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FEMS Microbiology Letters 186 (2000) 143–150

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

MiniReview

A new beginning with new ends: linearisation of circular chromosomes during bacterial evolution

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Received 20 December 1999; received in revised form 1 March 2000; accepted 1 March 2000

Abstract

Bacterial circular chromosomes have sporadically become linearised during prokaryote evolution. Unrelated bacteria, including the spirochete *Borrelia burgdorferi* and the actinomycete *Streptomyces*, have linear chromosomes. Linear chromosomes may have been formed through integration of linear plasmids. Linear chromosomes use linear plasmid strategies to resolve the 'end-of-replication problem', but they have generally retained from their circular ancestors a central origin of replication. *Streptomyces* linear chromosomes are very unstable and at high frequency undergo amplifications and large deletions, often removing the telomeres. At least in *Streptomyces*, chromosome linearity is reversible: circular chromosomes arise spontaneously as products of genetic instability or can be generated artificially by targeted recombination. *Streptomyces* circularised chromosomes are very unstable as well, indicating that genetic instability is not confined to the linearised chromosomes. Bacterial linear chromosomes may contain telomere-linked regions of enhanced genomic plasticity, which undergo more frequent genetic exchanges and rearrangements and allow differential evolution of genes, depending on their chromosomal location.

Keywords: Linear chromosome; Bacterial telomere; *Streptomyces*; *Borrelia*; Genetic instability; Chromosome circularization

1. Introduction

The distinction that prokaryotic chromosomes are circular and eukaryotic nuclear chromosomes are linear has been challenged by the discovery of linear chromosomes in several unrelated bacterial species. Pulsed-field gel electrophoresis allows the separation of megabase-sized DNA fragments in agarose gels [1]. Using this technique, the first linear bacterial chromosome was discovered in the spirochete *Borrelia burgdorferi*, the agent of Lyme disease [2]. The linearity of the chromosome of the antibiotic-producing actinomycete, *Streptomyces*, was first demonstrated in *Streptomyces lividans* [3] and then generalised to other species. *Agrobacterium tumefaciens*, the causal agent of crown gall disease, harbours one circular chromosome and one non-homologous large linear DNA molecule. This linear molecule, which is almost devoid of auxotrophic markers, was assumed to correspond to a linear chromosome [4]. In addition, the macrorestriction map of the chromosome of the obligate intracellular proteobacterium *Coxiella burnetii* suggests linearity [5]. In eukaryotes, mitochondria and chloroplasts generally have circular chromosomes, a conformation consistent with their prokaryotic origin. Interestingly, these organelles have occasionally linearised their chromosome as well [6,7]. Linear plasmids have been detected in *Borrelia* and *Streptomyces*, as well as in several bacteria with circular chromosomes [6].

Although chromosome circularity may have been selected during prokaryote evolution, the most parsimonious hypothesis explaining the distribution of linear and circular chromosomes in bacteria is to assume that circular chromosomes have been linearised independently in several bacterial lineages (Fig. 1). Chromosome linearisation might have interesting consequences for different DNA processes, including replication termination, replicative transposition and conjugation [8].

2. Replication origin

Generally, linear bacterial chromosomes have a central

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0378-1097/00/$20.00
PII: S0378-1097(00)00118-X

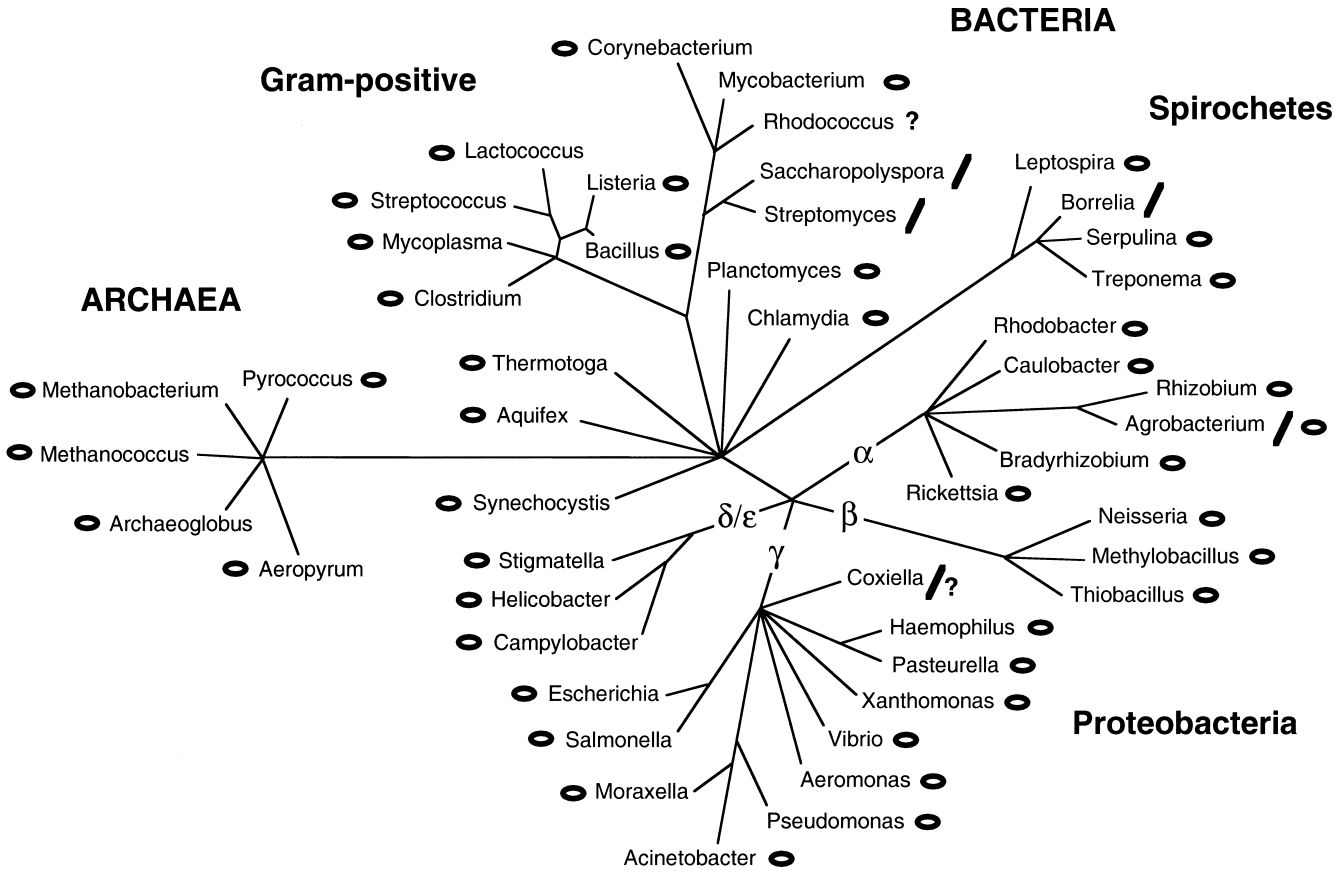

Fig. 1. Distribution of circular and linear chromosomes in prokaryotes. Symbols: circles, circular chromosomes; bars, linear chromosomes. Question marks indicate that the genome of *Rhodococcus fascians* has been reported alternately as linear and circular and that linearity of *Coxiella* chromosome has been suggested only by pulsed-field gel electrophoresis analysis. The number of chromosomes is not taken into account. The lengths of the branches of the tree do not reflect the degree of relationship between genera. References on circularity and linearity of chromosomes as well as on prokaryote taxonomy can be found on the server of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/Entrez/Genome/org.html, http://www.ncbi.nlm.nih.gov/Entrez/medline.html and http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html).

origin of replication, *oriC*, presenting a genetic organisation similar to that of circular chromosomes. In *B. burgdorferi*, replication proceeds bidirectionally from the *dnaA-dnaN-gyrB* region located in the middle of the linear chromosome [9]. In most *Streptomyces* species, functional evidence indicates that *oriC* is also located in the centre of the linear chromosome, close to the *dnaA-gyrB* region [10]. In the chromosome of *S. rimosus*, however, the *dnaA* region is located non-symmetrically [11]. The replication origin of the chromosome of *C. burnetii* has not been determined unequivocally. A putative *oriC* is flanked on one side by genes found in proximity to other bacterial chromosome origins, including *gidB*, *gidA*, *50k*, *rnpA* and *rpmH*. In contrast, genes such as *dnaA*, *dnaN*, *recF* or *gyrB* are not present on the other side. This putative origin is not located centrally on the chromosome but closer to one end [5]. On the other hand, *gyrA*, which is associated with several bacterial *oriC*, occupies a central position on the physical map of the *C. burnetii* chromosome. Hence, the geometry of the chromosome and the position of the major origin of replication need to be clarified in this bacterium.

3. Telomeres and chromosome replication

Because of the polarity of replication, linearised chromosomes are confronted with the new problem of copying their 3′ extremities. Bacterial linear chromosomes and plasmids have adopted at least two different strategies to complete their replication. While chromosome and plasmid telomeres are based on similar structural features within the same bacterial genus, *Borrelia* telomeres are clearly different from *Streptomyces* telomeres. *Borrelia* linear chromosomes have covalently closed hairpin structures at their termini that are similar to those reported for *Borrelia* linear plasmids and for *Escherichia coli* prophage N15 [6,12,13]. Such telomeric structures have also been found in some animal viruses (e.g. poxviruses [6]) and at the end of certain yeast linear mitochondrial DNA (e.g. in the closely related genera *Williopsis* and *Pichia* [7]).

Replication of *Borrelia* linear molecules might involve a circular intermediate [6,9,13]. Replication may proceed directly from the linear form of the chromosome, leading to the formation of a dimeric circular intermediate in which unit-length genomes are connected by duplicated hairpin

loop sequences and are subsequently resolved into monomers. Alternatively, terminal hairpins might be nicked in a way generating cohesive ends and allowing circularisation before replication [6,9,13].

In contrast, *Streptomyces* linear chromosomes and plasmids carry proteins bound to the 5′ end of the double-stranded telomeres. This was first revealed by electrophoretic retardation of linear DNA molecules when pulsed-field gel electrophoresis was performed without protease treatment [3]. Similar telomeric structures have been reported in several bacteriophages, including *Bacillus subtilis* phage Φ29, *E. coli* phage PRD1 and *Streptococcus pneumoniae* phage HB-3, in several fungal and plant mitochondrial linear plasmids, and in adenoviruses [6]. Telomeric terminal proteins are probably involved in the completion of replication [3,14]. The 166–168-bp terminal segments of *Streptomyces* linear replicons contain conserved palindromic sequences which have the potential to form complex secondary structures [15]. These conserved sequences are generally part of terminal inverted repeats that can be extremely large (e.g. 550 kb in *S. rimosus* [11]). Nothing is known to date about the structure of the telomeres of other prokaryotic linear chromosomes.

The structural and sometimes sequence similarities observed between linear chromosomes and linear plasmids within the same bacterial genus [3,12] indicate that these two categories of replicons are related. Mechanistically, linearisation of bacterial chromosomes might occur through integration of linear plasmids into circular chromosomes. It has been suggested that linear plasmids could have evolved from bacteriophages [6]. Consistently, the replication regions of *Streptomyces* linear plasmids pSLA2 and pSCL resemble those of temperate bacteriophages of Enterobacteriaceae and *Bacillus* [16]. The possibility that *Borrelia* telomeres have been acquired from a poxvirus has been discussed [13]. Linear chromosomes could continue to use the chromosomal bidirectional replication system but would require linear plasmid functions to replicate the telomeres. The central location of *oriC* in most linear chromosomes suggests either that linearisation occurred approximately at the same position relative to *oriC* in different bacteria, or that the central location of *oriC* has been selected, perhaps because it results in synchronisation of both replication forks.

4. Linear chromosomes with circular genetic maps

Hopwood, Sermonti and others established a genetic linkage map of *Streptomyces coelicolor* A3(2) in the 1950s and 1960s. This map, as well as those from other *Streptomyces* species, was consistent with a circular chromosome [17]. A possible explanation for a misleading linkage analysis such as this was given by Stahl and Steinberg [18]. These authors postulated that a strong selection toward even-numbered crossovers in recombination between linear molecules might create a circular linkage map. Markers located at opposite ends of each mating molecules tend in this way to end up in the same recombinant molecule, resulting in apparent genetic linkage. Indeed, Wang et al. provided evidence for such a phenomenon in *S. lividans-S. coelicolor* conjugation experiments [19]. These *Streptomyces* species are related sufficiently to allow recombination between their chromosomes but differ in their telomere sequences and macrorestriction patterns so that it can be determined which part of the recombinant chromosome comes from which parent. Recombinant chromosomes having one *S. coelicolor* telomere and one *S. lividans* telomere (mixed ends) should indicate an odd number of crossovers, while recombinant chromosomes with both ends originating from the same parent should have resulted from even numbers of crossovers. The results obtained by Wang et al. were compatible with a strong bias toward even-numbered crossovers [19]. An explanation might be that only small parts of the chromosome are transferred from the donor to the recipient, selecting double crossover events in a typical *Streptomyces* conjugation. Chromosomes with mixed ends would only appear when chromosomal ends are transferred. But markers from a chromosomal end were not selected in the experiments of Wang et al. [19]. The extreme rarity of recombinant chromosomes with mixed ends would also be understandable if the ends of the *Streptomyces* linear chromosomes strongly interact through their terminal proteins, giving to the chromosome a physically circular structure. Finally, there might be a selection against mixed ends. Nothing is known so far about the sequence specificity of the terminal proteins. If the *S. lividans* terminal protein does not recognise the chromosomal ends of the *S. coelicolor* chromosome and vice versa, mixed ends would only be possible if the terminal protein-encoding genes of both species are combined in a same chromosome.

5. Reversibility of linearity

At least in *Streptomyces*, linearity of the chromosome is a reversible state. *S. lividans* linear chromosomes can be circularised by artificial targeted recombination deleting either partially or totally the terminal inverted repeats [3,20,21]. Mutants with such chromosomes are viable and display either slight or no growth disadvantage compared to the wild-type. Circularisation also occurs spontaneously at high frequency under laboratory conditions [3,22]. At least some of these circularisation events, generally deleting both telomeres [23], occur through illegitimate recombination between sequences of low or no similarity [24]. Linearity in plasmids is reversible as well. Some *Streptomyces* linear plasmids can replicate in a circular form [16,25] and circular derivatives of linear plasmids have been observed in *Borrelia* [26]. Circularisation

of linear chromosomes has been observed in eukaryotes, too. Fission yeast mutants defective in telomerase or in two *ATM* homologues, whose products are involved in telomere maintenance, can survive with exclusively circular chromosomes [27,28]. These chromosomes support mitotic growth, but not meiosis [28].

6. Genetic instability of *Streptomyces* chromosomes

The linear chromosome of *Streptomyces* represents one of the most spectacular examples of genetic instability among prokaryotes [29]. On average, the chromosome of about 0.5% of germinating spores is affected by deletions removing up to 25% of the genome. For an average chromosomal size of about 8 Mb, this means that deletions can remove about 2 Mb of DNA, which exceeds by far the size of small bacterial genomes. Although internal rearrangements have been described, most of the deletions have been detected at or near the chromosome ends. Such deletions remove one or both telomeres and are associated in the latter case with chromosome circularisation. Inactivation of the *recA* gene or mutagenic treatment even increases the frequency of deletion [29,30]. Deletions are frequently accompanied by high-copy-number tandem amplification of specific sequences called amplifiable units of DNA (AUDs). Amplification of the best studied AUD, the AUD1 from *S. lividans*, requires RecA-catalysed homologous recombination between two large direct repeats and a DNA binding protein encoded by the AUD itself [30,31]. AUDs may correspond to elements able to 'capture' a replication fork by homologous or illegitimate recombination events taking place between a newly replicated sequence (downstream of the fork) and a non-replicated sequence (upstream of the fork). This might generate an 'amplification precursor' and lead to amplification by rolling-circle replication [32]. Alternatively, some AUDs might undergo overreplication because they correspond to the region where two replication forks meet in circularised chromosomes or, alternatively, after fusion of two linear chromosomes [20,29]. Interestingly, the 90-kb AUD2 from *S. lividans*, which is delimited by two copies of the insertion sequence IS*1373*, might be a giant mercury resistance transposon [33].

The discovery of the linearity of *Streptomyces* chromosomes was thought to provide the explanation for the observed high level of genetic instability, which could be due, for example, to degradation of non-protected telomeres. Spontaneous circularisation may lead, in this context, to disappearance of the free ends of the chromosome and hence to its stabilisation. Nevertheless, artificial and spontaneous circularised chromosomes were found to be at least as unstable as the corresponding linear chromosomes [20–22]. Particularly, the fusion region that may geographically correspond to the terminus of replication is a hot-spot for further deletions and amplifications. Hence, linearity is clearly not the reason for the high level of genomic rearrangement observed in streptomycetes. The hypothesis of chromosome stabilisation through circularisation postulated for *S. griseus* [24] is difficult to prove. If large deletions have already occurred and an essential gene is located near the deletion termini, an apparent stabilisation would be observed because all new deletions will be lethal.

Because of the enhanced frequency of rearrangements observed in *recA* mutants and after mutagenic treatment, it has been suggested that genetic instability of *Streptomyces* chromosomes is due to the collapse of replication forks at DNA single-strand breaks [29]. Most of the replication forks may be properly repaired by RecA, but a small number might be reconstructed by illegitimate recombination, leading to large deletions and chromosome circularisation. Such large deletions should be lethal in most bacteria and in the central region of the *Streptomyces* chromosome. An unusual feature of streptomycetes is the presence of a very large region on both sides of the chromosome that is completely devoid of housekeeping genes. For example, in *S. lividans*, the nearest auxotrophy marker *argG* is located about 800 kb away from the next chromosomal end. Therefore, streptomycetes might be unique in tolerating large deletions that do not affect the viability of the cells under laboratory conditions.

7. Adaptive value of linearity?

Linearity of eukaryotic chromosomes is thought to be essential for meiosis [34]. Telomere association might play an essential role in homologue pairing. Circular homologous chromosomes without telomeres might be randomly segregated to daughter cells because of lack of homologue pairing. If pairing and recombination occur by chance, an odd number of crossings-over between homologous chromosomes can result in the formation of dicentric circular chromosomes. In the absence of resolution into monomers, dicentric circular chromosomes will be pulled apart and broken or, alternatively, transmitted to only one daughter cell [34].

The reason(s) for the maintenance of chromosome linearity in some bacterial lineages remains unknown. It is particularly striking that, in *Streptomyces*, despite the high frequency of genetic instability-induced spontaneous circularisation, all natural isolates tested to date possess a linear chromosome. On the one hand, selection against *Streptomyces* circularised chromosomes under natural conditions may result from the large deletions usually accompanying circularisation. Generally, mutants generated by genetic instability are deficient for some traits of secondary metabolism including production of extracellular enzymes for degradation of polymers, antibiotic resistance, antibiotic production and sporulation. Such phenotypes might be deleterious in *Streptomyces* natural biotopes.

On the other hand, linear chromosomes should be more advantageous than circular chromosomes to avoid formation of multimers by interchromosomal homologous recombination in the polyploid mycelium of *Streptomyces* [29]. If such multimers are not resolved into monomers, they might be very difficult to replicate and extremely unstable. Moreover, *Streptomyces* can differentiate its polyploid aerial mycelium into haploid spores. On the analogy of meiosis of eukaryotic cells [34], circular chromosome multimers might be broken or unequally transmitted during partitioning in sporulation, leading to spores with abnormal chromosomal content and in some cases with rearranged chromosomes. The lack of an efficient site-specific recombination system restoring the monomeric state, an unfavourable position of the recognition sequence for the site-specific recombinase or the absence of efficient replication terminators typical of circular chromosomes may explain why *Streptomyces* circularised chromosomes are more frequently rearranged than their linear counterparts [20].

Linearity may facilitate the exchange of information between DNA molecules. Exchange of genetic material between linear molecules can occur by a single recombination event (Fig. 2A). In *S. rimosus*, exchange of ends between a linear plasmid and the linear chromosome involving a single cross-over has been observed [35]. This led to the formation of a linear prime plasmid containing one chromosomal end and the chromosomal oxytetracycline biosynthesis cluster. Conjugation of such prime plasmids can allow the transfer of chromosomal segments between species (horizontal gene transfer). Moreover, replicative transposition from a linear replicon to another one leads

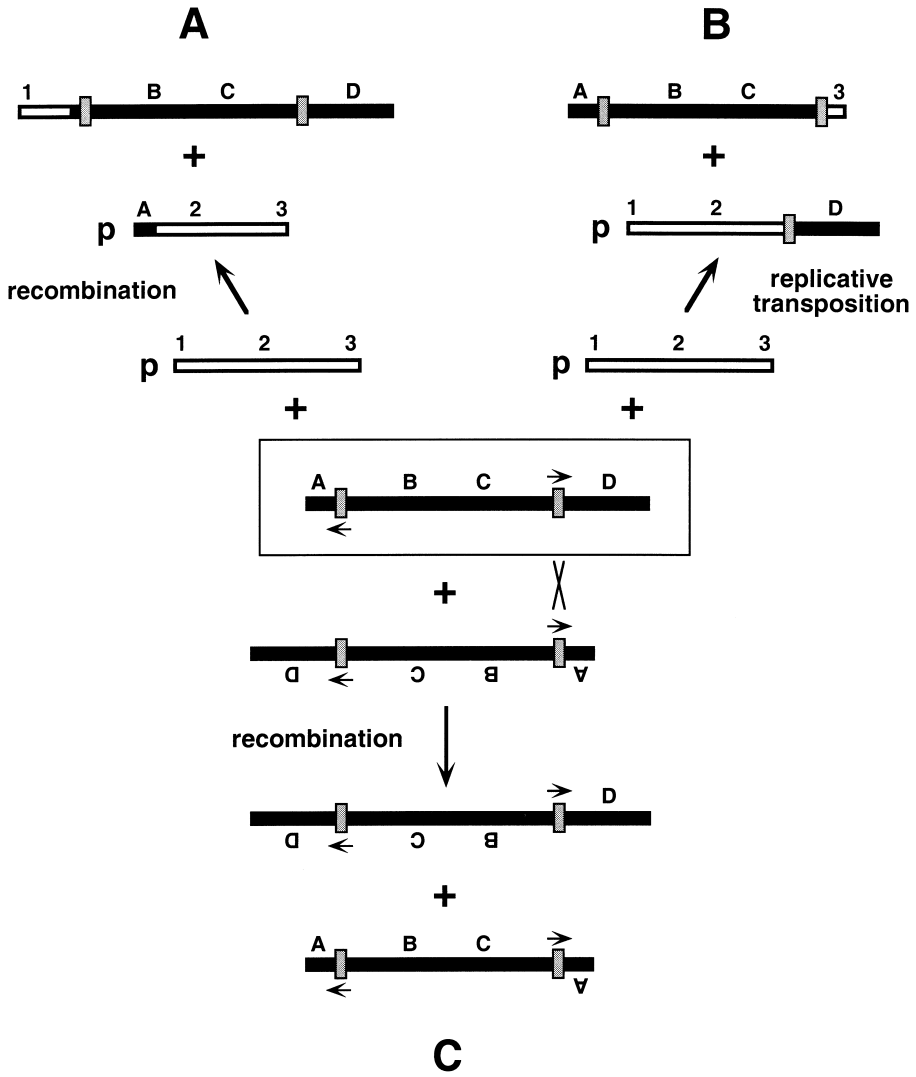

Fig. 2. Examples of genetic exchanges between linear DNA molecules. The original linear chromosome is boxed, 'p' indicates a linear plasmid, and the vertical boxes show two copies of a transposon in opposite orientations (shown by horizontal arrows). A: Exchange of ends between chromosome and plasmid through a simple recombination event (homologous or illegitimate). B: Exchange of ends between chromosome and plasmid through replicative transposition. C: Interchromosomal recombination between two non-allelic copies of a DNA sequence located in opposite orientation on different chromosome arms (in this case, recombination between two copies of the same transposon).

not only to transposon integration but also to exchange of ends (Fig. 2B) [8]. Such an event probably happened to the *Streptomyces* linear plasmid SLP2. One end of this plasmid is identical to a part of the terminal inverted repeats of the *S. lividans* chromosome and the sequence identity starts at a copy of transposon Tn*4811* [3]. *B. burgdorferi* chromosomes and linear plasmids have probably exchanged genetic information as well: the right terminal sequence of *B. burgdorferi* chromosome displays regions of considerable homology to several *Borrelia* linear plasmids [12,36,37]. Genes located near the ends of linear chromosomes are probably more mobile than centrally located genes, because they will be more frequently transferred after single cross-over recombination or replicative transposition between linear chromosomes and plasmids (Fig. 2A,B).

Another mechanism of telomere rearrangement is the replacement of a chromosomal arm by interchromosomal single cross-over between two non-allelic copies of duplicated genes located in opposite orientation on different chromosome arms, as described in *Streptomyces ambofaciens* (Fig. 2C) [38]. Similar rearrangements may occur by illegitimate recombination as well [29]. Such recombination events lead to the formation of new terminal inverted repeats and generate, together with a deletion, the duplication of sequences originating from one chromosomal arm onto the other. This may lead to gene duplication and genetic redundancy, which are important prerequisites for the appearance of novel gene functions. Genes located near the telomeres should be more frequently included into duplications and deletions generated by this mechanism than genes located far away from chromosome ends.

Hence, telomere-linked regions may represent areas of enhanced genomic plasticity undergoing genetic exchanges and DNA rearrangements more frequently than the rest of the chromosome. This might allow differential evolution of genes depending on their chromosomal location. Genes on linear chromosomes might underlie a kind of 'evolutionary gradient', with very rarely transferred/rearranged genes located at the centre of the chromosome, and highly mobile/rearranged genes located near the telomeres.

Consistently, conserved housekeeping genes are located far away from chromosomal ends in *Streptomyces coelicolor*. In contrast, analysis of deleted mutants generated by genetic instability suggests that insertion sequences and transposons, genes required for antibiotic resistance/biosynthesis and secondary metabolite production, as well as numerous genes encoding redundant functions involved, for example, in the degradation of polymers (e.g. chitinases), tend to be closer to the telomeres. Such genes might be preferentially transferred and rearranged without affecting the function and organisation of housekeeping genes. Transfer and rearrangement of genes involved in secondary metabolism and catabolism might be important for *Streptomyces* in order to respond to biotope variations. Particularly, transfer and rapid evolution of antibiotic production and resistance genes might be necessary under natural conditions to counter the appearance of antibiotic-resistant bacterial competitors.

An area of enhanced genomic plasticity might be present in the small genome of *B. burgdorferi* as well. The region including the right chromosome telomere, which contains sequences presenting homologies to linear plasmids, shows surprisingly few open reading frames compared to the open reading frame density elsewhere on the chromosome [36]. Moreover, a length polymorphism has been observed within this region between various natural isolates of *B. burgdorferi* [12]. Hence, this region may display a higher variability than the rest of the genome, and may interact more frequently than other regions with extrachromosomal linear molecules. *Borrelia* contains various linear and circular plasmids (or minichromosomes [13]), some of them involved in infectivity and virulence ([36,37] and references therein). They carry numerous genes encoding lipoproteins, substances that form the bacterium's coat [37]. These lipoproteins are probably important to survive attacks by the immune systems of different *Borrelia* hosts (arthropods and vertebrates). Infectivity and virulence genes might be transferred between chromosomes of different strains using a linear plasmid as vehicle. Such a plasmid could be integrated into the chromosomal 'area of enhanced genomic plasticity' of the recipient strain through recombination with plasmid-homologous chromosomal sequences. Interestingly, telomere-linked regions of the *Streptomyces* chromosome and (at least) several *Borrelia* linear plasmids share several common features: they are subject to extensive rearrangements [37] and are universally present in natural isolates but can be lost under laboratory conditions without affecting growth ([13] and references therein). Hence, *Borrelia* plasmid DNA may correspond to an 'extrachromosomal unstable region' having the potential to interact with one telomeric region of the chromosome.

Of course, *Borrelia* and *Streptomyces* plasmid genes probably can be expressed without integration into the chromosome. Nevertheless, integration of genes from plasmids into chromosomal sites might be advantageous by synchronising their replication with that of chromosomal genes, by ensuring their transmission to the next generation or even by modifying their expression. *Borrelia* may be able to selectively (and reversibly) integrate and thus favour some specific plasmid-carried virulence genes depending on its actual host. Furthermore, maintenance of an extrachromosomal plasmidic copy of a gene together with the integrated copy would lead to genetic redundancy. One copy of the gene could be 'saved' by integration into the chromosome, the second copy would be free to evolve. This may lead to the formation of new genes with modified properties or to new pathways by new gene combinations and might play a role in the evolution of infectivity and virulence factors, secondary metabolites and other important bacterial functions.

Acknowledgements

We are grateful to Manfred Schartl (University of Würzburg) for critical reading of the manuscript.

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