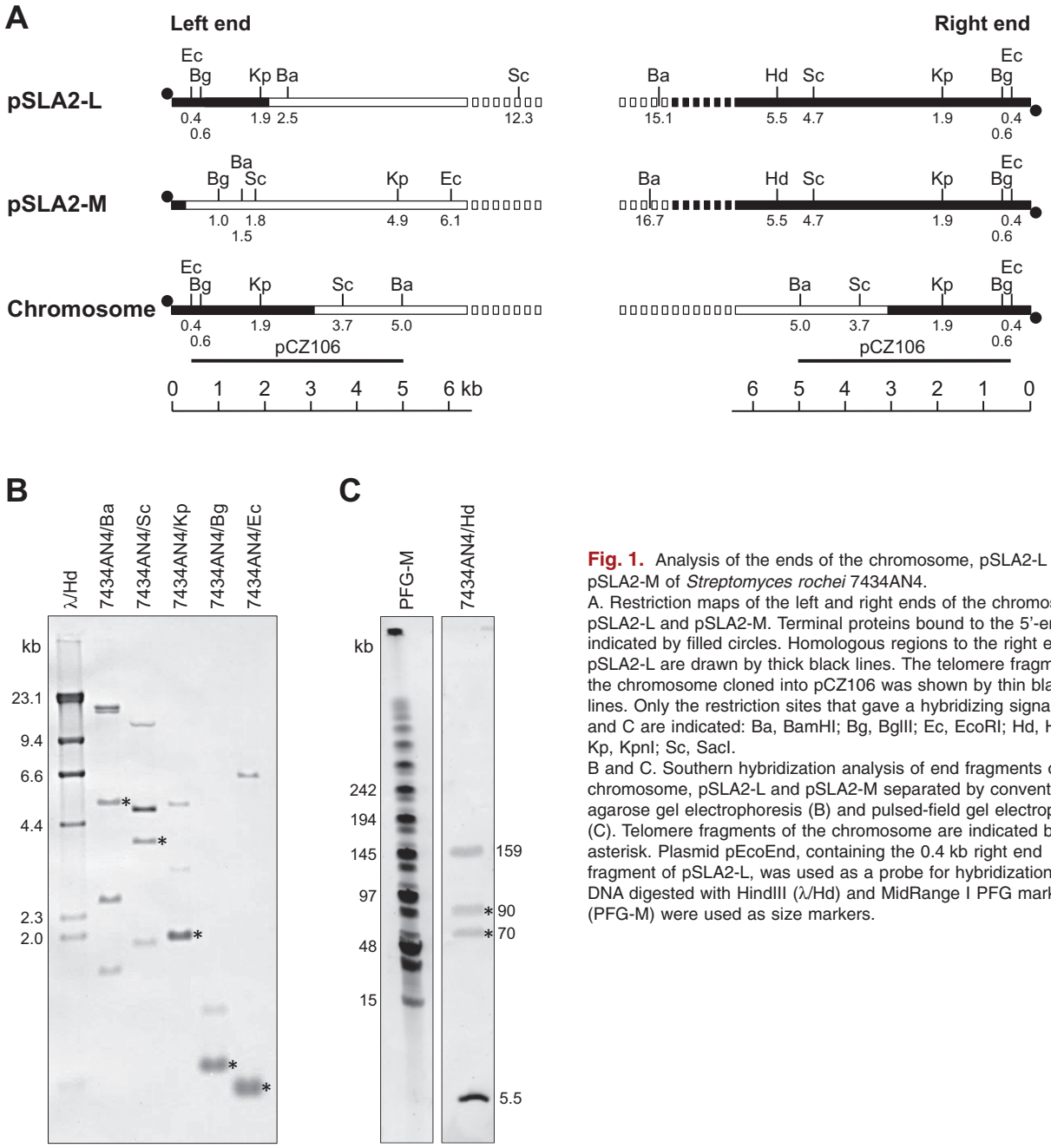


Fig. 1. Analysis of the ends of the chromosome, pSLA2-L and pSLA2-M of *Streptomyces rochei* 7434AN4.
A. Restriction maps of the left and right ends of the chromosome, pSLA2-L and pSLA2-M. Terminal proteins bound to the 5'-ends are indicated by filled circles. Homologous regions to the right end of pSLA2-L are drawn by thick black lines. The telomere fragment of the chromosome cloned into pCZ106 was shown by thin black lines. Only the restriction sites that gave a hybridizing signal in B and C are indicated: Ba, BamHI; Bg, BglII; Ec, EcoRI; Hd, HindIII; Kp, KpnI; Sc, SacI.
B and C. Southern hybridization analysis of end fragments of the chromosome, pSLA2-L and pSLA2-M separated by conventional agarose gel electrophoresis (B) and pulsed-field gel electrophoresis (C). Telomere fragments of the chromosome are indicated by asterisk. Plasmid pEcoEnd, containing the 0.4 kb right end fragment of pSLA2-L, was used as a probe for hybridization. λ DNA digested with HindIII (λ/Hd) and MidRange I PFG marker (PFG-M) were used as size markers.

and M-R, an adjacent 4.6 kb BamHI–EcoRI fragment, locating at 0.4 kb from the chromosomal end, was shotgun cloned (pCZ106) and sequenced (AB905441). Comparison of the sequences of pCZ106 and L-R revealed that their homology is extending up to 2.7 kb from the EcoRI site (2590/2636, 98.3 % identities). We speculate that the 0.4 kb extreme end sequences of the left and right telomeres of the chromosome (C-R and C-L), L-L, L-R and M-R are identical based on the following results: (i) C-R, C-L, L-L, L-R and M-R gave the same hybridizing signals when digested with KpnI, BglII and EcoRI (Fig. 1B). (ii) L-L, L-R and M-R have been cloned and sequenced, which revealed their identical end sequence (Hiratsu *et al.*, 2000; Mochizuki *et al.*, 2003; Yang *et al.*, 2011). (iii) In the ongoing genome project of strain 7434AN4, no sequence heterogeneity has been detected in the 0.4 kb extreme end region derived from the chromosome, pSLA2-L and pSLA2-M. Collectively, we concluded that the left and right telomere sequences of the chromosome are identical to each other and are

98.5% (3027/3073) identical to the right end sequence of pSLA2-L and pSLA2-M up to 3.1 kb from the ends (100% identical up to nt 1937).

Streptomyces linear chromosomes and plasmids have a conserved sequence at the extreme ends, and their 3'-leading-strand overhangs can possibly form a Y-shaped secondary foldback structure (Huang *et al*., 1998; Qin and Cohen, 1998). This structure may be recognized by Tap and TP to initiate a protein-primed DNA synthesis for end patching (Bao and Cohen, 2003). The telomere sequence of the *S. rochei* chromosome also shows high similarity to typical telomere sequences (Fig. 2A) and therefore its 3' single-stranded DNA can form a Y-shaped foldback structure (Fig. 2B), where sequences II and III and sequences IV and V form two hairpin loops and sequences I and VI form a base part (see later for further discussion).

Chromosomal circularization occurred in mutant 2-39 concomitant with a plasmid loss

The plasmid-less strain 2-39 has been obtained by protoplast regeneration of strain 51252, which carries only pSLA2-L (Kinashi *et al*., 1994). Strain 2-39 did not give a telomere signal when probed by the telomere clone pEcoEnd (data not shown), indicating terminal deletions of the chromosome. *Streptomyces* linear chromosomes frequently suffer deletions at both ends spontaneously or by various mutagenic treatments, leading to chromosomal circularization, arm replacement and amplification (Volff and Altenbuchner, 1998), among which circularization occurs most frequently.

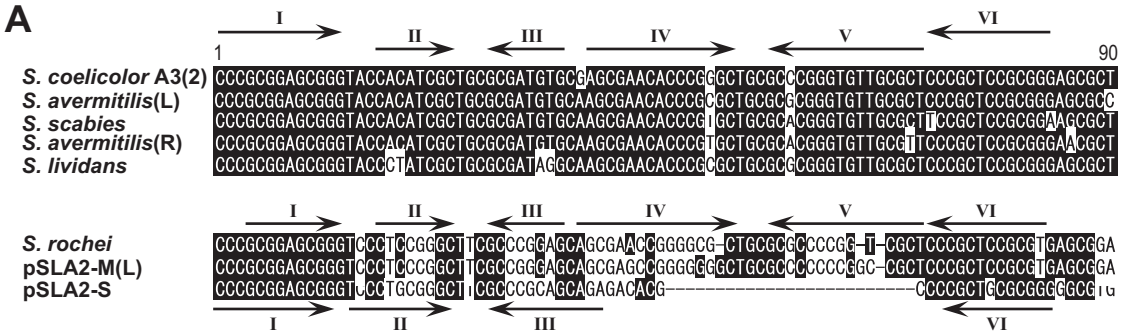
To know if chromosomal circularization also occurred in strain 2-39, we searched for a fusion sequence in its genome library and found a candidate contig 229. We considered this as a fusion contig, because it contains on each side two independent sequences of contigs 634 and 586 of strain 7434AN4. Based on the sequence of contig 229, a 2.3 kb fusion fragment was amplified and cloned from 2-39 DNA to give plasmid pCZ117. The nucleotide sequence of pCZ117 was identical to that of contig 229 (Fig. 3A), which indicates that this contig was not an artifact generated during construction of the library. Chromosomal circularization was further analyzed by Southern hybridization using pCZ117 as a probe. As shown in Fig. 3B, AflIII digest of 51252 DNA gave two hybridizing signals of the deletion end fragments at 4.8 and 1.7 kb, while that of 2-39 DNA gave a signal of the fusion fragment at 4.2 kb. Similar results were obtained for large fragments separated by PFGE. HindIII digests of 51252 and 2-39 DNAs showed two deletion end fragments at 500 and 450 kb and one fusion fragment at 650 kb respectively (Fig. 3C).

The fusion sequence of the 2-39 chromosome (contig 229) and the corresponding deletion end sequences of the 7434AN4 chromosome (contigs 634 and 586) are aligned and compared in Fig. 3A. Between two deletion ends of the chromosome, a 9 bp microhomology was identified, which is shorter than 20 bp required for homologous recombination in *Escherichia coli* (Watt *et al*., 1985). At the deletion end in contig 634, a cell division protein (FtsK, 1331 aa) is encoded. On the other hand, at the other deletion end in contig 586, a putative nucleotide-binding protein (NBP; 423 aa) is encoded. The generated fusion gene encodes for a protein (1162 aa), in which because of frame coincidence, N-terminal 422 aa of the FtsK protein was replaced by N-terminal 253 aa of the NBP.

To know whether chromosomal circularization was specific to mutant 2-39 or is general in plasmid-less mutants, strain 51252 was subjected to protoplast regeneration to cure pSLA2-L. By using the antibiotic-producing ability coded on pSLA2-L as a marker, two non-producers, YN-P7 and YN-P145, were selected from 192 regenerated colonies. When probed by the telomere clone, two colonies did not show the telomere signals of the chromosome or pSLA2-L (data not shown). This result indicates that two events, curing of pSLA2-L and terminal deletions of the chromosome, have occurred concomitantly in the newly obtained plasmid-less strains, too, which possibly led to chromosomal circularization. To analyze the deletion sizes in mutants, YN-P7 and YN-P145, their total DNAs were digested with AflIII and probed by the fusion plasmid pCZ117 cloned from mutant 2-39. As shown in Fig. 3B, mutant YN-P7 showed two intact signals (4.8 kb and 1.7 kb) of both deletion end fragments, while mutant YN-P145 gave only a signal (4.8 kb) of one deletion end fragment. Similar results were obtained for large fragments separated by PFGE. HindIII digest of YN-P7 DNA showed two hybridizing signals of both deletion end fragments at 500 and 450 kb, while that of YN-P145 showed a signal of one deletion end fragment at 500 kb. These results indicate that deletion sizes at the left and right chromosomal arms were different from strain to strain, and therefore there were no hot spots for terminal deletion.

Introduced tap-tpg *of pSLA2-M functioned to maintain a linear chromosome*

As described above, the genome project of the wild-type strain 7434AN4 is in progress. This strain carries all of the three linear plasmids, pSLA2-L, pSLA2-M and pSLA2-S. Consequently, we identified the *tap-tpg* gene pairs of pSLA2-L (*tapR1-tpgR1*) and pSLA2-M (*tapRM-tpgRM*) in the genome library of strain 7434AN4. However, we have not found an additional *tap-tpg* pair of the chromosome at this stage, where a draft assembly with ca. 450-fold genome coverage gave 340 contigs containing over 500 bp nucleotides (8999 kb in total size). Instead, we identified one gene in contig 95, which


A
I
II
III
IV
V
VI
1
90
S. coelicolor A3(2) CCCGCGGAGCGGGTACCACATCGCTGCGCGATGTGCGAGCGAACACCCGGGCTGCGCCCGGGTGTTGCGCTCCCGCTCCGCGGGAGCGCT
S. avermitilis(L) CCCGCGGAGCGGGTACCACATCGCTGCGCGATGTGCAAGCGAACACCCGCGCTGCGCCGGGGTGTTGCGCTCCCGCTCCGCGGGAGCGCC
S. scabies CCCGCGGAGCGGGTACCACATCGCTGCGCGATGTGCAAGCGAACACCCGTGCTGCGCACGGGTGTTGCGCTTCCGCTCCGCGGAAGCGCT
S. avermitilis(R) CCCGCGGAGCGGGTACCACATCGCTGCGCGATGTGCAAGCGAACACCCGTGCTGCGCACGGGTGTTGCGTTCCCGCTCCGCGGGAACGCT
S. lividans CCCGCGGAGCGGGTACCCTATCGCTGCGCGATAGGCAAGCGAACACCCGCGCTGCGCCGGGTGTTGCGCTCCCGCTCCGCGGGAGCGCT
S. rochei CCCGCGGAGCGGGTCCCTCCGGGCTTCGCCCGGAGCAGCGAACGCGGGGCG-CTGCGCCGCCCGG-T-CGCTCCCGCTCCGCGTGAGCGGA
pSLA2-M(L) CCCGCGGAGCGGGTCCCTCCGGGCTTCGCCGGGAGCAGCGAGCCGGGGGGGCTGCGCCGCCCCGGC-CGCTCCCGCTCCGCGTGAGCGGA
pSLA2-S CCCGCGGAGCGGGTCCCTGCGGGCTTCGCCGCAGCAGAGACACG----------CCCCGCTGCGCGGGGGCGTG

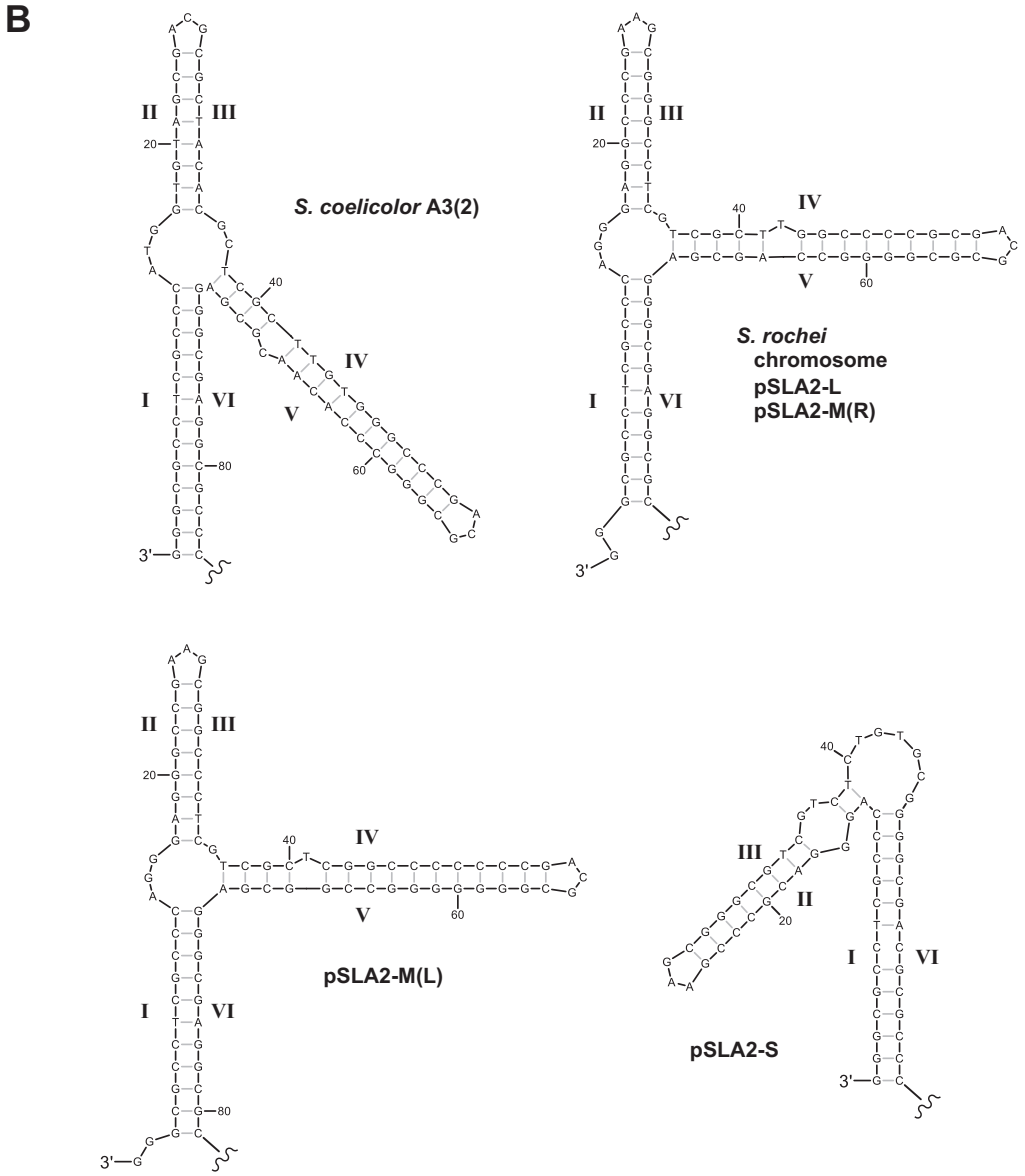

B
S. coelicolor A3(2)
S. rochei
chromosome
pSLA2-L
pSLA2-M(R)
pSLA2-M(L)
pSLA2-S
I
II
III
IV
V
VI
3'

Fig. 2. A. Alignments of the telomere sequences of representative *Streptomyces* species and *Streptomyces rochei* linear replicons: *Streptomyces coelicolor* A3(2) (NC_003888), *Streptomyces lividans* (AF194023), *Streptomyces avermitilis* MA-4680 (NC_003155), *Streptomyces scabies* 87.22 (FN554889), *S. rochei* 7434AN4 (this study), pSLA2-L (AB088224), pSLA2-M (AB597522) and pSLA2-S (AB905437). Highly conserved nucleotides in representative telomeres are drawn in white letters. The telomere sequences of *S. avermitilis* are different in the left (L) and right (R) arms. The telomere sequence of *S. rochei* is identical to that of pSLA2-L and to the right end sequence of pSLA2-M, while the left end sequence of pSLA2-M is a little bit different. Three pairs of inverted repeat sequences, I and VI, II and III, and IV and V, are indicated by arrowhead lines.
B. Secondary foldback structures formed by the 3'-single-stranded DNAs of the *Streptomyces coelicolor* A3(2) chromosome, the *S. rochei* chromosome [identical to pSLA2-L and pSLA2-M (R)], pSLA2-M (L) and pSLA2-S. Sequences II and III and sequences IV and V form a hairpin loop, while sequences I and VI form a base part.

has a relatively short sequence homologous to nt 117–221 of *tpgR1* of pSLA2-L (96/105, 91% identities) (Fig. 4, AB907705). We speculate that this gene was generated by at least two recombination events, which resulted in deletion of both 5'- and 3'-termini of the original *tpg* gene. Thus, a *tap* gene, which is always located upstream of *tpg*, was neither found around this truncated *tpg* homolog.

To further analyze the presence/absence of an additional *tpg* gene on the chromosome, we carried out Southern hybridization experiments using the *tpgR1* clone of pSLA2-L (pYN16) as a probe (Fig. 5). When digested with FspI, 7434AN4 DNA gave two hybridizing signals of *tpgR1* (6.5 kb) and *tpgRM* (3.4 kb), while 51252 DNA gave only a signal of *tpgR1* (6.5 kb). On the other hand, 2-39 DNA did not show any hybridizing signals. The signal of the truncated *tpg* homolog was neither detected here because of its short homology. These results led us to the idea that the chromosome does not contain a *tap-tpg* gene pair and instead pSLA2-L or pSLA2-M functions to maintain a linear chromosomal topology in strain 7434AN4. To support this hypothesis, we carried out the following complementation and curing experiments.

The *tapRM-tpgRM* gene pair of pSLA2-M was cloned into the *Streptomyces–E. coli* shuttle vector pRES18 (Ishikawa *et al*., 1996) to give plasmid pYN15, which was then introduced into strain 51252 carrying only pSLA2-L. The sequence of *tapRM-tpgRM* of pSLA2-M is a little bit different from that of *tapR1-tpgR1* of pSLA2-L, which was expected to avoid recombination between the two gene pairs during experiments. The obtained transformant, YN-T3, was analyzed by Southern hybridization using the telomere probe. As shown in Fig. 6A, strain YN-T3 gave three telomere signals at 12.3 kb (L-L), 4.7 kb (L-R) and 3.7 kb (C-L and C-R), and a signal of linearized pYN15 at 10.3 kb (homology of the vector part gave this signal).

Then, strain YN-T3 was subjected to protoplast regeneration to cure pSLA2-L in the presence of thiostrepton, which was added to maintain pYN15. Among 96 regenerated colonies tested, 8 colonies still kept pYN15 but lost pSLA2-L. All of the eight pSLA2-L-less strains (pYN15$^+$, pSLA2-L$^-$) did not show the signals of pSLA2-L at 12.3 (L-L) or 4.7 kb (L-R), but still showed the telomere signal of the chromosome at 3.7 kb and the pYN15 signal at 10.3 kb (Fig. 6B, four representative strains are shown here). This result indicates that *tapRM-tpgRM* of pSLA2-M could maintain a linear chromosome in place of pSLA2-L.

Finally, strain YN-C149, one of the pSLA2-L-cured strains carrying only pYN15, was subjected again to protoplast regeneration under no pressure of thiostrepton. A total of 192 regenerated colonies were tested for their thiostrepton sensitivity; 24 colonies were thiostrepton-sensitive and the remaining 168 colonies were still thiostrepton-resistant. All of the 24 sensitive colonies and 15 of the resistant colonies were subjected to Southern hybridization analysis. All colonies of the former group showed neither the pYN15 signal nor the telomere signal, while all colonies of the latter group showed both signals (Fig. 6C, six representative strains are shown here). Based on a complete correlation between the presence of the telomere and plasmid pYN15, we concluded that the *tapRM-tpgRM* of pSLA2-M functions to maintain a linear chromosome in strain YN-C149 in place of pSLA2-L. However, the intensity of the telomere signal was weak in most of the pSLA2-L-cured strains compared with the parent strain YN-T3, which suggests a possibility that an additional subsidiary gene(s) or region(s) is necessary for a full maintenance of the linear chromosome.

Discussion

In this study, we obtained the following results on the sequence and topology of the linear chromosome of *S. rochei* 7434AN4: (i) The left and right telomere sequences of the chromosome are identical to each other and are almost identical to the right end sequences of pSLA2-L and pSLA2-M up to 3.1 kb. (ii) The telomere sequence of *S. rochei* is similar to the typical telomere sequences of *Streptomyces* and could form a Y-shaped secondary foldback structure. (iii) Chromosomal circularization occurred in mutant 2-39 concomitant with a plasmid loss by terminal deletions followed by nonhomologous recombination of the deleted ends. (iv) The *tapRM-tpgRM* gene pair of pSLA2-M functioned to maintain a linear chromosome in plasmid-less strains in place of pSLA2-L.

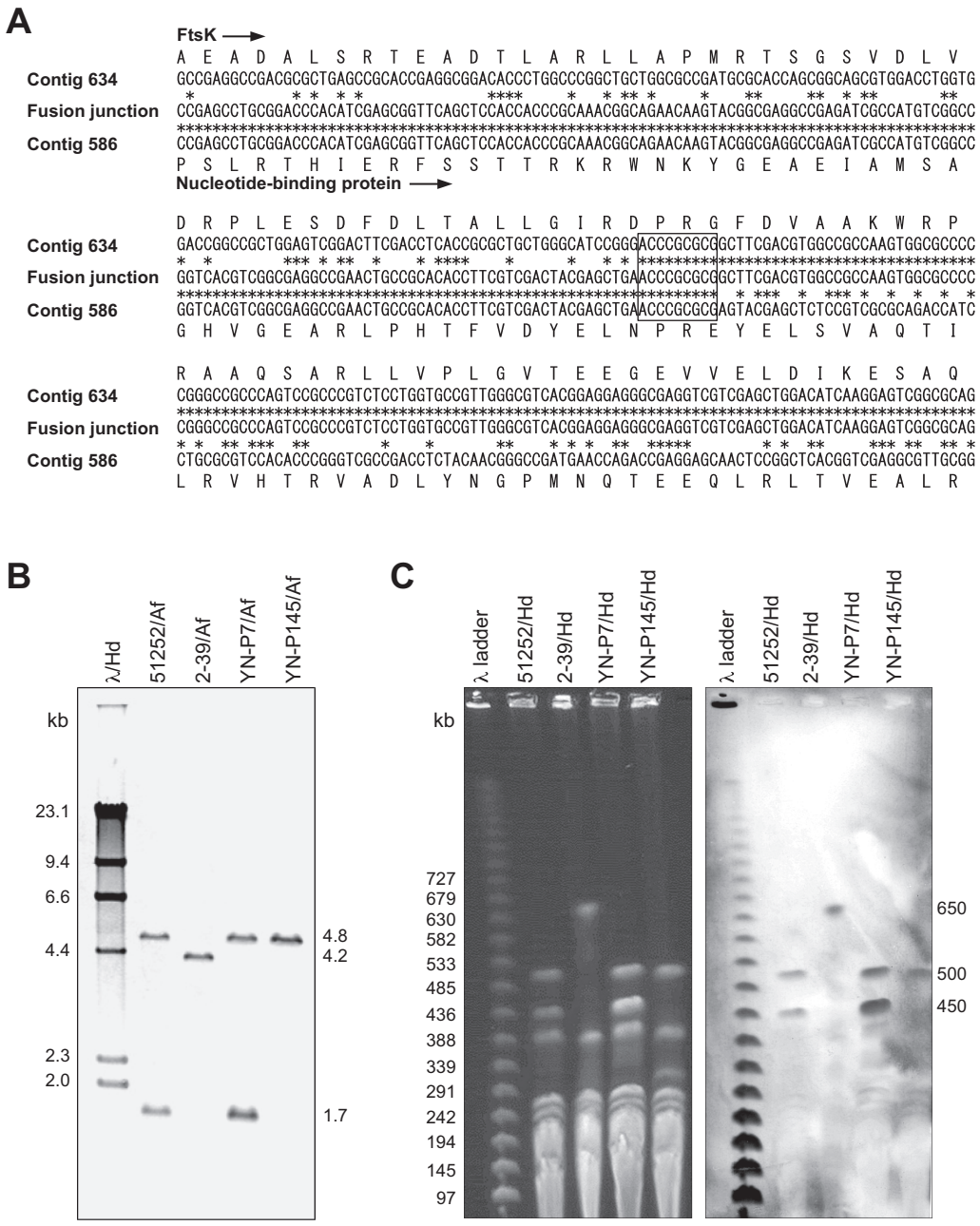


Fig. 3. A. Nucleotide sequence comparison of the deletion ends of the chromosome of strain 7434AN4 and the fusion junction of the plasmid-less mutant 2-39. Identical nucleotides are indicated by asterisk, and the 9 bp homologous sequences between the two deletion ends are enclosed by square. Amino acid sequences of the cell division protein (FtsK) and the nucleotide-binding protein are shown upper and under the nucleotide sequences.
B and C. Southern hybridization analysis of chromosomal deletions in three plasmid-less mutants, 2-39, YN-P7 and YN-P145. Total DNAs were digested with AflIII (B) and HindIII (C), separated by conventional (B) and pulsed-field (C) gel electrophoresis, and analyzed by Southern hybridization using the fusion clone pCZ117 as a probe. The EtBr-stained gel is also shown on the left side in C. Af, AflIII; Hd, HindIII.

As shown in Fig. 2B, the identical telomere sequences of the chromosome, pSLA2-L and pSLA2-M can form a Y-shaped foldback structure similar to that of *S. coelicolor* A3(2). Even the least homologous left end sequence of pSLA2-M shows high similarity in this region and forms a similar Y-shaped structure. On the other hand, the smallest linear plasmid pSLA2-S lacks sequences IV and V, although 14 nucleotides at the extreme end are identical to those of typical *Streptomyces* telomeres (Fig. 2A). Sequences I and VI and sequences II and III of pSLA2-S

```
                                  Homologous region of tpgR1 (96/105, 91% identities)
                                117
                                AGCCGTCGCCCGGCTTCTGAGGATCTCCCAGCGCACCGTGGAACGGTACGTGGCCGGCCAGCTCAAA
1                               ********* * *** *** *********** ** ******************************
ATGCGGTGTCCTCCACGTTGGACAGCCGTCGCTCAGCTCCTGGGGATCTCCCAGCACATCGTGGAACGGTACGTGGCCGGCCAGCTCAAG
M  R  C  P  P  R  W  T  A  V  A  Q  L  L  G  I  S  Q  H  I  V  E  R  Y  V  A  G  Q  L  K

                                                                                221
CGACCCCGCCGCGAGCTGCGCGACCGCATAGAGCGTGA
*******************************  *** **
CGACCCCGCCGCGAGCTGCGCGACCGCATAGGGCGCGATGAGGGTGGGGCCGGGAGCGCGGGGGAGACGCGGGTGCCGGTCCACGTTCGG
R  P  R  R  E  L  R  D  R  I  G  R  D  E  G  G  A  G  S  A  G  E  T  R  V  P  V  H  V  R

CCGGCGCCGGCACAGCATGTCCGGAGCGGCCGGAGGCGGTCCGCGAACGGCCCTTCCGGAAAGCTCCGCCGTCCCTGCCGAATCAGAGTA
P  A  P  A  Q  H  V  R  S  G  R  R  R  S  A  N  G  P  S  G  K  L  R  R  P  C  R  I  R  V

CGTGTGCTGAGGCGGTCACCCGGTTCGAACGGCCCGGTCTCTTGCCACCCGCCACCACCCGCCCCTCTCGCTACGCCCCGCGTGCCCTCC
R  V  L  R  R  S  P  G  S  N  G  P  V  S  C  H  P  P  P  P  A  P  L  A  T  P  R  V  P  S

                                                                                411
GCCGGAACGCCGTCGGCGTGGCGCCGGTGCGCTGCCGGAAGAACTTGGTGA
A  G  T  P  S  A  W  R  R  C  A  A  G  R  T  W  *
```

Fig. 4. Nucleotide sequence of the truncated *tpgR1* homolog found in contig 95 of strain 7434AN4. Nucleotides 117–223 of *tpgR1*, to which this gene shows high homology, are drawn upper the sequence.

can make a foldback structure with a single hairpin loop (Fig. 2B). All of the replication experiments of *S. rochei* linear replicons have been carried out using pSLA2-S or its derivative plasmids. These results suggest that the minimal structure essential for end patching is a foldback structure with a single hairpin loop rather than a Y-shaped structure with double loops.

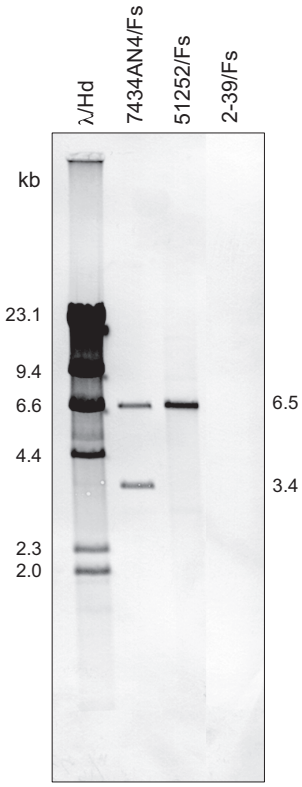


Fig. 5. Search for an additional *tpg* gene by Southern hybridization. Total DNAs of *Streptomyces rochei* 7434AN4, 51252 and 2-39 were digested with FspI and analyzed by Southern hybridization using the *tpgR1* of pSLA2-L as a probe. The *tpgR1* of pSLA2-L and the *tpgRM* of pSLA2-M gave hybridizing signals at 6.5 kb and 3.4 kb, respectively, but no additional *tpg* signals were detected. Fs, FspI.

Chromosomal circularization of *Streptomyces* species has been confirmed at a sequence level for *Streptomyces griseus* (Kameoka *et al*., 1999; Inoue *et al*., 2003), *Streptomyces avermitilis* (Chen *et al*., 2010) and *S. coelicolor* A3(2) (Nindita *et al*., 2013). In these cases, no homology or only 1 bp to 6 bp homology was identified between the left and right deletion ends. In this study, we identified 9 bp homology between the deletion ends for circularization of the *S. rochei* chromosome, which is still much shorter than the minimum size (20 bp) of homology required for homologous recombination (Watt *et al*., 1985). This result again supports nonhomologous recombination of deletion ends of *Streptomyces* linear chromosomes proposed previously (Kameoka *et al*., 1999).

Disruption of the *tpgL* gene on the chromosome of *S. lividans* showed that TP is essential to maintain a linear chromosome as well as the introduced pSLA2 plasmid (pSLA2-S) (Bao and Cohen, 2001). Complementation and curing experiments in this study proved that the *tapRM-tpgRM* of pSLA2-M functioned to maintain a linear chromosome in pSLA2-L-cured strains of *S. rochei*. The *tapR1-tpgR1* of pSLA2-L may function similarly, because their gene products have high sequence similarity to those of *tapRM-tpgRM*; TpgR1 and TpgRM are 98.4% identical (181/184 aa) and TapR1 and TapRM are 75.5% identical (558/739 aa). In addition, strain 51252, which carries only pSLA2-L, stably keeps a linear chromosome as strain K3A-12 does, which carries only pSLA2-M. We previously tried to cure pSLA2-L, pSLA2-M and pSLA2-S from strain 7434AN4 by protoplast regeneration and other curing methods. All of the mutants with a possible combination of the three linear plasmids have been obtained except for one that carries only pSLA2-S (Kinashi *et al*., 1994). We now realize that this result did not happen by chance; neither the *S. rochei* chromosome nor pSLA2-S

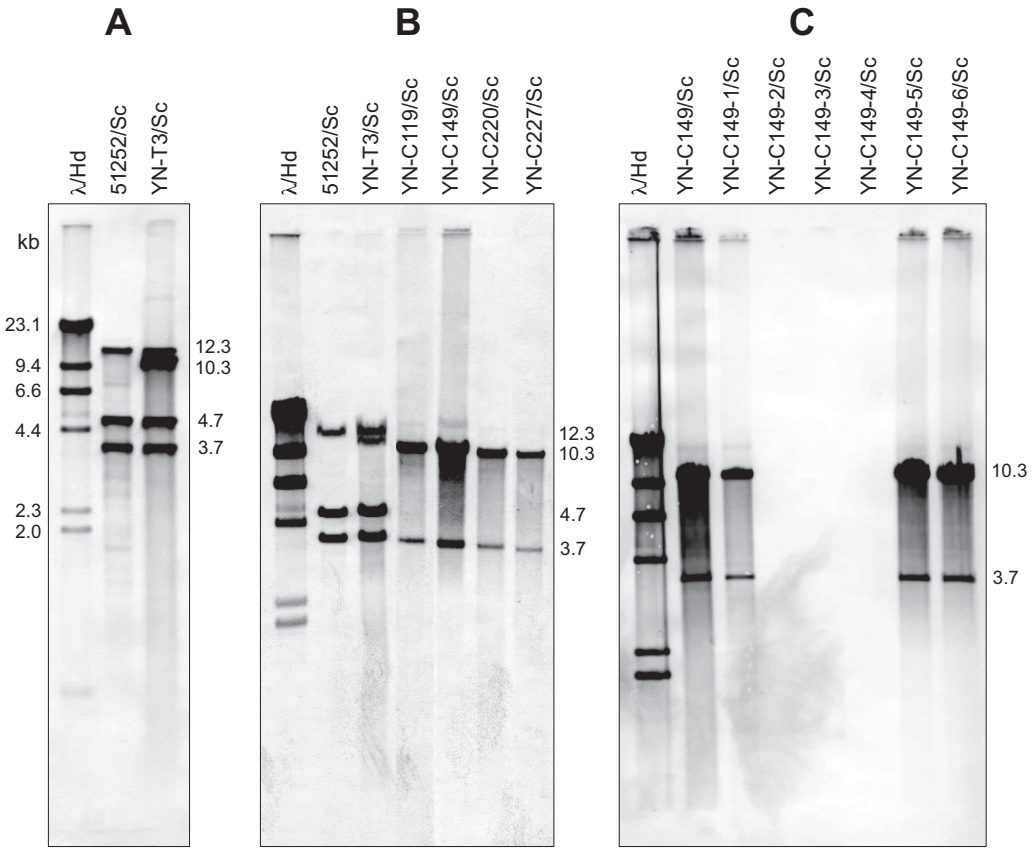


Fig. 6. Effects of plasmid pYN15 containing the *tapRM-tpgRM* gene pair of pSLA2-M on chromosomal topology of *Streptomyces rochei* strains. Total DNAs of various *S. rochei* strains with/without pYN15 were digested with SacI and analyzed by Southern hybridization using pEcoEnd as a probe.
A. Strain YN-T3 obtained by introduction of pYN15 into strain 51252. Hybridizing signals of the chromosome (3.7 kb), pSLA2-L (12.3 and 4.7 kb) and pYN15 (10.3 kb) are observed.
B. pSLA2-L-cured strains, YN-C119, YN-C149, YN-C220 and YN-C227 obtained by the first protoplast regeneration of strain YN-T3.
C. Strains YN-C149-1~6 obtained by the second protoplast regeneration of strain YN-C149. Sc, SacI.

supports end patching of itself or another without an intact *tap-tpg* gene pair.

A truncated *tpg* homolog was identified on the *S. rochei* chromosome (Fig. 4), which might have been generated by recombination. The truncated *tap-tpg* homologs (*SGR6987-6986*) were also found on the chromosome of *S. griseus* IFO13350 (Suzuki *et al*., 2008). The latter chromosome contains a novel type of gene pair (*gtpB-gtpA*) for end patching of the atypical telomere sequence. Thus, the deficiency of *tap-tpg* is rescued in different ways in two strains: by introducing linear plasmids, pSLA2-L and pSLA2-M, containing an intact *tap-tpg* pair in *S. rochei* 7434AN4 and by acquiring a novel set of the *gtpB-gtpA* genes and the atypical telomeres possibly from a certain linear plasmid in *S. griseus* IFO13350 (Suzuki *et al*., 2008). The fact that another atypical telomere sequence of *S. griseus* strain 2247 was totally different from that of strain IFO13350 (Goshi *et al*., 2002) supports the latter idea.

The number of examples where biosynthetic gene clusters are located on a giant linear plasmid is increasing gradually (Chater and Kinashi, 2007; Kinashi, 2011) since the discovery of SCP1 from *S. coelicolor* A3(2) (Kinashi *et al*., 1987); SCP1 carries the biosynthetic gene cluster for methylenomycin (Bentley *et al*., 2004). In addition, the methylenomycin cluster was found as an integrated form into the chromosome of *S. coelicolor* A3(2) (Hanafusa and Kinashi, 1992; Yamasaki *et al*., 2001) as well as into circular plasmid pSV1 of *Streptomyces violaceoruber* (Yamasaki *et al*., 2003). The gene clusters for the angucycline antibiotics were also identified on linear plasmid pSA3239 of *Streptomyces aureofaciens* (Novakova *et al*., 2013) as well as at both ends of the chromosome of *S. ambofaciens* (Pang *et al*., 2004). Recombination between a linear plasmid and a chromosome near the end of the latter causes end exchange, which transferred the oxytetracycline biosynthetic cluster from the *Streptomyces rimosus* chromosome to a 387 kb linear plasmid pPZG101

Table 1. Bacterial strains and plasmids used in this study.

Names	Description	Source/reference
Strains		
Streptomyces rochei 7434AN4	Wild-type strain (pSLA2-L, pSLA2-M, pSLA2-S)	Kinashi *et al*., 1994
S. rochei 51252	Ultraviolet irradiation of 7434AN4 (pSLA2-L)	Kinashi *et al*., 1994
S. rochei 1–2	Ultraviolet irradiation of 7434AN4 (pSLA2-L, pSLA2-M)	Kinashi *et al*., 1994
S. rochei K3A-12	High temperature culture of 1–2 (pSLA2-M)	Kinashi *et al*., 1994
S. rochei 2-39	Protoplast regeneration of 51252 (no plasmid)	Kinashi *et al*., 1994
S. rochei YN-P7	Protoplast regeneration of 51252 (no plasmid)	This study
S. rochei YN-P145	Protoplast regeneration of 51252 (no plasmid)	This study
S. rochei YN-T3	Introduction of pYN15 into 51252 (pSLA2-L, pYN15)	This study
S. rochei YN-C119	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C149	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C220	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C227	Curing of pSLA2-L from YN-T3 (pYN15)	This study
S. rochei YN-C149-1	Same genotype with YN-C149 (pYN15)	This study
S. rochei YN-C149-2	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-3	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-4	Curing of pYN15 from YN-C149 (no plasmid)	This study
S. rochei YN-C149-5	Same genotype with YN-C149 (pYN15)	This study
S. rochei YN-C149-6	Same genotype with YN-C149 (pYN15)	This study
Plasmids		
pEcoEnd	0.4-kb EcoRI end fragment of pSLA2-L in pUC19	Hiratsu *et al*., 2000
pCZ106	4.6-kb BamHI-EcoRI end fragment of chromosome in pUC19	This work
pCZ117	2.3-kb fusion fragment of 2-39 in pUC19	This work
pYN15	*tpgRM-tapRM* of pSLA2-M in pRES18	This work
pYN16	*tpgR1* of pSLA2-L in pUC19	This work

(Pandza *et al*., 1998). When recombination occurred between SCP1 and the center of the chromosome, two chimeric chromosomes were generated in *S. coelicolor* A3(2) strain 2106 (Yamasaki and Kinashi, 2004). This may be considered as a model of chromosomal duplication. In addition, we revealed in this study that linear plasmids, pSLA2-L and pSLA2-M, function even in maintaining a linear topology of the chromosome in *S. rochei*. Thus, the idea that giant linear plasmids have played critical roles in genome evolution and horizontal transfer of secondary metabolism was further supported.

Experimental procedures

Bacterial strains, plasmid and media

Streptomyces strains used in this study were derived from the wild-type strain *S. rochei* 7434AN4 and are listed in Table 1. Plasmid pUC19 was used for cloning in *E. coli* XL-1 blue and nucleotide sequencing, while the *E. coli–Streptomyces* shuttle vector pRES18 (Ishikawa *et al*., 1996) was used for DNA manipulation in *Streptomyces*. YM medium (0.4% yeast extract, 1.0% malt extract and 0.4% glucose, pH 7.3) and YEME medium (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose, 5 mM $MgCl_2$, 0.5% glycine) were used for growth and protoplast preparation of *S. rochei* strains respectively. *E. coli* XL-1 blue were grown in LB medium (0.5% yeast extract, 1.0% peptone and 1.0% NaCl, pH 7.0). When needed, ampicillin (50 μg ml^{-1}) or thiostrepton (10 μg ml^{-1} in liquid media and 50 μg ml^{-1} for agar media) was added to the media.

DNA manipulation and Southern hybridization

Streptomyces rochei strains were reciprocally grown in liquid YM medium in Sakaguchi flasks at 28°C for 2 days. DNA manipulation for *Streptomyces* (Kieser *et al*., 2000) and *E. coli* (Sambrook and Russell, 2001) was carried out according to standard procedures. Total DNA was digested with restriction enzymes, separated by conventional agarose gel electrophoresis or PFGE, and transferred to nylon membrane filters by the capillary method. MidRange I PFG Marker and Lambda Ladder PFG Marker were purchased from New England BioLabs (Ipswich, MA). DNA probes were labeled by digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) using random primers, and hybridization was carried out overnight at 70°C according to the manufacturer's protocol. After hybridization, washing was done twice for 5 min each in 2 × wash solution at room temperature and twice for 15 min each in 0.1 × wash solution at 70°C.

PCR, conventional nucleotide sequencing and analysis of DNA folding

Primer pairs listed in Supporting Information Table S2 were used for polymerase chain reaction (PCR) amplification. The reaction mixtures were subjected to heating (94°C for 2 min) followed by 35 cycles of PCR (94°C for 15 s, 64°C for 30 s and 68°C for 1 min) and a final extension step (68°C for 10 min). PCR was done on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan).

Nucleotide sequencing was performed by the dideoxy termination method, using BigDye Terminator v3.1/v1.1 Cycle

Sequencing Kits (Life Technologies, Carlsbad, CA) and a 3130xl Genetic Analyzer (Life Technologies). Genetyx-Mac 17.0.2 (Software Development, Tokyo) and FramePlot 4.0beta (Ishikawa and Hotta, 1999) were used for analysis of sequence data. All of the nucleotide sequences determined in this study have been submitted to DNA Data Bank of Japan (DDBJ), whose accession numbers are listed in Supporting Information Table S1. Folding of 3'-leading-strand overhangs at the telomeres was analyzed using the Mfold Web Server (http://bi.biopapyrus.net/app/mfold.html).

Whole shotgun genome sequencing of S. rochei *strains 7434AN4 and 2-39*

The sequencing library of strain 7434AN4 was prepared from 5 μg of total DNA with the GS General Library Prep Kit according to the manufacturer's protocol (all kits, softwares, and machines used for genome sequencing of strain 7434AN4 were products of Roche, Branford, CT). Two runs of sequencing were performed using the Genome Sequencer FLX with the GS Sequencing Kit and the GS PicoTiterPlate Kit 70 × 75. Imaging and signal processing were done using GS FLX SW v2.3 and GS Run Processor Full Processing. De novo assembly was performed using Newbler v2.3.

The sequencing library of strain 2-39 was prepared from 5 μg of total DNA with a median insert size of 500 bp for a multiplexed paired-end read format according to the Illumina (San Diego, CA) protocols. The final product was validated using the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The bar-coded library was sequenced as multiplexed paired-end 100 bp reads on a genome analyzer II system (Illumina). The quality of sequenced library was assessed by using FASTX-Toolkit http://hannonlab.cshl.edu/fastx_toolkit/ and the reads were trimmed to 80 bp. After trimming reads, de novo assembly was performed using Velvet (Zerbino and Birney, 2008) with parameters optimized by the VelvetOptimizer (http://www.bioinformatics.net.au/software.velvetoptimiser.shtml).

Construction of plasmids, pCZ106, pCZ117 and pYN16

The 4.6 kb BamHI–EcoRI fragment of the chromosome next to the 0.4 kb telomere EcoRI fragment was shotgun cloned from 7434A4 DNA into pUC19 to give plasmid pCZ106. The 2.3 kb fusion fragment of the chromosome was amplified from the 2-39 DNA using primers 2-39-229-f and 2-39-229-r, digested with EcoRI and PstI, and cloned into pUC19 to give plasmid pCZ117. The 0.9 kb fragment covering the *tpgR1* gene of pSLA2-L was amplified from 7434AN4 DNA using primers tpgR1-F4 and tpgR1-R4, digested with BamHI and EcoRI, and cloned into pUC19 to give plasmid pYN16.

Complementation and curing experiments

The *tapRM-tpgRM* gene pair of pSLA2-M was amplified by PCR using total DNA of strain K3A12, which carries only pSLA2-M, and primers tpgRM-f5 and tpgRM-r5. The resulting 4.5 kb amplified fragment was digested with EcoRI and BamHI and cloned into pRES18 to give the complementation plasmid pYN15. This plasmid was introduced into strain 51252, which carries only pSLA2-L, by polyethylene glycol (PEG)-assisted transformation to give strain YN-T3. Protoplast regeneration of strain YN-T3 and its pSLA2-L-less derivative YN-C149 was performed on R1M plates (Zhang *et al.*, 1997) with/without 50 μg ml^{-1} of thiostrepton.

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References

Bao, K., and Cohen, S.N. (2001) Terminal proteins essential for the replication of linear plasmids and chromosomes in *Streptomyces*. *Genes Dev* **15:** 1518–1527.

Bao, K., and Cohen, S.N. (2003) Recruitment of terminal protein to the ends of *Streptomyces* linear plasmids and chromosomes by a novel telomere-binding protein essential for linear DNA replication. *Genes Dev* **17:** 774–785.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.-M., Challis, G.L., Thomson, N.R., James, K.D., *et al.* (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417:** 141–147.

Bentley, S.D., Brown, S., Murphy, L.D., Harris, D.E., Quail, M.A., Parkhill, J., *et al.* (2004) SCP1, a 356,023 bp linear plasmid adapted to the ecology and developmental biology of its host, *Streptomyces coelicolor* A3(2). *Mol Microbiol* **51:** 1615–1628.

Chater, K.F., and Kinashi, H. (2007) *Streptomyces* linear plasmids: their discovery, functions, interactions with other replicons, and evolutionary significance. In *Microbiology Monographs, Vol. 7, Microbial Linear Plasmids*. Meinhardt, F., and Klassen, R. (eds). Berlin/Heidelberg: Springer, pp. 1–31.

Chen, C.W., Huang, C.H., Lee, H.H., Tsai, H.H., and Kirby, R. (2002) Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes. *Trends Genet* **18:** 522–529.

Chen, W., He, F., Zhang, X., Chen, Z., Wen, Y., and Li, J. (2010) Chromosomal instability in *Streptomyces avermitilis*: major deletion in the central region and stable circularized chromosome. *BMC Microbiol* **10:** 198.

Goshi, K., Uchida, T., Lezhava, A., Yamasaki, M., Hiratsu, K., Shinkawa, H., and Kinashi, H. (2002) Cloning and analysis of the telomere and terminal inverted repeat of the linear chromosome of *Streptomyces griseus*. *J Bacteriol* **184:** 3411–3415.

Hanafusa, T., and Kinashi, H. (1992) The structure of an integrated copy of the giant linear plasmid SCP1 in the chromosome of *Streptomyces coelicolor* 2612. *Mol Gen Genet* **231:** 363–368.

Hayakawa, T., Tanaka, T., Sakaguchi, K., Otake, N., and Yonehara, H. (1979) A linear plasmid-like DNA in *Streptomyces* sp. producing lankacidin group antibiotics. *J Gen Appl Microbiol* **25:** 255–260.

Hiratsu, K., Mochizuki, S., and Kinashi, H. (2000) Cloning and analysis of the replication origin and the telomeres of the linear plasmid pSLA2-L in *Streptomyces rochei*. *Mol Gen Genet* **263:** 1015–1021.

Huang, C.H., Lin, Y.S., Yang, Y.L., Huang, S.W., and Chen, C.W. (1998) The telomeres of *Streptomyces* chromosomes contain conserved palindromic sequences with potential to form complex secondary structures. *Mol Microbiol* **28:** 905–916.

Huang, C.H., Chen, C.Y., Tsai, H.H., Chen, C., Lin, Y.S., and Chen, C.W. (2003) Linear plasmid SLP2 of *Streptomyces lividans* is a composite replicon. *Mol Microbiol* **47:** 1563–1576.

Huang, C.H., Tsai, H.H., Tsay, Y.G., Chien, Y.N., Wang, S.L., Cheng, M.Y., *et al*. (2007) The telomere system of the *Streptomyces* linear plasmid SCP1 represents a novel class. *Mol Microbiol* **63:** 1710–1718.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., *et al*. (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat Biotechnol* **21:** 526–531.

Inoue, S., Higashiyama, K., Uchida, T., Hiratsu, K., and Kinashi, H. (2003) Chromosomal circularization in *Streptomyces griseus* by nonhomologous recombination of deletion ends. *Biosci Biotechnol Biochem* **67:** 1101–1108.

Ishikawa, J., and Hotta, K. (1999) FramePlot: a new implementation of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G + C content. *FEMS Microbiol Lett* **174:** 251–253.

Ishikawa, J., Niino, Y., and Hotta, K. (1996) Construction of pRES18 and pRES19, *Streptomyces-Escherichia coli* shuttle vectors carrying multiple cloning sites. *FEMS Microbiol Lett* **145:** 113–116.

Kameoka, D., Lezhava, A., Zenitani, H., Hiratsu, K., Kawamoto, M., Goshi, K., *et al*. (1999) Analysis of fusion junctions of circularized chromosomes in *Streptomyces griseus*. *J Bacteriol* **181:** 5711–5717.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich.

Kinashi, H. (1994) Linear plasmids from actinomycetes. *Actinomycetologica* **8:** 87–96.

Kinashi, H. (2008) Antibiotic production, linear plasmids and linear chromosomes in *Streptomyces*. *Actinomycetologica* **22:** 20–29.

Kinashi, H. (2011) Giant linear plasmids in *Streptomyces*: a treasure trove of antibiotic biosynthetic clusters. *J Antibiot* **64:** 19–25.

Kinashi, H., Shimaji, M., and Sakai, A. (1987) Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. *Nature* **328:** 454–456.

Kinashi, H., Shimaji-Murayama, M., and Hanafusa, T. (1991) Nucleotide sequence analysis of the unusually long terminal inverted repeats of a giant linear plasmid, SCP1. *Plasmid* **26:** 123–130.

Kinashi, H., Mori, E., Hatani, A., and Nimi, O. (1994) Isolation and characterization of linear plasmids from lankacidin-producing *Streptomyces* species. *J Antibiot* **47:** 1447–1455.

Mochizuki, S., Hiratsu, K., Suwa, M., Ishii, T., Sugino, F., Yamada, K., and Kinashi, H. (2003) The large linear plasmid pSLA2-L of *Streptomyces rochei* has an unusually condensed gene organization for secondary metabolism. *Mol Microbiol* **48:** 1501–1510.

Nindita, Y., Nishikawa, T., Arakawa, K., Wang, G., Ochi, K., Qin, Z., and Kinashi, H. (2013) Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2). *FEMS Microbiol Lett* **347:** 149–155.

Novakova, R., Knirschova, R., Farkasovsky, M., Feckova, L., Rehakova, A., Mingyar, E., and Kormanec, J. (2013) The gene cluster *aur1* for the angucycline antibiotic auricin is located on a large linear plasmid pSA3239 in *Streptomyces aureofaciens* CCM 3239. *FEMS Microbiol Lett* **342:** 130–137.

Ohnishi, Y., Ishikawa, J., Hara, H., Suzuki, H., Ikenoya, M., Ikeda, H., *et al*. (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J Bacteriol* **190:** 4050–4060.

Pandza, S., Biukovic, G., Paravic, A., Dadbin, A.J., Cullum, J., and Hranueli, D. (1998) Recombination between the linear plasmid pPZG101 and the linear chromosome of *Streptomyces rimosus* can lead to exchange of ends. *Mol Microbiol* **28:** 1165–1176.

Pang, X., Aigle, B., Girardet, J.M., Mangenot, S., Pernodet, J.L., Decaris, B., and Leblond, P. (2004) Functional angucycline-like antibiotic gene cluster in the terminal inverted repeats of the *Streptomyces ambofaciens* linear chromosome. *Antimicrob Agents Chemother* **48:** 575–588.

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

Sambrook, J.F., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Suzuki, H., Marushima, K., Ohnishi, Y., and Horinouchi, S. (2008) A novel pair of terminal protein and telomere-associated protein for replication of the linear chromosome of *Streptomyces griseus* IFO13350. *Biosci Biotechnol Biochem* **72:** 2973–2980.

Volff, J.N., and Altenbuchner, J. (1998) Genetic instability of the *Streptomyces* chromosome. *Mol Microbiol* **27:** 239–246.

Watt, V.M., Ingles, C.J., Urdea, M.S., and Rutter, W.J. (1985) Homology requirements for recombination in *Escherichia coli*. *Proc Natl Acad Sci USA* **82:** 4768–4772.

Yamasaki, M., and Kinashi, H. (2004) Two chimeric chromosomes of *Streptomyces coelicolor* A3(2) generated by single crossover of the wild-type chromosome and linear plasmid SCP1. *J Bacteriol* **186:** 6553–6559.

Yamasaki, M., Redenbach, M., and Kinashi, H. (2001) Integrated structures of the linear plasmid SCP1 in two bidirectional donor strains of *Streptomyces coelicolor* A3(2). *Mol Gen Genet* **264:** 634–642.

Yamasaki, M., Ikuto, Y., Ohira, A., Chater, K., and Kinashi, H.

(2003) Limited regions of homology between linear and circular plasmids encoding methylenomycin biosynthesis in two independently isolated streptomycetes. *Microbiology* **149:** 1351–1356.

Yang, C.C., Chen, Y.H., Tsai, H.H., Huang, C.H., Huang, T.W., and Chen, C.W. (2006) In vitro deoxynucleotidylation of the terminal protein of *Streptomyces* linear chromosomes. *Appl Environ Microbiol* **72:** 7959–7961.

Yang, Y., Kurokawa, T., Takahama, Y., Nindita, Y., Mochizuki, S., Arakawa, K., *et al.* (2011) pSLA2-M of *Streptomyces rochei* is a composite linear plasmid characterized by self-defense genes and homology with pSLA2-L. *Biosci Biotechnol Biochem* **75:** 1147–1153.

Zerbino, D.R., and Birney, E. (2008) Velvet algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* **18:** 821–829.

Zhang, H., Shinkawa, H., Ishikawa, J., Kinashi, H., and Nimi, O. (1997) Improvement of transformation system in *Streptomyces* using a modified regeneration medium. *J Ferment Bioeng* **83:** 217–221.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supplementary Materials

Table S1. Accession numbers of the nucleotide sequences described in the text

Accession Numbers	Description	Source/ref.
AB088224	pSLA2-L	Mochizuki *et al*., 2003
AB597522	pSLA2-M	Yang *et al*., 2011
AB905437	pSLA2-S	Takahashi *et al*., unpublished
AB905439	contig 229 of mutant 2-39, containing the fusion junction of the circularized chromosome	This study
AB905441	telomere of the 7434AN4 chromosome	This study
AB905442	contig 586 of strain 7434AN4, containing a nucleotide-binding protein gene at the fusion junction of the 2-39 chromosome	This study
AB905443	contig 634 of strain 7434AN4, containing a *ftsK* gene at the fusion junction of the 2-39 chromosome	This study
AB907705	contig 95 of strain 7434AN4, containing a truncated *tpg* homolog	This study

Table S2. DNA primers used in this study

taptpgRM-f5	TTGAATTCGCGTTCAGGTTCTGGGTTATAG
taptpgRM-r5	TTAGATCTACGTACTCGGAGTTGGTGTTG
2-39-229-f	TTGAATTCAAGTCCAGGCAGTAGAACTGGA
2-39-229-r	TTCTGCAGTTTATCACCGCTTCGGAGTAAG
tpgR1-F4	ATGGATCCAGAGCAGCAATTCGAGGAGCAC
tpgR1-R4	ATGAATTCTGGTCCAGATGGTGGAGGTCAC

The nucleotide sequences underlined are restriction sites introduced for cloning.

RESEARCH LETTER

Chromosomal circularization of the model *Streptomyces* species, *Streptomyces coelicolor* A3(2)

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linear chromosome; terminal deletion; genetic instability; genome evolution.

Abstract

Streptomyces linear chromosomes frequently cause deletions at both ends spontaneously or by various mutagenic treatments, leading to chromosomal circularization and arm replacement. However, chromosomal circularization has not been confirmed at a sequence level in the model species, *Streptomyces coelicolor* A3(2). In this work, we have cloned and sequenced a fusion junction of a circularized chromosome in an *S. coelicolor* A3(2) mutant and found a 6-bp overlap between the left and right deletion ends. This result shows that chromosomal circularization occurred by nonhomologous recombination of the deletion ends in this species, too. At the end of the study, we discuss on stability and evolution of *Streptomyces* chromosomes.

Introduction

The saprophytic filamentous soil bacteria, from the genus of *Streptomyces*, are well known to produce many clinically useful antibiotics. It is also a characteristic feature of this genus to contain an 8–9 Mb linear chromosome in place of a usual circular bacterial chromosome (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008). The linearity of *Streptomyces* chromosomes was first proved for *Streptomyces lividans* (Lin *et al.*, 1993), and the complete linear genome sequence of the model species, *Streptomyces coelicolor* A3(2), was determined in 2002 (Bentley *et al.*, 2002)

Streptomyces chromosomes are unusually unstable and are often subject to deletion and amplification spontaneously or by various mutagenic treatments (Volff & Altenbuchner, 1998). The size of chromosomal deletions reaches up to 2 Mb in some *Streptomyces* species (Fischer *et al.*, 1997; Chen *et al.*, 2010). Studies of this genetic instability of *Streptomyces* faced great difficulty previously, because *Streptomyces* chromosomes had been considered to be circular for a long time. We now have a correct

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idea of the linear structure of *Streptomyces* chromosomes and a powerful method, pulsed-field gel electrophoresis, for physical analysis of their rearrangements. We know at present that chromosomal deletions occur from both ends. However, instability of *Streptomyces* chromosomes has not been clarified well. For example, an amplifiable unit of DNA (AUD) was tandemly amplified several hundred times to form amplified DNA sequence (ADS) in *S. lividans* mutants (Altenbuchner & Cullum, 1985), but the gross structures of the mutant chromosomes have not been clarified.

The most frequent destination of *Streptomyces* chromosomes following terminal deletion is circularization. Chromosomal circularization was indicated by detection of a macrorestriction fragment in deletion mutants of *S. lividans* (Lin *et al.*, 1993; Redenbach *et al.*, 1993) and *S. ambofaciens* (Leblond *et al.*, 1996). It was finally confirmed in *Streptomyces griseus* by cloning and sequencing of the fusion junctions of the circularized chromosomes (Kameoka *et al.*, 1999; Inoue *et al.*, 2003). No sequence homology was found between the left and right deletion ends in two mutants, and only 1-bp and 6-bp homology was found in two other mutants. Accordingly, it was proposed that nonhomologous recombination between the left and right deletion ends caused chromosomal circularization (Inoue *et al.*, 2003). Microhomology was also detected at the fusion points of circularized chromosomes of *Streptomyces avermilitis* mutants (Chen *et al.*, 2010).

Chromosomal arm replacement is another destination of deleted chromosomes. When one chromosomal arm is deleted and the left and right arms carry a homologous sequence in an inverted orientation, homologous recombination between them causes arm replacement, which recovers a telomere and generates longer terminal inverted repeats (TIRs) at both ends. This phenomenon was first reported for *S. ambofaciens* (Fischer *et al.*, 1998) and was followed by *S. griseus* (Uchida *et al.*, 2003) and *S. coelicolor* A3(2) (Widenbrant *et al.*, 2007). Even the long TIRs formed by arm replacement suffer terminal deletion. When an inverted repeat sequence is present at the deletion end inside the long TIR, it could form a hairpin structure, which invades the opposite TIR strand during replication leading to a circular chromosome with an extremely large palindrome (Uchida *et al.*, 2004). Similar various rearrangements were observed in *Streptomyces* linear plasmids when deletions were introduced at specific locations within telomeres (Qin & Cohen, 2002).

In spite of these extensive analyses, chromosomal circularization has not been proved for the model species *S. coelicolor* A3(2) at a sequence level. In this study, we first report the cloning and sequencing of a fusion junction of a circularized chromosome of *S. coelicolor* A3(2) and show that chromosomal circularization occurred by nonhomologous recombination in this species, too. At the end of the study, we discuss on stability and evolution of *Streptomyces* chromosomes.

Materials and methods

Bacterial strains, plasmids, cosmid libraries, and medium

Streptomyces coelicolor A3(2) strain No. 4 used in this study is an *eshA* (named for a defect of extension of sporogenic hyphae; Kwak *et al.*, 2001) mutant obtained by cultivation of the wild-type strain 1147 at a high temperature (Kawamoto *et al.*, 2001). Strain M145 for which the genome project has been carried out (Bentley *et al.*, 2002) was used as a reference strain for comparison. The cosmid libraries of *S. coelicolor* A3(2) used in this study have been constructed and ordered by Redenbach *et al.* (1996) and Zhou *et al.* (2012). *Escherichia coli* XL1-Blue and pUC19 were used for cloning and sequencing of DNA fragments. Plasmid pLUS221 used as a probe for hybridization carries the 1.3-kb BamHI fragment at the extreme end of the *S. coelicolor* A3(2) chromosome (Huang *et al.*, 1998). Glucose-meat extract-peptone (GMP) medium contains 10 g of glucose, 4 g of peptone, 2 g of meat extract, 2 g of yeast extract, 5 g of NaCl, and 0.25 g of $MgSO_4 7H_2O$ L^{-1} (pH 7.0).

DNA manipulation and Southern hybridization

Streptomyces coelicolor A3(2) wild-type and mutant strains were reciprocally grown in liquid GMP medium in Sakaguchi flasks at 28 °C for 3 days. DNA manipulation for *Streptomyces* (Kieser *et al.*, 2000) and *E. coli* (Sambrook *et al.*, 1989) was carried out according to standard procedures. Total DNA was digested with restriction enzymes, separated by conventional agarose gel electrophoresis, and transferred to nylon membrane filters by the capillary method. DNA probes were labeled with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) using random primers, and hybridization was carried out overnight at 70 °C according to the manufacturer's protocol. After hybridization, washing was carried out twice for 5 min each in 2× wash solution at room temperature, and then twice for 15 min each in 0.1× wash solution at 70 °C.

PCR and nucleotide sequencing

Two primers for PCR amplification, del-L, 5′-CACCGAATTCTGAGCGATGGTCGTCGTGA-3′ (the EcoRI site is underlined) and del-R, 5′-ATACGGATCCTTCGCGATCGTCCCGCTGA-3′ (the BamHI site is underlined), were

designed based on Southern hybridization analysis of the left and right deletion ends of mutant No. 4. PCR was performed on a 2720 Thermal Cycler (Applied Biosystems, Foster city, CA) with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Nucleotide sequencing was performed by the dideoxy termination method, using BigDye Terminator v3.1/v1.1 Cycle Sequencing Kits (Life Technologies, Carlsbad, CA) and a 3130xl Genetic Analyzer (Life Technologies).

Results and discussion

Analysis of chromosomal deletion in mutant No. 4

Streptomyces coelicolor A3(2) strain No. 4 used in this study is an *eshA* mutant of the wild-type strain 1147 and shows several defective phenotypes (Kawamoto *et al.*, 2001). The *eshA* gene encodes a nucleotide-binding protein, disruption of which caused a loss of actinorhodin production due to a reduced level of ppGpp (Saito *et al.*, 2006). The *eshA* gene was identified as SCO7699 (nt 8535532–8536947 of the genome sequence) located at 131 kb from the right end (Bentley *et al.*, 2002), suggesting that at least the right chromosomal arm was deleted beyond this locus. To clarify whether only the right telomere was deleted or both telomeres were, Southern blot analysis was carried out using labeled pLUS221, which contains the 1.3-kb end BamHI fragment of the chromosome (Huang *et al.*, 1998). As shown in Fig. 1a, the reference strain M145 showed a positive signal at 1.3 kb, whereas no hybridizing signal was observed for mutant No. 4. As the *S. coelicolor* A3(2) chromosome has the 24-kb TIRs at the left and

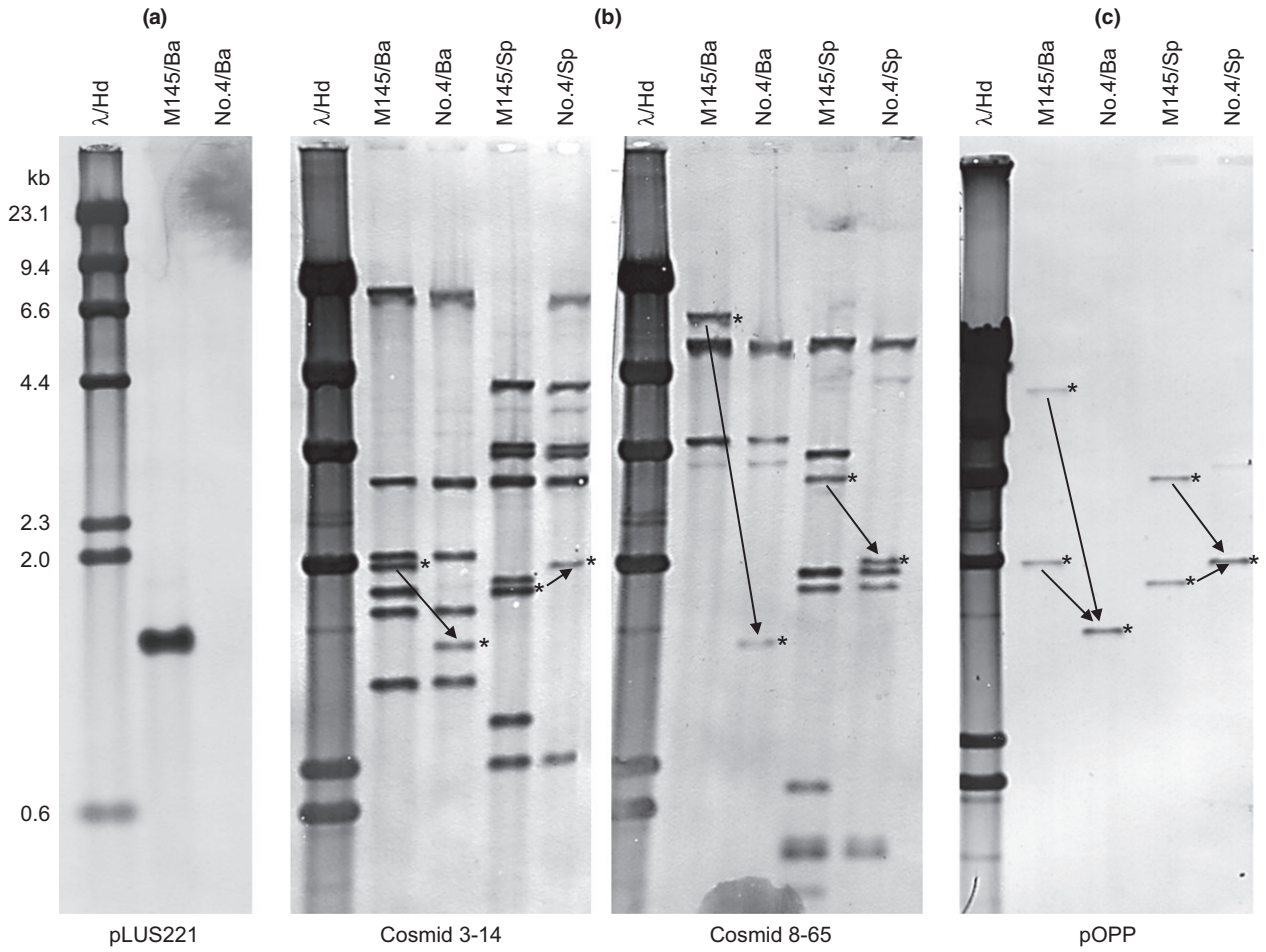

Fig. 1. Southern hybridization analysis of chromosomal deletion and circularization. (a) Analysis of telomere deletion using the telomere clone pLUS221. (b) Analysis of the deletion ranges using cosmids 3–14 and 8–64. Fragments at the deletion end and newly appeared fusion fragments are marked with asterisk and connected each other by arrow. (c) Analysis of chromosomal circularization using the fusion clone pOPP. Lambda DNA digested with HindIII was used as size markers, and their sizes are shown on the left side. The hybridization probes are indicated under each figure. λ, lambda DNA; Ba, BamHI; Hd, HindIII; Sp, SphI.

right ends, this result indicates that both telomeres were deleted in mutant No. 4.

To determine the range of deletions in the left and right arms, we used the original cosmid library constructed by Redenbach *et al.* (1996) as probe for hybridization, but could not identify the deletion-end cosmids. Therefore, we used another cosmid library recently constructed by Zhou *et al.* (2012) and finally identified the left deletion-end cosmid, 3–14, and the right deletion-end cosmid, 8–65. As shown in Fig. 1b, cosmid 3–14 (covers nt. 230,501-274,102 of the chromosome) and cosmid 8–64 (nt. 7,790,510-7,825,517) gave fewer hybridizing signals for mutant No. 4 compared with strain M145. Referring to the restriction maps of cosmids 3–14 and 8–64, the left and right deletion ends were delimited. It was revealed that the 4.2-kb BamHI fragment of cosmid 3–14 and the 13.6-kb BamHI fragment extending over the right end of cosmid 8–65 were fused to generate a 3.3-kb BamHI fragment (Figs 1b and 2a). Similarly, the 3.9-kb SphI fragment of cosmid 3–14 and the 6.2-kb SphI fragment of cosmid 8–65 were fused to generate a 4.2-kb SphI fragment. It should be noted that cosmids 3–14 and 8–65 hybridized to the same 3.3-kb BamHI fragments, and the hybridization intensity of the former was stronger than the latter. This result indicates that the 3–14 sequence in the 3.3-kb BamHI fragment is larger than the 8–65 sequence.

Based on these results, two primers, del-L and del-R, were synthesized and used for PCR amplification, which gave a 1.7-kb amplified fragment (data not shown). This fragment was digested with BamHI and EcoRI and cloned into pUC19 to give plasmid pOPP. As expected, the pOPP probe hybridized to both the left and right deletion-end fragments of strain M145 and to the fusion fragment of mutant No. 4 (Fig. 1c).

Sequence analysis of the fusion junction

Nucleotide sequencing of plasmid pOPP determined the fusion junction of the circularized chromosome of mutant No. 4. The sequences around the left and right deletion ends of strain M145 and the fusion junction of mutant No. 4 are aligned and compared in Fig. 2b. Between the left and right deletion ends, a 6-bp microhomology was identified, which is much shorter than the minimum size (20 bp) of homology required for homologous recombination (Watt *et al.*, 1985). This result again supports nonhomologous recombination of two deletion ends, which was proposed for chromosomal circularization of other *Streptomyces* species. At the left deletion

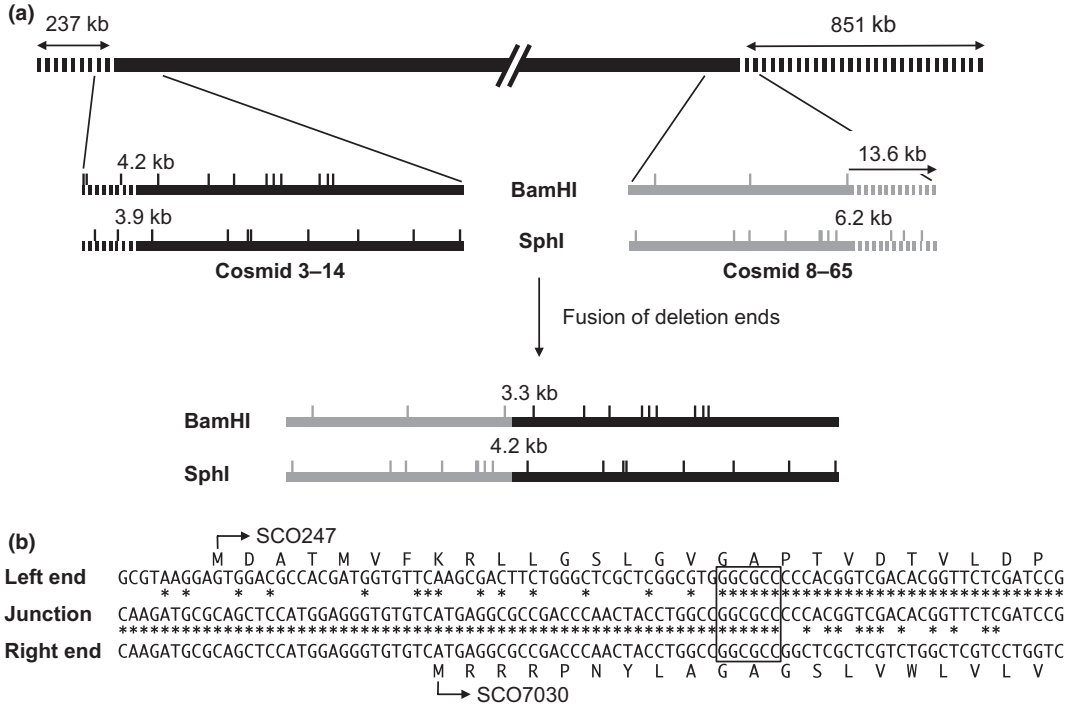

Fig. 2. Restriction maps of the deletion-end regions in strain 1147 and the fusion region in mutant No. 4 (a) and sequence alignments of the left and right deletion ends and the fusion junction (b). Deleted regions are indicated by broken lines. The 6-bp microhomology present at the deletion ends is indicated by a square. The aa sequences of the SCO247 and SCO7030 proteins are shown above and below each nucleotide sequence.

end, a putative sporulation control protein, SpoOM (SCO247), is encoded. On the other hand, at the right deletion end, a possible binding-protein-dependent transport protein (SCO7030) is encoded. The generated fusion gene encodes for a protein, in which due to frame coincidence, the N-terminal 16-aa of the SCO247 protein were replaced by the N-terminal 9-aa of SCO7030. As this protein carries most (325 aa/341 aa) of the SCO247 sequence, it may function as the SCO247 protein does in the parent strain 1147.

Mutant No. 4 lost a total of 1088 kb DNA (237 kb from the left end and 851 kb from the right end) during terminal deletion and circularization. Although mutant No. 4 shows several defective phenotypes, it grows normally (Kawamoto *et al.*, 2001). Thus, many genes located in the deleted terminal regions are not essential for survival. As the *eshA* gene is located at 131 kb from the right end, it is possible that deletion of other genes rather than *eshA* caused some of the defective phenotypes found in mutant No. 4.

Circularized *Streptomyces* chromosomes are stably maintained

There have been contradictory issues on stability of circularized *Streptomyces* chromosomes. Lin & Chen (1997) and Volff *et al.* (1997) independently constructed artificially circularized chromosomes of *S. lividans* and studied their genetic instability. In both cases, the circularized chromosomes showed higher frequencies of deletion and amplification. Thus, they claimed that the circularized chromosomes were more unstable than the parent linear chromosomes. However, it should be noted that in both cases, the deleted sequences in the artificially circularized chromosomes were restricted to the left and right TIR regions of about 30 kb each. Therefore, deletable genes such as the chloramphenicol resistance gene (*cmlR*) and the arginine biosynthetic gene (*argG*) and an amplifiable sequence such as AUD were still retained. Fischer *et al.* (1997) also prepared circularized chromosomes of *S. ambofaciens* with extremely large deletions (more than 2 Mb) by mutagenic treatments and reported their genetic instability, too. In this case, it may be possible that the extremely large deletions eliminated regions important for stable maintenance of the circularized chromosome.

In contrast to the examples described above, our and other groups obtained stably maintained circular chromosomes by mutagenic treatments of *S. griseus* (Kameoka *et al.*, 1999; Inoue *et al.*, 2003), *S. coelicolor* A3(2) (this work), and *S. avermitilis* (Chen *et al.*, 2010). In these cases, the sizes of deletions were 480 kb (130 kb at the left end + 350 kb at the right end), 580 kb (30 kb + 550 kb), and 300 kb (130 kb + 170 kb) for *S. griseus*, 1088 kb (237 kb + 851 kb) for *S. coelicolor* A3(2), and 1939 kb (1611 kb + 328 kb) for *S. avermitilis*, respectively. Thus, the deletable sizes seem to have some relation to the chromosomal size of each species: 7.8 Mb (calculated from AseI fragments) for *S. griseus* strain 2247 (Lezhava *et al.*, 1995), 8668 kb for *S. coelicolor* A3(2) (Bentley *et al.*, 2002), and 9026 kb for *S. avermilitis* (Ikeda *et al.*, 2003). Larger chromosomes may contain larger terminal regions dispensable for survival (Kirby, 2011). We speculate that terminal deletions proceed progressively until to appropriate points, where circularized chromosomes reach a stable state and are stably maintained. Thus, mutant chromosomes with small deletions or amplification may be intermediates in this process, which are finally converted to more stable circular chromosomes.

Linear replicons always have a problem of terminal replication, namely incomplete replication of the 5′ ends. *Streptomyces* linear chromosomes solve this problem as follows. Linear chromosomes are replicated bidirectionally from an internal origin (Musialoski *et al.*, 1994). This leaves single-strand overhangs at the 3′ ends (Chang & Cohen, 1994), which are filled by a novel patching synthesis primed by terminal protein (Qin & Cohen, 1998). It was suggested that *Streptomyces* linear chromosomes were generated by integration of a linear plasmid into a circular chromosome (Volff & Altenbuchner, 2000). If circularized chromosomes are stably maintained, why have *Streptomyces* kept a linear chromosome in the evolutionary history. The following advantages could be raised for linear chromosomes. (1) The genome sizes of *Streptomyces* are about two times larger than those of bacteria such as *E. coli* and *Bacillus subtilis*. Therefore, *Streptomyces* linear chromosomes could accommodate many genes that are indispensable for sophisticated morphological differentiation and adaptation to environmental changes of this soil-living genus. The size of circular chromosomes is limited, because large super-twisted circular replicons are difficult to be unwound and resolved to single strands during replication. (2) A single crossover with another linear replicon near the end of chromosome leads to end exchange (Pandza *et al.*, 1998), by which *Streptomyces* linear chromosomes could obtain genes necessary for secondary metabolism and environmental adaptation. In addition, when a single crossover occurs near the center of chromosome, it could give two chimeric chromosomes. This event actually occurred in *S. coelicolor* A3(2) strain 2106 (Yamasaki & Kinashi, 2004), which was considered as a model of chromosomal multiplication.

Structural comparison of *Streptomyces* linear replicons and studies of their genetic instability will give us impor-

tant hints how circular chromosomes have been converted to linear chromosomes in the evolutionary history (Volff & Altenbuchner, 2000; Chen *et al.*, 2002; Kirby, 2011).

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References

Altenbuchner J & Cullum J (1985) Structure of an amplifiable DNA sequence in *Streptomyces lividans* 66. *Mol Gen Genet* **201**: 192–197.

Bentley SD, Chater KF, Cerdeno-Tarraga AM *et al.* (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**: 141–147.

Chang PC & Cohen SN (1994) Bidirectional replication from an internal origin in a linear *Streptomyces* plasmid. *Science* **265**: 952–954.

Chen CW, Huang CH, Lee HH, Tsai HH & Kirby R (2002) Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes. *Trends Genet* **18**: 522–529.

Chen W, He F, Zhang X, Chen Z, Wen Y & Li J (2010) Chromosomal instability in *Streptomyces avermitilis*: major deletion in the central region and stable circularized chromosome. *BMC Microbiol* **10**: 198.

Fischer G, Decaris B & Leblond P (1997) Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA. *J Bacteriol* **179**: 4553–4558.

Fischer G, Wenner T, Decaris B & Leblond P (1998) Chromosomal arm replacement generates a high level of intraspecific polymorphism in the terminal inverted repeats of the linear chromosomal DNA of *Streptomyces ambofaciens*. *P Natl Acad Sci USA* **95**: 14296–14301.

Huang CH, Lin YS, Yang YL, Huang SW & Chen CW (1998) The telomeres of *Streptomyces* chromosomes contain conserved palindromic sequences with potential to form complex secondary structures. *Mol Microbiol* **28**: 905–916.

Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M & Omura S (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat Biotechnol* **21**: 526–531.

Inoue S, Higashiyama K, Uchida T, Hiratsu K & Kinashi H (2003) Chromosomal circularization in *Streptomyces griseus* by nonhomologous recombination of deletion ends. *Biosci Biotechnol Biochem* **67**: 1101–1108.

Kameoka D, Lezhava A, Zenitani H, Hiratsu K, Kawamoto M, Goshi K, Inada K, Shinkawa H & Kinashi H (1999) Analysis of fusion junctions of circularized chromosomes in *Streptomyces griseus*. *J Bacteriol* **181**: 5711–5717.

Kawamoto S, Watanabe M, Saito N, Hesketh A, Vachalova K, Matsubara K & Ochi K (2001) Molecular and functional analyses of the gene (*eshA*) encoding the 52-kilodalton protein of *Streptomyces coelicolor* A3(2) required for antibiotic production. *J Bacteriol* **183**: 6009–6016.

Kieser T, Bibb MJ, Buttner MJ, Chater KF & Hopwood DA (2000) *Practical Streptomyces Genetics*. The John Innes Foundation, Norwich.

Kirby R (2011) Chromosome diversity and similarity within the *Actinomycetales*. *FEMS Microbiol Lett* **319**: 1–10.

Kwak J, McCue LA, Trczianka K & Kendrick KE (2001) Identification and characterization of a developmentally regulated protein, EshA, required for sporogenic hyphal branches in *Streptomyces griseus*. *J Bacteriol* **183**: 3004–3015.

Leblond P, Fischer G, Francou FX, Berger F, Guérineau M & Decaris B (1996) The unstable region of *Streptomyces ambofaciens* includes 210 kb terminal inverted repeats flanking the extremities of the linear chromosomal DNA. *Mol Microbiol* **19**: 261–271.

Lezhava A, Mizukami T, Kajitani T, Kameoka D, Redenbach M, Shinkawa H, Nimi O & Kinashi H (1995) Physical map of the linear chromosome of *Streptomyces griseus*. *J Bacteriol* **177**: 6492–6498.

Lin YS & Chen CW (1997) Instability of artificially circularized chromosomes of *Streptomyces lividans*. *Mol Microbiol* **26**: 709–719.

Lin YS, Kieser HM, Hopwood DA & Chen CW (1993) The chromosomal DNA of *Streptomyces lividans* 66 is linear. *Mol Microbiol* **10**: 923–933.

Musialoski MS, Flett F, Scott GB, Hobes G, Smith CP & Oliver SG (1994) Functional evidence that the principal DNA replication origin of the *Streptomyces coelicolor* chromosome is close to the *dnaA-gyrB* region. *J Bacteriol* **176**: 5123–5125.

Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M & Horinouchi S (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J Bacteriol* **190**: 4050–4060.

Pandza S, Biukovic G, Paravic A, Dadbin AJ, Cullum J & Hranueli D (1998) Recombination between the linear plasmid pPZG101 and the linear chromosome of *Streptomyces rimosus* can lead to exchange of ends. *Mol Microbiol* **28**: 1165–1176.

Qin Z & Cohen SN (1998) Replication at the telomeres of the *Streptomyces* linear plasmid pSLA2. *Mol Microbiol* **28**: 893–903.

Qin Z & Cohen SN (2002) Survival mechanisms for *Streptomyces* linear replicons after telomere damage. *Mol Microbiol* **45**: 785–794.

Redenbach M, Flett F, Piendl W, Glocker I, Rauland U, Wafzig O, Kliem R, Leblond P & Cullum J (1993) The *Streptomyces lividans* 66 chromosome contains a 1 MB deletogenic region flanked by two amplifiable regions. *Mol Gen Genet* **241**: 255–262.

Redenbach M, Kieser HM, Denapaite D, Eichner A, Cullum J, Kinashi H & Hopwood DA (1996) A set of ordered cosmids

and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21**: 77–96.

Saito N, Xu J, Hosaka T, Okamoto S, Aoki H, Bibb MJ & Ochi K (2006) EshA accentuates ppGpp accumulation and is conditionally required for antibiotic production in *Streptomyces coelicolor* A3(2). *J Bacteriol* **188**: 4952–4961.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Uchida T, Miyawaki M & Kinashi H (2003) Chromosomal arm replacement in *Streptomyces griseus*. *J Bacteriol* **185**: 1120–1124.

Uchida T, Ishihara N, Zenitani H, Hiratsu K & Kinashi H (2004) Circularized chromosome with a large palindromic structure in *Streptomyces griseus* mutants. *J Bacteriol* **186**: 3313–3320.

Volff JN & Altenbuchner J (1998) Genetic instability of the *Streptomyces* chromosome. *Mol Microbiol* **27**: 239–246.

Volff JN & Altenbuchner J (2000) A new beginning with new ends: linearisation of circular chromosomes during bacterial evolution. *FEMS Microbiol Lett* **186**: 143–150.

Volff JN, Viell P & Altenbuchner J (1997) Artificial circularization of the chromosome with concomitant deletion of its inverted repeats enhances genetic instability and genome rearrangement in *Streptomyces lividans*. *Mol Gen Genet* **253**: 753–760.

Watt VM, Ingles CJ, Urdea MS & Rutter WJ (1985) Homology requirements for recombination in *Escherichia coli*. *P Natl Acad Sci USA* **82**: 4768–4772.

Widenbrant EM, Tsai HH, Chen CW & Kao CM (2007) *Streptomyces coelicolor* undergoes spontaneous chromosomal end replacement. *J Bacteriol* **189**: 9117–9121.

Yamasaki M & Kinashi H (2004) Two chimeric chromosomes of *Streptomyces coelicolor* A3(2) generated by single crossover of the wild-type chromosome and linear plasmid SCP1. *J Bacteriol* **186**: 6553–6559.

Zhou M, Jing X, Xie P, Chen W, Wang T, Xia H & Qin Z (2012) Sequential deletion of all the polyketide synthase and nonribosomal peptide synthetase biosynthetic gene clusters and a 900-kb subtelomeric sequence of the linear chromosome of *Streptomyces coelicolor*. *FEMS Microbiol Lett* **333**: 169–179.

参 考 論 文
（Thesis Supplements）

pSLA2-M of *Streptomyces rochei* is a composite linear plasmid characterized by self-defense genes and homology with pSLA2-L.
Yingjie Yang, Toru Kurokawa, Yoshifumi Takahama, Yosi Nindita, Susumu Mochizuki, Kenji Arakawa, Satoru Endo and Haruyasu Kinashi.
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pSLA2-M of *Streptomyces rochei* Is a Composite Linear Plasmid Characterized by Self-Defense Genes and Homology with pSLA2-L

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The 113,463-bp nucleotide sequence of the linear plasmid pSLA2-M of *Streptomyces rochei* 7434AN4 was determined. pSLA2-M had a 69.7% overall GC content, 352-bp terminal inverted repeats with 91% (321/352) identity at both ends, and 121 open reading frames. The rightmost 14.6-kb sequence was almost (14,550/14,555) identical to that of the coexisting 211-kb linear plasmid pSLA2-L. Adjacent to this homologous region an 11.8-kb CRISPR cluster was identified, which is known to function against phage infection in prokaryotes. This cluster region as well as another one containing two large membrane protein genes (*orf78* and *orf79*) were flanked by direct repeats of 194 and 566 bp respectively. Hence the insertion of circular DNAs containing each cluster by homologous recombination was suggested. In addition, the *orf71* encoded a Ku70/Ku80-like protein, known to function in the repair of double-strand DNA breaks in eukaryotes, but disruption of it did not affect the radiation sensitivity of the mutant. A pair of replication initiation genes (*orf1-orf2*) were identified at the extreme left end. Thus, pSLA2-M proved to be a composite linear plasmid characterized by self-defense genes and homology with pSLA2-L that might have been generated by multiple recombination events.

Key words: *Streptomyces*; linear plasmid; CRISPR; *ku70/ku80*; genome evolution

The saprophytic soil bacteria, *Streptomyces* carry a linear chromosome of 8–10 Mb in place of the usual circular bacterial chromosome.[1–3] In addition to a linear chromosome, *Streptomyces* species frequently contain several large linear plasmids.[4–6] *Streptomyces* linear chromosomes and plasmids have the same structural features in the main. They have terminal inverted repeats (TIRs) at both ends, and the 5′ ends are blocked by a terminal protein. Most *Streptomyces* linear replicons have conserved telomere sequences that can make a Y-shaped foldback structure at the extreme ends.[7] This structure may be recognized by the terminal protein and telomere-associated protein and function in protein-primed terminal replication.[8] The structural and sequence similarities of *Streptomyces* linear chromosomes and plasmids suggest a close relationship in evolutionary history.

We have been studying *Streptomyces rochei* 7434AN4, which contains three linear plasmids, pSLA2-L (211 kb), -M (113 kb), and -S (18 kb).[9] The complete nucleotide sequencing (210,614 bp) of pSLA2-L and extensive gene disruption experiments revealed that it contains biosynthetic gene clusters for lankacidin, lankamycin, a cryptic type-II polyketide, and a carotenoid.[10] In addition, pSLA2-L encoded many regulatory genes for secondary metabolism that make a complex γ-butyrolactone-dependent regulatory cascade.[11–13] The telomere sequence of pSLA2-L makes a typical Y-shaped foldback structure, while that of pSLA2-S does not.[14] pSLA2-L and -M are considered to be necessary for morphological differentiation, because the mutants carrying only pSLA2-L or -M make spores, while the plasmidless mutant 2–39 remains at the stage of aerial mycelium before spore formation.[9]

Thus the nucleotide sequencing of pSLA2-M is crucial in order to determine the biological function of pSLA2-M itself and the evolutionary relationships among pSLA2-L, -M, and -S and the chromosome of *S. rochei* 7434AN4. Here we describe the complete nucleotide sequence of pSLA2-M, *in silico* analysis of 121 open reading frames (*orf*s), and disruption of the *ku70/ku80*-like gene (*orf71*). The gene organization of pSLA2-M suggests that many recombination events have occurred to generate its present form. It is noteworthy that pSLA2-M contains multiple self-defense genes, such as a CRISPR (clustered regularly interspaced short palindromic repeats) cluster and a bacterial *ku* gene.

Materials and Methods

Bacterial strains and culture conditions. S. rochei strain K3A12, which carries only pSLA2-M,[9] was used to isolate pSLA2-M and to construct a cosmid library. *Streptomyces* strains were grown in YM liquid medium (0.4% yeast extract, 1% malt extract, and 0.4% glucose, pH 7.3). *E. coli* XL1-Blue was used for routine cloning and *E. coli* SURE2 in the construction of a cosmid library. *E. coli* strains were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/mL).

DNA manipulation and Southern hybridization. DNA manipulation of *Streptomyces*[15] and *E. coli*[16] were carried out by standard

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procedures. Plasmid pSLA2-M was separated by contour-clamped homogeneous electric fields (CHEF), which was done in 0.5 × TBE buffer using 1.0% agarose gel at 14 °C. Digested fragments were separated by CHEF or conventional agarose gel electrophoresis and transferred to nylon membranes. DNA probes were labeled with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany), and hybridization was carried out following the manufacturer's protocol.

Construction of the cosmid library and end cloning. A cosmid library was constructed for total DNA of strain K3A12 using the vector Supercos1 and Gigapack III Gold Packaging Kit (Stratagene, La Jolla, CA), and was probed with pSLA2-M DNA to obtain a sub-library. Twelve cosmids in the pSLA2-M sub-library were analyzed by restriction mapping, and finally three clones, 3A9, 4E5, and 7E3, were selected and aligned (see Fig. 2).

The left and right end fragments, *Bgl*II-I (1.1 kb) and *Pst*I-J (3.8 kb), were forced-cloned into pUC19 pre-digested with *Hin*cII and *Bgl*II and with *Pst*I and *Hin*cII respectively. The end fragments, which should still retain a small peptide covalently bound to the 5′ end after protease treatment, were ligated directly to the vector and subjected to transformation.

Nucleotide sequencing and analysis. The nucleotide sequences of the three cosmid regions were determined using *Bam*HI shot-gun clones and additional supplementary clones of the restriction and PCR fragments. The gap between the left-end fragment, *Bgl*II-I, and cosmid 3A9 was filled by cloning *Bgl*II-F and a PCR fragment linking *Bgl*II-I and *Bgl*II-F. The gap between cosmid 7E3 and the right-end fragment, *Pst*I-J, was filled by cloning of PCR fragments prepared on the basis of the terminal homology between pSLA2-M and -L. Consequently, overlapping sequences covering the entire region of pSLA2-M were read more than twice without any gaps. Nucleotide sequencing was done by the dideoxy termination method, using a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA) and BigDye Terminator v3.1/v1.1 Cycle Sequencing kits (Life Technologies). Sequence data were assembled by Sequencher 4.5 (Gene Codes, Ann Arbor, MI), and were analyzed with Genetyx-Mac 15.0.3 (Software Development, Tokyo), FramePlot[17] (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl), and MiGAP (Microbial Genome Annotation Pipeline ver1.05) (https://migap.lifesciencedb.jp/mgap/jsp/index.jsp). Gene prediction was further examined by homology search using the BLASTP program.

Disruption of orf71 *and radiation assay.* The 3.0-kb *Pst*I-*Bgl*II fragment carrying *orf71* (nt 54,696–55,625 of pSLA2-M) was cloned into pBluescript SK(+) (Stratagene) pre-digested with *Pst*I and *Bam*HI to give pYT-01. Into the *Bam*HI site at the 5′-terminus of *orf71* was inserted a 1.6-kb *Bam*HI fragment carrying a kanamycin resistance cassette from pUC4-KIXX (Pharmacia) to afford pYT-02. The vector part of pYT-02 was replaced with an *E. coli-Streptomyces* shuttle vector, pRES18,[18] to give a targeting plasmid, pYT-03.

Plasmid pYT-03 was transformed into protoplasts of *S. rochei* K3A12. The plasmid-integrated strains were selected with thiostrepton (10 μg/mL), and were serially cultured in the absence of thiostrepton to facilitate a second crossover. The double cross-overed strains were identified by Southern hybridization using pYT-01 as probe to give mutant KYT325.

Spore suspensions of strains K3A12 and KYT325 were subjected to various doses of gamma irradiation from the ^{60}Co source. The suspensions were diluted and spread on YM agar plates, and colonies were counted after 4 d at 28 °C.

Results and Discussion

Physical mapping of pSLA2-M

Previous experiments showed that pSLA2-M and -L were cross-hybridized to each other, but pSLA2-S did not hybridize to pSLA2-L or -M.[9] To analyze more precisely the homology between pSLA2-M and -L and to prepare for the nucleotide sequencing of pSLA2-M, restriction analysis was done. pSLA2-M was isolated by pulsed-field gel electrophoresis from mutant K3A12,

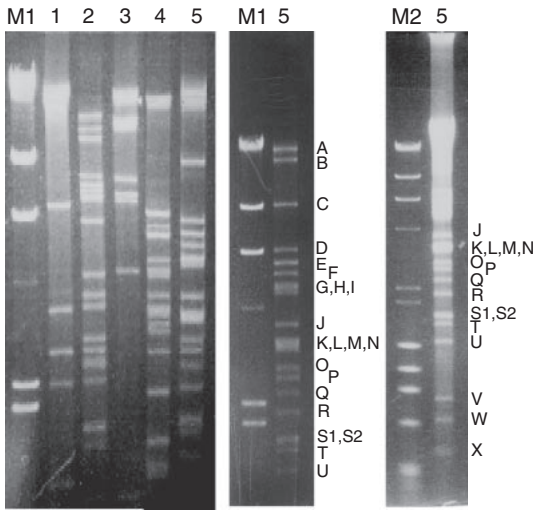

Fig. 1. Analysis of Restriction Fragments of pSLA2-M by Agarose Gel Electrophoresis.

pSAL2-M was digested with *Cla*I (lane 1), *Bcl*I (lane 2), *Bgl*II (lane 3), *Bam*HI (lane 4), and *Pst*I (lane 5). λ DNA digested with *Hin*dIII (M1) and φ × 174 DNA digested with *Hae*III (M2) were used as size markers.

which carries only pSLA2-M,[9] and was digested with various restriction enzymes. As shown in Fig. 1, *Bgl*II and *Pst*I gave 11 and 24 fragments respectively, and were selected for physical mapping. The sizes of the small *Pst*I fragments, J (3.8 kb), K (3.4 kb), S (1.7 kb), T (1.6 kb), U (1.4 kb), V (0.8 kb), and X (0.4 kb), were same as those of *Pst*I fragments, L (3.4 kb), Q2 (1.6 kb), T (0.4 kb), P (1.7 kb), R (1.4 kb), S (0.8 kb), and K (3.8 kb), aligned at the right end of pSLA2-L,[10] suggesting terminal homology between pSLA2-M and -L. The fragments obtained by double digestion with *Nde*I and *Spe*I were isolated and used as probes against the *Bgl*II and *Pst*I fragments for grouping. Furthermore, the isolated *Bgl*II fragments and the *Bam*HI shot-gun clones were hybridized against the *Pst*I fragments to construct *Bgl*II and *Pst*I restriction maps of pSLA2-M (Fig. 2).

The terminal location of fragments *Bgl*II-I and *Bgl*II-J, and *Pst*I-E and *Pst*I-J was confirmed by their retardation in gel electrophoresis due to the binding of a terminal protein when not treated with protease (data not shown). It is noteworthy that the sizes and order of *Pst*I fragments K-T-X-S2-U-V-J at the right end were identical to those of the coexisting linear plasmid pSLA2-L, *Pst*I-L-Q2-T-P-R-S-K.[10] Thus the terminal homology between pSLA2-M and -L extends over 14 kb from the right end into fragments *Pst*I-L of pSLA2-M and *Pst*I-Q1 of pSLA2-L.

Nucleotide sequencing and basic features of pSLA2-M

For complete nucleotide sequencing of pSLA2-M, a cosmid library was constructed for the total DNA of strain K3A12. The pSLA2-M sub-library was selected from the K3A12 library by colony hybridization with pSLA2-M DNA, which finally gave three ordered cosmid clones, 3A9, 4E5, and 7E3 (Fig. 2). The left- and right-end fragments, *Bgl*II-I (1.1 kb) and *Pst*I-J (3.8 kb), were forced-cloned. In addition, clones filling the gaps between the end clones and cosmids 3A9 and 7E3 were obtained to cover the entire region of pSLA2-

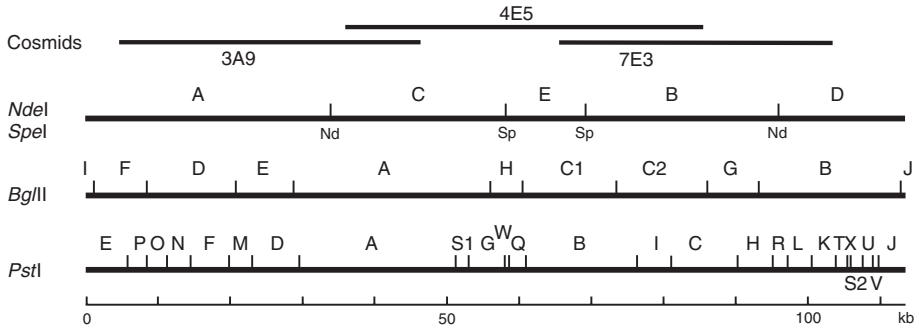

Fig. 2. Restriction Map of pSLA2-M.
The restriction sites for *Nde*I, *Spe*I, *Bgl*II, and *Pst*I are indicated. The positions of three cosmids, 3A9, 4E5, and 7E3, are also shown.

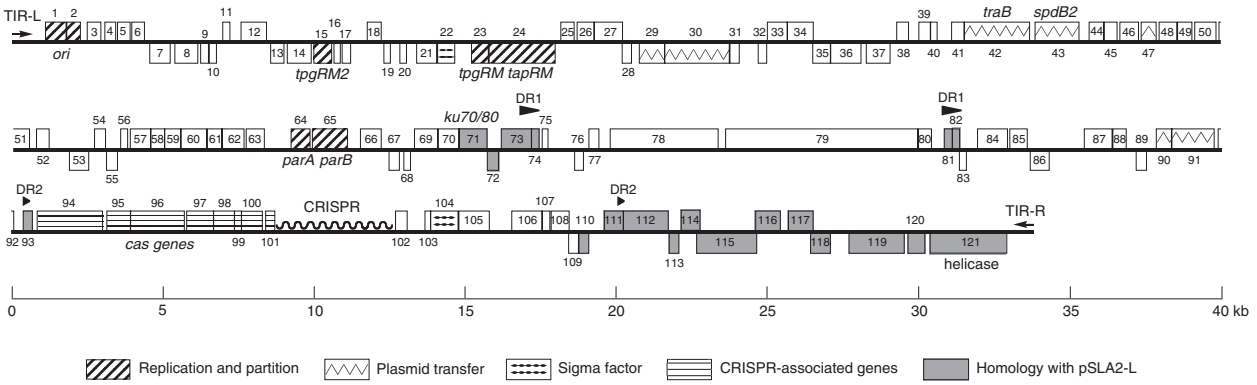

Fig. 3. Gene Organization of pSLA2-M.
Each *orf* is shown as a box above or below the DNA line based on its direction of translation (rightward or leftward). The *orf*s explained in the text and the homologous regions with pSLA2-L are indicated by different shadings. The left and right terminal inverted repeats (TIR-L and TIR-R) and two direct repeats (DR1 and DR2) are shown by arrows.

M. The complete sequence of pSLA2-M was determined using all of these clones and their subclones.

pSLA2-M was determined to be a 113,463-bp linear plasmid that contained 352-bp terminal inverted repeats (TIR) with 91% (321/352) identity at both ends. The GC content of pSLA2-M was 69.7%, comparable to the 356-kb linear plasmid SCP1 of *Streptomyces coelicolor* A3(2) (69.1%),[19] but slightly lower than the *S. coelicolor* A3(2) chromosome (72.1%)[1] and the coexisting linear plasmid pSLA2-L (72.8%).[10] pSLA2-M carried 121 *orf*s, shown in Fig. 3 and listed in Supplemental Table S1 (see *Biosci. Biotechnol. Biochem.* Web site). The TTA codon was found in 10 *orf*s (*orf3*, *orf8*, *orf20*, *orf28*, *orf45*, *orf63*, *orf94*, *orf104*, *orf116*, and *orf117*), making their translation dependent on the leucyl-tRNA coded by *bldA*.[20] Although TTA codons are present in 2–3% of the whole genome of *Streptomyces*,[21] they are more abundant (8%, 10/121) in pSLA2-M. In this respect, five TTA-containing *orf*s were found in pSLA2-L (3.5%, 5/143). Among the 10 *orf*s containing a TTA codon, *orf94* encoded a putative CRISPR-associated Cas3 family protein, and *orf104* encoded a putative RNA polymerase sigma factor. The complete nucleotide sequence of pSLA2-M has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AB597522.

Telomere structures and terminal homology with pSLA2-L

Most *Streptomyces* linear chromosomes and plasmids contain conserved telomere sequences of 160–170 bp, which can make multiple hairpin loops including a Y-shaped foldback structure at the extreme end.[7] pSLA2-M also contains conserved telomere sequences at both ends, which make a Y-shaped foldback structure (Fig. 4). Although the left and right TIR sequences are not identical, their secondary structures are similar, suggesting an important biological function.

It was found that the terminal homology between pSLA2-M and -L extends over 14,555 bp from the right end, with only 5 nt mismatches. This brings to mind the terminal homology between the linear plasmid SLP2 and the *S. lividans* chromosome,[22] which indicates that SLP2 was generated by single crossover of the wild-type chromosome and a linear plasmid. Similarly, single crossover between pSLA2-M and another linear replicon might have generated the right part of pSLA2-L (see below).

Replication origin

Two *orf*s, *orf1* and *orf2*, at the left extreme end of pSLA2-M, showed 99% and 93% identity in protein sequence to the replication initiation genes, pFRL2.1 and pFRL2.2, of linear plasmid pFRL2 from *Streptomyces* sp. FR1. Zhang *et al.*[23] found experimentally that pFRL2.1 and pFRL2.2 together with an adjacent non-coding sequence function as a replication origin in *S. lividans*. *Streptomyces* linear replicons display two different replication mechanisms, bidirectional replication from an internal replication origin and protein-primed terminal replication from the ends. The end locations of pFR2.1/pFR2.2 of pFRL2 and *orf1*/*orf2* of

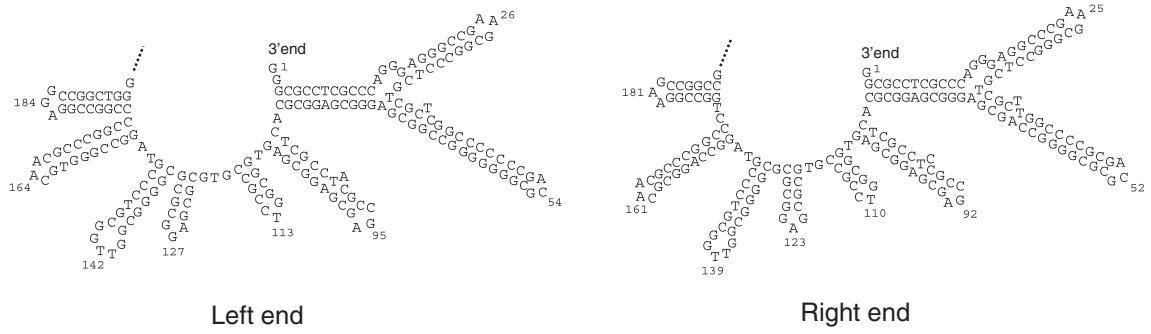

Fig. 4. Possible Secondary Structures at the Left and Right Extreme Ends of pSLA2-M.

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TpgRM     MDSLGDSLDRALEGAFTRRIPQSAQAQMKYLVKQLKG-TKATAQALGISQRTVERYVSGKLKRPRQDLRGRIEREVKKRWQPQVRAKARKK  90
TpgR2     ..........................................-.......................................................  90
TpgR3     ...I..G......S.....P.................-.R.V.RL.R.....................................................  90
TpgR1     ...I..G......S.....P.................-.R.V.RL.R..........A.Q.....RE..D......H.........R..RR  90
TpgSGR    .SLF..G..A.VHK....PV.K..S...R........-...V..M.R..........KEQI.K..P..AA.L.G.........I.....Q.  90
TpgC/TpgL .SLF.NG..A.VQK....PA.K..G...R........-...V..M.RV.........KNEI....P..AA.L.....A........R..Q.  90
TpgSLP2   .GII..G....VQ.....P..K..G...R........-.R.V.GL..V.........KDQIR...A..AQ.L.DA.RQ....R..DR...Q  90
TpgRM2        MEG.A..ERAL...PA.K..R....F.R.RE..S..RL.ER..V.RK..Q..L..ASTK.NKR..QALVQ.TEAE.......Q..QR  87

TpgRM     AASTDGLVVSTRARFGFTAAPGTTDDARIRDITQALPPEYADRLFTAREQGATEQQLQQIAADGLAQMYFRANNSRAHGLG-VEFTDIEQIQIEL 184
TpgR2     ..................................................H.................DT......-.........E.Q. 184
TpgR3     ..................................................H.................DT......-.........E.Q. 184
TpgR1     ..T.............................I......F.A............H.................DT......-.........E.Q. 184
TpgSGR    ..T.G.I.ID....I.Y..PI....ED.L.HL.V....R..A...D.Q.T...D.R......EA.KEV..QDSGR..GS.EE.R.A...HLEFD. 185
TpgC/TpgL ..T...I.ID....L.Y..PI.S..QD...HL.V....Q..G...D.HQA...D....G...EA.KEV..QDGGR..GS.EE.R.....HLEFD. 185
TpgSLP2   ..AST...IH...................HL.L....HH.A..LD.QDA..S....RGL..EA.GEV...DGGR..G-..E.....V..AEFD. 184
TpgRM2    .TTSG....KF..SL....N-.SS.......LSI.VS.SH.ALILA.Q.E...DTD.HKAV.EAI.DA...QAGGGRAS.V-...R.V.WLD..F 180
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Fig. 5. Alignment of Terminal Proteins of *S. rochei* 7434AN4 and Other *Streptomyces* Species.

Amino acids identical to TpgRM are shown by dots in the DDBJ style. The accession numbers of the proteins and their similarities to TpgRM are as follows: TpgR2 of *S. rochei*, AAL05042, 97% (179/184); TpgR3 of *S. rochei*, AAL05043, 92% (170/184); TpgR1 of *S. rochei*, NP_851524, 85% (157/184); TpgSgr of *S. griseus*, BAI23312, 61% (114/185); TpgL/TpgC of *S. lividans*/*S. coelicolor* A3(2), AAL05040, 61% (114/185); TpgSLP2 of *S. lividans*, NP_862058, 63% (116/184); TpgRM2, 46% (83/180).

pSLA2-M are unusual, because replication origins are usually located in the center of linear replicons for efficient functioning. This is true of other linear plasmids of strain 7434AN4. Chang and Cohen[24] reported bidirectional replication from an internal origin of pSLA2 (=pSLA2-S), and we identified three pairs of replication initiation genes (*orf87/89*, *orf93/94*, and *orf95/96*) among the 143 *orf*s of pSLA2-L.[10,25]

Conserved genes in Streptomyces *linear replicons: the* tpg-tap*,* parA-parB*, and helicase genes*

Most *Streptomyces* linear plasmids and chromosomes hitherto analyzed contain a pair of genes coding a terminal protein (*tpg*) and a telomere-associated protein (*tap*) for telomere patching, another pair for DNA partitioning (*parA* and *parB*), and a helicase gene. All of these genes were also found on pSLA2-M. Although the two former pairs are located close to each other in many linear replicons, they are separated on pSLA2-M: *tpgRM-tapRM* (*orf23-24*) and *parA-parB* (*orf64-65*). On the other hand, the helicase gene (*orf121*) was located at the right extreme end, as in other linear replicons. It is noteworthy that all five genes, with high similarity, were also found on pSLA2-L, where the *tpgR1-tapR1* (*orf102-101*) and *parA-parB* (*orf98-97*) genes were located nearby, and a truncated (*orf1*) and an intact (*orf143*) helicase gene were at the left and right extreme end respectively.

Concerning the *tpg* genes of *S. rochei* 7434AN4, we noticed an interesting discrepancy. Bao and Cohen[26] cloned and sequenced three *tpg* genes, *tpgR1*, *tpgR2*, and *tpgR3*, from strain 7434AN4, and located them on the chromosome, pSLA2-L, and pSLA2-M respectively, but nucleotide sequencing of pSLA2-L revealed that *tpgR1* is the pSLA2-L *tpg*.[10] To our surprise, pSLA2-M *tpg* (*tpgRM*) was not identical to *tpgR2* or *tpgR3*, although it was most similar to *tpgR2* (97% (179/184) identity in protein sequence). Why this discrepancy exists is unknown at present. In addition to *tpgRM*, another *tpg* gene (*orf15*) named *tpgRM2*, with a low similarity (46% (83/180) identity to TpgRM) was found on pSLA2-M, which does not make a pair with a *tap* gene. All five terminal proteins of strain 7434AN4 and typical *Streptomyces* terminal proteins are aligned and compared in Fig. 5.

Genes involved in plasmid transfer and spore formation

The mechanism of plasmid transfer in *Streptomyces* is different from and much simpler than those in other bacteria. Only TraB, a FtsK/SpoIIIE-family DNA translocase, suffices for intermycelial transfer of circular plasmids in double-stranded form.[27] In addition, Tiffert *et al.*[28] have suggested that TraB together with spread proteins such as SpdB2 form a channel at the septal cross walls and function in the intramycelial spread of plasmid DNAs, too. Although little is known about the transfer of linear plasmids, recent work by Hsu and Chen[29] suggests that TraB and SpdB2 also function in the transfer and spread of linear plasmid SLP2 in

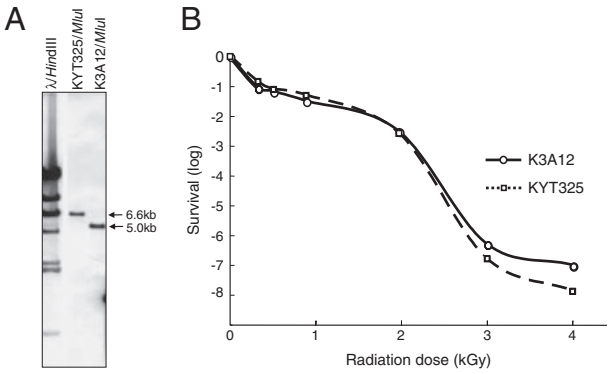

Fig. 6. Southern Hybridization Analysis of *orf71* Disruption (A) and the Effects of ^{60}Co Irradiation on Parent (K3A12) and Mutant (KYT325) Strains (B).

S. coelicolor. Two adjacent *orf*s of pSLA2-M, *orf42* and *orf43*, encode putative TraB and SpdB2 proteins that are most similar to TraB of *S. viridochromogenes* (accession no. EFL33809, 35% (242/702) identity) and SpdB2 of *S. natalensis* (CAB62260, 55% (67/123) identity) respectively. Additional possible transfer genes are marked by wavy lines in Fig. 3: *tra3* (*orf29*), *tra1* (*orf30*), *spdB3* (*orf47*), and *traA* (*orf90* and *orf91*) (see Supplemental Table S1).

Strain K3A12, which carries only pSLA2-M, and strain 51252, which carries only pSLA2-L, both produce spores, while strain 2–39, which has lost all three linear plasmids, remains at the stage of aerial mycelium formation.[9] This suggests that pSLA2-M and -L both code for a gene(s) essential to develop morphological differentiation from aerial mycelium to spore. The *parA-parB* genes (*orf64-65*) and two sigma factor genes (*orf22* and *orf104*) might be candidate genes, but we recently obtained data suggesting that strain 2–39 contains a circularized chromosome in place of a linear chromosome (unpublished results). Hence it is also possible that terminal deletions leading to chromosomal circularization caused a loss of the gene(s) indispensable for spore formation in mutant 2–39.

The bacterial ku *gene (*orf71*) and its disruption*

It is well known that in eukaryotes Ku70/Ku80 proteins function in the repair of double-strand DNA breaks caused by ionizing irradiation or other DNA-damaging agents.[30] The *orf71* of pSLA2-M encodes a Ku70/Ku80-like protein. In addition, strain 7434AN4 contains another *ku* gene on pSLA2-L (*orf121*). The gene products of these two *orf*s are 97% (300/312) identical to each other and do not contain a vWA extension domain at the N-termini, a common feature of bacterial Ku proteins. The progress of bacterial genome projects has revealed that *ku* genes are distributed in *Streptomyces* species including linear plasmids such as SCP1[19] and pSV2 (accession no. NC_004934). This suggests the possibility that the *ku* genes function in the integrity of *Streptomyces* linear replicons.

To determine the function of *orf71*, a kanamycin-resistant gene cassette was inserted into the gene, and mutant KYT325 was obtained. As shown in Fig. 6A, the 5.0-kb *Mlu*I fragment of parent strain K3A12 was shifted to 6.6 kb in mutant KYT325, which confirms gene replacement. The spore suspensions of both strains

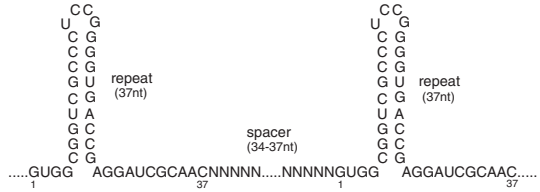

Fig. 7. Possible Hairpin Loop Structures of Precursor RNA Deduced from the CRISPR Sequence.

were subjected to ^{60}Co irradiation, and surviving spores were counted after spreading and growing them on YM plates. Against our expectations, the two strains showed similar survival curves (Fig. 6B), which indicates that *orf71* does not function in radiation resistance.

We had thought that strain K3A12 does not have a chromosomal *ku* gene, because the total DNA of strain K3A12 showed no additional signals when probed with *orf71* (Fig. 6A), but recently we identified two additional *ku70/ku80*-like genes in the ongoing genome project of *S. rochei* 7434AN4 (unpublished results). Hence it is necessary to do comprehensive targeting experiments including these possible chromosomal *ku* genes.

The CRISPR cluster

CRISPR sequences were found from nt 88,846 to 92,386 of pSLA2-M, which contain 49 copies of 37-bp identical or similar repeat sequences and 34–37 bp unique spacer sequences (Fig. 7 and Supplemental Table S2). On the left side of the repeat sequences, eight CRISPR-associated (*cas*) genes (*orf94-orf101*) were identified (Fig. 3). CRISPR sequences have been found in many prokaryotes, especially in archaea, and provide acquired resistance to phage infection.[31] It was found that precursor RNA transcribed from the repeated sequences is cleaved into small pieces of CRISPR RNAs by Cas proteins, which in turn repress the transcription of phages by a mechanism similar to that of RNAi. As shown in Fig. 7, the 37-bp repeat sequences of the precursor RNA form a hairpin structure, which may be recognized by Cas proteins. The CRISPR/*cas* clusters were grouped into several subtypes based on the organization of the *cas* genes.[32] Eight *cas* genes of pSLA2-M are aligned in the order *cas3*(*orf94*)-*cas5*(*orf95*)-*csd1*(*orf96*)-*csd2*(*orf97*)-*cas4*(*orf98*)-*cas1*(*orf99* and *orf100*)-*cas2*(*orf101*), which is similar to that of *Desulfovibrio vulgaris*.

Experimental infection of *Streptococcus thermophilus* with bacteriophages resulted in the addition of a new pair of repeat and spacer sequences to the CRISPR cluster.[33] Thus the CRISPR sequences progressively increase with every phage infection, and a new interspace sequence is derived from the infected phage DNA. The 37-bp repeat sequences are identical (GTGGCGGTCGCCCTCCGGGGTGACCGAGGATCGCAAC) from repeat-1 to repeat-34, except for repeat-20, and diverge to sequences with a 1–5 nt difference after repeat-35 (Supplemental Table S2). To determine the origins of the spacer sequences, a homology search was carried out on all 48 spacers, but no sequences with high homology were found in the databases. Nevertheless, the high GC contents of both the repeat and spacer sequences suggest that they were generated in actinobacteria.

Possible evolutionary pathway generating pSLA2-M and -L

S. rochei 7434AN4 carries three linear plasmids, pSLA2-L, -M, and -S. pSLA2-L contains the biosynthetic clusters for lankacidin, lankamycin, a cryptic type-II polyketide, and a carotenoid as well as many regulatory genes for secondary metabolism. The present study found that pSLA2-M carried multiple self-defense genes, such as the bacterial *ku* gene (*orf71*) and the CRISPR cluster. Although their activities have not been confirmed, these self-defense genes are located on linear plasmid pSLA2-M in place of the chromosome. How were these linear plasmids with a specific gene organization generated?

As for the extremely condensed biosynthetic clusters on pSLA2-L, we did not detect any transposition-related genes or sequences around the clusters, except for a few truncated genes.[10] This is true also for the methylenomycin cluster on SCP1.[19] Based on these results, the hypothesis was proposed that horizontal transfer did not occur by a direct transposition of the cluster, but involved multiple recombination events.[34] Several cycles of single crossover on both sides of the cluster generate a gene organization that gives the impression of having been generated by direct transposition of the cluster. Truncated genes frequently found around the clusters may be traces of nonhomologous recombination.

The situation is different for pSLA2-M. The region containing two large membrane proteins (*orf78* and *orf79*) is flanked by 566-bp direct repeats (DR1, nt 56,754–57,319 and nt 70,623–71,188, 98% (560/566) identity), and the region containing the CRISPR cluster and *orf102-orf111*, flanked by 194-bp direct repeats (DR2, nt 80,380–80,573 and nt 99,938–100,130, 91% (178/194) identity). This suggests the possibility that both regions were inserted by homologous recombination of a circular DNA. In both cases, the DR and neighboring sequences are homologous to pSLA2-L, suggesting that circular DNAs were inserted into pre-pSLA2-L to generate pSLA2-M. However, another possibility, that the DR sequences were also generated by multiple recombination events, cannot be ruled out.

The terminal homology between pSLA2-M and -L extends 14.6 kb from the right end, which means that pSLA2-L contains only the 1.2-kb right end of the 20-kb CRISPR region flanked by the two DR2 sequences. Thus a single crossover might have occurred at this point, removing the main part of the CRISPR region of pSLA2-M to afford the 14.6-kb homologous region at the right end of pSLA2-L. Thus many mutual recombinations might have generated the present forms of pSLA2-M and -L. Similarly, a single crossover between the wild-type linear chromosome and a linear plasmid generated the two chimeric linear chromosomes in *S. coelicolor*[35] and caused end-exchange in *S. rimosus*.[36]

We recently determined the nucleotide sequence of the smallest linear plasmid, pSLA2-S (unpublished results). No homology was found between pSLA2-S and pSLA2-L or -M. Therefore, to clarify the structural relationships among the three linear plasmids and the chromosome of strain 7434AN4 and to analyze their generation mechanism, it is crucial to determine the nucleotide sequence of the chromosome. The genome project of *S. rochei* 7434AN4 is now in progress. The completion of it ought to yield important hints as to the genome evolution of this strain and of *Streptomyces* species in general.

Acknowledgments

This report is dedicated to the late Dr. K. Sakaguchi, who first isolated *Streptomyces* linear plasmid pSLA2 (=pSLA2-S)[37] and analyzed its physical properties,[14] and has always encouraged our work on pSLA2 plasmids. We thank the Center for Gene Science, Hiroshima University, for nucleotide sequencing. This work was supported by Grant-in-Aids for Scientific Research on Priority Areas 18018028 (to H.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1) Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, and Hopwood DA, *Nature*, **417**, 141–147 (2002).
2) Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M, and Omura S, *Nat. Biotechnol.*, **21**, 526–531 (2003).
3) Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M, and Horinouchi S, *J. Bacteriol.*, **190**, 4050–4060 (2008).
4) Kinashi H, Shimaji M, and Sakai A, *Nature*, **328**, 454–456 (1987).
5) Chater KF and Kinashi H, "Microbiology Monographs, Vol. 7, Microbial Linear Plasmids," eds. Meinhardt F and Klassen R, Springer, Berlin/Heidelberg, pp. 1–31 (2007).
6) Kinashi H, *J. Antibiot.*, **64**, 19–25 (2011).
7) Huang CH, Lin YS, Yang YL, Huang SW, and Chen CW, *Mol. Microbiol.*, **28**, 905–916 (1998).
8) Qin Z and Cohen SN, *Mol. Microbiol.*, **28**, 893–903 (1998).
9) Kinashi H, Mori E, Hatani A, and Nimi O, *J. Antibiot.*, **47**, 1447–1455 (1994).
10) Mochizuki S, Hiratsu K, Suwa M, Ishii T, Sugino F, Yamada K, and Kinashi H, *Mol. Microbiol.*, **48**, 1501–1510 (2003).
11) Arakawa K, Mochizuki S, Yamada K, Noma T, and Kinashi H, *Microbiology*, **153**, 1817–1827 (2007).
12) Yamamoto S, He Y, Arakawa K, and Kinashi H, *J. Bacteriol.*, **190**, 1308–1316 (2008).
13) Suzuki T, Mochizuki S, Yamamoto S, Arakawa K, and Kinashi H, *Biosci. Biotechnol. Biochem.*, **74**, 819–827 (2010).
14) Hirochika H, Nakamura K, and Sakaguchi K, *EMBO J.*, **3**, 761–766 (1984).
15) Kieser T, Bibb MJ, Buttner MJ, Chater KF, and Hopwood DA, "Practical *Streptomyces* Genetics," John Innes Foundation, Norwich (2000).
16) Sambrook J, Fritsch EF, and Maniatis T, "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).
17) Ishikawa J and Hotta K, *FEMS Microbiol. Lett.*, **174**, 251–253 (1999).
18) Ishikawa J, Niino Y, and Hotta K, *FEMS Microbiol. Lett.*, **145**, 113–116 (1996).
19) Bentley SD, Brown S, Murphy LD, Harris DE, Quail MA, Parkhill J, Barrell BG, McCormick JR, Santamaria RI, Losick R, Yamasaki M, Kinashi H, Chen CW, Chandra G, Jakimowicz D, Kieser HM, Kieser T, and Chater KF, *Mol. Microbiol.*, **51**, 1615–1628 (2004).

20) Leskiw BK, Lawlor EJ, Fernandez-Abalos JM, and Chater KF, *Proc. Natl. Acad. Sci. USA*, **88**, 2461–2465 (1991).
21) Chandra G and Chater KF, *Antonie Van Leeuwenhoek*, **94**, 111–126 (2008).
22) Huang CH, Chen CY, Tsai HH, Chen C, Lin YS, and Chen CW, *Mol. Microbiol.*, **47**, 1563–1576 (2003).
23) Zhang R, Xia H, Guo P, and Qin Z, *FEMS Microbiol. Lett.*, **290**, 209–216 (2009).
24) Chang PC and Cohen SN, *Science*, **265**, 952–954 (1994).
25) Hiratsu K, Mochizuki S, and Kinashi H, *Mol. Gen. Genet.*, **263**, 1015–1021 (2000).
26) Bao K and Cohen SN, *Genes Dev.*, **15**, 1518–1527 (2001).
27) Reuther J, Gekeler C, Tiffert Y, Wohlleben W, and Muth G, *Mol. Microbiol.*, **61**, 436–446 (2006).
28) Tiffert Y, Götz B, Reuther J, Wohlleben W, and Muth G, *Microbiology*, **153**, 2976–2983 (2007).
29) Hsu CC and Chen CW, *J. Bacteriol.*, **192**, 307–315 (2010).
30) Critchlow SE and Jackson SP, *Trends Biochem. Sci.*, **23**, 394–398 (1998).
31) Sorek R, Kunin V, and Hugenholtz P, *Nat. Rev. Microbiol.*, **6**, 181–186 (2008).
32) Haft DH, Selengut J, Mongodin EF, and Nelson KE, *PLoS Comput. Biol.*, **1**, 474–483 (2005).
33) Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, and Horvath P, *Science*, **315**, 1709–1712 (2007).
34) Kinashi H, *Actinomycetol.*, **22**, 20–29 (2008).
35) Yamasaki M and Kinashi H, *J. Bacteriol.*, **186**, 6553–6559 (2004).
36) Pandza S, Biukovic G, Paravic A, Dadbin A, Cullum J, and Hranueli D, *Mol. Microbiol.*, **28**, 1165–1176 (1998).
37) Hayakawa T, Tanaka T, Sakaguchi K, Otake N, and Yonehara H, *J. Gen. Appl. Microbiol.*, **25**, 255–260 (1979).