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DNA double strand break end-processing and RecA induce RecN expression levels in *Bacillus subtilis*

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ABSTRACT

Bacillus subtilis cells respond to double strand breaks (DSBs) with an ordered recruitment of repair proteins to the site lesion, being RecN one of the first responders. In *B. subtilis*, one of the responses to DSBs is to increase RecN expression rather than modifying its turnover rate. End-processing activities and the RecA protein itself contribute to increase RecN levels after DNA DSBs. RecO is required for RecA filament formation and full SOS induction, but its absence did not significantly affect RecN expression. Neither the absence of LexA nor the phosphorylation state of RecA or SsbA significantly affect RecN expression levels. These findings identify two major mechanisms (SOS and DSB response) used to respond to DSBs, with LexA required for one of them (SOS response). The DSB response, which requires end-processing and RecA or short RecO-independent RecA filaments, highlights the importance of guarding genome stability by modulating the DNA damage responses.

1. Introduction

DNA damage is a serious threat to cellular homeostasis in all-living organisms [1,2]. In the first steps of damage response, cells alter their chromatin (nucleoid in bacteria) structure and utilize specific repair pathways (e.g., base excision repair, nucleotide excision repair, mismatch repair and direct reversal) to remove DNA lesions. If unrepaired, an intricate series of interlocking signaling reactions, known as the DNA damage response (DDR), coordinates cell cycle arrest (inhibition of DNA replication in bacteria), activation of transcriptional programs and initiation of DNA repair [1,3–8]. The DDR, which plays an essential role in ensuring rapid detection of DNA damage and selecting the repair pathway needed to repair the damage, can be divided into a series of distinct, but perhaps functionally interrelated, pathways defined by the type of intermediate accumulated: single strand [ss] DNA regions [SSRs], 3′-tailed duplex, and DNA ends or "DNA structures" [1,3–6].

In bacteria, DNA insults produced as by-products of normal metabolism usually do not trigger a DDR. In response to DNA insults produced by chemical or physical agents, which create SSRs without compromising nucleoid integrity, a surveillance mechanism specific for SSRs activates the SOS response [1,9–11]. Repression of SOS genes is mediated by the binding of LexA to operator sequences (termed SOS boxes) in the promoter regions of these genes [12,13]. Upon detection of DNA damage (e.g., from exposure to UV radiation), the replication fork stalls at the damage site generating SSRs coated by the single-stranded binding protein (termed SSB in *Escherichia coli* or SsbA in *Bacillus subtilis*). Note that henceforward in this paper, and unless stated otherwise, the indicated genes and products are of *B. subtilis* origin. The nomenclature used to denote the origin of proteins from other bacteria is based on the bacterial genus and species (e.g., *E. coli* SSB is referred to as SSB_{Eco}).

Mediator proteins (e.g., RecOR or RecFOR) promote partial dislodging of SSB/SsbA and facilitate RecA nucleation. Then, the modulators (e.g., RecF, RecX, RecU, etc.) facilitate the formation of a RecA·ssDNA filament of effectual length on the ssDNA [14–16]. In both *E. coli* and *B. subtilis* cells, the RecA·ssDNA filament interacts with and facilitates LexA auto-cleavage [10,11,17]. As the amount of LexA is reduced, dissociation of LexA from the operator regions leads to expression of 30–50 unlinked SOS genes both in *E. coli* [18–20], and *B. subtilis* cells [17,21,22]. The diversity of DDR is large among different bacterial species (e.g., only seven of the *E. coli* genes have their counterpart in *B. subtilis* genes), and the size of the core of the SOS regulon is even smaller when comparing a larger number of bacterial species [see 12].

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DNA damage that generates DNA double strand breaks (DSBs) compromises nucleoid integrity and triggers a complex DDR in bacteria. These damages generate two major classes of recombinogenic structures: one-ended DSBs, which are created when a replication fork encounters a DNA single-strand nick, or two-ended DSBs, which are created by direct fracture of a DNA duplex [1,2]. The former damages are repaired via homologous recombination (HR), which is an error-free mechanism, and two-ended DSBs are mainly repaired via HR, although when HR is impaired or an intact sister chromosome is absent (e.g., in spores), non-homologous end-joining (NHEJ) becomes operative [14,16]. One-ended DSBs mostly accumulate after exposing bacteria to H_2O_2 or mitomycin C (MMC), and two-ended DSBs after exposure to ionizing radiation [1,2]. Upon addition of MMC, there is a large set of genes (~140) whose expression is altered in a RecA-dependent manner, and among those, only 30–40 genes exhibit a LexA-dependent induction (a genuine SOS response) [21]. Similar results were observed when *E. coli* cells were exposed to MMC [20]. It is likely therefore that: (i) different types of DNA damage (UV and MMC) have different effects on the global transcriptional profile, (ii) UV irradiation triggers the SOS response and (iii) MMC-induced DNA damage triggers two different responses (SOS and DSB response) [17,20–22].

In response to DNA DSBs, the molecular machinery involved in DNA repair is recruited to the lesion site, with RecN being among the first responders [15,23]. Upon induction of DSBs, damage-induced RecN focus formation is faint in the absence of RecA when compared to the wt strain [23], suggesting that the RecN pool is increased in a RecA-dependent manner. It is thought that RecN, in concert with polynucleotide phosphorylase (PNPase, encoded by the *pnpA* gene), senses the damage site, contributes to basal end-resection, and works as a concentrator of 3′-ends upon long-range end-processing [15,16]. Recently it has been shown that in the presence of two-ended DSBs a null *pnpA* mutation (Δ*pnpA*) is epistatic with Δ*recN* and Δ*ku*, which by themselves are non-epistatic [24].

Previously it has been shown that RecN expression is induced upon transient exposure to H_2O_2 [25]. A protein–protein interaction network analysis suggested that RecN might be co-expressed and connected with subgroups of proteins involved either in DNA repair (PNPase, RecA), DNA replication (e.g., PolC, HolB and DnaX) or chromosome maintenance (TopA, TopB, Smc), among other functions [25,26]. PNPase, which co-purifies with RecN, controls mRNA turnover and promotes basal resection of DSBs [24,27]. In the presence of RecA, PNPase degrades clean and certain chemically modified 3′-ends in an Mn^{2+}-dependent manner [24], as reported for eukaryotic Mre11 [28,29]. This step is followed by long-range end-processing catalyzed by two separate machineries: RecJ in concert with a RecQ-like helicase (RecQ or RecS) and SsbA or the AddAB helicase-nuclease complex (counterpart of *E. coli* RecBCD, $RecBCD_{Eco}$). These complexes catalyze nucleolytic resection of the 5′-terminal end to reveal a 3′-tailed duplex DNA [30–34]. Then, RecN, in the ATP bound form, binds to the ssDNA ends and promotes end tethering [35,36].

Induction of RecN expression occurs by different mechanisms in different bacterial Phyla, and in many cases is independent of the SOS response [12,15]. In this study the signal(s) and mediator(s) that amplify the DDR to one- or two-ended DSBs were analyzed by measuring RecN expression levels. As a control we have measured the synthesis of RecA, representative of LexA-dependent and LexA-independent avenues. We report that MMC or H_2O_2 addition increased the levels of RecN by a mechanism that is independent of the LexA repressor or the phosphorylation state of RecA or SsbA. RecN induction depends on end-processing and on the RecA protein; however, it is independent of RecO, whose function is required for full RecA filament formation onto ssDNA. Our results identify functions that, as part of the DDR in bacteria, are required for induction of the ubiquitous RecN protein.

2. Materials and methods

2.1. Bacterial strains and plasmids

The BG214 or 168 strains and their isogenic derivatives are presented in Supplementary Table S1 [24,36–41]. The pCB422-borne *recN* gene, under the control of the *recA* promoter, was used to artificially increase the basal levels of RecN, and to overexpress the RecN protein for protein purification [42]. The *recN* alleles in the chromosome were replaced by the *recN-yfp* gene as previously described [23] and introduced into different genetic backgrounds by SPP1-mediated general transduction (see Table S1).

2.2. Protein analysis and chemicals

The RecA and RecN proteins were purified from BG214 cells bearing plasmid-borne *recA* and *recN* genes as described [42,43]. The molar extinction coefficients for RecA and RecN were calculated to be 15,200 and 30,600 $M^{-1}\,cm^{-1}$ at 280 nm, as previously described [43]. The protein concentrations were determined using the aforementioned molar extinction coefficients. Quantities of RecA and RecN are expressed as moles of monomeric protein. Rabbit polyclonal anti-RecN or anti-RecA antibodies were obtained using standard techniques as described elsewhere [24].

MMC was obtained from Sigma Life Sciences. The addition of MMC causes inter-/intra-strand crosslinks and it leads ultimately to one-ended DSBs, whereas H_2O_2, which reacts with ferrous iron to generate highly reactive hydroxyl radical, leads ultimately to one- and two-ended DSBs [1]. The increased accumulation of RecA protein, expressed from its native locus and promoter, began to be detected at 0.25 μM MMC and reached a plateau at 1.5–4 μM MMC [44], hence 3 μM MMC was used for further analysis. The wild type (wt) and its isogenic derivatives (see Table S1) were grown to an OD_{560} = 0.4 at 37 °C in LB. The culture was split and one half was untreated, whereas the other half was treated with MMC (3 μM) or H_2O_2 (2.5 mM) and growth was continued for 30 or 60 min. Cell growth was halted by addition of NaN_3 to 10 μM, and cells were harvested. The cultures for RecN determination were concentrated 20- or 50-fold by centrifugation. The cells were resuspended in buffer A (50 mM Tris–HCl [pH 7.5], 5% glycerol) containing 150 mM NaCl, and lysed by lysozyme treatment (0.5 μg/ml) and sonication.

Extracts containing equal protein concentrations were separated on 10% SDS-PAGE. Blots were probed with anti-RecN or anti-RecA antibodies. The RecN or RecA accumulation was determined by western blotting. As expected, the polyclonal anti-RecN or anti-RecA antibodies detected no signal in the absence of RecN or RecA, respectively (Supplementary Fig. S1(A and B)), suggesting that no cross-reactive signal could mask our studies.

For RecN determination, similar results were observed when the cultures were concentrated 20- or 50-fold (Fig. 1 and Table 1) or when the extracts were loaded onto a Q-Sepharose column, and the retained RecN protein was concentrated by total elution (data not shown). Different concentrations of purified RecN or RecA were used to construct standard curves. RecN or RecA protein bands on developed immunoblots were quantified with a scanning densitometer (Quantity One software). Purified RecN or RecA protein standards yielded a linear relationship between antibody signal and the RecN or RecA protein concentration. The amount of RecN or RecA protein in each induced sample was interpolated from the purified RecA protein standard curve. The in vivo concentration of RecN and RecA was estimated considering the cell volume of 1.2 fl, and based on the total number of cells/ml.

For RecN half-life determination, BG214 plasmid-free cells or cells carrying the pCB422-borne *recN* gene, or its derivatives lacking different protease genes (Δ*clpX*, Δ*clpE*, Δ*clpC*, Δ*clpP* or Δ*lonA*) were grown to an OD_{560} = 0.4 at 37 °C in LB. MMC (3 μM) was