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DisA and c-di-AMP act at the intersection between DNA-damage response and stress homeostasis in exponentially growing *Bacillus subtilis* cells

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This work is dedicated to the memory of Luisa Hirschbein.

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ABSTRACT

Bacillus subtilis contains two vegetative diadenylate cyclases, DisA and CdaA, which produce cyclic di-AMP (c-di-AMP), and one phosphodiesterase, GdpP, that degrades it into a linear di-AMP. We report here that DisA and CdaA contribute to elicit repair of DNA damage generated by alkyl groups and H_2O_2, respectively, during vegetative growth. *disA* forms an operon with *radA* (also termed *sms*) that encodes a protein distantly related to RecA. Among different DNA damage agents tested, only methyl methane sulfonate (MMS) affected *disA* null strain viability, while *radA* showed sensitivity to all of them. A strain lacking both *disA* and *radA* was as sensitive to MMS as the most sensitive single parent (epistasis). Low c-di-AMP levels (e.g. by over-expressing GdpP) decreased the ability of cells to repair DNA damage caused by MMS and in less extent by H_2O_2, while high levels of c-di-AMP (absence of GdpP or expression of sporulation-specific diadenylate cyclase, CdaS) increased cell survival. Taken together, our results support the idea that c-di-AMP is a crucial signalling molecule involved in DNA repair with DisA and CdaA contributing to modulate different DNA damage responses during exponential growth.

1. Introduction

DNA damage is a serious threat to cellular homeostasis in all-living organisms. In response to alterations in the chromatin/nucleoid structure or due to poor base pair stacking, cells activate an intricate series of interlocking signalling reactions, known as DNA damage responses (DDRs), that sense and selectively recognize different types of damage in concert with cell cycle arrest (inhibition of DNA replication in bacteria). Among those DDRs, specific pathways ensure correct lesion identification and removal of the different types of lesions [1,2]. If the DNA damage level is high or the lesions are not rapidly repairable, single strand (ss) DNA regions, nicks or breaks activate different orchestrated DDRs, via homologous recombination and/or non-homologous end-joining, to preserve genome integrity [3–5]. The DDRs, which play 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: ssDNA regions, 3′-tailed duplex, DNA ends, DNA structure or recombination intermediates [3–6].

In exponentially growing *Bacillus subtilis* cells, DNA insults produced by physical (UV radiation) or chemical agents (e.g. methyl methane sulfonate [MMS]), which do not compromise nucleoid integrity, stall replication fork progression, generate ssDNA regions, and activate the LexA- and RecA-dependent surveillance mechanism, termed SOS response [1,7,8]. On the other hand, DNA damage that generates one- or two-ended DNA double strand breaks (DSBs) compromises nucleoid integrity, collapses the replication fork(s) and triggers complex DDRs [9,10]. In response to one-ended DSBs, which are created when a replication fork encounters a DNA single-strand nick (which mostly accumulate after exposing cells to H_2O_2 or mitomycin C [MMC]), or two-ended DSBs, which are created by direct fracture of a DNA duplex after exposure to nalidixic acid (Nal) treatment, activates both the LexA-dependent (SOS response) and a LexA-independent DSB response [6,11]. This response, which is still RecA-dependent, induces RecN, which in concert with PNPase, is among the first responders to DSBs [6,12–14]. At the onset of sporulation, there is a different type of DDR. Here, the DisA checkpoint protein, which is a non-specific DNA-binding protein, forms a dynamic focus moving along chromosomes, and it scans for MMC-induced one-ended or Nal-induced two-ended DSBs [15]. When damage is encountered, DisA pauses at the lesion site and

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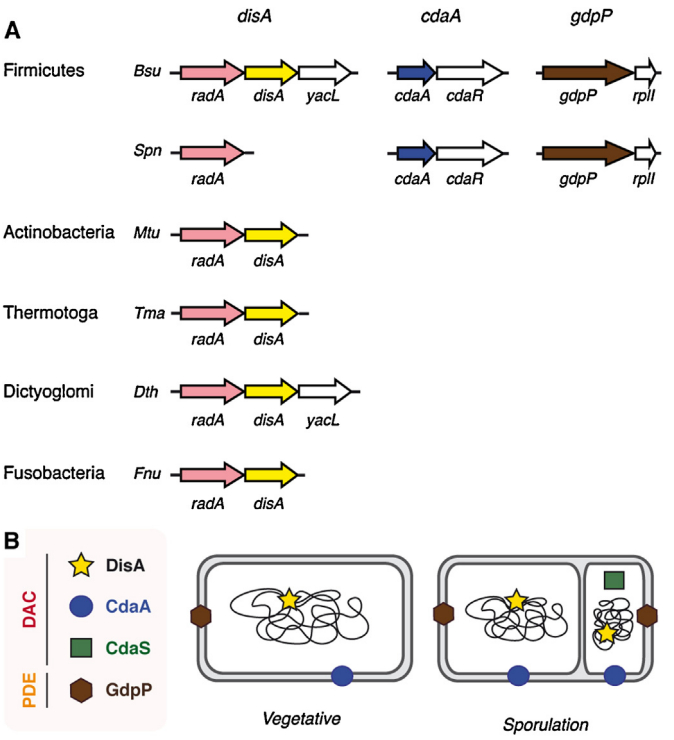

Fig. 1. Phylogenic distribution of DisA, CdaA and GdpP enzymes and their localization. (A) Conserved genomic organization of *disA*, in which the *radA* gene is always upstream. Except some Clostridia or Bacilli classes, other Firmicutes only contain the CdaA DAC enzyme. The nomenclature used to denote the origin is based on the bacterial genus and species (e.g. *B. subtilis* [*Bsu*], *Streptococcus pneumoniae* [*Spn*], etc.). Firmicutes encode for a PDE (GdpP) enzyme. (B) Localization of DAC and PDE enzymes in *B. subtilis* cells. DisA and CdaS are cytosolic, whereas CdaA and GdpP are membrane localized. DisA, CdaA and GdpP are expressed during exponential growth and during commitment to sporulation, while CdaS is only expressed during sporulation.

induces a cellular response that delays activation of the Spo0A central player, and culminates in a temporary block in sporulation before asymmetric division [15]. Furthermore, in spores lacking the AP endonucleases Nfo and ExoA, DisA might also provide a checkpoint control in a different cellular context [16]. In exponentially growing cells the role of DisA and/or of variations in the levels of c-di-AMP remains unknown. Since DisA is also encoded in non-sporulating bacteria of different phyla (Fig. 1A), it is possible that it might have a more general role. DisA has an N-terminal globular domain with diadenylate-cyclase (DAC) activity and a C-terminal RuvA-like (HhH) DNA-binding domain, separated by a central helical domain [17]. DisA converts a pair of ATPs into a cyclic diadenosine monophosphate (cyclic 3,5-diadenosine monophosphate, c-di-AMP), but when DisA binds to branched DNA structures (e.g. Holliday junction [HJ] recombination intermediates) c-di-AMP synthesis is suppressed [17], suggesting that c-di-AMP acts as a second messenger in response to HJs.

In *B. subtilis*, DisA and CdaA (also termed YbbP or DacA) contribute to maintain the intracellular c-di-AMP pool, which is essential for survival, while GdpP (also termed YybT) is a specific c-di-AMP phosphodiesterase that breaks down c-di-AMP into a linear di-AMP molecule [18]. *B. subtilis* encodes for a third DAC enzyme, CdaS (also termed YojJ) that is only expressed during spore development [19]. An increase in the levels of c-di-AMP is compatible with cell viability, but there seems to be a minimal level of c-di-AMP necessary for the cell to survive. Indeed, a *disA cdaA* double mutant strain could not be obtained without inducible expression of the sporulation-specific *cdaS* gene during vegetative growth [19,20].

DisA, CdaA and GdpP are expressed during vegetative growth and during sporulation, with DisA being cytosolic and CdaA and GdpP membrane-localized (Fig. 1B), whereas CdaS is only expressed in the spore compartment (Fig. 1B) [15,21,22]. Similarly, the unique DAC activity of CdaA, in other Firmicutes (Fig. 1A) (e.g. *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Mycoplasma genitalium*, etc.), is also essential for cell viability [23–27], suggesting a broader role of c-di-AMP in responding to environmental cues (cell wall homeostasis, responses to stress and cell proliferation) at least in bacteria of the Firmicutes phylum. Note that, unless stated otherwise, the indicated genes and products are of *B. subtilis* origin. The *radA* (also termed *sms*) gene lays upstream of the *disA* gene and forms an operon (Fig. 1A) [28]. For simplicity the gene was renamed as *radA* and the product RadA/Sms.

In this study, we have analyzed the role of DisA function in exponentially growing cells and extended these studies to understand the effect of increased or decreased c-di-AMP levels in the DNA damage response. We provide experimental evidence that exponentially growing Δ*disA* cells are sensitive to MMS, and that *radA* mutation might work in concert with *disA* for the repair of MMS-mediated DNA lesions. Over-expression of GdpP, which leads to decreased c-di-AMP levels, reduced survival of cells treated with MMS. In the absence of DisA or CdaA higher levels of c-di-AMP increased cell survival upon treatment with MMS or H_2O_2, respectively, suggesting that the c-di-AMP pool also contributes to DNA repair. Taken together, these data suggest a function for c-di-AMP acting as a signalling molecule that in concert with DisA (or CdaA) couples DNA integrity with cell wall homeostasis in exponentially growing cells.

2. Materials and methods

2.1. Bacterial strains and survival studies

The BG214, PY79 and 168 strains and their isogenic derivatives were listed in Supplementary Table S1 [15,19,22]. The selected agents were used to induce different types of DNA damage: MMS, H_2O_2 and MMC, were obtained from Sigma Aldrich, and Nal from SERVA. Small variations in drug concentrations were used. The drug concentration used in each experiment was the highest dose that allowed us to document the clearest phenotype.

Acute survival assays were performed as previously described [29]. Briefly, *B. subtilis* cells were grown to an OD_{560} = 0.4 at 37 °C in LB broth, and exposed to different concentrations of MMS. After 15 min of exposure, cells were diluted and plated on LB agar plates. The large majority of cells are one and two non-separated with an average of ~1.6 cells/colony forming unit (CFU), suggesting that the proportion of cells is roughly similar to CFUs, hence no correction was performed. CFUs were counted and plotted against the concentration of damaging agents, in order to obtain survival curves. For experiments in which external c-di-AMP was provided, the cells were pre-treated with polyamines, as previously described [15].

The sensitivity of cells to chronic exposure to MMS, H_2O_2, Nal or MMC was determined by growing cultures to an OD_{560} = 0.4 and spotting 10 μl of serial 10-fold dilutions (1×10^{-3} to 1×10^{-6}) on freshly prepared LB agar plates supplemented with the indicated concentrations of the DNA damaging agent, as described [30]. Plates were incubated overnight at 37 °C.

2.2. Quantification of c-di-AMP

B. subtilis strains were grown in minimal medium to an OD_{560} = 0.4 at 37 °C with agitation. The cells were centrifuged, washed with growth medium and resuspended in extraction buffer