Transcriptional and Mutational Analyses of the *Streptomyces lividans* recX Gene and Its Interference with RecA Activity

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The role of the 20,922-Da RecX protein and its interference with RecA activity were analyzed in *Streptomyces lividans*. The recX gene is located 220 bp downstream of recA. Transcriptional analysis by reverse transcriptase PCR demonstrated that recX and recA constitute an operon. While recA was transcribed at a basal level even under noninducing conditions, a recA-recX cotranscript was only detectable after induction of recA following DNA damage. The recA-recX cotranscript was less abundant than the recA transcript alone. The recX gene was inactivated by gene replacement. The resulting mutant had a clearly diminished colony size, but was not impaired in recombination activity, genetic instability, and resistance against UV irradiation. Overexpression of the wild-type recA gene in a *Pseudomonas aeruginosa* recA mutant (rec-2) was only tolerated if the recX gene was simultaneously expressed. Therefore, a regulatory role for recX in RecA activity was suggested (26). However, it was not clear whether it controls the expression of the recA gene or interacts directly with the RecA protein (26).

Various attempts have been made to generate recA deletion mutants in streptomyces. It was only possible to isolate disruption mutants with residual RecA activity (1, 21). Therefore, a crucial role of the recA gene in ensuring the viability of streptomyces was suggested. However, it could not conclusively be excluded in these experiments that a polar effect on downstream genes (e.g., recX) was responsible for the failure to generate recA-deficient *Streptomyces lividans* mutants. Such polar effects on recX have also been discussed by Papavinasaundaram et al. (24) to explain the inability to inactivate recX of *Mycobacterium smegmatis*.

In this paper, we report the transcriptional analysis of the *S. lividans* recX gene and the construction of a recX gene replacement mutant. The phenotypic characterization of the mutant suggested that RecX downregulates RecA activity by protein-protein interaction to overcome the toxic effects of RecA overexpression.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, and media. The *E. coli* strain used for subcloning and DNA sequencing was XL1-Blue (4). The parental *Streptomyces* strain was *S. lividans* TK64. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium (25). *Streptomyces* strains were cultured as described previously (6). Antibiotics were supplemented, where appropriate, at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 5 μg/ml; thiostrepton, 25 μg/ml; gentamicin, 5 μg/ml; chloramphenicol, 10 μg/ml. The plasmids used in this work are listed in Table 1.

DNA manipulations. Standard procedures were performed as described by Hopwood et al. (8) and Sambrook et al. (25). Hybridization used digoxigenin-labeled dUTP and a digoxigenin detection kit (Boehringer, Mannheim, Germany). Gene replacement mutants were selected as described by Wohlleben and Muth (37).

Expression of recX. The *S. lividans* recX gene was amplified by PCR with primers SrecX and TrecX (Table 2). Following restriction with NdeI and BamHI, the fragment was inserted into the *Streptomyces* expression vector pIJ4123 (30), yielding plasmid pSVX-his. In pSVX-his, the recX gene is expressed with an

RecA is a multifunctional protein that is involved in homologous recombination, DNA repair, and the induction of the SOS response (13, 35). The protein is highly conserved among all prokaryotes (12, 23), and homologues of RecA are also found in eukaryotes (2). Transcriptional regulation of recA by the SOS repressor LexA has been well studied in *Escherichia coli* and *Bacillus subtilis* (18, 36). Under normal growth conditions, the LexA protein binds to a specific DNA sequence, the SOS box, upstream of the promoter region and inhibits transcription. Following DNA damage, autocleavage of the LexA SOS box, upstream of the promoter region and inhibits transcription. Following DNA damage, autocleavage of the LexA

stem of recA (7). In mycobacteria, the recX gene overlaps with the coding region of recA, and the two genes are cotranscribed (24). Overexpression of the wild-type recA gene in a *Pseudomonas aeruginosa* recA mutant (rec-2) was only tolerated if the recX gene was simultaneously expressed. Therefore, a regulatory role for recX in RecA activity was suggested (26). However, it was not clear whether it controls the expression of the recX gene or interacts directly with the RecA protein (26).

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TABLE 1. Plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td>pUC18</td>
<td>lacZ, bla</td>
<td>31</td>
</tr>
<tr>
<td>pGM11</td>
<td>aphII; temperature-sensitive Streptomyces vector</td>
<td>37</td>
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<tr>
<td>pIF293.2</td>
<td>bla; PiptA</td>
<td>J. Altenbuchner, Stuttgart, Germany</td>
</tr>
<tr>
<td>pSVX1</td>
<td>recA displacement plasmid; pGM11 derivative carrying the 1,550-bp PsI-NcoI fragment; tsr insertion within the BclI site</td>
<td>This study</td>
</tr>
<tr>
<td>pSVQ1</td>
<td>Recombination test plasmid; pGM11 derivative carrying a 1,316-bp recQ PCR fragment, disrupted by the tsr gene</td>
<td>This study</td>
</tr>
<tr>
<td>pSVX2</td>
<td>pGM11 derivative carrying the 1,685-bp SalI fragment encoding RecX and the C-terminal half of RecA</td>
<td>This study</td>
</tr>
<tr>
<td>pSVAX2</td>
<td>recX complementation plasmid; pGM11 derivative carrying a 2,346-bp PCR fragment containing the complete recA-recX operon</td>
<td>This study</td>
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<tr>
<td>pIJ4123</td>
<td>Streptomyces His tag expression vector; tsr kan PiptA redD</td>
<td>30</td>
</tr>
<tr>
<td>pSVX-his</td>
<td>pIJ4123 derivative carrying a PCR fragment containing the recX gene</td>
<td>This study</td>
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<tr>
<td>p2004/1 i</td>
<td>Bifunctional SCP2 derivative, p15A E. coli ori, tsr cat</td>
<td>Unpublished data</td>
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<td>pEXRecA</td>
<td>recA expression plasmid; p2001/41 derivative; PiptA recA tsr aacC1</td>
<td>This study</td>
</tr>
<tr>
<td>pEXR169-H</td>
<td>recA expression plasmid; p2001/41 derivative; PiptA recA(R169-H)</td>
<td>This study</td>
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Preparation of S. lividans RNA. *S. lividans* was cultivated in 50 ml of YEME (composition given in reference 8) for 2 to 3 days. The culture was induced with methyl methanesulfonate (MMS; 25 μg ml⁻¹) for 20, 40, and 60 min. Cells were harvested and shock frozen at −70°C. An aliquot was resuspended in 100 μl of P-buffer containing 0.53 mg of lysozyme and incubated for 7 min at 37°C. RNA was extracted from uninduced and MMS-induced cultures by using the RNaseasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

RT-PCR analysis. RNA prepared from *S. lividans* was treated with 3 U of RNase-free DNase I (Promega, Mannheim, Germany) and precipitated according to standard protocols (25). The RNA concentration was photometrically determined by using a Genequant fixed-wavelength photometer (Pharmacia, Freiburg, Germany). The reverse transcription reaction was carried out with an enhanced avian reverse transcriptase PCR (RT-PCR) kit (SIGMA, Germany) according to the manufacturer’s instructions.

RESULTS

Identification of the *S. lividans* recX gene. At 220 bp downstream of the recA stop codon in *Streptomyces coelicolor* A3(2) (EMBL accession no. AL020958), an open reading frame with significant similarity to the recX genes of *Mycobacterium leprae* (43.5% identity, 161-amino-acid [aa] overlap) and *P. aeruginosa* (28.5%, 147 aa) was identified. To prove the presence of the recX homologue and the conservation of the gene organization in *S. lividans*, we amplified the corresponding *S. lividans* recX fragment with primers deduced from the *S. coelicolor* sequence. Partial sequence analysis (data not shown) resulted in sequences identical to that of *S. coelicolor*. The recA-recX intergenic region contains several direct repeats and has the potential to form secondary structures. A hairpin structure with 20 bases in the stem and 7 in the loop (ΔE = −26.2 kJ/mol) which could act as a transcriptional terminator of recA transcription is located 64 bp downstream of the recA stop codon. This putative termination structure is also present downstream of the *Streptomyces ambofaciens* recA gene (1).

recX is cotranscribed with recA after induction with the DNA-damaging agent MMS. The distance of 220 bp between recA and recX and the putative termination structure downstream of recA suggested that these two genes were transcribed independently in *S. lividans*. An RT-PCR analysis was performed to assess whether both genes were cotranscribed. Since recA of *S. lividans* is regulated by the SOS repressor LexA (unpublished results), RNA was isolated after induction with the DNA-damaging agent MMS (11). Primer pairs within recA (recA1 and recA2) and recX (recX1 and recX2) were used to detect the independent transcription of each gene. In order to prove the presence of recA-recX cotranscripts, primers corresponding to the 3' region of recA (recA3) and recX (recX2) were chosen (Table 2 and Fig. 1). The functionality of the primers for RT-PCR was demonstrated by PCR on genomic DNA as the template (Fig. 2, lane DNA). The absence of

<table>
<thead>
<tr>
<th>Primer Strand</th>
<th>Oligonucleotide sequence (5’→3’)</th>
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<tbody>
<tr>
<td>recA1</td>
<td>+ ATCGAAGGCTCATCCCGACCGGTTCT</td>
</tr>
<tr>
<td>recA2</td>
<td>− ATGTGTCGACAGCCGCCCTCG</td>
</tr>
<tr>
<td>recA3</td>
<td>+ ATCAGAGCAGAAGCTGGGCTGGG</td>
</tr>
<tr>
<td>recX1</td>
<td>+ TCCTGTCGAGGGCGCGAGAAG</td>
</tr>
<tr>
<td>recX2</td>
<td>− CCGTTGCTCCGCTCTCTTTTTC</td>
</tr>
<tr>
<td>glnA1</td>
<td>+ GGGAGAACGACTCTCCGCGACCC</td>
</tr>
<tr>
<td>glnA2</td>
<td>− GCCACAGGCTTCACGCCGGCTGCT</td>
</tr>
<tr>
<td>SrecX</td>
<td>+ GGAAATCTCCATATGGACGCTCTGCGCGAGA</td>
</tr>
<tr>
<td>3recX</td>
<td>− CGGGATCTCTCGAGAACCCTCTCGGCGAGG</td>
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TABLE 2. Oligonucleotides used for RT-PCR analysis
contaminating DNA in the RT-PCR was confirmed by a control PCR with RNA as a template (Fig. 2E). From uninduced cultures, no recX transcript and only a weak band indicating basal expression of the recA gene were detected (Fig. 2A to C, lane 1). Twenty minutes after induction with DNA-damaging MMS, the intensity of the recA-specific band increased, demonstrating induction of the recA gene during the SOS response. Transcription of the recX gene, however, was not detectable even 20 min after induction. A recA-recX cotranscript appeared only 40 and 60 min after induction (Fig. 2B, lanes 3 to 4), when expression of recA reached its maximum (Fig. 2A, lanes 3 and 4). This demonstrated that recX was cotranscribed with recA after induction of the SOS response. Probably due to the termination structure between recA and recX, the recA-recX cotranscript was produced only at a low level (less than 10%) compared to the recA transcript.

As a control for the quality of the RNA preparation, RT-PCR was also performed with a primer pair deduced from the glnA gene (glutamine synthetase I) which is not induced by DNA damage. In contrast to the recA and recA-recX message, the intensity of glnA transcription did not significantly change during MMS induction (Fig. 2D).

Construction of a recX gene replacement mutant in S. lividans TK64. To analyze the role of RecX, we intended to inactivate the recX gene. Therefore, the cloned recX gene was disrupted by the insertion of the thiostrepton resistance gene into the single BclI site located in the N-terminal half of recX. The temperature-sensitive replacement plasmid pSVX1 (Fig. 3A), which carries the disrupted recX gene, was transferred into S. lividans TK64, and colonies were selected with the thiostrepton resistance marker integrated into the chromosome. Subsequently, the colonies were picked on LB agar containing thiostrepton (25 μg ml⁻¹) or kanamycin (50 μg ml⁻¹). One out of 600 colonies was found to be thiostrepton-resistant and kanamycin sensitive, indicating gene replacement and plasmid loss.

To verify the gene replacement, genomic DNA of the mutant was isolated and subjected to Southern blot analysis with a probe encoding the C-terminal part of RecA and the complete RecX. The probe hybridized with a 3,168-bp fragment that is 1,060 bp larger than the fragment in the wild type (Fig. 3B). This increase in size was due to the insertion of the tsr cassette. In addition, the recX genotype was further confirmed by PCR. With primers (recX1 and recX2) flanking the insertion site within recX, a fragment from the mutant was amplified which was 1,060 bp larger than the corresponding wild-type fragment (Fig. 3B).

Phenotype of the S. lividans SVX1 mutant. The recX mutant showed normal wild-type morphology on agar plates and in liquid culture. Only when spores were plated on solid medium was the colony size of the mutant clearly reduced (about 30% of the wild-type area) compared to that of S. lividans TK64 (Fig. 4). In order to investigate the effect on RecA-related functions, the UV sensitivity, the ability to undergo homologous recombination, and the genetic instability of the mutant SVX1 were analyzed.

As demonstrated in Fig. 5, the UV sensitivity of SVX1 was not significantly affected, indicating that the mutant is still proficient in recombinational DNA repair and able to induce the SOS response.

To test the recombination activity of the SVX1 mutant, the

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**FIG. 2.** Transcriptional analysis of the recA-recX operon by RT-PCR. After induction with 25 μg of MMS ml⁻¹, RNA was isolated from S. lividans TK64 at different intervals and used for RT-PCR with different primer combinations. (A) recA-specific primers. (B) recA-recX cotranscript-specific primers. (C) recX-specific primers. (D) Control reaction using glnA-specific primers. (E) Control reaction without RT. Lanes: DNA, control PCR with genomic DNA as template; 0, without induction; 20, 40, and 60 min after induction, respectively; M, size standard (1-kb ladder [Boehringer]: 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).
ability to integrate the temperature-sensitive plasmid pSVQ1 (Table 1) into the chromosome was studied as described in Materials and Methods. The plasmid integrated into the SVX1 chromosome with an efficiency of 2 to 10%, similar to that in the parental TK64 chromosome, demonstrating that the recX mutant pSVX1 is recombination proficient and that inactivation of recX does not significantly affect the recombination activity of S. lividans.

The genetic instability of the recX mutant SVX1 was assessed by analyzing the segregation of chloramphenicol-sensitive colonies, which arise by the loss of the chromosomal end containing the chloramphenicol resistance gene (cml). The mutant SVX1 segregated chloramphenicol-sensitive mutants at wild-type frequency (0.8%), thus indicating that genetic instability was also not affected by the inactivation of recX.

The only visible effect of recX inactivation was the small colony size after plating spores on solid medium. To confirm that this phenotype was due to the inactivation of recX and was not caused by an additional mutation, it was necessary to show phenotypic reversal. The S. lividans recX gene was amplified by PCR and cloned into the high-copy expression vector pIJ4123 under control of the thiostrepton-inducible tipA promoter (pSVX-his). On thiostrepton-containing agar, the wild-type colony size of SVX1 carrying pSVX-his was fully restored. Mutant SVX1 could also be complemented by plasmid pSVAX2 containing the complete recA-recX operon, including the promoter region (Fig. 1). In contrast, plasmid pSVX2, which encoded the complete RecX and the C-terminal half of RecA but lacked a functional promoter sequence, was not sufficient to restore wild-type size.

Overexpression of RecA is toxic in the absence of RecX. To analyze the effects of recA overexpression in the recX mutant SVX1, the recA expression plasmid pEXrecA, an SCP2 (30) derivative which carried recA under control of the thiostrepton-inducible tipA promoter (20), was constructed. Transformants of S. lividans TK64(pEXrecA) and SVX1(pEXrecA) were grown for 2 days in liquid culture under uninduced conditions. Subsequently, the cultures were homogenized, and the mycelial fragments were plated on medium containing thiostrepton (20 μg ml⁻¹) and gentamicin (5 μg ml⁻¹), respectively, to compare the colony titers under induced and noninduced conditions.

For S. lividans TK64(pEXrecA), the colony titer on thiostrepton was about 60% of that on gentamicin-containing plates, indicating the inhibitory effect of recA overexpression. For the recX mutant, however, no single colony could grow on thiostrepton-containing medium. This demonstrated that in-
In order to analyze the mode of action of RecX, the influence of RecX on transcription of recA was investigated. RT-PCR with RNA isolated from the mutant SVX1 was performed. Only a faint band indicating the basal transcription of recA was detected without induction. Twenty minutes after administration of MMS, the intensity of the recA-specific band increased, and the maximum of recA transcription was reached after 60 min (Fig. 6). This clearly demonstrated that recA transcription is not significantly enhanced in the absence of RecX. Therefore, a role of RecX in repressing recA transcription is very unlikely.

**DISCUSSION**

The role of RecA in homologous recombination and in the induction of the SOS response has been elucidated in great detail (2, 13). However, very little is known about the recX gene, which is cotranscribed with recA and is supposed to be involved in modulating RecA activity (24, 26). By RT-PCR analysis, we showed that, as in other bacteria (7), the *S. lividans* recX gene is cotranscribed with recA following DNA damage, although the gene organization of the *Streptomyces recA* region suggested an independent transcription of recX. In contrast to *E. coli*, in which induction of the SOS response starts 1 min after UV irradiation and is completed after 4 to 5 min (28), expression of the *S. lividans recA* gene remained unchanged for the first 20 min. Only 40 min after induction, recA transcription reached its maximum. This delay in the induction of the SOS response in *Streptomyces* is difficult to understand. However, in *M. smegmatis* and *Mycobacterium tuberculosis*, the induction of the SOS response was also very slow, and the maximum levels of recA transcription were obtained 5 and 6 h after induction with DNA-damaging agents (19, 24). Simultaneously with the induction of recA, a recA-recX cotranscript appeared that was not detectable without induction. Two distinct transcripts are also formed in the recA-recX operon of *P. aeruginosa*. By Northern blotting, a 1.2-kb transcript representing the recA message and a 1.4-kb transcript comprising recA and recX were identified (9).

Although recA and recX form an operon in *S. lividans*, the transcription rates of both genes differ drastically. This is in contrast to *M. smegmatis*, in which both genes are transcribed with the same efficiency (24). Since the coding region of the *M. smegmatis recX* operon overlaps with the 3′ coding region of recA, it makes sense that the recX gene is always transcribed at the same level as recA. In *S. lividans* (and probably other *Streptomyces* strains), the weak termination structure between recA and recX might be responsible for transcription of recA without recX in the uninduced state and for the low level of recA-recX cotranscript in comparison to the level of recA transcript alone. To elucidate the possible function of RecX, a recX mutant was constructed. Our ability to construct a recX mutant clearly showed that the recX gene is dispensable in *Streptomyces*. Therefore, the failure to inactivate recA (21) must be due to the recA gene itself and is not due to a polar effect on the downstream recX gene, as was discussed for *M. smegmatis* (24).

Only a few very few data are available about the phenotypic effects of recX inactivation. The only published recX mutant represents a recA recX double mutant of *M. smegmatis* (24). Therefore, this mutant is not appropriate to analyze the function of RecX and its interference with RecA or RecA-related functions. For *P. aeruginosa*, a recX mutant was generated: in order to determine the coding region of the *P. aeruginosa recA* gene, several deletion mutants affecting only recA but also the downstream regions were generated in the chromosome (9). As can now be deduced from the nucleotide sequence (26, 27), a recX-containing fragment has been deleted in mutant PD07 recAΔ34. This deletion had only very slight effects on UV resistance. The recombination activity of PD07 recAΔ34 was not analyzed (9).

The *S. lividans* recX mutant SVX1 was not affected in any of the classical recA-related functions, but the small colony size in comparison to that of the wild type showed that RecX deficiency interferes with normal growth. A more drastic phenotype was observed when recA was overexpressed. Whereas induction of recA expression resulted in the reduction of the colony titer to about 60% in the wild type, indicating the toxic effect of recA overexpression, growth of the recX mutant was...
completely inhibited. A similar observation was previously published by Sano (26) for P. aeruginosa and Papainas-
sundaram et al. (24) for M. smegmatis. In these experiments, however, the authors intended to complement a recA (26) or a recA recX (24) double mutant and showed that recA could only be overexpressed if recX was coexpressed.

Since we could show that recA overexpression is lethal in a recX mutant, one would expect an impaired viability of the recX mutant after UV irradiation. DNA damage caused by UV irradiation should result in the induction of the SOS response and in overexpression of recA (17). Therefore, the recX mutant should not be inhibited directly by the UV irradiation, but due to the toxic action of RecA. However, the recX mutant was not significantly affected under these conditions. Probably, other SOS-induced genes are also involved in protecting the cell from RecA.

The nature of the toxic effect of recA overexpression is unknown. Since the recX mutant tolerated the overexpression of a mutated recA gene encoding an inactive protein, the toxic effects of RecA must be caused by one of the biochemical activities of RecA. The expression of several heterologous RecA proteins, e.g., from P. aeruginosa, B. subtilis, and Deinococcus radiodurans, and RAD51 from Saccharomyces cerevisiae has also been shown to be toxic to E. coli. In these cases, an enhanced affinity for DNA was suggested to be responsible for the toxicity (38). A mutant E. coli recA(E-96D) protein that was toxic has been shown to prevent proper chromosome segregation (5).

Overexpression of recA was only tolerated in the mutant SVX1 when recX was simultaneously highly expressed. In addition, the small colony size of the mutant was also complemented to the wild-type size. Obviously, the N-terminal 20-aa elongation containing the His tag that results from the Streptomyces expression vector pIJ4125 (30) did not substantially interfere with the activity of RecX.

About the mode of action of the RecX protein in controlling RecA activity, only speculations exist. Due to the basic character of the RecX proteins (Pl value of about 9 to 11) and the weak similarity to resolvases, a possible function of the P. aeruginosa RecX as a transcriptional repressor of RecA has been discussed (7, 26). However, transcription of recA was not affected in the S. lividans recX mutant. Following induction with MMS, the transcription rate after 60 min was the same as in the wild type. This demonstrates that RecX does not repress transcription of recA. Furthermore, it was shown by immunoblotting that the same amount of RecA protein was produced in the recX mutant as in the wild type (unpublished results). A very similar result was described for P. aeruginosa. Deletion of the recX-containing DNA fragment also did not influence recA transcription or production of RecA protein in P. aeruginosa PDO7 recA34 (9). Because RecX does not affect expression of recA, we propose an interaction of RecX with the RecA protein. This interaction could result in the inhibition of RecA activity to accelerate the shutdown of the SOS response.

Recently, it was suggested by Zaitsev and Kowalczykowski (38) that the function of RecA proteins from distinct bacteria is adapted to the specific needs of a given organism by the modulation of monomer-monomer interaction strength. Since all of the biochemical functions of RecA are directly affected by the DNA binding, an alteration of the binding characteristics might efficiently modulate the specific activity of RecA. The RecX protein might be a candidate protein for controlling RecA. RecX could interact with the highly variable and species-specific C terminus (12) of RecA, which is located at the outer site of the RecA filaments (29), explaining why the RecX proteins from the different bacteria show only low sequence conservation (20 to 43% identity).

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REFERENCES


