Evidence that an Additional Mutation Is Required To Tolerate Insertional Inactivation of the *Streptomyces lividans recA* Gene

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In contrast to *recA* of other bacteria, the *recA* gene of *Streptomyces lividans* has been described as indispensable for viability (G. Muth, D. Frese, A. Kleber, and W. Wohlleben, Mol. Gen. Genet. 255:420–428, 1997.). Therefore, a closer analysis of this gene was performed to detect possible unique features distinguishing the *Streptomyces* RecA protein from the well-characterized *Escherichia coli* RecA protein. The *S. lividans recA* gene restored UV resistance and recombination activity of an *E. coli recA* mutant. Also, transcriptional regulation was similar to that of *E. coli recA*. Gel retardation experiments showed that *S. lividans recA* is also under control of the *Streptomyces* SOS repressor LexA. The *S. lividans recA* gene could be replaced only by simultaneously expressing a plasmid encoded *recA* copy. Surprisingly, the *recA* expression plasmid could subsequently be eliminated using an incompatible plasmid without the loss of viability. Besides being UV sensitive and recombination deficient, all the mutants were blocked in sporulation. Genetic complementation restored UV resistance and recombination activity but did not affect the sporulation defect. This indicated that all the *recA* mutants had suffered from an additional mutation, which might allow toleration of a *recA* deficiency.

The RecA protein is the central enzyme in homologous recombination, DNA strand exchange, and recombinational DNA repair (reviewed in reference 15). In response to DNA damage, RecA becomes activated by the presence of single-stranded DNA and supports as a coprotease the autocatalytic cleavage of the SOS repressor LexA, UmuDC, and phage repressors (17). Digestion of LexA results in the induction of the SOS regulon, a set of more than 30 genes in *Escherichia coli* that are required for DNA repair, UV-induced mutagenesis, and inhibition of cell division (34). The *E. coli recA* gene has been analyzed in great detail. By three-dimensional structural, biochemical, and mutagenesis studies, protein regions have been proposed which are associated with distinct enzymatic activities of RecA (13). These regions include amino acid sequences for DNA binding, monomer-monomer interaction, filament formation, and LexA cleavage. Sequencing studies of more than 70 different procaryotic *recA* genes demonstrated that the deduced RecA proteins are highly conserved, with an overall similarity of between 43 and 100% (3). Only the N- and C-terminal regions, which are located on the outer surfaces of RecA filaments (32) and are involved in monomer interaction, display species-specific variety.

In *Streptomyces*, RecA is believed to be involved in genetic instability, manifested by the occurrence of large deletions comprising up to 1,000 kb, DNA amplifications, and DNA rearrangements (39). Treatment of *Streptomyces* cultures with agents inducing a SOS response enhances genetic instability (37). Although the *recA* genes of several *Streptomyces* strains have been cloned (1, 21, 26, 44), it was not possible to inactivate *recA* by targeted gene replacement. Only C-terminally truncated *recA* mutants with residual activity were isolated (1,

24). One of these mutants, FRECD3, missing the last 87 amino acid residues, was severely impaired in homologous recombination, highly UV sensitive, and defective in DNA amplification. The genetic instability of FRECD3 was about 70 times enhanced, and mutants that had lost the ends of the linear *Streptomyces lividans* chromosome (16) were segregated with a frequency of about 32% (38). Since a partial inactivation of *recA* had dramatic effects and since no completely defective *recA* mutants of *S. lividans* could be isolated, an essential role of *recA* for the viability of *Streptomyces* was suggested (24). A plausible hypothesis for a specific function of RecA in ensuring the viability of *Streptomyces* was proposed by Volff and Altenbuchner (38). In this model, RecA is required for the repair of single-stranded gaps which would cause the replication fork to collapse. Without the RecA-dependent reconstitution of the replication fork, a chromosomal end becomes lost (38). Recently, a *Streptomyces rimosus* mutant in which *recA* was disrupted was described (20). The mutant was UV sensitive, but its ability to perform homologous recombination was not analyzed. However, the presence of such a mutant indicated that at least in a specific strain background, *recA* could be inactivated without interfering with viability.

In this article, we address the question of what distinguishes the *Streptomyces* RecA protein from the RecA proteins of other bacteria, in our attempt to explain the different viability phenotypes of *recA* mutants. From gene inactivation studies in the presence of a second *recA* copy, we obtained evidence that *recA* could be inactivated only in strains that had suffered from an additional mutation, probably suppressing the lethal effects of RecA deficiency.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* strains used for subcloning and gene expression were XL1-Blue (4) and JM109 (43). The parental S*treptomyces* strain was *S. lividans* TK64 (12). *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium. *Streptomyces* strains were cultured as described previously (12). The plasmids used are listed in Table 1. Antibiotics were added supplementally,

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TABLE 1. Plasmids used in this work

Plasmid	Description	Reference(s)
4H8	*S. coelicolor* cosmid, carrying *recA* region; *aphII*	28
pUC18	*bla lacZ*	43
pUC18rec	pUC18, carrying *S. lividans recA* on a 2,820-bp fragment; *bla*	Present study
pGM8	Temperature-sensitive pSG5 derivative; *tsr aacC1*	25
pGMhyg	pSG5 derivative carrying the hygromycin phospho-transferase gene *hph*	19; Muth, unpublished
pSVXS	pUC18 derivative carrying the 3,271-bp *Xho*I-*Sal*I (partial digest) fragment (*recA*) of cosmid 4H8	28; present study
pEXrecA	*recA* expression plasmid; *tsr aacC1 cat recA*	36
pKOrecA	Temperature-sensitive *recA* replacement vector; *hph aphII*	Present study
pRErecA	Replacement vector for the reconstitution of *recA*; *tsr aacC1 bla*	Present study
pTWSl1	pSVB30 derivative carrying the 1,293-bp *Apa*I-*Bam*HI fragment containing the *S. lividans recA* gene	Present study
pSVB30	Cloning vector; *bla*	2
pIJ920	SCP2 derivative; *vph*	18
pCK3S	pSG5 derivative carrying a 550-bp fragment of *snpR*; *tsr*	24
pJOE2702	*E. coli* expression vector, rhamnose induction; *bla*	33
pJOE2702lexA	pJOE2702, carrying the *S. lividans lexA* gene	Present study

where appropriate, at the following concentrations: ampicillin, 150 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; thiostrepton, 25 μg ml^{-1}; gentamicin, 5 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}, hygromycin, 50 μg ml^{-1}; tetracycline, 15 μg ml^{-1}.

DNA manipulations. Standard procedures were as described by Hopwood et al. (12) and Sambrook et al. (30). Hybridization was performed with digoxigenin (DIG)-labeled dUTP and a DIG detection kit (Roche, Mannheim, Germany). Gene replacement mutants were selected as described by Wohlleben and Muth (42).

Assay for UV sensitivity. *E. coli* cultures were grown till they reached an optical density at 600 nm of 0.8, harvested by centrifugation, and resuspended in 0.8% NaCl. Serial dilutions were plated onto LB agar containing 1 mM isopropyl-β-D-thiogalactopyranoside and irradiated with UV light (VL115c, 254 nm, 730 μW/cm^2; Vilber Lourmat, Marne-La-Vallée, France) at a distance of 10 cm for various periods (2, 5, 10, 15, and 20 s), followed by incubation in the dark. UV resistance of *S. lividans* strains was determined as described by Muth et al. (24).

Assay for genetic instability. The genetic instability of *S. lividans* strains was measured as the ratio of chloramphenicol-sensitive colonies, as described by Vierling et al. (36).

Assay for homologous recombination. To assay the efficiency of homologous recombination in *E. coli*, matings with the Hfr donor strain KH500 (Hantke, Tübingen, Germany), which carries a tetracycline resistance marker (Tn*10*) near the F plasmid integration site, and a *recA* deletion strain, DK1 (14), harboring the plasmid pTWSl1 (Table 1), were performed. The mobilization frequency (Table 2) of the tetracycline resistance marker into DK1 was determined on isopropyl-β-D-thiogalactopyranoside-containing medium. Recombination activity in *S. lividans* was measured by its ability to integrate the temperature-sensitive plasmid pCK3S via homologous recombination into the chromosome as described by Vierling et al. (36).

Gel retardation experiments. The *lexA* gene of *S. lividans* TK64 was amplified by PCR using chromosomal DNA of *S. lividans* TK64 and the primers 5′-GGAATTCCATATGCACGCGATGAGCGACGC and 5′-CGGGATCCTCAGACGCGACGCAGTACGGCC, which were derived from the *Streptomyces coelicolor lexA* gene located on cosmid 5B8 (ftp://ftp.sanger.ac.uk/pub/S_coelicolor/sequences). PCR products were cloned under control of the rhamnose-inducible promoter in the expression plasmid pJOE2702 (33). *E. coli* JM109 (pJOE2702lexA) was grown at 37°C until it reached an optical density at 578 nm of 0.2 and induced with rhamnose (0.2%). Six hours after induction, the cells were harvested and disrupted with a French press. The upstream region of

recA containing the putative SOS box was amplified with the primers 5′-GGAATTCCGTACGCTCGGAAGTGC and 5′-CGGGATCCTCGACATCACCCGTCA. The resulting fragment was 3′-end labeled with DIG-11-dUTP (Roche) according to the manufacturer's instructions. Thirty femtomoles of the labeled fragment was incubated at room temperature for 15 min with 11 μg of LexA-containing soluble crude extract in a total volume of 20 μl (binding buffer, 4 μl; poly[d(I-C)], 1 μl; poly-L-lysine, 1 μl [Digoxigenin Gelshift Kit; Roche]). Subsequently the reaction mixture was run on a 5% polyacrylamide gel and transferred to a nylon membrane by Southern blotting, and the DIG-labeled DNA complexes were visualized using anti-DIG-alkaline-phosphatase-conjugated antibody.

Immunoblotting. Immunoblotting was performed as described by Engels et al. (9) using polyclonal rabbit antisera raised against purified His-tagged *S. lividans* RecA protein (Vierling and Muth, unpublished data).

Construction of the *recA* replacement plasmid. A pUC18 subclone (pUC18rec) carrying a 2,820-bp chromosomal fragment of *S. lividans* TK64 that contained *recA* with its upstream and downstream regions was digested with *Bam*HI and *Nco*I. Following Klenow treatment, the *recA*-containing *Bam*HI-*Nco*I fragment was replaced by an *aphII* cassette. The *Nco*I site overlaps the putative start codon of *recA*, while the *Bam*HI site is located 99 bp downstream of the *recA* stop codon in a noncoding region. The resulting plasmid was subsequently fused with pGMhyg (Muth, unpublished data), a hygromycin resistance-encoding, temperature-sensitive pSG5 derivative, yielding the *recA* replacement plasmid pKOrecA.

Construction of the replacement plasmid for the reconstitution of *recA*. A 3,271-bp fragment of the *S. coelicolor* cosmid 4H8 resulting from a *Xho*I/partial *Sal*I digest was subcloned into pUC18, resulting in pSVXS. In order to distinguish the reconstituted *recA* gene from the wild-type gene, the single *Bam*HI site located downstream of *recA* was eliminated by Klenow treatment. Subsequently the resulting plasmid was fused via *Eco*RI with the temperature-sensitive pGM8, yielding pRErecA.

Fixation of *Streptomyces* colonies for scanning electron microscopy. The *S. lividans* wild type, *S. lividans* SV64ΔrecA, and the reconstituted mutant SVRErecA were grown on R2YE agar for 5 days. Agar plugs were cut out with a cork borer and fixed for 10 min in 2.5% glutaraldehyde–100 mM cacodylate (pH 7.5). Subsequently the plugs were washed in 100 mM cacodylate (10 min) and H$_2$0 (10 min) and dehydrated (10 min) in 30, 50, 70, 90, and 100% EtOH. After critical point drying under CO$_2$, the mycelium was coated in a vacuum evaporator with

TABLE 2. Recombination activity of the *E. coli recA* mutant DK1, carrying the *S. lividans recA* gene

Recipient strain (plasmid)	Titers			Transfer frequency (%)	Relative recombination activity
	Recipient	Donor	Transconjugants		
JM83	8.20×10^8	3.59×10^9	1.81×10^7	2.20×10^{-2}	1
DK1 (pSVB30)	1.50×10^6	3.59×10^9	0	$<6.67 \times 10^{-7}$	<0.00003
DK1 (pTWSl1)	2.58×10^8	3.59×10^9	6.05×10^6	2.35×10^{-2}	1.06

a thin layer of Au-Pd. Observations were made with a Hitachi S-2460N scanning electron microscope with a secondary electron mode operating at 10 kV.

RESULTS

***S. lividans recA* complements the *E. coli recA* deletion mutant DK1 efficiently.** In order to analyze whether the *Streptomyces* RecA protein has the same activities as the well-characterized *E. coli* RecA protein, we attempted complementation of an *E. coli* mutant devoid of *recA*. The presence of the plasmid pTWSl1, containing the *recA* gene under control of the *lac* promoter, complemented the UV sensitivity of the *recA* deletion mutant DK1 to wild-type levels (data not shown). This indicated the proficiency of the *S. lividans* RecA protein for recombinational repair and the ability to support cleavage of the *E. coli* LexA repressor. The ability to perform homologous recombination was studied by Hfr matings using DK1 (pTWSl1) as the recipient. The outgrowth of tetracycline-resistant DK1 (pTWSl) colonies demonstrated that the *S. lividans recA* gene was able to restore recombination activity in DK1 (Table 2). Therefore, the *S. lividans* RecA protein possesses the same basic activities as the *E. coli* RecA protein.

***S. lividans recA* is regulated by the LexA repressor.** Next, we tested whether regulation of the *Streptomyces recA* gene differs from that for other bacteria. Previously it was shown that transcription of the *recA* operon in *S. lividans* was induced following treatment with the DNA-damaging methane methylsulfonate (36). This indicated that as in all other bacteria, *recA* is regulated by the SOS repressor LexA, which binds to so-called SOS boxes (Cheo box) in the promoter region of the genes of the SOS response. In the putative promoter region of the *S. lividans recA* gene, there is a sequence, **GAAC**ATCC**AT TC,** which resembles (as indicated by boldface type) the *Bacillus subtilis* SOS box **GAAC**NNNN**GTT(C/T)**. To analyze transcriptional regulation of *recA* by LexA, we expressed the *S. lividans lexA* gene in *E. coli* as described in Materials and Methods. A 109-bp fragment containing the putative SOS box of the *S. lividans recA* gene was amplified by PCR and 3′ labeled with DIG. After incubation with LexA, the reaction mixture was separated on a 5% Tris-borate-polyacrylamide gel, blotted onto a nylon membrane, and visualized with anti-DIG antibody conjugate. The retardation of the SOS box-containing fragment (Fig. 1) showed that the *Streptomyces* LexA protein is able to bind the proposed SOS box, indicating that LexA controls *recA* expression. When the respective fragments were incubated with an *E. coli* crude extract containing GlnR, a transcriptional regulator that binds in the promoter region of the *glnA* gene (N. Weissschuh and A. Engels, personal communication), no retardation was observed (data not shown).

The chromosomal *recA* gene of *S. lividans* TK64 can be deleted in the presence of a plasmid-borne *recA* copy. Since it was not possible to detect any significant difference between the activities conferred by the *S. lividans* and *E. coli recA* genes or their regulation, it was essential to confirm that the inability to remove *recA* from the genetic background was not the result of methodological complications. Therefore, we proceeded to demonstrate that the replacement plasmid was functional and that the homologous DNA fragments are sufficient in size to allow efficient recombination. To analyze whether the chromosomal *recA* fragment could be deleted while expressing a plas-
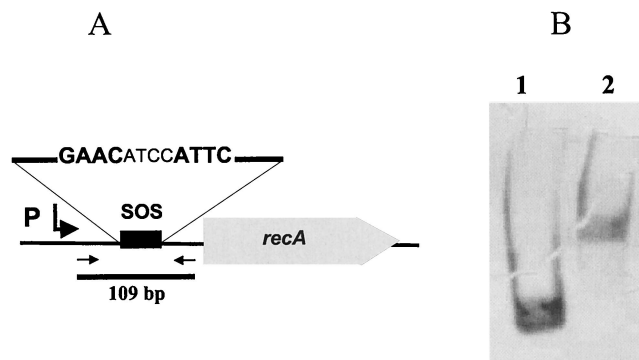

FIG. 1. Transcriptional regulation of the *S. lividans recA* gene. A 109-bp fragment containing the putative SOS box of the *S. lividans* TK64 *recA* gene was amplified by PCR (A), labeled with DIG, and incubated with *S. lividans* LexA-containing crude extract. Following electrophoresis on a 5% polyacrylamide gel and capillary transfer to a nylon membrane, the shifted and unshifted fragments were visualized by alkaline phosphatase-conjugated anti-DIG antibody (B) (Roche). Lane 1, 109-bp fragment, incubated with LexA-free crude extracts; lane 2, 109-bp fragment with LexA-containing crude extract.

mid-borne *recA* copy, the *recA* gene of *S. lividans* was cloned under control of the thiostrepton-inducible *tipA* promoter (23). Since *S. lividans* did not tolerate the transformation with *recA* on a multicopy plasmid (unpublished results), the expression cassette was inserted into a single-copy SCP2 derivative, yielding pEXrecA (Fig. 2).

In the temperature-sensitive *recA* gene replacement plasmid pKOrecA, the complete *recA* coding region was replaced with the *aphII* gene. The *aphII* gene was inserted in the same orientation as *recA* to minimize any polar effects on the downstream *recX* gene, which is cotranscribed with *recA* after induction of the SOS response (36). pKOrecA contained fragments of 858 and 925 bp, corresponding to the upstream and downstream regions of *recA* for recombination with the chromosome. It carried only a 75-bp region identical to pEXrecA to minimize the risk of recombination between the two plasmids. *S. lividans* TK64 was cotransformed with the plasmids pEXrecA and pKOrecA. Transformants carrying both plasmids were selected on gentamicin- and kanamycin-containing agar. Subsequently, colonies that carried the kanamycin resistance gene integrated into the chromosome were selected under inducing (thiostrepton-kanamycin) conditions at 39°C. From 400 picked colonies, four were found to be kanamycin resistant and hygromycin sensitive, indicating that the chromosomal *recA* gene was replaced. By PCR and Southern blotting experiments, the correct replacement of the chromosomal *recA* gene via double crossover and the loss of vector sequences were confirmed in all of these clones (data not shown). In contrast, if the replacement plasmid pKOrecA was introduced into *S. lividans* TK64 without pEXrecA, replacement of the chromosomal *recA* gene could not be achieved. From 3,000 picked colonies that were selected at 39°C on kanamycin-containing agar, all still carried the hygromycin resistance gene of the vector, indicating that the whole plasmid had integrated into the chromosome via a single crossover. This result confirmed our previous observations (24) that *recA* might be indispensable in *S. lividans* and that it was not possible to inactivate *recA* without concomitant expression of a *recA* copy.
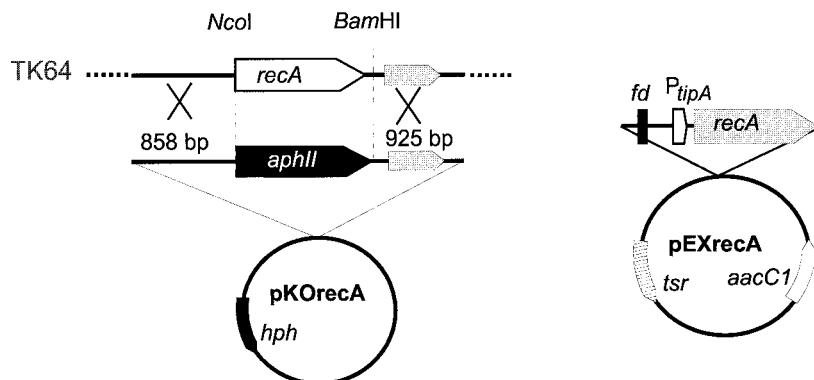
FIG. 2. Replacement of the *S. lividans* TK64 *recA* gene by the simultaneous expression of a plasmid-borne *recA* copy. Schematic maps of the *S. lividans* chromosomal *recA* region, gene replacement plasmid pKOrecA and the *recA* expression plasmid pEXrecA, carrying the terminator region of phage *fd* and the thiostrepton-inducible *tipA* promoter (P_{tipA}), are given. The sizes of the homologous regions and relevant restriction sites are indicated.

A mutant deficient for *recA* can be generated by curing the *recA* expression plasmid. To study the presumed detrimental effects of *recA* inactivation by switching the *tipA* promoter on and off, first the inducibility of *recA* expression in pEXrecA was analyzed. The *recA* gene of pEXrecA was replaced by the promoterless *aphII* gene from the transposon Tn5. Without induction, the *tipA* promoter mediated resistance to kanamycin (50 μg ml^{-1}). On 0.5-μg ml^{-1}, 1 μg-ml^{-1}, and 5-μg-ml^{-1} thiostrepton, respectively, a resistance to kanamycin at concentrations of 150, 200, and 400 μg ml^{-1} was observed. The highest level of resistance (at a kanamycin concentration of 600 μg ml^{-1}) was obtained by induction with 25 μg of thiostrepton ml^{-1}. Due to the basic activity of the *tipA* promoter even in the absence of thiostrepton, it was necessary to cure the *recA* mutant strains of plasmid pEXrecA in order to analyze whether the strains survived in the absence of RecA.

The four SV64ΔrecA strains were transformed with the plasmid pIJ920. pIJ920 is an SCP2 derivative containing the viomycin resistance gene *vph* (18) and is incompatible with the *recA* expression plasmid pEXrecA, which is also based on the SCP2 replicon. By selecting for the viomycin resistance gene of pIJ920, the pEXrecA plasmid could be displaced from the isolated *recA* replacement mutants. Five out of 80 tested viomycin-resistant transformants had lost the gentamicin and thiostrepton resistance of pEXrecA. Southern blotting and PCR experiments using internal *recA* primers confirmed the absence of *recA* (Fig. 3). Furthermore, immunoblots with RecA-specific antisera were negative (Fig. 4).

The *recA* mutant SV64 displayed a classical *recA* phenotype. To assay for recombinational activity, the SV64ΔrecA mutant was transformed with the plasmid pCK3S (24), a temperature-sensitive pGM derivative that carries a 550-bp fragment of the TK64 *snpR* gene, encoding the regulator of the metalloprotease SnpA. Following a temperature shift to 39°C to eliminate autonomously replicating plasmids, the cultures were homogenized, and serial dilutions of the mycelial fragments were plated in parallel on LB agar and LB containing thiostrepton. The ratio of the titer obtained on thiostrepton plates allowing the outgrowth only of colonies with pCK3S in their chromosome to the titer on LB agar revealed the recombination fre-

quency. While the plasmid pCK3S was integrated into the chromosome of TK64 at a frequency of about 54% (titer on LB agar, 4.5×10^6; titer on thiostrepton, 2.4×10^6), integration of pCK3S into the SV64ΔrecA chromosome did not occur (titer on LB agar, 5.0×10^6; titer on thiostrepton, 0). Furthermore, the *recA* deletion mutant *S. lividans* SV64ΔrecA was highly sensitive to UV irradiation. Although still more than 10% of the wild-type fragments survived a UV dose of 73 J/m^2, about 99.99% of *S. lividans* SV64ΔrecA mycelial fragments were destroyed (Fig. 5). To analyze the effects of *recA* deficiency on genetic instability, mycelial fragments of the *S. lividans* wild type and the *recA* mutant SV64ΔrecA were plated on soja-mannitol-agar. After 7 days, the mycelium was scraped off, homogenized, and replated. After three rounds, dilutions were plated and single colonies were subsequently picked and placed on chloramphenicol-containing and chloramphenicol-free medium. The *recA* mutant, SV64ΔrecA, had segregated chloramphenicol-sensitive colonies with a frequency of 6.2%, about 12.5 times that of the wild type.

The *recA* mutant SV64 represents a *whi* mutant. Besides the defects in homologous recombination, UV resistance, and genetic instability, all *recA* mutants were impaired in sporulation. On R5- or soja-mannitol-agar, a white aerial mycelium was formed that contained no spores. The aerial mycelium was further studied by scanning electron microscopy. The mutant SV64ΔrecA formed long straight unseptated hyphae with little or no curling (Fig. 6). Obviously, sporulation was blocked at an early time point in the life cycle of *S. lividans*. Thus, the *recA* mutant had a phenotype similar to that described for the *S. coelicolor whi* mutants (6).

Reconstitution of *recA* does not complement the sporulation defect. To analyze whether the sporulation defect was an effect of *recA* inactivation or the *recA* mutant had suffered from an additional mutation, we complemented the mutant by reconstituting a wild-type *recA* gene into the chromosome of *S. lividans* SV64ΔrecA: using the plasmid pRErecA, the *aphII* gene was replaced by the *S. coelicolor recA* gene, which differs from the *S. lividans recA* gene by two base-pair substitutions (see Discussion). The plasmid pRErecA carries a 3,172-bp chromosomal fragment of *S. coelicolor* A(3)2 with 1,161 bp
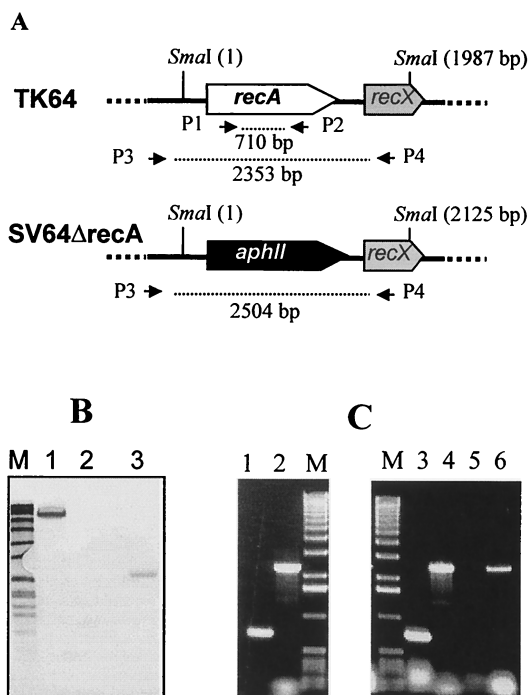
A

B   **C**

FIG. 3. Replacement of the *recA* gene of *S. lividans* TK64. A schematic drawing (A), Southern blot (B), and PCR analysis (C) are shown. (A) Relevant restriction sites, primers used for PCR amplification, and the sizes of the respective fragments are indicated. (B) *Sma*I-digested total DNA of *S. lividans* TK64 and the *recA* mutant SV64ΔrecA was hybridized against a DIG-labeled *recA* PCR fragment. Lane M, Bio-VII-Marker (Roche): 8,576, 7,427, 6,106, 4,899, 3,639, 2,799, 1,953, 1,882, 1,515, 1,482, 1,164, 992, 710, 492, and 359 bp; lane 1, SV64ΔrecA (pEXrecA); lane 2, SV64ΔrecA; lane 3, *S. lividans* TK64. (C) Agarose gel electrophoresis of PCR fragments. Lane M, 1-kb ladder (Roche): 12,216, 11,198, 10,180, 9,162, 8,144, 7,126, 6,108, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, 517, 506, 396, 344, 298, 220, 201, 154, 134, and 75 bp; lane 1, TK64, P1/P2; lane 2, TK64, P3/P4; lane 3, SV64ΔrecA (pEXrecA), P1/P2; lane 4, SV64ΔrecA (pEXrecA), P3/P4; lane 5, SV64ΔrecA, P1/P2; lane 6, SV64ΔrecA, P3/P4.
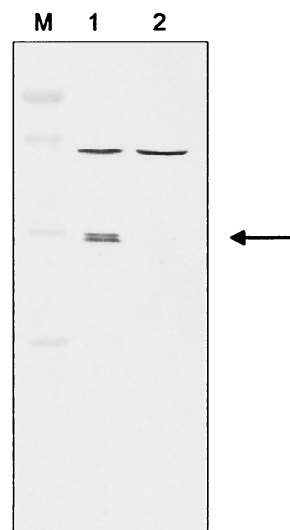

FIG. 4. Detection of RecA by immunoblotting. Total proteins were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and blotted to a nylon membrane. RecA was detected using a polyclonal antiserum raised against purified *S. lividans* RecA protein. The arrow indicates the RecA-specific band. Lane M, marker, Prestained Low Range Standard (Bio-Rad, Munich, Germany): 116, 80, 52.5, 34.9, 29.9, and 21.8 kDa; lane 1, *S. lividans* TK64; lane 2, SV64ΔrecA.

onstrates that the mutants have suffered from an additional mutation affecting morphologic differentiation and that the sporulation defect was not a consequence of inactivation of *recA*.

DISCUSSION

To investigate whether the *Streptomyces* RecA protein had functions different from those of other RecA proteins, we complemented an *E. coli recA* mutant with the *S. lividans recA* gene. Expression of the *S. lividans recA* gene in DK1 (14) restored UV resistance and recombination activity in Hfr mat-

upstream and 885 bp of the downstream region of *recA*. To distinguish the reconstituted *recA* gene from the wild-type *recA* gene, the *Bam*HI site located in the intergenic region 96 bp downstream of *recA* was eliminated by Klenow treatment. Following a temperature shift, transformants were picked on thiostrepton- and kanamycin-containing media to screen for *tsr* and *aphII* sensitive colonies that probably had replaced the *aphII* gene by a double crossover event. The correct replacement event was confirmed by Southern blot analysis and PCR. By reverse transcription-PCR analysis, the inducibility of *recA* transcription (data not shown) in response to the DNA-damaging methane methylsulfonate was found to be indistinguishable from that of the parent *S. lividans* TK64 strain (36). The reconstituted mutant was fully complemented with regard to UV sensitivity (Fig. 5) and recombination activity. The integration of the recombination test plasmid occurred with a frequency of 29%, which is on the same order as in the wild type. However, the reconstitution of *recA* did not affect the sporulation deficiency of *S. lividans* SV64ΔrecA. Scanning electron microscopy also revealed no difference in the *S. lividans* SV64ΔrecA mutant (data not shown). This clearly dem-
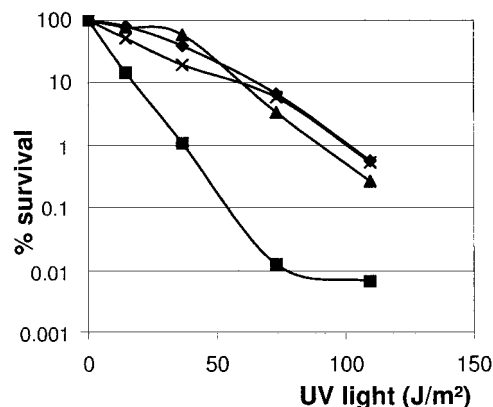

FIG. 5. UV sensitivity of the *S. lividans recA* mutant SV64ΔrecA. Spores or aerial mycelial fragments of *S. lividans* TK64 (♦), the *recA* mutant SV64ΔrecA (■), SV64ΔrecA carrying the *recA* expression plasmid pEXrecA (▲), and the reconstituted SVRErecA strain (✕) were plated on LB agar and irradiated with UV light (254 nm, 730 μW/cm²) for various periods. SV64ΔrecA(pEXrecA) was grown on LB containing thiostrepton for induction of *recA* expression.
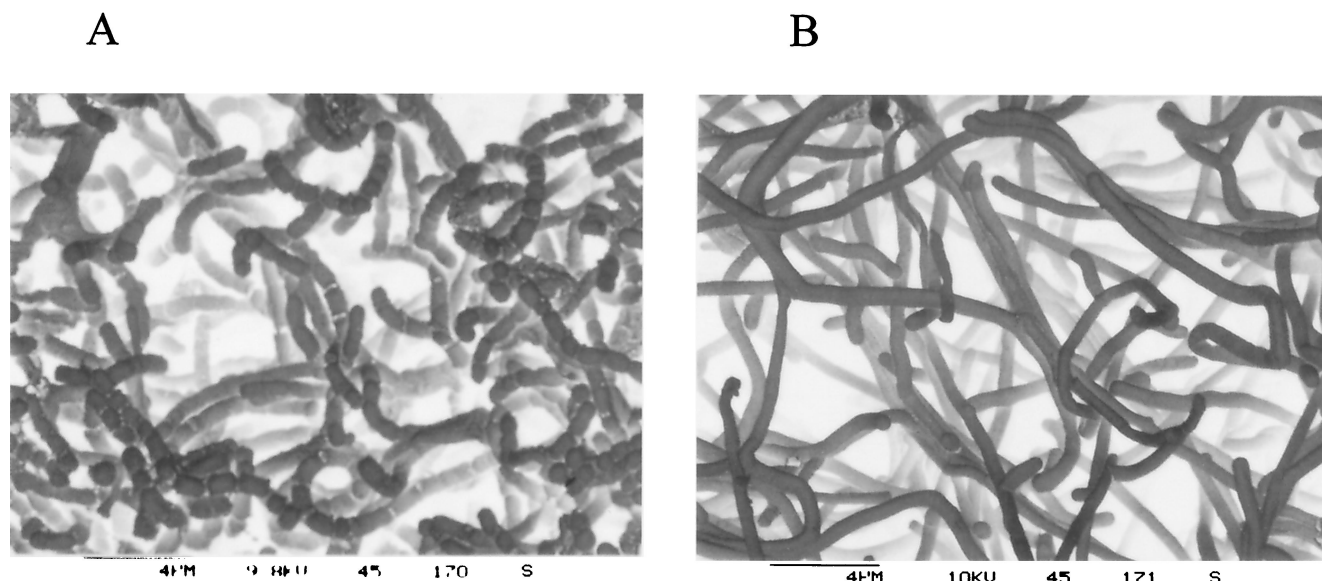
A

B

FIG. 6. Scanning electron micrographs of the surfaces of colonies of the wild-type strain *S. lividans* TK64 (A) and the *recA* mutant SV64ΔrecA (B). Colonies were grown for 5 days on soja-mannitol plates before being prepared for electron microscopy.

ings to the wild-type level. This suggests that the *S. lividans* RecA protein fulfills all the enzymatic activities that have been ascribed to *E. coli* RecA, namely protease activity to support cleavage of the LexA repressor, proficiency for recombinational DNA repair, and the ability to perform homologous recombination (13). Furthermore, there is no evidence that the *Streptomyces* RecA protein might have a distinct activity, since the deduced amino acid sequence of the *S. lividans* RecA protein is, besides the species-specific C terminus, highly similar to that of other bacterial RecA proteins (3).

In all bacteria, it has been shown that transcription of DNA damage-inducible genes is controlled by LexA, which binds to so-called SOS boxes in promoter regions (17). By sequence comparison and site-directed mutagenesis combined with gel retardation assays and hydroxyl radical footprint protection assays, Winterling et al. proposed a new consensus sequence (CGAACRNRYGTTYC) for SOS boxes of gram-positive bacteria (40, 41). Although the SOS box of the *S. lividans recA* gene (CGAACATCC**A**TTC**T**) differs from this consensus sequence in three positions (shown in bold) and does not form a perfect palindrome, the binding of *S. lividans* LexA and the inducibility by DNA damaging agents demonstrated its functionality. The same sequence, CGAACATC(C/T)ATTCT, is also found in front of all the other *Streptomyces recA* genes where sequence information is available (EMBL accession no. AL020958) (1). As in *Mycobacterium tuberculosis* and *M. smegmatis* (22), this SOS box overlaps with a consensus sequence of a heat shock promoter. This putative heat shock promoter of the *Streptomyces recA* genes has been postulated by sequence similarity (26), but in *M. smegmatis*, a transcriptional start site of the *recA* gene corresponding to this heat shock promoter was mapped by primer extension (27). The overlap of the SOS box with the −10 or −35 promoter region is a common feature and was described for various genes of the SOS response (10). A second putative SOS box (**TGAACG**(G/C)CA(G/A)**TTCG**)

(conserved bases shown in bold) is present within the N-terminal coding region of *Streptomyces recA* (amino acid position +18) (1). However, its involvement in SOS regulation has not been investigated. Two LexA binding sites have also been described for several other LexA-regulated genes, e.g., *B. subtilis dinC* and *dinR*, *recN* or *lexA* from *E. coli* (10). Although the *Streptomyces* SOS box is not a perfect palindrome, the presence of two binding sites may indicate tight regulation by LexA. Note that the promoter region of the *S. coelicolor* (EMBL accession no. AL022268) and *Streptomyces clavuligerus* (EMBL accession no. AJ224870) *lexA* genes also contain putative SOS boxes. These boxes lie 148 and 76 bp upstream of the putative translational start of *lexA*, respectively. The *lexA* SOS boxes differ from the *recA* SOS box in six positions (shown in bold) (CGAAC**GTGTG**TTTG) and fit perfectly the proposed consensus sequence. A gel retardation reaction performed with an 86-bp PCR fragment containing the putative SOS box of *lexA* showed that the *Streptomyces* LexA is able to bind the proposed SOS box (Vierling and Muth, unpublished results), indicating that LexA is autoregulative also in *S. coelicolor*.

Because the *S. lividans recA* gene neither conferred a function distinct from that of *E. coli recA* nor differed in its regulation from that of other bacteria, we tested whether it was possible to replace the chromosomal *recA* gene in the presence of a plasmid-borne *recA* copy. This turned out to be a successful approach. The chromosomal *recA* gene could be efficiently replaced with a frequency of about 1%, whereas it was not possible (>0.03%) without the simultaneous *recA* expression.

Since the *tipA* promoter in pEXrecA is not tightly repressed in the absence of thiostrepton, resulting in a basal level of *recA* expression, it was not possible to study the presumed toxicity of *recA* inactivation by switching the *tipA* promoter on and off. Therefore, we had to cure the *recA* expression plasmid to demonstrate the indispensability of *recA*. To our surprise, we observed that following the replacement of the chromosomal

recA fragment, it was possible to cure the *recA* expression plasmid pEXrecA without a lethal effect. Displacing the resident *recA* expression plasmid by the incompatible plasmid pIJ920 (18) was a very efficient curing technique. In contrast to other described curing methods, such as growth at elevated temperatures or treatment with intercalating dyes (8), plasmid curing by incompatibility is not associated with any mutagenic side effects.

There are two possible explanations of why the generation of a completely defective *recA* mutant succeeded only by this procedure whereas it was not possible by the classical protocol.

(i) For unknown reasons, the *recA*-containing DNA fragment is only a poor substrate for recombination enzymes. Overexpression of the RecA protein from the thiostrepton-inducible *tipA* promoter could confer an enhanced recombination activity that allowed even the recombination of poor substrates. A 10-fold stimulation of homologous recombination by the overexpression of a bacterial *recA* gene has already been reported for plant and mammalian cells (29, 31). However, in *S. lividans* (pEXrecA), induction of *recA* overexpression did not result in an enhanced recombination rate. Neither under inducing conditions nor under noninducing conditions was the integration rate of a test plasmid with a 540-bp fragment, suitable for homologous recombination, increased (unpublished results). In contrast, the presence of the *recA* expression plasmid had only negative effects on the integration frequency of the test plasmid. This was probably due to the detrimental effects of *recA* overexpression (36) interfering with the survival of the integrants.

(ii) The *recA* mutant had acquired an additional mutation which suppresses the toxic effects of *recA* inactivation. Since up to now no suppressor mutations for *recA* have been described (15), the mutation must affect a function that allows the cell to survive with *recA* deficiency. The plasmid-borne copy of *recA* which is under control of the *tipA* promoter might be just sufficient to override the lethal effect of RecA deficiency but might not be able to complement *recA* with wild-type efficiency. Therefore, selection pressure could exist to select for such suppressing mutations. The reasons for the lethal effects of inactivation of *recA* in *Streptomyces* are not known, but a role of RecA in the repair of damaged replication forks was suggested (38). In an alternative model, the recombination patching model, RecA activity is required for the replication of the ends of the linear chromosome. Since the *recA* mutant SV64ΔrecA still contained a linear chromosome, it was possible recently to disprove this model (C.-H. Huang, H.-H. Lee, S.-H. Chou, and C. W. Chen, personal communication). Although *E. coli recA* mutants are viable, they are also severely affected and show slower growth, probably due to the generation of up to 50% dead cells (5). If the lethal effect of *recA* inactivation reflects a defect in DNA repair, a suppressing mutation could stimulate RecA-independent repair mechanisms or delay cell division to provide more time for the repair. The requirement for a second mutation would explain why neither by classical mutagenesis (11) nor by conventional gene inactivation techniques (1, 24) has it been possible to isolate *recA* mutants of *S. coelicolor, S. lividans,* or *Streptomyces ambofaciens*. A *recA* mutant was described only for *S. rimosus* (20). Since the genotype of this strain was not characterized in

detail, there is no information available about the presence of any additional defects.

Beside the classical *recA* phenotype, UV sensitivity, deficiency in homologous recombination, and enhanced genetic instability, all the mutants were sporulation deficient. It should be stressed that some of the mutants were isolated in independent experiments. *S. lividans* SV64ΔrecA strains had the morphology of so-called *whi* mutants (6). This was confirmed by scanning electron microscopy of the *recA* mutant. Obviously, the differentiation was blocked in an early stage before the formation of septation. Thus, the mutant resembles *whiA, whiB, whiG, whiH, whiI, or whiJ* mutants of *S. coelicolor*, with which the formation of sporulation septa is essentially abolished (7). Since the *recA* mutant formed long straight hyphae with little to no curling, the morphology was similar to that described for *whiG* mutants (35).

All defects of the classical *recA* phenotype could be fully restored by the *S. lividans recA* gene when placed under control of the thiostrepton-inducible *tipA* promoter on a single-copy SCP2 derivative or by the reintroduction of the *S. coelicolor recA* gene at the original chromosomal position. The *S. coelicolor recA* gene differs from that of *S. lividans* by two base-pair substitutions (shown in bold): a C**G**T-C**G**G exchange that had no effect on the amino acid composition and a **G**CG to **A**CG substitution that changes an alanine to a threonine. This amino acid exchange is localized at position 369 in the C-terminal end, which is not conserved in bacterial RecA proteins (3). However, complementation did not affect the sporulation deficiency, demonstrating that the block in morphologic differentiation was not caused by the inactivation of *recA*. This might be a clear indication that *S. lividans* SV64ΔrecA had acquired an additional mutation that could suppress the toxic effects of *recA* deficiency. During vegetative growth of the *Streptomyces* substrate mycelium, only very few cross walls are formed in the growing hyphae. The formation of cross walls, which corresponds to the cell division of unicellular bacteria, occurs in the *Streptomyces* life cycle mainly during differentiation. The aerial mycelium erected from the substrate mycelium becomes fragmented into spore chains and finally is released (6). A mutation blocking the septation of the *Streptomyces* aerial mycelium could have an effect for *Streptomyces* similar to the inhibition of cell division by SulA during the SOS response in *E. coli*, in preventing *Streptomyces* from producing nonviable spores with damaged DNA.

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