REVIEW ARTICLE

Giant linear plasmids in *Streptomyces*: a treasure trove of antibiotic biosynthetic clusters

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Many giant linear plasmids have been isolated from *Streptomyces* by using pulsed-field gel electrophoresis and some of them were found to carry an antibiotic biosynthetic cluster(s); SCP1 carries biosynthetic genes for methylenomycin, pSLA2-L for lankacidin and lankamycin, and pKSL for lasalocid and echinomycin. Accumulated data suggest that giant linear plasmids have played critical roles in genome evolution and horizontal transfer of secondary metabolism. In this review, I summarize typical examples of giant linear plasmids whose involvement in antibiotic production has been studied in some detail, emphasizing their finding processes and interaction with the host chromosomes. A hypothesis on horizontal transfer of secondary metabolism involving giant linear plasmids is proposed at the end.
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INTRODUCTION

Giant linear plasmids are widely distributed in actinobacteria and are involved in secondary metabolism, such as antibiotic production, degradation of aromatic compounds and phytopathogenicity.[1–3] In particular, the involvement of linear plasmids in antibiotic production was reported in many *Streptomyces* species. In most cases, antibiotic biosynthetic genes have been studied in detail, but the gross structures of linear plasmids and their structural relationship with linear chromosomes have not, because chemists are mainly interested in biosynthetic mechanism. However, accumulated data suggest that giant linear plasmids have played critical roles in genome evolution and horizontal transfer of secondary metabolism. Thus, to clarify the mechanism of these biologically important phenomena, precise structural and sequence comparison of linear plasmids and chromosomes is necessary. I have been in Professor Hutchinson's laboratory, Wisconsin University, from 1984 to 1986 and studied biosynthesis of the polyether antibiotic lasalocid in *Streptomyces lasaliensis*. This experience after returning to Japan resulted in the discovery of giant linear plasmids from many antibiotic-producing *Streptomyces* species, including pKSL from *S. lasaliensis*.[4,5] In this review, I summarize giant linear plasmids involved in antibiotic production in *Streptomyces*, emphasizing their finding processes and interaction with the host chromosomes. At the end, possible mechanisms on how giant linear plasmids could function in horizontal transfer of secondary metabolism, are discussed.

SCP1 IN *STREPTOMYCES COELICOLOR*

Isolation and characterization of the giant linear plasmid of SCP1
It has long been suggested that plasmids are involved in antibiotic production in *Streptomyces* species, on the basis of genetic instability of the antibiotic-producing ability.[6] In particular, extensive genetic studies by Hopwood and colleagues[7,8] confirmed that the plasmid SCP1 carries the biosynthetic (*mmy*) genes for methylenomycin (Figure 1) in *S. coelicolor* A3(2). Furthermore, the *mmy* gene cluster was cloned by mutational cloning and analyzed in detail,[9] even though SCP1 itself was still resistant to isolation and its physical identity had not been clarified. The application of pulsed-field gel electrophoresis (PFGE) to *Streptomyces* DNA changed this situation and revealed that SCP1 is a giant linear plasmid of about 350 kb.[10] At the same time, many linear plasmids were detected in other antibiotic-producing strains, including *S. rochei* and *S. lasaliensis* (Table 1).[4] Furthermore, subsequent physical analysis revealed that *Streptomyces* chromosomes themselves are linear.[11,12]

Restriction analysis and treatment with exonuclease III and λ exonuclease showed that SCP1 contains terminal inverted repeats (TIRs) of about 80 kb at both ends and the 5′ ends are blocked by a terminal protein (Figure 2a).[13] These two features proved to be conserved in most of linear plasmids and chromosomes in actinobacteria. Cloning and sequencing of the terminal fragments revealed that the telomere sequence of SCP1 could not form a Y-shaped foldback structure,[14] a feature conserved in most *Streptomyces* linear replicons,[15] which may function in terminal replication.[16] However,

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Dedicated to the late Dr C Richard Hutchinson for his exceptional contributions to natural product biosynthesis, engineering and drug discovery.
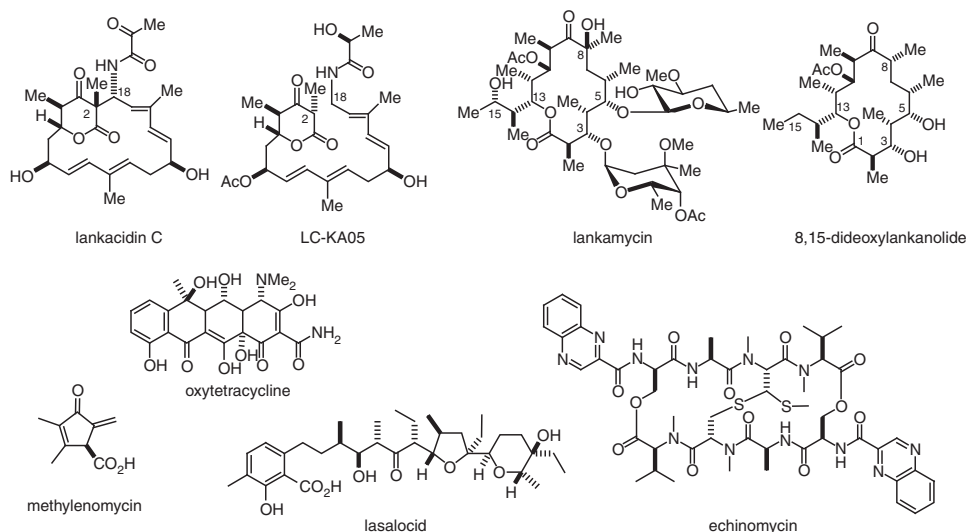
Figure 1 Structures of linear plasmid-determined antibiotics and some of their intermediates.

Table 1 Giant linear plasmids in antibiotic-producing *Streptomyces* strains

| Strains	Antibiotics	Sizes of plasmids (kb)	References
S. lasaliensis NRRL 3382R	Lasalocid, echinomycin	520 (pKSL)	4,5,57,59
S. coelicolor A3(2) 1147	Methylenomycin	356 (SCP1)	10,13,14,17
S. violaceoruber JCM 4979	Methylenomycin	plasmid ladder (390–630)	4,10
S. rochei 7434AN4	Lankacidin	17 (pSLA2-S), 100 (pSLA2-M)	4,42,43
	Lankamycin	210 (pSLA2-L)	
S. rimosus MV17	Oxytetracycline	1000	60,61
S. clavuligerus IFO 13307	Cephamycin, clavulanic acid	12 (pSCL1), 150 (pSCL2)	63–66
NRRL 3585		450 (pSCL3)	
S. clavuligerus ATCC 27064	Many antibiotics	1800 (pSCL4)	67
S. *carzinostaticus* ATCC 15944	Neocarzinostatin	880	70
S. atroolivaceus S-140	Leinamycin	900	71
S. globisporus	C-1027	160	72
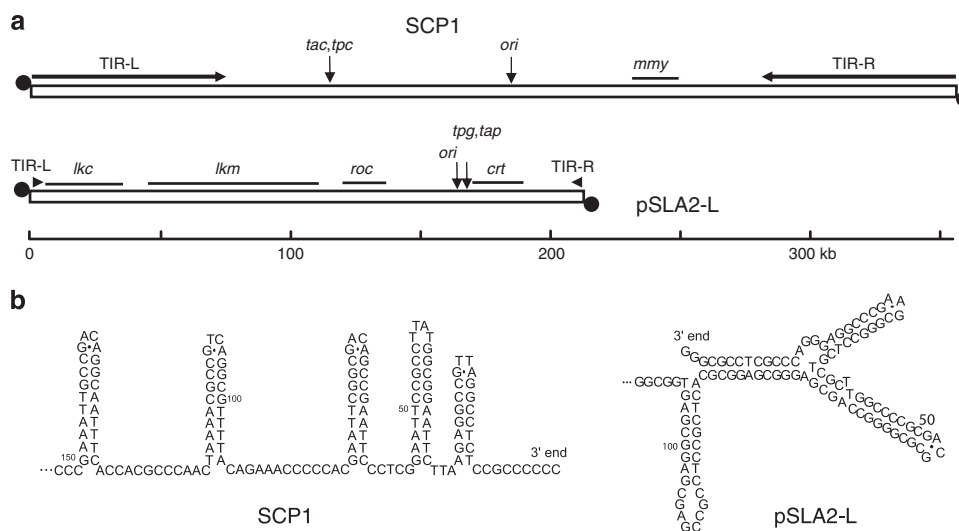
Figure 2 The gross structures of the linear plasmids, SCP1 and pSLA2-L (**a**), and possible secondary telomere structures deduced from their sequences (**b**).
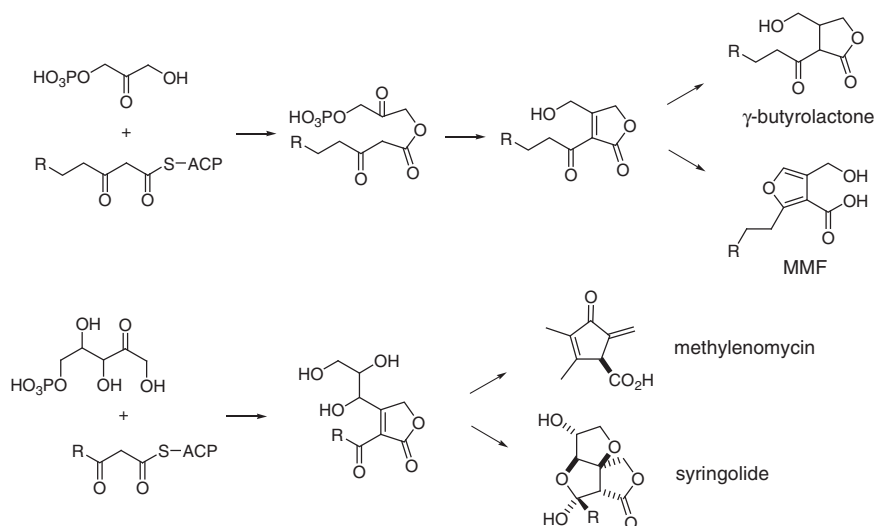
Figure 3 Similar biosynthetic pathways for γ-butyrolactone, methylenomycin furan, methylenomycin and syringolide.

several hairpin loop structures are still possible to be made (Figure 2b). Eventually, the complete nucleotide sequence (356 023 bp) of SCP1 was determined,[17] which confirmed the size of TIRs (75 122 bp) and located the *mmy* gene cluster, a replication origin, two partition genes (*parA* and *parB*) and others. However, a terminal protein gene (*tpg*)[18] was not identified from the sequence data, because of the unique telomere sequence of SCP1. Later, Huang et al.[19] isolated the terminal protein as a complex with SCP1 and finally identified the *tpc* (terminal protein of SCP1, *orf127*) and *tac* (telomere-associated protein of SCP1, *orf125*) genes of SCP1.

Biosynthesis of methylenomycin and autoregulators in S. coelicolor
In silico analysis of the SCP1 sequence together with gene disruption experiments identified the 19-kb *mmy* gene cluster consisting of 21 genes from *mmyR* to *mmyF*.[20] It is noteworthy that a new type of autoregulators, 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids named methylenomycin furans (MMFs) (Figure 3), were isolated from *S. coelicolor*,[21] in addition to SCB1,[22] a typical γ-butyrolactone autoregulator. Thus, *S. coelicolor* produces two different types of autoregulators; MMFs stimulates methylenomycin production,[20] whereas SCB1 stimulates the biosynthesis of an unknown polyketide.[23] Three genes in the *mmy* gene cluster, *mmfL*, *mmfH* and *mmfP*, constitute a biosynthetic cluster for MMFs.[20] The *mmfL* gene product shows 27% identity with AfsA, a key enzyme for A-factor biosynthesis in *S. griseus*,[24] whereas *scbA*, another homologue of *afsA* for SCB1 synthesis, is located on the chromosome.[25] The same precursors, dihydroxyacetone phosphate and β-ketoacyl CoA, were incorporated into both types of autoregulators.[26,27] Thus, γ-butyrolactones and MMFs are biosynthesized by identical butenolide intermediates, followed by reduction of a double bond (γ-butyrolactone) or recyclization of a butenolide ring (MMF) (Figure 3).

The incorporation pattern of [U-^{13}C] glycerol suggests that methylenomycin itself is also synthesized by a butenolide intermediate, which is formed by condensation of pentulose-5-phosphate with β-ketoacyl CoA (Figure 3).[28] It is noteworthy that the same butenolide compound has been proposed as an intermediate for syringolide biosynthesis in *Pseudomonas syringae*.[29]

Interaction of SCP1 with S. coelicolor chromosome and other replicons
SCP1 is known to interact with the *S. coelicolor* chromosome to generate various SCP1-chromosome hybrid structures. A 1850-kb linear DNA was found in *S. coelicolor* 2106, a *cysD* donor, which transfers a *cysD* phenotype in the mating with an SCP1-free strain.[30] It was revealed that this large linear DNA and the 7.2-Mb linear chromosome in strain 2106 were formed by a single crossover of SCP1 and the wild-type chromosome concomitant with short deletions.[31] As the telomere sequences of SCP1 and the host chromosome are totally different, both chimeric linear DNAs do not have TIRs. The 1850-kb DNA could not be cured by various mutagenic treatments. Thus, we concluded that the 7.2-Mb chromosome and the 1850-kb DNA are chimeric linear chromosomes and were named chromosomes I and II, respectively. The recombination event occurred in strain 2106 may be considered as a model for duplication and multiplication of a linear chromosome.[3,31]

It is also known that SCP1 is integrated in the central region or in other regions of the host chromosome. In the mating with SCP1-free partners, these SCP1-integrated strains showed either a bidirectional or unidirectional gradient of transfer of genetic markers with respect to the SCP1 integration site. The integrated structures of SCP1 in an NF (normal fertility) strain 2612[32] and an NF-like strain A634[33] were analyzed. Although both strains show a bidirectional gene transfer, the direction of SCP1 insertion and the deletions at both sides of SCP1 were different.[34,35] Thus, several rounds of recombination might have occurred at both sides of SCP1 to generate the present integrated forms of SCP1 in these strains.

S. violaceoruber JCM 4979, another methylenomycin producer, carries series of linear plasmids ranging from 390 to 630 kb with a size difference of about 35 kb (Table 1).[4] Restriction and sequencing analysis suggested that these linear plasmids were generated by insertion of a 31-kb circular plasmid SCP2 into SCP1 by homologous recombination between two copies of transposon Tn*5714* on each plasmid, followed by amplification of a 36-kb segment comprising of adjacent SCP1 and SCP2 regions (Kinashi et al.[36] and unpublished results).

On the other hand, *S. violaceoruber* SANK 95570, from which Haneishi et al.[37] first isolated methylenomycin, carries a 163-kb circular plasmid pSV1. It was revealed that pSV1 and SCP1 have

homology to each other only around the *mmy* and *parA, B* regions.[38] This result eliminated models in which pSV1 was generated by circularization of SCP1 or vice versa. It was rather suggested that the *mmy* gene cluster was horizontally transferred as a set together with the *parA, B* region from pSV1 to SCP1. The truncated gene (SCP1.216Ac) at the left side of the *mmy-parAB* segment on SCP1 might have been generated by non-homologous recombination (see later).

PSLA2-L IN *S. ROCHEI*

Isolation and characterization of pSLA2-L

Hayakawa *et al.*[39] first isolated a linear plasmid pSLA2 from *S. rochei* 7434AN4, which produces two structurally unrelated polyketide antibiotics, lankacidin and lankamycin (Figure 1). Physical analysis revealed that pSLA2 is 17 kb in size and is structurally similar to adenovirus and bacteriophage ϕ29 DNA; TIRs are present at both ends and a terminal protein is bound to the 5′ ends.[40,41] However, it was later found that pSLA2 was not involved in the production of lankacidin or lankamycin.

The application of PFGE to strain 7434AN4 revealed two linear plasmids, pSLA2-L and pSLA2-M, in addition to pSLA2-S (=pSLA2)[4] (Table 1). A complete correlation was observed between the presence of pSLA2-L and the production of lankacidin and lankamycin in mutants with a different plasmid profile.[42] Finally, nucleotide sequencing of pSLA2-L (210 614 bp) revealed the GC content (72.8%), the size of TIRs (1992 bp) and 143 open reading frames (*orfs*)[43] (Figure 2a). pSLA2-L has a conserved telomere sequence, which can form a typical Y-shaped foldback structure (Figure 2b).[44]

Homology searching of the 143 Orfs along with gene disruption experiments delimited the ranges of the biosynthetic clusters for lankacidin (*lkc*, *orf4-orf18*), lankamycin (*lkm*, *orf24-orf53*), a cryptic type-II polyketide (*roc*, *orf62-orf70*) and carotenoid (*crt*, *orf104-orf110*).[43] In addition, many regulatory genes were found; a γ-butyrolactone biosynthetic gene (*srrX*, *S. rochei regulatory gene* X), six *tetR* family repressor genes (*srrA*, *srrB*, *srrC*, *srrD*, *srrE*, and *srrF*) including the γ-butyrolactone receptor gene (*srrA*) and three *Streptomyces* antibiotic regulatory protein (SARP) genes (*srrW*, *srrY* and *srrZ*). Thus, two-thirds of pSLA2-L DNA is occupied by secondary metabolism-related genes.

The regulatory cascade for secondary metabolism

Disruption of the regulatory genes indicated that *srrX* has a positive effect on the production of lankacidin and lankamycin, and a negative effect on spore formation.[45] This effect is different from that of *afsA* in *S. griseus*, which shows positive effects on both streptomycin production and spore formation.[46] The receptor gene *srrA* has counteracting functions against both effects of *srrX*. On the other hand, *srrB* has a negative effect only on antibiotic production, whereas *srrC* has a positive effect on spore formation without affecting antibiotic production. Extensive transcriptional analysis revealed the main signaling pathway; *srrX* → *srrA* → *srrY-srrC* → *srrZ* → *lkmAI* (PKS gene) → lankamycin production.[47,48] The SARP gene *srrY* also activates lankacidin production through an unidentified pathway. We speculate that the cistron organization of *srrY-srrC* functions as a switch from antibiotic production to spore formation. The expression of *srrY-srrC* activates antibiotic production, whereas its cessation may turn the phase to spore formation. However, the details of the regulatory pathway downstream of *srrC* have not been clarified.

Biosynthesis of lankacidin and lankamycin

Lankacidin C is a 17-membered macrocyclic antibiotic (Figure 1), different from usual even-membered macrolides like erythromycin.

This unique structure and the gene organization of the lankacidin synthase (*lkc*) cluster raised two interesting questions. (i) How is the carbon–carbon linkage formed between C_2 and C_{18}. (ii) How can five ketosynthase domains in the *lkc* cluster accomplish eight condensation reactions necessary for lankacidin synthesis. The amine oxidase (*lkcE*) disruptant accumulated an acyclic intermediate LC-KA05 (Figure 1), which was converted to lankacidin C by other mutants.[49] This result indicates that LkcE oxidizes LC-KA05 to an unisolated imine ($C_{18}=N$), which in turn receives a nucleophilic attack by the enolic carbon (C_2) to form the C_2–C_{18} linkage. In regard to the second question, we proposed a hypothesis of modular-iterative mixed polyketide biosynthesis.[49] Although we isolated no confirmative metabolites, heterologous expression of the *lkc* cluster (*lkcA-lkcO*), gene fusion of *lkcF* and *lkcG*, and extensive disruption of the *lkc* genes supported this hypothesis.[50,51] Thus, LkcC may be used iteratively (four times) and LkcA, LkcF and LkcG modularly to accomplish eight condensation reactions for the lankacidin skeleton.

The 14-membered macrolide antibiotic, lankamycin contains a 3-hydroxy-2-butyl group at C-13 (Figure 1), which is different from an ethyl group in erythromycin. The feeding experiment of [3-^{2}H]-isoleucine indicates that isoleucine was oxidized to 2-methylbutyrate and incorporated as a starter.[52] Thus, a colinear relationship was conserved here between the *lkm*-PKS domains and biosynthetic reactions. On the basis of the structures of intermediates accumulated by mutants of the P450 hydroxylase and glycosyl transferase genes, a possible post-PKS pathway was proposed; 8,15-dideoxylankanolide (Figure 1) → 8-deoxylankanolide → 3-O-L-arcanosyl-8-deoxylankanolide → 3-O-L-arcanosyl lankanolide → 3-O-L-arcanosyl-5-O-D-chalcosyl lankanolide → lankamycin.[52,53]

Synergistic action of lankacidin and lankamycin

The close location of the lankacidin and lankamycin clusters on pSLA2-L and the co-regulation of their biosynthesis by the same unisolated γ-butyrolactone compound suggest that two antibiotics may have synergistic action.[2] This idea proved to be true by a collaborative work with Drs Yonath and Mankin.[54] Furthermore, crystallographic analysis and chemical probing showed that two antibiotics were bound to a different ribosome site, lankacidin to the peptidyl transferase center and lankamycin to the nascent peptide exit tunnel, leading to a synergistic inhibition of peptide formation. A similar super-cluster for cephamycin and clavulanic acid was found in *S. clavuligerus*,[55] where the two metabolites function synergistically as an antibiotic and a β-lactamase inhibitor, respectively, and their biosynthesis is regulated by the SARP gene *ccaR*.[56]

PKSL IN *S. LASALIENSIS*

As described above, we investigated lasalocid biosynthesis in *S. lasaliensis* NRRL 3382R in Professor Hutchinson's laboratory. Although we constructed many lasalocid-nonproducing mutants, cosynthesis experiments with many combinations, all failed to give positive-lasalocid production.[5] This result suggested that all of the mutants might have the same genetic defect in antibiotic production, for example, the loss of a plasmid carrying the biosynthetic gene cluster for lasalocid. The antibiotic-producing ability was transferred by protoplast fusion from producers to non-producers, supporting this hypothesis. However, we could not detect such a plasmid with conventional agarose gel electrophoresis.

A 520-kb linear plasmid pKSL was finally detected by PFGE analysis,[4] its presence and an antibiotic-producing ability being correlated in protoplast fusants.[5] The cosmid library of *S. lasaliensis*

ATCC 35852 (=NRRL 3382R) was constructed and screened with a ketosynthase probe, which afforded three overlapping cosmids. Their shotgun sequencing revealed an 82-kb region covering the entire lasalocid biosynthetic (*lsd*) cluster.[57] It is interesting that the *lsd* cluster contains a gene set for producing ethylmalonyl-CoA, different from other polyether clusters. This may be reasonable, because the biosynthesis of lasalocid requires three moles of ethylmalonyl-CoA (Figure 1). Although the disruption of the *lsd* genes has not been succeeded, the enzymatic activity of the epoxide hydrolase Lsd19 was confirmed by *in vitro* formation of lasalocid from a synthesized bisepoxide through cascade epoxide opening reactions.[58] The whole gene cluster for echinomycin biosynthesis was cloned and expressed in *E. coli*,[59] which also confirmed the function of the echinomycin cluster on pKSL.

LINEAR PLASMIDS IN S. RIMOSUS

S. rimosus R6, an oxytetracycline producer, carries pPZG101, a giant linear plasmid of 387 kb, which has a unique central region of 30 kb flanked by inverted repeats of about 180 kb.[60] During the study of genetic instability of *S. rimosus* R6, one overproducer (MV17) was found to carry a 1-Mb linear plasmid, pPZG103. This plasmid arose from a single crossover between pPZG101 and the chromosome, such that about 200 kb at one end is derived from pPZG101 and the other 800 kb is the chromosomal terminal sequence carrying the oxytetracycline cluster.[61] The chromosome had a parental structure, so there was an increased copy number of the oxytetracycline cluster due to the extra copy on the plasmid.

LINEAR PLASMIDS IN S. CLAVULIGERUS

β-Lactams are produced by a wide range of microorganisms including fungi and gram-positive and negative bacteria. β-Lactam biosynthetic genes form a cluster in which individual genes and their organizations are conserved beyond the barrier between prokaryote and eukaryote. From these results, horizontal transfer of the β-lactam cluster was proposed.[62]

Screening with PFGE for linear plasmids in β-lactam producing actinomycete strains was carried out by us and Roy's group, which revealed linear plasmids of 12–450 kb. Among them, *S. clavuligerus* NRRL 3585 (=IFO 13307), a producer of cephamycin and clavulanic acid, carried three linear plasmids, pSCL1 (12 kb), pSCL2 (120 kb) and pSCL3 (430 kb).[63,64] However, none of them hybridized to the biosynthetic probes, the *pcbC* gene for isopenicillin synthase or the *cefD* and *cefE* genes for isopenicillin N epimerase and deacetoxycephalosporin C synthase, respectively. Nucleotide sequencing of pSLC1 and pSLC2 neither revealed any genes for cephamycin or clavulanic acid biosynthesis.[65,66]

Recently, Madema *et al.*[67] isolated an extremely large linear plasmid, pSCL4, from *S. clavuligerus* ATCC 27064 and determined its 1.8-Mb nucleotide sequence. Surprisingly, this plasmid was densely packed with an exceptionally large number of gene clusters for the potential production of antibiotics, such as staurosporine, moenomycin, β-lactams and enediynes. Although strain ATCC 27064 was originally identical with strains IFO 13307 and NRRL 3585, it does not carry pSCL1, pSCL2, or pSCL3. Instead, its on going genome project revealed that only a 7-kb DNA in the assembly matched the pSCL1 sequence. Thus, it was suggested that pSCL4 was formed through recombination of pSCL1 with the arms of the host chromosome. The authors claimed that pSCL4 is a plasmid, but not a chromosome, on the basis of the fact that it does not code any essential genes for survival. This idea should be proved experimentally by curing of pSCL4 as well as pPZG103 in *S. rimosus*.

OTHER GIANT LINEAR PLASMIDS

The first application of PFGE to *Streptomyces* DNA enabled the detection of giant linear plasmids in additional antibiotic-producing *Streptomyces* strains; *S. fradiae* C373.1 (tylosin), *S. parvulus* JCM 4068 (actinomycin) and *S. venezuelae* JCM 4526 (chloramphenicol).[4] Since then, many giant linear plasmids have been isolated from antibiotic producers, for example, pSB1 from *S. bambergiensis* (moenomycin),[68] and pSA1 and pSA2 from *S. avermitilis* (avermectin).[69] However, their functions in antibiotic production have not been confirmed. In contrast, to our surprise Shen *et al.* (B. Shen, personal communication) recently found that the biosynthetic gene clusters for neocarzinostatin in *S. carzinostaticus*,[70] leinamycin in *S. globispora*[71] and C-1027 in *S. atroolivaceus*,[72] are all located on a giant linear plasmid (Table 1). The same situation may be present in other cases, where the antibiotic clusters have not been located in spite of extensive biosynthetic studies. Therefore, the analysis with PFGE and Southern hybridization will increase the number of giant linear plasmids involved in antibiotic production.

HORIZONTAL TRANSFER OF ANTIBIOTIC PRODUCTION

As described above, giant linear plasmids are widely distributed in *Streptomyces* and may be contributing to horizontal transfer of antibiotic production, directly and indirectly. First, the conjugal transfer of linear plasmids carrying an antibiotic cluster transfers a producing ability to the accepted strains. Ravel *et al.*[73] reported that the giant linear plasmids, pRJ3L (322 kb) and pRJ28 (330 kb), both encoding mercury resistance, were successfully transferred by conjugation from their streptomycete hosts to *S. lividans*.

Second, single crossover between linear plasmids and linear chromosomes causes end exchange, which transfers the antibiotic cluster from the plasmid to the chromosome or vice verse. As described above, the methylenomycin cluster was transferred from SCP1 to the *S. coelicolor* chromosome[31] and the oxytetracycline cluster from the *S. rimosus* chromosome to pPGZ3.[61] The linear plasmid SLP2 in *S. coelicolor* also proved to be a composite of the chromosome end and a linear plasmid,[74] although antibiotic production was not involved in this case. Comparison of the genome sequences of *S. coelicolor*, *S. avermitilis* and *S. ambofaciens* revealed that the central parts of the chromosomes are highly syntenic, whereas the terminal regions are species-specific.[75] This fact may also reflect end exchange of *Streptomyces* linear chromosomes. As essential genes are not coded in the terminal regions, they are exchangeable except for the extreme ends that are necessary for terminal replication. Even the extreme ends are exchangeable, as long as the machinery for terminal replication are linked to new ends.

Third, more complex interactions between linear plasmids and chromosomes were observed. In *S. coelicolor*, SCP1 is integrated in the central regions of the chromosome with different sizes of deletions at both sides.[34,35] pSCL4 of *S. clavuligerus* is composed of a 7-kb pSCL1 sequence flanked by the two chromosomal arms.[67] In this connection, I discussed on horizontal transfer of the extremely condensed biosynthetic clusters on pSLA2-L.[3] We could not detect any transposition-related genes or sequences surrounding the clusters, except for several truncated genes.[43] This is true for the methylenomycin cluster on SCP1.[17,20] On the basis of these results, I hypothesized that in most cases horizontal transfer has not occurred by a direct transposition of the cluster, but has involved multiple recombination events. Several cycles of single crossover at both sides of the cluster generate a gene organization that gives a probably misleading impression of having been generated by a direct transposition of the
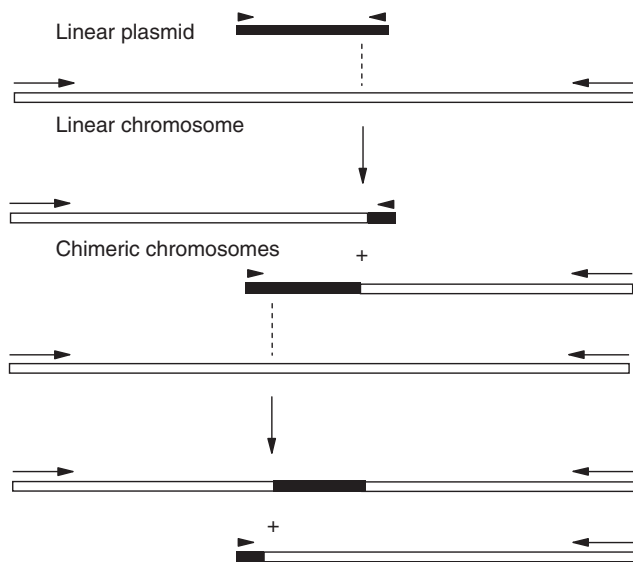
Figure 4 Recombination of linear replicons, which could cause end exchange and plasmid integration.

cluster. Truncated genes frequently found around the cluster may be traces of non-homologous recombination.

The same mechanism could be applied to horizontal transfer of much larger linear plasmids. The fact that two chimeric chromosomes are stably maintained in *S. coelicolor* strain 2106 (see ref.[31]) indicates that single crossover occurs even in more central regions of chromosome. Nevertheless, chimeric linear replicons without TIRs may have somewhat inferior properties, because most of *Streptomyces* linear replicons have TIRs. For example, linear replicons without TIRs cannot take a racket frame structure,[76] which might function in terminal replication or intramolecular replicational repair of terminal deletions.[3,31] Thus, chimeric DNAs without TIRs generated by single crossover might be followed by second and third homologous or non-homologous recombinations to recover TIRs. These series of recombination events result in a structure, which looks like a product of direct integration of a linear plasmid (Figure 4). Precise sequence comparison of giant linear plasmids and linear chromosomes on the basis of their complete sequencing will finally clarify the mechanism of horizontal transfer of secondary metabolism.

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