The Linear Chromosomes of *Streptomyces*: Structure and Dynamics

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(Received Oct. 17, 1994)

ABSTRACT

The linearity of the chromosome has been observed in several *Streptomyces* species, and thus may be characteristic of the genus. The termini of the chromosomes contain inverted repeats, and carry covalently-bound proteins at the 5' end, presumably the primer for replication. The terminal inverted repeats of the *Streptomyces lividans* chromosome are about 30 kb long, of which the extreme 16 kb is indistinguishable from that of the right end of the 50-kb linear plasmid SLP2. The terminal sequence of *S. lividans* exhibits abundant palindromes, resembling those of several linear *Streptomyces* plasmids for the first 12~17 bp. In spite of this, the terminal DNA sequences among different species are not highly conserved over long distances.

A functional *oriC* has been located at the center of the chromosomes of *Streptomyces coelicolor* and *S. lividans*. These chromosomes appear to replicate bi-directionally from the center and are presumably patched up at the ends by protein-primed replication. At least in *S. lividans*, the telomeres are dispensable and the chromosome could be circularized in viable cells by targeted recombination, which removed the telomeres and joined the two arms. The circularized chromosomes were unstable, undergoing further rearrangements, and resulted in relatively poor growth and sporulation.

Long stretches of DNA on various *Streptomyces* chromosomes are prone to spontaneous deletions and amplifications. In *S. lividans* and

S. coelicolor, the unstable regions correspond to the termini. Moreover, circularized chromosomes, containing deletions of several hundreds of kb in the terminal regions, were found in some spontaneous deletion mutants of *S. lividans* ZX7. These observations indicate the readiness of the linear chromosome to be circularized, and implicate the termini in the structural instability of *Streptomyces* chromosomes.

The linearity of the *Streptomyces* chromosomes

We have recently discovered that the chromosomes of most, if not all, the streptomycetes are linear¹⁾. The first discovery was made in *S. lividans* ZX7 (Fig. 1), a derivative lacking a DNA modification²⁾ and plasmids³⁾. Linear chromosomes were also demonstrated in two of its ancestral cultures, TK64 and 1326 (the wild type strain). The termini of the 8-Mb linear chromosome of ZX7 contain covalently-bound proteins, like some linear viral DNAs (such as adenoviruses and *Bacillus* phage ϕ 29)⁴⁾, and some linear plasmids (including those of *Streptomyces*)⁵⁾. Presumably, as in these other linear DNA molecules, the terminal proteins (TP) at the telomeres of the ZX7 chromosome also prime replication initiated at the termini.

The chromosome of ZX7 contains terminal inverted repeats (TIR). The TIR, based on the comparison of cloned cosmids in these regions, is about 30 kb in length⁶⁾ (Y. S. Lin and C. W. Chen, unpublished). Of this TIR, the terminal 16 kb was indistinguishable from the right end of the linear plasmid SLP2, which exists in two

Fig. 1. The linear chromosome of *S. lividans*. The 8-Mb linear DNA is modeled in a racket shape with the 30-kb TIR forming the handle. The possible interactions between the TIR, the TP (closed circles) and/or other binding proteins (open circles) are shown in the enlarged portion. The 'unstable region' (dashed arc) spans the two termini. The terminal 16-kb of the chromosome, including *Tn4811*, is homologous to one end of SLP2 plasmid (which exists in two copies per chromosome). The *oriC* sequence (open circle) is located at the center of the chromosome. Bi-directional replication initiated at *oriC* is depicted by the double-headed arrow (the possible extension of the continuous strand synthesis to the telomeres are shown in dashes). TP-primed replications are indicated by the two short arrows, which meet the discontinuous strands of the replication forks from *oriC* at the putative terminators (*ter*).

copies per chromosome in *S. lividans*^{1,7)}. The telomeres of the ZX7 chromosome also correspond to a discontinuity in the physical map constructed by Leblond *et al.*⁸⁾

The previously isolated *oriC* sequence of *S. lividans*, which can initiate DNA replication when cloned as a circular plasmid⁹⁾, is located at the center of the linear chromosome. Recent results of Musialowski *et al.*¹⁰⁾ indicated that replication of the *S. coelicolor* chromosome initiated in the region of *oriC* and proceeded bi-directionally towards the ends of the chromosome. It is likely that this applies to other *Streptomyces* species. Thus, the replication initiated at the telomeres primed by the TP probably only patches up the terminal sequences. Interestingly, this is comparable to the replication mode of the linear plasmids of *Streptomyces*^{11,12)}. Chang and Cohen¹²⁾ showed that most of the 17-kb linear plasmid pSLA2 of *Streptomyces rochei* was first replicated from an internal origin, and only a stretch of about 280 terminal nucleotides was subsequently filled in by the TP-primed replication. The dual modes of sequential replication raise the possibility of the existence of replication terminators (*ter*) on the linear chromosome that arrest the forks arriving from the internal origin before the onset of the terminal replication (Fig. 1).

The linear chromosome of ZX7 in Fig. 1 is drawn as a racket shape, based on the invertron model proposed by Sakaguchi⁵⁾, assuming non-covalent interactions between the TP, and possible other elements including the TIR and their binding proteins. The adenoviruses and ϕ 29 DNA have been shown to form circular shapes by interactions between their TP⁴⁾.

The chromosomes of *S. coelicolor* A3(2) derivatives M130 and M145 are also linear¹⁾, with their *oriC* sequences¹³⁾ similarly situated in the middle of the chromosome¹⁴⁾. The *AseI* restriction maps of *S. coelicolor* and *S. lividans* are also very similar and the corresponding fragments cross hybridized, as did as most of

the *AseI* linking clones (Fig. 2)⁸. The termini of the *S. coelicolor* chromosome also appear to contain TP. The interactions between the TP may be responsible for the circularity of the genetic maps in *S. coelicolor* as determined by heteroclone analysis¹⁵, by providing a link between the two arms of the chromosome during recombination (Circularity of the linkage maps of streptomycetes derived from the analysis of selected recombinants could also result from the requirement for double crossovers in

merozygotes¹⁶).

Pulsed-field gel electrophoresis (PFGE) analyses of the intact chromosome from *Streptomyces moderatus* ATCC 23443, *Streptomyces parvulus* 2283, *S. rochei* 7434-AN4, *Streptomyces antibioticus* IMRU 3720, *Streptomyces lipmanii* ATCC 27357, and *Streptomyces rimosus* R6 593 indicated that they were also 8-Mb linear DNA molecules containing TP^{1,17}. Mizukami *et al.*¹⁸ also reported that the chromosome of *Streptomyces griseus* was a linear molecule of about 7.8 Mb. The intact chromosome of *Streptomyces clavuligerus* ATCC 27064 (NRRL 3585) appeared to be a linear molecule of about 5.7 Mb¹⁷, but restriction analysis gave a larger (6.7 Mb) size estimate¹⁹.

Sequence homology and variations at the chromosomal termini

We have cloned the telomere DNA from ZX7 and sequenced the first 240 bp (Fig. 2). The sequence is identical to the nucleotide sequence of the right terminus of SLP2^{7,10} as expected. The terminal sequences of SLP2 contain only 44-bp imperfect inverted repeats⁷. The telomere sequence of ZX7 shares a short homology to that of the other linear plasmids, pSCL1 from *S. clavuligerus*²⁰, pSLA1 from *S. rochei*²¹, and SCP1 from *S. coelicolor*²². The first 200 bp at the ZX7 telomere contains an unusually high number of palindromic sequences, with sizes ranging from 11 to 25 bp, each including a 1- to 3-bp unpaired center. The palindrome-richness has also been observed in the termini of the characterized linear plasmids of *Streptomyces*, suggesting some common functions of these termini, such as recognition by TP, DNA polymerases, and possibly other proteins.

Yang¹⁷ has identified the chromosomal termini of a few other *Streptomyces* species. Using about 40 kb of DNA from one terminus of the ZX7 chromosome, she found significant hybridization only to the putative termini of *S. lipma-*

Fig. 2. Comparison of the *S. lividans* ZX7 and *S. coelicolor* M145 chromosomes. The two chromosomes were aligned (*S. lividans* ZX7 outside and *S. coelicolor* M145 inside) based on Leblond *et al.*⁸, following the coordinates of Kieser *et al.*¹⁴ for *S. coelicolor*. The DNA molecules are drawn as arcs with their TP shown in closed circles. The *AseI* restriction sites are shown as intersecting ticks. The terminal fragments in *S. lividans* ZX7 and *S. coelicolor* M145 were determined by Lin *et al.*¹, and H. M. Kieser and D. A. Hopwood (unpublished). Homology between the corresponding linking clones⁸ is indicated by the dashed lines. The approximate locations of *oriC* (open circles) on ZX7⁹ and M145 fragments¹⁴, and selected genes and DNA elements, are indicated. AUD, amplifiable unit of DNA.

Fig. 3. The first 240 bp of the *S. lividans* ZX7 chromosome. The telomeric DNA of ZX7 was cloned¹⁾ and the its first 240 bp was determined^{1,17)}. The palindromic sequences are boxed with their inverted repeats shown by the arrows. The thicker, hatched arrows indicate the repeat of the first palindrome within the fourth palindrome. Two nucleotides (GC) at positions 88 and 89 were missing in the previously published sequence¹⁾. A few 6-bp restriction sites are indicated.

nii and, to a lesser extent, to the putative termini of the chromosome or a linear plasmid in *S. parvulus*. No hybridization was seen in five other species, including *S. coelicolor* M130. Therefore, it appears that the terminal sequences of the *Streptomyces* chromosomes are highly variable. Our sequencing of the 10-kb terminus of the ZX7 chromosome showed a G+C content of about 65% (M.-F. Tsou, Y.-C. Tsai, Y.-L. Yang, and C. W. Chen, unpublished results), about 7–10% below the average G+C content for *Streptomyces* genomes. The relatively low G+C content as well as the sequence heterogeneity in the chromosomal termini of difference species suggest that these unstable sequences encode either extraneous proteins or no proteins at all.

Genetic instability in the streptomycetes

A remarkable finding was that the previously defined unstable regions of the linear chromosomes of *S. lividans* and *S. coelicolor* are located at the termini (Fig. 2). These unstable regions undergo large deletions spontaneously at

high frequencies (about three to four orders of magnitude higher than those for spontaneous mutations in typical genes). Genetic instability of *Streptomyces* has been reviewed^{23–25)}. These deletions have sizes up to about 2 Mb (in *Streptomyces ambofaciens*)²⁶⁾, and often result in simultaneous loss of genetic traits, such as aerial mycelium and spore formation, antibiotic biosynthesis and resistance, pigment production, production of extracellular enzymes, and arginine biosynthesis. All these mutations are relevant to secondary metabolism, except for arginine auxotrophy, which frequently but not always involves the loss of argininosuccinate synthetase encoded by the *argG* gene²⁷⁾.

Genetic instability is a universal property of all *Streptomyces* species studied. Exposure to some mutagenic agents such as ultraviolet irradiation, DNA intercalating compounds, and gyrase inhibitors, or simply cold storage increases the incidence of these deletions. The deletions are frequently accompanied by extensive tandem amplifications of specific DNA sequences adjacent to the deletion. The unstable

region corresponds to the genetically *silent* region of the *S. coelicolor* A3(2) chromosome around 3 o'clock on the genetic map, where very few genes have been mapped genetically²⁸. Unstable genetic elements, such as the *argG* gene, and the amplification units were later physically mapped to this region^{8,14}.

Despite all we know about the genetic instability of *Streptomyces* spp., we do not know its biochemical mechanism or its biological significance. Several laboratories, including ours, have for years searched unsuccessfully for clues to the mechanisms involved among candidates such as homologous recombination, site-specific recombination, and transposition. The establishment of the unstable regions at the termini of the linear chromosomes shed new light on the instability problem.

Spontaneous and targeted circularization of the *S. lividans* chromosome

Redenbach *et al.*⁶ recently isolated several chloramphenicol sensitive mutants of ZX7, in which the chloramphenicol resistance gene was deleted together with long stretches of chromosomal DNA. All the deletions were mapped to the terminal regions of the chromosome. The deletions in five mutants, MR01-MR05, appeared to span both termini of the chromosome, and included several hundreds of kb. The fact that these deletions span both termini suggested that they might have joined the two termini and circularized the chromosomes⁶. Indeed the chromosomes were shown to be circular, because they had lost the terminal DNA and the TP, and failed to migrate as a linear DNA in PFGE¹. These mutants grew and sporulated like the parent culture ZX7, indicating that the linear chromosome of *S. lividans* can undergo spontaneous circularization readily in viable cells.

We devised a scheme based on a targeted recombination procedure to circularize the *S. lividans* chromosomes¹. We constructed a

suicide plasmid based on an *Escherichia coli* vector. On this suicide vector, pLUS411, a copy of a 2.3-kb fragment from the TIR was placed on either side of the *aphII* gene (conferring resistance to kanamycin) in opposite orientations (Fig. 4). Outside of this cassette the thiostrepton resistance gene, *tsr*, was placed on the vector. pLUS411 was used to transform *S. lividans* ZX7, and kanamycin-resistant, thiostrepton-resistant transformants were isolated in which pLUS411 had integrated into the chromosome by a single crossover. From these cultures, kanamycin-resistant but thiostrepton-sensitive derivatives were readily isolated (designated YSC), in which the chromosomes presumably had been circularized by a second crossover (Fig. 4)¹. The circularity of the chromosomes in the YSC strains were confirmed by their inability to enter the gel in PFGE (as typical of all large open circular DNA molecules), and their failure to hybridize with the terminal DNA, Tn4811 sequence, the vector or *tsr* sequences¹. Most of the YSC cultures grew and sporulated more poorly than the parent ZX7.

Rearrangements accompanying circularization of the chromosome

Restriction analysis of the YSC chromosomes revealed an unexpected tandem amplification of a 20.2-kb DNA consisting of one of the remaining TIR plus the *aph* gene (Lin and Chen, unpublished; Fig. 4). The amplification must have happened after the second crossover event, because the vector sequences are not present: amplifications were not observed in strains isolated after only one crossover. This is quite different from the tandem insertions (or amplifications) of the suicide vector DNA frequently observed in targeted recombination experiments performed in *Streptomyces* (observation in our and several other laboratories). In the latter cases, the tandem repeats are spontaneously removed by the subsequent crossover

events. The amplification (containing *aphII*) was stable when the YSC cultures were kept under kanamycin selection. However, when kanamycin was removed, all the cultures lost

Fig. 4. Circularization of the ZX7 chromosome by targeted recombination, and subsequent amplifications and deletions. (Top) *Circularization* — pLUS411 DNA containing a 2.3-kb *Pst*I-*Eco*RI fragment (open box) from the TIR flanking the *aphII* gene (solid box) in inverted orientations, plus the *tsr* gene was used to transform *S. lividans* ZX7, and kanamycin-resistant, thiostrepton-sensitive transformants were selected¹⁾. In these cultures two homologous recombination events (dashed lines) had removed the telomeres and replaced them with a continuous DNA segment (the *aphII* gene), resulting in a circularized chromosome. (Middle) *Amplification* — The 20.2-kb DNA segment containing one of the remaining TIR plus the *aphII* gene was tandemly amplified (bracketed with the subscript *n*) in the circularized chromosomes of the YSC cultures. (Bottom) *Deletion* — On release from Km selection, all the amplified DNA (including *aphII*) was deleted together with long stretches of neighboring sequences, to the right and/or the left.

kanamycin resistance, as a result of the deletion of the amplified and the neighboring sequences (Lin and Chen, unpublished; Fig. 4, bottom). Therefore, it appeared that the circularized chromosomes with the amplifications were intrinsically unstable. Interestingly, many of these kanamycin-sensitive derivatives grew better than their kanamycin-resistant YSC parents. This suggests that the YSC strains were disadvantaged by their circular chromosomes. When selection for kanamycin resistance was removed, spontaneous deletions allowing faster growth quickly emerged.

Models for terminal rearrangements

The artificial circularization experiments have several implications. Firstly, circularization of the chromosome *per se* appears to enjoy relatively wide degrees of latitude: It occurs spontaneously or through artificial targeted recombination, involving deletion and joining of sequences at different locations of the chromosome arms. Secondly, the disadvantage caused by the circularized chromosomes does not seem to depend directly on the sizes of the deletions. The original deletions in the YSC strains were small, only about 32 kb, but the growth of many of them was poor. The spontaneous deletions in MR01-05 of Redenbach *et al.*⁶⁾, on the other hand, were much larger (hundreds of kb), but the growth of MR01-05 was better than that of most of the YSC strains. Thus, particular arrangements in the chromosome were responsible for the poor growth. Finally, artificially circularized chromosomes may be unstable, and subject to further rearrangements. The high efficiency of the deletional events that happened when kanamycin was removed was striking. Within a sporulation cycle in the absence of kanamycin, all the cultures were devoid of the amplification plus some neighboring sequences.

Why would the circularized chromosome in the YSC strains cause growth retardation? Why and how did the amplifications and deletions

occur? We speculate that the rearrangements may be the consequence of *stalled replication forks*. The natural terminator (*ter*) sites and damaged nucleotide sequences in bacterial chromosomes have been known to be hotspots of recombinations, including amplifications and deletions²⁹). Horiuchi *et al.*³⁰) showed that replication forks stalled at the *ter* site of the *E. coli* chromosome might present single-stranded daughter DNA for the entry of the RecBCD recombinase, and the recombination hotspot (Chi) sequences were involved in the high frequency of recombination.

According to the model of dual-mode replication of the *Streptomyces* chromosome, blockage of terminal replication would cause prolonged stalling of replication forks arriving from *oriC* at the terminators (Fig. 5). Such blockage of terminal replication might be caused by (i) inhibition of the particular enzymatic reactions, (ii) loss of TP through hydrolysis of the phosphodiester bond, (iii) loss of the terminal DNA sequences necessary for interactions with TP and/or DNA polymerase (presumably of family B, different from the replisomes of family C)³¹). The loss of TP, even transient, may leave the terminal DNA open to exonuclease attack.

Circularization of the *Streptomyces* chromosome as shown above removes both TP and the telomeres. In the artificial targeted circularization experiments of Lin *et al.*¹), the DNA deleted during the circularization was only 32 kb. If the terminators were still present, the prolonged arrest of the replication forks arriving from *oriC* would induce rearrangements. Furthermore, in order for these cells to survive, these replication forks would have to bypass the (leaky) terminators and complete replication. This handicap might be the cause of the slow growth of the YSC strain. The large deletions that occurred in the YSC strains on the relief of kanamycin selection presumably removed one or both hindering terminators, and the resulting deriva-

tives regained normal, or near-normal, growth rates. In contrast, the spontaneous mutants, MR01-05, with much larger deletions that presumably had removed one or both terminators, grew better than the YSC strains. It is possible that these spontaneous mutants also have undergone multiple stages of deletions before arriving at the final structure, as frequently observed in deletions mutants³²).

The fact that most of the boosters of structural instability of the *Streptomyces* chromosomes are known to arrest the progression of replication forks is in accordance to this model. Clearly there is a great need to study interactions of

Fig. 5. Model for terminal structural instability caused by blocked terminal replication. The telomeres are lined up in the center with TP indicated by the closed circles. The main replication forks, shown with the continuous and discontinuous daughter strands (with the arrows indicating the 5' → 3' polarity), are arrested at the putative terminators (*ter*) depicted by the double open triangles in gray. Blockage of terminal replication by various factors leaves the main replication forks stalled at *ter*, which supposedly generates deletions and amplifications (See text for details).

the internally-initiated and terminally-initiated replication, in addition to the machinery for terminal replication.

Linear vs. circular bacterial chromosomes

If the chromosomes of *Streptomyces* circularize spontaneously at relatively high frequencies in a process that appears to be irreversible, how can linearity persist in nature? Perhaps there are advantages for a linear chromosome in *Streptomyces* species in the wild. The linear chromosomes of the streptomycetes have evolved into specialized structures and replication mechanisms that may not adapt effectively to circularized forms. The termini of the chromosome may interact to assume a special structure that is important for normal maintenance, replication, and segregation. The dual-mode replication of the linear chromosomes may have fine tuned to the particular type of growth regulation for the streptomycetes. Removal of the termini by circularization interferes with these putative cellular mechanisms and may result in subtle disadvantages. For example, circularized chromosomes may have slightly lower rates of replication, or segregate less well into the spores. The possible physiological changes resulting from the circularization of the *Streptomyces* chromosomes beg for investigation.

However, there are no reasons to assume categorically that linear chromosomes are advantageous over circular chromosomes (or vice versa). Each topological type has probably evolved independently into balanced structures for which any changes (like most mutations) are likely to be detrimental. Bacteria (such as *E. coli*) with time-tested circular chromosomes would almost certainly suffer if their chromosomes were linearized.

Although TP-initiated replication is theoretically sufficient to duplicate the complete chromosome (as in $\phi 29$ and adenoviruses), these chromosomes nonetheless possess a typical internal replication origin. The replica-

tion system initiated at *oriC* is highly complex with its multitude of proteins involved — DNA polymerases (more than one kind), RNA polymerase, RNase, ligase, helicases, and other accessory proteins. Most of these enzymes (except for the primary DNA polymerase) play other important metabolic roles in the cell. Replication from an internal origin also demands a complicated scheme — continuous synthesis of a leading strand and discontinuous synthesis of a lagging strand. In contrast, replications initiated from the ends are much simpler — fewer and simpler proteins (TP, DNA polymerase, helicase(s) and one or more accessory proteins) and a more simplistic scheme (with only continuous synthesis and no discontinuous synthesis). It is thus easy to postulate that the more primitive bacterial chromosomes are replicated from the telomeres, and that the complex machinery for internal initiation came later by recruiting existing enzymes with other cellular duties. It is an open question whether this type of bacterial chromosome that replicates exclusively from the telomeres still exists in living bacteria.

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