Influence of Disruption of the *recA* Gene on Genetic Instability and Genome Rearrangement in *Streptomyces lividans*

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Received 11 September 1996/Accepted 30 January 1997

Streptomyces lividans TK23 gives rise to chloramphenicol-sensitive (Cml^s) mutants at a frequency of about 0.5%. This is due to the frequent occurrence of very large chromosomal deletions removing the corresponding chloramphenicol resistance gene. A mutant in which the *recA* gene has been disrupted (*S. lividans* FrecD3 [G. Muth, D. Frese, A. Kleber, and W. Wohlleben, personal communication]) segregated about 70 times more chloramphenicol-sensitive mutants than the parental strain. An enhancement of the deletion frequency was responsible for this mutator phenotype. The amplifiable locus *AUD1* has a duplicated structure in some *S. lividans* strains and is frequently highly amplified in some mutants generated by genetic instability. The chromosomal *AUD1* is not amplified in strain TK23 because of the lack of one duplication. Nevertheless, *AUD1*-derived amplifiable units presenting the typical duplicated organization amplified very well in TK23 when carried on a plasmid. No amplification of these units was observed in the *recA* mutant. The ability to amplify was restored when the wild-type *recA* gene was introduced into the plasmid carrying the amplifiable unit. These results suggest that the RecA protein plays a role in reducing the level of genetic instability and chromosomal deletions and show that the *recA* gene is necessary to achieve high-copy-number amplification of *AUD1*.

Genetic instability in *Streptomyces lividans* is manifested by the occurrence of chloramphenicol-sensitive (Cml^s) mutants at a frequency as high as 0.5% (1, 2). Cml^s mutants themselves segregate arginine auxotrophs (Arg[−]) at high frequency (circa 25%). Both mutant phenotypes are due to spontaneous deletions of the corresponding chromosomal genes *cmlR* and *argG* (3, 4). More than 10% of the chromosome can be spontaneously removed without affecting the viability of the cells under laboratory conditions. Deletions include very frequently one or both ends of the chromosome, which is linear in the *Streptomyces* species so far tested and has long terminal inverted repeated DNA sequences at its ends (10–12, 18, 19). But *Streptomyces* chromosomes can also exist as circular forms in naturally occurring Cml^s mutants or after artificial circularization (12, 19, 24).

High-copy-number amplification of specific chromosomal sequences called amplifiable units of DNA (AUD) is frequently associated with large spontaneous deletions. At least two categories of amplified DNA sequences have been reported in *Streptomyces* (8). The first includes the so-called class I amplified sequences, which arise from the same chromosomal region but differ in size and endpoints depending on the mutant. Such amplifications have been observed under special conditions in *S. lividans* (18).

In contrast, class II amplifications are highly reproducible between different isolates and arise from sequences flanked by direct repeats. Probably the best-studied example is the amplification of the chromosomal locus *AUD1* in *S. lividans* TK64. This amplification corresponds to a highly amplified 5.7-kb fragment and is found in about 90% of Cml^s Arg[−] double mutants (1, 2). In strain TK64, *AUD1* has a duplicated structure consisting of three 1-kb and two 4.7-kb repeats alternately organized in direct orientation (2). The 1-kb repeats encode

DNA binding proteins which are required for *AUD1* amplification (16, 22). The 4.7-kb repeats may encode a chitinase and are necessary for amplification just as repeated DNA. They can be mutated or replaced by DNA repeats from *Escherichia coli* without affecting the ability to amplify. A minimal length of the repeats is necessary for amplification (22). No amplification is detected when a 4.7-kb repeat is lost, as is the case in strain TK23, used in this work (6). This suggests the involvement of homologous recombination in the amplification of *AUD1*.

The RecA protein is a key protein for homologous recombination, DNA repair, and genetic rearrangement in bacteria (5, 20, 26). The *recA* gene has been cloned from *S. lividans* (15) and *Streptomyces venezuelae* (27). We investigated the role of RecA in genetic instability by studying the *recA* mutant FrecD3 obtained by Muth et al. from *S. lividans* TK23 (14). This mutant expresses a RecA protein lacking 87 amino acids, is much more sensitive to UV light and methyl methanesulfonate than the parent, and presents a dramatically reduced but probably not totally abolished homologous recombination activity (RecA^d phenotype, for RecA deficient [14]). The frequency of Cml^s mutants and chromosomal deletions in FrecD3 has been determined. As TK23 cannot amplify *AUD1*, we have used an extrachromosomal amplification system (22) to test the ability of the genome of the *recA* mutant to undergo DNA amplification.

Genetic instability of TK23 and the *recA* strains. In strain FrecD3 (RecA^d) (Table 1), the *recA* gene was disrupted by a single crossover (14). The plasmid used confers resistance to kanamycin. The original strain FrecD3 was subcloned twice on HT medium (17) plus kanamycin (10 µg/ml) at 30°C. Spores from a kanamycin-resistant colony were then plated on medium without antibiotic. From this progeny, five colonies were randomly chosen and plated once again on kanamycin-free medium, giving rise to strains Jni8, Jni9, Jni10, Jni11, and Jni12 (Table 1). Five subclones of parental strain TK23 (RecA⁺) were isolated at the same time and called Jni3, Jni4, Jni5, Jni6, and Jni7. The genomic DNA of all these strains was isolated, digested with *Bam*HI, and hybridized with the labeled *recA* gene of *S. lividans* (15) used as a probe in Southern blot

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TABLE 1. *S. lividans* strains used in this work

Strain(s)	Phenotype ^a	Progenitor	Reference
TK23	RecA ⁺ Cml ^r Arg ⁺	1326	7
Jni3, Jni4, Jni5, Jni6, Jni7	RecA ⁺ Cml ^r Arg ⁺	TK23	This work
Jni3C1, Jni3C2	RecA ⁺ Cml ^s Arg ⁺	Jni3	This work
Jni5C1, Jni5C2	RecA ⁺ Cml ^s Arg ⁺	Jni5	This work
Jni6C1, Jni6C2	RecA ⁺ Cml ^s Arg ⁺	Jni6	This work
Jni7C1, Jni7C2	RecA ⁺ Cml ^s Arg ⁺	Jni7	This work
FrecD3	RecA ^d Cml ^r Arg ⁺ Km ^r	TK23	14
Jni8, Jni9, Jni10, Jni11, Jni12	RecA ^d Cml ^r Arg ⁺ Km ^r	FrecD3	This work
Jni8C1, Jni8C2	RecA ^d Cml ^s Arg ⁺ Km ^r	Jni8	This work
Jni9C1, Jni9C2	RecA ^d Cml ^s Arg ⁺ Km ^r	Jni9	This work
Jni10C1, Jni10C2	RecA ^d Cml ^s Arg ⁺ Km ^r	Jni10	This work
Jni11C1, Jni11C2	RecA ^d Cml ^s Arg ⁺ Km ^r	Jni11	This work
Jni12C1, Jni12C2	RecA ^d Cml ^s Arg ⁺ Km ^r	Jni12	This work

^a Arg⁺, prototrophy for arginine; Cml^r and Cml^s, resistance and sensitivity to chloramphenicol; Km^r, resistance to kanamycin; RecA⁺, presence of the wild-type *recA* gene; RecA^d, deficiency in RecA due to the disruption of *recA* (14).

experiments as described elsewhere (22). No band corresponding to the wild-type *recA* gene was detected in Jni8, Jni9, Jni10, Jni11, and Jni12 (not shown). This indicated a relative stability of the disruption of the *recA* gene in the FrecD3 subclones under the culture conditions that we have used.

Spores of the RecA⁺ and RecA^d strains were plated on HT medium without antibiotic, and the frequency of Cml^s (Arg⁺ and Arg⁻) was determined (Table 2) as described previously (1). The five RecA⁺ strains Jni3 through Jni7 segregated Cml^s Arg⁺ mutants at an average frequency of 0.4% ± 0.2% (mean of the frequencies ± standard error). Only one Cml^s Arg⁻ double mutant was found in the progeny of a Cml^r RecA⁺ strain (in Jni6). The frequency of Cml^s mutants was much higher in the offspring of the five RecA^d strains tested: Cml^s Arg⁺ and Cml^s Arg⁻ mutants were detected at average frequencies of 24.2% ± 5.6% and 3.7% ± 2.2%, respectively. In all, RecA^d strains segregated about 70 times more Cml^s mutants than the RecA⁺ strains (27.9% ± 5.7% versus 0.4% ± 0.2%). Arg⁻ mutants were detected in the offspring of the two RecA⁺-derived Cml^s mutants tested (4 of 77 = 5.1% for Jni3C2; 7 of 95 = 7.4% for Jni6C1). In contrast, no Arg⁻ mutant was found in the RecA^d strain-derived Cml^s mutants Jni8C2, Jni9C1, Jni10C1, Jni11C1, and Jni12C1 (36, 40, 55, 100, and 66 colonies tested, respectively).

Chromosomal deletions in the Cml^s mutants. The chromosomal DNA of the RecA⁺ and RecA^d strains as well as that of some Cml^s mutants was hybridized in Southern blot experiments with labeled fragments of the unstable region (Fig. 1). No rearrangements were detected in the five RecA⁺ and five RecA^d strains. Particularly, the terminal inverted repeats revealed by pLUS449 (12) and pJOE2424 (25) were found to be present, indicating that the chromosome of the RecA^d mutants is linear like that of the wild-type strains.

Among eight RecA⁺ strain-derived Cml^s mutants, two showed no detectable rearrangement in the regions homologous to the probes used. Especially, the terminal inverted repeats and the chloramphenicol resistance gene *cmlR* did not seem to be rearranged, as observed in some Cml^s mutants gained from *S. lividans* TK64 (22). This indicated the presence of mutations not detected by the method used. On the other hand, large deletions removing *cmlR* were detected in the six remaining Cml^s mutants. The minimal size of the deletion was

estimated to be between 100 and 800 kb depending on the mutant.

Large deletions ranging from at least 200 to more than 800 kb were found in all the RecA^d strain-derived Cml^s mutants studied. The *cmlR* gene was deleted in all cases. Moreover, deletions were usually larger than in the Cml^s mutants isolated from the RecA⁺ strains. For example, both terminal inverted repeats were absent in the 10 mutants tested, while 6 of 8 RecA⁺-derived mutants retained at least a part of these repeats. The reduced frequency of Arg⁻ auxotrophs in the progeny of RecA^d strain-derived Cml^s mutants may be explained by a more frequent codeletion of *argG* and essential genes due to the mutator phenotype.

AUD1-derived DNA amplifications. The chromosomal locus *AUD1* is not amplified in strain TK23, because of the lack of the necessary duplicated structure (6, 22) (Fig. 2). Southern blot experiments showed that the *AUD1* locus of the RecA^d strain FrecD3 has the same structure (not shown). Therefore, we used an extrachromosomal amplification system (22) to study the role of RecA in *AUD1* amplification. This system contains different *AUD1*-derived modified AUDs integrated in a low-copy-number circular plasmid (SCP2) (13). This has been used previously in strain TK64 to demonstrate the role of the 1-kb repeat-encoded DNA binding protein in amplification (22). The modified amplifications have the same characteristics of reproducibility and high copy number as the chromosomal ones. Amplification of the plasmid-located AUDs does not require any interaction with the chromosome via homologous recombination because it occurs at high frequency in mutants having a complete deletion of the whole *AUD1* locus. *AUD954* has two truncated 4.7-kb repeats with 2.7 kb in common and the right 1-kb repeat (22) (Fig. 2). In *AUD2390*, both truncated 4.7-kb elements have been replaced by foreign DNA repeats originating from transposon Tn5. The selection gene *tsr* confers resistance to thiostrepton (50 µg/ml) (21). *S. lividans* TK23 and FrecD3 protoplasts were prepared, and transformations were performed as described elsewhere (22). The cultures of FrecD3 that were transformed consisted of a mixture of spores of the five strains Jni8, Jni9, Jni10, Jni11, and Jni12. Both plasmid-located AUDs were found to amplify at high frequency in the RecA⁺ strain TK23 (Fig. 2). In contrast, no amplification of *AUD954* or *AUD2390* was found in strain FrecD3. The presence of the plasmids in 24 FrecD3 transformants was shown by Southern blot hybridization (not shown). Also, the disrupted *recA* gene was revealed by DNA hybridization in the 24 FrecD3 transformants tested, but the wild-

TABLE 2. Genetic instability of the RecA⁺ and RecA^d strains

Strain	Phenotype ^a	No. of colonies tested	Frequency (%)		
			Cml ^r	Cml ^s Arg ⁺	Cml ^s Arg ⁻
Jni3	RecA ⁺ Cml ^r	1,172	99.4	0.6	ND ^b
Jni4	RecA ⁺ Cml ^r	1,356	99.8	0.2	ND
Jni5	RecA ⁺ Cml ^r	1,501	99.5	0.5	ND
Jni6	RecA ⁺ Cml ^r	2,071	99.75	0.2	0.05
Jni7	RecA ⁺ Cml ^r	1,730	99.6	0.4	ND
Jni8	RecA ^d Cml ^r	100	70.0	23.0	7.0
Jni9	RecA ^d Cml ^r	167	82.0	16.8	1.2
Jni10	RecA ^d Cml ^r	186	70.5	26.3	3.2
Jni11	RecA ^d Cml ^r	116	64.7	33.6	1.7
Jni12	RecA ^d Cml ^r	90	73.4	21.1	5.5

^a Abbreviations are the same as in Table 1.

^b ND, not detected.

FIG. 1. The unstable region in the *RecA*⁺ and *RecA*⁻ strains and in their *Cml*^R derivatives. (A) *RecA*⁺ strains Jni3 to Jni7 and *RecA*⁻ strains Jni8 to Jni12; (B) *RecA*⁺ strain (Jni3 to Jni7)-derived *Cml*^R mutants; (C) *RecA*⁻ strain (Jni8 to Jni12)-derived *Cml*^R mutants. The extent of deletions was determined by Southern blot hybridization of the genomic DNA of the different strains with various digoxigenin (Boehringer, Mannheim, Germany)-labeled DNA probes. Cosmids 78, 85/3, 54, 26, and A6 contain 30- to 40-kb parts of the unstable region of *S. lividans* (19). Insert of pLUS449 corresponds to one cloned extremity of the chromosome (12). The chloramphenicol resistance gene *cmlR* was amplified by PCR (16) from the genomic DNA of strain TK64 by using primers derived from the sequence previously published (4). Plasmid pJOE2424 reveals the junctions of the terminal inverted repeats of the chromosome (25). The absence of a line or an arrow indicates the presumed deletion. The chromosome is shown with discontinuities; the sizes of most nonrepresented regions are given. Thick arrows show the terminal inverted repeats; the heads of the arrows represent the sequence homologous to pLUS449.

type gene was not found. The ability of *AUD954* to amplify was restored by insertion of the wild-type *recA* gene into the corresponding plasmid pJOE954. This indicates that *recA* is necessary to achieve the high-copy-number amplification in our extrachromosomal system. The results are consistent with the observation that two direct repeats of a certain length (>1 kb) are necessary for class II AUD amplification. The amplifications obtained in our extrachromosomal system present the same features as those arising from the chromosomal *AUD1* (22). We therefore assume that they occur by the same mechanism and suggest that *RecA* is involved in the amplification of *AUD1* in *S. lividans*.

Young and Cullum have proposed a model for the amplification of class II AUDs (28). When a replication fork reaches *AUD1*, a homologous recombination event takes place between one newly replicated and one nonreplicated repeat. This may form an amplification precursor with the replication fork entrapped. The tandem amplification is achieved by rolling-circle replication. Hence, according to this model, the recombination between the repeats may be catalyzed by *RecA*.

The high instability of the *S. lividans recA* mutant may be caused by frequent unrepaired collapses of the replication forks. In this paper, we report that the *recA* mutant FrecD3 of *S. lividans* presents a mutator phenotype resulting from a

FIG. 2. Behavior of *AUD1*-derived artificial class II AUDs in *RecA*⁺ strain TK23 and *RecA*^d mutant FrecD3. (A) Structure of the locus *AUD1* in *S. lividans* TK64 and TK23 and of the artificial AUDs. Plasmids pJOE954 and pJOE2390 contain *AUD954* and *AUD2390*, respectively, inserted in the SCP2 derivative pEI16 (13, 22). Plasmid pJOE2806 was constructed by cloning a 1.5-kb *Bam*HI-*Sma*I fragment from pUC19Brec containing the *recA* gene of *S. lividans* (15) in pJOE954. The numbers in parentheses indicate the strains with amplified DNA versus the total number of strains analyzed. ND, not determined. (B) Behavior of *AUD954*. Genomic DNAs were digested with *EcoRV* and *Pst*I, both enzymes cutting in *AUD954*. Lanes: 1, λ *Hind*III size standard; 2, TK23; 3, FrecD3; 4 and 5, TK23 plus pJOE954 (transformants 1 and 2, respectively); 6 and 7, FrecD3 plus pJOE954 (transformants 1 and 2, respectively); 8 and 9, FrecD3 plus pJOE2806 (transformants 1 and 2, respectively); 10, λ *Bgl*I size standard.

higher frequency of large chromosomal deletions. This may indicate that the *RecA* protein plays a role in reducing the level of genetic instability. We suggest a model of genome rearrangement in *Streptomyces* (Fig. 3) derived from that proposed for the repair of collapsed replication forks in *E. coli* (9). The replication proceeds bidirectionally from the origin of replication *oriC* to the chromosomal ends. A single-stranded interruption of the DNA located between *oriC* and chloramphenicol resistance gene *cmlR* may cause the collapse of the right replication fork (Fig. 3A to C). In the presence of *RecA*, the broken end would invade the intact homologous duplex

(Fig. 3D). The resolution of the Holliday junction would repair the replication fork (Fig. 3D and E). Two chromosomes carrying *cmlR* result from the completion of the replication (Fig. 3F). In contrast, in the absence of *RecA* and therefore of recombinational repair, the completion of the replication will lead to the formation of two chromosomes (Fig. 3G), one of them presenting the deletion of the *cmlR* gene and of the adjacent chromosomal end. Illegitimate recombination events or residual *RecA* activity leading to the circularization of the chromosome or to fusions of two chromosomes in an inverted duplicated manner may save the chromosome from complete

FIG. 3. A role for RecA in the repair of the chromosomal lesions leading to genetic instability. See the text for further explanations. Black circles are the proteins bound to the chromosomal ends of the linear chromosome. Black arrows in panel D show the resolution of the Holliday junction.

degradation and hence generate the type of rearrangements observed in the chloramphenicol-sensitive mutants isolated from the *recA* strain.

In general, the deletions in the *Cml^R* mutants derived from FrecD3 seemed to be larger than the ones from TK23 and different in the preferred endpoints. This might be just a matter of statistics due to the low number of mutants tested. On the other hand, it could also reflect additional deletion mech-

anisms acting in the *RecA⁺* strain, for example, chromosomal circularization and concomitant deletions of the ends by homologous recombination at direct repeated sequences (like insertion elements) present in the unstable region.

DNA-damaging agents such as UV light stimulate the occurrence of rearrangements in *Streptomyces* (23). DNA lesions are removed in *E. coli* by excision. This generates a single-stranded region of about 12 nucleotides which, if not sealed,

could lead to the collapse of a replication fork (9). A similar phenomenon occurring in *Streptomyces* may explain the stimulation of chromosome rearrangement by some genotoxic agents.

The high frequency of mutants and rearrangements especially in the RecA^d strains implies frequent collapses during replication. This phenomenon should occur in *Streptomyces* at least as frequently as in *E. coli*, where 25% of wild-type cells could experience a collapse during exponential growth (9). The frequency may be much higher in the large G+C-rich genome of streptomycetes, making the RecA protein necessary for viability by preventing fragmentation of the essential region of the chromosome. This could explain the difficulty, and probably the impossibility, of completely inactivating the *recA* gene in *Streptomyces* (14). The residual homologous recombination activity observed in mutant FrecD3 (14) may be sufficient to perform a basal recombinational repair allowing the viability of the strain.

J.-N. Voff has been supported by a long-term fellowship from the European Molecular Biology Organization.

We gratefully thank G. Muth and W. Wohlleben for the gift of *recA* and FrecD3 and for communication of results prior to publication. We are grateful to C. W. Chen, U. Rauland, M. Redenbach, J. Cullum, and D. A. Hopwood for plasmids and strains and to R. Mattes for helpful discussions and support. Many thanks go to P. Viell for excellent technical assistance and to C. Müller for her help during the preparation of the manuscript.

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