**Abstract**

*Streptomyces* RecA proteins are characterized by a conserved, positively charged extension of unknown function appended at their C-terminals. To investigate the function of this element, we introduced the *Streptomyces rimosus* recA gene and its mutant form encoding the protein with a C-terminal deletion into *S. rimosus*. Both transcript and protein levels were dramatically increased in the strain expressing the truncated gene compared to the strain bearing the wild-type recA, indicating involvement of the characteristic C-terminal extension in regulating the recA expression in *Streptomyces*. Considering that RecA acts as a major regulator of DNA damage response in bacteria, this mode of regulation is expected to have broader implications and significance that outreaches our current understanding of RecA autoregulation.

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**Keywords:** RecA; *Streptomyces*; C-terminus; Transcription; DNA damage response; Promoter

1. Introduction

RecA is a multifunctional protein that plays a central role in the process of homologous recombination, recombinational DNA repair and regulation of the DNA damage response [1]. It is amongst the most conserved bacterial proteins with an overall similarity between 43% and 100% [2], and is universally distributed within the bacterial kingdom, with the exception of a few obligate endosymbionts [3]. The most conserved part of RecA is its central domain that binds ATP and DNA, while its N- and C-terminal regions, involved in monomer interaction [4], display species-specific variety.

The role of RecA protein in regulating DNA damage response has been well established for the *Escherichia coli* system: it acts as an indirect regulator of a number of genes, including the recA itself, that are normally repressed by the LexA protein. The RecA nucleates onto single-stranded DNA produced as a result of DNA damage, forming an activated RecA filament. The filament in turn interacts with the LexA protein and stimulates its autocatalytic cleavage, thereby inducing the genes of the SOS-regulon [5]. In particular, binding of the RecA to both DNA and protein regulators is mediated by the negatively charged C-terminus of the RecA protein [6,7]. Consequently, this structural element is an important determinant of cellular RecA quantity control: it was demonstrated that removal of 25 residues from C-terminus increased levels of the RecA production and led to constitutive SOS induction [6].

Whereas *E. coli* and majority of bacterial RecA proteins possess shorter and acidic C-termini, a characteristic ~20 amino acid extension rich in alanines and lysines
is present at the C-termini of Streptomyces RecAs [8,9] (Fig. 1A). The function and structure of this appendix are unknown, but deletion of this region in the *Streptomyces lividans* RecA has been shown not to affect major RecA functions (judged by the ability of truncated protein to restore UV-resistance and homologous recombination in a RecA-deficient strain) [10]; the same was confirmed for the C-terminal deletion in the *Streptomyces rimosus* RecA [11]. Moreover, our understanding of DNA damage response in *Streptomyces* is obscure: although the upstream regions of the streptomycete recA reveal the presence of a putative recA promoter overlapped by an imperfect LexA-binding site [12], transcriptional analysis of the *S. rimosus* recA showed that this putative promoter has only a very weak activity and is not significantly induced upon DNA damage [13] (Fig. 2). Instead, the major, DNA damage inducible recA promoter was identified 94 bp downstream (Fig. 2) as a novel type of promoter [13]. It has been experimentally demonstrated for the homologous *Mycobacterium*
tuberculosis recA promoter (Fig. 2) that its transcription is LexA-independent [14]. Genomic analyses suggest that this type of promoter regulates majority of DNA repair genes in Actinomycetales [15]. Considering these observations we investigated the role of the streptomycete RecA appendix in mediating the RecA expression levels; we show that C-terminal deletion of 21 amino acids in the *S. rimosus* RecA leads to a dramatic increase of the cellular RecA in vivo. This effect is at least partly due to the transcriptional deregulation of the major recA promoter and its constitutive expression.

2. Materials and methods

2.1. Strains and plasmids

To compare expression levels of the RecA proteins, *S. rimosus* R6-593 strain and *Streptomyces lividans* TK23 strain were both used as hosts for the plasmids pZGRecA and pZGRecA 1–355, which, respectively, encoded the wild-type RecA protein and its truncated form with a 21 amino acid deletion at its C-terminus [11]. Both plasmid encoded recA genes included the identical 228 bp long recA promoter region upstream of their coding sequences.

2.2. Purification of the recombinant *S. rimosus* RecA

*Streptomyces rimosus* recA gene [9] was cloned into the expression plasmid pET11d (Novagen). *E. coli recA* deletion strain FR333 [16] was used as a host for overproduction of the RecA protein. Cotransformation with the plasmid pGP1-2 provided the host with the T7 RNA polymerase.

*Escherichia coli* FR333 harboring both plasmids was grown in LB medium, supplemented with ampicillin (100 µg/mL) and kanamycin (50 µg/mL), at 30 °C to OD$_{600}$ of 0.6. Induction of T7 RNA polymerase was achieved by incubating cells at 42 °C for 40 min. The cells were further grown at 37 °C for 2 h before harvesting. Cell biomass, resuspended in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 1 mM DTT (Buffer A), was disrupted by sonication. Cell debris was removed by centrifugation at 12,000 g for 30 min and then at 55,000 g for 30 min, all at 4 °C. The RecA protein in cell-free extract represented >10% of the total proteins. Crude extract was loaded on the hydroxylapatite column equilibrated with 20 mM potassium-phosphate, pH 7.4, 20 mM KCl, 10% glycerol, 0.1 mM EDTA, 5mM β-mercaptoethanol and 0.2 mM PMSF; proteins were eluted with linear gradient of potassium phosphate (20–250 mM, pH 7.4). The eluates were analyzed by SDS–PAGE. Fractions with the RecA were collected and dialyzed against 20 mM Tris–acetate, pH 7.5, 10% glycerol, 1 mM DTT and 0.1 mM EDTA. The RecA protein was precipitated with 7 mM spermidine–acetate, pH 7.5. The precipitate, recovered by centrifugation (12,000 g for 15 min. at 4 °C), was dissolved in Buffer A.

This procedure yielded about 2 mg of >95% pure RecA protein per 150 ml of induced *E. coli* culture.

2.3. Preparation of antibodies against the *S. rimosus* RecA protein

Anti-RecA antibodies were prepared in rabbits. Animals were immunized four times with 30 µg of the RecA dissolved in 100 µl of the complete Freud adjuvant, at intervals of 4 weeks. Bleeding of animals and preparation of the antiserum were performed according to the standard clinical procedure.

2.4. Western analysis

Cell free extracts were prepared as described above. Protein concentrations were determined by the Bradford
assay and additionally checked by SDS–PAGE. For the Western analysis, proteins (total 20 μg of proteins per lane) were resolved by SDS–PAGE under reducing conditions on 12% gel and blotted on PVDF membrane (Dupont). Membranes were incubated with anti-RecA serum (diluted 1:10000) and immunocomplexed bands were visualized by chemiluminescent detection (Super signal CL-HP Substrate System, Pierce). Signals were quantified using Image Master® VDS Software (Pharmacia Biotech).

2.5. Primer extension analysis

Freshly fragmented mycelium of S. rimosus R6-593 was irradiated with a Philips 30 W (254 nm) low-pressure mercury lamp at 200 J m⁻² or mock irradiated as described previously [11]. Total cellular RNA was prepared from the mycelia using hot phenol extraction as published [17].

Primer extension reaction was performed as follows: 10 pmol of the oligonucleotide 5'-GCCGAATTGGCC- GTCAA TTCTG-3', complementary to the sequence 46–66 bp downstream of the recA translational start (Fig. 2), was incubated at 37 °C for 60 min with 0.6 MBq of [γ-32P]ATP (Amersham) and 10 U of T4 polynucleotide kinase (NEB). The reaction was stopped by the addition of EDTA and the labeled primer was separated on a Sephadex-G25 mini column. 1 pmol of primer was annealed to 40 μg of the total RNA at 65 °C for 90 min. The extension was carried out at 42 °C for 60 min using AMV reverse transcriptase (Life Technologies). Sequencing reactions (Sequenase ver. 2.0, USB protocol) of the recA gene were carried out with the same primer. The extension and sequencing reactions were ethanol-precipitated, resuspended in 4 μl of loading dye, denatured at 70 °C for 5 min and loaded on a 6% (20:1) acrylamide gel.

2.6. Sequence analysis

RecA protein sequences were obtained from publicly available databases. Selected sequences were aligned using CLUSTALX [18] and analyzed by the neighbor-joining method from the same package. GeneDoc [19] was used to display sequence alignments for figures.

3. Results and discussion

3.1. Positively charged C-terminal extension is restricted to the Streptomyces and Bifidobacterium RecAs

RecA proteins bearing alanine and lysine rich C-terminal appendix have as yet been noted in several bacterial species of Streptomyces genus, whereas RecAs in other bacteria lack such appendix and are readily shorter. Our survey of available genomic databases confirmed that all seven streptomycete RecA sequences possess this characteristic element (Fig. 1A). Conservation between C-terminal extensions is fairly high and strikingly includes several lysine and alanine residues, possibly suggesting a specific function (Fig. 1A). On the other hand, analysis of all available bacterial RecA sequences revealed that only a single organism outside of Streptomyces genus – Bifidobacterium longum – possesses a homologous extension. As our analysis shows that RecAs phylogenetically more related to the streptomycete RecAs, such as proteins from mycobacteria or Amycolatopsis mediterranei and Propionibacterium acnes, do not include a similar extension (Fig. 1A and B), it is plausible to speculate that this appendix is an evolutionary old element, lost in most members of Actinobacteria.

3.2. Effect of C-terminal truncation on the RecA expression levels in S. rimosus

To investigate the role of C-terminal extension in expression of the recA gene in vivo, we compared expression of the plasmid-encoded wild type RecA and its truncated form in S. rimosus R6-593 strain. The results are shown in Fig. 3A. The basal amount of the RecA protein in non-induced wild type S. rimosus strain is low and was estimated to ~0.03% of the total cellular proteins (lane 5), whereas UV-exposure induced about 10-fold increase in the RecA expression (lane 6). S. rimosus cells transformed with pZG5RecA (bearing the wild type S. rimosus recA) produced a similarly strong signal in their non-induced state (lane 1), likely due the presence of the multiple plasmid copies. Strikingly, non-induced cells transformed with pZG5RecA1-355 (expressing the truncated S. rimosus recA) showed a

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Fig. 3. (A) Western blot analysis of the RecA protein levels in different S. rimosus strains. Lane 1: total protein extract of S. rimosus + pZG5RecA; lane 2: ~15 ng of pure E. coli RecA protein (352 amino acids; 37,842 Da); lanes 3 and 4: total protein extract of S. rimosus + pZGRRecA1-355 (in lane 4, total amount of loaded protein extract is reduced by 5-fold, compared to lane 3); lane 5: total protein extract of the wild-type S. rimosus cells before UV-induction; lane 6: total protein extract of the wild-type S. rimosus cells after UV-induction; lane 7: ~5 ng of pure S. rimosus RecA protein (376 amino acids; 39,702 Da). Predicted molecular weight of RecA1-355 protein is 37,794 Da. (B) Western blot analysis of the RecA protein levels in different S. lividans strains. Lane 1: ~5 ng of each pure S. rimosus RecA protein (39.7 kDa) and E. coli RecA (37.8 kDa); lane 2: total protein extract of S. lividans; lane 3: total protein extract of S. lividans + pZG5RecA; lane 4: total protein extract of S. lividans + pZGRRecA1-355.
more dramatic increase (~80-fold) of the RecA amount (lanes 3 and 4) compared to the S. rimosus cells transformed with pZG5RecA. Moreover, stimulated production of the wild type S. rimosus RecA synthesized from the chromosomal gene could be observed, in addition to the increase in the truncated, plasmid encoded RecA (indicated by the presence of two distinct protein bands in lane 4). Such observation suggests that the presence of the truncated RecA leads to a deregulation of the recA expression through a mechanism that functions both in cis and trans. Furthermore, this demonstrates that observed differences in expression levels are not due to the differential plasmid stability between the two strains. It is worth mentioning that S. rimosus cells support excessive amounts of RecA protein, since their growth rate is only moderately slower compared to the parental strain (data not shown).

We also tested whether deregulation of the truncated S. rimosus recA gene could be seen in heterologous background: transformation of S. lividans with the plasmids pZGRecA and pZGRecA1–355 showed the effect similar to the one observed in S. rimosus (Fig. 3B), albeit upregulation in this case was less dramatic. This finding suggests that the same mechanism operates in other streptomycete species.

3.3. Truncated S. rimosus recA is constitutively transcribed

We performed primer extension analysis of the RNA extracts from the two S. rimosus strains bearing the plasmids pZGRecA and pZGRecA1–355 to determine whether the increase in the RecA levels is due to the increase in transcription of the recA genes. The result shown in Fig. 4 demonstrates that RNA extracted from the strain bearing the truncated recA gene possesses notably higher amounts of the recA transcript compared to the one bearing the full-length gene. The transcription is upregulated from the major S. rimosus recA promoter, while no increase of the transcription from the weak promoter was observed (data not shown). Furthermore, in the case of S. rimosus pZGRecA1–355 the signal is not additionally increased following the treatment with UV light, suggesting that, in this case, transcription is constitutively upregulated. In contrast, RNA extract from strain bearing plasmid pZG5RecA showed a significant increase in the amount of the recA transcript following DNA damage induction (lanes 1 and 2), but even then the transcription level was considerably lower than in cells bearing S. rimosus pZGRecA1–355.

Taken altogether, recA transcription in Streptomyces seems deregulated by the C-terminal RecA deletion, via unknown mechanism. Our preliminary data suggest that the mechanism does not require an intact RecA protein, since we also observed deregulation in the RecA-deficient S. rimosus strain (data not shown). Importantly, the observed upregulation operates in cis and trans, and proceeds through the same promoter as recA upregulation induced by DNA damage [13], suggesting that the specific RecA C-terminal extension acts as a mediator of DNA damage response in Streptomyces. At this point we can only speculate how this basic module rich in alanines and lysines relates to the transcriptional regulation of the inducible recA promoter. However, it is interesting to note that the second RecA protein of proteobacterium Myxococcus xanthus, although shorter than the streptomycete RecAs, possesses an alanine- and arginine-rich C-terminus [20]. Moreover, the promoter region of the corresponding M. xanthus recA2 gene possesses a motif highly homologous to the major recA promoter of Streptomyces (our unpublished data), suggesting a possible link between the basic RecA C-terminus and this unusual type of promoter, which precedes majority of DNA repair genes in Actinomycetales [15]. We can hypothesize that the basic RecA C-terminus does not bind to the promoter directly, but that it rather interacts with the negative regulator RecX, as suggested earlier [21]. Possibly, RecX acts at the level of nucleoprotein filament formation and, analogously to E. coli...
system, inhibits coprotease and recombinase activities of RecA protein [22,23]. However, this possibility remains a mere speculation until the basic aspects of Streptomyces DNA damage response, such as activation of RecA coprotease activity by ssDNA and LexA-dependency of the process, are clarified.

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References