Characterization of two *Streptomyces ambofaciens* recA mutants: identification of the RecA protein by immunoblotting

Bertrand Aigle 1,a, Anne-Catherine Holl a, Jaime F. Angulo b, Pierre Leblond a, Bernard Decaris a,*

a Laboratoire de Génétique et Microbiologie, UA INRA 952, Université Henri Poincaré, Nancy I, Boulevard des Aiguillette, B.P. 239, 54506 Vandoeuvre-lès-Nancy, France

b Laboratoire de Génétique de la Radiosensibilité, Direction des Sciences du Vivant, CEA, B.P. 6, 92265 Fontenay-aux-Roses, France

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Abstract

The recA gene was isolated from *Streptomyces ambofaciens* DSM40697. Its nucleotide sequence predicted a protein of 372 residues. Two recA mutants, NSAR1001 and NSAR57, obtained by gene disruption encoded a RecA protein lacking respectively 30 and at least 62 amino acids from the C-terminal end. NSAR1001 showed a wild-type sensitivity to UV light and oxolinic acid. In contrast, NSAR57 was highly sensitive to these agents and the loss of the inserted DNA restored the wild-type phenotype. Western blot analysis using antiserum to *Escherichia coli* RecA showed that overproduction of RecA was correlated with overtranscription of recA in an *S. ambofaciens* amplified mutant derived from genetic instability.

Keywords: *Streptomyces ambofaciens*; recA; Gene disruption; Overtranscription; Immunodetection

1. Introduction

*Streptomyces ambofaciens*, a Gram-positive filamentous bacterium, exhibits a high level of genetic instability, characterized by large genomic rearrangements (deletions and/or amplifications of DNA sequences) within a region of approximately 2000 kb, which includes the extremities of the linear chromosome [1].

Voll et al. [2] have shown that genetic instability in *S. ambofaciens* can be stimulated by treatments known to induce the SOS system. The SOS response is likely to be present in streptomycetes [3,4]. In *Escherichia coli*, induction of the SOS system requires the RecA protein, which is also essential for homologous recombination and SOS mutagenesis (for review see [5]). The RecA protein may therefore play a role in genetic instability.

The recA genes of *Streptomyces lividans* TK24 and *Streptomyces venezuelae* ISP5230 were characterized [6,7]. Here, we report the isolation and characterization of the *S. ambofaciens* recA gene, its product and the construction of two recA mutants.

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Table 1
Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURE</td>
<td>mcrA, D(mcrCB-hsdSMR-mrr)171, endA1, supE44, thi-1,1-,-, gyrA96, relA1, lac, recB, recJ, bceC, unsuC::Tn5 (KanR), uvrC, [F'; proAB, lacF'ZAM15, Tn10 (tetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>ET12567</td>
<td>dam dem hsdS cm R</td>
<td>[8]</td>
</tr>
<tr>
<td>GY7066</td>
<td>Δ(srl+recA)306::Tn10 metB thi pyrE lac MS286 F80dIIlacBK1 sfiB114</td>
<td>[9]</td>
</tr>
<tr>
<td>GY7107</td>
<td>as GY7066 but miniF recA+KanR (pGY5887)</td>
<td>R. Devoret and A. Balone (pers. com.)</td>
</tr>
<tr>
<td>S. lividans ZX7</td>
<td>derivative of JT46, defective in DNA modification</td>
<td>[10]</td>
</tr>
<tr>
<td>DSM40697</td>
<td>used as wild-type strain</td>
<td>[11]</td>
</tr>
<tr>
<td>NSAR1001</td>
<td>as DSM40697 but recA::pDHR1</td>
<td>This work</td>
</tr>
<tr>
<td>NSARS77</td>
<td>as DSM40697 but recA::pRM2 and deleted for the unstable region of the chromosome</td>
<td>This work</td>
</tr>
<tr>
<td>RP181110</td>
<td>derived from the wild-type strain ATCC23877 after UV light mutagenesis</td>
<td>Rhône-Poulenc</td>
</tr>
<tr>
<td>NSA205</td>
<td>derived from RP181110 after ethidium bromide treatment</td>
<td>[12]</td>
</tr>
</tbody>
</table>

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The strains and plasmids are listed in Table 1. Streptomyces and E. coli strains were cultured in media described in [16,17]. E. coli ET12567 and S. lividans ZX7 were the hosts for pDHR1 and pRM2 before transformation of S. ambofaciens. Spores treated with UV light or mitomycin C (MMC, Sigma) as in [2] were grown at 37°C on HT medium [18]. For the oxolinic acid treatments, colonies were grown at 37°C on HT plates with oxolinic acid (Sigma; range 2.5–10 μg ml⁻¹).

2.2. Gene disruption of recA

Protoplast transformation was performed as described [16]. Transformants were selected with thiostrepton (30 μg ml⁻¹). When pRM2 was used, transformants were selected at the permissive temperature (28°C). Cultures were allowed to sporulate on selective HT plates at 28°C and then grown on HT with thiostrepton at the restrictive temperature of 39°C for 10 days.

2.3. DNA and RNA manipulations

Total DNA and plasmid extractions from Streptomyces and E. coli were performed as in [16]. DNA manipulations were performed using kits: ligations and fill-ins (Stratagene), nested deletions (Pharma-cia), DNA labelling (Multiprime, Amersham), sequencing (Taq Track, Promega). [α-32P]dCTP and nylon membrane were purchased from Amersham. An oligonucleotide was used to achieve the full
recA sequence. RNA extractions and S1 nuclease protection experiments were performed according to [16,17].

2.4. Protein extraction, Western blotting and immunodetection

Proteins from E. coli cultures or from Streptomyces strains were extracted as described [12] and separated on 12% SDS-PAGE, stained with Coomassie blue or transferred to nitrocellulose together with a size marker (Sigma). For immunodetection of RecA, membranes were washed with TBS (50 mM Tris-HCl pH 8.0, 150 mM NaCl) and incubated in TBS+10% milk powder for 1 h at room temperature (RT). Rabbit polyclonal antibodies raised against E. coli RecA [19] were added (incubation for 1 h at RT). Membranes were further incubated for 30 min with goat anti-rabbit IgG (dilution 1/7500) coupled to alkaline phosphatase (Promega). The blots were stained with 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium (Boehringer Mannheim) in alkaline phosphatase buffer. The BioImage System (Millipore) was used to quantify the intensity of the signals and the proteins on gels.

3. Results and discussion

3.1. Characteristics of the S. ambofaciens DSM40697 recA gene

The S. ambofaciens recA gene was isolated by screening a genomic library with an internal fragment of the S. cattleya recA gene [6] as a probe. The sequence homologous to the probe was located in a 3 kb PstI fragment (Fig. 1). A 1572 bp nucleotide sequence from this fragment (EMBL accession number Z30324) revealed an ORF encoding a protein of 372 aa which displayed 94.4% and 89.9% identity with the predicted RecA proteins of S. lividans TK24 [6] and S. venezuelae ISP5230 [7], respectively (data not shown). The start codon (ATG) of this ORF was chosen in comparison with the start codon of the already described recA genes and because a potential ribosomal binding site is located upstream of this codon. A putative transcriptional terminator was located 125 bp after the stop codon. No E. coli σ70-like promoter sequence was found but only a putative E. coli heat shock-like promoter as in S. lividans TK24 [6].

Two domains similar to SOS operator sites (also

Fig. 1. Restriction map of the 3 kb PstI fragment containing the S. ambofaciens DSM40697 recA gene. The sequenced region is shown by the double arrow. The recA coding region is represented by the hatched box and the orientation is given by the position of the start (ATG) and stop codons. Length is given in base pairs. The bar represents the homologous region to the S. cattleya recA fragment. The dotted double arrows represent the 1.5 kb SacII and 0.8 kb RsrII fragments, used as probes in low-resolution S1 mapping (see text and Fig. 3A, top).
called ‘Cheo’ box; 5′GAAC-N4-GTTC3′) located within DNA damage-inducible promoters in Bacillus subtilis [20] were identified 170 bp upstream and 54 bp downstream of the start codon. Although only one domain was previously reported in S. lividans TK24, our comparison revealed two ‘Cheo’ boxes as well as in S. venezuelae ISP5230. Consequently, the putative Streptomyces SOS system would be more similar to the B. subtilis than to the E. coli one.

3.2. Disruption of the S. ambofaciens recA gene

pDHR1 and pRM2 plasmids which contain internal fragments of recA were constructed (Fig. 2). They were used to disrupt recA by a single crossover event and generate strains NSAR1001 and NSAR57, respectively, as confirmed by Southern hybridization (not shown). Hybridization revealed that in NSAR57, a deleted form of pRM2 (pRM2d, Fig. 2), was integrated in recA and that, in NSAR1001, pHR1 was integrated as a tandem repeats (circa 4 copies). The deletion of pRM2 occurred in S. lividans ZX7 (not shown) before transformation S. ambofaciens. The NSAR1001 and NSAR57 recA genes encode RecA proteins lacking 30 and at least 62 aa, respectively.

The stability of the insertions in S. ambofaciens NSAR1001 and NSAR57 was tested by growing the bacteria in liquid or solid cultures without antibiotic. NSAR1001 was stable, unlike NSAR57 in which the insertion was lost at high frequency. The excision of pRM2d was confirmed by hybridization and PFGE analysis which revealed that most of the unstable region had been deleted in NSAR57 including both DNA extremities (not shown). Thus, the homologous recombination function of RecA seems altered in NSAR1001. In NSAR57, instability of the pRM2d insertion could result from the replication mechanism of the pGM160, i.e. rolling circle, known to promote frequent recombination events.
Fig. 3. Transcriptional and traductional analysis of recA. A: Low-resolution S1 nuclease mapping of recA mRNA of the RPI81110 and NSA205 strains. The protected DNA is the 1.5 kb SacI fragment of pSAR1a (see Fig. 1). After transfer, the DNA component of the DNA-RNA hybrids was detected with the \(^{32}\)P-labeled 0.8 kb RsaI fragment. Negative controls were the 1.5 kb SacI fragment with non-homologous RNA (i.e. yeast tRNA), treated or untreated with S1 nuclease. The exposure time was 8 days at \(-70\)°C with two intensifying screens. The 1.5 kb signal corresponds to the renatured DNA-DNA probe. B (top): Immunodetection of RecA protein with E. coli RecA antibodies (dilution 1/4000) in S. ambifaciens and E. coli strains. E. coli proteins were obtained from 1.8×10^7 cells. Arrows indicate the position of RecA. Bottom: One-SDS-PAGE gel of proteins from S. ambifaciens strains stained (7.5 μg each track) with Coomassie blue and detection with E. coli RecA antibodies (dilution 1/40000).

3.3. DNA damage sensitivity of NSAR1001 and NSAR57 strains

The sensitivity of NSAR1001 and NSAR57 to UV light, MMC and oxolinic acid was tested. NSAR1001 and the WT strain were found to show the same sensitivity to these agents. In contrast, NSAR57 was much more sensitive to UV light and
to oxolinic acid than these strains but not to MMC. The analysis of four revertants of NSAR57 showed that the loss of the insertion restores a WT-like sensitivity (data not shown). Thus, the mutant phenotype is due to recA disruption and not to the large deletion detected in NSAR57. These agents are known to induce the SOS system [21,22]. Therefore, the deletion of 30 C-terminal amino acids (NSAR1001 mutant) would not alter the RecA efficiency in SOS repair. In contrast, in NSAR57, which produces a RecA protein truncated by at least 62 C-terminal amino acids, the SOS system induction would be impaired. Similar results were also described in S. lividans [23] and in E. coli [24]. In this hypothesis, the absence of sensitivity to MMC in NSAR57 suggests the presence of a specific repair system for MMC damage.

3.4. Overtranscription of recA and overproduction of RecA in a mutant derived from genetic instability

Low resolution S1 nuclease mapping was performed with RNA from the RP181110 strain (reference strain) and one of its derivatives, NSA205 (Table 1), with a 1.5 kb SacII fragment of pSAR1a as protector DNA (Fig. 1). NSA205 is amplified for AUD205 [12], one of two amplifiable loci of the S. ambofaciens unstable region. Using equal amounts of total RNA, the NSA205 track showed a stronger signal compared to RP181110 (Fig. 3A) indicating overtranscription of recA. This result was confirmed by Northern blot analysis using total RNA extracted from exponential and stationary culture phases. As recA did not map in the unstable region [1], overexpression of recA did not result directly from the rearrangement of this region.

RecA production was then tested with an E. coli RecA antiserum on Western blots. Antibodies detected the E. coli RecA protein (as a single 39 kDa protein in strain GY7107) but also the S. ambofaciens DSM40697 RecA protein (Fig. 3B, top) as a 41 kDa protein, a size compatible with a 372 aa protein. In contrast, a 38 kDa protein was detected in NSA1001, consistent with the 346 aa protein predicted for RecA1001. Indeed, when pDHR1 was inserted in recA (NSAR1001), a new stop codon was created in frame with recA five codons downstream (Fig. 2C). In NSAR57 no strong signal was detected. In this strain, RecA which lacked at least 62 aa would not be recognized by the antibodies since the major antigenic determinant is located in RecA carboxy terminus [25].

In addition, all S. ambofaciens strains gave two bands of slightly different molecular mass. The lower band could correspond to a proteolytically degraded protein or result from post-translational modification. Alternatively, translation may start from two different start codons.

To quantitate the RecA amount in RP181110 and NSA205 strains, equal quantities of proteins (7.5 µg) were electrophoresed onto two different gels, one was blotted and the other stained with Coomassie blue to check equal loading of protein. Serial dilution of the antibodies showed that a 1/40000 dilution gave an optimal result (Fig. 3B, bottom). The RecA yield was 2.4-fold higher in NSA205 than in RP181110 as demonstrated by scanning the bands using the BioImage System.

So, recA overtranscription and overproduction were correlated in strain NSA205. The high level of RecA may be due to the constitutive expression of the SOS system which therefore would be involved in genetic instability. However, the occurrence of a mutation independent of genetic instability responsible for the overexpression of recA cannot be ruled out, since NSA205 was derived from RP181110 by treatment with ethidium bromide [12].

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References


