Understanding the DNA repair mechanics of germination regulator DisA in *Bacillus subtilis* spores

by David

Abstract

Spores have a limited ability to fend off DNA damage. They are known to employ strategies during dormancy that minimize damage, but the only capacity to repair accumulated damage is between germination and outgrowth of a vegetative cell. During this time the spore regains metabolic activity and DNA repair capabilities. The regulatory protein DisA has previously been shown to delay sporulation when DNA damage is found, and have now recently been shown in a similar role during germination of spores.

Through a recent paper by Campos et al. (2014) we see DisA expression occurs during the ripening stages before outgrowth of *Bacillus subtilis* spores. We explore the connection DisA has with the base excision repair pathway and find that an inability to proficiently repair DNA from an inhibited repair pathway causes a slowing of the spore outgrowth by DisA. Gandara and Alonso (2015) help us understand DisA causes slow growth in germinating *Bacillus subtilis* spores by changing the concentrations of cyclic di-AMP second messenger. Finally, we see how the specific mechanics of DisA remain elusive through inconclusive attempts by Raguse et al. (2017) to explore connections with other proteins and alone on stalled replication forks.

DisA and similar regulators are important proteins that can be used to better understand ways in which organisms deal with DNA damage at different life cycles. Understanding these mechanics more in depth can help us construct ways to inhibit these damage mitigating pathways or enhance their ability to find and repair damage in organisms.

Introduction

Sporulation is a powerful survival strategy many bacteria utilize successfully. It has benefits to survival against environmental stresses in typical environments, but survival over long periods of time represents a different problem. Permafrost represents a unique, unchanging environment with frozen matrices of soil dating back thousands of years. Many samples taken from permafrost contain both spores that have remained dormant since ancient times and metabolically active bacteria from ancient lineages. Damage to DNA is known to accumulate over time, especially over such time scales as thousands to millions of years. Phylogenetic studies sometimes seek to PCR and sequence ancient DNA from extinct species but generally the DNA damage accumulation limits these studies to DNA younger than 100 or even 10 thousand years (Mitchell et al. 2005).

DNA is inherently unstable with bases being lost periodically at a rate directly related to temperature. Degradation can also occur from environmental factors such as radiation, chemical agents that cause damage to genetic information, pH, and temperature (extremely high or low). The effects on DNA can include strand breaks, base loss, base mutation, deamination, and depurination. The main inducers of DNA damage in permafrost are not environmental factors but intrinsic instability of DNA and background radiation (Mackelprang et al. 2017).

Bacteria that remain metabolically active in permafrost are able to utilize their DNA repair mechanisms, the most common mechanism being homologous recombination. Other mechanisms include base or nucleotide excision repair, mismatch repair, and non-homologous end joining. It is known that when spores are dormant they cannot utilize the classic DNA repair mechanisms available to active bacteria. It is only when they begin to germination do they have a renewed ability to undergo DNA repair (Campos et al 2014). The steps of germination are

regulated and DNA repair is shown to play a direct role in whether a spore is allowed to fully germinate or if germination will be halted due to an excess of accumulated damage. Some of the mechanisms available for the spore to utilize are similar to the ones available to the vegetative, active bacterial form such as non-homologous end joining.

It is worth noting that spores also have strategies for reducing DNA damage while in a dormant state. Small acid-soluble proteins are a class of proteins that form in a developing spore and bind to the DNA of the spore. In Bacillus subtilis, these proteins have been shown to offer the DNA greater protection again heat and UV damage than non-spore DNA of the same species (Stelow 2007). The fact that these proteins are created during sporulation and can only attempt to prevent DNA damage shows the limited capabilities spores have in the battle against DNA damage. These small acid-soluble proteins are degraded in germination where DNA repair mechanisms then take over the job. It is only then does the spore finally have some capacity to repair damage.

This paper will focus on a specific regulatory mechanism by the *Bacillus subtilis* protein DisA that is responsible for scanning DNA damage before allowing a germinating spore to continue forming into a vegetative cell. Exploring recent publications will show how DNA repair systems interact with this regulatory checkpoint, how this DisA is hypothesized to communicate its regulation, and attempts to understand its specific mechanics. These new discoveries in spore mechanics give great insight into regulation of important life cycles in bacteria and essential strategies that help the spore repair accumulated DNA damage.

Main Section

1. DNA Repair occurs before Vegetative Cell Outgrowth

When a spore has sensed it is in its preferred environment with safety and nutrients for survival in bacterial form, it will begin a process involving a few different phases that result in the formation of a final vegetative cell (Sinai et al. 2015). First the spore will undergo germination, a process similar to the sporulation process to form a spore, involving a number of morphological changes with shedding of protective layers. The next phase is called ripening where transcriptional and translational machinery is produced along with proteins necessary for DNA repair mechanisms. This ripening phase before the subsequent outgrowth phase marks an important period where metabolic activity is restored and mechanisms begin to repair DNA that has been damaged throughout dormancy. Outgrowth follows involving the growth of the final vegetative cell from the dissolving layers of the spore.

During the process of sporulation, a number of proteins are known to regulate the formation of the spore and ensure certain criteria are met. The protein DisA has been studied in sporulation and is thought to delay the bacterial cell's construction of a spore if DNA damage is detected (Lenhart et al. 2012). This protein has recently been studied taking on a similar role in another differentiation phase, regulating the spore's formation from dormancy into a vegetative cell.

Campos et al. (2014) applied oxidative stress to spores of Bacillus Subtilis to create two forms of DNA damage, AP sites and oxidized DNA bases such as 8-oxo-G. These types of damage are known to be repaired through the base excision repair pathway, one of a number of DNA repair mechanisms employed by the spore before vegetative cell differentiation. This mechanism utilizes AP endonucleases to clear regions of affected bases to make way for DNA

polymerase to add the appropriately functional bases. Wild type spores and mutant spores lacking the *nfo* and *exoA* genes, which code for AP endonucleases in the base excision repair pathway, were treated with oxidative stress during their dormancy. The concentration of AP sites and oxidized bases in both groups were measured in their dormancy and later in their outgrowth phase. Most of the AP sites and oxidized bases seemed to be repaired between the dormancy measurement and outgrowth measurement in both wild type and mutant, indicating repair probably occurs during the period before outgrowth. Mutant spores showed less of their damage to be repaired when compared with wild type spores (Fig. 1). We may expect this result without the base excision repair pathway fully functional in the mutant spores but more specific testing would be required for a conclusive link between mutants with these missing genes, an attenuated base excision repair pathway, and lower capability for DNA repair.

2. Growth Rate slowed by DisA during DNA Damage

In order to test if our suspected DisA regulator protein delays the transition from spore to vegetative cell, growth rate in germination and outgrowth were measured by Campos et al. (2012) in spores following heat shock. The same mutants lacking the AP endonuclease-coding genes *nfo* and *exoA* were compared with wild type. The mutants were found to have significantly slower growth rate compared to wild type (Fig 2A,D,E). When the *disA* gene was also knocked out in the mutants the growth returned to higher levels similar to wild type (Fig 2B,D,E). The Δ*nfo exoA disA* mutants then had *disA* gene function restored through a separate promoter, which restored the slow growth to levels significantly below wild type (Fig 2C). This may indicate that the DisA protein plays an important role in slowing growth when the base excision repair mechanism is missing important endonucleases. With the base excision repair pathway not fully functional and less repair of DNA damage occurring, as suggested by the previous experiment,

the spore may be relying on other pathways or other AP endonucleases in an attempt to mediate this accumulated damage. DisA would then play an important role in delaying outgrowth until DNA damage has been sufficiently repaired by any means. This is an untested hypothesis but a plausible one based on the results of this experiment. This direction of thinking would be worthy of further investigation. Wild type spores also had their *disA* over expressed producing no change in growth rate. Wild type spores may not have changed growth rate because they did not have missing endonucleases and could repair their damaged DNA at normal rates, unlike the slow mutant spores which might have had to take more time to repair their DNA. A link between DisA and slow growth under DNA damage is conclusive, but the role of the specific result of the missing AP endonuclease genes is still uncertain.

To specifically find the time period where slowed growth occurs the concentration of transcribed DisA was measured by Campos et al. (2012) in the germination phase and separately in the outgrowth phase following induced DNA damage. Spatial and temporal expression of DisA was measured using DisA-GFP and *disA-lacZ* fusion, where transcription is directly related to measured beta-galactosidase activity. The results showed much larger synthesis of DisA during the outgrowth phase, after metabolic activity has been restored to the spore but before the vegetative cell has grown (Fig. 3). This is the last phase before the bacterial cell forms and a key time point for regulation of this differentiation. This is a relatively simple experiment and clear evidence of the time period DisA becomes functional.

The ability of DisA to slow growth with missing AP endonucleases was studied more in depth with a final experiment by Campos et al. (2012). This experiment was meant to explore the cell division and chromosome segregation cycles in relation to DisA following induced DNA damage. This DNA replication was measured using staining to AT regions of DNA and

fluorescence microscopy, allowing general visualization of the replication dynamics. This experiment showed significantly less cell division and chromosome segregation in mutant spores missing *nfo* and *exoA* genes compared to wild type spores or mutant spores missing *nfo*, *exoA*, and *disA* (Fig. 4). DisA is involved in a pathway regulating DNA replication in response to DNA damage, as indicated by this experiment. The specific cause and mechanics that govern this regulation are still unknown.

3. DisA regulates sensitivity to damage using Cyclic di-AMP

During spore revival, DisA has been shown to convert two ATPs into cyclic di-AMP, but such synthesis is suppressed when DisA binds to Holliday junctions. Cyclic di-AMP is a second messenger often used in signal transduction suggesting this may be a way DisA communicates regulatory messages when DNA damage is detected. To explore whether this cyclic di-AMP affects DNA repair capacity, Gandara and Alonso (2015) conducted an experiment with knocked out and separately over expressed *gdpP* (using promoter P-*gdpP*), a gene that codes for an enzyme known to degrade cyclic di-AMP. These mutants were exposed in dormancy to varying concentrations of either methyl methane sulfonate or H2O2 in order to induce DNA damage. The knocked out *gdpP* mutant had similar or lower sensitivity to the induced DNA damage when compared to wild type, while the over expressed cyclic di-AMP had much higher sensitivity to the DNA damage than wild type (Fig. 5). This is exactly what we might expect. If low cyclic di-AMP concentrations work as a regulatory message for the spore to slow growth and modulate DNA repair mechanisms, we can expect that the low concentrations produced after degradation by over expressed GdpP to show the increased sensitivity that resulted from the experiment. Similarly, if high cyclic di-AMP levels signal normality, we can expect that high concentrations due to lack of degradation from GdpP will produce the results obtained. DisA may keep a

constant moderate to high level of cyclic di-AMP until DNA damage is detected, at which point it binds to the Holliday junction of the repairing DNA and stops producing cyclic di-AMP. The lowered levels of cyclic di-AMP signal to the spore to slow growth until the DNA damage has been repaired. The results of this experiment give strong evidence for cyclic di-AMP having this role, but the connection with DisA is not conclusive. Other proteins are known to influence cyclic di-AMP concentrations and DisA is not the only one.

In order to be more conclusive and clearly show a link between DisA and cyclic di-AMP regulation, Gandara and Alonso (2015) used a mutant *disA* gene unable to synthesize cyclic di-AMP and compared its response to DNA damage with and without the addition of external cyclic di-AMP. The exact experiment was done on wild type as a control. Results showed clear increased sensitivity to damage without functional *disA* with the ability to return to normal sensitivity with the addition of cyclic di-AMP (Fig 6). Wild type had the same sensitivity with and without additional cyclic di-AMP. This experiment shows a clear connection between DisA and the second messenger cyclic di-AMP. Combined with the previous experiment we have strong evidence regarding how DisA communicates its regulatory responses.

4. Specific mechanics and pathways still elusive

Recent research papers continue to study the regulatory functions of DisA after induced DNA damage. Raguse et al. (2017) attempted to study the mechanisms of DisA further by attempting to explore the proteins involved in the same DNA repair pathways as the ones thought to be regulated by DisA. RecA is a protein involved with DNA repair and is known to reduce survival of spores if absent. One experiment constructs mutant spores without *disA*, *recA*, or with both absent. These spores were exposed to ionizing radiation or ultra-high vacuum desiccation, both of which produce strand breaks and damage template bases. Double mutant

spores that underwent ionizing radiation had similar sensitivities to their corresponding single mutants, which were more sensitive than wild type (Fig. 7A,C). Double mutant spores that underwent desiccation had much lower tolerance to damage than their corresponding single mutants (Fig. 7B,D). The experiment indicates DisA and RecA are both necessary for proper spore response to DNA damage and likely work together to a repair pathway but the difference in response between two treatments suggests DisA has a role in other pathways depending on the type of damage that is present.

To explore more, Raguse et al. (2017) wanted to see if DisA delayed overall DNA replication when damaged sites were found. In order to study the effect on DNA synthesis by bound DisA in a controlled way, an in vitro assay was constructed with the necessary DNA replication proteins and DNA substrate to mimic transiently paused replication forks. The assay was initiated with the addition of ATP and dNTP in the absence or presence of bound DisA. Bound DisA did not affect initiation or DNA synthesis, and no competition with transcriptional proteins or assistance in the replication was suggested when compared to the assay without DisA. Okazaki fragments were noticeably longer with DisA bound, which may indicate inhibition of the primase DnaG or some effect on template switching that allows longer fragments to be generated (Fig. 8). This experiment showed DisA does not affect in vitro DNA replication for arrested replication forks, at least under the conditions tested. The unique design of this experiment allowed it to be very conclusive but only under its specific set up, so it should not be considered to rule out the ability of DisA to delay DNA replication under other factors.

Conclusion

DisA accumulates before outgrowth of the vegetative cell and tends to slow growth rate if DNA damage is detected. It is suggested that dysfunctional DNA repair mechanisms may increase this growth delay as the spore's capacity to repair its DNA is attenuated, but this hypothesis requires more testing. An interesting direction of study to later investigate would be if the spore attempts to use other pathways or similar proteins to replace dysfunctional repair mechanisms or proteins. Experimentation shows DisA delays DNA replication in response to DNA damage. In an attempt to step one layer deeper and understand how DisA communicates to delay growth in response to DNA damage, two experiments were set up to study a second messenger synthesized by DisA. The second messenger cyclic di-AMP was shown to directly modulate sensitivity to DNA damage depending on its concentration. The link between DisA and cyclic di-AMP was conclusive. It would be interesting to find other proteins that modulate cyclic di-AMP for similar or other regulatory purposes, given that cyclic di-AMP is a common second messenger employed by other proteins. Finally, recent experiments show that DisA's specific mechanics are still elusive and require more investigation, even as its general function is known.

The inability of spores to repair DNA damage during dormancy makes sporulation seem unfavorable. In fact, recent surveys have estimated an age limit after which spores tent to have accumulated too much DNA damage to germinate into vegetative cells. A survey of spores and active bacteria in permafrost samples from Siberia, Canada, and Antarctica estimate a range of 400 to 600 thousand years as a limit to spore lifespan (Johnson 2007). Species of active bacteria in the same samples date as far back as 740 thousand years old.

Spores may have some strategies to decrease the amount of DNA damage that can accumulate during dormancy, but in order to repair already accumulated damage they must rely

on the DNA repair mechanisms that become active before vegetative cell outgrowth, including the checkpoint regulated by DisA. Inability to properly prepare DNA for vegetative life can lead to cancerous bacteria or death. The strategies to deal with damage during germination are especially interesting because some act both in sporulation and germination. It is possible that similar checkpoints can act in specific cell cycle stages within any cell. Better understanding of these checkpoints can help us better understand regulation and DNA damage repair mechanics in other phases of life, bacterial or otherwise. This knowledge could also be used to construct antibiotics that target these damage mitigating proteins so bacteria cannot repair themselves, or can oppositely be used to construct methods for enhancing an organism's natural ability to detect DNA damage and prevent cancer.

References

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Figures

	Avg % band intensity ± SD remaining after enzyme treatment of strain[b]:					
	WT		*nfo exoA*		*nfo exoA disA*	
Enzyme	DS	OG	DS	OG	DS	OG
EndoIV	87 ± 9.8	94 ± 8.8	86 ± 7.6	75 ± 8.6	47 ± 5.1	12 ± 0.7
Fpg	18 ± 2.1	97 ± 9.2	9 ± 1.1	67 ± 7.3	10.5 ± 1.2	8.5 ± 0.94

Figure 1 (Campos et al. 2015) quantifies the percentage of chromosomal DNA remaining after inducing DNA damage in spores while they are still in dormancy (DS) and later during their outgrowth phase (OG). This shows wild type spores were able to retain more of their DNA than the double mutant, and the triple mutant had the worst retention of DNA.

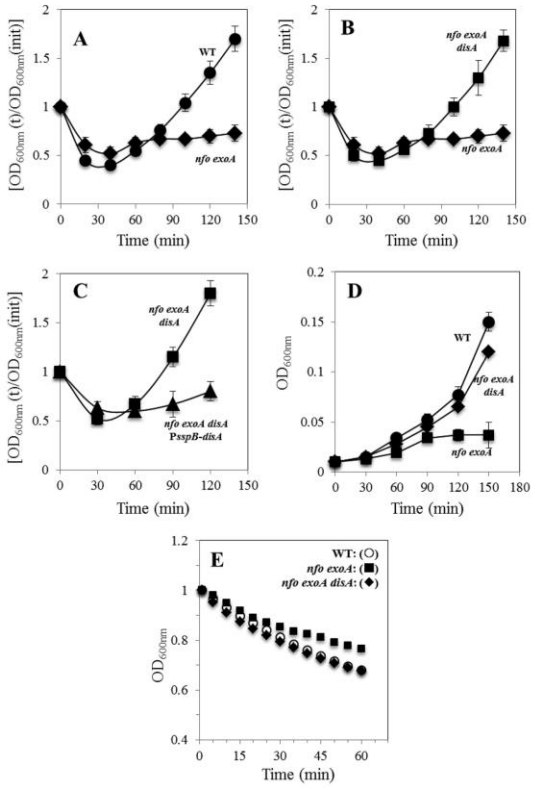

Figure 2 (Campos et al. 2015) measures growth rate by optical density (OD) after DNA damage. (A) shows wild type growth compared to a slower growth for mutant lacking *nfo* and *exoA* genes.

(B) shows the same slowed double mutant along with a faster growing triple mutant lacking *disA* gene, where DisA would normally slow growth rate.

(C) shows the fast growing triple mutant compared to slower growing triple mutant with *disA* reintroduced through a separate P*ssp* promoter, re-establishing DisA function.

(D) shows the results of (A) and (B) specifically for spores in the outgrowing phase

while (E) shows the results of (A) and (B) specifically for spores in the germinating phase, showing growth is slowed during germination and regained later during outgrowth.

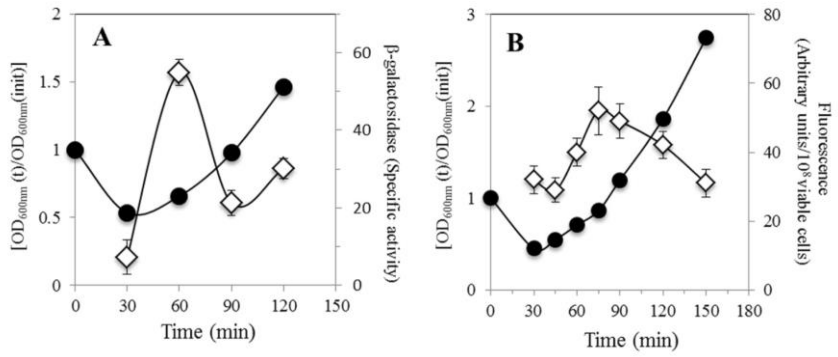

Figure 3 (Campos et al. 2015) quantifies beta-galactosidase activity (A, clear diamond points), which is directly related to the transcription of *disA-lacZ* construct, and the concentrations of DisA-GFP (B, clear diamond points). This shows the peak expression of DisA occurs between 60 and 90 minutes after the onset of germination, during the ripening and outgrowth phase but before the vegetative cell was produced.

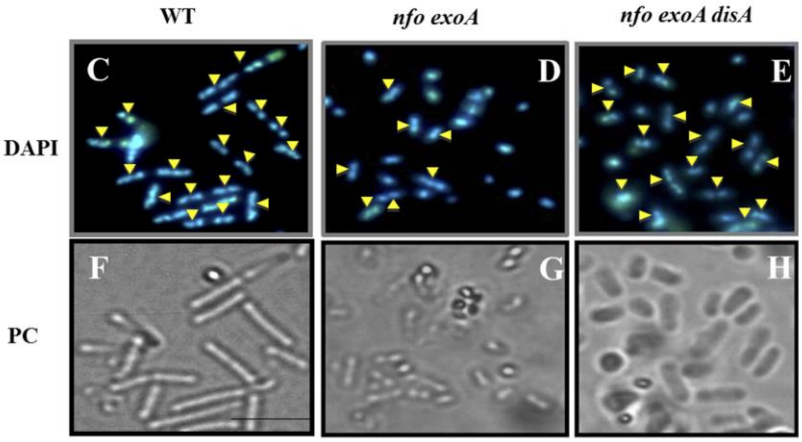

Figure 4 (Campos et al. 2015) shows many less replicated and segregated spores (yellow triangles) of double mutant *nfo exoA* when compared with wild type and triple mutant *nfo exoA disA* using DAPI florescent microscopy and phase-contrast (PC) microscopy.

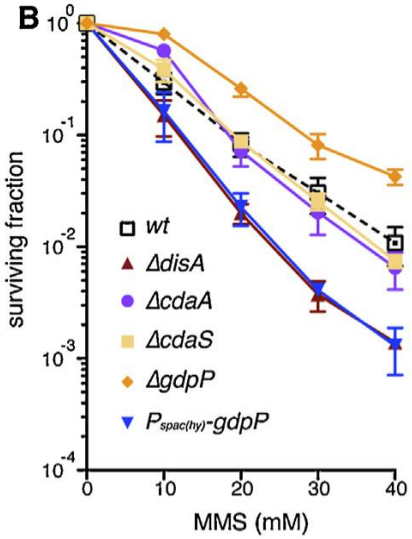

Figure 5 (Gandara and Alonso 2015) shows higher sensitivity and lower survival following induced DNA damage for mutants lacking *disA* or over expressing cyclic di-AMP degradation enzyme GdpP (P-*gdpP*) when compared with wild type or spores with knocked out *gdpP* where cyclic di-AMP concentrations will not be degraded to the same extent.

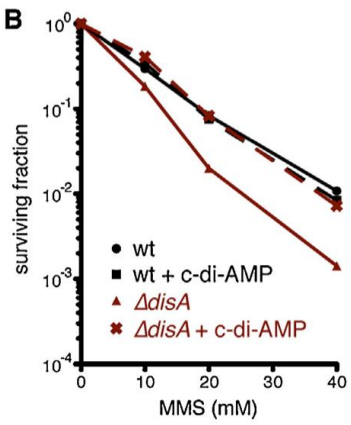

Figure 6 (Gandara and Alonso 2015) shows the change in sensitivity between two groups, the control wild type spores and experimental mutant spores lacking *disA* gene, to induced DNA damage. These two groups were tested each under two different conditions, one condition without changes to naturally occurring cyclic di-AMP and the other with increases to cyclic di-AMP concentrations. Only the mutant lacking *disA* greater sensitivity to DNA damage could be reversed with the addition of more cyclic di-AMP.

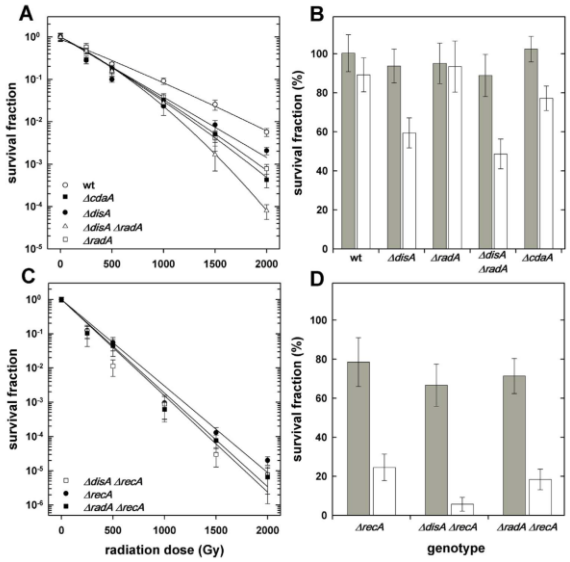

Figure 7 (Raguse et al. 2017) shows the sensitivity and survival after induced DNA damage by ionizing radiation for wild type (A) and mutants lacking *disA*, lacking *recA*, and double mutant lacking both (C). They also show the results as a percentage without (grey) and with induced DNA damage by ultra-high vacuum desiccation (white) for wild type (B) and the mutants (D).

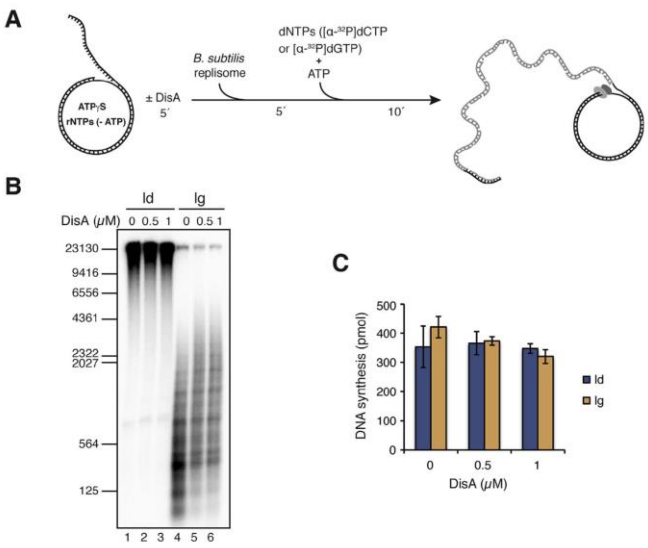

Figure 8 (Raguse et al. 2017) shows the set up for the stalled replication fork assay (A) and the resulting leading (Id) and lagging (Ig) lagging strand lengths (B) with increasing concentrations of DisA. These lengths are quantified (C) and show decreasing lengths for the lagging strand with increasing DisA.