UNIVERSITY OF L'AQUILA

DEPARTMENT OF BIOTECHNOLOGICAL AND APPLIED CLINICAL SCIENCES

Doctoral school in EXPERIMENTAL MEDICINE

Curriculum Biochemical Science

XXXIII cycle

Doctoral Thesis

Is suramin truly a good inhibitor of the bacterial RecA protein? Inhibition and activation, two sides of the same coin

SSD BIO/12

Candidate

Cracchiolo Salvatore

Programme coordinator

Prof.ssa Mariagrazia Perilli

Tutor

Prof. Giuseppe Celenza

A.A. 2019/2020

Table of contents

List of figures

List of tables

List of schemes

List of equations

Abstract

Bacteria live in a hostile habitat and are continuously exposed to a multitude of environmental stress that can compromise the survival of the cells in the absence of an efficient adaptation system. To survive and adapt to these “hard” conditions, bacteria have developed a global response system that modulates the expression of specific genes and, consequently, the cellular metabolism, allowing the microorganisms to respond to the modification of the environment. This inducible system is termed SOS response, which is mainly triggered by lesion to DNA. The SOS response entails the induction of multiple proteins that promote the integrity of DNA and cell survival. SOS response includes more than 50 genes that carry out diverse functions in response to DNA damage. These genes encode for proteins involved in DNA reparation, including excision repair, homologous recombination, translesion DNA replication and cell division arrest. In addition, the response is regulated depending on the severity of the DNA damage and the time elapsed since the damage was detected. In the early phase of SOS response, the error-free repair and maintenance process repairs minor DNA lesions without introducing mutations on the genome. If the DNA damage cannot be repaired, the SOS response actives all those processes that permit to repair DNA at the expense of genome integrity. In those emergencies, the error-prone DNA process operates, causing elevated mutation levels but allowing for continuous replication and cell survival

The role of SOS response is not limited to DNA damage reparation; it also promotes processes that allow the bacteria to resist other stressed abuses. In this manner, microorganism develops tolerance towards the stimuli that triggered the response; this is the mechanism by which bacteria acquire resistance to antibiotics. Many antibiotics trigger the SOS response, either directly through DNA damage or indirectly, e.g., damaging protein or cell wall and membrane or producing toxic intermediates. The resistance and low susceptibility to antimicrobics is a consequence of an overexpression of SOS inducing proteins. The SOS response also contributes to spreading the resistance by promoting horizontal dissemination of antibiotic resistance genes. Stress-induced by antibiotics can promote acquisition and expression of resistance genes by mobile DNA elements, promoting resistance development

Only two proteins regulate the SOS expression: a transcriptional repressor LexA and a recombinase enzyme RecA. In a physiological state, LexA inhibits specific gene expression by binding specific SOS-boxes sequences, which are included in the SOS gene promoter region. When cells undergo stress pressure, particularly when lesion on DNA occurred, single-strand DNA (ssDNA) is formed. RecA binds to ssDNA to form a nucleoprotein filament. This complex is a flexible structure and represents the active form of RecA; it can perform three

separate functions: homologous recombination, SOS induction and SOS mutagenesis. RecA induces the SOS response by promoting the autocatalytic cleavage of LexA and release from the SOS box, thus inducing the expression of the SOS genes.

The SOS response is a formidable strategy operated by bacteria against a myriad of environmental stress and has an active role in promoting survival and resistance to antibiotics. Targeting the SOS pathway represents an attractive strategy to reduce the tolerance toward the antibiotics and block the spread of resistance; this can be achieved by the inhibition of the two central proteins of the SOS pathway. RecA nucleofilaments are central for SOS response induction; focus on the inhibition of this protein seems to be one of the best strategies to slow down the development of resistance and make bacteria susceptible to antibiotics.

Our laboratory identified several potential inhibitors for RecA and developed a high throughput assay for screening potential inhibitors of the SOS response. Nautiyal et al. identify the suramin as a potent inhibitor for all biochemical activities for RecA. This work has investigated the mechanism of interaction between RecA and suramin, elucidating the binding site and the inhibition mechanism. Surprisingly the suramin has a dual effect on RecA activity: at high concentration inhibits the ATPase activity of RecA, as previously demonstrated, whereas, at low concentration, it leads to an increase in ATPase activity. Moreover, in the presence of suramin, RecA ATPase activity becomes independent from ssDNA.

The activity of suramin has been further investigated in the RecA-LexA interaction. As previously observed, the activity of suramin depends on its concentration; in fact, suramin performs as an activator at low concentrations (<1 μM) even in the absence of ssDNA, while acts as an inhibitor at higher concentrations.

To better understand how suramin modulates the RecA activity, molecular docking and molecular dynamic simulations were performed.

This study can provide further information regarding a new potential binding site for modulating RecA activity.

Chapter 1

The SOS response

The English naturalist Charles Darwin developed the idea of *natural selection* after five years of studying plants and animals (Darwin 1859). However, Darwin did not know that bacteria and microorganism follow the same theories deduced in his postulates. Indeed, bacteria have a powerful predisposition to adapt and survive against several environmental aggressions.

Bacteria are ubiquitous, and they can colonise every biotic and abiotic niche in our ecosystem. Due to their universal presence, bacteria are continuously exposed to various stress. Extreme conditions of temperature, pH, salinity, and natural selection can disrupt the crucial interaction that keeps biomolecules functional and destroys cellular integrity. For every extreme environmental condition investigated, microorganisms not only can tolerate these conditions, but they need those conditions to live in an optimal state (Rampelotto 2010). Bacteria must overcome various stresses that suppress their ability to grow and replicate. For achieving this aim, microbes develop numerous strategies, including changes in the cellular membrane, expression of repair enzyme, the organisation in biofilm (Haruta and Kanno 2015). In addition to environmental and physical aggression, bacteria must compete with other microorganisms and major organisms. Even bacteria can produce toxic chemical compound or rather "antibiotics". This competitive pressure drives bacteria to develop antibiotic resistance mechanisms. Initially, it was thought that antibiotic resistance is exclusively associated with the use and overuse or misuse of antibiotics in humans and animals. This is true for those resistance mechanisms developed by mutation and a strong positive selection of mutants. However, most antibiotic resistances are acquired through horizontal gene transfer from other ecologically and taxonomically distant bacteria (Aminov 2009; Aminov and Mackie 2007; Finley et al. 2013).

These organisms evolved with well-equipped molecular tools to resist numerous aggressions. The most important feature possessed by bacteria is the spread of this resistance. Abuse of large quantities of antibiotics to therapeutic and other use in human and animals has created an abnormal pressure condition, result in mobilisation of resistance element in bacterial populations (Brown and Wright 2016).

1.1 The hypothesis of SOS response

The concept of SOS response was formulated more than 50 years ago by direct observation of distinct phenomena. First of all, Jean Weigle had observed that the reactivation of UV-irradiated phage λ increase when the phage was plated on previously irradiated *E. coli* host cells. Moreover, lysis induction by phage λ and filamentous growth of *E. coli* cells was observed in response to UV irradiation. These data suggested a relationship between these phenomena. Indeed, Miroslav Radman concludes that, in *E. coli*, there is a DNA repair system induced by DNA damages (Janion 2008). Radman's hypothesis is: "*E. coli* possesses a DNA repair system which is repressed under normal physiological condition, but which can be induced by a variety of DNA lesions".

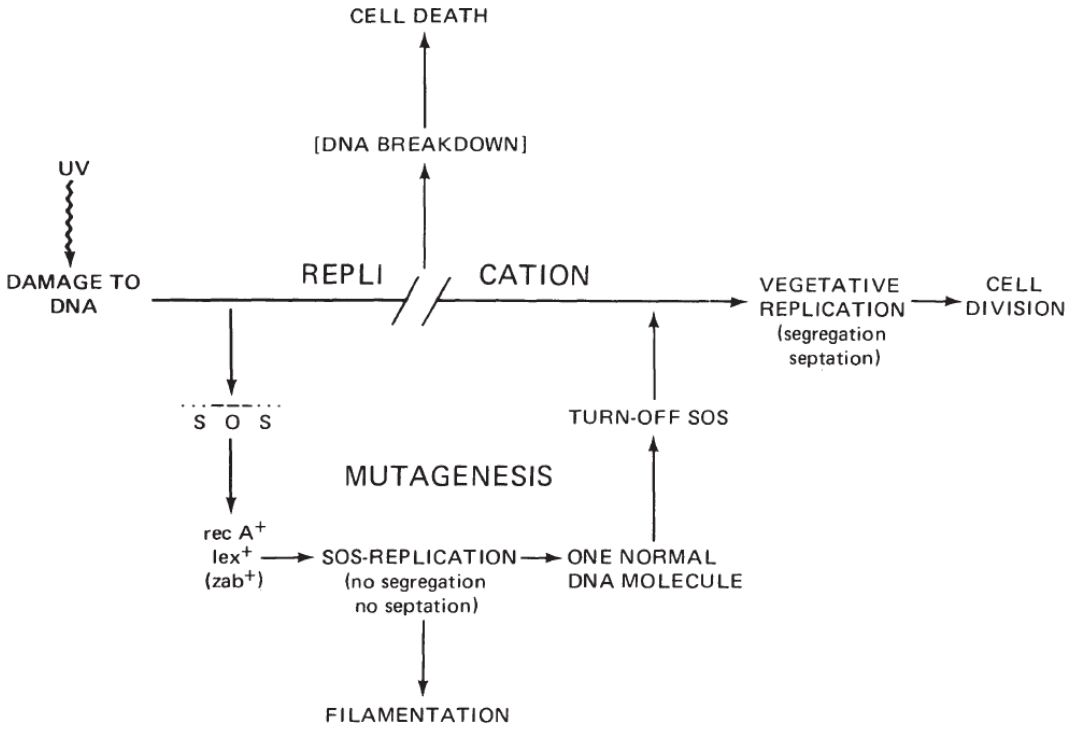


Figure 1. Radman's SOS repair pathway hypothesis.
(Radman 1975)

In his work describes the SOS repair system as a response induced by a blockage of DNA replication or DNA lesions in the cell. He demonstrated that this response leads to repair the damage on DNA allowing bacteria to survive. Furthermore, he was also able to identify the two principal actors of this pathway, or RecA and LexA proteins (Radman 1975).

1.2 Trigger of SOS response

The main stimulus that triggers the SOS response is bacterial DNA damage, which can occur both exogenously and endogenously. Several endogenous pathways can cause lesions on DNA, leading to DNA replication arrest (Fig. 2a). When replication is impaired, double-strand damage (DSBs) on DNA can occur. Replication arrest or stalling leads to the formation of single-strand DNA (ssDNA) on the lagging strand template, representing the main component of SOS induction. Replication arrests also occur upon transcription and replication collisions (Fig. 2b), as occurs when bulky factors block the DNA polymerase complex. DSBs are also formed in nonreplicating bacteria during transcription due to the presence of R-loops (Fig. 2c). Those complexes are RNA-DNA hybrids formed when RNA polymerase is stalled on DNA, and the RNA in synthesis anneals to the homologous transcribed ssDNA template. R-loops can be formed in growing cells and cause replication-transcription conflicts that lead to genomic instability. (Baharoglu and Mazel 2014).

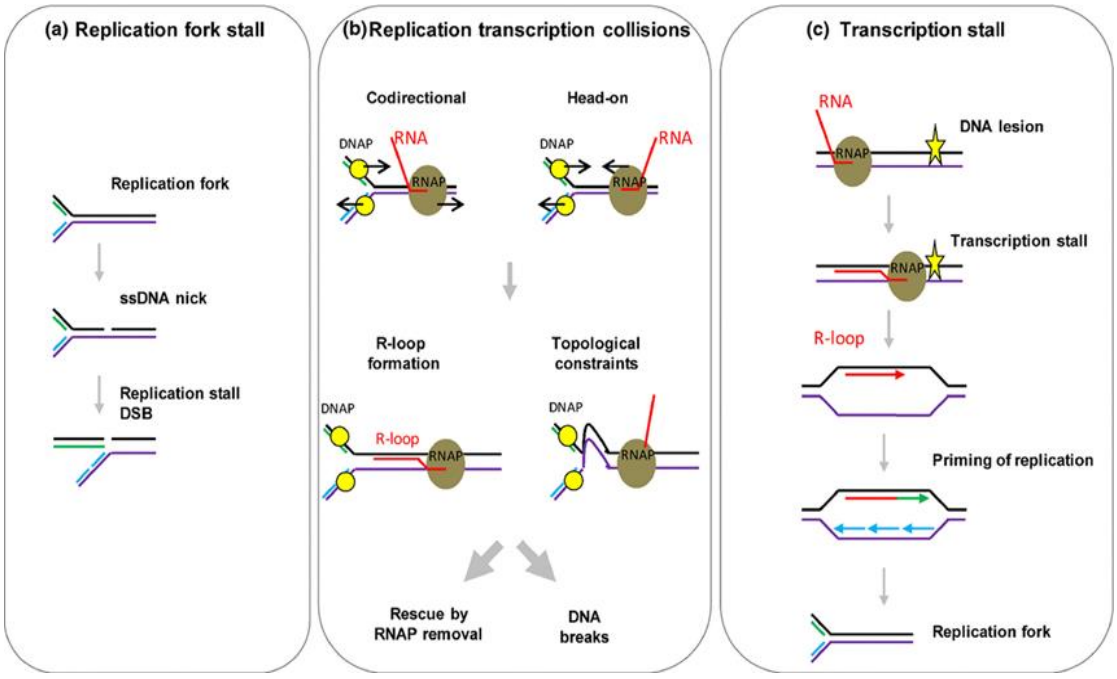


Figure 2. Origins of ssDNA.
(Baharoglu and Mazel 2014)

ssDNA can be transiently generated during the horizontal gene transfer process, including conjugation and transformation. Other endogenous stimuli trigging the SOS response are defects in recombination or chromosome segregation and some cellular by-products such as reactive oxygen species (ROS) (Memar et al. 2020), which are the most common endogenous insults to DNA. Those molecules can arise as a by-product of the metabolic process, as during aerobic cellular respiration, superoxide (O_2^-), hydrogen peroxide

(H_2O_2) and hydroxyl radicals (·OH) are formed. These insults to DNA commonly lead to the generation of 7,8-dihydro-8-oxoguanine (8-oxoG), which is repaired by an enzymatic complex called GO system. However, this repair mechanism is not perfect, and this kind of lesion can increase mutation rates. Moreover, many of these genomic lesions lead to stall the replication and inducing the SOS response. These pathways often involve the use of error-prone DNA polymerases, known as translesion-DNA (TLS), which have high error rates and thereby increase mutations rates (Merrikh and Kohli 2020).

Diverse external agents can originate ssDNA, for instance, UV irradiation. The reparation of this lesion was made by homologous recombination (HR) or TLS that required RecA recruitment, therefore SOS induction. Other physical factors can induce SOS by DSB formation, such as high pressure; in this case, SOS response is trigger through the Mrr protein. The resistance at this factor can arise spontaneously through mutations inactivating the *mrr* gene. High concentrations of free ferrous ion lead to ROS production through the Fenton reaction, damaging the DNA. Moreover, external ROS can indirectly cause DNA damage by the attack on lipids and proteins (Baharoglu and Mazel 2014)

1.3 SOS pathway

The SOS response was initially described as an inducible DNA repair system that allows bacteria to survive abrupt increases in DNA damage. To date, it is known that SOS response is a complex gene network that acts on several bacteria activities, including DNA repair, promotes mutagenesis, horizontal gene transfer, pathogenesis mechanism, and biofilm formation. SOS response plays a central role in the maintenance of the genetic variability to stressful conditions. Furthermore, the SOS system is responsible for contrasting the selective pressure of antibiotics and develops resistance against these drugs (Memar et al. 2020).

The induction of SOS response leads to increase expression of over 50 genes that carry out diverse functions mentioned above. All of the SOS genes account for almost 0.76% to 1.58% of genes of the entire genome of the *E. coli* chromosome (that count 4300 genes) (Janion 2001). The expression of genes involved in the response is fine regulated either in time of expression and quantity. This regulation depends on the entity of damage and by the time elapsed since the damage was detected. To minor endogenous DNA damage, only a few genes are expressed, while upon severe DNA damage and persistent inducing signal, other genes involved in more desperate actions will be expressed. The time, level, and duration of SOS response genes depend on the strong affinity for a specific consensus sequence named SOS-box. This sequence is a perfect palindrome: $TACTG(TA)_5CAGTA$; the access to the promoter is regulated by a transcriptional repressor named LexA. The affinity of LexA to different SOS boxes regulates the expression of genes involved in SOS response. The deviation of SOS boxes

from the consensus sequence defines the heterology index (HI) (Maslowska, Makiela-Dzbenska, and Fijalkowska 2019). The value of HI is a measure of the relative strength which LexA repressor binds to SOS box: at low HI, the gene is tightly repressed, and vice versa (Janion 2008).

Error-free repair systems are involved in the early phase of SOS response (<1 min after SOS induction). In this phase, all processes aim to maintain the integrity of the bacterial genome. The first genes induced are the *urv* genes (*uvrA*, *uvrB* and *uvrD*) and *cho*, resulting in DNA reparation for excision of damaged nucleotides (NER). Even *lexA* is also expressed in this phase of SOS response: if DNA damage is quickly repaired, LexA repressor can suppress the SOS induction by preventing the bind of polymerase in SOS boxes. Other genes involved in DNA damage repair expressed in this phase are *ruvA* and *ruvB* involved in homologous recombination DNA repair. Error-free DNA replication systems are expressed in this phase, enabling DNA synthesis resumption when replication is stalled.

DNA synthesis, in this phase, is performed by Polymerase II and Polymerase IV, encoded by *polB* and *dinB*, respectively; these polymerases replicate the DNA with a high-fidelity and low error rate. Furthermore, for maintaining the integrity of the bacterial genome, *dinI* is express at this phase. This gene encodes DinI, which retards Polymerase V formation by inhibiting the processing of UmuD to UmuD'. The Middle phase of SOS response starts about five minutes after SOS induction. In this phase, the *recA* gene is expressed. RecA protein participates in the recombination of homologous DNA repair and acts as an enhancer of the SOS response. The genes expressed in this phase can repair large amounts of DNA damage. If the DNA is still unrepaired, an error-prone system acts, allowing for continuous replication and cell survival at the cost of elevated mutation levels. The most error-prone DNA polymerase is Polymerase V (PolV), encoded by *umuC* and *umuD* genes. This polymerase copies with a base substitution error rate of about 10^{3}-10^{4}, while Pol IV have an accuracy 10-15-fold greater. Pol V, also named Translesion Synthesis polymerases (TLS), can accommodate mismatched base pairs due to more spacious on active site. Hence, it can bypass lesions by filling the gap with any nucleotide allowing DNA replication. Furthermore, in the late stages of SOS response, *sul*A is expressed, arresting cell division by binding to FtsZ, a key cytoskeletal protein responsible for cell scission (Janion 2008) (Maslowska, Makiela-Dzbenska, and Fijalkowska 2019) (Wigle et al. 2009).

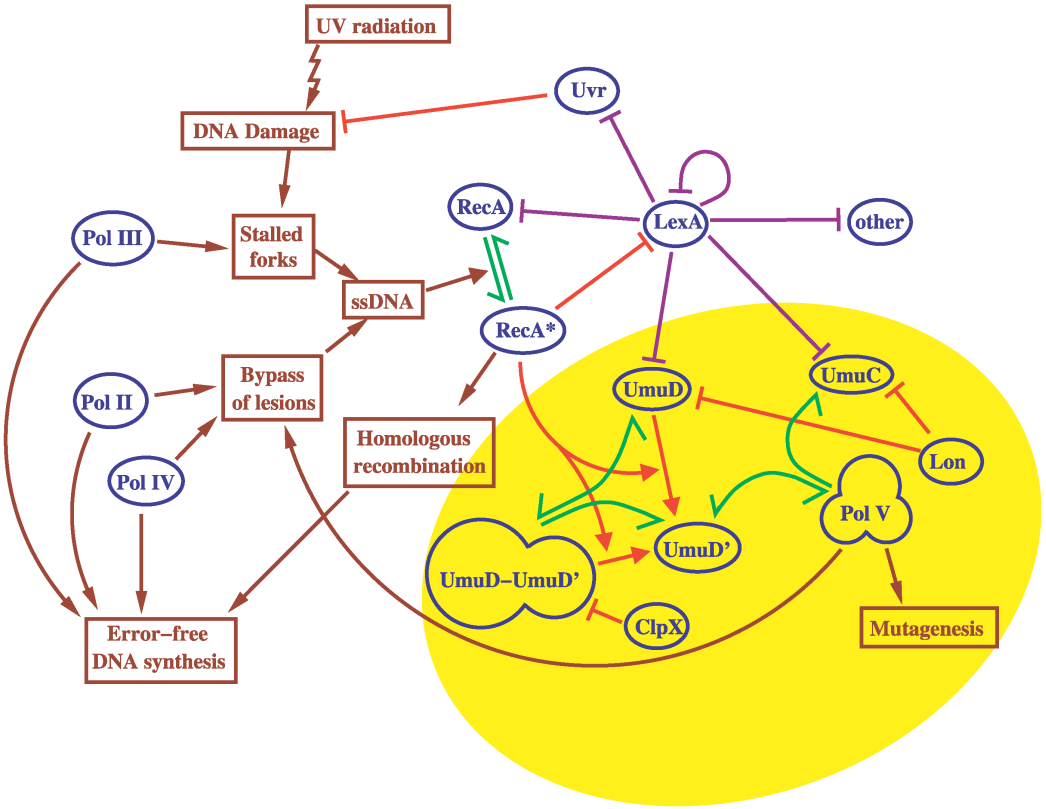


Figure 3. Schematic representation of the SOS Network in E. coli (Krishna, Maslov, and Sneppen 2007)

	Gene	Function/gene product	Copy number		HI
			non-induced	induced	
Early phase	*lexA*	Repressor of SOS genes	1300	7540	6.34/7.02
	uvrA	NER system	20	250	6.98
	uvrB	NER system	250	1000	6.11
	uvrD	NER system	5000/8000	25000/65000	
	polB	Polymerase II	40	300	12.09
	dinB	Polymerase IV			
	ruvA	RuvAB-helicase	700	5600	9.19
	ruvB	Recombinational repair system	200	1600	9.19
	dinI	Inhibitor of UmuD processing	500	2300	6.24
Middle phase	*recA*	SOS inductor/recombination repair	1000/10000	100000	4.31
	recN	Recombinational repair system			5.16/11.47
Late phase	*sulA*	Cell division inhibitor		+125-fold	4.65
	umuD	Part of Pol V	180	2400	2.77
	UmuC	Part of Pol V	0	200	2.77

Table 1 Gene expressed at different phase of SOS response (Janion 2001)

Gene	Function	Gene	Function
Cho	Endonuclease of nucleotide excision repair	*ftsK*	Cell division protein
dinB	Pol IV	*hokE*	Toxic polypeptide
dinD	DNA-damage inducible protein	*molR*	Molybdate metabolism regulator
dinF	DinF MATE transporter	*rmuC*	Predicted recombination limiting protein
dinG	ATP dependent helicase	*sbmC*	DNA gyrase inhibitor
dinI	DNA damage inducible protein	*sulA*	Cell division inhibitor
dinQ	Toxic inner membrane peptide	*umuDC*	Pol V
dinS	DNA damage inducible protein	*uvrA-B-D*	NER system
lexA	Regulator of SOS	*ybfE*	DNA damage inducible protein
polB	Pol II	*ydjM*	Inner membrane protein
recA	SOS inductor	*yebG*	DNA damage inducible protein
renN	Recombinational repair system	*symE*	Toxic-like protein
recX	RecA inhibitor	*tisAB*	Toxic peptide
ruvAB	Recombinational repair system	*ssb*	ssDNA binding protein

Table 2. *Escherichia coli* LexA-regulated gene
(Maslowska, Makiela-Dzbenska, and Fijalkowska 2019)

1.4 SOS response and antimicrobials

Antimicrobials or antibiotics are low molecular weight molecules produced by a multitude of organisms included bacteria. These molecules are metabolites produced afterward specific cellular signalling or specific conditions such as nutrient limitation.

Antibiotics can be grouped based on their chemical structures or their mechanism of action. According to their biological effects, these molecules can be divided into two major classes: bactericidal compounds that kill bacteria (e.g., β-lactam, fluoroquinolones, and aminoglycosides) and bacteriostatic compounds that inhibit bacterial growth (e.g., macrolides, tetracyclines). (Laureti, Matic, and Gutierrez 2013).

Antibiotic-mediated cell death is a multifaceted process that begins with the physical interaction between a drug molecule and its specific target on the bacterium, leading to biochemical, molecular and ultrastructural alterations (Kohanski, Dwyer, and Collins 2010). Based on these actions, antimicrobial agents can be divided into several groups: agents that inhibit cell wall synthesis, depolarise cell membrane, inhibit protein synthesis, inhibit nucleic acid synthesis, and inhibit metabolic pathways (Laureti, Matic, and Gutierrez 2013).

1.4.1 Inhibition of DNA replication

During DNA synthesis, replication, and cell division, chromosomal supercoiling requires the modulation from a specific class of enzyme named topoisomerase that is essential for cell viability. This enzyme catalyses strand breakage and rejoining reactions that lead to relaxing the supercoiling of DNA. These reactions represent the target for a synthetic class of antimicrobials agents named quinolones. Those molecules are derivatives of nalidixic acid, introduced in 1960. The more widely used antimicrobials of this class are fluoroquinolones, levofloxacin, and ciprofloxacin (Kohanski, Dwyer, and Collins 2010).

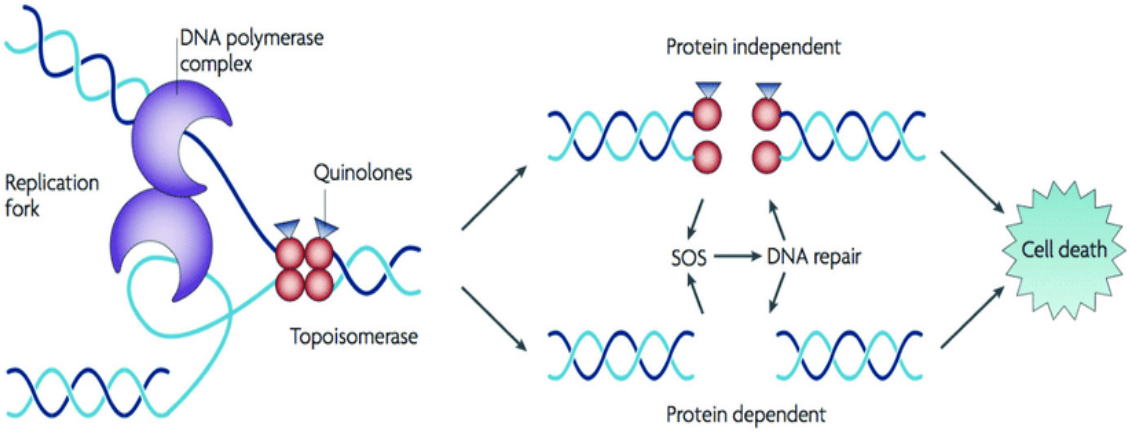


Figure 4. Quinolones mechanism of actions
(Kohanski, Dwyer, and Collins 2010)

The quinolone class mechanism of action is based on a stable interaction complex with topoisomerases and cleaved DNA. This complex generates double-stranded DNA breaks, these are trapped by covalently (but reversibly) linked topoisomerases. The net damaging effect of quinolones is the arrest of DNA replication machinery and inhibit the DNA synthesis due to blocked replication fork on this complex. The inhibition of topoisomerase function by quinolone antibiotics negatively affects the cell, leading to block the bacterial replication and eventually cell death (Kohanski, Dwyer, and Collins 2010).

1.4.2 Inhibition of RNA synthesis

The effect of inhibition of RNA synthesis is similar to DNA synthesis inhibition and leads to a catastrophic effect on prokaryotic nucleic acid metabolism and inducing bacterial cell death. Rifamycin is a class of semi-synthetic bactericidal antibiotics that inhibit DNA transcription by stably binding, with high affinity, to the β-subunit of a DNA-bound and active RNA polymerase.

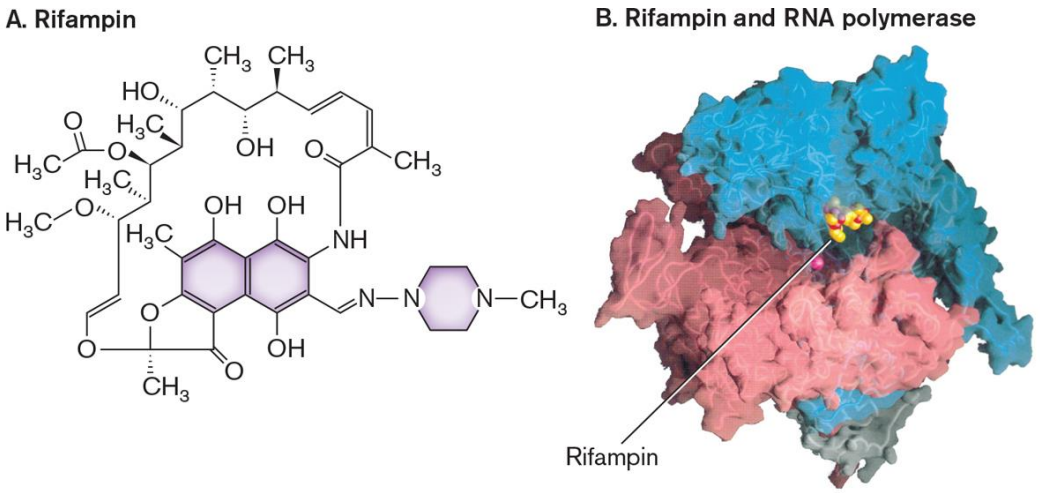


Figure 5. A. Rifamycin molecule; B. RNA polymerase rifamycin complex.

Rifamycin is bactericidal against Gram-positive bacteria and bacteriostatic against Gram-negative bacteria, a difference that has been attributed to the specific drug uptake (Kohanski, Dwyer, and Collins 2010).

1.4.3 Inhibition of cell wall synthesis.

Bacteria can be distinguished into two large groups according to their molecular composition. Gram-positive groups can retain the crystal violet dye during the Gram staining, and Gram-negative that release the dye. Both types of cells are encased by a peptidoglycan (PG) layer (also called murein), thicker and multi-layered in the Gram-positive bacteria and thin on Gram-negative bacteria. However, Gram-negatives are provided with an outer membrane containing lipopolysaccharides, which creates an additional permeability barrier. The cell wall of bacteria provides shape and rigidity as a result of a matrix composed of an alternation of N-Acetylmuramic acid (MurNac) and N-Acetylglucosamine (GlcNac) residues that allow bacteria to survive even in the presence of osmotic pressure (Scheffers and Pinho 2005). Targeting the cell wall represents an efficient strategy because it is specific to prokaryotes. Two types of antimicrobials interfere with several steps of homeostatic cell wall biosynthesis, β-lactams and glycopeptides; both can bring on loss of bacteria structural integrity, change to cell shape and, sometimes, culminating in cell lysis (Mohr 2016). Maintenance of the peptidoglycan layer is under the control of transglycosylases proteins, which add disaccharide pentapeptides to extend the glycan strand of existing peptidoglycan molecules; and penicillin-binding proteins (PBPs) that cross-link adjacent peptide strands of immature peptidoglycan unit.

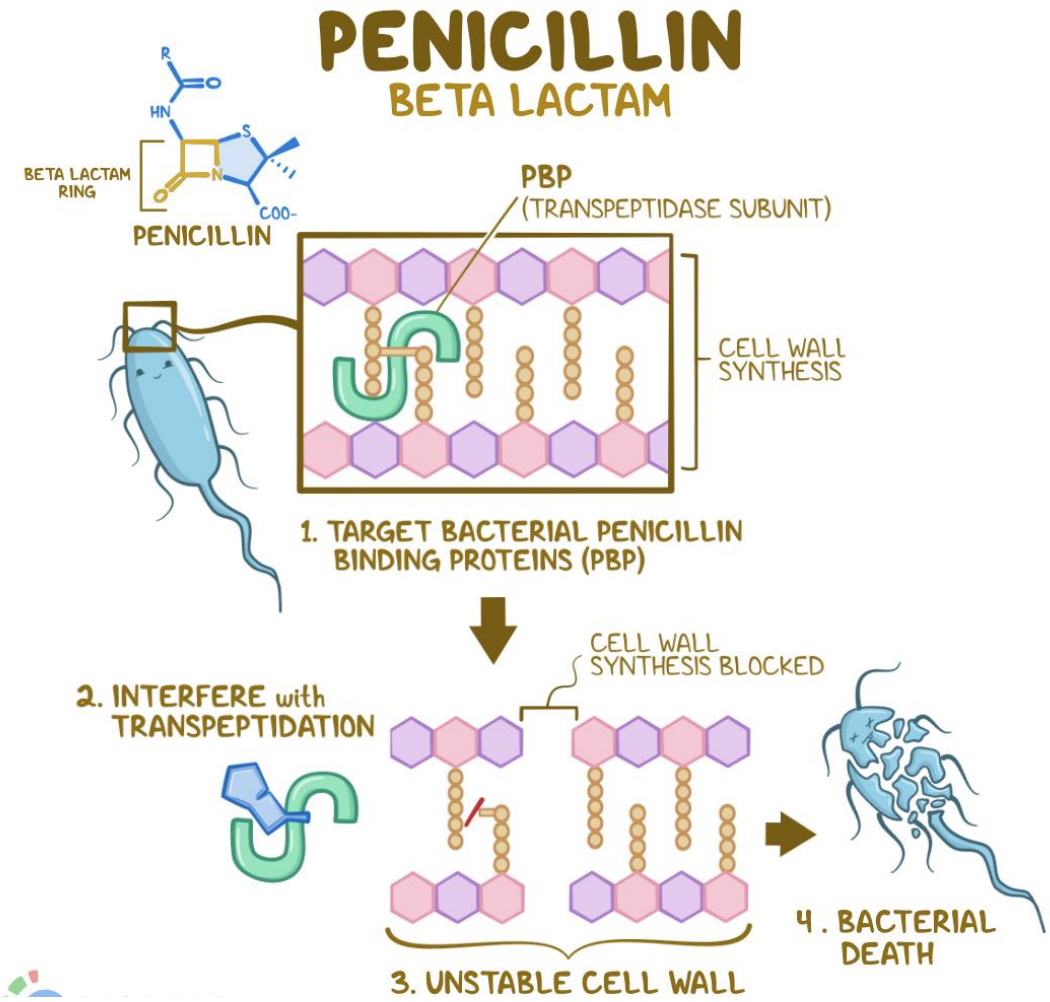


Figure 6. β-lactam antibiotics mechanism of action.

The β-lactam antibiotics containing a cyclic amide ring that mimics the terminal D-alanyl-D-alanine dipeptide of peptidoglycan. This similitude allows β-lactams to act as a substrate for the PBP during cross-link formation, preventing peptidoglycan units' cross-linking formation.

Glycopeptide antibiotics act as steric inhibitors of peptidoglycan synthesis by binding peptidoglycan units, causing a reduction of the mechanical strength of the cell.

Other types of antibiotics that inhibit cell wall formation are derivatives of glycopeptides. These compounds prevent the synthesis and transport of individual peptidoglycan. Moreover, they can insert themselves into the cell membrane, altering the structural integrity of the bacterial cell wall (Kohanski, Dwyer, and Collins 2010).

1.4.4 Inhibition of protein synthesis

Bacterial protein synthesis occurs in three sequential phases (initiation, elongation, and termination) that involve a multiprotein complex named ribosome. The ribosome comprises two ribonucleoprotein subunits, the 50S and 30sS, representing the target for inhibitors protein synthesis antibiotics. Macrolides, lincosamides, streptogramins, amphenicols, and oxazolidones inhibit the 50S subunit by physically blocking either initiation of protein translation or translocation of peptidyl tRNAs.

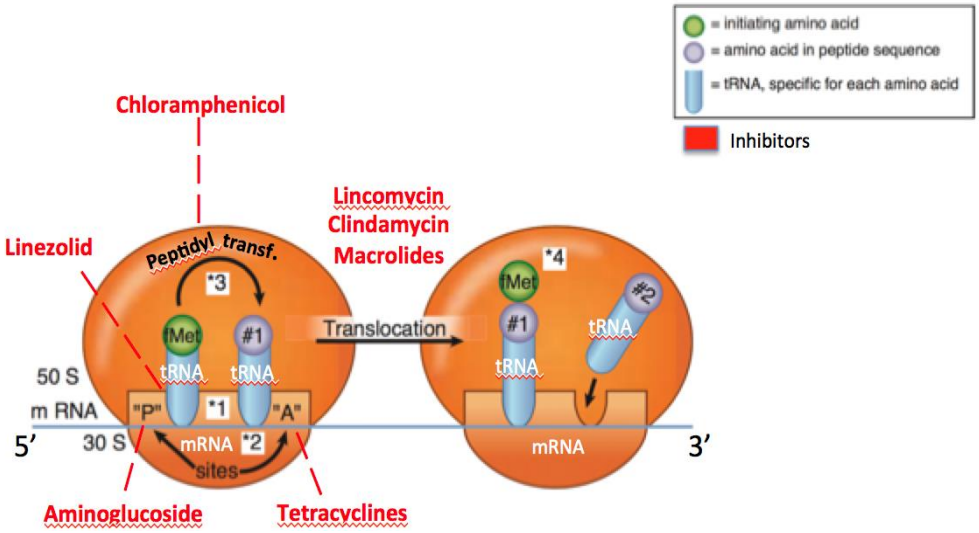


Figure 7. Protein synthesis inhibitors mechanism of actions.

These compounds block the access of peptidyl tRNA to the 50S subunit of the ribosome by steric inhibition and eventually trigger the dissociation of peptidyl tRNA.

30S ribosome inhibitors include tetracyclines and aminocyclitols. Tetracyclines block the access of aminoacyl tRNAs to the ribosome, while the aminocyclitols, which comprises spectinomycin and aminoglycosides, bind the 16S rRNA component of the 30S ribosome subunit. Spectinomycin interferes with the stability of peptidyl tRNA binding to the ribosome but does not cause protein mistranslation, whereas aminoglycosides can induce an alteration in the complex conformation, promoting tRNA mismatch and protein mistranslation (Kohanski, Dwyer, and Collins 2010).

1.5 Mechanisms of antibiotics resistance

In 1945 Sir Alexander Fleming made a prophecy that proved to be stunningly accurate, he warned that misuse of penicillin could lead to developing resistant bacteria strain that causes

more serious infection in the host. Everyday common and uncommon bacteria, previously susceptible to common antimicrobials, are reported to be resistant to different antibiotics. The resistance phenomenon was observed since 1940, after the introduction of the first class of antibiotics, sulphonamides, and penicillin. Common bacteria, such as *Staphylococcus aureus*, develop resistance to these classes very quickly. Nowadays, 17 different classes of antibiotics have been produced; unfortunately, for each class of these, bacteria became resistant; furthermore, in some cases, these bacteria can develop simultaneous resistance to two or more antibiotic classes (Alanis 2005).

The origin of resistance in bacteria can be distinguished in natural resistance and acquired resistance. Natural resistance might be intrinsic (always expressed in the species) or induced (the genes are naturally occurred in the bacteria but are expressed only in the presence of an inductor, such as antibiotics). Intrinsic resistance is independent of previous antibiotics exposure, a trait that is shared within a bacterial species. For instance, bacteria that lack a cell wall, such as *Mycoplasma* are intrinsically resistant to all of the classes of antibiotics that target the cell wall, including β-lactams and glycopeptides. In the acquired resistance, bacteria must acquire genetic material that confers the resistance. This acquisition can occur by transformation, transposition, and conjugation (all termed as horizontal gene transfer HGT). In addition, bacteria may induce mutations to their own chromosomal DNA. The acquisition can be temporary or permanent; the temporary acquired resistance occurs when a plasmid contains a gene that confers resistance allowing the cell to survive. Acquired resistance becomes permanent when a mobile genetic element is inserted in the chromosomal DNA. Those mobile elements may contain the genes that confer resistance to one or more antibiotics. In addition, those genes will be inherited in successive bacterial generations (vertical gene transfer). The permanent acquired resistance can also occur independently from external DNA elements: the resistance can emerge from spontaneous mutations in chromosomal DNA. These mutations are permanent and are spread in successive bacterial generations (C Reygaert 2018). Acquisition of external DNA elements can also occur via a vector, such as viruses capable of infecting bacteria (bacteriophages). The virus, containing the bacterial gene for antibiotic resistance, introduces the genetic material into the receiving bacteria (Alanis 2005).

Antimicrobials resistance mechanism can be grouped in four categories: limiting the uptake of the drug, modifying the target, inactivation of the drug, and active drug efflux. Gram-negative bacteria and Gram-positive present different structure that reflect distinct types of resistance mechanisms used. For instance, Gram-negative bacteria use all four mechanisms, while Gram-positive bacteria commonly use limiting the uptake of the drug due to lack of lipopolysaccharides (LPS) outer membrane and consequently cannot have drug efflux mechanisms (C Reygaert 2018).

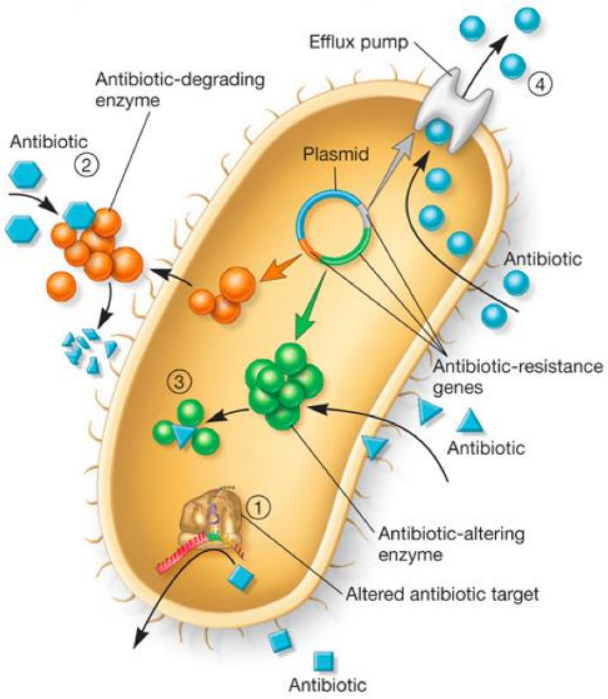


Figure 8. General antimicrobial resistance mechanisms.
(C Reygaert 2018)

1.5.1 Limiting drug uptake

The limitation of drug uptake is the common natural intrinsic form of resistance in bacteria. The structure and functions of LPS in Gram-negative bacteria give an innate resistance to a multitude of antimicrobial agents and provides a barrier to different types of molecules. Mycobacteria present a high lipid content membrane that makes them impermeable to hydrophilic drugs, and, as mentioned above, their lack of cell wall makes them resistant to all drugs that target the LPS metabolic pathway. Both Gram-negative bacteria and Gram-positive bacteria present porin channels that allow access to hydrophilic molecules, which can be exploited for drugs uptake. Bacteria can act in two possible ways to limit drug uptake from hydrophilic porin channels: decrease the number of porins present and mutate the porin channel to change the selectivity. Another way is the formation of a bacterial biofilm community. The biofilm matrix contains polysaccharides and protein that form a thick and sticky matrix that limits the entrance of the antimicrobial agents into bacteria. Furthermore, bacteria in biofilm tends to reduce their metabolism and cell division, so antibiotics that target growing and dividing cell have a reduced effect against bacteria included in biofilm (C Reygaert 2018).

1.5.2 Modification of drug targets

Antimicrobial agents present different targets in the bacterial cell, and all these targets can undergo modification to resist those drugs. B-lactam drugs, for example, target the PBPs in the cell wall. Bacteria can resist by increasing the PBPs to maintain the normal function or decrease the PBPs to reduce targets. Alteration in the number of PBPs impacts the amount of drug that can bind to the target. Furthermore, in *Staphylococcus aureus*, PBPs can change their structure (by the acquisition of the *mecA* gene) and alter the ability of the drug to bind. Several enterococci and *Staphylococcus aureus* (vancomycin-resistant enterococci VRE, and MRSA for staphylococcus, respectively) can become vancomycin-resistant through the acquisition of *van* gene, which results in changes in the structure of peptidoglycan precursors, causing a decrease in the binding ability of vancomycin.

Ribosomal subunits can be altered to resist the action of drugs; these modifications may occur via ribosomal mutation (aminoglycosides, oxazolidinones), ribosomal subunit methylation (macrolides, streptogramins) and ribosomal protection (tetracycline).

Mutations in the enzymes involved in the nucleic acid synthesis, such as DNA gyrase or topoisomerase, change their structure and decrease or eliminate the ability of the drugs (fluoroquinolones) to bind to these components (C Reygaert 2018).

1.5.3 Drug inactivation

Bacteria inactivate drugs in two possible ways: by degrading the drug or by transferring a chemical group to the drug. A wide group of enzymes that degrade antibiotics is the β-lactamases. These hydrolysing enzymes inactivate β-lactam drugs by hydrolysing the β-lactam ring structure, which causes alteration of the drug structure and the ability to bind their target. Gram-negative bacteria use β-lactamase enzymes against lactam drugs such as penicillin and cephalosporin. The genes that transcribe for these enzymes are included on chromosomal DNA of bacteria or may be acquired from plasmids.

The inactivation of drugs can also occur by the transfer of acetyl, phosphoryl, and adenyl groups. This transfer occurs via several transferases; for instance, acetylation is used to inactivate aminoglycosides, chloramphenicol, streptogramins, fluoroquinolones, phosphorylation, and adenylation are used for inactivating aminoglycosides (C Reygaert 2018).

1.5.4 Drug efflux

Bacteria present several efflux pumps, some are constitutively expressed, and others are induced or overexpressed afterwards environmental stimuli or when a substrate is present.

The main function of these pumps is excreting toxic compounds from the cell, although many of these pumps can transport a large variety of compounds (multi-drug MDR efflux pumps). Bacterial efflux pumps can be classified into five main families based on structure and energy resources: ATP-binding cassette (ABC) family, multi-drug and toxic compound extrusion (MATE) family, small multi-drug resistance (SMR) family, and resistance-nodulation-cell division (RND) family (C Reygaert 2018).

Chapter 2

RecA as the main protagonist of SOS response

RecA is the ancestral protein of a group of proteins that bind and hydrolyse the adenosine triphosphate (ATP) to make a mechanical work. This family includes DNA helicases, F1-ATP synthase, ATP-binding cassette (ABC) membrane transporters and the DNA repair enzyme Rad50. This class of protein shares a common core fold that contains the ATP-binding site. The binding and hydrolysis of ATP orchestrate a conformational change which leads to large reorientation of the subunits (C. E. Bell 2005). RecA is a high conserved protein discovered over 50 years ago by Clark and Margulies (Clark and Margulies 1965). This protein belongs to a group originated from a single progenitor essential for homologous recombination and includes three homologues family: RecA, RAD51 and Rad51-paralogs. These homologous proteins carry out related functions in several organisms as archaea, eukaryotes and bacteria. The archaeal homologue is called RadA, while in eukaryotes RAD51 and Dmc-1. Bacterial RecA is one of the most conserved protein, and its sequence is shared by ~ 60-70% of the entire bacterial domain life (J. C. Bell and Kowalczykowski 2016).

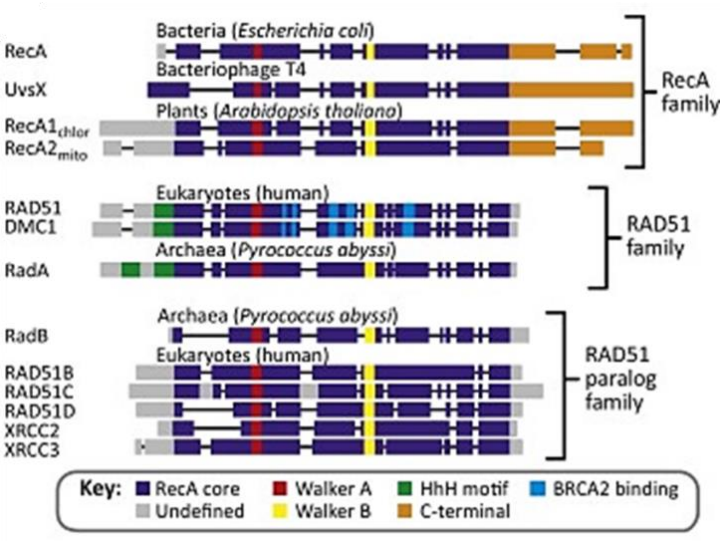


Figure 9. Conserved domains of the RecA, RAD51, and RAD51 paralog families. (J. C. Bell and Kowalczykowski 2016)

In a physiological state, RecA has a monomeric form with no relevant biological activity, but in the presence of ssDNA, a hallmark of DNA damage, hundreds to thousands of monomer homopolymerize with ATP to form a nucleoprotein filament (NPF) that represent the active form of RecA (RecA*) (Wigle et al. 2009).

2.1 Relevance of nucleofilament protein

The RecA protein of *Escherichia coli* (EcRecA) is a multifunctional protein that carries out three essential functions in the bacterial cell (Piero R Bianco and Kowalczykowski 1998):

- General genetic recombination and DNA repair.
- Regulation of expression of over unlinked genes in response to DNA damage (SOS induction).
- Regulation of activity of specialised DNA polymerases to facilitate error-prone DNA synthesis.

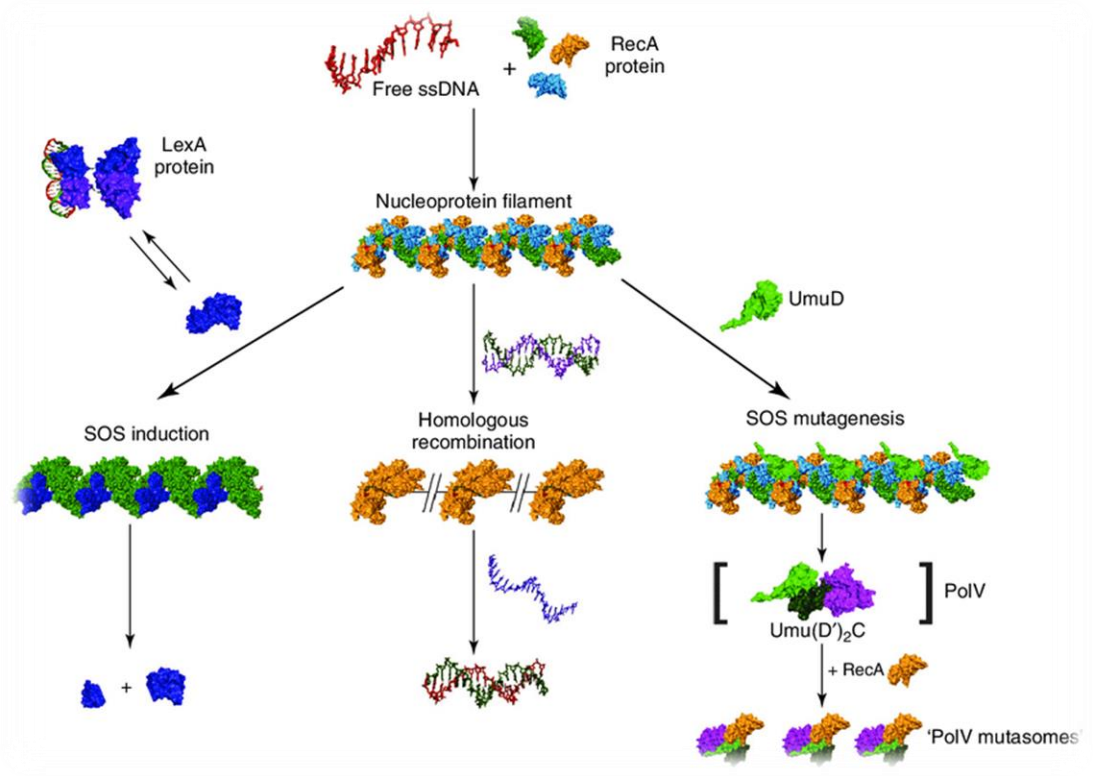


Figure 10. Schematic image of three essential functions of RecA.
(Piero R Bianco 2018)

RecA must be assemble on ssDNA to form a nucleoprotein filament, known as presynaptic complex, to mediate these functions. In this complex, RecA is capable of

performing three separate biochemical functions (Figure 10): homologous recombination, SOS induction, and SOS mutagenesis (Piero R Bianco and Kowalczykowski 1998).

The presynaptic filament is formed in two steps, slow initial nucleation followed by a rapid and cooperative extension of the filament in the 5' to 3' direction. Nucleation starts with five monomers of RecA bound to ssDNA. Filamentation requires ATP binding but no hydrolysis: when ATP hydrolysis occurs, it causes filament disassembly in the 5' to 3' direction (Patel et al. 2010). The ATPase activity of RecA is DNA dependent, and the binding of ATP increases RecA affinity for ssDNA. Therefore, RecA nucleofilament is a ternary complex (Nayak and Bryant 2015).

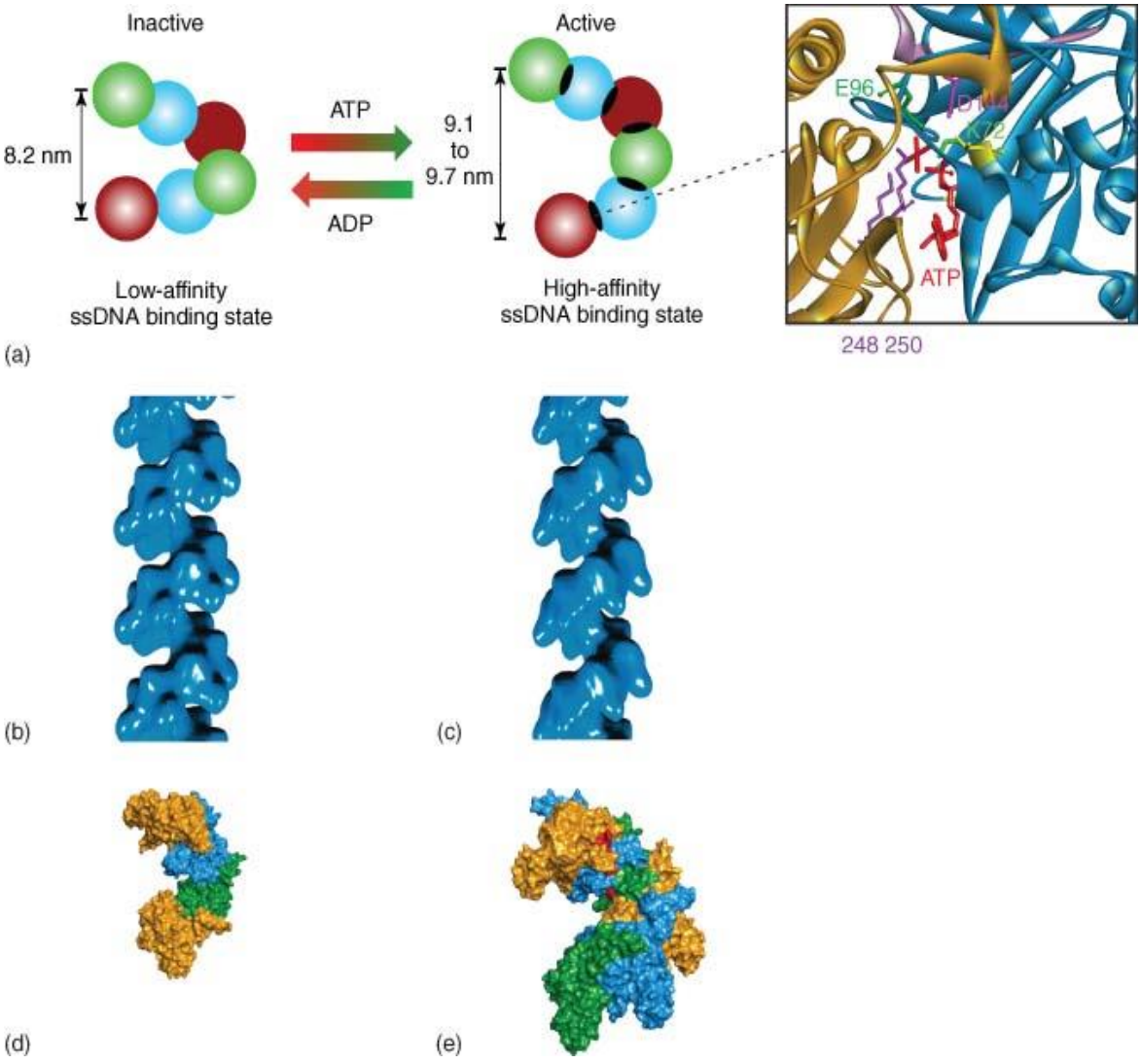


Figure 11. Nucleoprotein filament in inactive form (left) and active form (right).
(Piero R Bianco 2018)

The nucleoprotein filament is a helical complex of RecA protein monomers wrapped around ssDNA; one monomer binds three nucleotides, with about six monomers per helical

turn. RecA can form filament also on double-strand DNA (dsDNA) in a specific condition but with low affinity than ssDNA. RecA nucleofilament can exist in two forms (figure 11a):

- Inactive form, formed in the absence of a nucleoside triphosphate or in the presence of adenosine diphosphate (ADP)
- Active form, formed in the presence of ATP

Inactive filament (figure 11b-d) has a collapsed conformation with a helical pitch of approximately 7 ±1 nm. When RecA binds ATP (figure 11c-e), the protein increases the ssDNA affinity and change the conformation in the active form. The active form has an extended conformation with a helical pitch of 9.4 ±3 nm. The complete filament has a regular right-handed helical structure with a large helical groove. One side of the groove is smooth, and the other is penetrated by the protrusion of the individual monomers. This last groove represents the binding site for the main targets of RecA, or rather LexA and UmuD, and it seems that it is also involved in the binding of dsDNA. A recent study revealed that the binding of LexA and dsDNA to RecA is competitive, indicating binding at the same overlapping site on the filament (Piero R Bianco 2018).

Dissociation of RecA to the filament depends on the level of hydrolysation of ATP. If ATP is not regenerated, the level of ADP increases, destabilising the RecA monomers. Rapid dissociation of RecA from the filament can occur when ADP/ATP ratio is near 1.0. RecA disassembly also occurs in the presence of a regeneration system. A large unidirectional dissociation is observed when ATP hydrolysis occurs at an ATP concentration sufficient to operate at V_{max}. The rate of end-dependent (5'to 3') filament disassembly is 60-70 monomers min^{-1} when RecA binds on ssDNA, while 120 monomers min^{-1} when RecA is bind on dsDNA at neutral pH (Cox 2007).

ATPase activity of RecA has a K_m for ATP on the order of 50-100 μM, variables for conditions and cofactors. At ATP saturation, the k_{cat} is about 30 min^{-1} on ssDNA, while it is 20 min^{-1} on dsDNA. The hydrolysis of ATP occurs along the entire nucleofilament, and all subunits participate. However, only a few contiguous monomers of RecA dissociated from filament after ATP hydrolysation. RecA monomers hydrolyse 2-3 molecules of ATP per second without dissociating; this activity is important for RecA function *in vivo*. Indeed, mutations that alter ATP hydrolysation compromise key activities such as forming the nucleoprotein filaments, promoting DNA pairing and exchange, and facilitating the autocatalytic cleavage of LexA protein (Cox 2007).

The assembly of RecA monomers on the filament is not spontaneous, and it is regulated by other protein. For dsDNA break, the assembly is regulated by RecBCD, a helicase/nuclease that process dsDNA breaks and directly loads RecA onto ssDNA afterwards encountering a specific sequence called χ (*crossover hotspot instigator*, Chi, 5'-GCTGGTGG-3'), those sequence is spaced approximately every 4-5 kb throughout the *E. coli* genome. If the

χ sequence is not present, RecBCD rapidly degrades the linear dsDNA (up to 30 kb *in vitro* and 10 kb *in vivo*) to defend against bacteriophage infection. For the DNA lesions formed during DNA replication, RecA monomers are assembled on ssDNA by the combined action of RecQ helicase and RecJ nuclease, which produced long regions coated by ssDNA binding protein (SSB). Neither RecQ nor RecJ interacts with RecA; in fact, the assembly of RecA monomers on ssDNA filament occurs due to RecF, RecO and RecR proteins action. These proteins form two complex, RecFOR and RecOR, that regulate the RecA filament assembly by enhancing nucleation and growth of the filament (J. C. Bell and Kowalczykowski 2016).

An important characteristic of nucleoprotein filament that affects the stability of the filament is the ssDNA length. RecA, bonded with ATP, does not form a stable filament on ssDNA lengths shorter than about 30 nucleotides. Moreover, the nucleation frequency drops drastically when the ssDNA length is below 21 nucleotides, and no nucleation is observed with 13 and 15 nucleotides (Joo et al. 2006). RecA monomers can bind oligonucleotides 9-20 nucleotides length, but for obtaining maximum stimulation of ATPase activity, more longer chain lengths are required (Piero R. Bianco and Weinstock 1996)(Katz and Bryant 2001).

2.3 The role of RecA protein in homologous recombination

Homologous recombination (HR) is a conserved pathway that contributes to important cellular processes such as double strand-break repair (DSBR), the rescue of collapsed or stalled replication forks, horizontal gene transfer, and exchange of genetic information between DNA molecules. These processes are fundamental for genome integrity and enhance genetic diversity (Kaniecki, De Tullio, and Greene 2018). General genetic recombination leads to the exchange of homologous regions between two chromosomes or double-stranded DNA molecules. The resulting DNA contains genetic information originally present in each of the parental molecules. In *E. coli*, RecA protein is essential for the primary pathway of homologous genetic recombination. It performs two important reactions: ATP-stimulated DNA strand annealing between complementary single strands of DNA and ATP-dependent DNA strand invasion (exchange) between ssDNA and a homologous sequence within dsDNA.

Biochemical mechanisms of HR can be separated into four stages: initiation, homologous pairing and DNA strand exchange, DNA heteroduplex extension, and resolution. The scheme illustrated in Figure 12 is the DSBR model applied to homologous recombination mechanisms in several organisms. DBS, in most cases, is necessary for the initiation of the process. It is introduced due to a programmed *in vivo* process or environmental circumstances (P. R. Bianco, Tracy, and Kowalczykowski 1998).

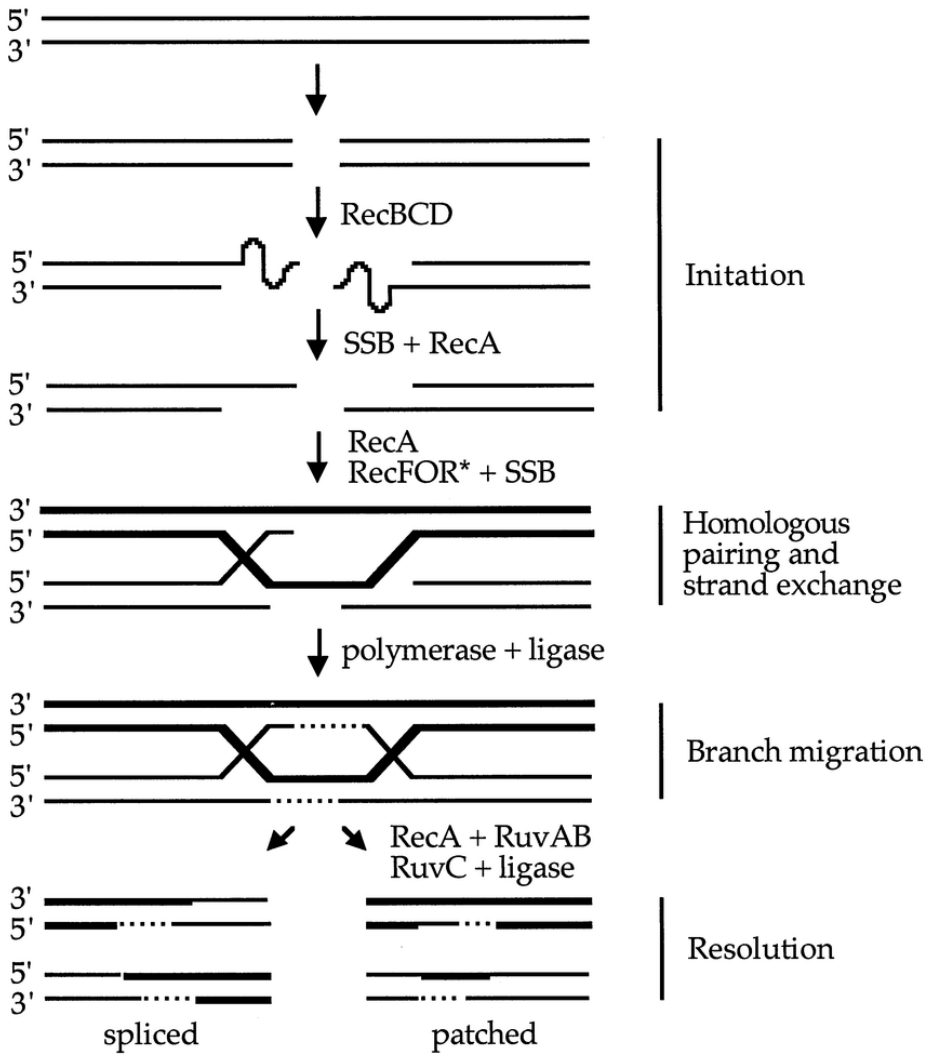


Figure 12. Model of recombinational repair of DNA double-strand breaks.
(McGrew and Knight 2003)

Besides external stress, in E. coli, DBS can be formed during physiological processes such as conjugation or transduction. Participation of RecA protein in these pathways is essential. A mutation that alters RecA function reduces conjugation and transduction by as much as 50,000-fold. HR is initiated at the dsDNA end that is processed to form ssDNA suitable for RecA. In *E. coli,* initiation of HR involves RecBCD complex to create ssDNA, which binds the end of linear dsDNA and simultaneously unwinds the DNA and degrades the 3'-terminal strands of DNA. RecBCD enzyme complex binds on a specific χ sequence (as described above) and form a 3'-terminal ssDNA tail terminating at the χ sequence. During translocation and unwinding, RecBCD complex loads RecA protein on this terminal.

RecA has a high affinity for χ sequence. RecA protein, combined with ATP and SSB protein, binds ssDNA to form a continuous presynaptic filament. The presence of SSB facilitates a load of RecA protein by destabilising the secondary structure of DNA and prevents

the attack from other nucleases. Furthermore, the RecF, RecO, and RecR proteins stimulate the formation of the joint molecules (or D-loop) by helping RecA protein to overcome the inhibition of binding due to the presence of SSB proteins. Once a joint molecule is formed, RecA protein, with other proteins, catalyses pairing of the remaining two DNA strands to produce a Holliday junction (P. R. Bianco, Tracy, and Kowalczykowski 1998). The next phase is the homology search, RecA nucleofilament opens the tethered target double-stranded DNA molecule forming a protein-bound intermediate (van der Heijden et al. 2008).

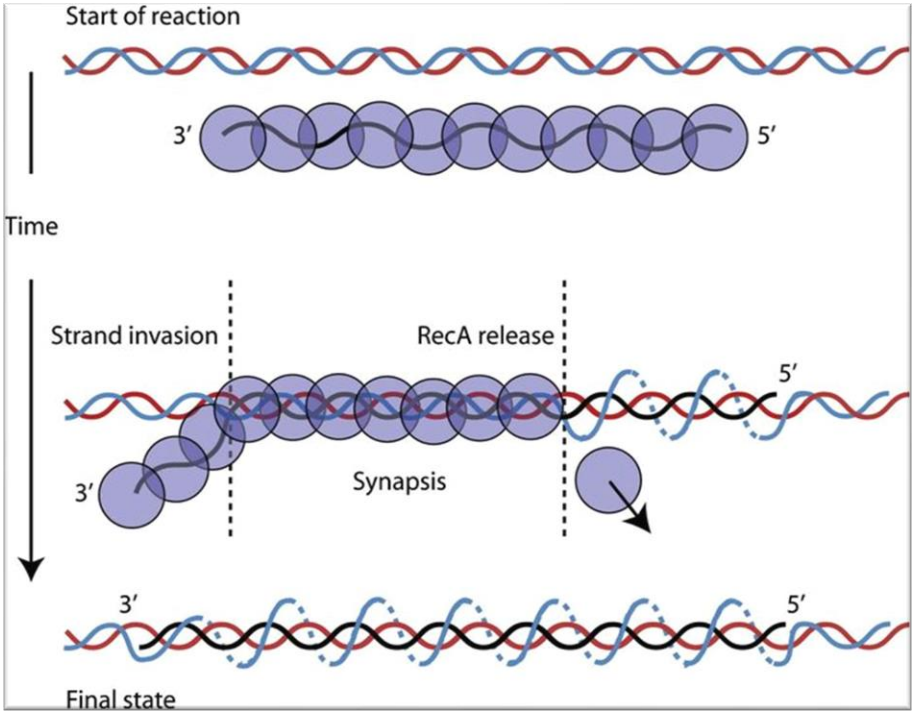


Figure 13. Strand invasion and exchange reaction by RecA.
(van der Heijden et al. 2008)

The incoming DNA molecule binds to the secondary DNA binding site of RecA in a sequence-independent manner. The specificity of the homology sequence is determined by the sequence of the ssDNA within the nucleoprotein filament. Even if the entire nucleoprotein filament is involved in search of homology, the minimum length of homology required for recognition is only 9 nucleotides *in vitro* and about 23-40 bp *in vivo* (Piero R Bianco 2018).

Strand invasion and exchange reaction in the presence of ATP move forward 5'-3' direction. Indeed, the nucleoprotein filament extends at the 3' end, and dissociation occurs at 5' end when ATP is hydrolysed. Lack of ATP hydrolysis, such as in the presence of ATPγS or mutations that alter this function, causes a loss in distinct polarity (van der Heijden et al. 2008). The presence of ATP increases affinity for DNA and is essential for strand exchange, but ATP hydrolysis is not required for the exchange of DNA strands. However, hydrolysis is required

to dissociate RecA proteins from the filament once the reaction is complete. Furthermore, dissociation of RecA from the filament facilitates the bypass of structural barriers and maintains the polarity of the reaction.

The result of RecA nucleoprotein invasion is the formation of a complex formed by RecA, ssDNA e dsDNA, named DNA heteroduplex or Holliday junction. This complex undergoes branch migrations with the aid of RecA activity that proceeds in a unidirectional direction 5'-3' at a rate of 2-10 bp/sec.

The final step of recombination is the resolution of the heteroduplex to produce a recombinant progeny. In *E. coli*, the resolution of the Holliday junction is performed by the RuvC protein, in concert with the RuvAB proteins, which cleaves the junction symmetrically to produce both spliced and patched recombinant (P. R. Bianco, Tracy, and Kowalczykowski 1998).

2.4 Co-protease activity of RecA in SOS pathway

RecA complexed in nucleoprotein filament (RecA*) has a co-protease activity which can stimulate the self-cleavage of specific proteins or rather LexA repressor, UmuD, and phage repressor proteins (CI) (Piero R Bianco 2018). LexA and UmuD are proteins involved in SOS, where LexA regulates the expression of genes involved in SOS response while UmuD promotes mutagenesis in the bacterial genome. Phage repressor (CI) is a transcriptional repressor of λ phage that regulate the vital cycle of phage (lytic or lysogenic) based on the health status of bacteria (Adikesavan et al. 2011).

LexA protein is a transcriptional repressor belonging to a class of particular enzymes that show a self-cleavage activity as a part of their physiological function (Mo, Birdwell, and Kohli 2014).

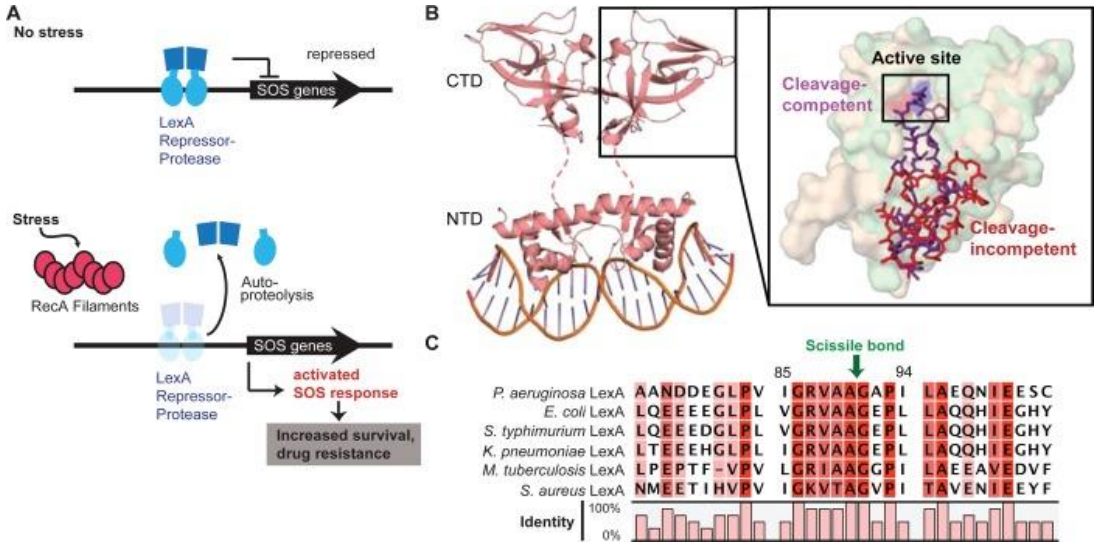


Figure 14. A. Autoproteolysis of LexA repressor induced by RecA nucleofilament; B. Structure of LexA dimer form *E. coli*; C. Conserved cleavage regions from different families.
(Mo, Birdwell, and Kohli 2014)

LexA is a 22 kDa protein with two functional domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), separated by a short flexible linker. The NTD contains the DNA binding site, while the CTD contains protease activity. LexA, in a homodimer form, interacts in specific DNA sequences, named SOS box, via a helix-turn-helix motif. CTD mediates the dimerisation; this domain contains a latent serine proteinase activity which mediates the self-cleavage activity (Culyba, Mo, and Kohli 2015). Cleavage occurs between Ala84 and Gly85, which lie in the cleavage site region (residues 79-95); this cut does not destroy the DNA binding domain but separates it from the CTD, altering the DNA binding of NTD. Crystal structures of LexA show that the cleavage site region can assume two possible conformations. In the non-cleavable state (NC), the cleavage site is distant 20 Å from the active site, whereas in the cleavable conformation, the cleavage site is positioned near the catalytic dyad Ser119 and Lys156 (Kovačič et al. 2013)(Giese, Michalowski, and Little 2008).

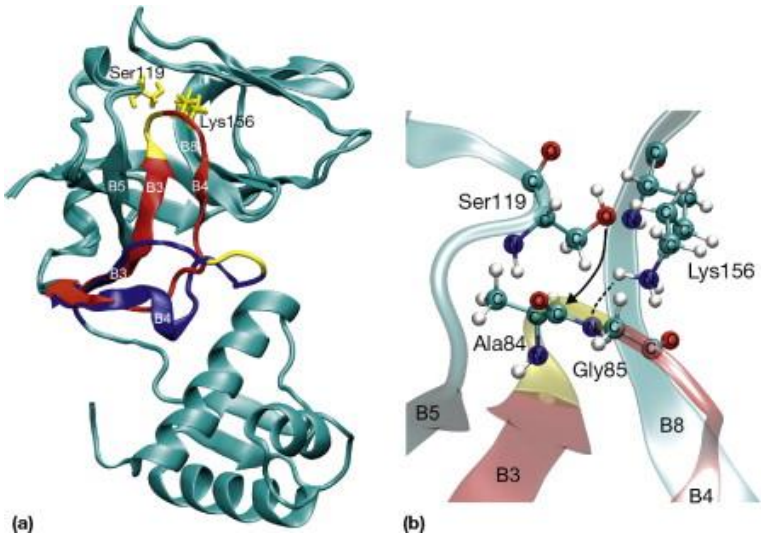


Figure 15. LexA in the two conformational states. (a) non cleavable state. (b) cleavable state and self-cleavage mechanism.
(Butala, Žgur-Bertok, and Busby 2013)

RecA, in his active form (RecA*), can bind LexA in both conformational states, although preferentially interact and stabilise LexA at the cleavable state. In the non-cleavable conformation, the residue Lys156 is exposed to the solvent in a protonated state to prevent autodigestion (Kovačič et al. 2013). The binding of activated RecA to LexA causes a dual allosteric effect: the approach of the cleavage site (Ala84-Gly85) to the catalytic dyad (Ser119-Lys156) and the burying of the Lys156, causing its deprotonation. The uncharged form of Lys156 removes a proton from the Ser119 hydroxyl group, which then acts as a nucleophilic centre that attacks the cleavage site. Hence, the charge state of Lys156 determines the cleavage reaction. In fact, this reaction can occur *in vitro* at alkaline pH, even in the absence of RecA (Butala, Žgur-Bertok, and Busby 2013).

Besides the induction of SOS response, RecA* has an active role in the SOS pathway, specifically in the mutagenic pathway. Mutagenesis in SOS response is performed by Polymerase V (PolV), a complex formed by UmuD'$_2$C. This complex is produced through a series of steps depending on the active form of RecA. The precursors of PolV are UmuD$_2$ and UmuC, which are encoded by *umuDC* gene. The operon that regulates the transcription has a high-affinity binding site for LexA, making it a late expressed gene (~ 30 min after DNA damage). However, despite being expressed, UmuD and UmuC are inactive for SOS mutagenesis.

Furthermore, the intracellular level of these proteins is kept minimum through Lon-mediated proteolytic degradation. Here RecA* plays an active role as co-protease since RecA* mediates the autocatalytic cleavage reaction of UmuD, removing 24 amino acid residues N-terminal to generate the active form of this protein (UmuD'). Thus, the persistence of DNA

damage leads to the accumulation of RecA* that increases the active form of UmuD' preventing degradation. However, this step requires a persistent damage-inducing signal because if the reaction is inefficient, it leads to the formation of UmuD/D' heterodimers which is rapidly targeted for degradation by the ClpXP protease. Thus only UmuD'$_2$ homodimer is the active form (Robinson et al. 2015).

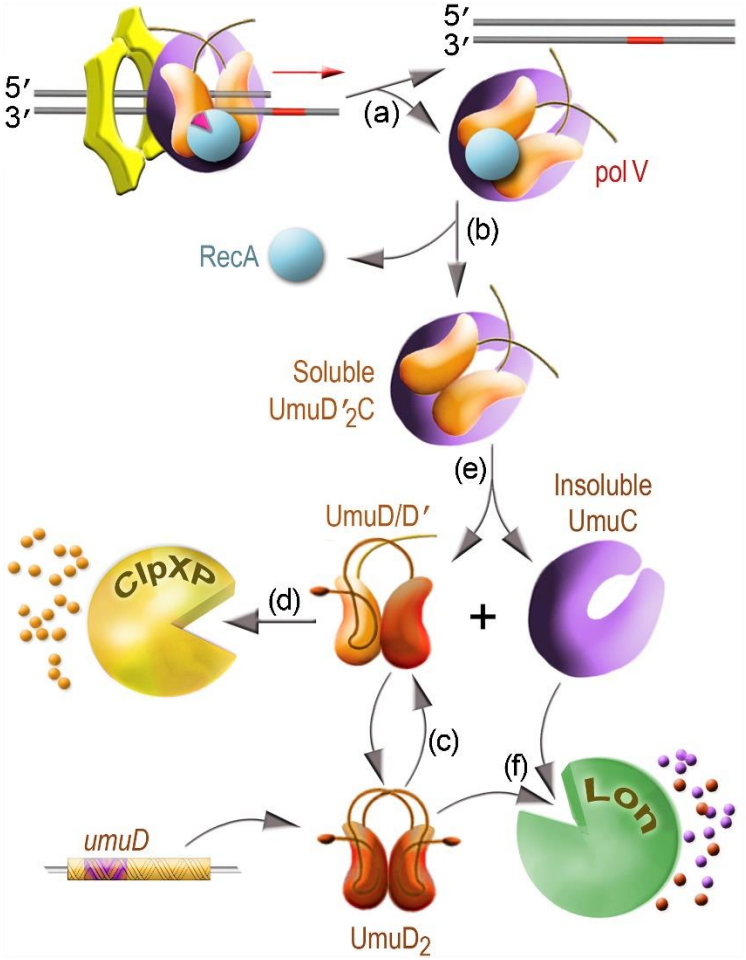


Figure 16. Polymerase V complex formation.
(Goodman et al. 2016)

UmuD'$_2$ associates with UmuC to form PolV (UmuD'$_2$C), a step that only occurs when the damage persists for at least 45 minutes. However, PolV cannot promote translesion DNA synthesis *in vivo* and has a weak catalytic activity *in vitro* in the absence of RecA. Highly active PolV requires the presence of RecA*. PolV interacts with RecA* and removes a single RecA-ATP molecule from the 3'-proximal end of the filament to form the active complex of PolV Mut (UmuD'$_2$-UmuC-RecA-ATP). However, to perform translesion synthesis, RecA* is no more needed (Goodman et al. 2016) (Jiang et al. 2009).

2.5 Structure of the damage sensor RecA

Bacterial RecA protein is a highly conserved protein, and its primary sequence has a huge grade of homology between diverse bacterial species. *E. coli* RecA shares a range of identical amino acid homology from 49% for *Mycoplasma pulmonis* to 100% for *Shigella flexneri* (Lusetti and Cox 2002).

RecA is a 38 kDa globular protein of 352 amino acids, involved in many different cellular functions (Sattin and Goh 2004). The RecA structures are organised into three domains: an N-terminal domain, NTD (residues 1-33), a core domain (residues 34-269) and a C-terminal domain, CTD (residue 270-352). NTD consists of an α-helix and a β-strand arranged against the core domain of a near monomer in the filament. The core domain includes the ATP binding site (Walker A motif or P loop, residues 66-73), the Mg^{2+} binding site (Walker B motif, residues 139-144) and the two putative DNA binding loops L1 and L2. The core region is composed of a β-sheet flanked by α-helices on either side. The CTD comprises three-stranded β-sheet and three α-helices; this domain seems to regulate the binding of the dsDNA to the RecA-ssDNA filament.

The crystal structure of RecA protein showed that residues 1-5, 329-352, and the putative DNA binding loops are disordered. The last few residues of CTD (329-352) presents a prevalence of negatively charged residues. It is thought that these residues are involved in forming salt bridges with various basic residues of the protein to produce a closed structure that does not consent to the entry of dsDNA into the nucleoprotein filament. Mg^{2+} (6-10 mM) breaks these salt bridge allowing the entry of dsDNA into the RecA-ssDNA filament. Furthermore, residues Trp-290 and Gly-301 to Asn-304 in CTD are involved in binding dsDNA before it interacts with the nucleoprotein filament. So, CTD may act as a "gateway" for dsDNA entry into the RecA-ssDNA filament (Rajan 2007).

To perform its many roles, RecA has to assemble with ssDNA into nucleoprotein filament. However, in addition to the monomeric form, RecA exists in the cell as aggregates of inactive filaments called bundles. The form that RecA assumes depends on the presence of ATP and/or ssDNA. In the absence of ssDNA, RecA tends to form inactive aggregates as rod and bundles. It is thought that these different forms are a storage form of RecA to keep this protein available but inactive in the cell. The balance between these forms may play a role in regulating RecA activity (AJ 1998).

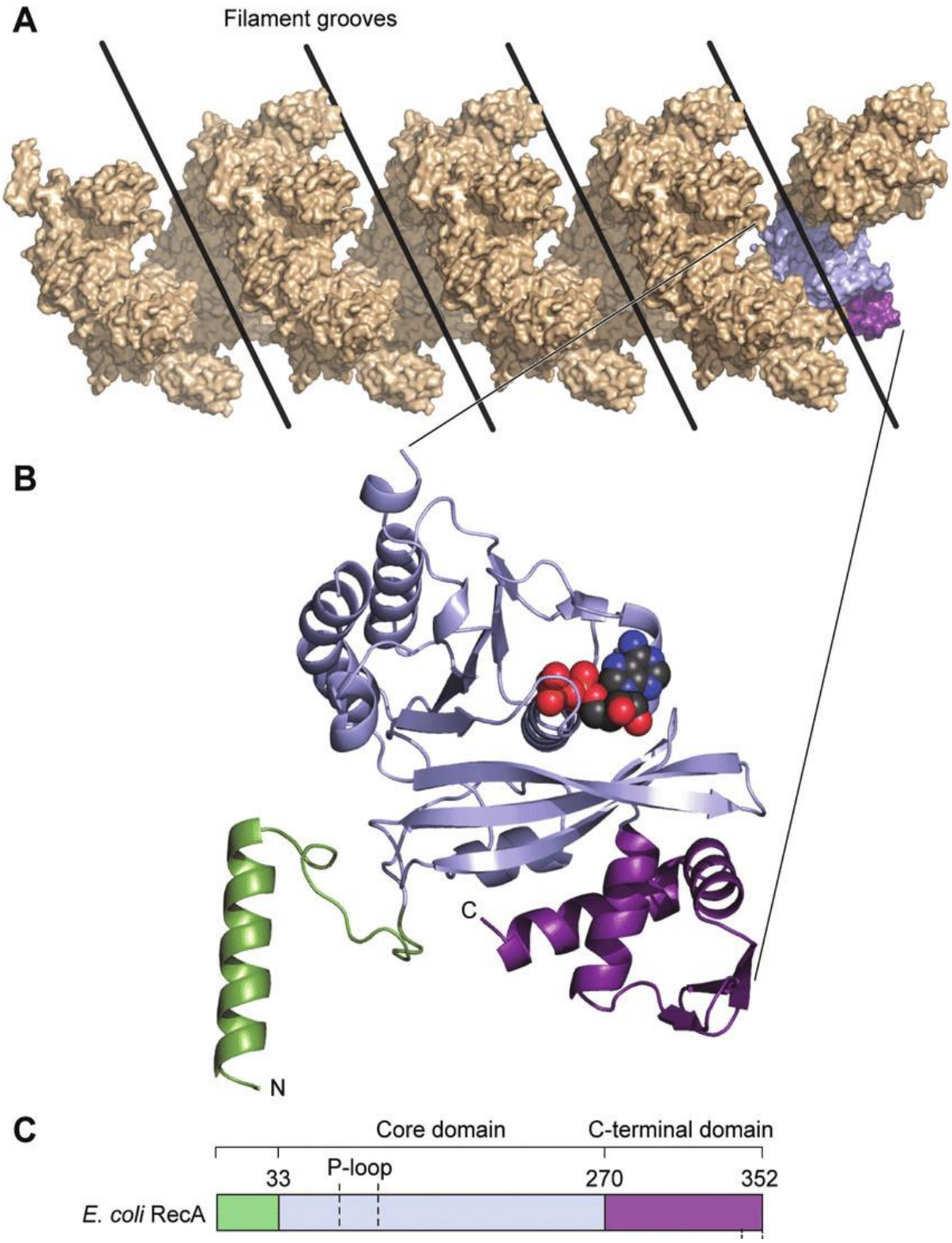


Figure 17. RecA protein structure: primary structure to complex form.
(Cox 2007)

2.5.1 ATP binding domain

The core domain of RecA protein contains other DNA binding loops, the Walker A motif (or P loop) which binds ATP and Walker B motif that binds Mg^{2+} (McGrew and Knight 2003).

The Walker A motif (66-73) lies on the inside surface of the protein filament. This motif has a specific sequence defined as GXXXXGK(T/S), highly conserved and identical in 61 of 64 bacterial RecA proteins. This sequence frequently occurs in ATP and GTP binding proteins, and its function is involved in stabilising the binding of NTP. Structurally is organised in loop-like secondary structures (hence the name P loop) that make specific interactions with the phosphate groups of bound NTPs (McGrew and Knight 2003). In the crystal structure of the filament, the ATP binding site lies between neighbouring RecA subunits and is oriented toward the interior of the filament (VanLoock et al. 2003).

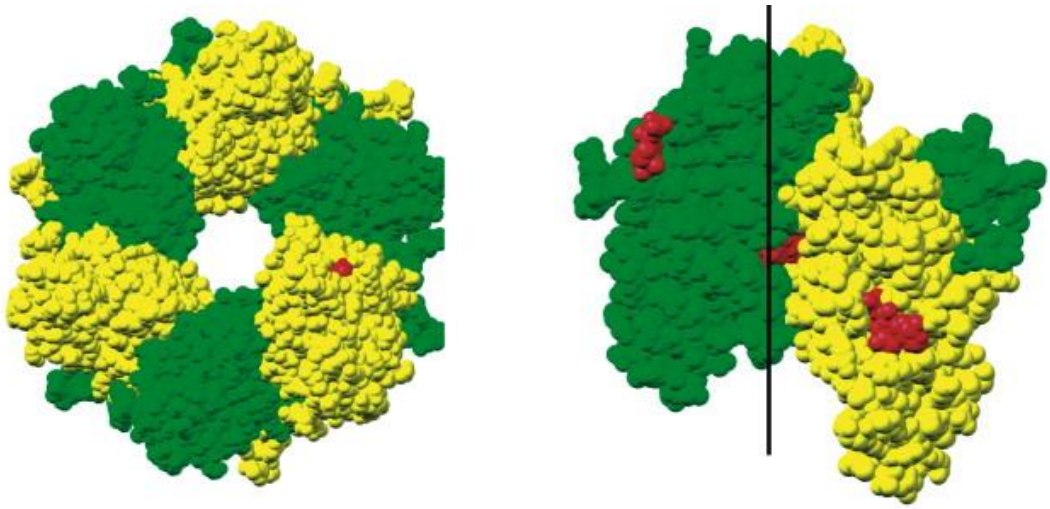


Figure 18. RecA model filament with ATP binding site (red).
(VanLoock et al. 2003)

The Walker B motif consists of residues from 139 to 144 surrounding four hydrophobic residues. Residues 144 contains an Asp, which is identical in 64 bacterial RecA sequence. The crystal structure of RecA-ADP has shown that this residue is involved in the binding of Mg^{2+}. The residues 145-149 represent the C-ending of the B motif and are identical in 63 RecA sequences. It was suggested that this region is functionally or structurally involved in ATP hydrolysis. The structural model of the RecA/NTP complex showed that residues Val146 and Ala 147 form hydrogen bonds with flanking L2 loops. Moreover, this sequence may act as a "connector" segment involved in NTP-induces structural changes in L2 to L1, therefore coordinating the DNA binding to the primary and secondary sites (McGrew and Knight 2003).

ATP binding to RecA induces a large reorientation, enhancing affinity for DNA binding, increasing cooperative filament assembly, promoting communication between primary and secondary DNA binding sites, and increasing association with other proteins co-protease substrate and the PolV complex. These induced conformational changes are made possible by the interactions among specific residues, defined MAW (Make ATP Work; residues 42-65), and regions of the structure on either side of the ATP binding site (McGrew and Knight 2003). Conversely, the hydrolysis of ATP causes a reverse reorientation of the subunit and release of ADP-P_i. This change leads to destabilisation of the interface within two monomers, where the binding site of ATP is located (C. E. Bell 2005).

2.5.2 DNA binding domain

RecA has two binding sites for DNA, the L1 region formed by a short helix followed by a turn and an extended segment, and L2 region formed by β-hairpin, which bind dsDNA and ssDNA, respectively (Chen, Yang, and Pavletich 2008). In the crystal structure, these two loops lie close to the polymer axis and are arranged in the inner surface where the DNA lies. Mutational studies have demonstrated the binding specificity of these loops: single-point mutant RecA E207Q (L2 region) lacks SOS response induction and homologous recombination activities *in vivo*. Whereas single point mutant at L1 region has constitutive co-protease activity (indicating that RecA is capable to binds ssDNA) but lack recombination *in vivo*. However, it was found that double mutation at the L1 region reduces the level of recombination and constitutive co-protease *in vitro*, suggesting that this loop is involved in both primary ssDNA and secondary dsDNA binding (Moya 2006).

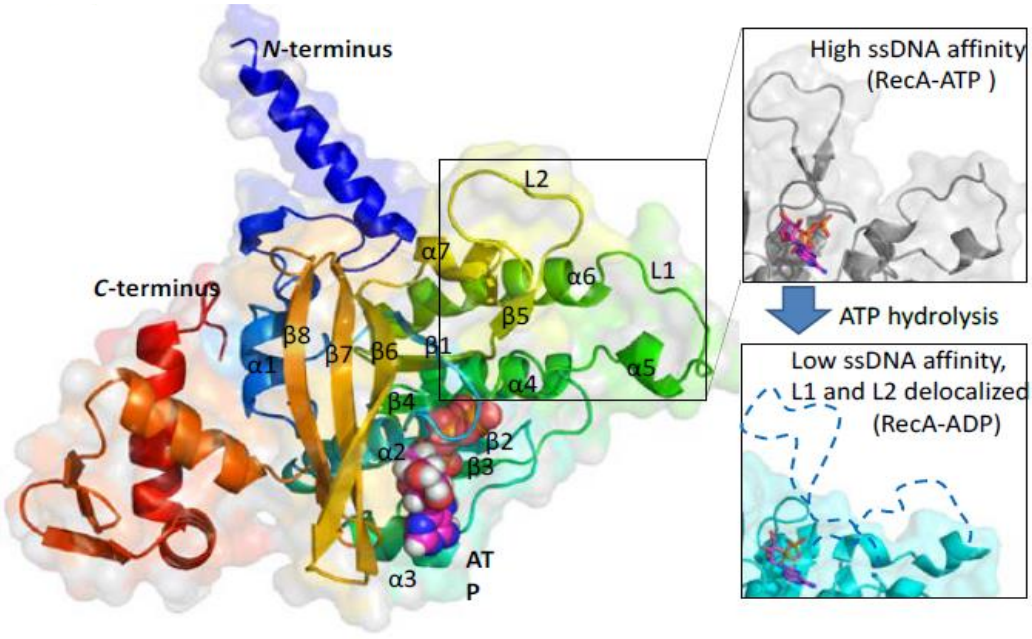


Figure 19. RecA DNA binding domain in high and low affinity for ssDNA.
(Zhou et al. 2021)

2.5.3 Structural interaction between RecA and LexA

Studies of different amino acid substitution in RecA protein have observed that the residue Pro67 is involved in making specific contacts with co-protease substrates of RecA. Pro67 is located in a region of the RecA structure that makes direct contact with LexA and UmuD. RecA and LexA interaction occurs within two points: the helical filament groove and a second including the L1 region. Considering that the recombination and co-protease functions of RecA are mutually exclusive and L1 represents the secondary DNA binding site, the induction of LexA cleavage is competitive with DNA binding in recombination reaction (McGrew and Knight 2003).

RecA does not participate directly in the chemistry of cleavage reaction. It was suggested that the active form of RecA stabilises LexA in the cleavable state through an allosteric mechanism, facilitating self-cleavage. Functional LexA is a homodimer that interacts with specific DNA sequences. The interaction with RecA* occurs between one subunit of the LexA dimer and seven successive RecA protomers. The second subunit interacts only with the edge of the nucleoprotein filament's deep helical groove (Kovačič et al. 2013).

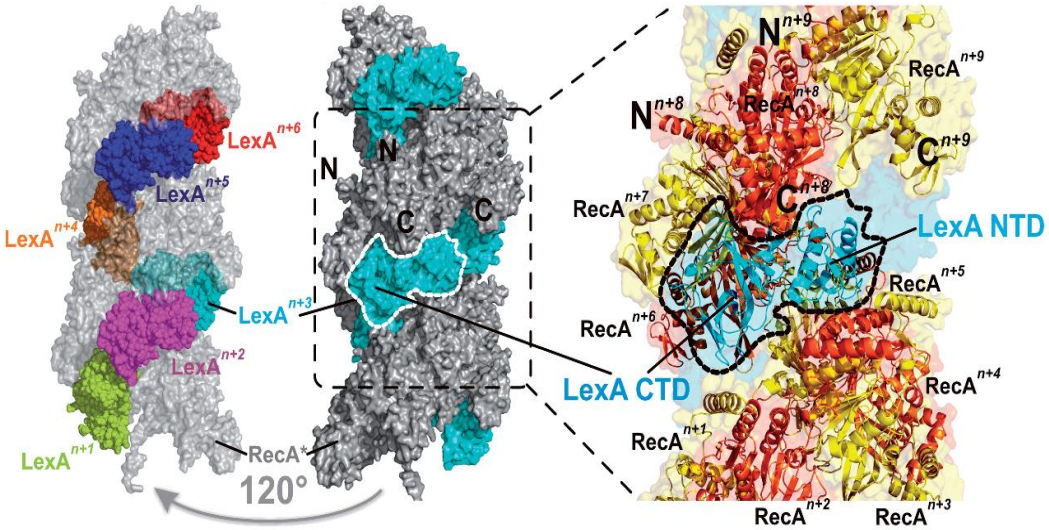


Figure 20. Model of LexA-RecA* three-dimensional structure.
(Kovačič et al. 2013)

Chapter 3

Inhibition and modulation of RecA activity

RecA has a central role in bacterial genomic stability and survival. Inhibition of his biochemical activities leads to DNA repair defects and general genomic instability (Kim et al. 2015). However, the high efforts to inhibit the RecA activities are due to its main role in the SOS network. RecA is involved in both inductions of SOS response and SOS mutagenesis. Bacterial strains that lack RecA functions are hypersensitive to antibiotics and less prone to acquired resistance. RecA represents a critical component of the response, making it an attractive target for slowing the evolution of antibiotic resistance (Wigle et al. 2009) (Culyba, Mo, and Kohli 2015).

3.1 Endogens modulator of RecA

The activity of RecA protein is regulated at different levels. It regulates both its activity and its expression. Several proteins are involved in the regulation of RecA activities; two have opposing activities in the modulation of RecA function, named RecX and DinI.

RecX protein is expressed under regulation from the *recA* promoter downstream of the RecA gene. The main function of this protein is to reduce the deleterious effects of overexpression of RecA protein. RecX is a negative modulator of the RecA function. Overexpression of RecX reduces the induction of SOS response, while the deletion of its gene in E. coli does not affect the phenotype. RecX *in vitro* can inhibit ATPase and strand exchange activities of RecA protein. The inhibitory effect of RecX is due to the block of RecA filament extension during assembly by capping the filament (Cox 2007).

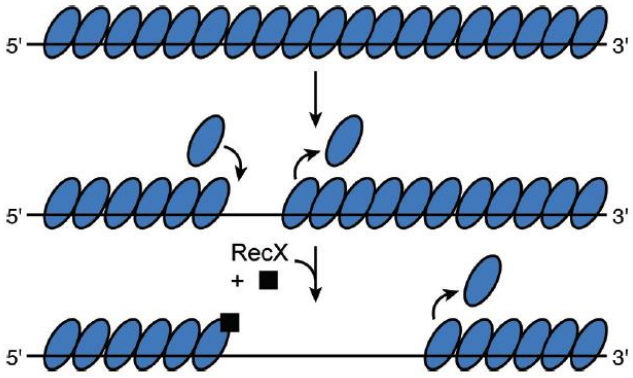


Figure 21. RecX inhibition of filament extension.
(Cox 2007)

DinI protein is a small polypeptide induced in the early phase of SOS response. Initially, it was thought that his role was to carry out the SOS response. Overexpression of DinI in *E. coli* leads to more sensitivity from UV and inhibits the induction of the SOS response. DinI inhibits the RecA-mediated cleavage of UmuD protein. However, it does not affect either strand exchange or induction of autocatalytic cleavage of LexA (Cox 2007).

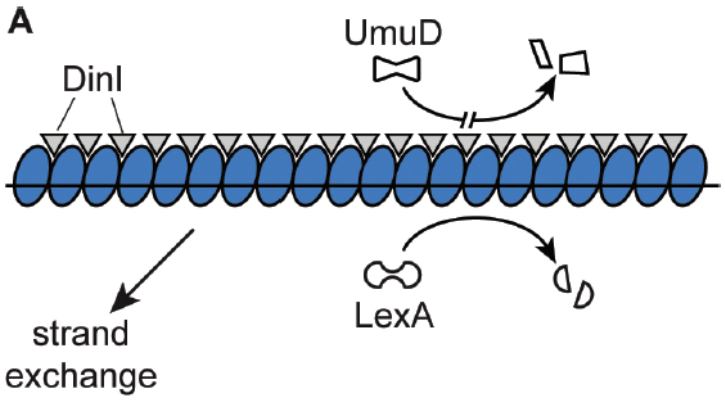


Figure 22. Effect of DinI on RecA activities.
(Cox 2007)

The presence of DinI in the early phase of SOS response suppresses the premature activation of PolV but leaves most other RecA activities unaffected. RecA C-terminus regulates the interaction between DinI and RecA; deletion of this region increases the interaction between these proteins.

RecX and DinI have opposite biochemical effects on RecA filament. RecX has a negative effect and destabilising on RecA nucleofilament, while DinI promotes stability of RecA filament. Furthermore, when DinI has a stoichiometric concentration with RecA, it has a strong stabilising effect and disassembly is almost completely suppressed (Cox 2007).

3.2 RecA inhibitors

RecA has a central role in the survival of bacteria, especially against antimicrobial agents. *E. coli* strains that are deficient in RecA function are more sensitive to bacterial antibiotics. The role of RecA is essential for acquired tolerance to antibiotics treatment by enhancing DNA repair that occurs by antibiotics-induced DNA damage or indirectly from metabolic and oxidative stress (Alam et al. 2016).

RecA represents an attractive target for delaying the development of resistance to antibiotics and making bacteria more susceptible to existing antimicrobial molecules (Wigle et al. 2009).

Several studies are focused on inhibiting RecA ATPase activity due to its importance for carrying out its biochemical functions. Moreover, only the ATP presence and binding, but no hydrolysis, is required for RecA co-protease activity on LexA and SOS induction. Blocking ATP binding on RecA might have strong effects on RecA activities and SOS response activation (Bellio et al. 2017). Several compounds have been discovered that inhibit RecA ATPase activity in vitro, including polysulfunated naphtylurea, metal cations, nucleotide analogues, small organic compounds (Sexton et al. 2010), and natural compounds such curcumin and lichen secondary metabolites (Bellio et al. 2014) (Bellio et al. 2017). However, most of these compounds do not have specific RecA biological activities *in vivo*, probably due to the impermeability of bacterial membrane towards these compounds (Alam et al. 2016). Only curcumin exhibits inhibition of induced SOS response in *E. coli* (Ojha and Patil 2019).

3.3 Suramin as inhibitor and modulator of RecA activity

Suramin is a molecule that belongs to naphthalene poly-sulfonated compounds. This molecule has shown to be a strong inhibitor of the ATPase activity of RecA (Zhou et al. 2021). Nautiyal *et al.* showed that suramin was able to inhibit the main biochemical function of RecA, or rather ATP binding, ATPase activity and DNA strand exchange. Furthermore, suramin inhibits the co-protease activity of RecA but not influence the *recA* expression. However, they observed a lack of correlation in the concentration of IC_{50} between *in vitro* biochemical activities of RecA and *in vivo* bactericidal activity of suramin. Indeed, a higher concentration was necessary to observe the inhibitory effects in *Mycobacterium tuberculosis* (Nautiyal, Patil, and Muniyappa 2014).

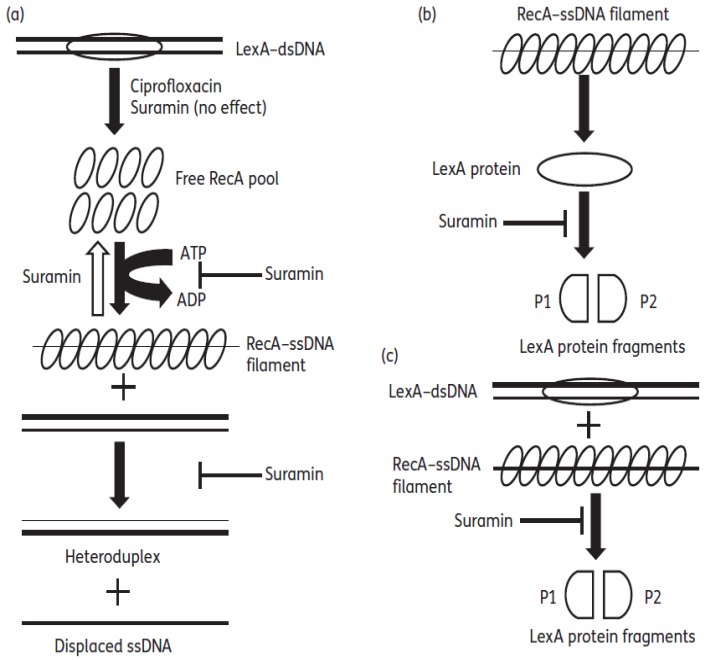


Figure 23. Suramin inhibitory effects on RecA**.**
(Nautiyal, Patil, and Muniyappa 2014)

Suramin has a negative molecular charge which may be the cause of lack of *in vivo* activities. In addition, its poor action is due to expulsion by bacterial efflux pump (Nautiyal, Patil, and Muniyappa 2014).

In their work, Nautiyal *et al.* describe suramin as a potent inhibitor of RecA activity. However, in our work, we observed that *in vitro* has a different effect on biochemical activities of RecA depending on concentration. Moreover, several studies and experimental tests have demonstrated that RecA work as a trimeric complex in combination with ssDNA and ATP. The absence of one of these components compromises the biochemical activity of RecA. Nonetheless, we have reported that in the presence of suramin, RecA can perform most of its biochemical activities even in the absence of ssDNA.

AIM OF THE WORK

The bacterial SOS response is an amazing strategy by which bacteria contrast every kind of stress, both exogenous and endogenous. Moreover, the SOS response stimulates the bacteria mutagenesis, facilitating bacterial adaptation to stress that induce the response, including antibiotic pressure. However, the SOS response consists of a complex network that includes more than 50 genes involved in several functions. This pathway is regulated by only two proteins: a recombinase (RecA) and a transcriptional repressor (LexA). In this regard, these two proteins represent an attractive target for contrasting the activation of SOS response. Preventing the activation of SOS response means blocking all those bacterial mechanisms of resistance and tolerance to stress, including antimicrobial agents.

This study aims to define the mechanism of inhibition of suramin towards the biochemical activities of RecA. Even though RecA represents an ideal target to inhibit the SOS response induction, it must be considered that this protein is a part of an ancient and evolutionarily widespread protein family. Indeed, RecA has up to seven important eukaryotes homologues (Rad51 family) and sharing a similar protein structure. Therefore, a low specifics inhibitor may have potential toxicity with a wide range of side effects in human as well (Culyba, Mo, and Kohli 2015) (Yakimov, Bakhlanova, and Baitin 2021).

Nautiyal *et al.*, in their study, demonstrate the inhibitory effects of suramin on RecA biochemical activities, although the mechanism of action remains not yet conclusively determined. Thus, this thesis aims to elucidate the inhibition mechanism of suramin for study the biochemical behaviour of RecA.

To achieve this objective were performed the following experiments: (1) cloning of *recA* gene in an over-expression vector; (2) producing and purification of a functional RecA protein; (3) determining the inhibition mechanism of suramin; (4) identify the potential binding site of suramin on RecA by *in silico* analysis; (5) verify other potential inhibitory effects of suramin toward principal biochemical activities of RecA.

MATERIALS AND METHODS

Reagents

Adenosine triphosphate (ATP), dimethyl sulfoxide (DMSO), isopropyl-β-D-thiogalactopyranoside (IPTG), Tris-HCl, imidazole, glycerol, turbonuclease, β-Mercaptoethanol, 1,4-dithiothreitol (DTT) and kanamycin were purchased from Sigma-Aldrich Chemical Co. The oligonucleotide named poly$(dT)_{36}$ was purchased from Integrated DNA Technologies, BIOMOL Green was obtained from Enzo Life Sciences Inc., Lysozyme from Euroclone and RecA protein from New England Biolabs inc.

Media Preparation

Media culture, used for experiments, was prepared by dissolving commercially prepared powder in distilled water and then autoclaved at 15 psi and 121 °C for 15 minutes. If needed, antibiotics were added to cooled autoclaved broth before culturing the cells.

Agar medium was prepared by adding 15 g of bacteriological agar (Biotec) to 1 L of Luria-Bertani (LB, Oxoid) broth.

Preparation of DNA construct

The *recA* gene was amplified by PCR from genomic DNA of *Escherichia coli* clinical isolate with specific oligonucleotides (Table 3) and ligated into two over-expression vectors, pET-28b and pET-24a (both purchased from Novagen).

Cloning in pET28b (Fig. 24a) allowed to have protein with an N terminal six-His tag. Instead, with pET24a cloning (Fig. 24b), the obtained protein shows a C terminal six His tag.

The amplification was carried out in 100 µL reaction: 2 ng of DNA template, 800 pmol of each oligonucleotide, 2 µL of 10 mM dNTPs mix, 2 units of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs inc.), 1X of Q5 Reaction Buffer and 1X of Q5 High GC Enhancer.

Name	Sequence (5'-3')	Restriction site
	pET28b cloning	
RecA_NdeI_for	GGG GGG **CAT ATG** GCT ATC GAC GAA AAC	NdeI
XhoI_RecA_stop_R	GGG GG**C TCG AG**T TAA AAA TCT TCG TTA GTT T	XhoI
	pET24a cloning	
BamHI_recA_F	GGG GG**G GAT CC**A TGG CTA TCG ACG AAA AC	BamHI
XhoI_recA_His_R	GGG GG**C TCG AG**A AAA TCT TCG TTA GTT TC	XhoI

Table 3. Sequences of the primers used for cloning of *recA*. Restriction cutting sites and the coding sequences are shown in bold and italic letters, respectively. All the oligonucleotides were purchased from Eurofins Genomics.

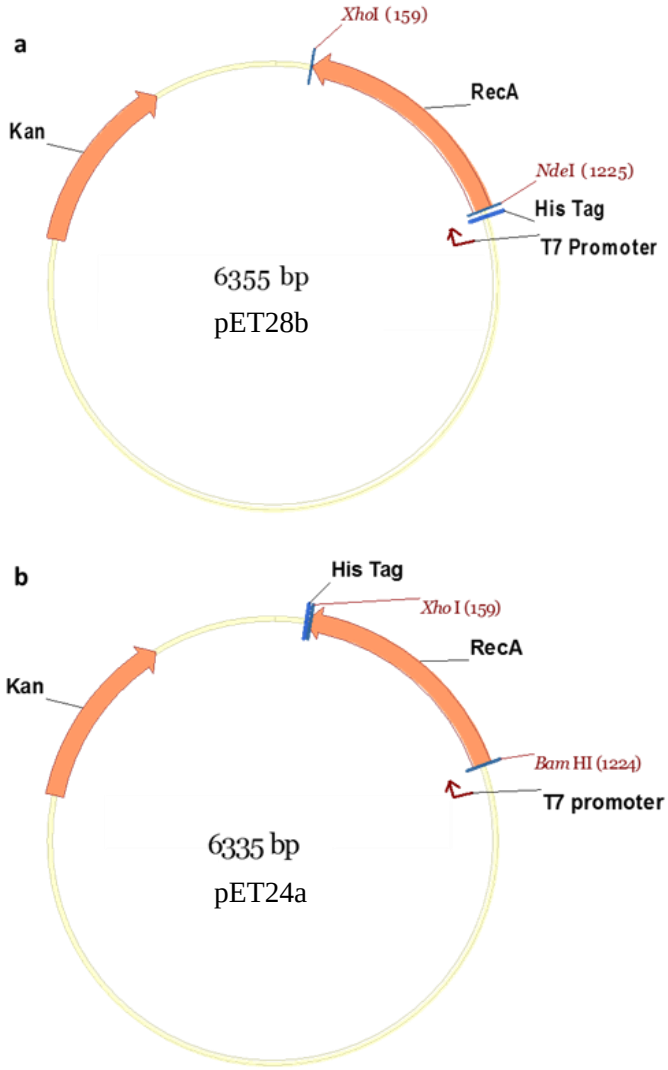


Figure 24. Plasmid vector map and cloning region in the vector. a) The 6.3 kb pET28b bacterial protein expression vector containing *recA* gene. b) The 6.3 kb pET24a bacterial protein expression vector containing *recA* gene.

Vectors were digested and dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (Thermo scientific) (1 U/µL) [in 40 µL of total reaction: 1 µg of plasmid vector, 2 µL FastAP, 4 µL buffer 10X].

PCR products, pET28b and pET24a were subjected to double enzymatic digestion using the restriction enzymes reported in table 3 [in 30 µL reaction: 1 µg of DNA template, 1 µL of BSA, 3 µL Buffer 3 10X, 3 µL XhoI, 0.5 µL NdeI (for pET28b-*recA* ligation) or 3 µL BamHI (for pET24a-*recA* ligation). All enzymes were purchased from Biolabs]. These reactions were incubated 1 hour at 37°C and subsequently extracted from 1% agarose gels (w/v) and purified with the Expin Gel SV kit (GeneAll Biotechnology).

The cut vector was then ligated to the cut PCR products by T4 DNA ligase (New England Biolabs inc.) [2 µL Ligase buffer 10X, 1 µL T4 Ligase, 1 µL ATP 10 Mm, 0.5 µL MgCl2 30 ng digested fragments and 50 ng digested vector, incubated overnight at 16°C].

E. coli JM109(DE3) competent cells, having genotype endA1, recA1, gyrA96, thi, hsdR17 (rk-, mk+), relA1, supE44, λ–, Δ(lac-proAB), [F´, traD36, proAB, lacIqZΔM15], λDE3, were transformed with the pET28b-recA plasmid and with the pET24a-recA plasmid by heat shock method. The reaction was carried out in this way: defrosted cells were incubated with 5 µL of ligation for 10 minutes in ice, 50 seconds at 42°C and 2 minutes in ice again. After that, the mixture was incubated for 1 hour at 37°C, in SOC medium [20 g Bacto-Tryptone (Biolife), 5 g Yeast extract (Biolife), 0.5 g NaCl (Sigma-aldrich), 2.5 mL of 1 M KCl (Sigma-aldrich), ddH2O to 1 L] and successively transferred to LB agar plates containing 50 µg/mL kanamycin (spread plate method) and grown overnight at 37°C. The gene for kanamycin resistance helps the clone selection during cloning process.

The colonies grown on the selective medium were transferred in LB broth and incubated overnight at 37°C. The constructs were extracted and purified from these cells using Exprep Plasmid SV mini kit (GeneAll) and the integrity of the cloned fragment was confirmed by DNA sequencing by sending the DNA samples to the Eurofins Genomics S.R.L.

Agarose gel electrophoresis

100mL of 1-1.5% (w/v) agarose gels were prepared in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The solution was boiled until the agarose was completely dissolved in the buffer. 5µL of EuroSafe Nucleic Acid Stain Solution (20,000x) was added and mixed properly when the agarose solution cooled down. The cooled, liquified gels were then allowed to set in the gel cast by pouring and the cascade was properly sealed to prevent leaking. DNA samples were mixed with 1X loading buffer [0.04% (w/v) bromophenol blue, 5% (v/v) glycerol]. Different volumes of samples were loaded on the gels depending on the

type of sample and purpose of the gel. DNA samples were resolved on the agarose gel at 100 V for 30 min in 1X TAE buffer. The gel resolved samples were visualized and analyzed with a UV illuminator. For the DNA gel extraction procedure, samples were viewed with a UV lamp and cut from the gel for purification.

Expression and purification of RecA protein

Transformed recombined bacterial strains were grown at 37°C overnight in a shaking incubator in 50 mL of Brain-Heart Infusion (BHI, Oxoid) medium containing 50 mg/L kanamycin.

Three flasks containing 650 mL of Terrific Broth medium with 50 mg/L kanamycin were inoculated with 5mL of the overnight cultures [to prepare 1 litre of medium: in 900 mL of distilled water add 12 g of Bacto Tryptone (Biolife), 24 g of Bacto-yeast extract (Biolife) and 4 mL of glycerol; separately, in 90ml of distilled water add 2.31 g KH_2PO_4 monobasic (Fluka) and 12.54 g K_2HPO_4 dibasic (Fluka); adjust the volume; sterilize each solution separately by autoclaving]. The cultures were incubated at 37°C in an orbital shaker at 220 rpm. When the optical density at 600 nm reached 0.4-0.5, 1 mM of IPTG was added to induce protein expression, and the cultures were incubated overnight at 20°C.

Cells were pelleted (4,000 rpm, 30 min, 4°C) and washed twice with washing buffer containing 20 mM Tris-HCl (pH 8.0). The obtained pellet was resuspended in Lysis buffer [20 mM Tris-HCl, 45 mM Imidazole, 10% Glycerol, at pH 8.0] added with Turbonuclease [1µL/7mL] and Lysozyme [2mg/mL] (Euroclone) and incubated at room temperature for 1 hour. Crude protein extract was obtained by sonication, followed by centrifugation (20,000 rpm, 1 hour, 4°C). The supernatant containing the soluble protein fraction was added with 500 mM NaCl and then stored at 4°C.

Protein purification was performed using two in series of His Trap FF 1 mL columns (GE Healthcare) pre-equilibrated with Binding Buffer [20 mM Tris-HCl, 45 mM Imidazole, 10% Glycerol, 500 mM NaCl, at pH 8.0]. Bound proteins were eluted in a single step with Elution Buffer containing a high Imidazole concentration (500 mM) and the protein quantified by Bradford assay.

To evaluate the purity of RecA protein, the highest concentration fractions were resolved on an SDS-Page and those containing highly purified RecA were pooled and dialyzed against 20 mM Tris-HCl, 5% Glycerol, 5 mM β-Mercaptoethanol, pH 8.0.

After dialysis, the sample was further concentrated up to 1 mg/mL using Corning Spin-X UF Concentrators (Sigma-Aldrich) and frozen stored at −80 °C.

SDS-PAGE

Protein samples were prepared before running the protein gels. 20 µg of proteins were mixed with 5X loading buffer [10% SDS (w/v), 10 mM β-Mercaptoethanol, 20% Glycerol (v/v), 0.2 M Tris-HCl, pH 6.8, 0.05% Bromophenol blue] to reach a final concentration of 1X. The protein samples were boiled for 10 minutes after adding the loading buffer and then loaded in the gels. Mini-PROTEAN TGX Stain-Free Precast Gel (BioRad) were used, and the runner condition were 200V for 30-40 minutes. 10X Tris/Glycine/SDS Electrophoresis Buffer (BioRad) was used as running buffer. The gels were stained with staining buffer [0.25% (w/v) Coomassie Brilliant Blue R-250, 40% methanol and 10% glacial acetic acid] for 2 hours and then de-stained with de-staining buffer [10% methanol and 10% glacial acetic acid] overnight.

Colorimetric assay for determination of ATPase activity

The ATPase activity of RecA protein was quantified by BIOMOL Green, an analogue of the malachite green reagent. This method allows the detection, in a single point step, of free phosphate in solution. Reactions were performed as previously described (Lee, Wigle, and Singleton 2007) using an Assay Buffer containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, pH 8.0.

Comparison of RecA proteins activity

Both purified proteins were tested to evaluate whether the presence of His-tag, at the two different ends, could cause loss of their ability to hydrolyse ATP or determine an increase or decrease of this activity.

Different concentrations of ATP (ranging from 30 to 600 µM) have been used. Besides RecA protein with the His-tag at N-terminal level ($RecA_{N\text{-}Ter}$) and the one, tagged at C-Terminal level ($RecA_{C\text{-}Ter}$), has also been used purchased RecA protein as control ($RecA_{wt}$). $[ATP]_{50}$ parameters have been calculated as the result of a dose-response plot obtained using a saturating $poly(dT)_{36}$ concentration (2 µM). Enzymatic efficiency was evaluated as the total amount of free phosphate products.

Data were analysed using the software OriginPro 8.5.1 and Microsoft Excel.

Determination of RecA parameters

In order to describe RecA activity, the parameters $[ATP]_{50}$ and $[poly(dT)_{36}]_{50}$ were determined. They are defined as the half maximal effective concentrations for ATP and $poly(dT)_{36}$, respectively.

$[ATP]_{50}$ has been calculated as the result of a dose-response plot obtained using a saturating $poly(dT)_{36}$ concentration (2 µM) and a concentration of ATP ranging from 30 µM to 600 µM. $[poly(dT)_{36}]_{50}$ has been determined fixing the concentration of ATP at 300 µM and a concentration of $poly(dT)_{36}$ ranging from 0.04 µM to 2 µM.

RecA (0.5 µM) protein was incubated in the Assay Buffer and the ATPase activity monitored in clear, flat-bottom 96-well microplate, after 7 minutes from starting of reaction (Freitag and McEntee 1988).

Data were analysed using the software OriginPro 8.5.1 and Microsoft Excel.

Inhibition assay and mechanism of inhibition

The inhibition assays were conducted with a variable concentration of suramin ranging from 0 to 100 µM. Reactions were performed in a 96-well microplate using 0.5 µM of $RecA_{N\text{-}Ter}$, 300 µM of ATP and 2 µM of $poly(dT)_{36}$, an additional microplate was added as control with 0 µM of $poly(dT)_{36}$. The ATPase activity in each well was revealed by BIOMOL Green, following manufacturer instructions, and quantified spectrophotometrically at 655 nm by microplate reader iMark, BioRad (Milan, Italy). The percentage of inhibition was calculated as the percentage residual activity of $RecA_{N\text{-}}$Ter respect to the untreated reaction.

The possible mechanism of inhibition was determined by fixing $poly(dT)_{36}$ at a saturating concentration (2 µM), suramin ranging from 0 to 10 µM, and ATP ranging from 30 to 600 µM. $RecA_{N\text{-}Ter}$ at 0.5 µM was incubated with suramin in 96-well microplate and the ATPase activity was spectrophotometrically monitored at 655 nm, an additional microplate was added as control with 0 µM of $poly(dT)_{36}$.

Data were analysed using the software OriginPro 8.5.1 and Microsoft Excel.

Competitivity assay

Competition assay was conducted by fixing suramin at 0.62 µM, ATP at 200 µM and varying $poly(dT)_{36}$ concentration from 0.0078 µM to 2 µM. $RecA_{N\text{-}Ter}$ at 0.5 µM was incubated with suramin in 96-well microplate and the ATPase activity was spectrophotometrically monitored at 655 nm.

Data were analysed using the software OriginPro 8.5.1 and Microsoft Excel.

Molecular modelling

In silico bind prediction for suramin *vs* $RecA_{N\text{-}Ter}$ was performed by using Autodock4 (version 4.2.6). Docking was conducted with the x-ray crystallographic coordinates of *E. coli* RecA protein (PDB code 3CMX). Crystallographic waters were removed. Docking parameter-imposed flexibility for the catalytic pocket residues while the remaining part of the protein was kept rigid. Dynamic molecular simulation was performed with Orac software.

RecA co-protease activity assay

RecA-dependent cleavage of LexA was performed in reaction buffer (200 mM Tris-HCl pH 7.4-7.5,80 mM $MgCl_2$, 10 mM DTT). Reaction was conducted with 2.5 µM of $RecA_{N\text{-}Ter}$, 11.5 µM of $poly(dT)_{36}$, 5 µM of LexA, 1 mM of ATP and a ranging from 0.5 µM to 50 µM of suramin. The co-protease activity was revealed by analysing the autodigestion product of LexA in an SDS-page electrophoresis.

Gels were acquired and analysed by Image Lab software (BioRad).

RESULTS AND DISCUSSION

RecA protein purification

In order to investigate how the suramin influences the biochemical activity of RecA protein, RecA was cloned into two overexpression vectors, pET28b and pET24a, so purified by His Trap column as described in materials and methods. The amount obtained for pET28b-*rec*A purification was about 30 mg of protein per litre of culture with a degree of purity higher than 95% as determined by SDS-PAGE (Fig. 19). With pET24a-*rec*A vector were obtained roughly 25 mg of protein from the same amount of culture, also in this case, the degree of purity was higher than 95% considering the first eluted fraction from the column (Fig. 20). In terms of amount of protein obtained, it was not observed no difference using two different cloned vectors.

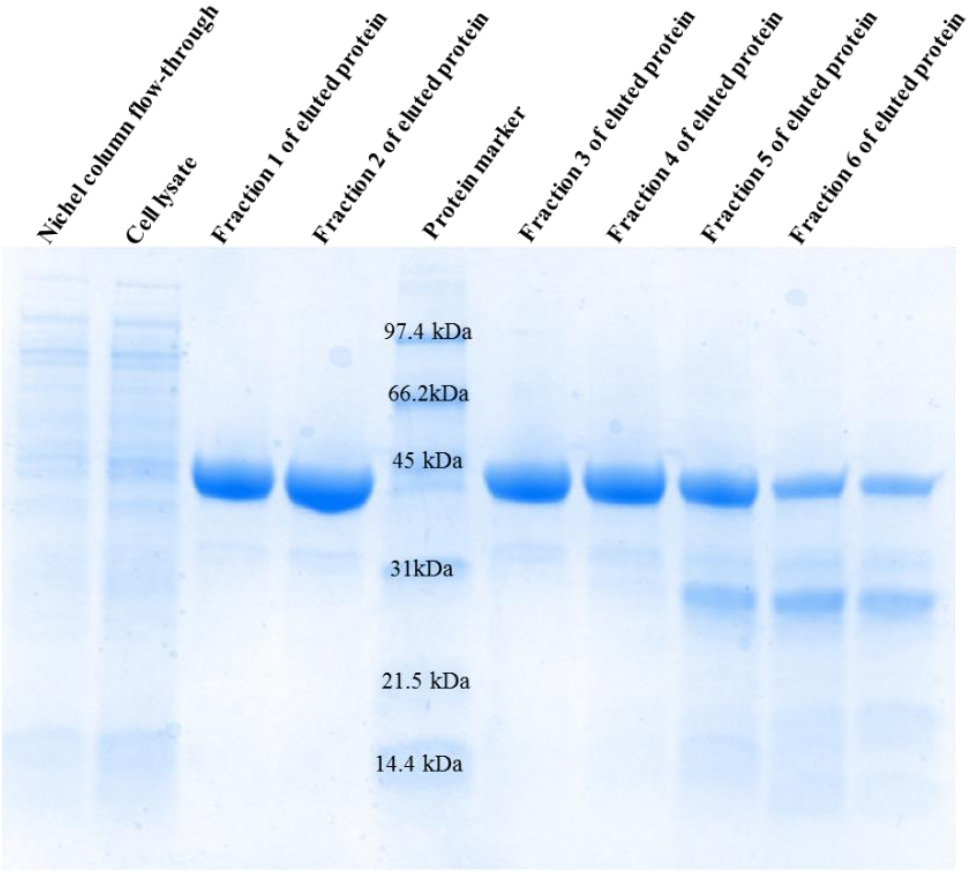


Figure 25. SDS-PAGE gel of the $RecA_{N\text{-}Ter}$ purification step. 12,5% Glycine-SDS polyacrylamide gel stained with Coomassie Brilliant Blue R250. 20 µg of samples were loaded in each well. Gel showing different eluted fractions of $RecA_{N\text{-}Ter}$ protein from purification column. Molecular weight of $RecA_{N\text{-}Ter}$ is about 40.0 kDa.

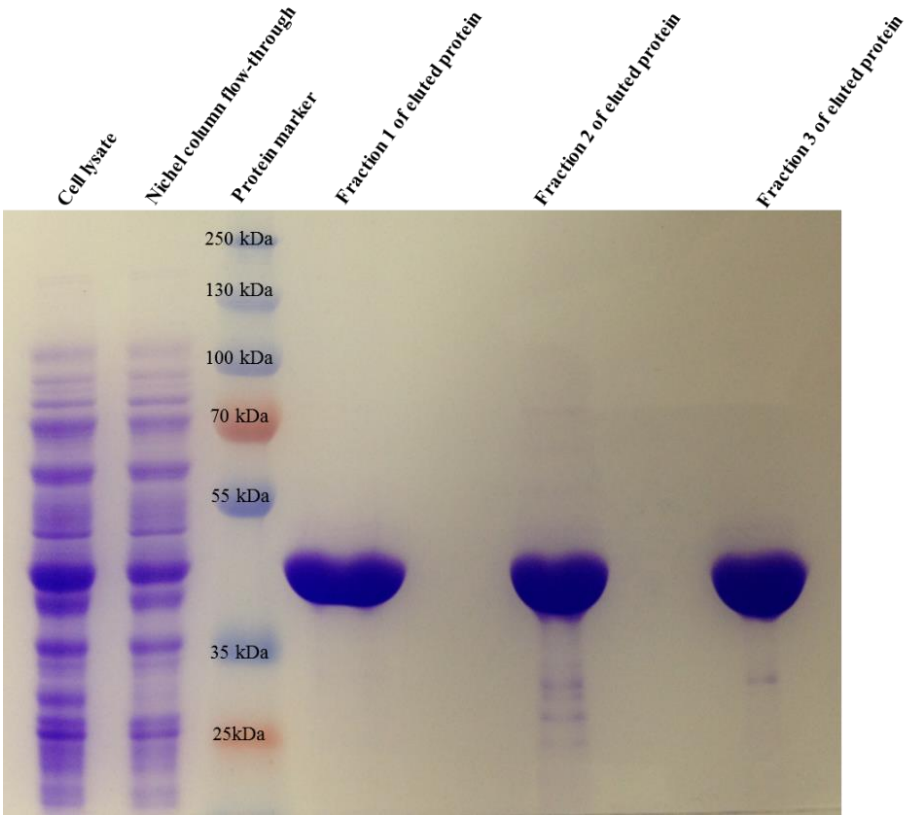


Figure 26. SDS-PAGE gel of the $RecA_{C\text{-}Ter}$ purification step. 12,5% Glycine-SDS polyacrylamide gel stained with Coomassie Brilliant Blue R250. 20 µg of samples were loaded in each well. Gel showing three different eluted fractions of $RecA_{C\text{-}Ter}$ protein from purification column. Molecular weight of $RecA_{C\text{-}Ter}$ is about 38.0 kDa.

Comparison of ATPase activity

Considering that both end-domains of RecA protein are important for the correct performance of its functions, the proteins obtained from the purifications were tested to compare their activity levels. Purchased RecA protein was used as a control of the maximum level of activity. In this regard were performed two different tests.

The reaction was conducted with a variable concentration of ATP; it was observed that $RecA_{N\text{-}Ter}$ protein and $RecA_{wt}$ show a comparable activity level and a negligible difference of P_i production after the appointed time (table 4 and Fig. 27). Conversely, $RecA_{C\text{-}Ter}$ protein presents a maximum amount of P_i production by approximately 80% lower than $RecA_{N\text{-}Ter}$ (table 4 and Fig. 27).

	Free phosphate (uM)		
ATP (uM)	**$RecA_{N\text{-}Ter}$**	**$RecA_{C\text{-}Ter}$**	**$RecA_{wtl}$**
600	29.31	6.54	24.63
300	26.57	6.55	24.67
200	22.17	6.34	22.97
100	11.21	6.55	16.24
50	3.27	2.64	5.51
30	1.35	1.06	1.84

Table 4. P_i produced after 7 minutes of reaction.

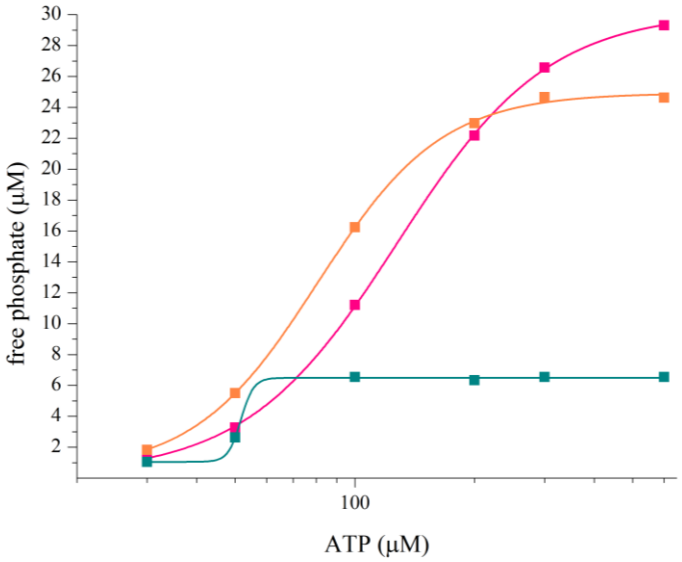


Figure 27. Semi-log plots of free phosphate produced by RecA. The figure shows in green, pink, and orange curves the total free phosphate produced, using different concentrations of ATP, by $RecA_{C\text{-}Ter}$, $RecA_{N\text{-}Ter}$ and $RecA_{wt}$, respectively.

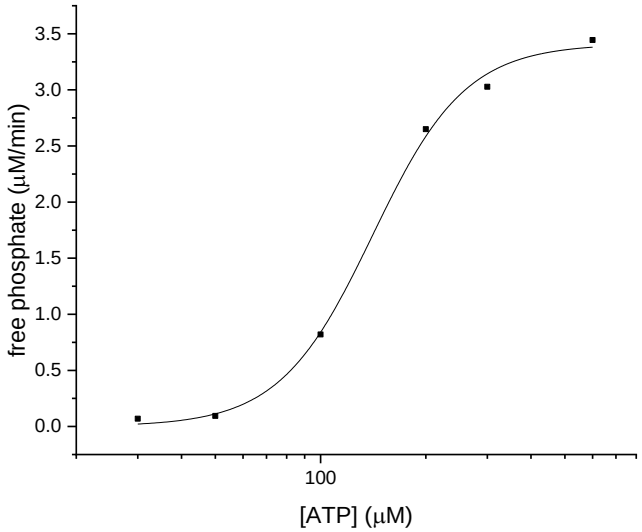
Having regard to the results, $RecA_{N\text{-}Ter}$ has activity comparable to the wild type of protein and represents the ideal candidate for the study.

Determination of RecA parameters

RecA protein works as a polymeric complex in combination with ATP and ssDNA. The ATPase activity of RecA depends on the binding with DNA. RecA activity has been described by defining the half-maximal effective concentration ($S_{0.5}$) of both substrates.

The values obtained from the experiments were 140.91±5.63 µM for ATP ($[ATP]_{50}$) and 0.32±0.01 for poly$(dT)_{36}$ ($[poly(dT)_{36}]_{50}$).

A)



B)

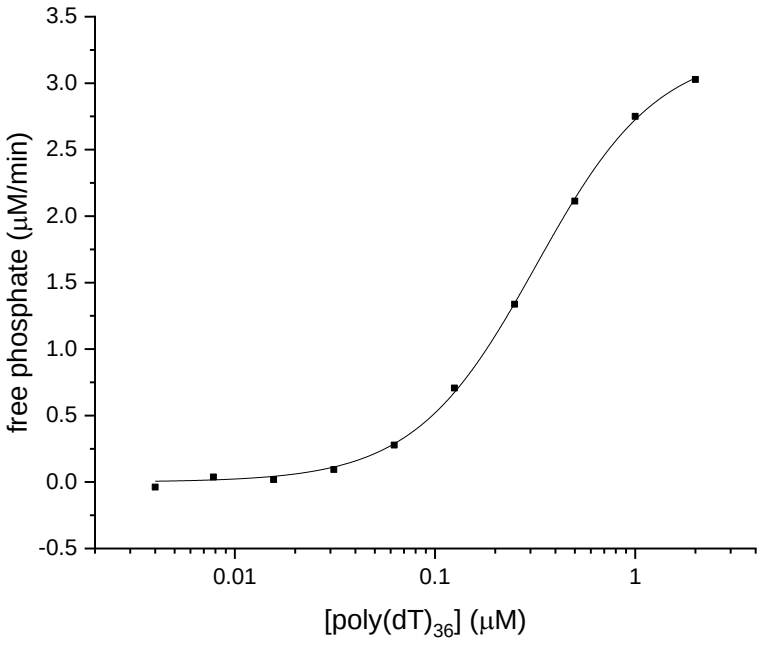


Figure 28. Semi-log plot of free phosphate produced per minute by RecA ATPase activity as function of A) concentrations of ATP, B) concentrations of poly(dT)$_{36}$.

As show in Figure 28, RecA follow a cooperative kinetic model with both substrates, this mean that the binding with poly(dT)$_{36}$ promotes the binding of ATP and vice versa.

Suramin inhibition assay

Suramin is a polysulphonated napthylurea compound synthesized in 1916 by Bayer and introduced in 1922 as antitrypanosomal drug. After a hundred year, this compound is still used in a wide array of applications, from parasitic and viral diseases to cancer (Kaur et al. 2002), as an antidote and autism treatment (Wiedemar, Hauser, and Mäser 2020). Furthermore, recently several studies investigated the use of suramin to treat the current disease of coronavirus SARS-CoV-2 (Salgado-Benvindo et al. 2020; Yin et al. 2021).

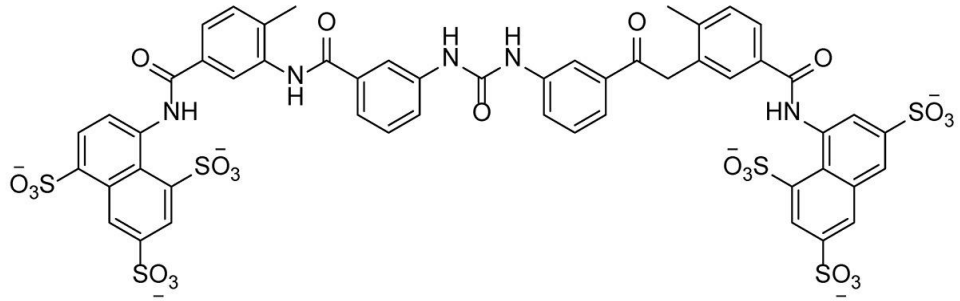
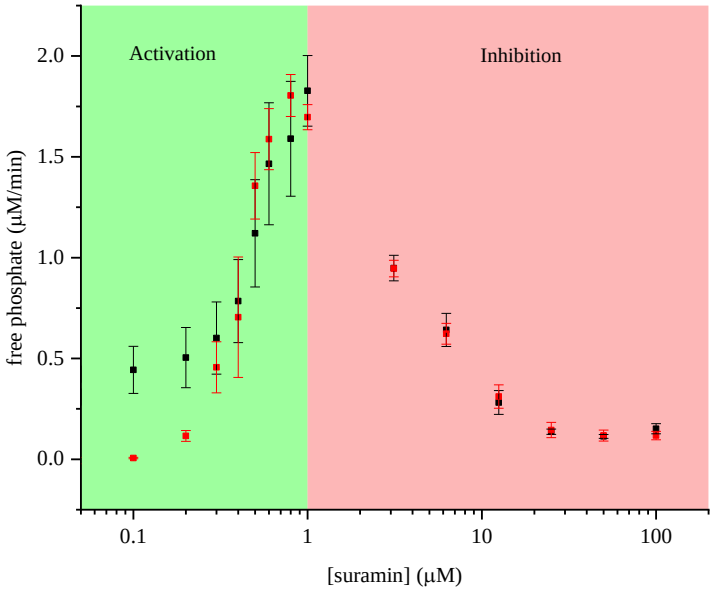


Figure 29. Chemical 2D structure of suramin
(Zhou et al. 2021)

Suramin does not show an antimicrobial activity when used as a single component. However, it has a synergic effect when combined with ciprofloxacin against *Mycobacterium tuberculosis* (Culyba, Mo, and Kohli 2015). Conversely, suramin generated interest as an inhibitor of RecA biochemical activity *in vitro*. Several studies describe that suramin was able to inhibit the ATPase activity of RecA and interfere with the three crucial functions of this protein in SOS response (Nautiyal, Patil, and Muniyappa 2014; Zhou et al. 2021).

Suramin was tested for its ability to inhibit the RecA production of free phosphate coming from the hydrolysis of ATP, which was quantified by measuring the quantity of free phosphate after seven minutes of reaction in the presence of a variable concentration of suramin ranged from 0 to 100 µM and fixing the ATP at 300 µM and poly(dT)$_{36}$ at saturating concentration of 2 µM. In addition, a negative control was conducted by a reaction without poly(dT)$_{36}$.



.

Figure 30 Semi-log plot of free phosphate product per minute by RecA ATPase activity. Free phosphate produced in presence of different suramin concentration (0-100 µM), 300 µM ATP, with 2 µM of poly(dT)36 (black solid squares), or without poly(dT)36 (red solid circle).

The presence of suramin has a dual effect on RecA ATPase activity in a concentration-dependent manner. The data revealed that at a concentration below 1 µM, suramin acts as an activator and increases the ATPase activity, as shown in Figure 30 (green zone). Above 1 µM, suramin operates as an inhibitor of RecA ATPase activity (red zone). However, the most unexpected data is the activity of RecA in the absence of poly(dT)$_{36.}$ As previously mentioned, RecA binds ssDNA to carry out its biochemical functions, included ATPase activity. In the presence of suramin, RecA becomes independent from ssDNA (Figure 30 red solid circle), and the activity follows the same trend of reaction as observed in the presence of poly(dT)$_{36}$.

To better understand how suramin modulates the ATPase activity of RecA, the reaction was ideally separated into two phases: the activation phase, for concentrations of suramin lower than 1 µM, and the inhibition phase, for concentrations of suramin higher than 1 µM.

Suramin as activator

At low concentrations, suramin promotes the RecA ATPase activity. The highest activity is observed at a concentration of 0.62 µM of suramin, approximately 60% higher than the control without suramin. It is noteworthy that the concentration of RecA in solution is about 0.5 µM, almost equivalent to the concentration of suramin at the maximum activation.

It is possible to conclude that suramin, in the activation phase, acts as a potent activator in a 1:1 stoichiometric ratio.

RecA ATPase activity in the absence of ssDNA

The effect of suramin on the RecA ATPase activity was investigated at concentrations of ATP ranging from 30 to 600 µM without poly(dT)$_{36}$.

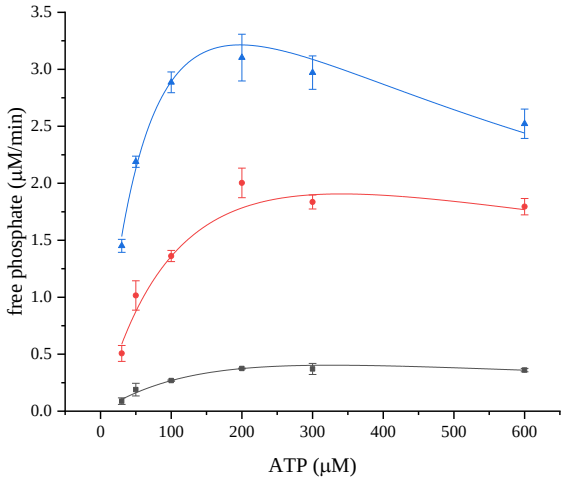


Figure 31. Free phosphate/minute produced at different ATP concentrations (from 30 to 600 µM) and different concentrations of suramin without poly(dT)$_{36}$. The reaction was conducted at three different concentrations of suramin: 0.156 µM black solid square; 0.312 µM red solid circle; 0.625 µM blue solid triangle.

As shown in Figure 31, free phosphate/minute produced in the reaction increases as a function of suramin concentration. Two main differences can be observed for the plot in the absence of suramin and the presence of ssDNA (Figure 28):

- the plot curve of free phosphate/minute produced follows a hyperbolic trend, as opposed to a sigmoidal trend in the presence of ssDNA, which characterises a cooperative model. It means that in the presence of suramin, RecA might change its cooperativity kinetic model.
- At high concentrations of substrate (ATP, the free phosphate/minute produced by RecA decreases. The shape of the curve can be interpreted as a substrate inhibition, an effect that is not observed in the presence of ssDNA.

Based on this remark, suramin seems to induce a change in the enzymatic model of RecA ATPase activity, from a cooperative model (Figure 28 A) to a non-cooperative or Michaelis-Menten model-like. Furthermore, the presence of suramin might induce a decrease of activity at a high concentration of ATP as the effect of substrate inhibition.

The effect of substrate inhibition can be quantified as a non-competitive inhibition model assuming [S] as substrate and inhibitor as reported in Equation 1, where Kis represents the substrate inhibition constant.

$$v_0 = \frac{V_{max}[S]}{K_m + [S](1 + \frac{[S]}{K_{is}})}$$ **Equation 1**

The results of the non-linear fitting of Equation 1 with the data reported in Figure 31 are reported in Table 5.

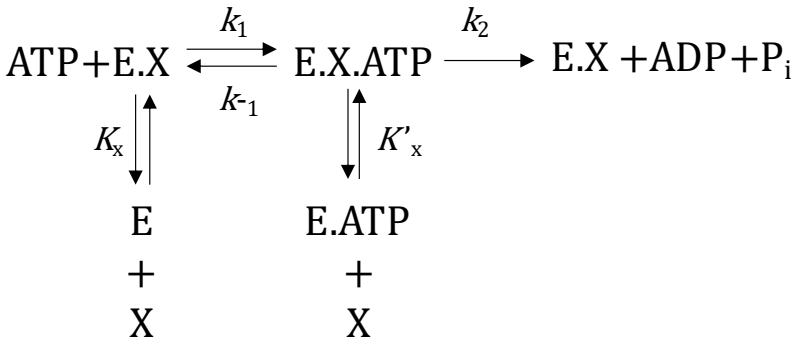
[suramin] (µM)	V_{max} **(µM*min^{-1})**	K_m **(µM)**	V_{max}/K_m **(min^{-1})**	K_{is} **[ATP] (µM)**
0.625	5.3+/-0.67	70.7+/-16.6	0.074	587.1+/-177.8
0.312	4.11+/-1.12	171.2+/-70.88	0.024	568.3+/-342.2
0.156	0.80+/-0.34	181.1+/-117.2	0.004	628.1+/-581.6

Table 5. Kinetic parameters of RecA vs suramin reaction without poly(dT)$_{36}$.

Suramin exerts several effects on the enzymatic kinect of RecA:

- the maximal velocity of reaction (V_{max}) increases with the increase of suramin concentration.
- The coefficient K_m of the reaction decreases with increasing of the concentration of suramin. It means that the affinity for the substrate (ATP) grows with increasing the suramin concentration.
- The catalytic efficiency, represented by the ratio V_{max}/K_m, increases almost 18-fold between 0.156 µM of suramin and 0.625 µM of suramin.
- K_{is} slightly decreases with increasing concentrations of suramin.

When suramin acts as an activator, the mechanism of reaction can be plausibly represented by Scheme 1, where suramin can bind both the free enzyme and RecA bonded to ATP.



Scheme 1. RecA and suramin activation reaction model (suramin is represented as X).

Equation 2 describes a mixed model of activation as represented in Scheme 1.

$$v = \frac{V'[S]}{K_m'(1 + \frac{K_x}{x}) + [S](1 + \frac{K'_x}{x})};\ Mixed\ model \qquad \textbf{Equation 2}$$

Equation 2 can be processed to determine the equilibrium dissociation constants of suramin in both cases. K_x and K'_x represent suramin equilibrium dissociation constants that bind the free RecA and the RecA-ATP complex, respectively.

The dissociation equilibrium coefficients of suramin can be determined by rearranging Equation 2 as a function of V_{max} to determine Kx (Equation 3) and in function of the ratio Vmax/Km to determine K'_x (Equation 4).

$$V_{max}^{app} = \frac{V}{1 + {K_x}/{X}} \qquad \textbf{Equation 3}$$

$${V_{max}^{app}}/{K_m^{app}} = \frac{{V}/{K_m}}{1 + {K'_x}/{X}} \qquad \textbf{Equation 4}$$

The value of K_x and K'_x represents the concentration of suramin when V_{max} and the catalytic efficient (V_{max}/K_m) reach half of the maximal value, respectively. However, in the reaction without poly(dT)$_{36}$, the plot can be represented with only 3 points of suramin concentration because below 0.156 µM the activity is too low, whereas over 0.625 µM the activity reaction is in the inhibition phase.

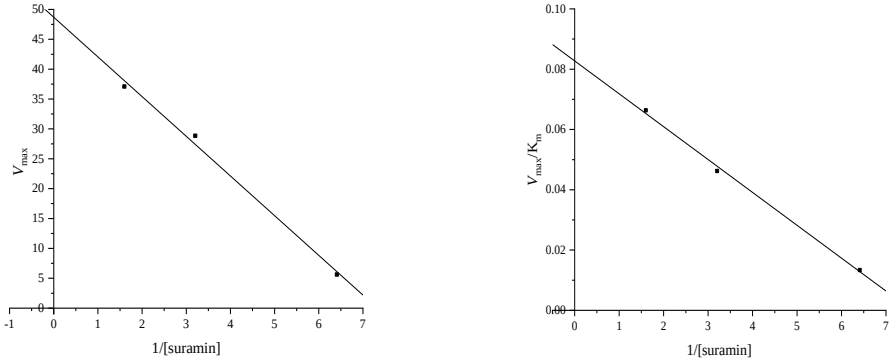


Figure 32. Kinetic parameters of RecA vs suramin in the activated phase without poly(dT)$_{36}$.

The value of K_x and K'_x has been determined through the linear regression equation by fixing the ordinate value (y) as half of its maximum value. The value obtained is 0.31 µM for each coefficient, which is coherent with the concentration of suramin at which the maximum activation of RecA is observed Figure 30.

RecA ATPase activity in the presence of ssDNA

The effect of suramin on the RecA ATPase activity was investigated at concentrations of ATP ranging from 30 to 600 µM with 2 µM poly(dT)$_{36}$.

In the reaction in which poly(dT)$_{36}$ is present, RecA shows a basal activity that is increased with the presence of suramin.

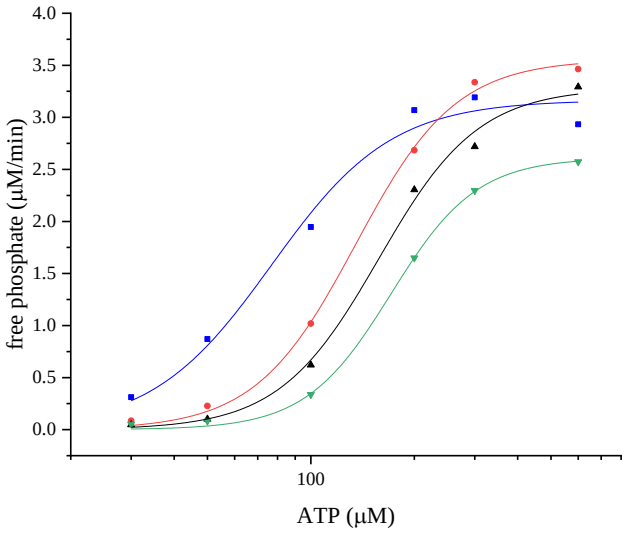


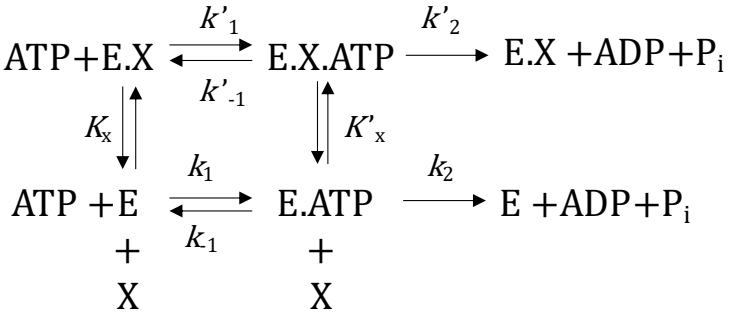
Figure 33. The semi-log plot of free phosphate was produced at different concentrations of ATP (from 30 to 600 µM) and different suramin concentrations with 2 µM poly(dT)$_{36}$. The reaction was conducted at three different concentrations of suramin: 0.156 µM black solid up triangle; 0.312 µM red solid circle; 0.625 µM blue solid square, and without suramin in the green solid down triangle.

As shown in Figure 33, the free phosphate/minute produced from the RecA activity without suramin (green solid down triangle) increases with suramin concentration. The substrate inhibition phenomenon which characterises the reaction in the absence of ssDNA (Figure 31) can be observed only at the maximum concentration of suramin (0.62 µM) when poly(dT)$_{36}$ is present in the reaction medium.

To describe the reaction of RecA ATPase activity in the presence of both suramin and poly(dT)$_{36}$, the model must consider that:

- RecA shows ATPase activity even in the absence of suramin
- Suramin can bind both free RecA and RecA complexed with ATP
- In the presence of ssDNA, RecA behaves as a cooperative enzyme.

In this case, the determination of the activation mechanism and its kinetic parameters is more complex because RecA has an activity even in the absence of the activator. It should be noted that this is not necessarily a mechanism for activation at all; indeed, if RecA bonded with suramin is less reactive than RecA bounded with ssDNA, suramin act as an inhibitor of the reaction. Furthermore, the conditions that decide whether suramin is an activator or an inhibitor may differ in different substrate concentrations. In the situation that $k_2 > k'_2$ suramin acts as an inhibitor at high concentrations of substrate. Therefore, the behaviour of suramin is much more complex since it can have different actions depending on both its concentration and substrate concentration. Suramin can be defined as a *general modifier*, where "*modifier"* is a term that embraces both inhibitors and activators. The *general modifier* mechanism is described in Scheme 2.



Scheme 2. General modifier mechanism.

As previously described (Figure 28), in the presence of poly(dT)$_{36}$ RecA follows a cooperative kinetic model, which can be described by the Hill equation. However, must be considered the effect of substrate inhibition observed at the highest concentrations of suramin. The Hill equation that takes into account the effect of substrate inhibition is represented in Equation 5.

$$v_0 = \frac{V_{max}[S]^h}{K_{0.5}^h + [S]^h(1 + \frac{[S]}{K_{is}})}$$ **Equation 5**

[suramin] (µM)	V_{max} (µM*min^{-1})	$K_{0.5}$ (µM)	V_{max}/K_m (min^{-1})	K_{is} [ATP] (µM)
0.625	4.482+/-0.48	109.12+/-13.42	0.041	1218.11+/-401.36
0.312	4.489+/-0.17	161.95+/-5.18	0.027	2318.56+/415.11
0.156	3.282+/-0.16	157.61+/-10.78	0.020	>10^4
0.078	3.591+/-0.35	179.84+/-11.80	0.019	>10^4
0.039	3.134+/-0.05	178.56+/-3.79	0.017	>10^4
0.0192	3.086+/-0.04	184.29+/-3.33	0.016	>10^4
0.0098	3.077+/-0.39	184.88+/-15.52	0.016	>10^4
0	2.725+/-0.34	175.59+/-13.89	0.015	>10^4

Table 6. Kinetic parameters of RecA vs suramin reaction in the presence of poly(dT)$_{36}$.

The kinetic parameter of the reaction schematised in Scheme 2 and determined by Equation 5 are reported in Table 6.

As expected, the coefficient $K_{0.5}$ decreases with suramin concentration, while V_{max} and the catalytic efficiency ($V_{max}/K_{0.5}$) increases. The constant K_{is} at the maximum concentration of suramin has a value comparable at the twice of the maximum substrate concentration. At low concentrations of suramin, the K_{is} value is so high that it is insignificant in Equation 5.

The dissociation equilibrium coefficients of suramin (K_x and K'_x) can be determined as previously done (by plotting Equation 3 and Equation 4 against the reciprocal of suramin concentration).

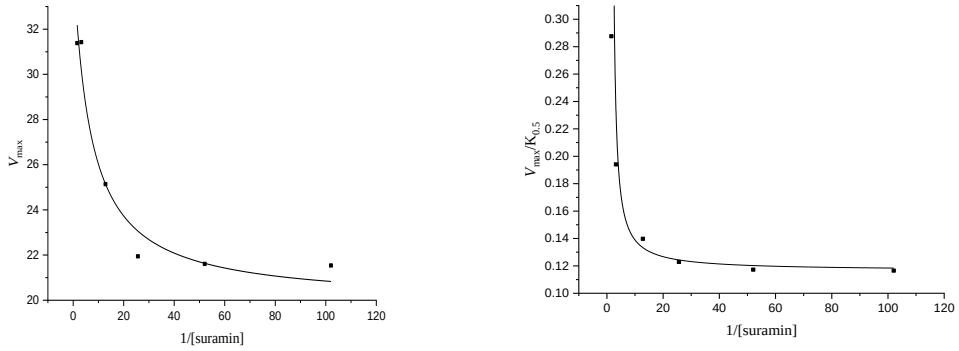


Figure 34. Kinetic parameters of RecA vs suramin in the activation reaction with 2 µM of poly(dT)$_{36}$.

The coefficient K_x and K'_x has been determined through a non-linear fitting of the plot, and the value is 0.19 µM and 0.15 µM, respectively.

Suramin as inhibitor

As shown in Figure 30 (red zone), once exceeded 0.62 µM of suramin, a further increase in suramin concentration leads to decreased ATPase activity of RecA protein. Besides, both reactions, in the presence and without the poly(dT)$_{36}$, follow the same trend. It is plausible that once suramin reaches the stoichiometric concentration of 1:1 with RecA, additional molecules of suramin bind on another site, inducing the inactivation of ATPase activity. To verify this hypothesis, the kinetic parameters of both reactions were determined as previously done.

RecA ATPase activity in the absence of ssDNA

The inhibition phase of suramin was investigated from 1.25 µM to 10 µM at different concentration of ATP ranged from 30 µM to 600 µM.

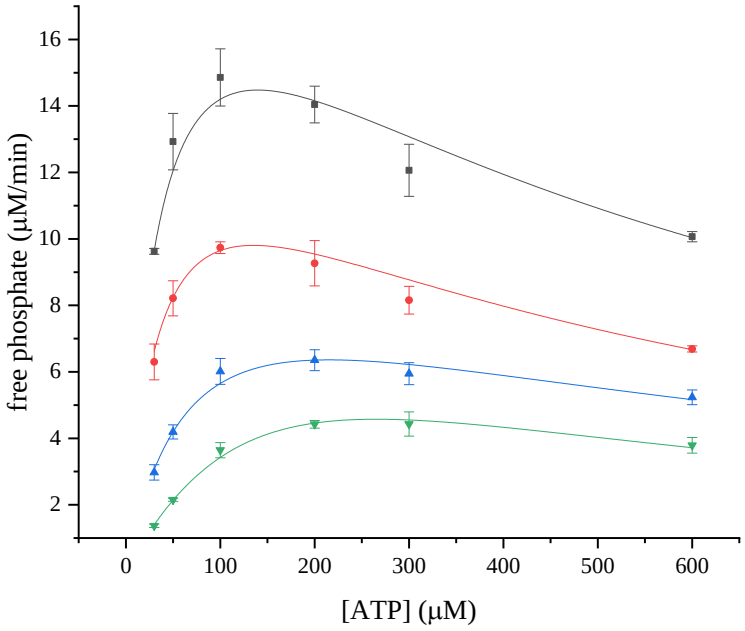


Figure 35. Free phosphate/minute produced at several ATP concentrations (from 30 to 600 µM) and several concentrations of suramin without poly(dT)$_{36}$. The reaction was conducted with four different concentrations of suramin: 1.25 µM black solid square; 2.5 µM red solid circle; 5 µM blue solid up triangle, and 10 µM green solid down triangle**.**

As opposed to the activation phase, starting from a concentration of 1.25 µM of suramin, the RecA ATPase activity decreases with increasing suramin concentration. Moreover, even at this phase, an evident substrate inhibition effect is observed, except for the highest suramin concentrations 5 µM and 10 µM.

[suramin] (µM)	V_{max} (µM*min^{-1})	K_m (µM)	V_{max}/K_m (min^{-1})	K_{is} [ATP] (µM)
10	1.657±0.45	204.616±78.08	0.008	339.753±156.6
5	1.564±0.23	74.355±20.60	0.021	580.946±207.9
2.5	2.169±0.28	38.259±11.34	0.056	459.019±140.5
1.25	3.330±0.43	37.253±11.03	0.089	430.820±128.2

Table 7. Kinect parameters RecA vs suramin inhibition without poly(dT)$_{36}$.

The kinetic parameters were calculated with Equation 1 which considers the presence of substrate inhibition. To ascertain the inhibition model, the kinetic parameters were plotted against the concentration of suramin.

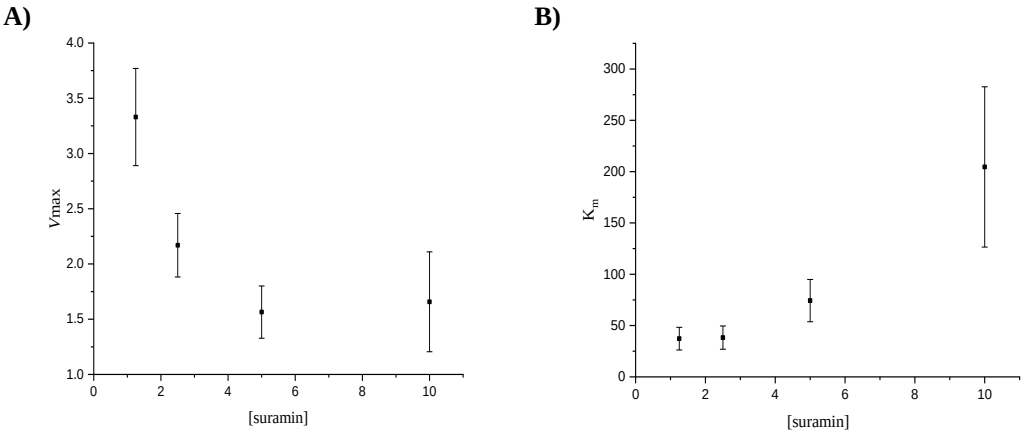


Figure 36. Kinect parameters RecA vs suramin inhibition without poly(dT)$_{36}$. A) Plotting of Vmax against suramin; B) Plotting of Km against suramin.

As shown in Figure 36, both K_m and V_{max} change with increasing suramin concentration. It means that the presence of suramin alters both the reaction rate and the substrate affinity. Therefore, considering these results, the model of inhibition is a non-competitive model or mixed model.

RecA ATPase activity in the presence of ssDNA

The same reaction was conducted in the presence of poly(dT)$_{36}$ to verify if at several range of ATP the results follow the same trend of reaction without ssDNA.

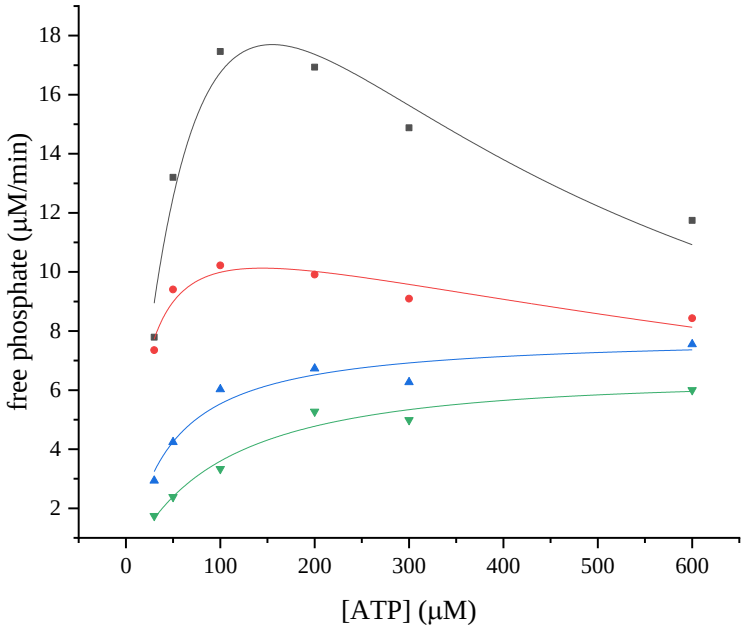


Figure 37. Free phosphate/minute produced at different ATP concentrations (from 30 to 600 µM) and different suramin concentrations with 2 µM poly(dT)$_{36}$. The reaction was conducted with four different concentrations of suramin: 1.25 µM black solid square; 2.5 µM red solid circle; 5 µM blue solid up triangle, and 10 µM green solid down triangle**.**

The resulting plot of reaction is superimposable with the plot of the reaction without poly(dT)$_{36}$. These results confirm what has been observed in Figure 30. Furthermore, the substrate inhibition effect is evident at the same concentrations of suramin as those observed in the absence of ssDNA.

However, even if the plot shows the same trend, to determinate the kinetic parameters, must be considered the cooperativity of ssDNA with RecA. Hence, the kinetic parameters were calculated with the Equation 5.

[suramin] (µM)	**V_{max} (µM*min^{-1})**	**K_m (µM)**	**V_{max}/K_m (min^{-1})**	**K_{is} [ATP] (µM)**
10	0.967±0.15	88.083±34.53	0.010	>10^4
5	1.036±0.08	38.770±7.33	0.026	>10^4
2.5	1.544±0.05	22.320±1.92	0.069	2003.96±513.6
1.25	3.226±0.15	40.112±2.03	0.080	635.17±82.3

Table 8. Kinect parameters RecA vs suramin inhibition in the presence of poly(dT)$_{36}$.

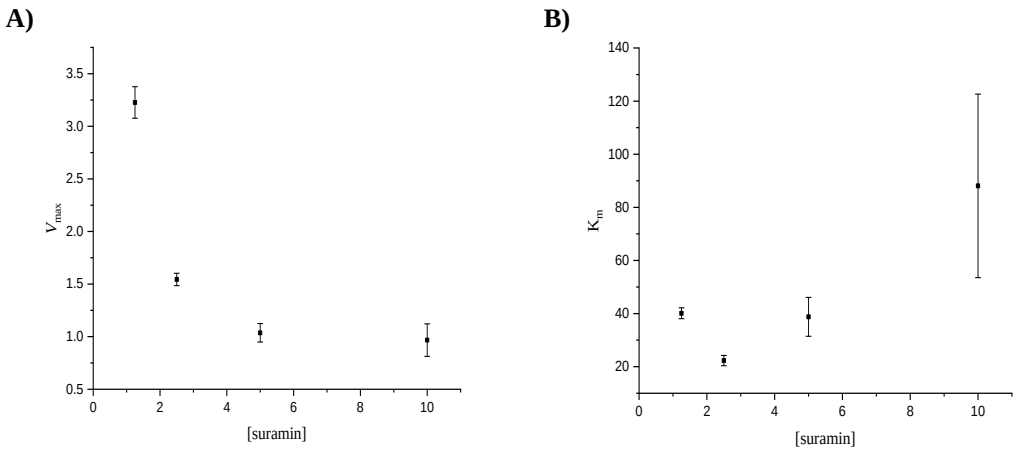
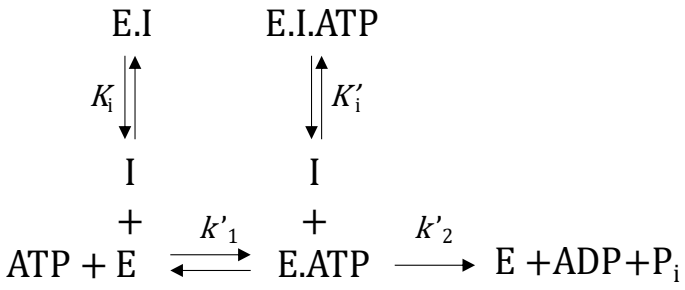


Figure 38. Kinect parameters RecA vs suramin inhibition in the presence of poly(dT)$_{36}$. A) Plotting of V_{max} against suramin; B) Plotting of K_m against suramin.

The kinetic parameters observed in Figure 38 follow the same trend of the reaction without ssDNA. It means that suramin induces the same inhibition model on RecA ATPase activity even when poly(dT)$_{36}$ is present.

Inhibition model

Regarding the results, it is reasonable to suppose that the presence of ssDNA is irrelevant in the reaction in the inhibition phase. Indeed, the inhibition model is the same for both reactions, hence, the possible mechanism of inhibition can be schematised as reported in scheme 3



Scheme 3. Reaction model of inhibition of RecA ATPase activity by suramin (I).

Scheme 3 can be represented with Equation 6, which describes a mixed model of inhibition, where both V_{max} and K_m are affected by inhibition.

$$v_0 = \frac{V_{max}[S]}{[S]\left(1+\frac{I}{K_i}\right)+K_m\left(1+\frac{I}{K'_i}\right)} \qquad \textbf{Equation 6}$$

The dissociation equilibrium coefficients of suramin can be determined by rearranging **Equation 6** as a function of V_{max} for determining K_i (**Equation 7**) and as a function of the ratio V_{max}/K_m to determine K'_i (**Equation 8**).

$$V_{max}^{app} = \frac{V}{1 + {I}/{K_i}} \qquad \textbf{Equation 7}$$

$$V_{max}^{app}/K_m^{app} = \frac{V/K_m}{1 + {I}/{K'_i}} \qquad \textbf{Equation 8}$$

The value of K_i and K'_i can be determined by the non-linear fitting of the plot of V_{max} and V_{max}/K_m against the concentration of suramin reach half of the maximal value, respectively.

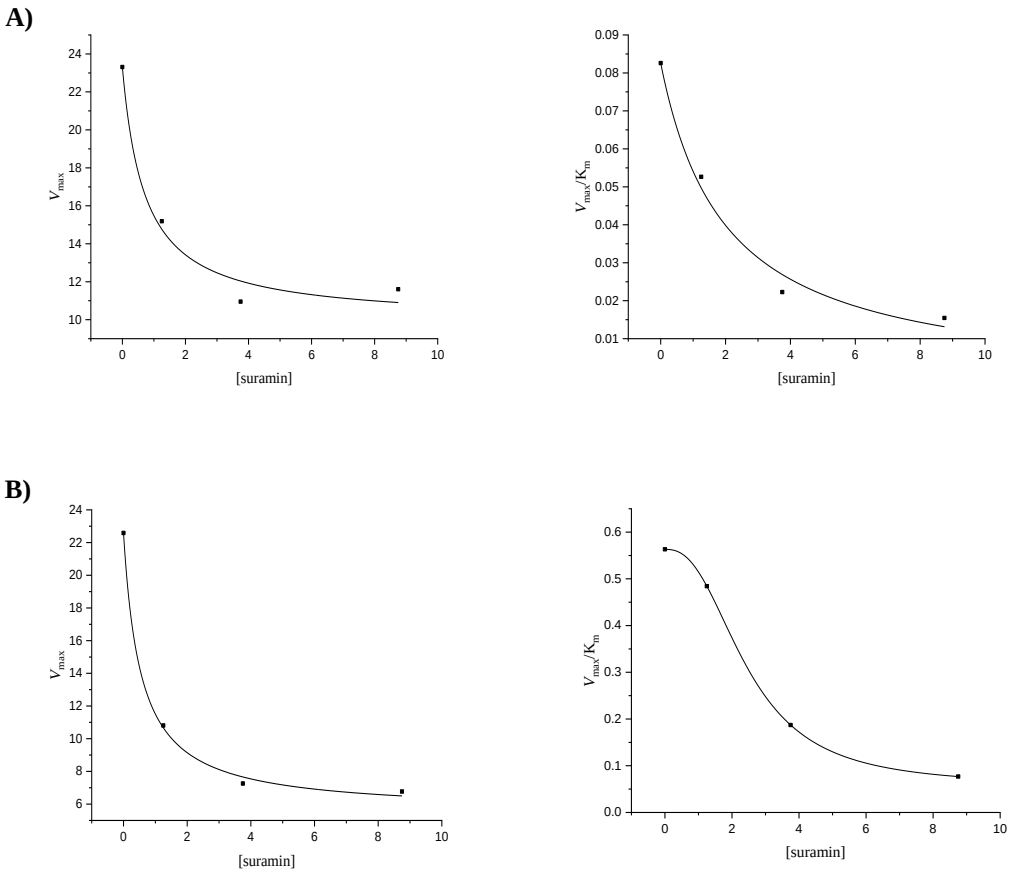


Figure 39. Kinetic parameters of RecA vs suramin. A) reaction without poly(dT)$_{36}$; B) reaction with 2 µM of poly(dT)$_{36}$.

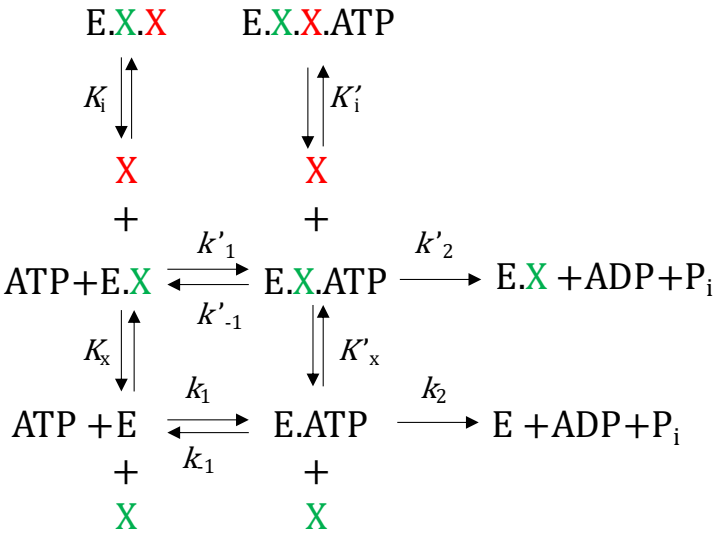
The values of the dissociation equilibrium coefficients have been determined to be:

- K_i = 1.96 µM and K'_I = 3.23 µM for the reaction without poly(dT)$_{36}$.
- K_i = 1.79 µM and K'_I = 3.7 µM for the reaction with 2 µM poly(dT)$_{36}$.

In summary, the suramin exerts several effects on the ATPase activity of RecA:

- At low concentrations (<1 µM) increases the ATPase activity
- Allows RecA to carry out its ATPase activity regardless of the presence of ssDNA
- High concentrations of substrate induce substrate inhibition
- High concentrations inhibit ATPase activity.

All these observations reveal that suramin does not act exclusively as an inhibitor of the ATPase activity of RecA. Indeed, depending on its concentrations and substrate concentrations, it can have diverse effects. Therefore, it might be more accurate to conceive the suramin as a modulator of ATPase activity of RecA. This model of modulation of RecA activity by suramin can be schematised as reported in Scheme 4



Scheme 4. General modifier model; suramin has represented as X. The suramin that acts as an activator is represented in green, while in red represents the suramin when acting as an inhibitor.

Analysing all the results and as represented in Scheme 4, it can be deduced that suramin might have two possible binding sites on RecA. The binding of suramin to these two sites leads to the opposite effect or activation and inhibition of ATPase activity of RecA. It can be asserted that the affinity for the site that leads to increase ATPase activity is higher than the site that decreases the activity: K_x and $K'_x < K_i$ and K'_i. Generally, it reasonable to suppose that suramin occupies first the activation binding site on RecA. When the concentration of suramin reaches the stoichiometric ratio of 1:1 with RecA, suramin binds the second site that inhibits the activity.

Competition assay

In order to evaluate the possibility that suramin can bind the ssDNA binding site on RecA and investigate the mechanism by which suramin induce the ATPase activity of RecA even in the absence of poly(dT)$_{36}$, a competition assay was performed.

The competitivity between suramin and poly(dT)$_{36}$ for the ssDNA binding site on RecA was evaluated by fixing the ATP concentration at approximately [ATP]$_{50}$, suramin at a concentration where maximum activity is observed, and increasing concentration of poly(dT)$_{36}$ (ranging from 0.0078 µM to 2 µM). Comparing the ratio between the velocity of reaction in the presence and without suramin (V_a and V_0, respectively) against the concentration of poly(dT)$_{36}$, it is possible to assess if poly(dT)$_{36}$ acts as a competitor for the binding site of the suramin on RecA.

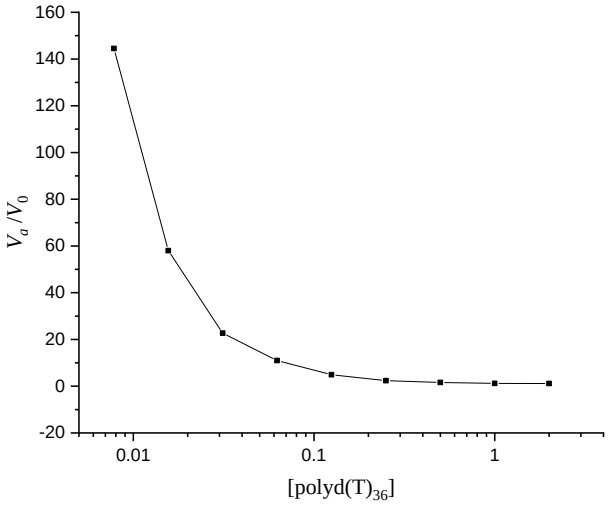


Figure 40. Semi-log plot of the ratio of the V_a (in the presence of suramin) and V_0 (in the absence of suramin) against poly(dT)$_{36}$ concentration. The reaction was performed fixing [ATP] at 200 µM, [suramin] at 0.625 µM and different range of poly(dT)$_{36}$ ranging from 0.0078 µM to 2 µM.

As previously mentioned, RecA preferentially binds ssDNA forming a nucleoprotein filament, and its active form is in a polymeric complex. In the presence of ssDNA, RecA shows a high ATP hydrolytic activity, which is critical for nucleofilament assembly and disassembly. Several studies have demonstrated that the binding site of ATP is located at the monomer-monomer interface; therefore, the only active form able to hydrolyse ATP is the nucleofilament. However, as can be seen from the results, in presence of suramin RecA hydrolyses ATP even in the absence of ssDNA. As observed in Figure 40, the ratio V_a/ V_0 decreases with increasing poly(dT)$_{36}$. As poly(dT)$_{36}$ concentration gradually increases, suramin is displaced from the binding site, and the reaction rate is comparable with the rate of

reaction in the absence of suramin. It is possible to conclude that suramin binds on ssDNA binding site on RecA (L2 region).

Molecular modelling

As previously described RecA possesses four binding sites on the core domain: ATP binding site (Walker A motif, residues 66-73), Mg^{2+} binding site (Walker B motif residues 139-144), ssDNA binding site (L2 loop, residues 195-209) and dsDNA binding site (L1 loop, residues 157-164) (Zhou et al. 2021).

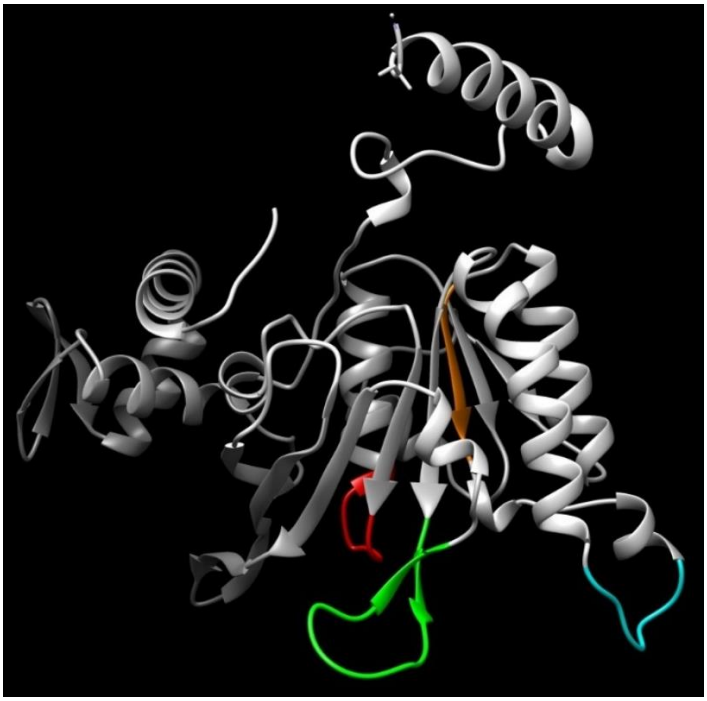


Figure 41. RecA protein model. In red the ATP binding site; in orange Mg2+ binding site, in green ssDNA binding site, and in blue dsDNA binding site.

The biochemical activity of RecA depends on the presence of ssDNA, included ATPase activity. However, it has been experimentally demonstrated that in the presence of suramin, RecA can hydrolyse ATP even in the absence of ssDNA. From kinetic data was observed that suramin might compete with the ssDNA binding site. It is reasonable to suppose that suramin can bind the L2 loop on RecA, inducing a conformational change in the protein, allowing the hydrolysis of ATP. A docking simulation was performed between RecA and suramin to verify this hypothesis.

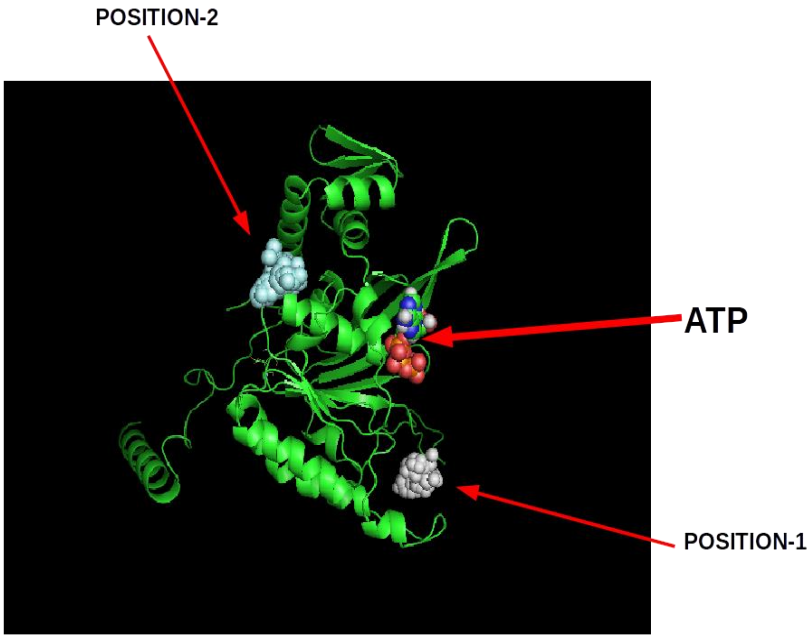
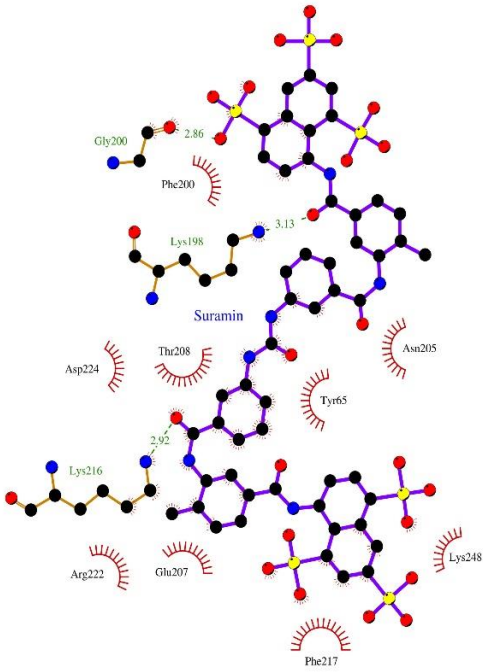


Figure 42. Molecular model of RecA protein with the possible sites for suramin.

The *in-silico* analysis reveals that suramin forms stable interaction with 2 sites on RecA, this result confirms what was predicted with the kinetic experiments. One of these (on position 1 in Figure 42) overlaps the L2 loop on RecA, representing the binding site for ssDNA. Suramin interacts with the L2 loop on RecA *via* three hydrogen bonds: with the ε-amino group of Lys-198 and Lys-216, and with the hydrogen group of Gly-201, furthermore makes several hydrophobic interactions with different amino acid in the L2 loop.

A)



B)

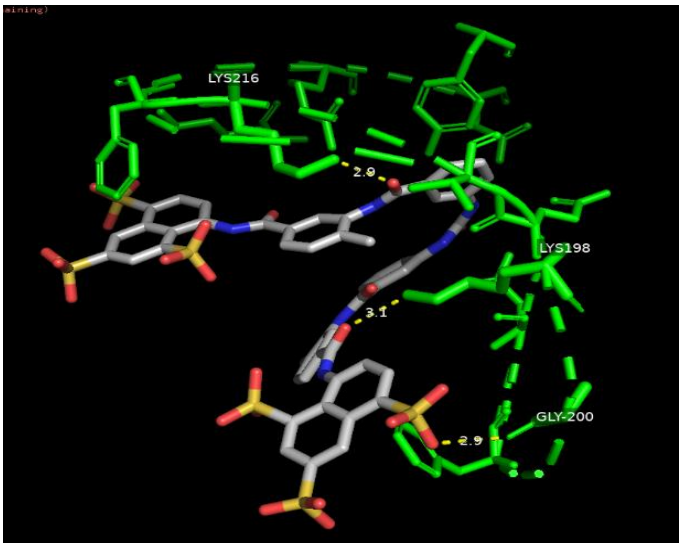
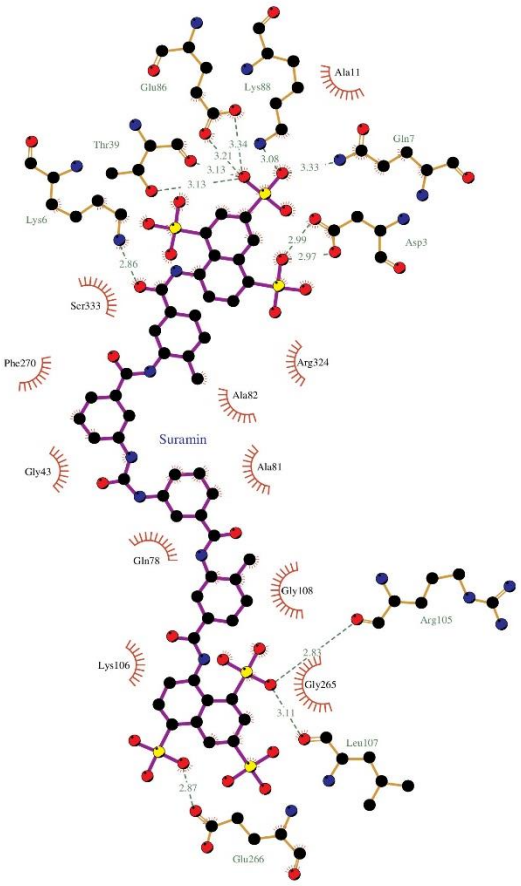


Figure 43. Docking simulation of the binding of suramin to RecA in position 1. A) ligand interaction between RecA L2 loop and suramin; B) molecular modelling of RecA L2 loop and suramin.

The second site (position 2) does not overlap to knowing sites, confirming that suramin acts as non-competitive inhibitor. In this position suramin makes several hydrogen bonds with a large number of residues.

A)



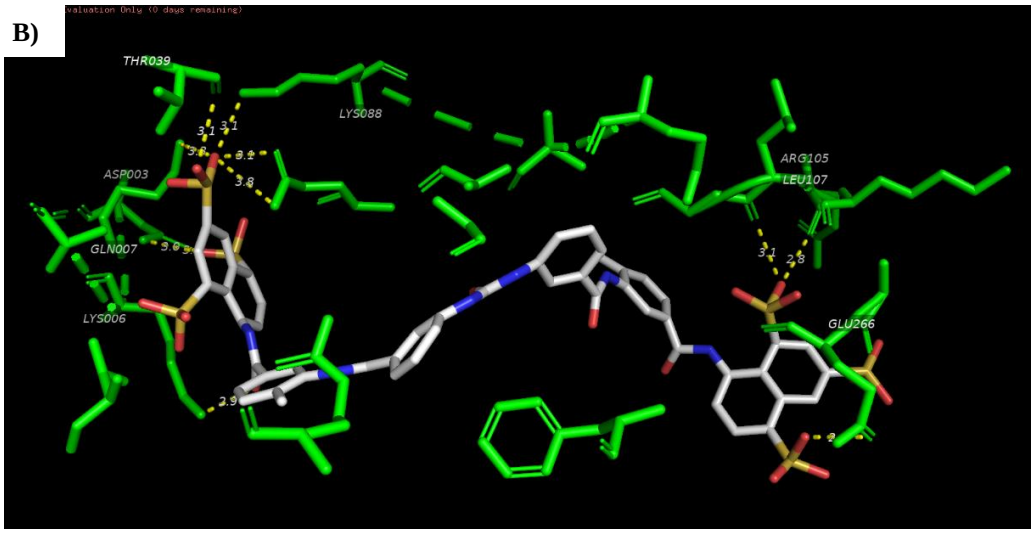


Figure 44. Docking simulation of the binding of suramin to RecA in position-2. A) ligand interaction between RecA L2 loop and suramin; B) molecular modelling of RecA L2 loop and suramin.

RecA co-protease activity assay

The direct involvement of RecA in the SOS response is given by the fact that it is the main responsible for the induction of LexA autoproteolysis. LexA is a transcriptional repressor that binds the SOS box sequences and inhibits the expression of the genes involved in the SOS response. RecA protein interacts with LexA to promote its autoproteolysis and consecutively release the promoter, promoting the expression of genes involved in SOS response.

In order to investigate if suramin can alter the co-protease activity of RecA, the cleavage reaction of RecA was conducted at several concentrations of suramin, ranging from 0.5 µM to 50 µM.

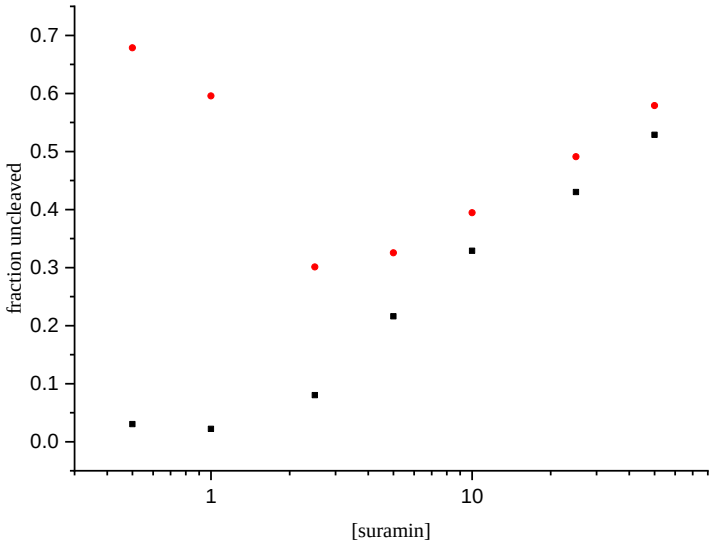


Figure 45. Semi-log plot of the fraction of LexA un-cleaved in function of the suramin concentration. The reaction was conducted in presence of poly(dT)36 (black solid square); and without poly(dT)36 (red solid circle).

As shown in Figure 45, suramin alters the co-protease activity of LexA in the same fashion observed in RecA ATPase activity or rather depends on suramin concentration. Moreover, in this assay, RecA shows activity in the presence of suramin, even in the absence of poly(dT).

CONCLUSION

The SOS response represents an extraordinary strategy by which bacteria resist environmental stresses and is responsible for the development of antibiotic resistance. Hence, targeting this pathway represents an interesting approach to contrast the bacterial evolution of drug resistance.

Another reason why the SOS response represents an attractive target is that is regulated mainly by two proteins, the RecA/LexA dyad. The inhibition of one of these two proteins might impair the mechanism of bacterial resistance evolution.

This work focused on the investigation of RecA biochemical activities and the clarification of suramin inhibition mechanism. Previously studies demonstrated that suramin is able to inhibit the biochemical activities of RecA (Nautiyal, Patil, and Muniyappa 2014; Zhou et al. 2021), although the inhibition mechanism was not determined.

In this thesis, in addition to defining the mechanism of inhibition of suramin, it was reported a behaviour of RecA never previously observed. In presence of suramin, RecA shows ATPase activity even in the absence of ssDNA. This effect is interesting since several studies have fully demonstrated that RecA activities are depending on the presence of ssDNA and nucleoprotein filament formation. Furthermore, the effect of suramin on RecA depends on its concentration: at low concentration suramin acts as potent activator of the ATPase and co-protease activity while, once exceeded a threshold value, suramin acts as a moderate inhibitor.

This dual effect of suramin introduces a challenge in the discovery of RecA inhibitor. In fact, what appears to be a moderate inhibitor at high concentrations, at low concentration dramatically increases RecA activity. Besides, the most severe problem is that in the presence of suramin RecA can induce the SOS response even in the absence of ssDNA, which is the trigger signal for SOS response initiation, promoting bacterial mutagenesis.

Docking simulations showed that suramin competitively binds the ssDNA binding site (L2 loop) when it acts as an activator and, a further and never described binding site, which appears to be responsible for the inhibition phase, at higher concentrations. This site can be exploited as target for the development of new potential inhibitors to contrast the SOS response induction.

References

Adikesavan, Anbu K. et al. 2011. "Separation of Recombination and SOS Response in Escherichia Coli RecA Suggests LexA Interaction Sites" ed. Diarmaid Hughes. *PLoS Genetics* 7(9): e1002244. https://dx.plos.org/10.1371/journal.pgen.1002244 (February 2, 2021).

AJ, Mustard. 1998. "A Genetic Analysis of RecA-LexA Protein Interactions in Escherichia Coli." University of Arizona.

Alam, Md Kausar et al. 2016. "RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance." *Cell Chemical Biology* 23(3): 381–91. https://pubmed.ncbi.nlm.nih.gov/26991103/ (January 13, 2021).

Alanis, Alfonso J. 2005. "Resistance to Antibiotics: Are We in the Post-Antibiotic Era?" *Archives of Medical Research* 36(6): 697–705.

Aminov, Rustam I. 2009. "The Role of Antibiotics and Antibiotic Resistance in Nature." *Environmental Microbiology* 11(12): 2970–88. http://doi.wiley.com/10.1111/j.1462-2920.2009.01972.x (January 3, 2021).

Aminov, Rustam I., and Roderick I. Mackie. 2007. "Evolution and Ecology of Antibiotic Resistance Genes." *FEMS Microbiology Letters* 271(2): 147–61.

Baharoglu, Zeynep, and Didier Mazel. 2014. "SOS, the Formidable Strategy of Bacteria against Aggressions." *FEMS Microbiology Reviews* 38(6): 1126–45. https://pubmed.ncbi.nlm.nih.gov/24923554/ (December 18, 2020).

Bell, Charles E. 2005. "Structure and Mechanism of Escherichia Coli RecA ATPase." *Molecular Microbiology* 58(2): 358–66.

Bell, Jason C., and Stephen C. Kowalczykowski. 2016. "RecA: Regulation and Mechanism of a Molecular Search Engine." *Trends in Biochemical Sciences* 41(6): 491–507.

Bellio, Pierangelo et al. 2014. "Curcumin Inhibits the SOS Response Induced by Levofloxacin in Escherichia Coli." *Phytomedicine*.

———. 2017. "SOS Response in Bacteria: Inhibitory Activity of Lichen Secondary Metabolites against Escherichia Coli RecA Protein." *Phytomedicine* 29: 11–18. https://pubmed.ncbi.nlm.nih.gov/28515022/ (December 22, 2020).

Bianco, P. R., R. B. Tracy, and S. C. Kowalczykowski. 1998. "DNA Strand Exchange Proteins: A Biochemical and Physical Comparison." *Frontiers in bioscience : a journal and virtual library* 3. https://pubmed.ncbi.nlm.nih.gov/9632377/ (February 1, 2021).

Bianco, Piero R., and George M. Weinstock. 1996. "Interaction of the RecA Protein of Escherichia Coli with Single-Stranded Oligodeoxyribonucleotides." *Nucleic Acids Research* 24(24): 4933–39. https://pubmed.ncbi.nlm.nih.gov/9016663/ (February 7, 2021).

Bianco, Piero R. 2018. "RecA Protein." In *ELS*, Chichester, UK: John Wiley & Sons, Ltd, 1–12. http://doi.wiley.com/10.1002/9780470015902.a0000584.pub3 (February 4, 2021).

Bianco, Piero R, and Stephen C Kowalczykowski. 1998. *RecA Protein*.

Brown, Eric D., and Gerard D. Wright. 2016. "Antibacterial Drug Discovery in the Resistance

Era.” *Nature* 529(7586): 336–43.

Butala, M., D. Žgur-Bertok, and S. J.W. Busby. 2013. “LexA Regulatory System.” *Encyclopedia of Biological Chemistry: Second Edition* (4): 706–11.

C Reygaert, Wanda. 2018. “An Overview of the Antimicrobial Resistance Mechanisms of Bacteria.” *AIMS Microbiology* 4(3): 482–501. /pmc/articles/PMC6604941/?report=abstract (January 17, 2021).

Chen, Zhucheng, Haijuan Yang, and Nikola P. Pavletich. 2008. “Mechanism of Homologous Recombination from the RecA-SsDNA/DsDNA Structures.” *Nature* 453(7194): 489–94. https://pubmed.ncbi.nlm.nih.gov/18497818/ (February 17, 2021).

CLARK, A. J., and A. D. MARGULIES. 1965. “ISOLATION AND CHARACTERIZATION OF RECOMBINATION-DEFICIENT MUTANTS OF.” *Proceedings of the National Academy of Sciences of the United States of* 53(2): 451–59. https://pubmed.ncbi.nlm.nih.gov/14294081/ (February 2, 2021).

Cox, Michael M. 2007. “Regulation of Bacterial RecA Protein Function.” *Critical Reviews in Biochemistry and Molecular Biology* 42(1): 41–63.

Culyba, Matthew J., Charlie Y. Mo, and Rahul M. Kohli. 2015. “Targets for Combating the Evolution of Acquired Antibiotic Resistance.” *Biochemistry*.

Darwin, Charles. 1859. Darwin *On the Origin of the Species*.

Finley, Rita L et al. 2013. “The Scourge of Antibiotic Resistance: The Important Role of the Environment.” https://academic.oup.com/cid/article/57/5/704/311765 (January 2, 2021).

Giese, Kim C., Christine B. Michalowski, and John W. Little. 2008. “RecA-Dependent Cleavage of LexA Dimers.” *Journal of Molecular Biology* 377(1): 148–61.

Goodman, Myron F., John P. McDonald, Malgorzata M. Jaszczur, and Roger Woodgate. 2016. “Insights into the Complex Levels of Regulation Imposed on Escherichia Coli DNA Polymerase V.” *DNA Repair* 44: 42–50.

Haruta, Shin, and Nanako Kanno. 2015. “Survivability of Microbes in Natural Environments and Their Ecological Impacts.” *Microbes and Environments* 30(2): 123–25. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4462920/ (January 1, 2021).

van der Heijden, Thijn et al. 2008. “Homologous Recombination in Real Time: DNA Strand Exchange by RecA.” *Molecular Cell* 30(4): 530–38.

Janion, Celina. 2001. “Some Aspects of the SOS Response System - A Critical Survey.” *Acta Biochimica Polonica* 48(3): 599–610. https://ojs.ptbioch.edu.pl/index.php/abp/article/view/3894 (January 27, 2021).

———. 2008. “Inducible SOS Response System of DNA Repair and Mutagenesis in Escherichia Coli.” *International Journal of Biological Sciences* 4(6): 338–44. https://pubmed.ncbi.nlm.nih.gov/18825275/ (January 5, 2021).

Jiang, Qingfei et al. 2009. “The Active Form of DNA Polymerase v Is UmuD′ 2 C-RecA-ATP.” *Nature* 460(7253): 359–63.

Joo, Chirlmin et al. 2006. “Real-Time Observation of RecA Filament Dynamics with Single Monomer Resolution.” *Cell* 126(3): 515–27. https://pubmed.ncbi.nlm.nih.gov/16901785/ (February 7, 2021).

Kaniecki, Kyle, Luisina De Tullio, and Eric C. Greene. 2018. “A Change of View: Homologous Recombination at Single-Molecule Resolution.” *Nature Reviews Genetics* 19(4): 191–207. https://www.nature.com/articles/nrg.2017.92 (February 8, 2021).

Katz, F. S., and F. R. Bryant. 2001. “Interdependence of the Kinetics of NTP Hydrolysis and the Stability of the RecA - SsDNA Complex.” *Biochemistry* 40(37): 11082–89. https://pubmed.ncbi.nlm.nih.gov/11551205/ (February 7, 2021).

Kaur, Maninderjeet et al. 2002. 20 Investigational New Drugs *Suramin’s Development: What Did We Learn?*

Kim, Taejin et al. 2015. “Directed Evolution of RecA Variants with Enhanced Capacity for Conjugational Recombination.” *PLoS Genetics* 11(6).

Kohanski, Michael A., Daniel J. Dwyer, and James J. Collins. 2010. “How Antibiotics Kill Bacteria: From Targets to Networks.” *Nature Reviews Microbiology* 8(6): 423–35. https://pubmed.ncbi.nlm.nih.gov/20440275/ (January 13, 2021).

Kovačič, Lidija et al. 2013. “Structural Insight into LexA-RecA* Interaction.” *Nucleic Acids Research* 41(21): 9901–10.

Krishna, Sandeep, Sergei Maslov, and Kim Sneppen. 2007. “UV-Induced Mutagenesis in Escherichia Coli SOS Response: A Quantitative Model.” *PLoS Computational Biology* 3(3): 0451–62.

Laureti, Luisa, Ivan Matic, and Arnaud Gutierrez. 2013. “Bacterial Responses and Genome Instability Induced by Subinhibitory Concentrations of Antibiotics.” *Antibiotics* 2(1): 100–114. /pmc/articles/PMC4790301/?report=abstract (January 13, 2021).

Lee, Andrew M., Tim J. Wigle, and Scott F. Singleton. 2007. “A Complementary Pair of Rapid Molecular Screening Assays for RecA Activities.” *Analytical Biochemistry* 367(2): 247–58. https://pubmed.ncbi.nlm.nih.gov/17601483/ (March 26, 2021).

Lusetti, Shelley L., and Michael M. Cox. 2002. “The Bacterial RecA Protein and the Recombinational DNA Repair of Stalled Replication Forks.” *Annual Review of Biochemistry* 71: 71–100.

Maslowska, Katarzyna H., Karolina Makiela-Dzbenska, and Iwona J. Fijalkowska. 2019. “The SOS System: A Complex and Tightly Regulated Response to DNA Damage.” *Environmental and Molecular Mutagenesis* 60(4): 368–84. https://onlinelibrary.wiley.com/doi/abs/10.1002/em.22267 (January 26, 2021).

McGrew, Dharia A., and Kendall L. Knight. 2003. “Molecular Design and Functional Organization of the RecA Protein.” *Critical Reviews in Biochemistry and Molecular Biology* 38(5): 385–432. https://www.tandfonline.com/doi/abs/10.1080/10409230390242489 (December 27, 2020).

Memar, Mohammad Yousef et al. 2020. “The Central Role of the SOS DNA Repair System in Antibiotics Resistance: A New Target for a New Infectious Treatment Strategy.” *Life Sciences*.

Merrikh, Houra, and Rahul M. Kohli. 2020. “Targeting Evolution to Inhibit Antibiotic Resistance.” *The FEBS Journal* 287(20): 4341–53. https://onlinelibrary.wiley.com/doi/10.1111/febs.15370 (December 30, 2020).

Mo, Charlie Y., L. Dillon Birdwell, and Rahul M. Kohli. 2014. “Specificity Determinants for Autoproteolysis of Lexa, a Key Regulator of Bacterial SOS Mutagenesis.” *Biochemistry* 53(19): 3158–68.

Mohr, Kathrin I. 2016. “History of Antibiotics Research.” *Current Topics in Microbiology and Immunology* 398: 237–72. https://pubmed.ncbi.nlm.nih.gov/27738915/ (January 19, 2021).

Moya, I. A. 2006. “The Thermodynamic Model for the RecA/LexA Complex Formation.” *undefined*.

Nautiyal, Astha, K. Neelakanteshwar Patil, and K. Muniyappa. 2014. “Suramin Is a Potent and Selective Inhibitor of Mycobacterium Tuberculosis RecA Protein and the SOS Response: RecA as a Potential Target for Antibacterial Drug Discovery.” *Journal of Antimicrobial Chemotherapy* 69(7): 1834–43. https://pubmed.ncbi.nlm.nih.gov/24722837/ (December 27, 2020).

Nayak, Sunil, and Floyd R. Bryant. 2015. “Kinetics of the ATP and DATP-Mediated Formation of a Functionally-Active RecA-SsDNA Complex.” *Biochemical and Biophysical Research Communications* 463(4): 1257–61. https://pubmed.ncbi.nlm.nih.gov/26100204/ (December 27, 2020).

Ojha, Debika, and K. Neelakanteshwar Patil. 2019. “P-Coumaric Acid Inhibits the Listeria Monocytogenes RecA Protein Functions and SOS Response: An Antimicrobial Target.” *Biochemical and Biophysical Research Communications* 517(4): 655–61. https://pubmed.ncbi.nlm.nih.gov/31416617/ (December 27, 2020).

Patel, Meghna et al. 2010. “A New Model for SOS-Induced Mutagenesis: How RecA Protein Activates DNA Polymerase V.” *Critical Reviews in Biochemistry and Molecular Biology* 45(3): 171–84. https://pubmed.ncbi.nlm.nih.gov/20441441/ (January 5, 2021).

Radman, M. 1975. “SOS Repair Hypothesis: Phenomenology of an Inducible DNA Repair Which Is Accompanied by Mutagenesis.” *Basic life sciences* 5 A: 355–67. https://pubmed.ncbi.nlm.nih.gov/1103845/ (December 30, 2020).

Rajan, Rakhi. 2007. “STRUCTURAL AND FUNCTIONAL STUDIES OF THE.”

Rampelotto, Pabulo Henrique. 2010. “Resistance of Microorganisms to Extreme Environmental Conditions and Its Contribution to Astrobiology.” *Sustainability* 2(6): 1602–23.

Robinson, Andrew et al. 2015. “Regulation of Mutagenic DNA Polymerase V Activation in Space and Time.” *PLoS Genetics* 11(8). https://pubmed.ncbi.nlm.nih.gov/26317348/ (February 14, 2021).

Salgado-Benvindo, Clarisse et al. 2020. “Suramin Inhibits SARS-CoV-2 Infection in Cell Culture by Interfering with Early Steps of the Replication Cycle.” *Antimicrobial Agents and Chemotherapy* 64(8). /pmc/articles/PMC7526844/ (May 2, 2021).

Sattin, Bernie D., and M. Cynthia Goh. 2004. “Direct Observation of the Assembly of RecA/DNA Complexes by Atomic Force Microscopy.” *Biophysical Journal* 87(5): 3430–36. https://pubmed.ncbi.nlm.nih.gov/15315951/ (February 22, 2021).

Scheffers, Dirk-Jan, and Mariana G. Pinho. 2005. “Bacterial Cell Wall Synthesis: New Insights from Localization Studies.” *Microbiology and Molecular Biology Reviews* 69(4): 585–607. https://pubmed.ncbi.nlm.nih.gov/16339737/ (January 18, 2021).

Sexton, Jonathan Z. et al. 2010. “Novel Inhibitors of e. Coli Reca Atpase Activity.” *Current Chemical Genomics* 4(1): 34–42. /pmc/articles/PMC2905775/ (March 11, 2021).

VanLoock, Margaret S. et al. 2003. “ATP-Mediated Conformational Changes in the RecA Filament.” *Structure* 11(2): 187–96.

Wiedemar, Natalie, Dennis A. Hauser, and Pascal Mäser. 2020. “100 Years of Suramin.” *Antimicrobial Agents and Chemotherapy* 64(3). /pmc/articles/PMC7038244/ (May 2, 2021).

Wigle, Tim J. et al. 2009. “Inhibitors of RecA Activity Discovered by High-Throughput

Screening: Cell-Permeable Small Molecules Attenuate the SOS Response in Escherichia Coli." *Journal of Biomolecular Screening* 14(9): 1092–1101.

Yakimov, Alexander, Irina Bakhlanova, and Dmitry Baitin. 2021. "Targeting Evolution of Antibiotic Resistance by SOS Response Inhibition." *Computational and Structural Biotechnology Journal* 19: 777–83.

Yin, Wanchao et al. 2021. "Structural Basis for Inhibition of the SARS-CoV-2 RNA Polymerase by Suramin." *Nature Structural and Molecular Biology* 28(3): 319–25.

Zhou, Ziyuan et al. 2021. "Structural Insights into the Inhibition of Bacterial RecA by Naphthalene Polysulfonated Compounds." *iScience* 24(1): 101952.