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# Study of the Physiological Response of NucS to Genotoxic Stress in Actinobacteria

Anaïs Bayard

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# Study of the physiological response of NucS to genotoxic stress in Actinobacteria

Thèse de doctorat de l'Institut Polytechnique de Paris  
préparée à l'École Polytechnique

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## List of Abbreviations

Abbreviation	Meaning
ADP	Adenosine Diphosphate
Alt EJ	Alternative End Joining
AMR	Antimicrobial Resistance
APO	Apoptosis
ARN	Acide Ribonucléique (RNA in French)
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
BioGRID	Biological General Repository for Interaction Datasets
BIR	Break Induced Replication
BLM (helicase)	Bloom Syndrome Protein
CASP (protein structure prediction)	Critical Assessment of protein Structure Prediction
CHARMM	Chemistry at HARvard Macromolecular Mechanics
CIP	Ciprofloxacin
CLUSTALW	A tool for multiple sequence alignment
CPR (resistant mutant)	Carbapenem-Resistant
CRE	Carbapenem-Resistant Enterobacteriaceae
CSB (archaea)	Cockayne Syndrome Group B Protein
DAM	DNA Adenine Methyltransferase
dHJ	Double Holliday Junction
DMT transfer family	Drug/Metabolite Transporter
DNA	Deoxyribonucleic Acid
DOI	Digital Object Identifier
DSB	Double-Strand Break
DSBR	Double-Strand Break Repair
DTL (duplication transfer system loss)	Duplication-Transfert-Loss
EDTA	Ethylenediaminetetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay
EPPs	Error-Prone Polymerases
FCB (Fibrobacteres, Chlorobi and Bacteroidetes)	A phylum group including Fibrobacteres, Chlorobi and Bacteroidetes
FP	Fluorescent Protein
GGR	Global Genome Repair
GNT	Genome Neighborhood Tool
GPU	Graphics Processing Unit
GRP	Glutathione-Related Proteins
H-NS	Histone-Like Nucleoid Structuring Protein
HEJ	Homologous End Joining
HIV	Human Immunodeficiency Virus
HR	Homologous Recombination
ICL repair	Interstrand Cross-Link Repair

IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
IQTREE	A software for phylogenetic tree construction
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kn	Kanamycin
LACA	Last Archaeal Common Ancestor
LB	Luria-Bertani Broth
MINT	Microhomology-Mediated End Joining
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum Inhibitory Concentration
MMC	Mitomycin C
MMEJ	Microhomology
MMR	Mismatch Repair
MRS	Multi-Resistant Strains
mRNA	Messenger RNA
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NAD	Nicotinamide Adenine Dinucleotide
NAPs	Nucleoid-Associated Proteins
NCBI	National Center for Biotechnology Information
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
OD	Optical Density
PBP	Penicillin-Binding Proteins
PDB	Protein Data Bank
rRNA	Ribosomal RNA
SDSA	Synthesis-Dependent Strand Annealing
SMC	Structural Maintenance of Chromosomes
SSA	Single-Strand Annealing
SSB	Single-Strand Break
SSBP	Single-Strand Binding Protein
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SUMO	Small Ubiquitin-like Modifier
TCR	Transcription-Coupled Repair
WHO	World Health Organization
UV	Ultraviolet Radiation

**Introduction:**

**Structure and Maintenance of the Chromosomal Bacterial DNA**

## General introduction

Wherever our gaze falls on the surface of our planet, the likelihood of encountering life is high. Life has extended its reach from the surface of plains to the depths of oceans, actively shaping the Earth's crust. The formation of sedimentary basins can be pointed within all the products of direct or indirect biological activity, with rivers moulding the landscapes as the Seine. Consequently, life on Earth has modified its history and influenced its future course. Our atmosphere oxygen-rich serves as a tangible record of the ongoing narrative of biological activity throughout the planet (Berner, 1999). In a similar vein, the lone wanderer will be able to observe the remarkable array of life populating the Earth. This diversity manifests not only in size and shape but is also expressed through the fulfilment of essential life functions such as nourishment, reproduction, growth and responsiveness to the environment.

In various aspects, one can, in turn, observe the diversity of life, whether it takes the form of trees with lifespans extending across centuries, ephemeral beings evolving for mere hours in the air before their demise, or among the most intriguing organisms –those eluding our sight due to their small sizes, bacteria and archaea. Microorganisms constitute most of our planet's living mass (Hood, 2012). Although they evade observation without specialised equipment, their presence becomes evident during the spread of diseases they may generate within the human population or in the deterioration of improperly stored food. Despite this apparent reality of diversity, a microscopic approach can illustrate fundamental similarities in the structural organisation of living beings on Earth. A case in point is the cytoplasm and genetic material contained within an intracellular environment bound by a membrane and the critical functions of the DNA molecule, serving as the information carrier from generation to generation (Miller, 1971).

Almost six decades ago, elucidating the DNA's double helical structure (Watson *et al.*, 1953), as detailed in Watson and Crick's paper, marked a watershed moment in the scientific realm. The enthusiasm of researchers dedicated to unravelling the mysteries of DNA since the mid-20<sup>th</sup> century has yielded significant breakthroughs, notably acknowledging the DNA molecule as the predominant form of genetic material in living organisms and recognising its pivotal role as the carrier of genetic information (Hershey *et al.*, 1952. Wolf *et al.*, 2003). The public has become familiar with various techniques for storing information, from calligraphy to the broad usage of computers and electronics. These techniques range from the use of

ancient writing tablets to the recording of sounds on vinyl records. Surprisingly, despite being shaped by 3.5 billion years of evolution, living cells exclusively rely on DNA as the repository of hereditary information (Cox, 2001). Consequently, all living cells utilise this double helix structure, comprising four elementary monomers. These monomers are intricately threaded together in a long linear sequence that encodes the genetic information (Forsdyke *et al.*, 2000). Significant strides have revolutionised genome reading techniques since developing pioneering sequencing methods by Sanger, Maxam and Gilbert in the 1970s (França *et al.*, 2002). At this point, it is possible to cover the entire human genome—which consists of millions of nucleotides—and decode it in a few hours with a 99.9 % accuracy rate (Nurk *et al.*, 2021).

Moving beyond the initial considerations of DNA, which explore its structure and stability inside the cell, contemporary biology stands at the nexus of computational sciences and artificial intelligence (AI). It concentrates on the incredible information storage capacity of DNA. By reimagining the initial discovery of Deoxyribonucleic Acid as a carrier of hereditary genetic information, scientific teams have developed DNA Digital Data Storage (Church *et al.*, 2012). This innovation enables the utilisation of the DNA molecule to encode and decode binary information synthetically. The potential appears remarkable due to its tightly packed information storage capacity, echoing the efficiency of DNA within cells.

Although the DNA molecule is considered one of the most stable, it is still susceptible to damage, such as base deamination, which could occur during the storage and incorporation of errors during the synthesis of the polynucleotide chain. To address these issues, researchers must develop innovative systems to monitor DNA integrity and ensure the fidelity of the sequence relative to the template. DNA repair pathways, such as the Mismatch Repair (MMR) that corrects errors in DNA by recognizing base-pairing errors, excising the nascent DNA strand around the lesion, and resynthesizing the segment using the parental strand as a template, perform these functions in cells. The protein central to this study, NucS, is a critical player in these DNA repair mechanisms.

Significant progress has been achieved in this area since 2019. Examples include encoding large datasets, such as the cryptographic key for a Bitcoin and 19GB of Wikipedia. The twenty-first century will be remembered as the DNA engineering era, where DNA-based technology is used for scientific purposes and to advance human society. Owing to the DNA molecule's widespread presence in living organisms, it is possible to compare different creatures when their DNA sequence is established. Consequently, this makes it possible to estimate their respective position in the Tree of Life. (House, 2009).

Before introducing DNA's crucial role in life's diversity and function, it is essential to understand its foundational structure. DNA's helical structure is not just a remarkable feat of molecular architecture but also the key to its function as the carrier of genetic information.

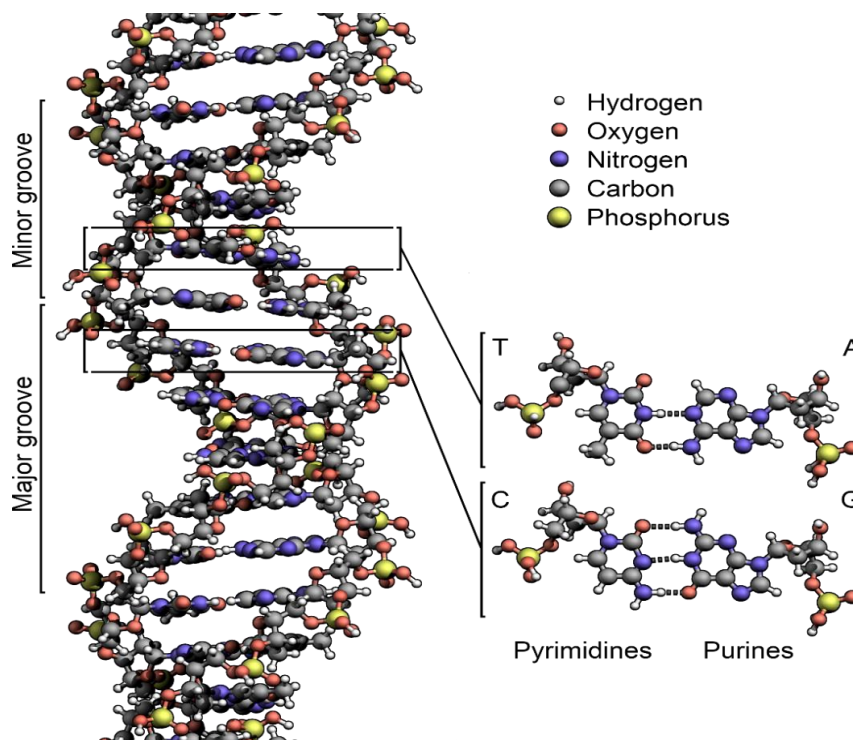
## **1. DNA: blueprint for life's diversity and function**

### **1. A DNA's helical structure enables genetic encoding**

The essential function of DNA is intricately tied to its structure, particularly the sequence of nucleotides that constitute it. Hence, the very configuration of these monomers plays a pivotal role in the mechanisms that underpin life. Each monomer can be decomposed into two constituents: a phosphate group attached to a sugar, a deoxyribose and a base (Rudolph, 1994). The base, distinct for each nucleotide, could be an A for Adenine, T for thymine, C for cytosine and G for guanine. Each nucleotide is connected to the next through via the phosphate group of the deoxyribose (Wittung *et al.*, 1994). At the molecular level, this succession of monomers manifests as a repetitive polymeric chain, forming a sugar-phosphate backbone that imparts structural support to the molecule, as represented in **Figure 1** (Sponer *et al.*, 2012).

Due to the chemical and structural properties inherent in the two polynucleotide chains that constitute DNA, mainly owing to the hydrogen bonds maintaining cohesion between the two DNA strands, the bases are situated within the interior of the double helix (Albert *et al.*, 2002). At the same time, the sugar-phosphate backbone is positioned on the exterior of the structure. As the literature on eukaryotes highlights, the two antiparallel strands of DNA wind around each other in a right-handed helix known as the B-form, maintaining a consistent distance along the molecule (Rich A, 1983). A complete turn can be observed for every ten pairs of nucleotide bases (Levitt M, 1978). This configuration protects the bases against potential external hazards. In every instance, a purine base is associated with a pyrimidine.

During elongation, nucleotides are added to one of the ends of DNA, owing to their assembly through the phosphate group. This polymerisation procedure disregards the distinctive base of each nucleotide, allowing, in theory, their addition in any order. However, within the context of DNA replication, a nascent DNA strand is synthesised from the template provided by a pre-existing parental strand. This process follows the principles of base pair complementarity, ensuring the faithful preservation of genetic information (Bell *et al.*, 2013; Reece *et al.*, 2011). Watson and Cricks’s interpretations underscored the structural complementarity between adenine, thymine, cytosine and guanine (Topal *et al.*, 1976).



**Figure 1:** The structure of DNA showing with detail the structure of the four bases, adenine, cytosine, guanine and thymine and the location of the major and minor grooves (by Zephyris, Wikimedia Commons, licensed under CC BY-SA 3.0).

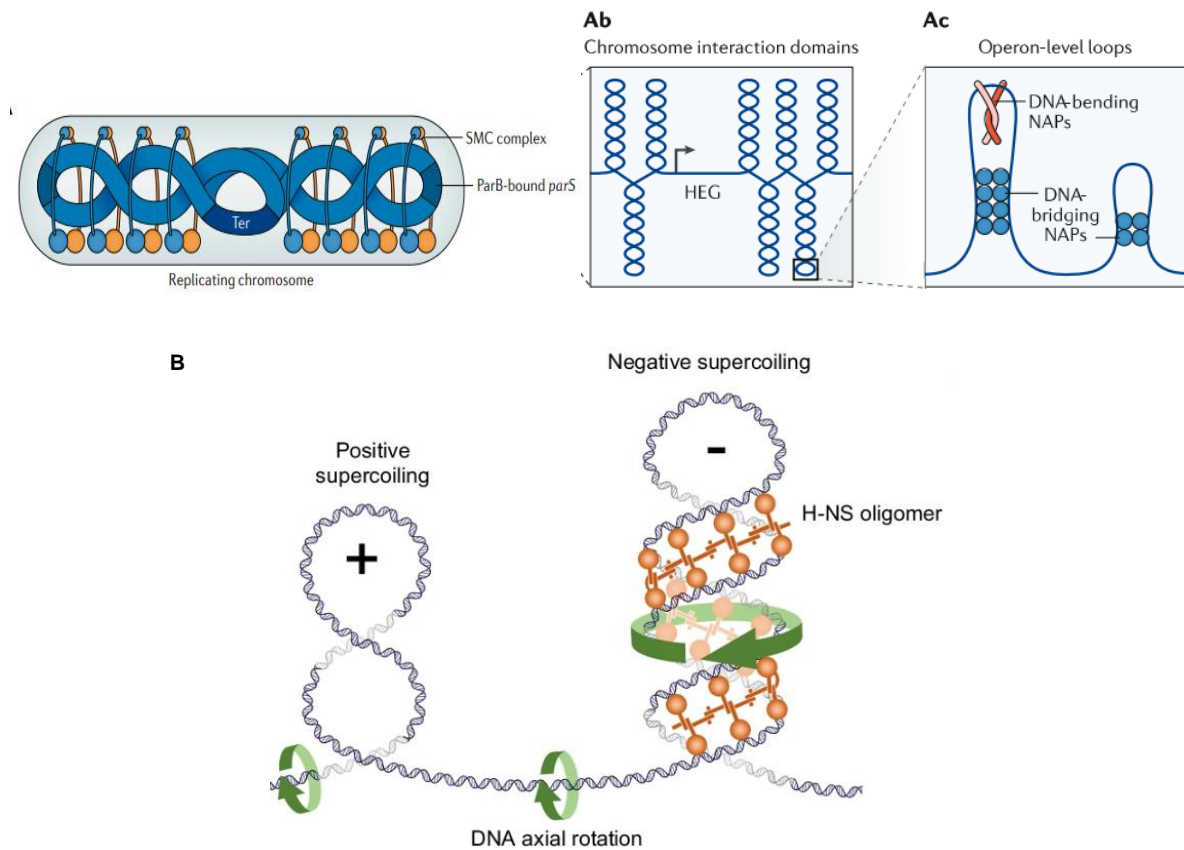
A distinctive feature of the DNA molecule lies in the weak interaction between its bases compared to the robustness of the backbone. This attribute enables the two DNA strands to dissociate without causing a break in the DNA molecule (Matta *et al.*, 2006). Moreover, this specificity highlights that the removal of a base from a nucleotide, resulting in an abasic site, or the incorporation of a non-complementary base during the replication can be tolerated for a particular duration without inducing a break in the DNA molecule (Petria *et al.*, 2020). Observing DNA within the cell under the electronic microscope and after inducing cell lysis through osmotic shock has unveiled the importance of DNA compaction within the cell. Once

released into the extracellular environment, the volume occupied by DNA significantly surpasses the initial size of the bacterium (Jin *et al.*, 2023). The length of the *E. coli* bacterial chromosome is approximately 4,6 million base pairs, equivalent to 1,5 mm packed in a 6  $\mu\text{m}$  long cell (Verma *et al.*, 2019). In eukaryotes, DNA is arranged in a linear configuration, organised into chromosomes, which may or may not be condensed, depending on the cell cycle stage (Campos *et al.*, 2009). This intricate organisation is essential for the survival of the cell. It involves elaborate compaction and epigenetic mechanisms to contain several million deoxyribonucleotides inside a cell while orchestrating their organisation and accessibility to enzymes for adequately reading the information they contain. DNA in most bacteria is not linear; instead, it adopts a circular structure, regarded as a single chromosome (Wu *et al.*, 2018). Even though bacteria include proteins similar to those found in eukaryotes and have been identified for their role in DNA compaction, the dynamics seen in eukaryotes cannot be directly applied to bacteria. Bacterial chromosomes respond to Nucleoid-Associated Proteins (NAPs) that facilitate the folding of the chromosome into different conformations (Browning *et al.*, 2010). This process depends on the binding of Histone-Like Nucleoid protein (H-NS), as presented in **Figure 2**, which attaches to the minor groove of the AT-rich regions of DNA and organises into a chain, polarising the formation of protein filaments alongside the DNA molecule (Dillon *et al.*, 2010). Simultaneously, the bacterial chromosome is also compacted into loops by Structural Maintenance of Chromosomes (SMC) complexes, formed by a tripartite ring (Badrinarayanan *et al.*, 2012). The interaction generated is anchored and stable within the cell. However, these loops remain dynamic and can respond to environmental stimuli (Jeppsson *et al.*, 2014).

Thus, the structure of the bacterial chromosome undergoes tolerated changes over time and in various cellular circumstances. This adjustment has been noted in parallel to low osmolarity conditions, promoting the formation of lateral filaments along the DNA. Additionally, temperature can influence the configuration of the structure of the bacterial chromosome. An elevated temperature favours H-NS dissociation and diminishes their assembly in the minor DNA groove. At a more intricate level of observation, modulations in the compaction of the bacterial chromosome also appear to be contingent upon the bacterial cell cycle phase, following the nutrient richness of the environment or amid unfavourable conditions Meyer *et al.*, 2013). This becomes notably apparent during periods of starvation and upon entering the stationary phase, where the bacterial chromosome and the plasmic membrane appear much denser (Pietnev *et al.*, 2015).



This regulatory process of chromosome packaging relies on the expression of specific essential proteins, namely the DNA-binding proteins from starved cells (Dps-like), whose expression undergoes a noteworthy increase during the stationary phase while absent in other phases. These proteins bind to DNA and each other, instigating an increase of chromosome compaction. These Dps-like complexes have enhanced resistance to cell damage induced by genotoxic compounds (Haikarainen *et al.*, 2009).



**Figure 2:** Organisation of the bacterial chromosome. **Aa.** ParAB–parS–mediated chromosome segregation, structural maintenance of chromosomes (SMC) complexes loaded at ParB-bound parS sites in the origin domain. **Ab.** The chromosome is subdivided into chromosome interaction domains (CIDs) **Ac.** The smallest structural unit of organisation of the bacterial chromosome may correspond to loops formed at the level of individual operons by nucleoid-associated proteins (NAPs) and its coiling into loop form thanks to the intervention of NAP's proteins. (Dame *et al.*, 2019). **B.** Model of H-NS-bound negatively supercoiled DNA (Figueroa-Bossi *et al.*, 2024).

Understanding the intricate structure of DNA is crucial as it forms the basis for how damage can affect genetic information and how repair mechanisms function. While the helical structure of DNA enables the encoding of genetic information, this information is vital for guiding cellular functions. Understanding how DNA stores and transmits this information illuminates its role in the continuity of life.

## **1. B DNA stores genetic information, guiding cell function and heredity**

Concurrently with studying the DNA structure, DNA's cellular function has emerged as a dynamic area of biology research. When defining the bacterial genome as the organism's genetic repository, it is essential to highlight its dissimilarity to eukaryotes. Unlike the latter, the bacterial genome is not enclosed within a nucleus but freely resides within the cytoplasm. Archaea are also described as organisms without envelopes, even if more complicated cases can be pinpointed (Islas-Morales *et al.*, 2023). The bacterial genome comprises various forms, including the bacterial chromosome described earlier and megaplasmid, plasmid or chromid (Harrison *et al.*, 2010). Both plasmids and mega-plasmids constitute non-essential elements, as they are devoid of genes essential for growth. Bacteria can survive even if they lose a plasmid. The distinction between a plasmid and a mega-plasmid lies in the length of the DNA molecule. Chromids represent a hybrid structure combining characteristics from both chromosomes and plasmids (Harrison *et al.*, 2010).

Understanding the nucleotide sequence of a chromosome and its transcription profile by RNA polymerases provides details regarding the presence and location of genes. In this context, genes refer to nucleotide sequences whose expression influences the organism's characteristics. Coding genes entails the information requisite for specific protein expression. Gene mapping and functional studies across various living organisms have revealed that genes with similar sequences and functions are found in multiple classes and families of organisms. (Martin *et al.*, 2003).

These discoveries disclosed that specific genes exhibit conserved sequences even among organisms significantly distant from the Tree of Life. Genes are considered homologous when their similarity in nucleotide sequence and function indicates they originate from a common ancestor (Webber *et al.*, 2004). Although the percentage of identity between two DNA sequences does not guarantee functional similarity in the resulting proteins, since protein structures are more conserved than primary sequences, identifying similarities in DNA segments has proven to be a compelling indicator for researchers when inferring a gene's hypothetical function. (Pearson, 2013). Researchers have shown ubiquitous genes can be transferred and exchanged between organisms like yeast and bacteria or between humans and yeast, despite the vast phylogenetic distance created by billions of years of evolution. Approximately 5 % of the human genome corresponds to conserved multi-species sequences (Siepel *et al.*, 2005).

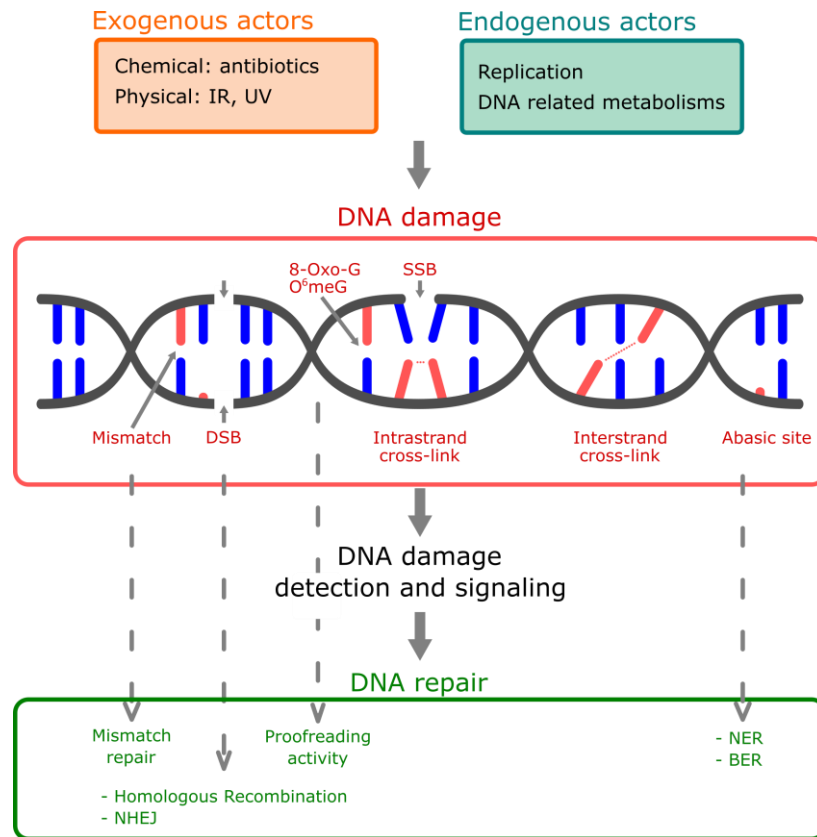
## 1. C DNA narrates evolution's history through genetic variation

As the custodian of the evolutionary history of life and the repository of genetic information, DNA undergoes a finely balanced oscillation between preserving its integrity and modifying its sequence (Astley D, 1992). In these terms, DNA can be seen as the focal point of evolution. As there are currently no known mechanisms to generate utterly random DNA sequences *de novo*, scientists have observed that a gene is never entirely new, even if new functions or forms may be attributed to it. These modifications are thus attributable to other mechanisms that can manifest in various situations (Albert *et al.*, 2002).

The causes of DNA damage are diverse. Environmental factors and the cell's internal activity collectively contribute to introducing 1,000 to 1,000,000 damages per day in the human body (Alhmod *et al.*, 2020). Initially, the environment, as specified by the concept of the Red Queen, constantly exposes an organism to exogenous assaults. As represented in **Figure 3**, these include Ionising Radiation (IR), Ultraviolet radiation (UV), the agents with antibiotic effects secreted by other organisms, or variations in the growth condition, such as the pH or the temperature (Guan *et al.*, 2017). Among the most common damages introduced in the DNA, the deamination and mismatched pairs are flagships and can occur spontaneously due to chemical reactions. Cellular metabolism can also be considered an endogenous source of damage. This can occur, for instance, through the production of Reactive Oxygen Species (ROS) or the incorporation of errors in DNA during nutrient deprivation. Such errors can also be related to the processivity and fidelity of the enzymes involved in DNA replication and repair (Pluskota-Karwatka, 2008).

Thus, chromosomes can become the target of both endogenous and exogenous factors, whether during DNA replication or translation or simply due to the effects of ageing and variations in living conditions. These factors can lead to the introduction of random accidents or errors. Although the introduction and fixation of a mutation in a population may span multiple generations, at the scale of a cell division, an analysis of the genomic sequence will reveal differences within the DNA sequences of two sister cells (Falconer *et al.*, 2010). Therefore, they are not entirely identical to their parent. These errors can, in uncommon instances, manifest as advantageous for the organism and its survival in its environment (Schneider *et al.*, 2011; Hartfield *et al.*, 2010). Nevertheless, these events are more frequently associated with severe damage to the DNA, either in its structure, such as introducing a

Double-Strand Break (DSB), a profoundly toxic incident, or disrupting a sequence encoding a protein essential for survival. The organism's repercussions may manifest as the onset of disease development (Roemhild *et al.*, 2022). These incidents are particularly critical for microorganisms, as the cell's death also means no progeny.



**Figure 3:** Schematic representation of the DNA molecule and the different types of endogenous or exogenous actors that can damage it, associated with the representation of said damage in the structure and the existing repair pathways to fix them.

Ultimately, within the very context of a competitive environment and iteration of trial and error, the natural selection process translates into Darwinian evolution. Whether neutral or advantageous, incorporated errors in the genome will be transmitted to subsequent generations. These genetic mutations can confer a competitive advantage in harnessing the environment regarding reproduction or nutrition. Thus, genetic variability across the genome is not uniform (Hodgkinson *et al.*, 2011; Pemberton *et al.*, 2012). DNA segments unrelated to genes or their regulation may change, contingent upon the likelihood of error incorporation in that specific genome portion. Conversely, a gene critical to the cell's operation will undergo few modifications over time. This holds for the 16S rRNA used in genetics to position bacteria within the Tree of Life. Certain DNA regions near replication initiation sites or telomeric levels will also exhibit varying propensities to record mutations (Hodgkinson *et al.*, 2011).

In addition to DNA base incorporation repair errors, large-scale genome modifications can occur during the copying, transposition, or deletion of entire genome segments. Consequently, a duplicated gene, even if it performs a crucial cellular function, can, if retained, be subject to mutation and diverge without affecting the survival of the offspring organisms (Pegueroles *et al.*, 2013). Likewise, a transposable element can, under specific circumstances, be excised from the genome and reinserted in a different location, creating a novel genomic sequence, disrupting the function of a gene, or altering its regulation. On rare occasions, it can create a new gene (Fedoroff, 2012). Through this lens, evolution appears to be shaped by accidents and errors subsequently subjected to Darwinian selection. DSBs in bacterial genomes play a pivotal role in the survival and adaptation of bacteria under antibiotic pressure. This type of damage is implicated in genomic mobility and reshuffling (Lottersberger *et al.*, 2015).

### **1. D Implication of a DSB in the genome for the survival of bacteria**

Among the different types of cellular damage, DSBs are the most harmful to cells. If the damage is not repaired quickly, it can significantly impact the DNA structure and lead to unexpected free 5' and 3' ends. This is particularly important in bacterial cells, where DNA takes on circular shapes. These freed ends can potentially cause genomic instability, preventing replication and ultimately resulting in cell death. Genetic instability can manifest through chromosome translocations or rearrangements among genes with similar sequences or functions (Schubert, 2021).

There are numerous reasons why DSBs occur. First, an endogenous break can be introduced due to cellular metabolic activities. Second, if this damage halts the replication, it can lead to the introduction of DSBs, see **Figure 4** (Okafor, 2023).

DNA polymerisation stops when the replication machinery encounters a break in one of the DNA strands (Muraki *et al.*, 2018). If replication does not resume, the replication fork can collapse and regress in a backward movement, resulting in the formation of a chicken-fork structure similar to the DNA configuration observed during the Holliday junction formation. This specific structure requires cleavage by Holliday junction resolvases, which results in the formation of a DSB during the process (Seigneur *et al.*, 1998). Subsequently, a mechanism of DSB Repair can also be activated to restore the molecule either through homologous recombination (HR) a highly reliable process- or through microhomology Non-Homologous End Joining (NHEJ), which is significantly more mutagenic (Stinson *et al.*, 2021).

In response to nutrient deprivation and metabolic shifts, an increase in the frequency of DSBs can also be observed (Chiodi *et al.*, 2019). This process should be considered, especially when nucleotides or essential factors for metabolic functions such as replication are reduced. A shift towards more rNTPs can thus cause DNA damage and lead to the formation of DSBs.

Additionally, DSB occurs following cell exposure to exogenous events like ionising radiation (IR). IR generates direct DSBs in the genome through collision between high-energy particles or photons with one of the DNA strands, leading to the breakage of the DNA sugar-phosphate backbone (Cannan *et al.*, 2016). This Single-Strand Break (SSB) can be converted into a DSB during this phase of structural instability. Approximately 10-50 DSBs occur in nucleated human cells daily (Vilenchik *et al.*, 2003).

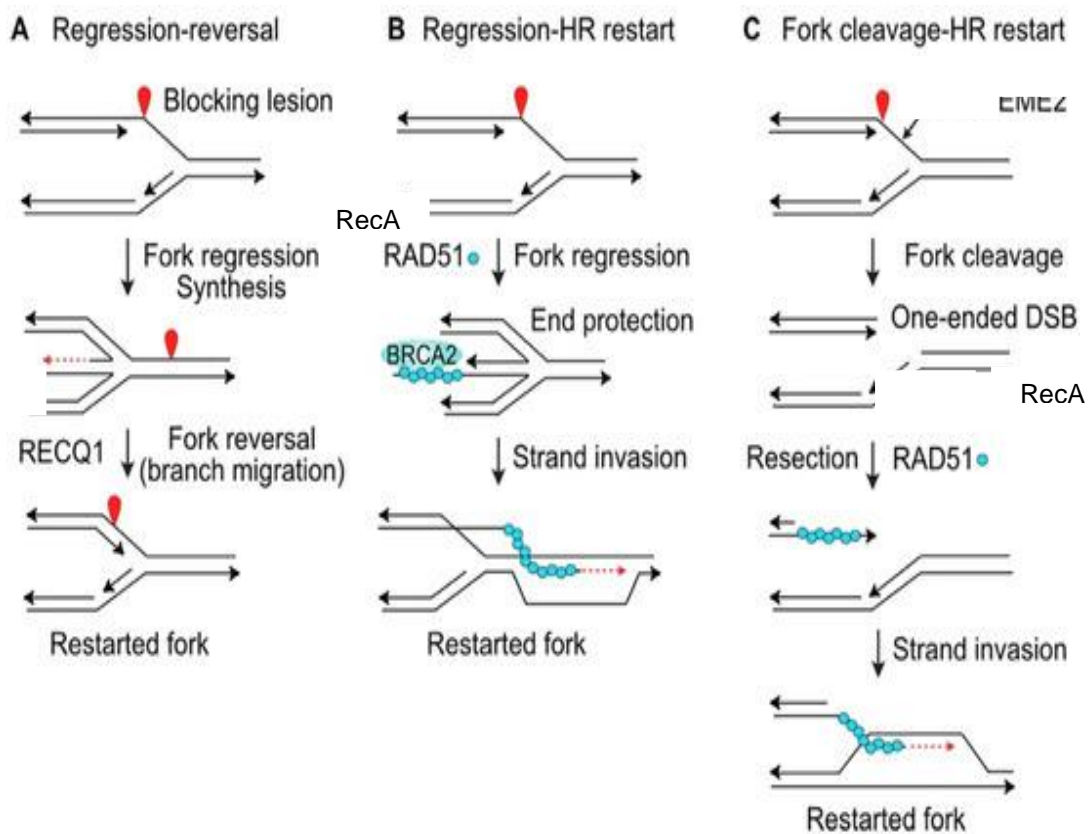
The repair machinery for IR-induced lesions can also generate DSBs in the cell. The first experimental observation of DNA breaks after exposure to IR dates back to the late 1930s. This involves Base Excision Repair (BER), which will be further developed later. If this DNA mechanism acts simultaneously on two sites with nearby DNA lesions, it can form double-strand single breaks, which is considered a DSB in this study. Several *in vitro* studies have demonstrated the formation of DSBs following BER (Sage *et al.*, 2011).

DNA breaks generally occur following exposure to chemical agents or exposure to IR. The concentration and duration of the administered treatment are particularly decisive parameters for the frequency and incidence of breaks within cells. An acute treatment spans a few minutes to several hours of exposure to the genotoxic agent, whereas a chronic treatment extends over several (Vitor *et al.*, 2020). These variables elicit different responses at the bacterial population level under pressure.

When targeting crucial replication enzymes such as topoisomerase II (Gomez-Herreros, 2019) or gyrases (IV), genotoxic antibiotics can indirectly introduce single or DSB by impeding the enzyme's activity or deregulating its function. This is notably observed with fluoroquinolones such as Ciprofloxacin (Cip) (Hooper *et al.*, 2016). Alternatively, other agents directly modify the DNA structure that will be converted into DSB during later episodes in the cell, such as during the replication. For example, Mitomycin C (MMC) induces crosslinks in the DNA that can lead to the formation of DSB during the replication as they instigate the collapse of the replication fork (Niedernhofer *et al.*, 2004). The subsequent

**Figure 4** details the sequential steps involved in the fork regression and introduction of DSB to repair a blocking lesion. Finally, other agents, including Phleomycin (Phleo), directly interact with DNA and, similarly to the effect of ionising radiation, lead to the formation of DSB.

These agents leave different traces in the DNA by introducing DSB at various stages of the cell's life cycle and also at distinct cellular locations. Thus, drugs targeting topoisomerase II will produce lesions preferentially in locations near or associated with transcriptionally active zones. (Canela *et al.*, 2017, 2019; Gothe *et al.*, 2019).



**Figure 4:** Fork restart mechanisms. **A** Blocked forks may regress to a Holliday junction-like structure and a one-ended DSB. DNA synthesis (dashed/red arrow) using newly replicated strands as a template allows the end to extend beyond the blocking lesion. The fork is restarted once RecA drives fork reversal by branch migration. **B** The one-ended DSB at regressed forks is protected. Fork restart is mediated by ssDNA filament invasion. **C** Stalled forks may be cleaved, creating a one-ended DSB. Resection creates ssDNA that is first protected and then RecA-ssDNA filament catalyses strand invasion to restart the fork (Nickoloff, 2022).

The implications of DSBs and antibiotic resistance extend to public health, particularly concerning infectious diseases. The rise of resistant bacterial strains highlights the urgent need for adequate treatments to combat these threats.

As we delve deeper into the significance of DNA, it is crucial to explore how genetic information impacts health and disease. One critical area is the acquisition of antibiotic resistance in bacteria, which poses a significant challenge to modern medicine.

## **2. Antibiotic resistance and bacterial defence mechanisms**

### **2. A Infectious diseases and antibiotic resistance**

Among the top 10 causes of death, tuberculosis is one of the three infectious illnesses that pose a significant threat to humankind. Since 1993, the World Health Organization (WHO) has deemed it a worldwide emergency (Huang *et al.*, 2022). Although one-fourth of the world's population may harbour the bacilli that cause tuberculosis, people with weakened immune systems, such as those living with HIV infection, are more vulnerable to the disease's effects (Cohen *et al.*, 2019). Current therapies propose giving four antibiotics simultaneously for six months: Rifampicin (Rif), Isoniazid, Pyrazinamide and Ethambutol (Paramasivan *et al.*, 1993).

There are challenging problems due to the increasing emergence of Multi-Resistant Strains (MRS), particularly to Rif and fluoroquinolones (Prasad *et al.*, 2018). Growing health concerns are particularly associated with bacterial species that belong to the Actinobacteria phylum, such as *Mycobacterium tuberculosis*. This is especially true for Corynebacteriaceae, which includes *C. diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis*, *C. rouxii* and *C. belfantii* (Prygiel *et al.*, 2022). The Resistance of Microorganisms to Antibiotics (AMR) is one of the top 10 priorities that represent a threat to humankind, with AMR contributing to the death of 4,95 million people in 2019 (Walsh *et al.*, 2023).

The conservation of DNA repair systems across living organisms, particularly within bacteria, designates these systems as pivotal targets for antibiotics. This strategic approach has permitted the treatment of pathogens using the same drug despite their distance in the Tree of Life. This paradigm is a significant time-saver when opposed to creating customised treatments for every bacterial infection.

All experimental procedures within this study were conducted on *Corynebacterium glutamicum*, chosen as a model for *M. tuberculosis* and other Corynebacteria with health-related concerns. It appears that the superorder of Corynebacteria includes agents responsible for leprosy, diphtheria and tuberculosis. More recent studies have highlighted an increase in



prevalence in samples of patients suffering from lung or skin infection of *Mycobacterium abscessus*, with a surge in multi-drug resistant cases (Novosad *et al.*, 2016). *C. glutamicum* has a much shorter doubling time than *M. tuberculosis*, 1.2 hours for the first organism versus 24 hours for the human pathogen. Finally, NucS has been shown to modulate the emergence of antibiotic resistance in *M. abscessus*, pinpointing the interest of this enzyme in DNA repair mechanisms (Cardoso *et al.*, 2022).

## **2. B Mechanisms of acquisition of resistance**

In this section, we will delve deeper into the acquisition of antibiotic resistance by bacteria, enhancing the earlier discussion on genomic alterations driven by diverse events and actors. It was in 1928 that Sir Alexander Fleming made the first observation of the effect of penicillin on a bacterial culture. He then describes the inhibition of the growth of a *Staphylococcus* culture when in contact with *Penicillium notatum*. The subsequent isolation of this molecule has enabled its identification as the active principle and facilitates its importation for use in the healthcare field (Tan *et al.*, 2015).

Intricate genetic complexities characterise the exploration of antibiotic resistance emergence and transmission in bacteria. When all the organisms with a shared history exhibit resistance to an antibiotic, this resistance is deemed innate or natural and corresponds to a Wild-Type (WT) resistance phenotype. In this scenario, antibiotic resistance directly arises from the bacterial chromosome and is vertically transmitted across generations (D’Costa *et al.*, 2011; Gillings *et al.*, 2017). However, the transmission mechanism is likely a horizontal phenomenon if the distribution of resistant organisms in the phylogenetic tree is sporadic or if the resistance is undergoing phases of emergence and reduction (Wang *et al.*, 2021).

When a bacterium acquires multiple resistance genes against various antibiotic classes, “multi-resistance” is employed (Woodford *et al.*, 2007). As a matter of definition, persistence characterises bacterial cells that remain impervious to the drug despite lacking a gene that codes for factors giving resistance to this antibiotic. This phenomenon is not confined to a specific locus of the genome. However, it is linked to stationary growth, where certain cells within the population may enter dormancy. Most antimicrobial agents are ineffective when a cell is not actively growing anymore. The occurrence of these persister cells is estimated at approximately 1 % in a culture experiencing a stationary phase (Pontes *et al.*, 2019).

There exist diverse mechanisms by which antibiotics target bacteria. The bacterial cell wall is a prime antibiotic target due to its constant resynthesis and dynamic remodelling, governed by a fine balance in peptidoglycan synthesis. By inhibiting the activity of critical enzymes involved in membrane renewal,  $\beta$ -lactams, including penicillins, disrupt the dynamic equilibrium of the membrane barrier. Consequently, the membrane loses its ability to effectively withstand the osmotic pressure exerted by the environment, resulting in bacterial lysis. This mechanism occurs at the extracellular level and manifests through a bacteriolytic effect (Zhou *et al.*, 2022).

Other antibiotics target intracellular components. This is the case with the three other mechanisms of antibiotic action that will be described. The permeability of the bacterial cell to these agents can be explained, for instance, by their diffusion through specific porins located in the membrane, facilitating the passage of smaller molecules (Bajaj *et al.*, 2014). A secondary target for antibiotics is the protein synthesis. This is exemplified by aminoglycosides such as Gentamicin (Gen). By binding to the catalytic site or altering the conformation of subunits of the 16S rRNA, these molecules disrupt the translation of mRNA into amino acid chains (Vaiana *et al.*, 2009). This disruption of the translation machinery leads to defective protein synthesis through its complete cessation or the introduction of errors during mRNA reading. This results in the accumulation of aberrant proteins within the cytoplasm. These proteins can then exert diverse harmful effects on the cell. In the case of their integration into the cytoplasmic membrane, they may compromise the membrane integrity (Lin *et al.*, 2018). During the exponential phase, a deficiency for specific key proteins, such as topoisomerases, can introduce breaks in the DNA molecule. This kind of antibiotic is said to be bacteriostatic (Yang *et al.*, 2009; Poddevin *et al.*, 1993).

Another example is Trimethoprim, an antibiotic that inhibits the enzyme Dihydrofolate Reductase (DHFR). DHFR is essential for synthesising tetrahydrofolate, a cofactor required for the production of thymidylate, purines and certain amino acids. By inhibiting DHFR, trimethoprim reduces the production of tetrahydrofolate, leading to a decrease in thymidylate synthesis (Dale *et al.*, 1995). This reduction in thymidylate availability can impair DNA synthesis and repair because cells cannot produce sufficient TTP for DNA replication and maintenance. Consequently, this inhibition affects bacterial cell division and growth, making

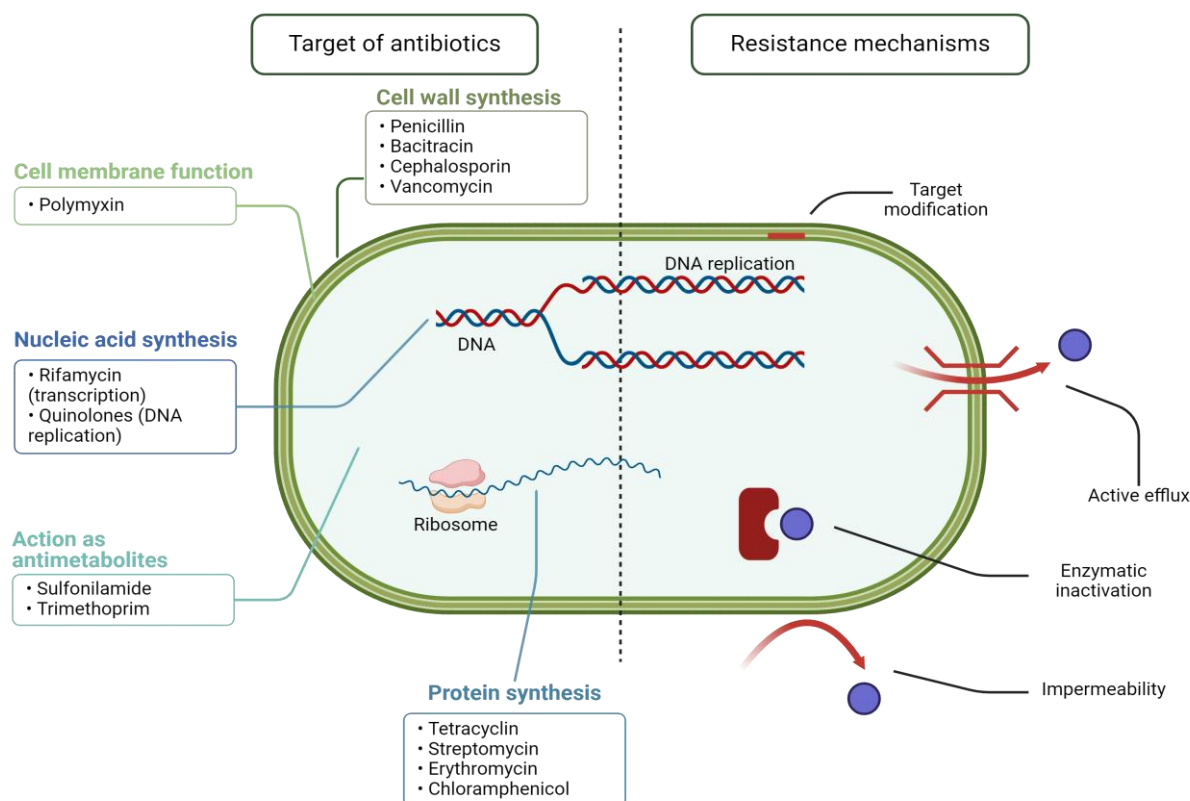
Trimethoprim an effective antibacterial agent. This mechanism also highlights the potential impact of Trimethoprim on DNA-related processes within the cell (Giroux *et al.*, 2017).

In a similar vein, antibiotics may also target the transcription of a gene into mRNA, as is notably the case with —Rifamycins, which inhibit the transcription. The observed impact resembles that of antibiotics that hinder protein synthesis, resulting in a bacteriostatic outcome (Kavčič *et al.*, 2020).

Finally, the last class of antibiotics presented pertains to those influencing the integrity of the DNA molecule such as those presented within Table 1. Typically, this type of function manifests through interaction with crucial enzymes involved in nucleic acid synthesis during replication, such as topoisomerases, or direct intercalation at the level of the DNA bases. This leads to the disruption of the backbone of the DNA and the introduction of single or DSBs (Maxwell *et al.*, 2018). These last breaks represent some of the most toxic events during cellular life. Generally, their occurrence is succeeded by DNA repair and re-joining. However, if the break remains irreparable, it culminates in cell death. Antibiotics such as Phleo, MMC, or CIP are examples of drugs with this type of impact on the cell (Shi *et al.*, 2023). To better illustrate these concepts, **Figure 5** provides a schematic representation of the target and mode of operation of different classes of antibiotics, Table 1 list the antibiotics of this class used in this study.

This paragraph will expound upon four bacterial resistance mechanisms, similar to the mechanisms of action outlined for antibiotics. As mentioned earlier, certain antibiotics target intracytoplasmic sites, implying that their entry into the intracellular environment is required for efficacy. Bacterial impermeability is, therefore, a fundamental aspect of the natural resistance of bacteria, notably Gram-negative bacteria (Leus *et al.*, 2023).

In the case of glycopeptides, these high-molecular-weight molecules cannot enter through the porins present in the outer membrane of Gram-negative bacteria due to size exclusion. Additionally, polar molecules face difficulty traversing the cell wall of enterococci, giving them intrinsic resistance to aminoglycosides (Reygaert, 2018). Another resistance mechanism involves reducing the number of porins an antibiotic would pass. This phenomenon can occur in response to the loss of a gene, as observed in *Pseudomonas aeruginosa* with Imipenem and certain Cephalosporine due to the loss of the D2 porin (Munita *et al.*, 2016).



**Figure 5:** Schematic representation of the different intracellular or membranous targets of antibiotics associated with their function and the mechanisms of bacterial resistance.

Antibiotics only carry out their activity within the cell through interaction with their target, relying on a 3D structural complementarity. However, the antibiotic's target can be modified in its folding or the organisation of its binding sites. In that case, it can result in a loss of the efficacy of the antibiotic. A mutation near the active site of the targeted molecule could induce structural changes leading to steric hindrance (Lopatkin *et al.*, 2021; Savage *et al.*, 2012). Similarly, alterations in a porin conformation can restrict the passage of specific molecules, such as  $\beta$ -lactams or fluoroquinolones (Pagès *et al.*, 2008). Hence, the structure and the number of Penicillin-Binding Proteins (PBP) directly impact the drug's binding capacity to its target (Sainsbury *et al.*, 2011; Lambrinidis *et al.*, 2015).

The third mechanism of action corresponds to efflux pump systems, which, in contrast to porins allowing passive antibiotic entry into the cell, actively expel antibiotics towards the extracellular environment (Li *et al.*, 2015). This type of resistance mechanism is considered natural, as bacterial genes encode the efflux pumps. The expression of these pumps can be either constitutive or responsive to environmental stimuli or under the presence of a specific substrate (Nikaido *et al.*, 2008). Efflux pumps play a role in the efflux of a large variety of

compounds, including toxic substances produced by the internal bacterial metabolism itself, as it can be influenced by the carbon source available (Varela *et al.*, 2021).

Finally, the last mechanism arises from the inactivation of the antibiotic within the cell, notably exemplified by  $\beta$ -lactamases that proceed to hydrolyse  $\beta$ -lactams. Bacteria employ two main pathways to inactivate or neutralise a drug. This can occur during the antibiotic's degradation or in response to the transfer of a chemical group to the antibiotic (Wright G, 2005). Typically, this mechanism involves the transfer of a phosphoryl, acetyl, or adenylyl group. Such a pathway for drug inactivation has been observed in response to Chloramphenicol or Fluoroquinolones (Reygaert, 2018). Plasmid carriers of  $\beta$ -lactamase genes are most commonly found in Enterobacteriaceae, participating in the emergence of Carbapenem-Resistant Enterobacteriaceae (CRE). Notably, infections caused by CRE have been associated with in-hospital mortality of up to 71 % (Pfeifer *et al.*, 2010; Friedman *et al.*, 2016).

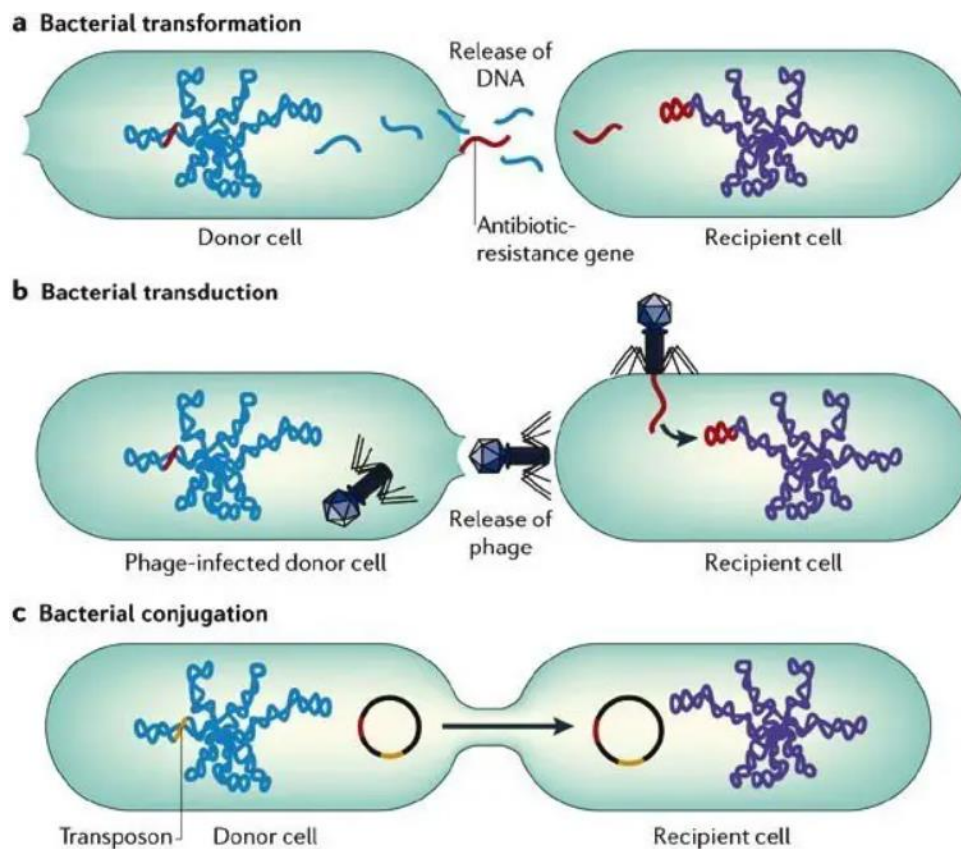
Antibiotic	Family	Mechanism of Action
Phleomycin	Bleomycin Family	Causes DNA strands to break by binding to DNA and inducing oxidative damage.
Ciprofloxacin	Fluoroquinolone	Inhibits DNA gyrase and topoisomerase IV causes DNA strand breaks, preventing DNA replication and transcription.
Mitomycin C	Mitomycins	Cross-links DNA, Causes DNA strand breaks, inhibiting DNA synthesis and function.
Gentamicin	Aminoglycoside	Binds to the 30S ribosomal subunit, causing misreading of mRNA and inhibiting protein synthesis.
Streptomycin	Aminoglycoside	Binds to the 30S ribosomal subunit, causing misreading of mRNA and inhibiting protein synthesis.
Rifampicin	Rifamycin	Inhibits DNA-dependent RNA polymerase, blocking RNA synthesis.
Ampicillin	Beta-lactam	Inhibits transpeptidase, preventing bacterial cell wall synthesis.

**Table 1:** Antibiotics used in this study, their families and mechanisms of action.

On an individual scale, the emergence of natural antibiotic resistance often relates to the processivity of key replication enzymes, exemplified by the polymerase Pol I, which is responsible for incorporating nucleotide bases into DNA during replication.

Concurrently, enzymes like Pol II, specialised in proofreading newly synthesised DNA, exhibit error excision activities and DNA resynthesis capabilities, thereby diminishing the likelihood of incorporating DNA base errors (Carey, 2015).

This type of chromosomal mutation conserved is relatively infrequent; bacteria have an average mutation rate of 1 for every  $10^6$  to  $10^9$  per nucleotide incorporation and most of these modifications will be deleterious to the cell and discarded (Reygaert, 2018). In addition, as exemplified by the resistance against Methicillin acquired in *Staphylococcus aureus*, the resistance can come with a cost to the organism, which, in this case, presents a significant decrease in its growth rate (Rungelrath, 2021). The surge in the number of bacteria resistant to one or multiple antibiotics is a testament to the plasticity of their genome. It is predominantly ascribed to mechanisms distinct from chromosomal mutations.



**Figure 6:** Mechanisms of horizontal DNA transfer into bacteria **A.** Transformation: Transfer of cell-free or “naked” DNA from one cell to another **B.** Transduction: Transfer of genes from one cell to another by a

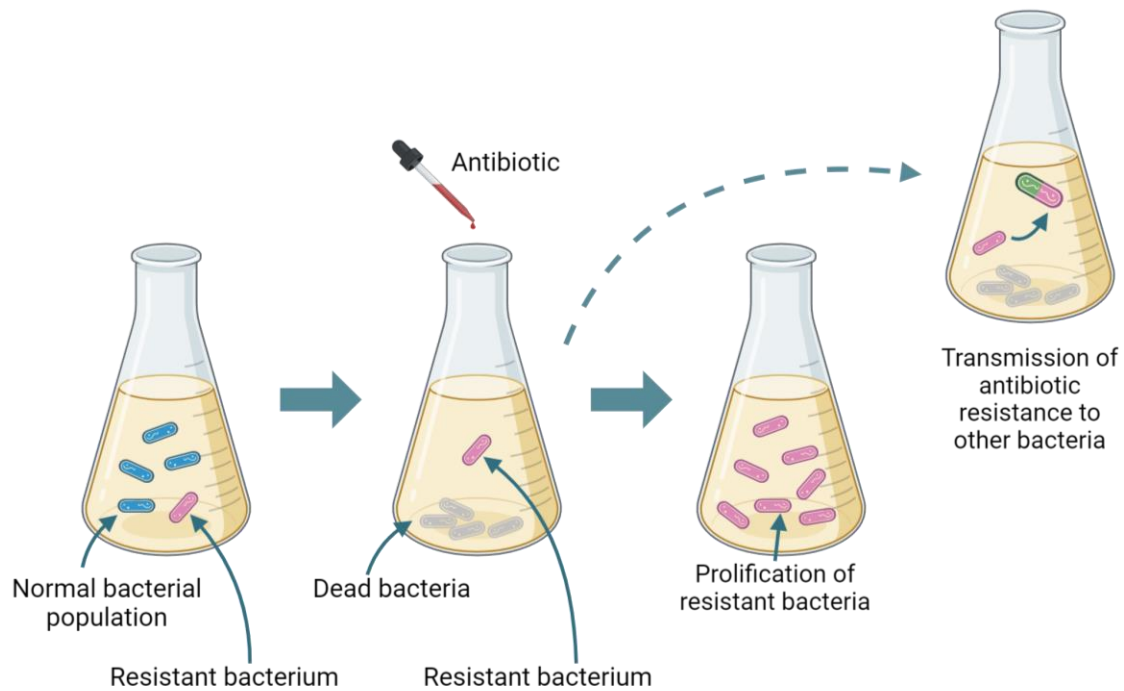
bacteriophage C. Conjugation: Transfer of genes between cells that are in physical contact with one another. (Acharya, T. (2024). *Molecular Biology*).

Unlike the rarity of chromosomal mutations, the exchange and acquisition of mobile genetic elements carrying antibiotic resistance genes can be found in up to 1 bacterium out of 100, a significantly higher occurrence. For illustration, the analysis of *Neisseria gonorrhoeae* genomic sequences revealed a growing prevalence of the spread of individuals resistant to Penicillin and other antibiotics over the past 40 years (Abrams *et al.*, 2017). On a different time scale, examining the *E. coli* genome across the last 100 million years reveals that at least 18 % of its genome would originate from horizontal gene transfer events with other species (Brown, 2003).

Hence, individuals of the same species can engage in horizontal transfer of DNA segments during conjugation. This process involves the direct transfer of circular DNA portions, often manifesting as plasmids. Moreover, through bacterial transformation, DNA segments from the surrounding environment can be taken up into the cytoplasm by naturally competent bacteria such as *Acinetobacter* spp, potentially adding antibiotic-resistance genes to their genomic repertoire (Veening *et al.*, 2017). Transport of exogenous DNA to naturally competent bacteria can also be seen during the transformation process, soliciting a great variety of proteins, as represented in **Figure 6** (Chen *et al.*, 2004).

Furthermore, viral vectors like bacteriophages can transmit linear DNA sequences during the transduction, shuttling bacterial DNA containing resistance genes from one bacterial host to another. These mechanisms underpin the swift dissemination of antibiotic-resistance vectors among different organisms. Following its entry into the cytoplasm, these plasmids can persist across generations as small circular DNA entities or integrate into the bacterial chromosome (Colavecchio *et al.*, 2017).

Specific loci in bacterial DNA are particularly prone to integrating such sequences, accumulating within operons. These sequences may conserve mobility and transposition capabilities within the genome (Costa *et al.*, 2009; Brandi G, 2021) . Fundamentally, the environmental pressure exemplified by the presence of a specific antibiotic directs the preservation of these sequences, allowing bacteria to survive under such conditions (Partridge *et al.*, 2018; Zhao *et al.*, 2020)).



**Figure 7:** Schematic representation of the selection and establishment of antibiotic-resistant organisms within a bacterial population with dissemination to other bacteria.

Bacteria that have acquired a gene coding for antibiotic resistance introduced in their environment will thus gain a competitive advantage over other non-resistant homologues (Gerardin *et al.*, 2016). This advantage supports a natural selection favouring individuals capable of survival, leading to the proliferation of antibiotic-resistant bacterial strains exposed to the specific antibiotic (Vogwill *et al.*, 2014). At the population level, these strains will become the most represented. This scenario can be observed within the intestinal microbiota or at mucosal locations such as the oral or genital areas. Refer to **Figure 7** for a detailed schematic overview of this system.

In the context of antibiotic treatment within the concentration range between the Minimum Inhibitory Concentration (MIC) and the concentration preventing the emergence of resistant mutants (CPR), only the bacteria-bearing resistance gene will survive, promoting horizontal gene exchanges encoding antibiotic resistance factors (Warsi O, 2024).

This phenomenon is conducive to the proliferation of resistant bacteria, inducing noteworthy variations in the existing bacterial flora and the commensal relationships with the host



organism (Waele *et al.*, 2018). Therefore, concentration and duration play critical roles in preserving a diverse and rich bacterial community within the microbiota during antibiotic administration. Alterations in the microbiota may carry implications for the onset of autoimmune, inflammatory and allergic diseases (Patangia *et al.*, 2022).

The interactions within the bacterial community become increasingly complex during the biofilm development process when many layers of bacteria are exposed to varying quantities of nutrients, oxygen and antibiotics. According to some research, under these conditions, resistance genes propagate more widely and the population has particularly effective DNA exchange zones (Michaelis *et al.*, 2023).

In response to extensive DNA damage, cells activate the SOS response, a crucial regulatory network. This system links various DNA repair mechanisms, enhancing the cell's ability to cope with genetic stress and maintain stability (Janion C, 2008).

## **2. C SOS response: a crucial link to DNA repair mechanisms**

Bacterial cell cycles do not follow the typical G1/S/G2 division stages, in contrast to the highly coordinated cell growth system of eukaryotic cells, which is governed by checkpoints that react to the internal condition of the cell (Reyes-Lamothe *et al.*, 2019). Nevertheless, bacteria possess dynamic response pathways that can modulate the cell cycle, especially in the presence of harmful agents. This adaptive response, known as the SOS pathway, becomes pivotal when the cell faces an exposition to genotoxic events, where DNA replication cannot maintain its usual cadence, requiring the activation and recruitment of appropriate repair mechanisms (Miller *et al.*, 2004 Alcasabas *et al.*, 2001).

The SOS mechanism depends on activating and controlling genes essential to intracellular signalling pathways and cell cycle regulation. The literature delineates this cascade as dependent upon the synergistic roles of two essential enzymes: RecA and LexA (Erill *et al.*, 2007; Mo *et al.*, 2018). In its basal state, LexA is weakly transcribed, dimeric and represses approximately forty genes (including its own and that of RecA) by binding to the SOS boxes close to their promoters (Zhang *et al.*, 2010). This interaction obstructs the RNA polymerase access to the site, inhibiting transcription.

As previously mentioned, RecA plays a dual role in HR, other DNA repair mechanisms and the SOS response. Exposure to genotoxic stress, especially ssDNA, activates RecA, forming

nucleoprotein filaments on these single-strand structures. Upon activation, RecA exhibits a protease activity that permits LexA cleavage. This process initiates the self-catalysis of LexA, thereby inhibiting its dimerisation and reducing its cellular abundance. Given that LexA's repressing activity is contingent upon its dimeric state, the 40 genes previously repressed show a subsequent escalation in their expression (Egelman E 2023). The SOS response is self-activating; it maintains its active state as long as the RecA protein is attached to ssDNA in a nucleofilament structure. Until the ssDNA anomalies are fixed, this activation enhances this pathway, which increases RecA expression and activity (Maslowska *et al.*, 2019). This model demonstrates how interspecies recombination is enhanced by the SOS response, mainly through the upregulation of RecA (Maslowska *et al.*, 2019).

These observations have been nuanced in bacteria, specifically within *M. tuberculosis*, by identifying a DNA damage-inducible promoter that upregulates RecA transcription (Movahedzadeh *et al.*, 1997; Bhattar *et al.*, 2014). Furthermore, studies indicate that even in the absence of *recA* in the genome of the organisms observed, DNA damage alone may activate directly genes implicated in repair pathways (Rand *et al.*, 2003; Davis *et al.*, 2002).

Checkpoint mechanisms safeguard the genome integrity of bacteria that are ready to enter the sporulation phase. By suppressing the transcription of specific genes, these pathways ensure that cells progressing to this stage are free of genomic abnormalities, such as by preventing genome compaction during sporulation (Walter *et al.*, 2015).

### **3. NucS, a player of interest in the DNA repair**

#### **3. A Key steps of the DNA repair**

Replication addresses a significant challenge in bacteria's life: the accurate duplication of genetic material to produce identical progeny to the mother cell. As previously discussed, DNA is susceptible to several agents and events that may compromise its structure and sequence. Maintaining this level of organisation requires monitoring mechanisms for newly synthesised DNA relative to the template (Burby *et al.*, 2019).

The DNA structure made up of two antisense strands polymerised in the 5' to 3' orientation, plays a pivotal role in its subsequent repair. Specifically, a 3' → 5' proofreading activity can hydrolyse incorrectly incorporated bases and then continue synthesising the produced 3' end,

creating phosphodiester bonds between adjacent nucleotides (Dodd *et al.*, 2020). In conjunction with the 3' → 5' proofreading exonuclease activity, MMR can be activated to accurately correct base incorporation errors with an accuracy of 1 error per 10<sup>3</sup> nucleotides. Considering both the exonuclease activity and MMR, in *Saccharomyces cerevisiae*, the probability of a base being incorporated incorrectly in the DNA decreases by a factor of 100,000 as low as one mistake per 1 billion nucleotides (Johnson *et al.*, 2000; Pray, 2008).

An aspect underscoring the significance of DNA repair lies in the significant fraction of the DNA's coding capacity devoted to encoding proteins exclusively involved in DNA repair. Despite DNA's inherent stability, it is still prone to spontaneous modifications, such as cytosine deamination into uracil (Bekesi *et al.*, 2021). An uncorrected and replicated DNA base sets the door for an error to be incorporated into the offspring's DNA.

Regardless of the specificity of the DNA repair mechanism to different damages, these processes involve a finely regulated cascade of protein interactions undergoing crucial steps. Firstly, the timely detection of DNA damage precedes the repair process that requires the presence of proteins responsible for monitoring the integrity of the genome (Kamat *et al.*, 2024; Kraithong *et al.*, 2020). Once identified, the damage must be signalled within the cell to recruit the appropriate repair machinery at the damage site and the cellular activities affected by this error must also be conveyed (Lin *et al.*, 1997; Thrall *et al.*, 2017).

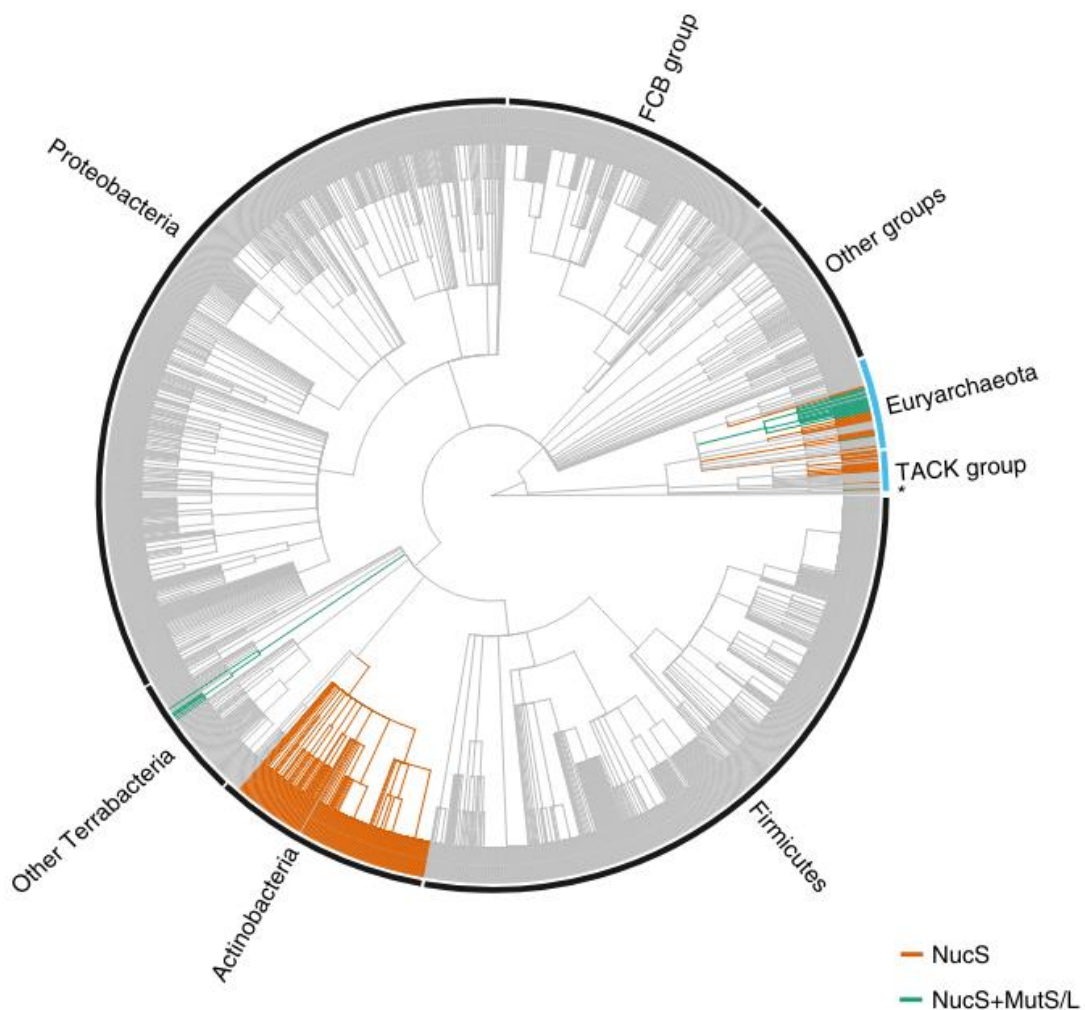
The diverse DNA repair systems diverge in the enzymatic activities observed following signalling and partner recruitment (Lyama *et al.*, 2013). In most DNA repair systems, either single or DSBs are induced in the damaged strand, followed by the excision of sequences spanning several nucleotides at the damaged site. Subsequently, the DNA is restored using the undamaged strand as a template. Then, a ligase sealed the repaired strand. These events can take place concurrently with DNA transcription, involving an even more substantial recruitment of proteins at the site and occasionally the introduction of additional errors, such as a second break in the initially undamaged strand of DNA, generating a DSB (Hanawalt *et al.*, 2008). While several well-known repair pathways, such as Nucleotide Excision Repair (NER) and MMR, handle different types of DNA damage, recent research has identified a protein that plays a significant role in a unique DNA repair mechanism. This protein, NucS (also known as EndoMS in archaea), has garnered attention for its ability to resolve mismatches and other forms of DNA damage in organisms that lack the conventional MMR system (Castañeda-García *et al.*, 2017).

### 3. B Historical discovery and phylogenetic history of NucS

Historically, NucS—also known as EndoMS in archaea—was discovered in *Pyrococcus abyssi* during a study on the rectification of branched DNA structures. Before its discovery, the XPF protein was thought to be the only source of the nucleolytic activity required to resolve these DNA conformations. However, there was no evidence of its ability to process Y-shape DNA structures. This observation implies that another gene may code for an endonuclease with similar selectivity. The activity of the isolated NucS/EndoMS proteins on Y-shaped and branched structures was confirmed by further biochemical investigations. The association of NucS/EndoMS with the RecB nucleases family steered its identification as a promising candidate for this role (Ren *et al.*, 2009).

A comparative study of the genomes bearing genes encoding for MutS/L alongside those encoding for NucS has highlighted a correlation: the absence of *mutS/L* coincides with the presence of *nucS*, especially within the entire Actinobacteria phylum as presented in **Figure 8**. These results have reinforced the initial hypothesis of NucS involvement in DNA repair, opening up the prospect of NucS activity in a new MMR pathway. In Archaea, organisms have been reported to have a co-presence of both *mutS/L* and *nucS*. This aspect suggests that traditional repair mechanisms and those based on NucS tend to be mutually exclusive, highlighting their independent roles in ensuring genomic fidelity and stability (Castañeda-García *et al.*, 2017; Ishino *et al.*, 2018).

The evolutionary history of NucS is crucial in understanding its role in DNA repair. The literature indicates that NucS likely originated in archaea and spread to certain bacteria, particularly within the phylum Actinobacteria, through Horizontal Gene Transfer (HGT). This hypothesis is supported by the gene's presence in many archaea, absence in eukaryotes and predominant distribution in Actinobacteria. Two possible HGT events are proposed to have facilitated the dissemination of NucS among Actinobacteria and the Deinococcus-Thermus group, potentially leading to the loss of MutS/L in these lineages. Further exploration into the evolutionary history of *nucS* suggests that it is a fusion of two distinct domains: the N-terminal domain, associated with DNA binding and the C-terminal RecB-like domain, which harbours endonuclease activity. This complex history, involving fusion events, shaped the current structure and function of the *nucS* gene (Castañeda-García *et al.*, 2017).



**Figure 8:** Phylogenetic profiling of NucS. The NCBI taxonomic tree comprises 2,186 species of bacteria (black outer label) and archaea (blue outer label). Orange branches: NucS only; green branches: NucS and MutS–MutL (Castañeda-García *et al.*, 2017).

Gaining structural insights into NucS is essential for comprehending its role in DNA repair. Detailed structural analyses reveal how NucS interacts with DNA and other proteins to perform its critical functions.

### 3. C Structural insights into NucS

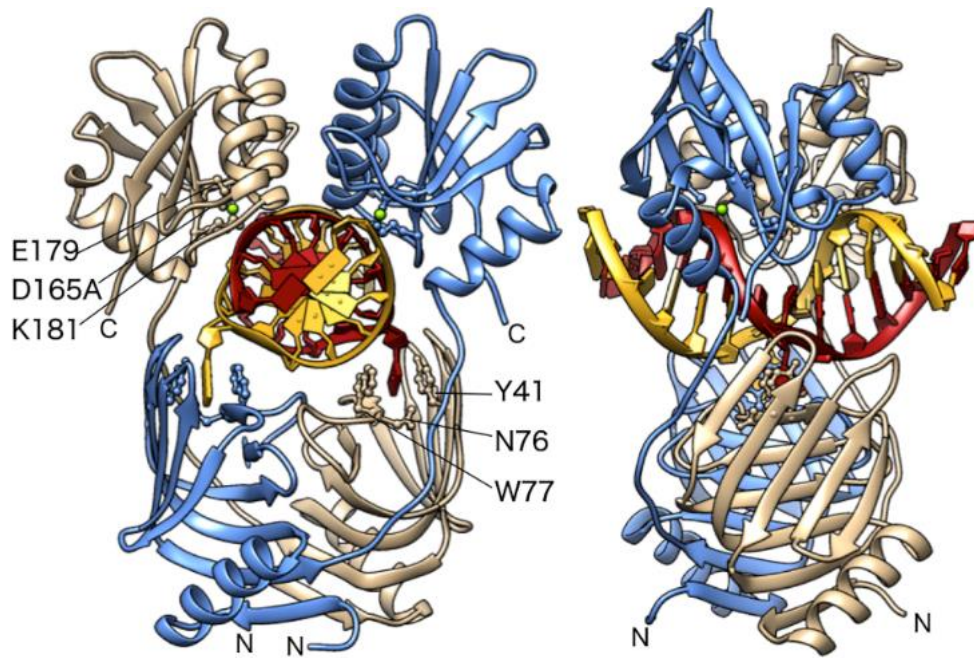
The study of protein structures delves into the intricacies of amino acid sequences that compose the polypeptide and the spatial requirements dictated by atomic radii, setting boundaries on the three-dimensional conformations adopted. However, theoretically, the protein backbone remains flexible, allowing for diverse folding (Ramachandran plot). The folding trajectory of an amino acid sequence is also influenced by non-covalent interactions between sequentially distant segments, although spatially proximate (Mikhonin *et al.*, 2006;

Irback *et al.*, 2000). These interactions determine the stability of the conformation adopted by the protein. The immediate environment of the protein, such as hydrophobicity, further sculpts the folding landscape for specific amino acid chains. Ultimately, the protein assumes a final conformation that optimally minimises the free energy required for the structure (Huang *et al.*, 2012; Mobley *et al.*, 2007).

Analyses of the NucS structure have highlighted its role as a homodimer see **Figure 9**, with dimerisation facilitated by its N-terminal domain. The structure of NucS was initially resolved by X-ray crystallography in *P. abyssi* (Ren *et al.*, 2009). These initial analyses were conducted on a stabilised structure of NucS in co-crystallization with a 15-mer dsDNA with a mismatch in the middle of the sequence. NucS has been described in 2 conformations: the closed form of NucS on the DNA and an open or apo conformation. The analysis of NucS from *P. abyssi* by X-ray has shown very different results; here, we focus on the predictions obtained from *TkoNucS*. (Ren *et al.*, 2009; Nakae *et al.*, 2016).

Research into NucS's structure has been carried out across both the archaeal and bacterial domains, yielding diverse insights from the collected literature. However, it is possible to highlight similarities across these different organisms. Structurally, a conserved domain is found in archaea and bacteria, exhibiting specialised activity in cleaving DNA mismatches. (Ishino *et al.*, 2018, Ahmad *et al.*, 2020).

The catalytic site of NucS consists of a series of amino acids with specific residues essential for its endonucleolytic function, particularly a combination of lysine, aspartate and histidine. These amino acids are critical for chelating divalent cations such as Mg<sup>2+</sup> or Mn<sup>2+</sup>. In microorganisms like *C. glutamicum*, the literature notes a mutation at the 144<sup>th</sup> amino acid from D to A, resulting in the loss of NucS's ability to exercise its activity on a mismatched DNA substrate (Ishino *et al.*, 2018; Takemoto *et al.*, 2018).



**Figure 9:** Structure of TkoEndoMS in Complex with Mismatched dsDNA. Model of the TkoEndoMS-dsDNA complex in front and side views. Critical residues for catalytic activity (Asp165Ala, Glu179 and Lys181) and mismatch base binding (Tyr41, Asn76 and Trp77) are shown as ball and stick models ( Nakae *et al.*, 2016).

NucS's affinity for DNA is facilitated by its DNA-binding domain, which also enables the enzyme to detect mismatches or aberrant base pairs within the DNA's double helix. This precise targeting is achieved through the formation of hydrogen bonds and electrostatic interactions between NucS and the damaged DNA, a process enhanced by the strategic positioning of amino acids within the DNA-binding domain and the exposure of nucleotides outside the DNA structure (Nakae *et al.*, 2016 and Zhang *et al.*, 2019).

In addition to its catalytic activity and DNA-binding site, NucS/ EndoMS exhibits a domain crucial for protein-protein interactions. The only partner found to date to interact with this C-terminal domain in bacteria in the literature is DnaN, also found under the name beta clamp or PCNA. DnaN anchors DNA polymerase to the DNA, enhancing processivity during replication. This protein-protein interaction domain corresponds to the last 12 residues of NucS/EndoMS in *P. abyssi* and the five last amino acids in *Thermococcus kodakarensis* and many members of the Actinobacteria, including *C. glutamicum* (Cebrián-Sastre *et al.*, 2021; Nakae *et al.*, 2016; Takemoto *et al.*, 2018; Ishino *et al.*, 2018; Ren *et al.*, 2009). Such interaction should be pivotal for coordinating the non-canonical MMR with other DNA repair processes or replicative machinery. This partnership could play an essential role in modulation of NucS's processivity and action, as DnaN has been shown to increase the

endonuclease activity of *Cg*/NucS significantly. This finding suggests a connection between the non-canonical MMR and the replication, particularly in the context of replication inaccuracies. Finally, examining the functional mechanisms of NucS/EndoMS and its activity in DNA repair provides a comprehensive understanding of its contributions to genomic stability. This knowledge is crucial for leveraging NucS in biotechnological and medical applications.

### **3. D Functional mechanisms of NucS/EndoMS and its DNA repair activity**

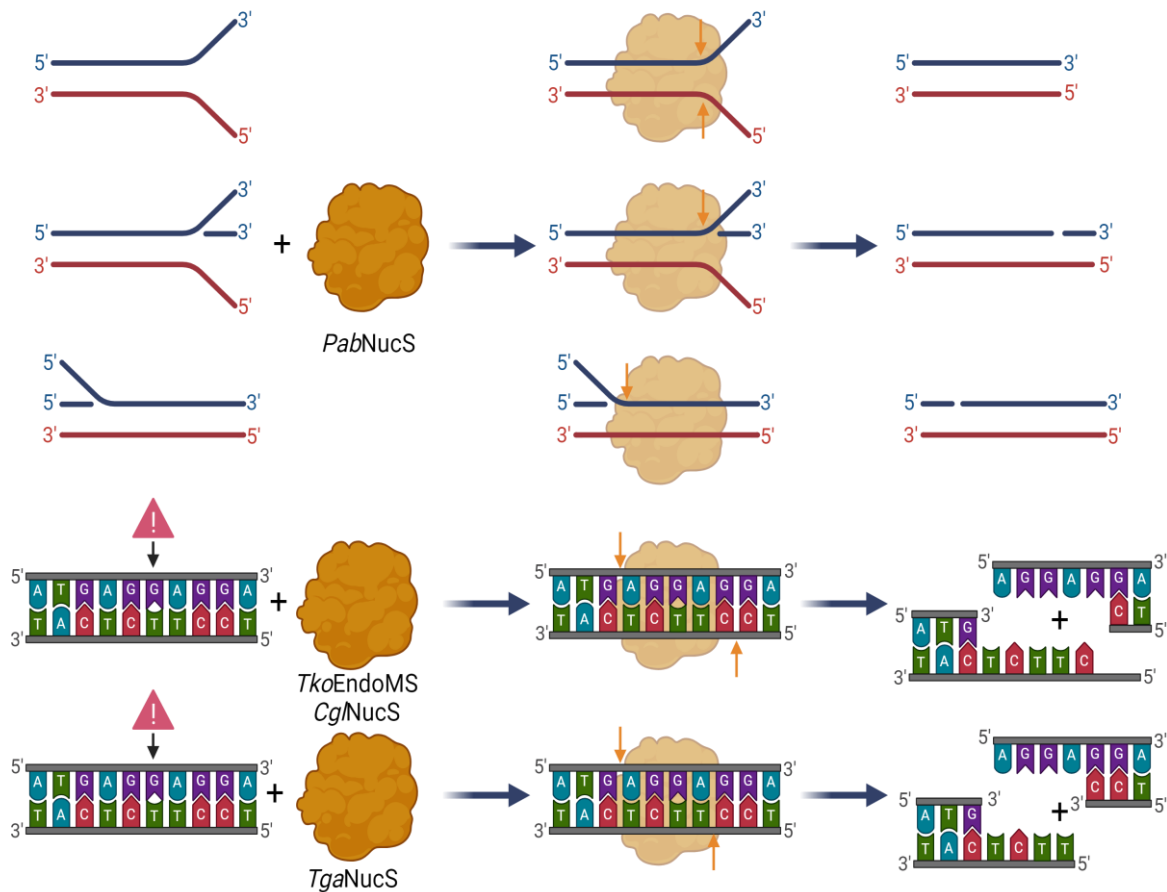
The literature on NucS underscores its critical role in the DNA repair mechanisms across bacterial and archaeal domains. Research highlights NucS's ability to identify and cleave not only Y-shape and branched DNA structure but also specific mismatches, including G/T, G/G and T/T types (Takemoto *et al.*, 2018), as summarised in **Figure 10**. This enzyme is characterised by its specificity of substrates and cleavage activity, resulting in a double-strand cut at the mismatch site. These features distinguish the MMR mediated by MutS/L from the non-canonical MMR involving NucS. Hypothesis tends to suggest that this alternative to the MMR should be considered in the context of organism adaptation to extreme growth conditions, ensuring the preservation of genome integrity (Castañeda-García *et al.*, 2017; Ishino *et al.*, 2018).

Bacterial studies have especially delved into NucS's involvement in the non-canonical MMR. This function is notably associated in Mycobacteria with the emergence of antibiotic resistance, highlighting NucS's critical influence regarding the evolution of pathogenicity and antibiotic resistance emergence in bacterial populations (Cebrián-Sastre *et al.*, 2021). Additionally, the deletion of NucS from bacterial genomes has even been associated with hypermutator phenotypes, emphasising its role in genomic maintenance (Ahmad *et al.*, 2020). A recent study reveals a specific distribution of transition mutations along the chromosome, suggesting that NucS activity is higher towards the chromosomal ends, thereby playing a crucial role in *Streptomyces*' evolutionary dynamics and genome plasticity (Dagva *et al.*, 2024).

However, studies have also acknowledged that NucS/EndoMS can identify DNA sequences with deaminated base pairs, as shown in *Thermococcus gammatolerans*. A result that suggests NucS's potential role in the maintenance of DNA integrity in the presence of



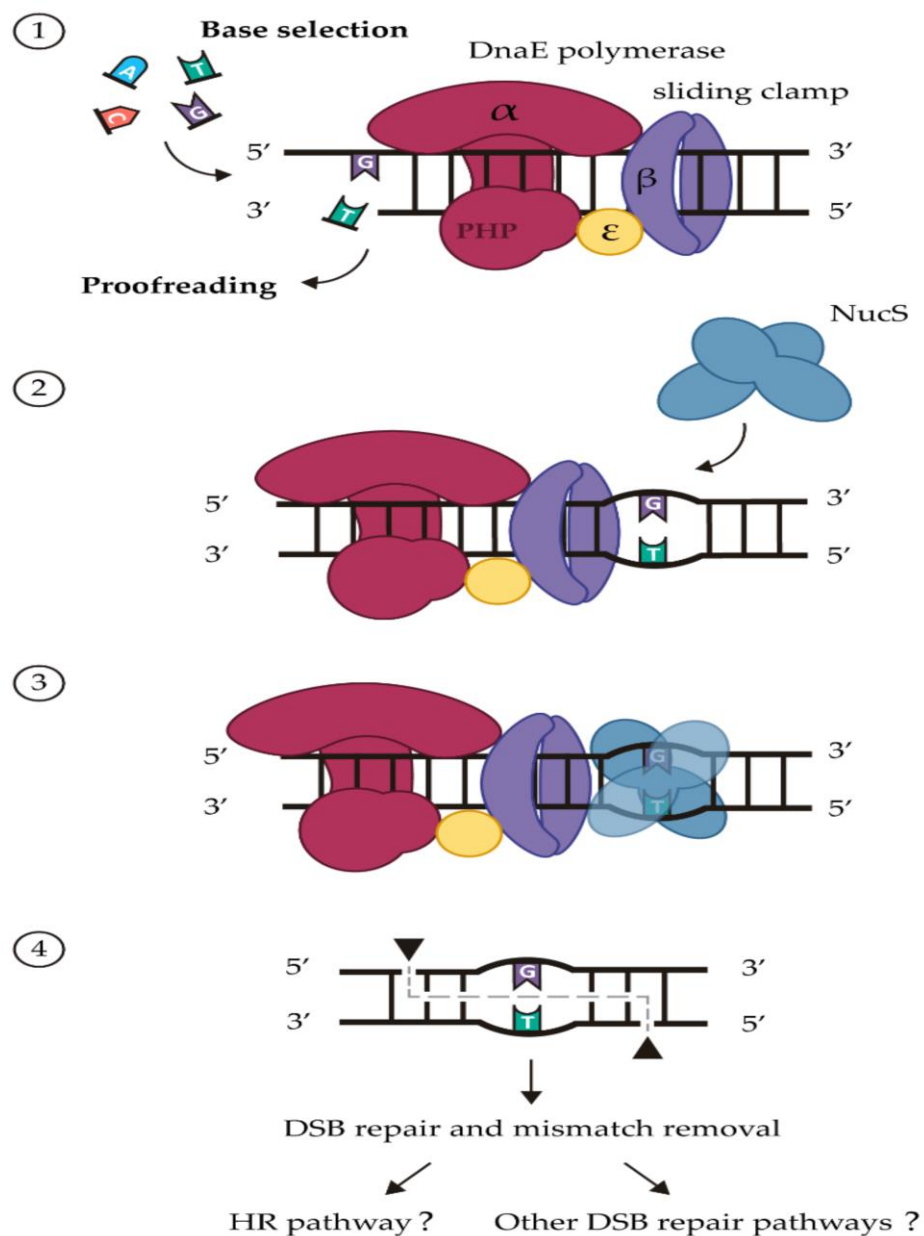
extreme conditions, such as elevated temperatures, which increase the rate of spontaneous mutations, U- and I-containing dsDNA (Suzuki *et al.*, 2019 and Zhang *et al.*, 2020). Furthermore, in *P. abyssi* and *T. gammatolerans*, NucS/EndoMS has been shown to interact with recombination intermediary DNA conformations (Ren *et al.*, 2009; Zhang *et al.*, 2020).



**Figure 10:** Graphical representation of NucS activity (from *P. abyssi*, *T. kodakarensis*, *T. gammatolerans* and *C. glutamicum*) based on DNA substrates in Y-shaped or forked configurations, as well as with mismatches or deaminations. Indication of the activity profile of NucS on these substrates with the final production containing a DSB or an SSB.

The cleavage activity of NucS entails introducing a double-strand incision within the DNA, precisely producing 5' protruding simple strand DNA of 5 nucleotides each. The literature suggests that this ssDNA product could facilitate the recruitment and loading of partners, ultimately removing the damage and restoring the DNA to its naive state.

This result highlights NucS's versatility and critical role in DNA repair. *TgaNucS* demonstrates a selective affinity for the excision of Uracil (U) and hypoxanthine (I): U/G and I/T mismatches in DNA, pinpointing its specialised function in addressing DNA lesions resulting from deamination (Zhang *et al.*, 2019). A function that is usually attributed in the literature to BER. Moreover, *TgaNucS* stays proficient at elevated temperatures, reaching up to 80 °C and across different pH levels. This remarkable resistance portrays NucS as a multifunctional enzyme that plays a pivotal role under diverse environmental conditions (Zhang *et al.*, 2019).



**Figure 11:** Model of action of the non-canonical MMR pathway in Actinobacteria. When DnaE the core polymerase represented in red and yellow arrives at the site of a mismatch, NucS (in blue) is recruited and interact with the sliding clamp in purple. Once bound onto the damage NucS activate its double-strands break activity (Cebrián-Sastre *et al.*, 2021).

Recent studies have unveiled a novel mechanism by which the mycobacterial NucS protein contributes to DNA mismatch repair, diverging from the canonical long-patch repair mechanisms (MMR) observed in other bacteria. Instead, NucS in Mycobacteria facilitates a unique short-patch repair process. Through experiments involving oligonucleotide recombination, it was demonstrated that NucS specifically targets and repairs mismatched nucleotides within a confined region of approximately 5-6 base pairs around the mismatch site, significantly differing from the extensive nucleotide degradation and resynthesis seen in long-patch repair (Islam *et al.*, 2023).

**Figure 11** shares the model of the activity of NucS on mismatch proposed in the literature; it suggests in the first steps the recruitment of NucS to the site of the DNA containing a mismatch, followed by the binding of NucS to this portion of the DNA. The presence of its partner, DnaN the sliding clamp, enhance its activity. NucS endonucleasic activity leaves a DSB in the DNA, cleaving both strands around the mismatch. Finally, the DSB and the mismatch may be repaired through the HR pathway or other DSB repair mechanisms (Cebrián-Sastre, *et al.*, 2021).

Hypotheses remain open about the release of NucS, either immediately after the cleavage or following an extended phase of lateral sliding along the DNA.

The discovery of NucS/EndoMS redefines the traditional understanding of MMR by proposing an alternative perspective on DNA repair strategies. It suggests an integrated repair system potentially leveraging HR-Double Strand Break Repair to correct mismatches, a deviation from the cell-characterized MutS/MutL-mediated pathway.

This thesis aims to explore NucS's structural and functional mechanisms in DNA repair, its interactions with other proteins and its implications for antibiotic resistance in bacteria.

In the following section, six DNA repair mechanisms will be mainly described: Mismatch Repair (MMR), NER, HR, NHEJ, Single-Strand Annealing (SSA) and finally, Micro Homology End Joining (MMEJ). All these mechanisms could directly involve NucS or be impacted by its activity.

## **4. The different pathways to repair the damage of the DNA**

### **4. A Deciphering mismatch repair: key players and regulatory synergies**

MMR is a highly conserved process across living organisms and has been considered ubiquitous by researchers over several decades. This mechanism displays a substantial level of conservation among organisms, positioning *E. coli* as an excellent model for studying this process (Groothuizen *et al.*, 2016). The similarities observed extend beyond the high percentage of identity between the two pivotal proteins, MutS/L, present in almost all phyla. They also encompass shared activity, such as recognised error specificity, bi-directionality and the specific recognition of the damaged strand versus the unmodified one (Sachadyn, 2010). However, in certain bacteria and archaea, the canonical MutS/L-dependent pathway is absent, suggesting the presence of alternative MMR mechanisms. One such alternative mechanism involves the protein NucS, also known as EndoMS in archaea, previously introduced (Castañeda-García *et al.*, 2017; Ishino *et al.*, 2018). The discovery of NucS has broadened our understanding of MMR, highlighting the evolutionary adaptability of DNA repair mechanisms and the intricate strategies cells employ to maintain genomic integrity. By investigating the structural and functional aspects of MMR, researchers aim to make parallels on how NucS compensates for the absence of MutS/L and contributes to the fidelity of DNA replication in diverse organisms.

A deletion in one of the genes supporting MMR activity is characterised by an escalation in the number of spontaneous mutations within a bacterial population. This mechanism can be described through various steps. Its primary role involves surveilling DNA integrity, specifically discerning among millions of base pairs to any incorrect pairings. In simpler terms, it detects bases that deviate from the Watson-Cricks interaction rule and insertions or small deletions (Bouchal *et al.*, 2020).

MMR targets correcting errors introduced into the DNA during replication and DNA Repair events. Not all DNA polymerases exhibit the same level of processivity or fidelity compared to the DNA template. This is particularly evident for Error-Prone Polymerases (EPPs) such as Pol V or Pol IV, which are implicated in repairing other types of DNA damage than mismatches (Tang *et al.*, 2000). These polymerases contribute to damage-induced mutagenesis due to their low fidelity regarding the naive strand (Lewis *et al.*, 2021).

Not all errors share an equal probability of incorporation in the DNA. Hence, T: G mismatches are the most prevalent, related to steric hindrance represented by the misincorporation of other bases (Allawi *et al.*, 1998). Moreover, methylcytosine deamination to thymine is a spontaneous chemical alteration mainly observed in CpG dinucleotides where cytosine is methylated (short patch repair). The MMR harbours a specificity of recognition and activity increased for this mismatch and the G: G and T: T (Marinus, 2012).

Initially, the mismatch is detected by the MutS homodimer, which possesses an ATP hydrolysis activity. The MutS homodimer distinguishes between the two DNA strands based on the presence of methyl groups on the parental strand compared to the newly synthesised strand, which lacks methylation at the d(GATC) site (Lahue *et al.*, 1987).

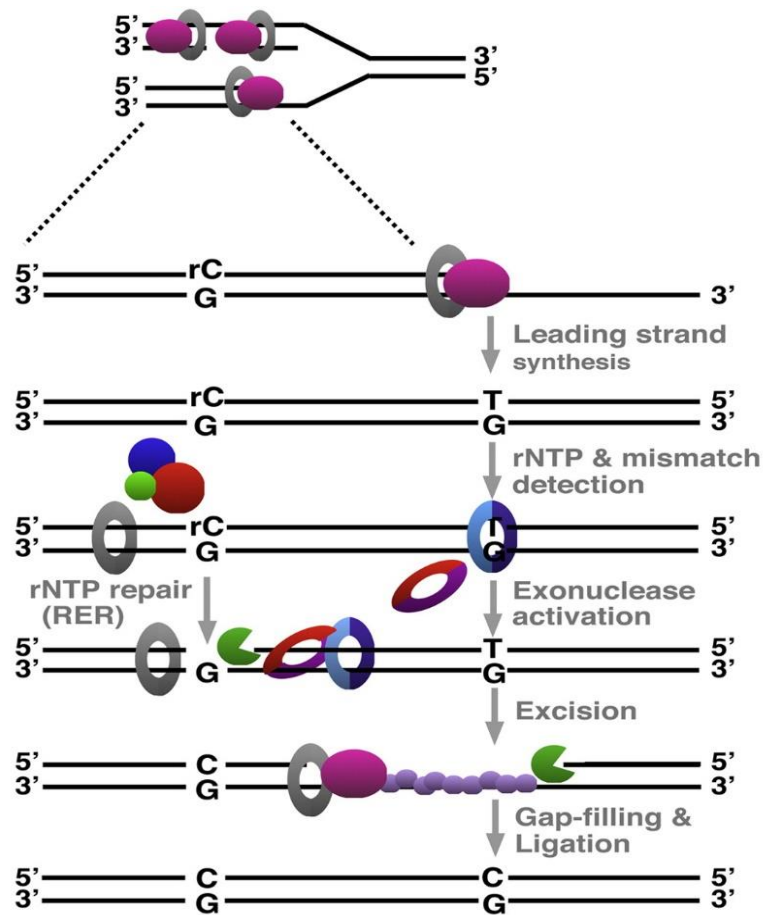
In certain organisms, MutS triggers the recruitment and activation of MutH. Even though this molecule is absent in certain study models, such as *E. coli* and seems to lack an ATPase activity, MutH has been implicated in enhancing the loading and processivity of Helicase II, necessary for the completion of MMR. Some studies emphasise an interaction between MutL and PCNA, the beta-subunit of DNA Polymerase III, suggesting a coupling between replication and MMR (Lyer *et al.*, 2010; Ban *et al.*, 1998; Junop *et al.*, 2003).

Three models in the literature interpret the different stages governing the function of the MMR. They unanimously agree that upon recognising the mismatch, the MutS homodimer does not linger at the damage site but continues its progression along the DNA. Although there are variations in describing this movement, the molecule seems to be sliding along the DNA. This migration is vital for discriminating between the newly synthesised and parental strands (Groothuisen *et al.*, 2015).

The discrimination process between the two DNA strands relies on the presence of a gene encoding the Dam protein in the genome. This protein adds a methyl group to an adenine in a dGATC sequence in the chromosome. However, during DNA replication, as the action of the Dam is not immediate, it may take a few minutes to mark the newly synthesised strand. Hence, at this point, there is a distinction between the methylated template strand and the newly synthesised strand that has not yet undergone modification (forming a hemimethylated DNA) (Robertson *et al.*, 2006).

However, the gene encoding this enzyme has not been reported in many organisms, surprisingly harbouring a functioning MMR. A hypothesis in the literature suggests that the

interaction of MutS/L with the replication fork, eventually mediated by the beta-clamp (PCNA), could explain how the discrimination between the newly synthesised and parental strands occurs. Furthermore, in the literature, articles emphasise the endonuclease activity of MutL homologs in eukaryotes, leading to the formation of SSB on mismatched substrates. This type of cleavage, independent of methylation recognition, could resemble an initiating signal for the mismatch signalling and repair pathway (Putnam, 2021). Finally, following the incision of the newly synthesised strand, the intervention of helicase II facilitates the opening of the DNA double helix. The recruitment of an exonuclease, the repolymerization of DNA at the gap formed and its subsequent ligation conclude the repair process. Each significant step of this mechanism is represented in **Figure 12** (Reyes *et al.*, 2015).



**Figure 12:** Schematic representation of MMR, showcasing the presence of an rNTP close to the mismatch site and the co-activation of different DNA repair mechanisms in eukaryotes (Ghodgaonkar *et al.*, 2013).

MMR has been described in eukaryotes for its anti-recombinase role within the cell. While forming DNA loops between dissimilar genomic segments, it is plausible that not all bases are correctly paired. The MMR machinery can detect these low homology sites, leading to the recruitment of the MSH proteins (homologous to MutS/L in yeast) at the loop formation. This step destabilises the loop, preventing its elongation and the recruitment of all the partners necessary for the HR (Calmann *et al.*, 2005; Surtees *et al.*, 2004). The same observation has been made in *E. coli* with MutS (Tham *et al.*, 2016). This activity is believed to prevent the integration of foreign genomic sequences, such as viral sequences, or recombination between divergent genome segments.

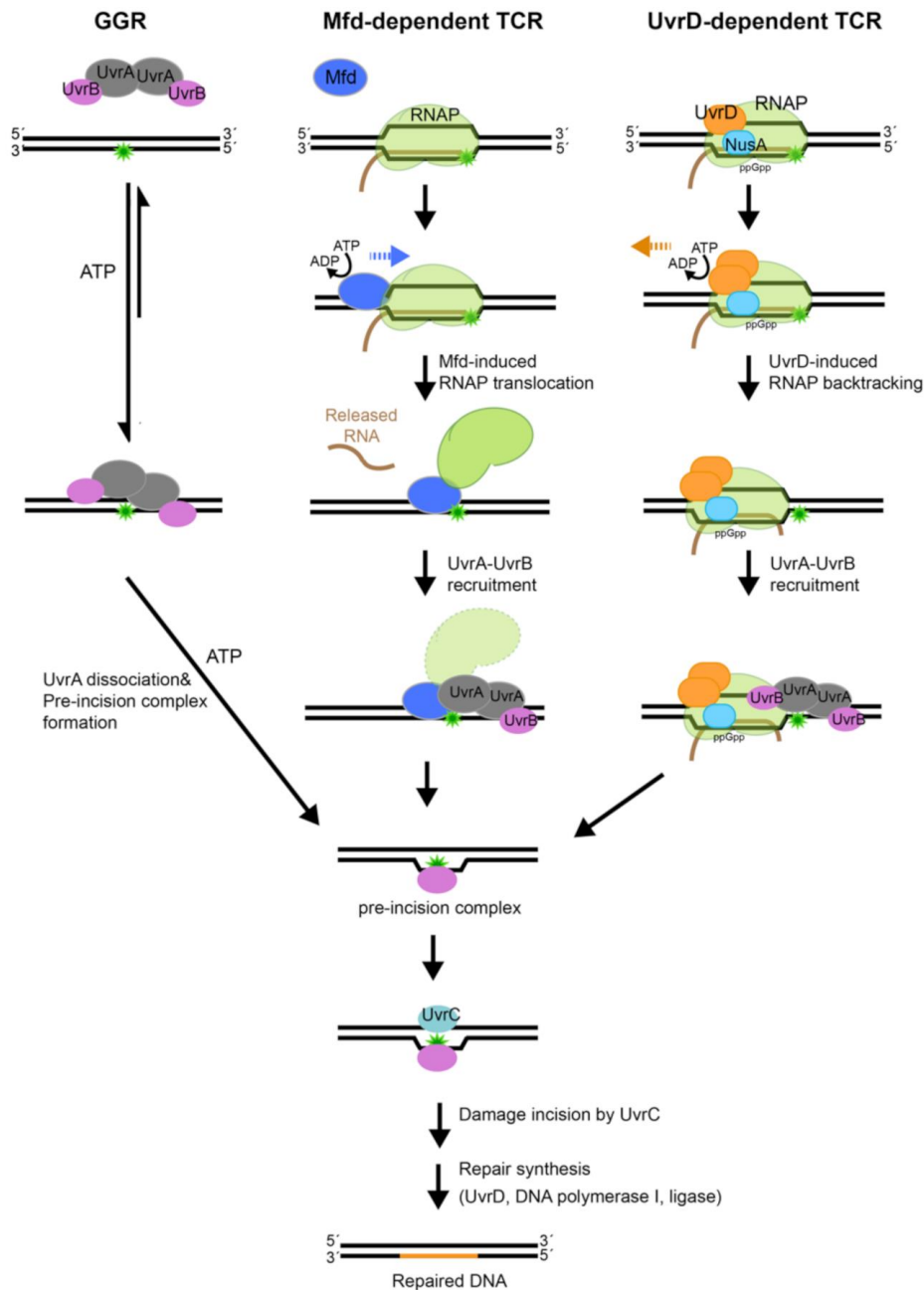
Analysis of the phylogenetic history supporting this DNA repair mechanism suggests that MMR may have been lost, inactivated and subsequently reacquired in bacteria at various points in the evolution (Muthye *et al.*, 2021). Given that the loss of MutS/L is directly correlated with the emergence of a hypermutator phenotype, these episodes may have supported bacterial adaptation phases to their environment, particularly during stress periods such as starvation (Prunier *et al.*, 2005; Mérimo *et al.*, 2002).

#### **4. B Decoding the dynamics of the nucleotide excision repair pathway**

NER is a DNA repair pathway thoroughly documented in the literature. It is a mechanism with a strong affinity for recognising damage related to DNA bases, like bulky helix-distorting lesions that can be caused by UV or intrastrand crosslinks such as thymidine dimers due to exposure to genotoxic substances (Zhang *et al.*, 2022). The NER prominently takes the stage, displaying superior activation compared to other repair pathways such as BER. The literature distinguishes two main DNA repair pathways by NER: GGR for Global-Genome Repair and TCR for Transcription-Coupled Repair. These pathways diverge in terms of enzymes implicated in recognising the damage, involving UvrA<sub>2</sub>B<sub>2</sub> for the GGR and in the specificity of the activation situation, such as during DNA transcription (Marteiijn *et al.*, 2014).

This DNA repair mechanism is activated upon signalling the presence of DNA distortion inherent to the damages, along with a short stretch of single-stranded DNA (ssDNA). Mutations resulting in the inactivation of this DNA repair pathway prove to be highly detrimental to the organisms carrying them. This is notably exemplified in humans by the genetic pathology Xeroderma pigmentosum, linked to a mutation inhibiting the GGR,

characterised by increased sensitivity to UV and a subsequent 1000-fold increase in the development of skin cancer compared to general rates registered (Steege *et al.*, 1999). A mutation causing defective TCR is at the root of the Cockayne syndrome, marked by premature death, growth retardation and neurodegeneration (Page *et al.*, 2000).



**Figure 13:** Representation of the DNA repair by NER pathways in bacteria. In global genome repair (GGR), the DNA damage is recognised by the UvrA<sub>2</sub>B<sub>2</sub> damage sensor. Transcription-coupled repair (TCR), which removes lesions from the DNA strand that is being transcribed, is mediated by the Mfd protein. Mfd binds to RNA polymerase (RNAP) stalled by the lesion, pushing it forward to expose the lesion and recruiting the NER machinery (Kraithong *et al.*, 2021).



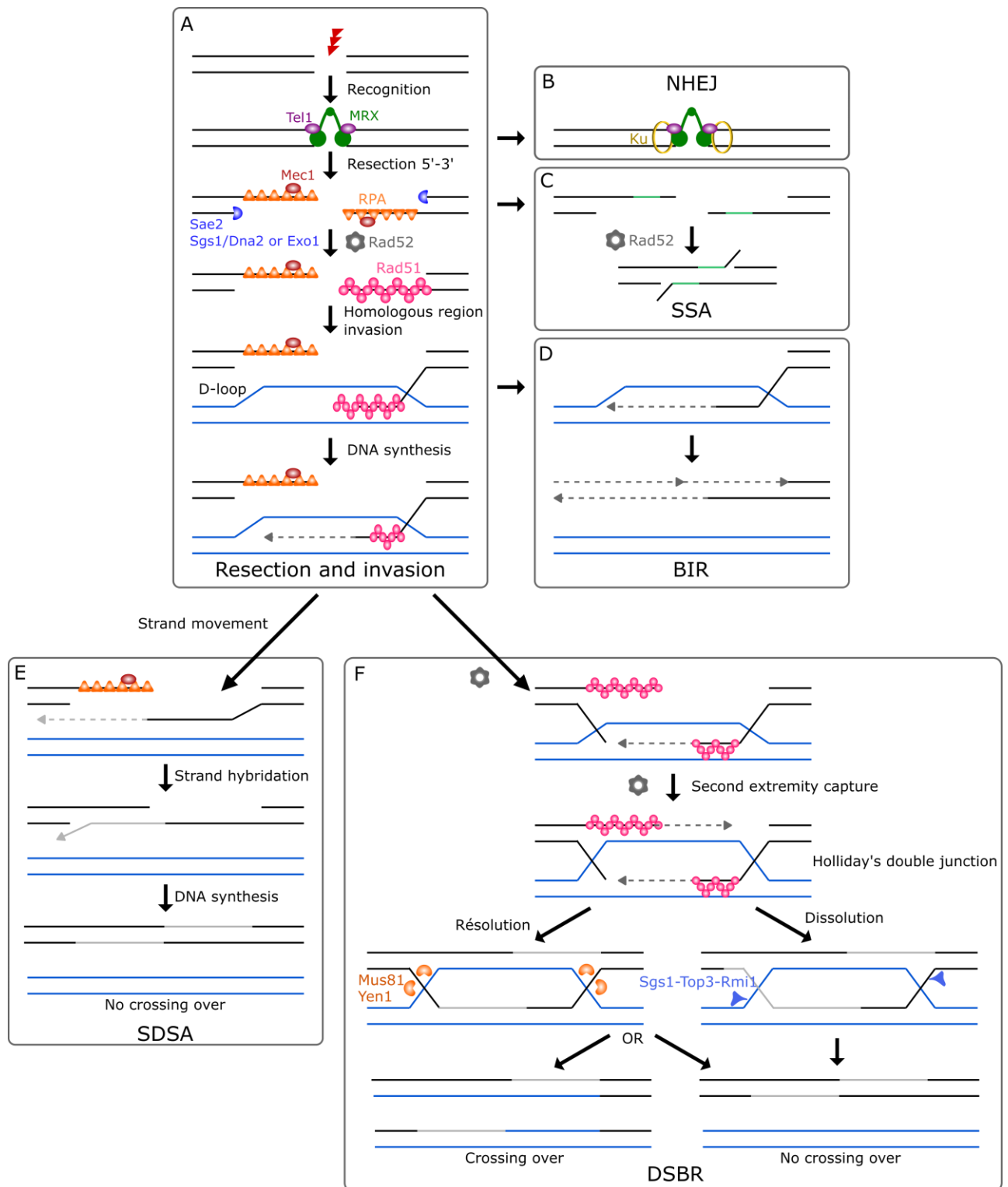
Regardless of the specificity of the DNA repair mechanism to different damages, these processes involve a finely regulated cascade of protein interactions. Firstly, the timely detection of DNA damage precedes the repair process that requires the presence of proteins responsible for monitoring the integrity of the genome. Once identified, the damage must be signalled within the cell to recruit the appropriate repair machinery at the damage site and the cellular activities affected by this error must also be conveyed (Mocquet *et al.*, 2008).

TCR takes precedence when dealing with lesions at the level of actively transcribed genes (Hanawalt *et al.*, 2008). Each step of both the GGR and TCR is represented in **Figure 13**. In *E. coli*, the recognition of the damaged strand is mediated by the protein Mfd (Mutation Frequency Declined), which initially binds to the stalled RNA polymerase (RNAP) due to the lesion. Mfd subsequently exerts a push requiring DNA hydrolysis to translocate RNAP forward, access the damaged site and recruit UvrA and the rest of the NER machinery (Ho *et al.*, 2018). The *uvrABCD* genes have all been identified in *Corynebacteria* spp. The sequences exhibited a high level of conservation. When DNA damage results in DSBs, HR is crucial in resolution. This process repair breaks and ensures chromosomal harmony, preventing genetic instability.

#### **4. C Double-Strand Breaks resolution: recombination as the keeper of chromosomal harmony**

HR serves as a pathway for repairing DSBs and a central mechanism for disseminating antibiotic-resistance genes among individuals of the same species or between phylogenetically distant organisms (Jachimowicz *et al.*, 2019; Van Hoek *et al.*, 2011; Hanage *et al.*, 2009).

HR involves proteins that stabilise sections of the bacterial chromosome as a filament. A bacterium initially possessing a single chromosome can transition to a multi-chromosomal state due to these filaments. The fate of these copies can vary: if preserved or reintegrated elsewhere in the genome, decreased pressure on these sequences can lead to mutations and antibiotic resistance. Alternatively, these filaments may favour a niche microenvironment, where the SOS response and episodes of HR between homologous chromosome segments can form new alleles. These sequences can then be fixed in the population through horizontal transfers (Bos *et al.*, 2014; Hanage *et al.*, 2009).



**Figure 14:** Coordinated Response to DSBs and Associated Repair Pathways in eukaryotes. **A.** The DSB is recognised and stabilised. **B.** Following the recognition and signalling of the break, the two ends can be directly repaired by NHEJ by joining the ends. **C.** in the context of SSA, microhomologies serve as a foundation for pairing the two DNA strands with the protruding ends. When a D-loop is formed, several repair pathways become possible. **D.** BIR involves replication. **E.** SDSA resolves the loop by displacing the hybridised strands, destabilising the structure. **F.** DSBR entails the loop migration and the resolution of the formed Holliday junctions (Emilie Fallet, Planet Vie ENS, under CC-BY-SA).

HR stands out as a crucial DNA repair mechanism for offering high-precision repair of DSB, even in cases where a portion of DNA has been degraded or lost. This distinguishes this mechanism from MMEJ or alternative end joining (Alt-EJ), emphasising its conservation across living organisms. This system relies on pairing two DNA strands that exhibit high similarity, analogues or homologues (Michel *et al.*, 2012). These sequences, with a high frequency of identity to the broken DNA segment, can originate from genes within the same family, possibly arising from genomic duplication or, in other instances, from foreign DNA segments introduced by a viral agent or acquired from an external plasmid (Ambur *et al.*, 2009). These aspects underscore the crucial role of HR as a voice of genetics shuffling mechanism and, in particular, facilitating the integration of exogenous sequences into the bacterial genome (Gonzalez-Torres *et al.*, 2019; Torrance *et al.*, 2024).

DNA DSB repair involves various steps and DNA conformation depending on the scenario. Several repair pathways can be used, each requiring a distinct degree of fidelity, energy and repair speed. **Figure 14** represents these several DNA repair processes, showing the intermediates and DNA structures at each step.

### **Pre-synaptic stage**

This first stage involves processing the free DNA ends generated by the break. Similar to previously described DNA repair systems, the initial steps of HR rely on recognising the presence of this highly toxic DNA damage. In bacteria, this function has been exemplified by the protein Ku. The interaction of Ku with the break stabilises the free ends of DNA (Habber J, 1999). Subsequently, it initiates the repair process through a signalling pathway, indicating in the cell the occurrence of this event (Lee *et al.*, 2015).

This activation leads to the recruitment of a helicase, which separates the DNA strands and a nuclease that creates a 3' overhanging end. To accomplish this function, several protein complexes, such as AdnAB in *M. tuberculosis* or AddAB in *Bacillus subtilis*, have been identified in the literature (Wigley, 2012). To avoid the resection of the ends of the two cleaved DNA strands, an alternative DSB repair system, the NHEJ is promoted (Kass *et al.*, 2010). The generated single strands are then stabilised by the recruitment of Single-Strand Binding Protein (SSB), with proteins like RecA or Rad51 in Eukaryotes (Liu *et al.*, 2011; Yang *et al.*, 2020).

## Synaptic phase

The protein RecA guides and facilitates the movement of these sequences within the DNA region, stabilising and recognising a homologous or analogous region (Prentiss *et al.*, 2015; Shibata *et al.*, 1981). This identified region serves then as a template for elongating the single strand. The enhanced mobility of the single-stranded DNA segment in the cell supports the similarity search. The pairing of the two complementary DNA segments forms a D-loop involving the displacement of the non-complementary strand and the association of the two homologous regions (Piazza *et al.*, 2019). HR encompasses three distinct processes that enable the repair of DSBs. There are the Double-Strand Break Repair (DSBR), the Synthesis-Dependent Strand Annealing (SDSA) and the Break-Induced Replication (BIR). While these three mechanisms share the same initial steps up to the formation of the D-loop, they diverge afterwards (Pham *et al.*, 2021; Li *et al.*, 2019).

In the DSBR, the D-loop stabilises and forms a double Holliday Junction (dHJ), potentially resulting in crossing overs. DNA synthesis co-occurs on the two free ends of the DNA in the 5' to 3' direction. A polymerisation that is facilitated by the stable interaction of the 3' end within the DNA loop. Specifically, the free 3' end generated by the break invades the D-loop. The other end, resulting from the break, pairs with the free strand of the D-loop through sequence complementarity, forming the Dhj (Bzymek *et al.*, 2010).

During the dHJ resolution, the simultaneous cleavage of both junctions is required, increasing the risks of genetic shuffling through inversion during the ligation between the template DNA strands and the neo-synthesized DNA (Punatar *et al.*, 2017 and Chan *et al.*, 2015). An alternative to cleavage involves the convergent slipping of the two HJs.

Regarding the SDSA, the free 3' end invades the D-loop and undertakes a template-directed extension step. After this synthesis is finished, the newly polymerised strand is moved outside of the D-loop and the D-loop loses its stability. Following this resection and until the chromosome is fully synthesised, the neo-synthesized strand hybridises with the second end of the break to act as a template for synthesising the complementary strand (Miura *et al.*, 2012).

Lastly, BIR occurs when only one end of the DSB can find a homologous sequence to invade. The 3' end invasive strand expands in a replication fork-like pattern, which can result in long tracts of new DNA synthesis and guarantee the other DNA strand's synthesis (Malkova *et al.*,

2013). BIR is often associated with genetic instability and can lead to large-scale genomic changes. These three processes emphasise the competition between these HR pathways once a DSB is generated, requiring the engagement of multiple distinct proteins. This competition persists past the D-loop formation and emphasises how dynamic the interplay between these proteins is.

### **Post-synaptic.stage**

During the DSBR process, the D-loop is established and stabilised with the help of translocases like RecG in bacteria, allowing DNA synthesis to proceed using the complementary strand as a template and ultimately requiring the resolution of the double Holliday junction (dHJ) structure. The dissolution of this complex DNA structure is enabled by resolvases such as RuvC. (Sharples *et al.*, 1999) Moreover, it involves recruiting new molecules such as FANCM, which destabilises the D-loop, or complexes like the BLM helicase and TopoIII topoisomerase (Panday *et al.*, 2020).

Recombination maintains a dual relationship with the DSB. On the one hand, it contributes to the formation of a DSB in the genome during the resolution of a dHJ. However, HR is also initiated by DSBs in the genome. Several studies assess the number of DSB within an organism's genome in response to various stresses to estimate the frequency of recombination events (Puchta *et al.*, 1996; Galli *et al.*, 1998; Taghian *et al.*, 1997).

This assumption should be considered in the context of the organism's growth phase (Gynna *et al.*, 2020). In bacteria, size constraints are such that, unlike eukaryotic organisms, they limit the tolerance for multiple copies of the genomes outside of replication. Thus, unlike diploid organisms, bacteria typically have a single chromosome and there are few copies of the genome in duplicate. This limitation leaves few opportunities for sequence homology to facilitate HR outside replication phases, particularly during the stationary phase. In such cases, the repair of DSBs in bacteria during the stationary phase relies on activating alternative repair mechanisms independent of homology (Pitcher *et al.*, 2007; De Vega M, 2013).

## **4. D NHEJ: pathway and regulation in DNA repair**

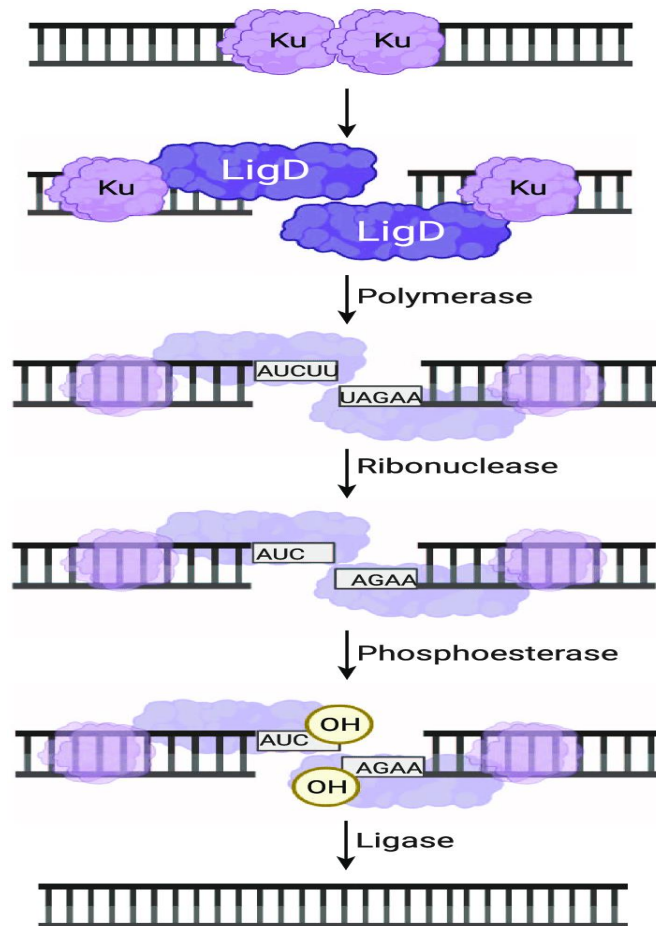
The NHEJ was initially identified in the 1980s while observing the integration of DNA from certain viruses into the genomes of eukaryotic cells (Bowater *et al.*, 2006). Although this DNA repair pathway was long studied only in eukaryotes, Mycobacteria have

also been identified as possessing functional NHEJ machinery. This was substantiated by monitoring the incorrect circularisation of linear transforming DNA molecules (Shuman *et al.*, 2007). The NHEJ pathway is crucial for resolving the DSB formed during the stationary phase. Moreover, this mechanism distinguishes itself from other DSB repair mechanisms as it does not rely on the presence of homology (Chiruvella *et al.*, 2013).

The NHEJ heavily relies on the presence of a critical protein, Ku. While Ku exhibits sporadic distribution among bacteria (Aravind *et al.*, 2001), including Actinobacteria, it is notably absent in Corynebacteria but present in Mycobacteria. In the initial stages, the Ku protein, functioning as a homodimer, identifies and binds to the DNA damage, stabilising the free ends of the DNA (Davis *et al.*, 2013). According to this observation, engineered Ku proteins fused with fluorescent proteins have been employed in certain studies to mark DSB within the organism's genome (Shee *et al.*, 2013).

As presented in **Figure 15**, following the Ku protein's fixation and signalling of the break, LigD is recruited and comes into play. This enzyme allows the addition of nucleotides, without needing a template, to the free 3' ends of DNA. It can also perform DNA repolymerisation following a complementary template. Studies have underscored the predominant incorporation of rNTPs rather than dNTPs, a deviation from the norm observed in most DNA polymerisation enzymes (Amare *et al.*, 2021). This observation correlates with the significantly increased presence of rNTPs during the stationary phase, conducive to NHEJ activation (Stephanou *et al.*, 2007). Some findings also suggest that this enzyme may possess DNA ligation activity.

Together, these diverse elements offer insights into the role of NHEJ in genome mutagenesis, especially in response to an energy-depleted and stressful environment, culminating in spore formation during the stationary phase (Ory *et al.*, 2014). Treating the free ends generated by the DSB and the nucleotide incorporation without a template can prove particularly mutagenic (Paris *et al.*, 2015). Despite DNA ligation occurring in the presence of microhomology, resection, resynthesis and spatial rearrangement of ends may also contribute to this mutagenesis.



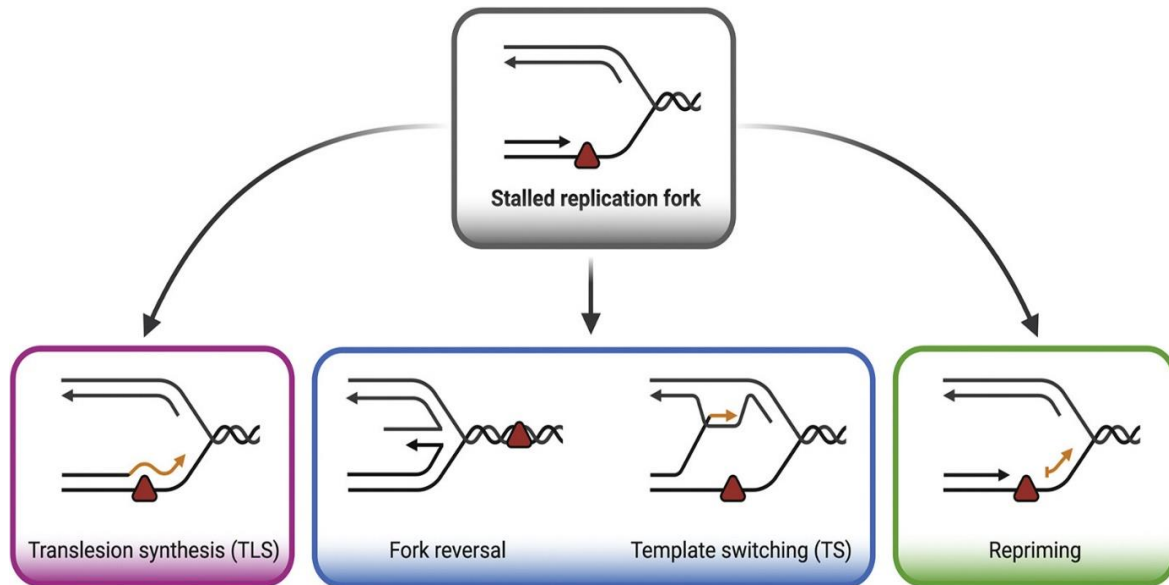
**Figure 15:** General representation of Non-homologous End Joining Repair in bacteria (*M. tuberculosis*, *P. aeruginosa* and *B. subtilis*). Ku homodimers recognize and bind to the free ends generated by the DSB. Following LigD is recruited at the site of the damage. LigD adds nucleotides from 5' to 3'. This enzyme can convert a 3'-phosphate to a 3'-hydroxyl and remove nucleotide. In the last step, the LigD ligase domain seals the phosphodiester backbone (Amare *et al.*, 2021).

There are several different ways for replication to restart in bacteria, including after a replication fork collapses. This resumption requires the recruitment at the DNA fork of critical proteins such as PriA or PriC (Windgassen *et al.*, 2017). Even in the presence of SSB, these molecules help to modify the aberrant DNA structure through their interactions with it. After the recruitment of the PriA helicase, other proteins, such as DnaB helicase and DnaT, come into play for the replication to restart (Abe *et al.*, 2020).

Replication encountering damage can still proceed without blocking in circumstances involving lesions other than DSB or SSB, as summarised in **Figure 16**, such as during Template Switching, Repriming, or Translesion Synthesis (Boiteux *et al.*, 2013).

However, in the presence of a break, bypass requires the involvement of DnaG. This enzyme has been described as priming both strands ahead of a lesion. Even though the lesion cannot

be resolved, DnaG permits the DnaB helicase to progress downstream of the damage and recruits Pol III, which can continue the polymerisation of the DNA (Kurth *et al.*, 2009; Marians K 2018). Ultimately, several studies underscore the importance of maintaining replication active in bacteria, emphasising its dependency on interactions within the replisome and DNA repair mechanisms. This type of replication restart operates independently of the presence of the replication origin and the involvement of DnaA (Quinet *et al.*, 2021).



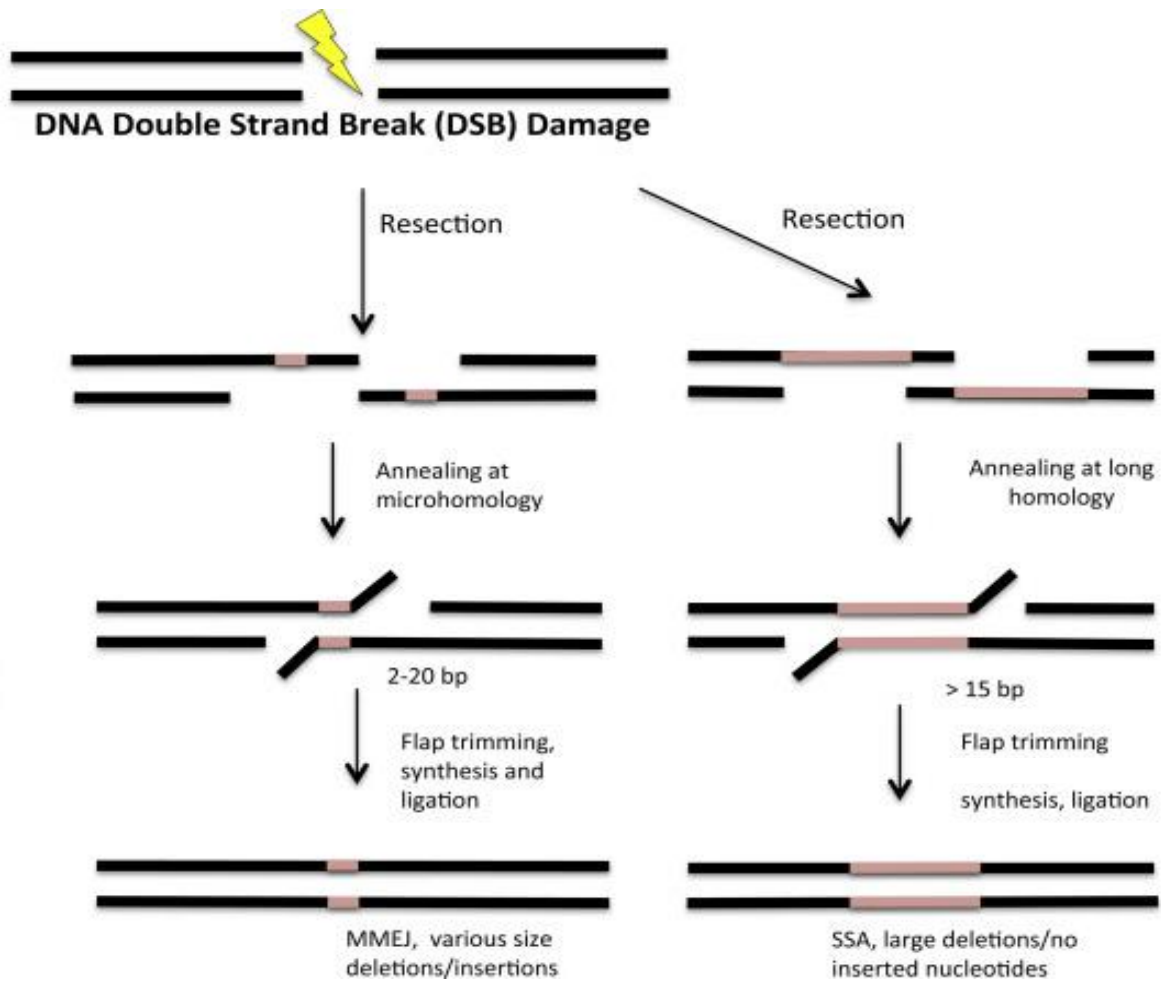
**Figure 16:** DNA replication stress response mechanisms Replication obstacles (represented by the red triangle) transiently stall fork progression. Replication obstacles can be “tolerated” by three distinct pathways to allow the resumption of replication fork progression: translesion synthesis (left), template switching or fork reversal (middle) and repriming (right) (Quinet *et al.*, 2021).

Given the various types of DNA damage, efficient repair mechanisms are essential. Proteins like NucS are pivotal in these processes, especially in organisms with high mutation rates. SSA and MMEJ are two other mechanisms involved in mutagenic and fast-acting DSB repair. By exploring them, we can appreciate cells' diverse strategies to maintain genomic integrity in the face of DNA damage.

#### 4. E Exploring single strand annealing and micro homologous end joining

Researchers have discovered a remarkable DNA restoration mechanism while studying bacterial strains resilient against UV radiation, such as *D. radiodurans* (Slade *et al.*, 2011). This mechanism, known as SSA, stands out for its ability to repair DNA even after it has shattered into many fragments (Bhargava *et al.*, 2016; Zahradka *et al.*, 2006).





**Figure 17:** Schematic representation of the SSA and MMEJ in eukaryotes. Once a DSB is generated and following a step of resection of the free ends, if the protrusive sequences present microhomologies, the MMEJ and SSA machinery can stabilise the free ends and anneal the microhomologies. Then the flap formed is processed and removed, the gap filled and the DNA ligated (Sinha *et al.*, 2016).

In contrast to previously described repair pathways, SSA facilitates swift DNA reconstruction without requiring the creation of intermediate DNA configurations or the recruitment of multiple partners as presented in **Figure 17** (Sinha *et al.*, 2016).

Notably, in eukaryotes, the MMR has been shown to play a mediator role in the activation of the SSA. In *S. cerevisiae*, the MSH2/MSH3 complex appears to mediate 3' ssDNA processing in concert with RAD10/RAD1. In the case of sequences that are not perfectly homologous (called homeologous sequences) like those processed in SSA, the MMR machinery can suppress such events via a process called heteroduplex rejection (Bhargava *et al.*, 2016). It orchestrates the DNA reassembly process independently of RecA, instead relying only on the presence of microhomology between broken DNA fragments. This

ultimately arises from the resection of longer non-homologous segments or identical repeated DNA segments originating from distinct regions of DNA.

DNA synthesis is restarted when homologies align and are paired, then a polymerase such as Pol  $\theta$  and a helicase are recruited. During ligation, the tail's homologous sequences are digested by exonuclease activity (Chang *et al.*, 2017). Therefore, since the homologous sequences encounter exonucleasic activity and the polymerase recruited harbour low fidelity, SSA emerges as a repair pathway with a high propensity for mutagenesis.

MMEJ acts in bacteria on short homologies of 5-25 base pairs at break sites that guide the repair course. The pathway starts by resectioning the DSB ends to lay single-stranded DNA regions bare. This way, the microhomologies anneal and the broken DNA ends can be aligned, with DNA polymerases filling in the gaps between them. DNA ligases fill the nicks and seal them, thus completing the repair (Truong *et al.*, 2013; Sharma *et al.*, 2015; Wang *et al.*, 2021).

This junctional process is generally considered more error-prone than NHEJ and HR because MMEJ uses short homologous sequences, which may lead to small insertions, deletions, or mutations at the repair junctions. The mutagenic potential of this process is high and, in turn, contributes to genetic diversity and, ultimately, evolution among bacterial populations (Sfeir *et al.*, 2024). In another context, MMEJ could be exploited in bacterial antibiotic resistance to enable the integration of resistance genes into the bacterial chromosome by repairing DSBs induced during horizontal gene transfer (Zhang *et al.*, 2016). Microhomologies within a plasmid or transposon sequences would bestow more capability of successful integration upon them and increase dissemination of antibiotic resistance (Partidge *et al.*, 2018; Bennett P M, 2008).

In the MMEJ, the DNA resection can be catalysed by nucleases like RecJ or exonuclease III, which generate overhangs of ssDNA that are required to discover microhomology (Sfeir *et al.*, 2015). This filling of the gapped structure is usually done with the help of DNA polymerase I and nicks are closed with the assistance of DNA ligase A to complete the repair. Since the overactivity of MMEJ leads to genomic instability, it is strictly regulated (Jiang Y 2022; Deng *et al.*, 2014).

While various DNA repair mechanisms exist, one protein of particular interest in recent research is NucS due to its unique role in organisms lacking the canonical MutS/L mechanism. Understanding its function holds the promise of providing new insights into genomic stability and antibiotic resistance. As the MutS/L proteins are known to participate in other critical DNA repair processes, such as HR and SSA, this study aims to explore whether NucS also contributes to the repair of DSBs. Unravelling the multifaceted roles of NucS could deepen our comprehension of DNA repair pathways and pave the way for novel therapeutic strategies against antibiotic-resistant pathogens.

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## **Presentation of the Thesis Objectives**

The primary objective of this thesis is to elucidate the physiological role of NucS in response to genotoxic stress in *Corynebacterium glutamicum*, focusing on its involvement in DSBR and its potential regulatory functions on DNA repair pathways. This research is grounded in the broader context of understanding genomic integrity mechanisms essential for species survival and adaptation. The study specifically aims to address several key hypotheses related to the function and regulation of NucS within bacterial cells.

### **Investigating NucS's Role in DSB Repair Pathways**

One of the central hypotheses of this thesis is that NucS plays a significant role in the homeostasis of DSBs, a critical type of DNA damage that can lead to severe genomic instability if not properly addressed. Indeed, NucS could create DSBs during MMR or, alternatively, could be recruited to DSBs, reflecting the anti-recombinase activity of this enzyme. By leveraging advanced microscopy techniques and genetic tools, this research aims to observe and characterise the recruitment and activity of NucS at sites of DSBs. This study will explore whether NucS facilitates the repair of DSBs through pathways distinct from HR, such as MMEJ. This investigation includes creating a system to visualise DSBs and HR activation in living cells, using fluorescent protein fusions, as well as tracking the dynamics of NucS' recruitment in response to genotoxic agents.

### **Examining the Interaction with DNA Repair Proteins**

Another important objective is to explore the interactions between NucS and other proteins, such as those involved in DNA repair. Given the established interaction between NucS and the beta-clamp (DnaN), which is crucial for DNA replication, this aspect of the research aims to uncover the potential regulatory mechanisms linked to NucS, particularly under conditions of stress. To do so, we used Bioinformatic tools to analyse the predicted interactome of NucS and neighbourhood genes.

### **Elucidating the Effects of NucS Deletion on Cellular Growth and Stress Response**

This thesis aimed to investigate the phenotypic consequences of NucS deletion in *C. glutamicum* to understand its functional significance. By comparing the growth and survival of WT and NucS-deficient strains under various stress conditions, including exposure to

DNA-damaging agents, this study seeks to determine how the absence of NucS affects cellular responses to genotoxic stress. This involves detailed growth curve analyses and competition assays to reveal any competitive advantages conferred by NucS deletion, particularly in environments that challenge genomic integrity.

### **Bioinformatics and Evolutionary Perspectives**

In addition to experimental approaches, this thesis will employ computational biology and bioinformatics tools to analyse the evolutionary conservation and functional domains of NucS. By comparing NucS sequences across different bacterial and archaeal species, the study aims to infer the evolutionary pressures that have shaped its function. This analysis will provide insights into the potential origins of NucS and its conservation across extremophiles, shedding light on its evolutionary adaptations to various environmental stresses.

This thesis aims to comprehensively understand NucS's multifaceted role in maintaining genomic stability, regulating DNA repair pathways and facilitating cellular adaptation to genotoxic stress. The findings from this research could have broad implications for biotechnology, medicine and our understanding of microbial evolution and resilience.

**Chapter 1 Study of the Role of NucS in Cell Growth in the Presence of**  
**Antibiotics that Mimic DSB**



## Abstract

Understanding species survival and diversification necessitates an awareness of genetic integrity. DNA repair processes are essential for cells to remain viable and avoid mutations. Even though canonical MMR systems like MutS/L have a long history, NucS (EndoMS) has emerged as a viable alternative mechanism in recent studies, especially for organisms that lack conventional MMR proteins. According to our study's hypothesis, NucS is involved in MMR, DSB repair and the regulation of other DNA repair pathways like HR and MMEJ.

- **NucS's role in the Cell Cycle Without Stress:** Growth measurements comparing the *Cgl*WT and *Cgl* $\Delta$ *nucS* revealed no appreciable differences in generation times or growth curve profiles.
- **Competition Assays under Normal Conditions:** *Cgl* $\Delta$ *nucS* did not exhibit a competitive advantage in a 24-hour co-culture with *Cgl*WT.
- **Reaction to Genotoxic Agents:** The *Cgl* $\Delta$ *nucS* strains showed minor sensitivity to Phleo and CIP, suggesting a modified DSBR.
- **Growth Advantage under Genotoxic Stress:** When antibiotics that produce DSBs, such as CIP, MMC and Phleo, are introduced, *Cgl* $\Delta$ *nucS* strains demonstrate a considerable competitive advantage over WT strains; however, this advantage does not hold in the presence of Gen, which does not induce DSBs.

Our results suggest NucS could play a role in DSBR, like in MMEJ. Under genotoxic stress, the loss of *nucS* may lead to dysregulated DSBRs and elevated HR, giving rise to a competitive growth advantage over *Cgl*WT.

## Introduction

Understanding genomic integrity is crucial for studying how offspring survive and contribute to species diversity across generations, making it one of the most important aspects of life sciences. All living organisms have a large set of DNA repair systems to repair genomic damage and prevent mutations. Some organisms are renowned for their resilience in mutagenic environments, such as *D. radiodurans*, whose robustness against damage is a hallmark of this species (Minton, 1994). For instance, MMR is critical for genomic maintenance and is conserved mainly across living beings. MMR also regulates HR in bacteria by interrupting this mechanism in the context of pairing between diverged DNA sequences (Spies *et al.*, 2015; Zahrt *et al.*, 1997). One of the roles of MMR is to correct mismatched or unpaired bases that escape the exonucleasic proofreading activity of the DNA polymerases during chromosomal duplication (Friedberg *et al.*, 2006). Although DNA polymerases, such as *E. coli*'s DNA pol III, are accurate due to base selection and proofreading activities (Kunkel *et al.*, 1992), errors still occur. *E. coli*'s DNA pol III may incorporate a mismatched nucleotide every one or two replication cycles when we take into account the inherent error rate of  $10^{-7}$  per base pair per replication cycle and a genome size of around 4.6 megabases,

The increased focus on the emergence of antibiotic-resistant strains, especially those deleterious to human health, such as *M. tuberculosis*, has instilled an even more urgent perspective into the study of preserving genomic integrity (Gygli *et al.*, 2017). Research in this area has highlighted a delicate equilibrium between DNA repair and damage tolerance mechanisms, where a fine regulation of these systems is essential for the progeny's survival (Salem *et al.*, 2009; Karran, 2001).

Recent studies have pointed out the full extent of the consequences of disabling DNA repair pathways, such as the MMR, on the emergences of antibiotic resistance and diseases (Evans *et al.*, 2000; Tham *et al.*, 2016). Impairment of MMR genes results in an increased susceptibility to cancers of the colon and other tissues in human beings (Leach *et al.*, 1993; Wind *et al.*, 1999; Pećina-Šlaus *et al.*, 2020). The cross-talk between different DNA repair pathways has shown that the inactivation of one system may affect the functionality of the other. This is observed by the regulatory function of MMR against HR. This surveillance system prevents inappropriate recombination events, which could result in genomic



instability or the introduction of undesired mutations. Inactivation or attenuation of MMR may further facilitate bacterial adaptation in a hostile environment as the intestinal tract of a host by elevating the spontaneous mutation rate (Silayeva *et al.*, 2020; Wang *et al.*, 2018). It has thus been reported that inhibition of the MMR induces an increased rate of HR, underlining the predisposition to the acquisition of foreign DNA segments in MMR-impaired bacteria (Tham *et al.*, 2013; Harris *et al.*, 1997; Matic *et al.*, 1995).

Such a background sets the scene for investigating the role of NucS. The lack of canonical MMR proteins, such as MutS/L, is correlated with the presence of *nucS* in the genomes of specific phyla of bacteria, *e.g.* Actinobacteria and some archaea. (Castañeda-García *et al.*, 2017). Despite the absence of conventional MMR pathways, these organisms do not exhibit a change in the frequency of mutations, suggesting an alternative mechanism at play. Further proof has been given that this uncanonical pathway to repair mismatches depends on the activity of NucS (Evans *et al.*, 2000; Tham *et al.*, 2016).

Both NucS and MutS/L are pivotal in DNA MMR, albeit through distinct mechanisms (Castañeda-García *et al.*, 2020). While in the literature, the dynamics and activity of MutS/L have been thoroughly investigated across both eukaryotes and bacteria —highlighting its partners for this mechanism and its interactions in the regulation of other cellular pathways—the intricacies of NucS's function and its regulatory framework remain largely elusive. The first observation of NucS/EndoMS activity on Y-shaped and branched substrates, as reported in the literature (Ren *et al.*, 2009; Takemoto *et al.*, 2018) suggests that NucS, much like MutS/L, could intervene in the HR

This hypothesis substantially complicates the existing assumptions surrounding the activity and function of NucS within the cell. Indeed, while NucS is associated with a non-canonical MMR pathway that likely involves the introduction of DSBs in the genome, we assume that NucS could also be recruited at the site of DSBs for their repair (Castañeda-García *et al.*, 2017) and participate in the MMR occurring in the vicinity of DSBs. In the context of this new hypothesis and through this study without questioning the DSB activity of NucS on a mismatched substrate, we are interested in understanding the alternative roles of NucS in repairing other types of DNA damage, specifically DSBs that are shown to promote the activation of HR (Nath *et al.*, 2022). We aim to examine the function of NucS in regulating other DNA repair mechanisms beyond MMR rather than its catalytic activity.

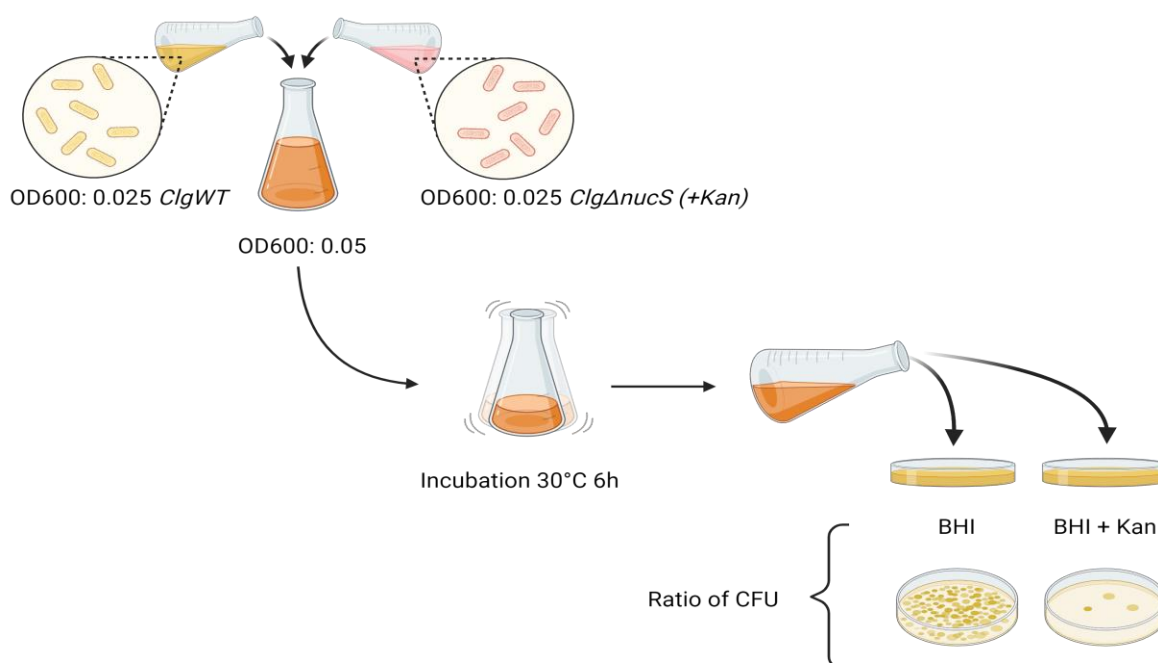
The study of bacterial growth is a common approach often employed to characterise the effects of gene mutations across bacterial populations. By focusing on gene deletion or inactivation, researchers gain insights into the roles of proteins or RNAs encoded by these genes in cellular viability and growth. Identifying phenotypes associated with genes of undetermined function facilitates the formulation of hypotheses regarding their biological activities (Klumpp *et al.*, 2009; Klumpp *et al.*, 2014). In the case of NucS, this approach is particularly relevant as, to date, its only protein partner validated experimentally in bacteria is DnaN, a protein described in the literature for its role in the replication of the genetic material. DnaN functions as a clamp around the DNA, enabling the loading of other molecular players involved in replication (Naktinis *et al.*, 1995; Barsky *et al.*, 2005).

We have focused our investigations on the role of NucS in the context of the DSBs repair by looking into the characterisation of the growth of *Cgl*/WT or *Cgl* $\Delta$ *nucS* genetic backgrounds under treatment inducing this type of damage. Without the introduction of genotoxic stress in the medium, no differences could be observed between the two genetic backgrounds. However, when stressed by these agents and cultivated in competition for the resources, it appears that the bacteria deleted for *nucS* presents an advantage against the WT one.

## Results

### A. Study of the role of NucS in the cell cycle without stress

In this study, we employed the ATCC 13032 *C. glutamicum* strain from which the *nucS* gene had been removed, an alteration previously engineered for the research conducted by Ishino *et al.*, (2018). This strain acted as a foundation for investigating the role of NucS within the cellular environment. As a starting point, we focused on independently measuring the growth of both the *CglWT* and *CglΔnucS* genetic backgrounds. We evaluated their respective generation time and growth curve profile, which revealed no significant differences (data not shown). The conditions enabled the measurement of a doubling time of 1.2 hours for both genetic backgrounds.

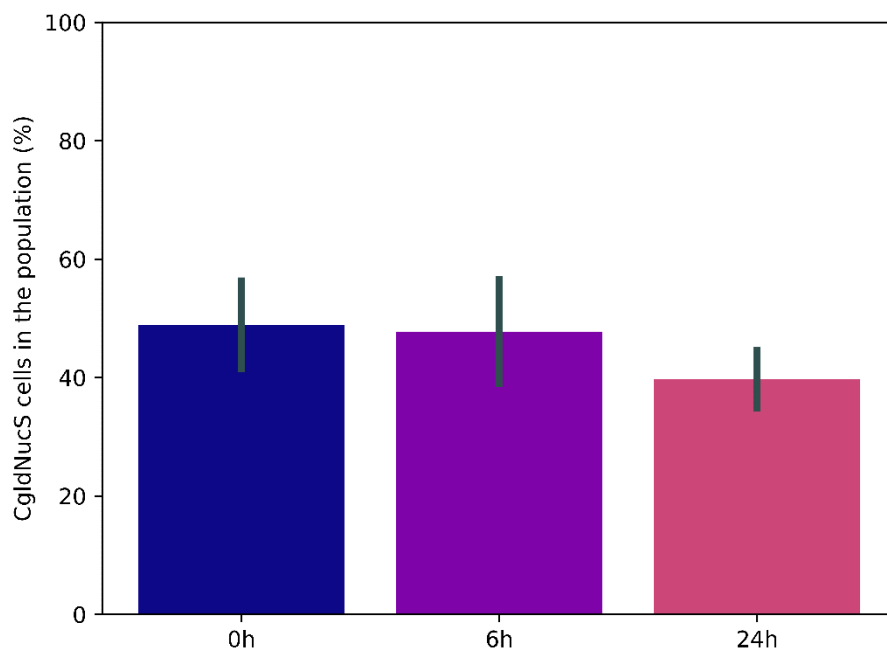


**Figure 18:** Schematic representation of the competition assay between *CglWT* and *CglΔnucS* under normal growth conditions for 6 hours.

As indicated in **Figure 18**, another way to fathom the role of NucS in the growth of *C. glutamicum* was to initiate a co-culture in the same growth medium without antibiotic with the WT genetic background and the deleted one. In this assay, competitive advantages that may not be evident in standard growth measurements could be highlighted. In a competition test, a minimal difference of 5 % in the growth rate of one bacterial population became perceptible through changes in the two genetic backgrounds ratio in the culture. To ensure the experiment is operated practically, the initial ratio of bacteria added into the Erlenmeyer flask

was 1:1, with an OD of 0.05. Any dataset that had thrown off this initial benchmark was discarded. We chose a 24 hours duration to perform our analysis, a sufficient period to observe all the phases of the bacterial cell cycle of *C. glutamicum*: lag phase, exponential growth and stationary phase.

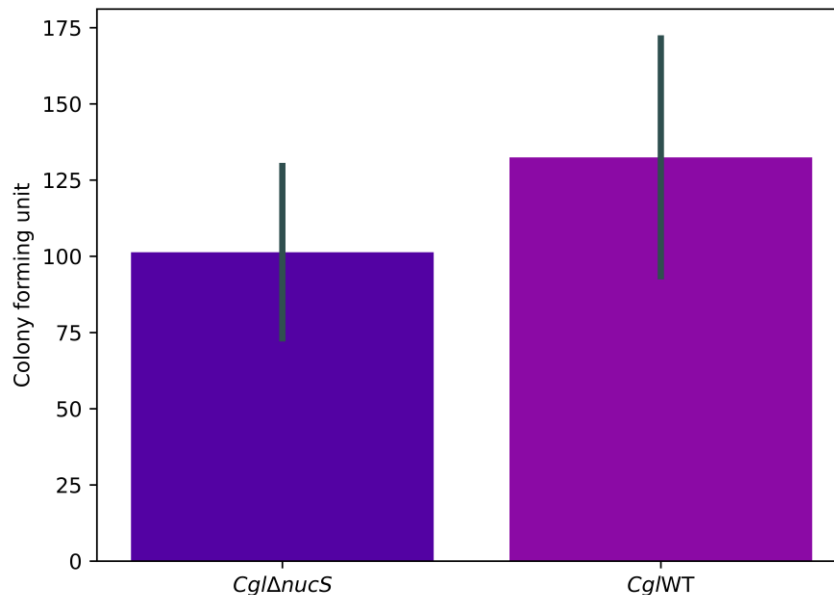
To differentiate between the two genetic backgrounds, we chose to detect specifically in the bacterial population the  $\Delta nucS$  bacteria, as the gene encoding NucS was replaced by the insertion of the Kanamycin (Kn) resistance cassette *apha3*. This modification facilitated the distinction between the mixed populations of the two genetic backgrounds through replicaplating on BHI agar containing Kn, in contrast to replica-plating on BHI agar alone. This method facilitates the calculation of the ratio between the two populations and enables the assessment of each strain's proportion.



**Figure 19:** Histogram of the frequency of *CglΔnucS* cells in a co-culture with *CglWT* cells at 0h, 6h and 24h. 0h:  $\mu = 48.92$ ,  $\sigma = 7.998$  6h:  $\mu = 47.77$ ,  $\sigma = 9.382$  24h:  $\mu = 39.73$ ,  $\sigma = 5.392$  T-Student analysis: 0h vs. 6h: p-value = 0.8231 0h vs. 24h: p-value = 0.0378 n=4.

After conducting four independent experiments with biological triplicates and two dilutions per condition for each medium—both with and without Kn—the results consistently demonstrated no difference in cell growth between the two tested genetic backgrounds, as represented in **Figure 19**. This indicates that NucS may have a minimal impact on cellular growth and survival under standard conditions.

We also investigated the response of these two genetic backgrounds to UV. We made a system to illuminate only half of our agar pad to compare the growth of bacteria in normal conditions or in the presence of UV. We concluded that UV had no direct impact on *CglΔnucS* regarding *CglWT* see **Figure 20**.



**Figure 20:** Histogram of colony forming unit of *CglWT* cells and *CglΔnucS* after 45s exposure to 112.5J/m<sup>2</sup>: *CglWT*  $\mu = 132,44$ ,  $\sigma = 40,0$  *CglΔnucS*  $\mu = 101,33$ ,  $\sigma = 29,3$  T-Student analysis: p-value = 0.76 n = 8.

## B. Study of the role of NucS in the cell cycle in presence of genotoxic agents

According to existing studies, there are indications that NucS may function as a multi-enzyme, involved not only in the non-canonical MMR but also in other DNA repair pathways (Takemoto *et al.*, 2018; Suzuki *et al.*, 2019; Zhang *et al.*, 2019). Moreover, in light of the DSBs it can introduce in the genome, it is even more interesting to look into interactions of NucS with this type of damage and indirectly its implication in other DNA repair pathways, especially those involved in processing this type of damage, such as the HR or the MMEJ (Ren *et al.*, 2009; Nakae *et al.*, 2016; Cebrián-Sastre *et al.*, 2021).

Various endogenous or exogenous agents can induce DSBs in the genome. We pursued the hypothesis that NucS acts as a regulatory agent in DSBR by conducting experiments with drugs known to cause such damage either directly or indirectly, spanning different families of antibiotics. To this end, we selected three antibiotics, Phleo, CIP and MMC, known for their roles in DSB formation, SOS response promotion and increased HR frequency, as

documented in the literature (Nath *et al.*, 2022). Indeed, the detection of a DSB is the initial signal required for the activation of the HR and several studies used the frequency of DSBs in the genomes of organisms to estimate the HR rates (Sugawara *et al.*, 1992; Richardson *et al.*, 1998).

## **B.1 Selection of genotoxic antibiotics**

The first antibiotic selected was Phleo, first reported in 1956 for its antibacterial and tumor-suppressive properties. This agent disrupts DNA synthesis in *E. coli* without affecting protein synthesis or the transcription machinery. Phleo is a member of the natural glycopeptide family and induces both SSBs and DSBs, thereby halting DNA synthesis. This antibiotic attaches to DNA through various binding modes. Initially, when an SSB is introduced in the DNA by the fixation of a first Phleo molecule, the same particle—or potentially a second one—can intercalate its bi-thiazole tail and, without detaching itself from the DNA, induce a second break, effectively creating a DSB (Chen *et al.*, 2008).

CIP, a member of the fluoroquinolone class, was the second antibiotic selected for this study. It inhibits DNA replication by interacting with enzymes such as topoisomerases, particularly GyrA. Breaks can be formed when the gyrase does not release tensions and coils in the DNA double helix during the replication. The efficacy of CIP is especially notable in gram-negative bacilli, including members of the Enterobacteriaceae family, such as *E. coli*, though it is also effective against gram-positive bacteria (Thai *et al.*, 2023).

The choice of CIP was motivated by our goal to contribute to the broader research effort addressing antibiotic resistance in pathogenic bacteria within the phylum Actinobacteria, including *M. abscessus* and *M. tuberculosis*. Fluoroquinolones are frequently used in combination with other therapeutic agents in drug therapy. They are considered an alternative in cases where traditional treatments for *M. abscessus* prove ineffective. Especially according to Castaneda *et al.*, (2022), if the WT genetic background of *M. abscessus* does not exhibit detectable resistance to CIP, the  $\Delta nucS$  one shows a notable increase in its resistance rates against this antibiotic. These findings suggest that the inactivation of *nucS* may contribute to the emergence of drug-resistant isolates, particularly in the presence of CIP.

The third antibiotic tested in this study, MMC, has been recognised since 1963 for its direct impact on DNA integrity, specifically for its ability to introduce cross-links between complementary strands and with a pronounced affinity for CpG sequences (Iyer *et al.*, 1963). This interaction leads to the inhibition of DNA synthesis. MMC has also been documented to inhibit protein and RNA synthesis at elevated concentrations. Additionally, this antibiotic promotes HR, chromosome breakage and sister chromatid exchange and triggers the SOS response in bacteria (Tomasz, 1995).

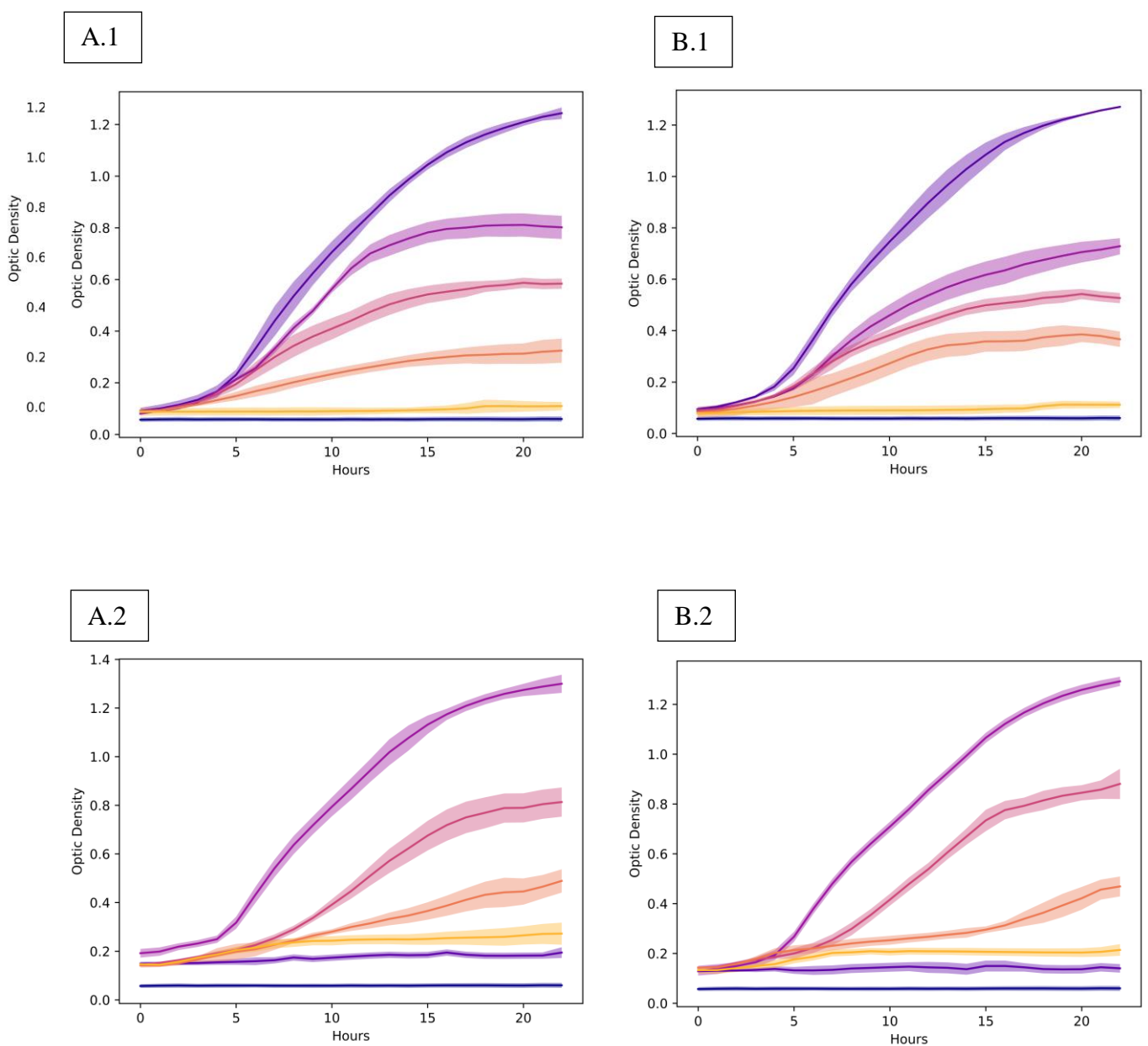
These three antibiotics induce DNA breaks in different ways and with varying frequencies depending on the concentrations used. Regarding the mechanisms by which Phleo and CIP introduce DSBs, it appears that the probability of DSBs under their treatment is higher than for MMC, which requires indirect processes.

Recently, the study conducted by Nath *et al.* (2022) confirmed the role of these three drugs in elevating the HR rate within *E. coli*. Amongst these drugs, only CIP was previously known for its effects on the HR rate in bacteria (Lopez *et al.*, 2009). This research emphasised the impact of two new drugs on the HR rate in *E. coli* and established a positive correlation between the number of DSBs introduced into the genome and the frequency of the HR. The result analysis infers that HR is essential in repairing DSBs caused by these antimicrobial agents. Since HR also enables chromosomal re-arrangement, gene duplication and amplification, these findings indicate that using these genotoxic antibiotics in the treatment of patients could heighten the risk of emerging antibiotic resistance due to the increased HR. In light of this publication in 2022, we also decided to use Gen as a control in our experiments because it is an antibiotic that does not have direct genotoxic effects.

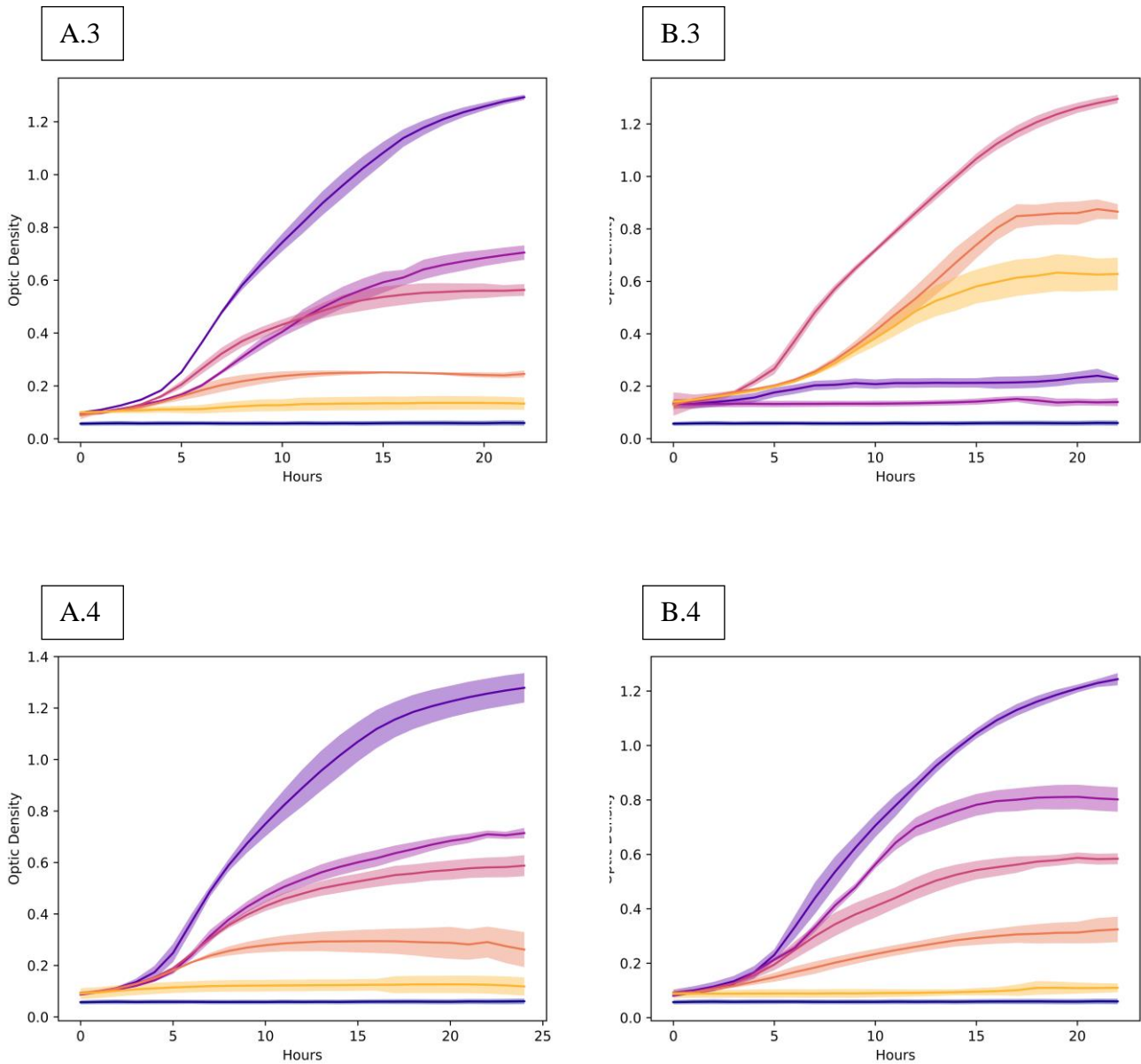
To refine and optimise our procedures, before engaging in more complex competition assays in the presence of antibiotics, we conducted a series of tests to determine the MIC of *C. glutamicum* against these antibiotics due to the lack of data within the literature. These experiments aimed to ensure that the collected data were of good quality and that the protocols were cost-effective. Initially, we compared the growth of *Cgl*WT and *Cgl* $\Delta$ *nucS* across an increasing range of antibiotics concentrations for 24 hours in BHI medium at 30 °C, using a 96-well plate format, taking on measurement of the OD every hour with an Infinite M1000Pro Tecan. Subsequently, the growth curves of these two genetic backgrounds were analysed by monitoring optical density fluctuations starting from 0.05 OD. We also carried

out a spot test that enabled us to compare the size and aspect of the colonies and the comportment of the two genetic backgrounds in the presence of different concentrations of antibiotics. No notable differences to characterise the deletion of *nucS* regarding *Cgl*/WT were noted from this experiment.

We observed dose-dependent responses with the four targeted antibiotics (Phleo, CIP, MMC and Gen), as presented in Figure 21, for the 2 genetic backgrounds. **Table 2** shows the MIC values deduced from these results for *Cgl*/WT ATCC13032 and *Cgl* $\Delta$ *nucS* for the different antimicrobials used in this work.







**Figure 21:** Growth curve of *Cgl*/WT ATCC13032 and *Cgl* $\Delta$ *nucS* ATCC13032 in presence of increasing concentration of antibiotic **A.1** *Cgl*/WT with Phleo (0  $\mu$ g/mL, 2  $\mu$ g/mL, 5  $\mu$ g/mL 8  $\mu$ g/mL, 10 $\mu$ g/mL) **B.1** *Cgl* $\Delta$ *nucS* with Phleo (0  $\mu$ g/mL, 2  $\mu$ g/mL, 5  $\mu$ g/mL 8  $\mu$ g/mL, 10 $\mu$ g/mL), **A.2** *Cgl*/WT with CIP (*Cgl*/WT 0  $\mu$ g/mL, 0,2  $\mu$ g/mL, 0,5 $\mu$ g/mL 0,8  $\mu$ g/mL, 1 $\mu$ g/mL and **B.2** *Cgl* $\Delta$ *nucS* with CIP 0  $\mu$ g/mL, 0,6  $\mu$ g/mL, 0,8 $\mu$ g/mL 1  $\mu$ g/mL, 1.5 $\mu$ g/mL), **A.3** *Cgl*/WT with MMC (0  $\mu$ g/mL, 0,2  $\mu$ g/mL, 0,3  $\mu$ g/mL 0,4  $\mu$ g/mL, 0,5 $\mu$ g/mL) **B.3** *Cgl*/WT with MMC (0  $\mu$ g/mL, 0,2  $\mu$ g/mL, 0,3  $\mu$ g/mL 0,4  $\mu$ g/mL, 0,5 $\mu$ g/mL) and **A.4** *Cgl*/WT with Gen (0  $\mu$ g/mL, 0,2  $\mu$ g/mL, 0,5  $\mu$ g/mL 0,8  $\mu$ g/mL, 1 $\mu$ g/mL) **B.4** *Cgl*/WT with Gen (0  $\mu$ g/mL, 0,2  $\mu$ g/mL, 0,5  $\mu$ g/mL 0,8  $\mu$ g/mL, 1 $\mu$ g/mL) incubated for 23 hours, n = 5.

Antibiotic	MIC <i>Cgl</i> WT ATCC13032	MIC <i>Cgl</i> $\Delta$ nucS ATCC13032
Ciprofloxacin	1 $\mu$ g/mL	1.5 $\mu$ g/mL
Phleomycin	10 $\mu$ g/mL	10 $\mu$ g/mL
Mitomycin C	0,5 $\mu$ g/mL	0,5 $\mu$ g/mL
Gentamicin	1 $\mu$ g/mL	1 $\mu$ g/mL

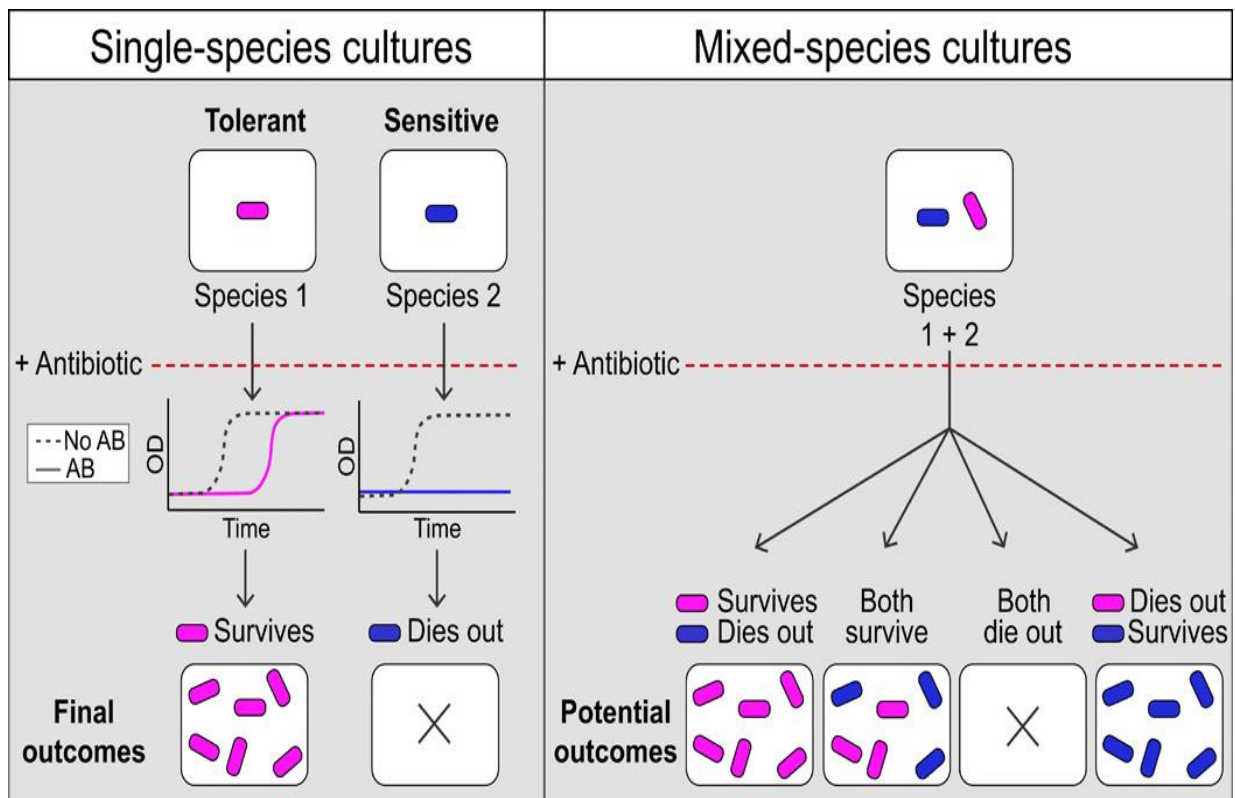
**Table 2:** Minimum Inhibitory Concentrations (MIC) of *Cgl*WT ATCC13032 and *Cgl* $\Delta$ nucS ATCC13032 in the Presence of Ciprofloxacin, Phleomycin, Mitomycin C, and Gentamicin.

No significant differences in MIC values were observed between the *Cgl*WT ATCC13032 and *Cgl* $\Delta$ nucS ATCC13032 genetic backgrounds, except in the presence of ciprofloxacin. In this case, the MIC for *Cgl* $\Delta$ nucS was 1.5  $\mu$ g/mL, compared to 1  $\mu$ g/mL for *Cgl*WT, indicating a slight increase in ciprofloxacin resistance in the  $\Delta$ nucS strain.

## **B.2 Competition test between *Cgl*WT and *Cgl* $\Delta$ nucS in the presence of CIP, MMC, Phleo, or Gen**

To deepen these analyses, we designed a competition assay between the *Cgl*WT and the *Cgl* $\Delta$ nucS genetic backgrounds, as presented before and in the presence of each antibiotic previously listed, to ascertain whether *Cgl* $\Delta$ nucS exhibits a growth advantage in the presence of drugs that induce DSBs. **Figure 22** illustrates the four potential outcomes of this experimental setup in the competition tests.

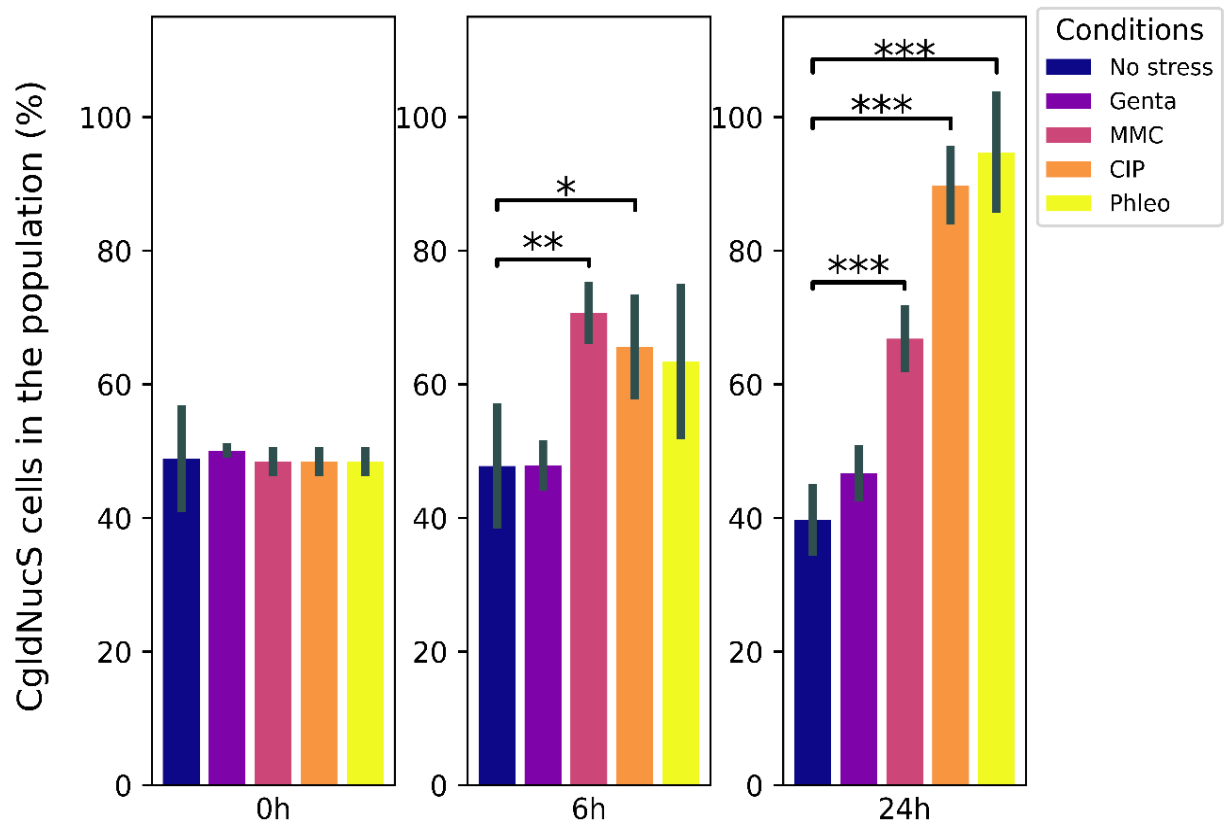
Indeed, while a traditional growth test reveals how a bacterial culture responds to an antibiotic, a competition assay between two different genetic backgrounds or bacterial species can yield various outcomes. In such tests, a bacterial population initially resistant to an antibiotic may quickly outcompete the other one and become the sole survivor within the environment. However, more intricate interactions between the two bacterial populations may also arise, influenced by disparities in their growth rates, metabolic activities, detoxification mechanisms, or virulence. Consequently, a bacterial population initially sensitive to an antibiotic could eventually prevail when co-cultured with a resistant strain and in the presence of the antibiotic become the dominant, surviving group.



**Figure 22:** Schematic representation of the competition test performed by mixing the same amount of *C. glutamicum*, WT and  $\Delta nucS$  in the presence of antibiotics (Galera-Laporta *et al.*, 2020).

At time point T0, the 1:1 ratio between *Cgl*/WT and *Cgl* $\Delta nucS$  was established at an OD of 0.05, reflecting equal inoculation for each strain into the Erlenmeyer flask. **Figure 23** illustrates the ratio derived from cell counts, confirming each genetic background's correct initial equivalent cell proportion.

When exposed to an antibiotic that causes DSBs in the genome, either CIP, Phleo or MMC, we observed a trend at 6 hours that suggests a competitive advantage over the *Cgl*/WT of the *Cgl* $\Delta nucS$  bacteria. After 24h of co-culture, this phenomenon was even more noticeable and significant, with almost 100 % (90 %  $\pm$  5,9 with CIP and 95 %  $\pm$  9,1 with Phleo) of the bacteria represented being *Cgl* $\Delta nucS$  after a CIP or Phleo treatment, confirming that this genetic background has a competitive edge over the *Cgl*/WT across these 18 growth cycles in the presence of these genotoxic antibiotics.



**Figure 23:** Graphical representation of the competition between *Cgl*WT or *CglΔnucS* in co-culture with CIP (0,2 μg/mL), MMC (0,2 μg/mL), Phleo (2 μg/mL) or Gen(0,5 μg/mL) or no antibiotic during 0, 6h and 24h at 30 °C. **0h** No stress:  $\mu = 48.92$ ,  $\sigma = 7.99$  Gen:  $\mu = 50.09$ ,  $\sigma = 1.13$  MMC:  $\mu = 48.41$ ,  $\sigma = 2.16$  CIP:  $\mu = 48.41$ ,  $\sigma = 2.16$  Phleo:  $\mu = 48.41$ ,  $\sigma = 2.16$  **6h** No stress:  $\mu = 47.77$ ,  $\sigma = 9.38$  Gen:  $\mu = 47.83$ ,  $\sigma = 3.80$  MMC:  $\mu = 70.69$ ,  $\sigma = 4.62$  CIP:  $\mu = 65.60$ ,  $\sigma = 7.85$  Phleo:  $\mu = 63.41$ ,  $\sigma = 11.67$  **24h** No stress:  $\mu = 39.73$ ,  $\sigma = 5.39$  Gen:  $\mu = 46.64$ ,  $\sigma = 4.21$  MMC:  $\mu = 66.83$ ,  $\sigma = 5.03$  CIP:  $\mu = 89.77$ ,  $\sigma = 5.91$  Phleo:  $\mu = 94.74$ ,  $\sigma = 9.07$ . T-Student analysis **0h**: No stress vs. Gen: p-value = 0.7984, No stress vs. MMC: p-value = 0.9250, No stress vs. CIP: p-value = 0.9250, No stress vs. Phleo: p-value = 0.9250, **6h**: No stress vs. Gen: p-value = 0.9910, No stress vs. MMC: p-value = 0.0025, No stress vs. CIP: p-value = 0.0174, No stress vs. Phleo: p-value = 0.0556, **24h** No stress vs. Gen: p-value = 0.0766, No stress vs. MMC: p-value = 0.0002, No stress vs. CIP: p-value = 2.63e-06, No stress vs. Phleo: p-value = 5.38e-06, n=4.

This advantage became statistically significant in the presence of MMC and CIP as early as 6 hours (71 %  $\pm$  4,6 for the MMC and 66 %  $\pm$  7,9 for the CIP). It stabilised by the 24<sup>th</sup> hour at the same ratio for the MMC (67 %  $\pm$  5,0), with the  $\Delta nucS$  bacteria comprising roughly 70 % of the population. In contrast, with the introduction of Gen, no statistical differences were observed at the 6 or 24-hour time points compared to the initial ratio.

These new experimentations have enriched our understanding of the *CglΔnucS* genetic background's behaviour under non-stress conditions and revealed its response to genotoxic antibiotic exposure. The results of this test suggest that the competitive advantage exhibited

by the *CglΔnucS* bacteria is not reliant on the latency phase but likely stems from a DNA repair mechanism active during the exponential phase and the stationary one. This observation could suggest that NucS activity in the DSBR does not require the presence of multiple copies of the bacterial chromosome, like the MMEJ.

## Discussion

The regulation and competition between DNA repair pathways and their connection to cell growth and division are topics that still harbour uncertainty in *Corynebacteria* and, more broadly, within the phylum of *Actinobacteria*. The literature suggests that these factors could play significant roles in the virulence and pathogenicity of certain species (Gonzalez *et al.*, 2012; Covacci *et al.*, 1997). Although *nucS* is conserved across *Actinobacteria*, its involvement in DNA repair mechanisms beyond the MMR remains largely unknown.

The literature suggests that NucS could be a multi-enzyme, interacting with the replication through its association with DnaN. To uncover new insight into the roles of NucS in DNA damage repair, it is essential to characterise the phenotypes associated with *nucS* mutants, particularly in a genetic background deleted for *nucS*. To address this ambition of characterising NucS's function, we conducted a phenotypic study of the colonies and the cellular growth of *CglΔnucS*. Although these tests did not reveal significant differences neither in morphological characterisation nor in cell growth, a competition growth essay in the presence of DNA-damaging agents provided valuable data. Several antibiotics were tested to establish a consistent dataset and highlight the connection between NucS and DSBs.

The quantitative data and observed phenotypes support the hypothesis that the deletion of *nucS* confers an advantage to the bacteria under conditions of genotoxic stress, notably when exposed to agents known to induce genomic instability, such as DSBs. This advantage is hypothesised to stem primarily from an altered DSB repair mechanism. We hypothesise that NucS could be required in an alternative system to the HR, like the MMEJ and subsequently compete with the HR for the DNA-free ends generated. It could also be further considered for an indirect inhibiting effect on the HR. This also suggests that NucS may be recruited to DSBs to control HR. This anti-recombinase activity of NucS may slow down bacterial growth, thus explaining why the deletion of *nucS* seemingly provides a competitive edge for the cells, at least in short-term laboratory experiments.

Following this hypothesis, the deletion of *nucS* could dysregulate and alter the DSB repair, either through the reparation of mismatches in the vicinity of DSBs or regarding an alternative mechanism involved in the DSBR. Moreover, if its inhibitory activity on the HR is removed, the HR rate could be elevated. However, when the antibiotic stress does not involve

the induction of DSBs, as with Gen, the competitive edge of the *CglΔnucS* bacteria is not evident, suggesting that the advantage is specifically linked to the genetic background's enhanced ability to repair complex DNA damage rather than a general growth superiority.

When compared, the DSB pathways are not equivalent in energy consumption or the number of actors required, nor is their overall fidelity for the initial sequence altered. If NucS allows the DSBs to be repaired by an alternative system, such as the MMEJ that competes with the HR, the outcome could imply a difference in the genetic stability and the overall bacteria survival due to variation in the energy balance required for the cell's survival. The *ΔnucS* genetic background may favour a repair process that is more accurate or more energy-efficient, potentially leading to increased genetic mobility and rearrangement but better survival under genotoxic stress.

A heightened HR ability could allow *ΔnucS* bacteria to more effectively repair DNA damage caused by antibiotics like Phleo and CIP, which disrupt the DNA double-helix, accumulating fewer mutations in their genome. Moreover, this hypothesis of an increased rate of HR in the *CglΔnucS* could allow these bacteria to adapt to hostile environments, where antibiotics would be added more swiftly than the WT genetic background. By facilitating an accelerated genetic reshuffling, the *ΔnucS* bacteria could quickly acquire and fix both beneficial mutations and new alleles from the HR of slightly divergent DNA sequences, which could, in turn, confer resistance.

However, considering a scenario involving an extreme environment where the duration exceeds that of treatment with an antibiotic, it is conceivable that NucS, by favouring a more mutagenic DNA repair pathway that is less accurate than HR, could benefit these organisms.

Further studies, such as acquiring a more precise dataset on tracking the differential growth and variation between the two bacterial populations, would help us refine our understanding of the mechanism underlying competitive advantage in the *CglΔnucS* bacteria. This could be done by transforming each genetic background tested with a plasmid expressing a different fluorescent marker specific for each strain, such as GFP for the first and mScarlet for the second. This type of experimentation would provide more details on the timing of the occurrence of the competitive advantage. Other conditions such as introducing new genotoxic agents that cause DNA damage in the medium, would be helpful to include. These assays could further our understanding of NucS's substrate specificity.

Another possible line involves pre-stressing cells in a medium containing genotoxic agents and comparing the response of the two genetic backgrounds in competition essays after removing the external stress. Moreover, observing the competition between these two bacterial populations during the stationary phase with and without antibiotics could highlight their survival strategies regarding the genome availability and compaction level.



## Material and Methods

### Bacterial strains and growth

**Table 3** lists the strains and plasmids used in the study. Unless otherwise stated, cells were grown at 30 °C in a BHI agar medium. When appropriate, supplements were used in the following concentrations: Kn 40 µg/mL.

Organism / Plasmide	Genotype	Origin
<i>E. coli</i> genetic backgrounds		
XL10-Gold (Cam <sup>R</sup> , Tet <sup>R</sup> )	Tet <sup>R</sup> Δ ( <i>mcrA</i> )183 Δ( <i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i> )173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte</i> [F' proAB <i>lacIqZ</i> ΔM15 <i>Tn10</i> (Tet <sup>R</sup> ) Amy Cam <sup>R</sup> ] <i>endA1, recA1, gyrA96, thi, hsdR17</i> ( <i>rk-</i> , <i>mk+</i> ), <i>relA1, supE44, (lac-proAB)</i> , [ <i>FtraD36, proAB,</i> <i>laqIqZM15</i> ]	Agilent
JM109 (Cam <sup>R</sup> )		Takera Bio
<i>C. glutamicum</i> genetic backgrounds		
<i>Cgl</i> ATCC 13032	Wild type strain	Ikeda <i>et al.</i> , 2003
<i>Cgl</i> (Δ <i>nucS</i> )	<i>Cgl</i> ATCC 13032 (Δ <i>nucS</i> :: <i>aphA-3</i> )	Ishino <i>et al.</i> , 2018
<i>Cgl</i> (Δ <i>rapZ</i> )	<i>Cgl</i> ATCC 13032 (Δ <i>rapZ</i> :: <i>aphA-3</i> )	This study
Plasmids		
pMC5S (Kan <sup>R</sup> , Amp <sup>R</sup> )	<i>ori pMB1 (colE1), plac, lacZα, Amp<sup>R</sup>, MCS</i> from pUC18	MoBiTec
pXMJ19 (Cam <sup>R</sup> )	<i>ori pMB1 (colE1), ptac, lacI<sup>q</sup>, Cam<sup>R</sup>, MCS</i> from pUC18	Wendisch <i>et al.</i> , 2016
pG-KJE8 (Cam <sup>R</sup> )	<i>ori p15A, paraB, dnaK, dnaJ, grpE, Cam<sup>R</sup></i> <i>nucS</i> from <i>Cgl</i> ATCC 13032 cloned in pQE80L	Takera Bio
pHM449 (Amp <sup>R</sup> )	( <i>Bam</i> HI / <i>Pst</i> I)	Ishino <i>et al.</i> , 2018
pHM481 (Kan <sup>R</sup> , Amp <sup>R</sup> )	Upstream (469 pb) and downstream (459 pb) regions of <i>rapZ</i> in <i>pMCS5</i>	This study
pHM483 (Amp <sup>R</sup> )	From pHM449 with <i>nucS</i> mutated in D144A	This study

**Table 3:** Information on the genetic backgrounds and plasmids used in this study

### Growth curves

Growth curves were performed to determine the MIC of CIP, Phleo, MMC and Gen on *Cgl*WT and *Cgl*Δ*nucS*. Cells were grown overnight in BHI medium at 30 °C cells were diluted to an OD600 of 0.05 and inoculated into a 96-well plate, with each well containing 200 µL of BHI medium with varying concentrations of antibiotics. The cultures were monitored every 1 hour and grown for 24 hours in biological and experimental triplicate, using a TECAN Infinite M1000 Pro plate reader with measurements taken at 600nm, results are based on 5 independent repetitions.

### **Competition test**

For the competition test, overnight cultures of *Cgl*WT and *Cgl* $\Delta$ *nucS* were diluted to an OD600 of 0.05 and mixed in a 1:1 ratio to achieve a final OD600 of 0.05. The mixed cultures were grown in shaking Erlenmeyer flasks at 30 °C until reaching an OD600 of 0.5 for the initial sample. The cultures were then incubated for an additional 24 hours. During this period, the cultures were then exposed to antibiotic treatments with CIP (2 µg/mL), Phleo (2 µg/mL), MMC (0,2 µg/mL) and Gen (0,5 µg/mL) or left untreated as a control. Samples were collected at specified time points (0, 6 and 24 hours), serially diluted and 100 µL were spotted onto BHI agar plates and BHI agar plates supplemented with 40 µg/mL Kn. The plates were incubated at 30 °C for 48 hours and CFUs were determined.

### **UV Irradiation test**

To assess the viability of *Cgl*WT and *Cgl* $\Delta$ *nucS* after UV irradiation, cells were grown overnight in a BHI medium at 30°C. The culture OD600nm was measured, and the cultures were serially diluted to achieve a final concentration of 10<sup>-7</sup> CFU/mL. A 100µL aliquot of the 10<sup>-6</sup> and 10<sup>-7</sup> dilutions was spread onto BHI agar plates. The plates were then exposed to UV irradiation using a 244 nm UV-C bulb, applying doses of 45 seconds (112,5 J/m<sup>2</sup>) and incubated overnight at 30°C. After two days of incubation, the number of CFUs was determined and standardised based on the initial overnight culture spread on agar plates with treatment. The results are based on 8 independent repetitions.

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**Chapter 2: Characterisation of the localisation of NucS and its  
physiological response to DSBs**





## Abstract

Genomic integrity is central to the survival and adaptability of any living organism. Thus, DNA repair mechanisms are of central importance in correcting endogenous and exogenous genomic damage, among which is the highly toxic DSB. In this regard, effective and timely repair of DSBs is crucial to preserve cellular integrity and functionality. In this chapter we will make hypotheses that NucS could also repair DSBs through HR regulation and promote alternative repair pathways, such as MMEJ.

- **Increased DSB number in *CglΔnucS*:** Using a Gam-meGFP DSBs reporter system, we observed that cells lacking *nucS* present more foci than WT bacteria, indicating that NucS may facilitate a timely, efficient DSBR pathway.
- **Increased RecA Recruitment in *CglΔnucS*:** *CglΔnucS* cells showed higher RecA recruitment, suggesting NucS's role in regulating HR. Either through direct inhibition or competition with another DSBR pathway.
- **NucS Localization:** NucS forms polar foci in response to DNA damage, similar to other DNA repair proteins. Under genotoxic stress, the number of foci per cell in the bacterial population also increased, indicating active involvement in DNA repair. Complex filamentous-like networks of foci were observed in apoptosis-like cells, as well as ring-like recruitment of mScarlet1-NucSd144A.
- **Response to Antibiotics:** In the presence of genotoxic antibiotics CIP, Phleo and MMC, cells showed a dose-dependent increase of mScarlet1-NucSD144A foci. This result further pointed out the role of NucS in responding to DNA damage. Aminoglycosides also seem to trigger such physiological responses.
- **Physiological Conditions:** More mScarlet1-NucSD144A foci were formed upon starvation and stress, indicative of maintained DNA damage response during this phase.

Our data suggest that NucS could be crucial in regulating DSBR pathways, potentially favouring MMEJ over HR and playing a part in maintaining genome stability under stress. These results seem to indicate that NucS activities balance DNA repair mechanisms, by promoting efficient and accurate repair and preventing an excess of HR and optimising bacterial adaptation to and survival to different kinds of stress.

## Introduction

All living organisms encounter genomic damage daily from both internal and external sources. Their survival hinges on activating DNA repair mechanisms, which respond to detecting and signalling these errors (Kimball, 1978). Among the various types of damage, DSBs are particularly detrimental to cellular integrity. When such breaks occur, replication halts and metabolic pathways are disrupted. Death is inevitable for the impacted cell without timely and effective repair (Gordenin, 2012; Krasin *et al.*, 1978).

Numerous systems are tasked with mending these breaks, each responding to different cell cycle phases and specific signals (Arnould *et al.*, 2021; Kanaar *et al.*, 1998; Pitcher *et al.*, 2007). One of the most intricate mechanisms is the HR. This pathway involves many factors and ensures the highest fidelity regarding the original template, though time-consuming (Chapman *et al.*, 2012. Wright *et al.*, 2018). The formation and stabilisation of a D-loop is a critical initial step in the HR, aligning the broken DNA sequence with a homologous segment for accurate repair. Before the formation of the D-loop, other pathways can be initiated such as the NHEJ or the MMEJ, which offer alternatives for DSBR (Piazza *et al.*, 2019).

The literature highlights that HR repairs DNA and facilitates genetic diversity. This diversity occurs when the sequence used to restore the break contains a different allele, a diverged gene or even exogenous DNA like viral DNA, allowing for a rich reshuffling of genetic material (Bürger, 1999). Consequently, regulating HR's activation and the wise selection of the pathway in DSBR is vital for organisms' survival and environmental adaptation (Denef *et al.*, 2009; Charlesworth, 1976).

The significance of HR is highlighted by its widespread preservation across diverse species (Cromie *et al.*, 2001). In humans, genomic regions characterised by low HR rates are associated with increased harmful mutations, which are sometimes linked to diseases and can influence susceptibility to various conditions (Hussin *et al.*, 2015; Zhu *et al.*, 2019). MutS/L and RecQ are well-documented for their antirecombinase activities (Hanada *et al.*, 1997). These crucial proteins prevent inappropriate HR between genomic segments that are either excessively divergent or analogous. Following such regulation, the DSB is repaired by a specific HR pathway, such as the SDSA. This scenario highlights that different DSBR mechanisms compete for the same substrate and necessitate regulatory proteins for effective functioning (Dupaigne *et al.*, 2008; Liu *et al.*, 2017).

NucS has been recognised in the literature for its endonuclease activity that induces DSBs, specifically at sites of mismatches (Takemoto *et al.*, 2018; Ishino *et al.*, 2018). Additionally, earlier studies have also highlighted its SSB activity on substrates that are described to be intermediates of HR (Suzuki *et al.*, 2019; Crézé *et al.*, 2011). A prominent line of inquiry from these studies is to question the involvement of NucS in other DNA repair mechanisms and further explore the frequently drawn parallel between MutS/L and NucS.

Technological advances, such as confocal microscopy and microfluidic chips, enhance the connections with biochemical investigations or bioinformatics predictions. These tools enrich the documentation of molecular pathways dynamics in living single cells. In the context of DNA repair, technological advances offer new perspectives by integrating the complexity of interactions among competing pathways that respond to the same damage signals. These technologies also allow detailed observation of the involvement of proteins and cofactors in response to various conditions in real time (Zentout *et al.*, 2021).

While the resolution limits of diffraction sometimes impede our ability to observe elements smaller than cellular compartments, fluorescence has opened up new perspectives by enabling the study of molecular dynamics within living cells, including processes like replication or DNA repair (Houseal *et al.*, 1989). From the second half of the 20<sup>th</sup> century, advancements in microscopy techniques have enabled detailed observations of bacterial phenotypes at the level of individual cells. For example, the filamentation of *E. coli* in response to genotoxic agents was first described by Adler *et al.* in 1965. Additionally, Donachie and Begg, (1970) detailed the morphological variations associated with different stages of the bacterial cell cycle and division.

Bacteria rank among the smallest living organisms on Earth, presenting unique challenges in observing their biological mechanisms. The advent of super-resolution technologies such as 3D Structured Illumination Microscopy (3D SIM) has significantly enhanced the contrast and resolution of these observations (Goodwin, 2013). This technology enables the distinction between molecular complexes within a bacterium and provides more detailed insights into the morphology of the structures observed (Dan *et al.*, 2013; Li *et al.*, 2015).

These improvements have enhanced the sensitivity of the collected signals, permitted co-labelling to visualise multiple cellular structures concurrently and even achieved resolutions that allow for the detection of individual molecules (Garini *et al.*, 2005; Vainrub *et al.*, 2006).

For this study, confocal microscopy was particularly adapted for observing the three-dimensional structure of molecular foci within a cell. This technique involves scanning the sample with a focused laser beam, point by point, to excite FPs expressed which then emit light at a specific filtered wavelength. This emitted light is subsequently amplified by photomultipliers and analysed to produce an image of the light emitted by the sample. This imaging method is ideal for optimising the signal-to-noise ratio sensibility and enhancing the precision of observations, especially for signals originating from within the cell rather than on its surface.

In our study, we opted to use Fluorescent Proteins (FPs) as markers, a decision driven by the significant advancements in this field since the discovery of Green Fluorescent Protein (GFP) from *Aequorea Victoria* in 1962 (Amsterdam *et al.*, 1995). Originating from this foundational FP, which is excited by blue light and emits in green at a peak wavelength of 504nm, several genetic modifications have led to a diverse array of variants with different excitation and emission spectra, such as Yellow Fluorescent Protein (eYFP) and Red Fluorescent Protein (eRFP) (Hentschel *et al.*, 2012; Campbell *et al.*, 2002; Ilagan *et al.*, 2010).

These diverse spectral properties now facilitate the optimisation of the FPs of interest based on macromolecular conditions such as steric hindrance, observation duration, or the need to colocalize with other fluorescent markers (Lambert *et al.*, 2020). Specifically, we selected mGFP, mVenus and mScarlet1 for our experiments (Albán *et al.*, 2004; Tao *et al.*, 2011).

These three FPs are monomeric, which is essential for molecular observation in living cells. This is a wanted property when they are fused to proteins of interest that are intended to be tagged (Bindels *et al.*, 2016). The monomeric nature of these FPs minimises spatial interference and reduces the risk of improper protein folding and potential steric hindrance that could inhibit interactions with other molecular partners. However, a downside to using these FPs is a possible reduction in the intensity of the emitted signal. To address this limitation, we selected FPs documented in recent literature for their enhanced emission properties compared to older versions. Notably, mScarlet1 emits in the red spectrum and was chosen for its superior luminosity (Bindels *et al.*, 2016; Hoi *et al.*, 2010).

One crucial factor for the success of these experiments on living cells is establishing reproducible bacterial growth conditions, which entails maintaining the cells under optimal physiological conditions for the duration of observation. In our study, observing cells for more than 2 or 3 hours was unnecessary; thus, the agar pad system proved adequate. However, the main limitation of this method becomes apparent after more than 3 hours, as the medium begins to dry out, cutting off the supply of oxygen and nutrients essential for bacterial growth and typically resulting in cell death (Joyce *et al.*, 2011).

A promising approach for extending the observation periods involves using microfluidic devices. Although there is an ongoing debate within the scientific community about the potential stress that microfluidics chips may display on bacteria, the consensus supports its advantages and the new research fields it opens. This technique facilitates the continual renewal of the medium, thereby sustaining bacterial growth over several hours to days. In the case of the study of the MMR, the mother machine, a microfluidic device, has been used to assess this mechanism in real time through the observation of MutS/L foci (Elez, 2021). Moreover, these systems allow for the introduction of antibiotics or other molecules, enabling accurate time monitoring of bacterial responses. When integrated with microscopic analysis, microfluidics also supports the investigation of more complex biological structures than those possible with agar pads, including bacterial communities and biofilm development (Allard *et al.*, 2022; Pérez-Rodríguez *et al.*, 2021; Kim *et al.*, 2018).

Although genetic and biochemical methods have traditionally been preferred for studying molecular mechanisms, the organisation and dynamics of the systems within complex environments often elude such analyses. The work presented in this manuscript section was conducted using confocal laser scanning microscopy and 3D SIM. The employment of fusions between FPs and proteins of interest allowed us to obtain information regarding DSB resolution mechanisms linked with NucS in *C. glutamicum*, such as forming and localising foci in real time in response to diverse conditions. Employing a genetic background deleted for *nucS* and fusions of mScarlet1-NucS, RecA-mVenus and Gam-meGFP, we explored how NucS interacts with DSBs under stress, potentially offering insights into its broader roles in DNA repair and cellular adaptation. Additionally, microscopy offered insights into cell morphology, enhancing our understanding of various cellular processes beyond DNA repair, including cell wall synthesis and the cell cycle phases of the observed cells.

## Results

### A. Study of the role of NucS in the processing of DSBs

#### A. 1 Selection and design of a system to track the DSBs in living cell

For this part of the study, we aimed to further investigate the parallels drawn between the functions of NucS and MutS/L as highlighted in the literature. When looking into these key enzymes' activity in other DNA repair pathways, research sheds light on their roles as anti-recombinase. This activity is mediated by destabilising the invading DNA strand in the D-loop, specifically by recognising mismatches in the complementary sequence. Building upon these insights, our hypothesis suggests that NucS might also regulate the HR by limiting the formation of the D-loop, eventually promoting an alternative way to repair the DSBs, like the MMEJ.

To initiate our live investigations into the role of NucS in regulating the HR, we chose to evaluate the baseline level of DSBs in *Cgl*/WT and contrast it with observations in *Cgl* $\Delta$ *nucS*. This decision was based on the connection between the activation of DSBR pathways and the introduction of DSB within cells. This correlation has notably been pointed to as the positive correlation between HR and the number of DSBs in a cell (Nath *et al.*, 2022).

By examining DSB levels in both *Cgl*/WT and *Cgl* $\Delta$ *nucS* genetic backgrounds, we aim to shed light on how NucS influences the time remanence of DSBs in the cell and indirectly how it could interact with DBS repair processes like its potential impact on HR efficiency. This approach directly explores NucS's role in maintaining genomic stability and modulating DNA repair, thereby advancing our understanding of bacterial adaptive mechanisms (Nath *et al.*, 2022).

The array of tools for detecting and analysing DSBs in genomes has significantly diversified in recent years, since the initial tests by electrophoresis. These experiments vary widely in terms of execution complexity, precision in DSB detection and the information gathered for analysis. We chose a real time visualisation technique based on microscopy and a fusion of an FP and the protein Gam. This technique allows for refining information regarding the spatial distribution of the DSB's foci and their mobility or stability within the cell (Atkinson *et al.*, 2024).

Since 2013, numerous research groups have expanded on the pioneering work of Shee *et al.*, which introduced a novel method for detecting DSBs in cells using a fluorescent marker. In this study, the marker named Gam, was fused to the GFP. This protein is orthologous to Ku and is known for its debated reliability in marking DSBs.

However, the cellular function of Gam and Ku widely differs. Meanwhile, Ku is directly involved in DSBR mechanisms because it is part of the bacterial endogenous machinery and stabilises the free ends DSBs create via the NHEJ. In contrast, the Gam protein, derived from the Mu bacteriophage, binds to the free ends of the linear viral DNA during the infection of a bacterium. This binding inhibits the activity of the bacterium's exonucleases, thereby safeguarding the viral DNA without being associated with the cell's molecular repair mechanisms or with another endonuclease (Fagagna *et al.*, 2003; Fukushila *et al.*, 2001; Akroyd *et al.*, 1986).

Furthermore, the phage-derived Gam protein can bind to sticky and blunt-ended DNA without discriminating between different forms of DSBs (Abraham *et al.*, 1990).

Additionally, research shows that Gam-GFP fusion colocalises with  $\gamma$ H2AX and partially with 53BP1, two proteins also interacting with the DSBs. The literature suggests this discrepancy may arise from Gam-GFP's enhanced speed and specificity in recognising DSBs. A primary limitation of this technique involves competition between Ku and Gam-GFP for DSB substrates. However, since our model organism is *C. glutamicum* and does not code for Ku, such concerns are irrelevant in our case (Fagagna *et al.*, 2003).

The literature highlights that a cell ceases to divide after forming Gam-FP foci. Hence, there is no benefit in monitoring the persistence duration of foci marked by this protein. Moreover, Gam, being exogenous, competes for the free ends of DNA formed during a DSB with the proteins of the endogenous DNA repair systems. This new actor could destabilise existing relations between DSBR pathways. Recent literature suggest it could enhance the NHEJ (Murphy, 2007; Bhattacharyya *et al.*, 2018).

Following our hypothesis, NucS could interact with DSBs and either facilitate or inhibit the recruitment and activation of specific DSBR pathways at the site of the damage, regardless of whether the breaks originate from external sources.

Therefore, if NucS demonstrates this activity, the number of DSBs identified by the Gam-meGFP fusion in untreated *CglΔnucS* should differ compared to *CglWT*. Specifically, considering that HR is a DNA repair pathway involving numerous factors over a prolonged period spanning several hours and assuming that NucS inhibits HR and facilitates DSBR through alternative mechanisms, the absence of *nucS* from the genome could promote homologous HR. According to this slower but more accurate mechanism, this could lead to a longer persistence of the DSBs within the cell.

Taking into account these various elements, we engineered a fusion of the Gam protein with an FP (Akroyd *et al.*, 1986; Abraham *et al.*, 1990) by genetically linking a fluorescent marker, mVenus, to the Gam coding sequence using a long and flexible spacer sequence: GPGLSGLGGGGSLG. This fusion protein was expressed in either *CglWT* or *CglΔnucS* and integrated in trans within a PXMJ19 plasmid that confers resistance to Chloramphenicol. An IPTG-inducible tac promoter regulates the expression of this chimeric construct.

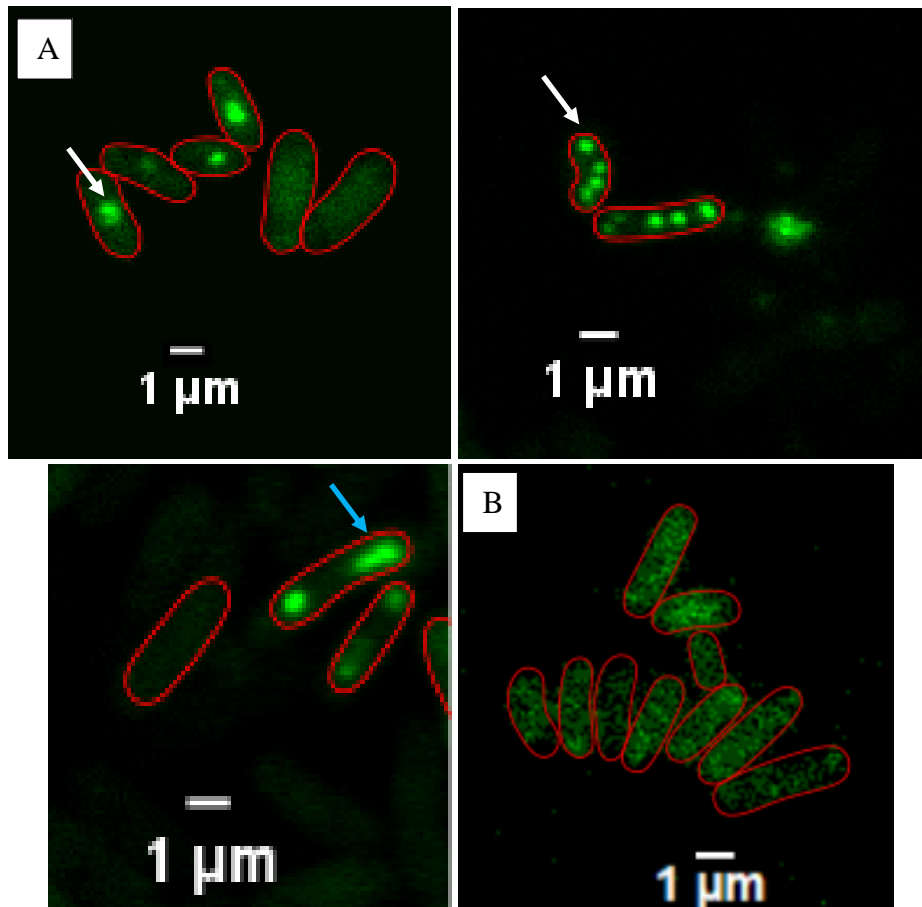
## **A. 2 Number of Gam-meGFP foci formed without stress in either *CglWT* or *CglΔnucS***

These experiments enabled us to observe the formation of Gam-meGFP foci in both *CglWT* and *CglΔnucS* genetic backgrounds, exhibiting variable localisations within the cells, as shown in **Figure 24**. While the precise positions of the foci were not studied in detail, we noted that they appeared at both the poles and the centre of the cells and less frequently at the periphery of the cytoplasm. When bound to the free ends of DNA, the Gam construct could compete with DNA repair systems and interfere with the mechanisms of DNA break movement and recruitment within the cell. Literature suggests that the recruitment of Gam-mVenus at the site of DSBs could also impede the normal progression of DNA repair (Murphy *et al.*, 2007).

The biological response of this construct was confirmed through its dose-dependent response in forming Gam-meGFP foci when exposed to genotoxic stress inducers such as CIP or Phleo. Upon such treatment, it was observed that the Gam-meGFP fusion was recruited to an increased number of foci within the bacterial populations, occasionally revealing multiple foci within a single cell or complex assemblies, a phenotype only observed under high genotoxic treatment. This observation underscores the physiological stimuli necessary for



forming the foci and is not dependent on the expression of the chimeric molecule. Consequently, we could count the basal foci number for both genetic backgrounds, revealing a significant difference between them. Hence, *CglΔnucS* exhibited a higher number of foci compared to the WT genetic background see **Figure 25**.



**Figure 24:** Confocal microscopy image of *Cgl/WT* expressing the Gam-meGFP construct **A.** after 5 hours of IPTG induction, treated with CIP (1 μg/mL) for 30 minutes. The arrows indicate the presence of foci formed on the free ends of the DNA induced by the antibiotic and detected by the Gam-meGFP construct. Up to 4 foci can be counted per cell and some more complex assemblies on the DNA are also present, as indicated by the blue arrow. **B.** Without antibiotic treatment.

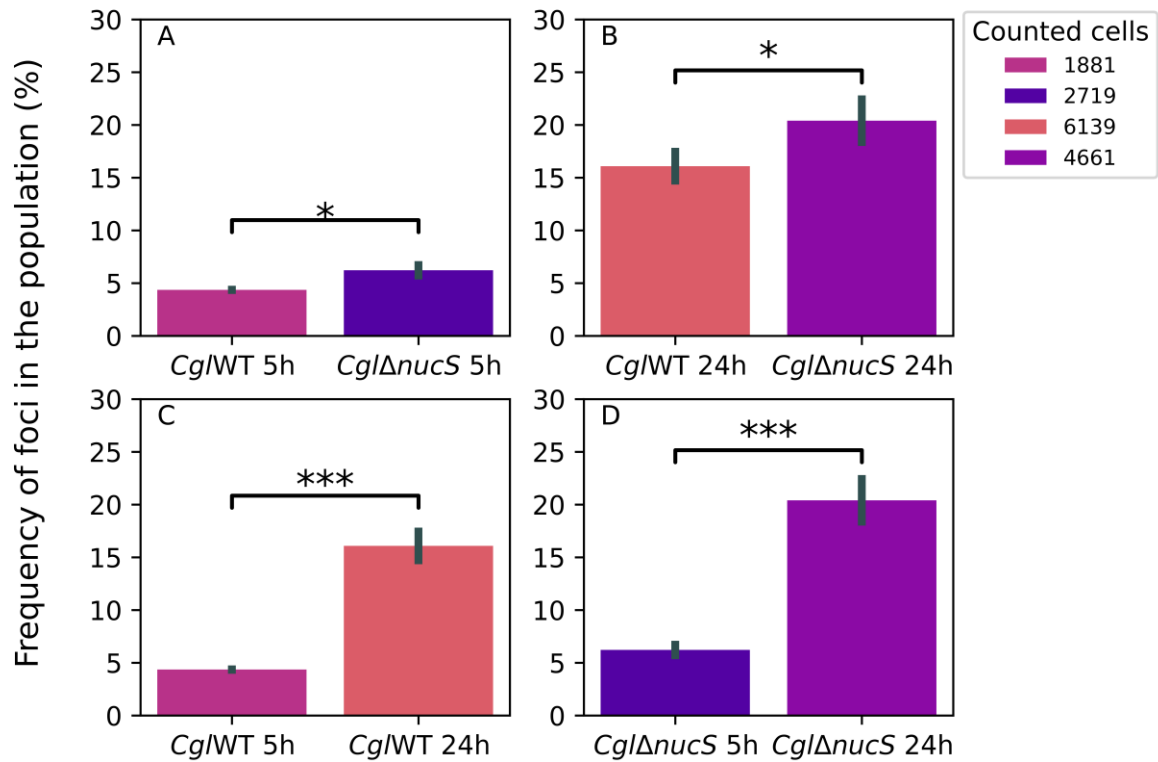
At the bacterial population scale, we noticed differences in fluorescence intensity and variations in the appearance of the foci. It is essential to consider that while the antibiotic treatments administered are known to introduce DSBs into the genome of their target, these molecules can also generate other types of lesions in the genome through secondary effects of their primary action. Such errors may cause DSBR factors to accumulate at the sites of the lesion, being a source of explanation for the variations in the size and appearance of the foci detected by confocal microscopy (Elowitz *et al.*, 2002). Moreover, the variations in the cytoplasmic intensity could represent stochastic variations in transcription and mRNA

accumulation. No treatment was administered to the bacterial populations to ensure optimal and consistent growth conditions, except for the pre-culture of the  $\Delta nucS$  genetic background, where Kn was added. This experiment was reproduced three independent times and the number of cells analysed in each condition approached 2,000. Automating cell counting per image has enhanced our process' accuracy and efficiency. Omnipose was selected as the optimal tool to match the resolution of our images.

During the exponential growth phase, the Gam-meGFP fusion's fluorescence was observable in most cells. Since the construct was housed on a plasmid under IPTG inducible promoter, its expression levels initially required optimisation through a series of IPTG concentration experiments.

In approximately 4.4 % ( $0.044 \pm 0.004$ ) of the analysed *Cg*/WT bacteria, a distinct Gam-meGFP foci was visible against the cytosolic fluorescence background. In the *Cgl* $\Delta nucS$  genetic background, nearly 6.2 % ( $0.062 \pm 0.009$ ) of the cells displayed a foci, a notable difference. Our results reveal a consistent frequency of cells exhibiting a single foci in both genetic backgrounds, with no instances of more than one foci per cell. Statistical analysis showed that the deletion of *nucS* implies a 40.9 % increase in the number of Gam-meGFP foci, indicating a higher presence of free DNA ends accessible to the chimeric molecule, representing the rate of DSBs in the bacterial population.

Cell divisions were indeed observed in the field for cells that did not exhibit foci. However, we could not observe complete cell divisions for cells displaying foci. Nonetheless, daughter cells showing typical post-replication spatial organisations appeared to display mirror-image foci regarding position and number. This observation suggests that in the case of breaks occurring during replication, the proteins associated with DNA repair can be preserved in the form of foci and passed on to the progeny. Alternatively, although unrepaired, DNA could be transmitted to a daughter cell if it is stabilised by the proteins of the preliminary phases of DSBR and repaired subsequently.



**Figure 25:** Statistical analysis of the number of Gam-meGFP foci present in *C. glutamicum* WT or  $\Delta$ *nucS* after 5 hours of growth or 24 hours. **Panel A:** Comparison of Gam-meGFP foci between *Cgl*WT and *Cgl*Δ*nucS* after 5 hours of growth. *Cgl*WT ( $\mu = 4.36$ ,  $\sigma = 0.39$ ) vs. *Cgl*Δ*nucS* ( $\mu = 6.22$ ,  $\sigma = 0.87$ ), p-value = 0.015. **Panel B:** Comparison of Gam-meGFP foci between *Cgl*WT and *Cgl*Δ*nucS* after 24 hours of growth. *Cgl*WT ( $\mu = 16.09$ ,  $\sigma = 1.74$ ) vs. *Cgl*Δ*nucS* ( $\mu = 20.41$ ,  $\sigma = 2.40$ ), p-value = 0.045. **Panel C:** Comparison of Gam-meGFP foci in *Cgl*WT between 5 and 24 hours of growth. *Cgl*WT 5 hours ( $\mu = 4.36$ ,  $\sigma = 0.39$ ) vs. *Cgl*WT 24 hours ( $\mu = 16.09$ ,  $\sigma = 1.74$ ), p-value =  $2.73 \times 10^{-5}$ . **Panel D:** Comparison of Gam-meGFP foci in *Cgl*Δ*nucS* between 5 hours and 24 hours of growth. *Cgl*Δ*nucS* 5 hours ( $\mu = 6.22$ ,  $\sigma = 0.87$ ) vs. *Cgl*Δ*nucS* 24 hours ( $\mu = 20.41$ ,  $\sigma = 2.40$ ), p-value =  $7.16 \times 10^{-5}$ . Each condition was tested in triplicate. The statistical test performed was t-student.

Consequently, the ratio of foci can be interpreted as the rate of foci formation per cell division. Thaler *et al.* 1987; Rosenberg *et al.*, 2013, analysed this rate in their studies after considering a correction for approximately 71 % detection efficiency of DSBs detected by GamGFP as foci in *E. coli*. Given this information, the number of DSBs should equal  $0.063 \pm 0.004$  for *Cgl*WT and  $0.089 \pm 0.009$  for the mutant strain. A t-student test has verified the significance of these findings, as detailed in **Figure 25**. No previous analysis has been conducted on the basal number of DSBs inside *C. glutamicum*. However, this result matches the number of DSBs shared about *E. coli* and *B. subtilis*, which is around 4 to 10 % of the bacterial population of these species (Pennington *et al.*, 2007; Simmons *et al.*, 2008).

### **A. 3 Number of Gam-meGFP foci formed in the starvation condition in either *Cgl*WT or *Cgl* $\Delta$ *nucS***

The growth conditions of bacteria significantly influence the integrity of their genome. Notably, high temperatures or acidic pH levels can trigger DNA breaks. During periods of nutrient deprivation, specific molecular processes, such as mycolic acid synthesis in *M. tuberculosis*, are decelerated (Jamet *et al.*, 2015). Resource scarcity within the cell increases rNTPs to dNTPs ratio, which impedes replication and induces stress (Itsko *et al.*, 2016). Moreover, studies have documented the cessation of bacterial growth accompanied by a reduction in cytoplasmic volume and have associated starvation with the induction of genomic damage and breaks (Nakayama *et al.*, 1975; Shi *et al.*, 2020).

To enhance our study, we introduced a starvation condition by monitoring the culture in the stationary phase after 24 hours of growth. Our hypothesis posits that NucS plays a role in regulating DSBR. Then, under conditions that promote increased DNA breaks, the knock-out organisms for *nucS* should display a consistent difference in the number of DSBs relative to the WT, as observed under optimal growth conditions. This difference is estimated to be around 30 % to 40 %. Should the variation in the number of foci deviate from this initial estimate, it might suggest that NucS also contributes to introducing these breaks.

As anticipated, the incidence of DSBs increases significantly when the growth medium of the bacteria is not refreshed after 24 hours of incubation, rising from 4.4 % foci in the population of bacteria WT to 16.1 % ( $0,016 \pm 0,002$ ). Remarkably, the foci in *Cgl* $\Delta$ *nucS* also increased to 20 % ( $0,020 \pm 0,002$ ). The disparity in the number of DSBs between the two genetic backgrounds is 27 % after 24h of growth, showing a decrease in the difference in the number of foci registered in the *Cgl* $\Delta$ *nucS* genetic background regarding earlier observations after 5 hours of incubation (40.9 %). These differences have been statistically validated by a t-student test, as illustrated in **Figure 25**.

These findings suggest that the presence of functional NucS proteins within *C. glutamicum* impacts the number of foci of Gam-meGFP formed in the cell during exponential growth and starvation. Since the presence of NucS influences the apparent number of DSBs within the cell, NucS likely intervenes in a pathway involved in the repair of DSBs.

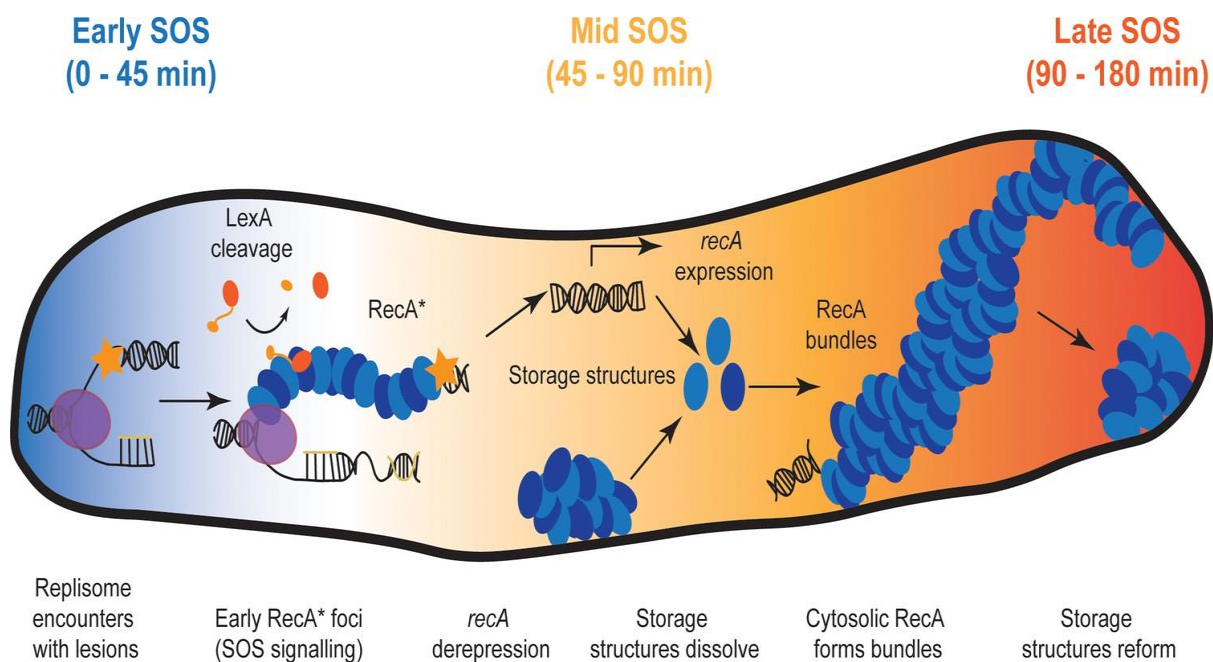
Furthermore, we observed the responsiveness of our system of DSB detection to variations in cell growth conditions: nutrient deprivation. In this context, we recorded a significant increase in DSBs in both bacterial populations. We also noted a decrease in the difference in the foci between *CglΔnucS* and *CglWT*. These results show that this disparity in foci is not due to their spontaneous formation but is somewhat attributable to specific biological mechanisms.

Thus, the variation in the observed number of DSBs may be more closely associated with the DNA repair processes recruited. Indeed, NucS could promote a specific DNA repair pathway for these breaks, thereby influencing their persistence in the cell rather than their genesis. By favouring a quicker DNA repair pathway over others, NucS could influence the competition between DSBR mechanisms and, subsequently, the time remanence of the DSBs in the cell. Given that the number of DSBs and the rate of HR are correlated, this initial experiment supports the hypothesis of NucS having an anti-recombination role.

## **B. Study of the role of NucS in the recombination/ SOS response**

### **B. 1 Selection and design of a system to track the activation of the recombination in living cells**

Recent studies have proven the limits of the Gam-FP system and mitigated its specificity for DSBs, indicating that it can also bind to other DNA structures (Atkinson *et al.*, 2022). To address the possibility that another hypothesis could explain the variation in the number of DSBs than the implication of NucS in DSBR mechanisms, we have opted to develop an alternative system for monitoring and visualising DSBR. This new approach enables us to track and quantify the recruitment of RecA directly fused to an FP to damage sites in real time, providing another depiction of the cellular repair mechanisms at play. We designed a flexible linker GPGLSGLGGGGSLG to ensure the 3D folding of the chimeric protein. Previous studies have extensively examined the behaviour of RecA, revealing several critical stages of its activity within the cell. Notably, it has been demonstrated that RecA is recruited to the cell poles via a sophisticated mechanism (Ghodke *et al.*, 2019). Through this experiment, we hoped to gain deeper insights into the dynamics of DNA repair pathways, specifically how NucS interacts with these pathways and influences cellular response to DSBs. This would help clarify whether the observed variations in DSBs are due to differences in the rate of HR or other underlying mechanisms.



**Figure 26:** Model for organisation of RecA complexes after DNA damage. The SOS response in *E. coli* is composed of three stages. Detection of UV damage leads to the formation of ssDNA-RecA filaments at sites of replisomes, triggering the early stages of the SOS response. In this stage, ssDNA-RecA (RecA\*) filaments catalyse auto-proteolysis of LexA to induce SOS and deregulate expression of the SOS inducible genes, including *recA*. Cells stop dividing and instead begin to filament. Storage structures of RecA dissolve in response to DNA damage to make RecA available for repair and recombination in the middle stage of the SOS response (45 to 90 min). RecA forms membrane-associated bundles starting in mid-SOS. These bundles mature in the middle-late stages of the SOS response. Finally, these are disassembled, RecA storage structures are reformed and cell division resumes in the late stages of the SOS response (Ghodke *et al.*, 2018).

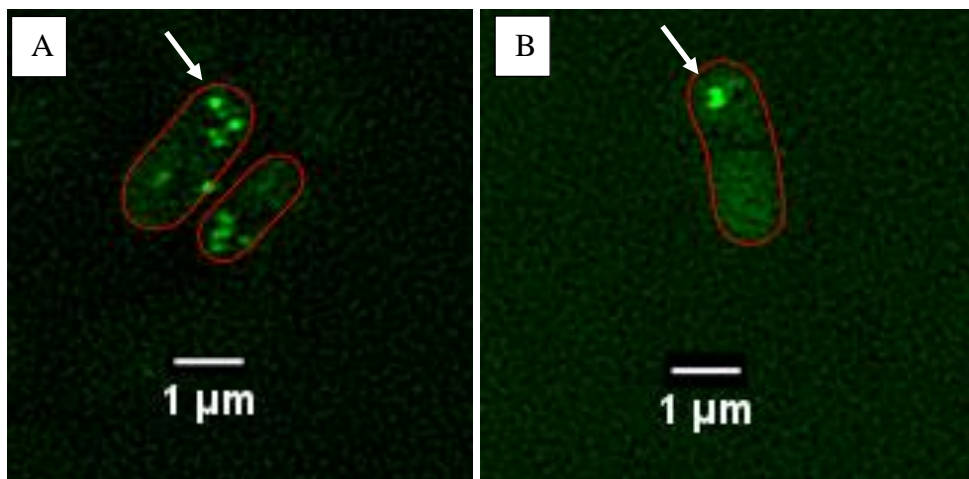
The detection of DNA damage, specifically DSBs, often initiates with the replisome's arrival at the cell poles. This event triggers the recruitment of RecA to ssDNA, marking the onset of the DNA damage response. RecA forms filaments on the ssDNA, as depicted in **Figure 26**, which catalyses the self-cleavage of LexA, leading to the activation of the SOS response.

Cells displaying RecA foci, indicative of an active SOS response, typically cease division and may undergo filamentation, a well-documented phenomenon in *E. coli* (Marceau *et al.*, 2011). In this context, RecA is integral to DNA repair mechanisms, typically operating between 45 and 90 minutes after the initial damage. Following this period, from 90 to 180 minutes, RecA is attracted to the membrane where it forms bundles (Ghodke *et al.*, 2018). This dynamic behaviour of RecA highlights its crucial role in initiating the SOS response and facilitating subsequent DNA repair processes, ensuring genomic stability and cell survival under stress conditions.

The specificity of RecA in HR is put in evidence through its ability to form nucleoprotein filaments on ssDNA that arise at DSB sites. These filaments are integral to the HR, as they promote the search for homologous sequences and the subsequent strand invasion necessary for accurate DNA repair (Lusetti *et al.*, 2002). The formation of these filaments is highly regulated, ensuring that the HR occurs efficiently and only in the appropriate contexts (Bell *et al.*, 2016). Indeed, our interest in RecA was further bolstered by the findings of Tham *et al.*, 2013, which discussed the cross-activity of MutS/L on HR. The proteins involved in canonical MMR can attach to the D-loop and exert a rotational constraint on RecA-mediated strand exchange. This action destabilises the D loop and results in an anti recombinational activity (Carrasco *et al.*, 2019).

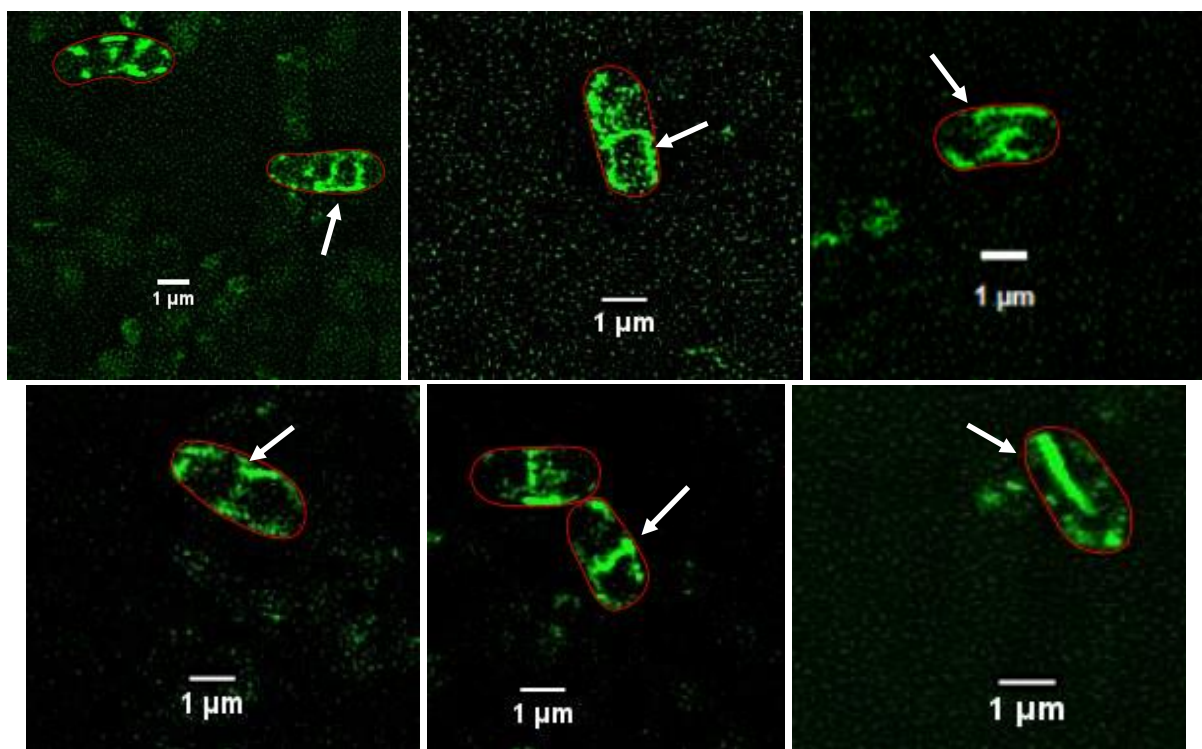
To create a fusion of RecA with the mVenus, we drew upon systems documented in the literature for tracking RecA in living cells. Given the absence of prior research on *C. glutamicum*, we proceeded to a codon optimisation guided by an established library (Chen *et al.*, 2004; Kidane *et al.*, 2012; Serrano *et al.*, 2018; Carrasco *et al.*, 2019).

This fusion was incorporated into the same IPTG inducible plasmid as the Gam-meGFP construct, PXMJ19. Initial observations confirmed the presence of foci in a subset of the bacteria examined without stress induction, see **Figure 27B** and a higher number of foci in the cells treated with a genotoxic antibiotic, see **Figure 27A**. A cytosolic signal was also detected, aligning with findings reported in the literature (Simmons *et al.*, 2007).



**Figure 27:** 3DSIM microscopy images of *Cg/WT* expressing the RecA-mVenus construct after 5 hours of IPTG induction, **A.** treated with CIP (1 µg/mL) for 30 minutes **B.** without stress. The arrows indicate the foci formed on the ssDNA induced by the damage caused by the antibiotic and detected by the RecA-mVenus construct.

To further characterise this system, we aimed to assess its responsiveness to antibiotics, as it has been done for the Gam-meGFP system and particularly to observe whether it could induce the formation of filaments or bundles in the cells a hallmark of the construct's activity corresponding to the later phases of the SOS response. After subjecting the cells to a 2-hour incubation period following the introduction of genotoxic stress with Phleo or CIP in the liquid medium, we noted the development of filaments of RecA-mVenus within the bacteria. However, this was infrequent (affecting less than 1 % of the cells). These filaments were typically anchored at polar points on the membrane and extended towards the centre of the cell, sometimes reaching up to 2  $\mu\text{m}$ , as presented in **Figure 28**.



**Figure 28:** 3DSIM microscopy image of *Cg/WT* expressing the RecA-mVenus construct after 5 hours of IPTG induction and treated with CIP (1  $\mu\text{g}/\text{mL}$ ) for 2 hours. The arrows indicate the filament formed on the ssDNA induced by the damage caused by the antibiotic and detected by the RecA-mVenus construct.

These observations were consistent with the literature on RecA, whether within eukaryotes with a nuclear envelope or bacteria. The scaffolding of repair enzymes at specific sites in the cell is a strategy conserved by many organisms (Gad *et al.*, 2021; Ochs *et al.*, 2019). This allows, on the one hand, the stabilisation of a DNA structure that could pose problems for the cell and, on the other hand, the enhancement of the probability of recruiting key enzymes at the repair site within an optimal and regulated microenvironment for their activity. This strategy likely improves the repair process' speed and reliability. (Kirkland *et al.*, 2015;



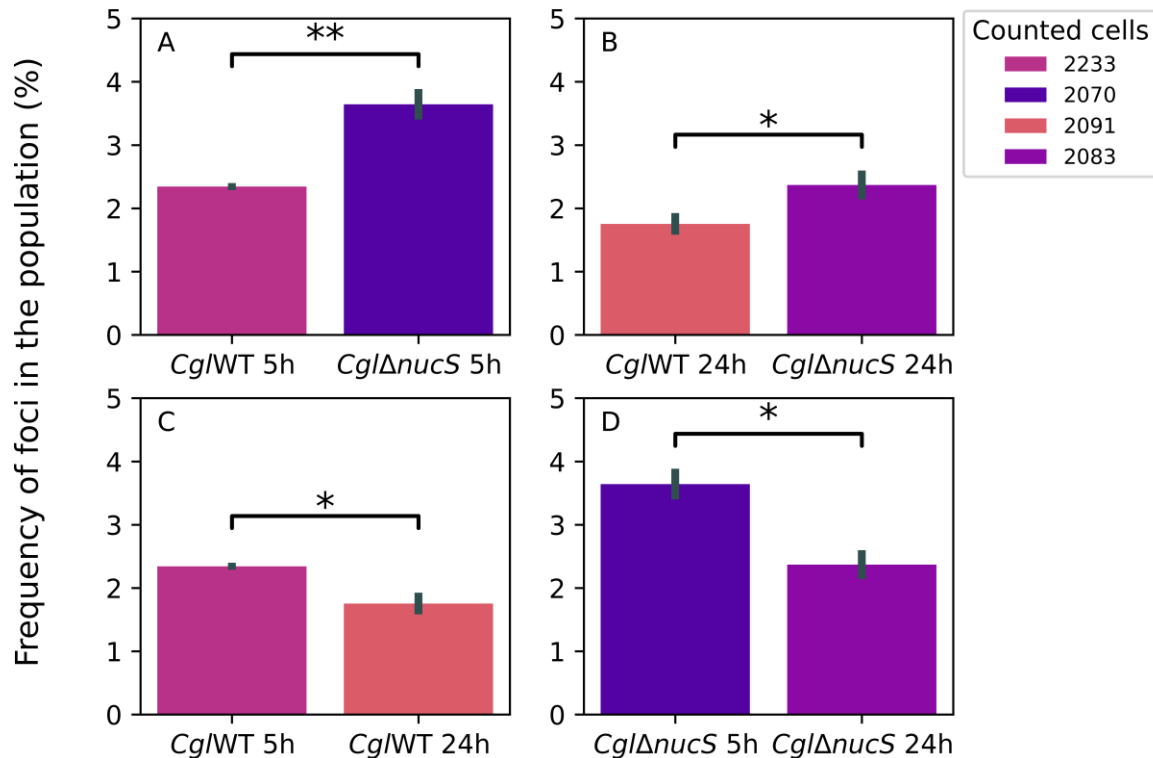
Kidane *et al.*, 2012). Hence, these results align with the descriptions provided in the literature regarding the functioning of RecA within the cell (Ghodke *et al.*, 2018), both in terms of the timing of the formation of the filament after a genotoxic stressor and the positioning within the cell. In our case, as the RecA-mVenus construct is not integrated into the genome but is controlled by an inducible promoter, the filaments and bundles observed are likely mixtures of the cell's endogenous, unmarked RecA protein and the fluorescent construct.

## **B. 2 Number of RecA-mVenus foci formed without stress in either *Cgl*WT or *Cgl* $\Delta$ *nucS***

Observations revealed fewer foci in the bacterial population imaged during the exponential growth phase after 5 hours of IPTG induction, without antibiotic treatment, compared to the experiments conducted with the Gam-meGFP fusion. Compared with this experimentation, almost half as many foci were detected within the bacterial populations. This finding is consistent with the literature indicating that RecA is not directly an indicator of the DSBs but rather reports the initial stages of HR and SOS response. As there are multiple pathways to repair DSBs other than the HR, it is unsurprising to observe fewer foci than the expected number of DSBs, with a reporter system specific to one of these pathways. The choice between these pathways involves a complex interplay of factors and regulatory proteins. For instance, the binding of Ku proteins to DSB ends promotes NHEJ. In contrast, the ssDNA generated by resection promotes the binding of RecA, thereby steering the repair process towards HR (Ciccina *et al.*, 2010).

Consequently, 1.75 % ( $0.018 \pm 0.006$ ) RecA-mVenus foci in the bacterial population under normal growth conditions versus 4.4 % of foci Gam-meGFP in *Cgl*WT cells aligns with our expectations. Analysis of the foci indicated that none of the cells contained more than one foci in drug-free conditions, as detailed in **Figure 29. A**. Analysis of the three iterations of this experiment highlights several 3,6 % ( $0.036 \pm 0.002$ ) foci for the *Cgl* $\Delta$ *nucS* bacterial population in the same conditions of culture. Hence, during the exponential phase, the cells' knock-out for *nucS* displayed a significantly higher number of foci —108 % more — than the *Cgl*WT genetic background. This marked difference could highlight a disparity in DNA repair dynamics between the *Cgl* $\Delta$ *nucS* and the *Cgl*WT cells. This observation is consistent with the higher number of Gam-meGFP foci noted in the *Cgl* $\Delta$ *nucS* bacterial population, suggesting that the increase in the number of Gam-meGFP foci could be due to a longer time

remanence of the foci. It would likely be a consequence of a heightened activation of the HR, that is more time consuming to repair DSBs than other DSBR pathways. Consequently, the deletion of *nucS* seems to induce a peculiar cellular response to DNA damage, particularly DSBs.



**Figure 29:** Statistical Analysis of the Number of RecA-mVenus Foci Present in *C. glutamicum* WT or  $\Delta$ *nucS* After 5 hours and 24 hours of Growth **Panel A:** Comparison of RecA-mVenus foci between *Cgl*WT and *Cgl* $\Delta$ *nucS* after 5 hours of growth. *Cgl*WT ( $\mu = 2.34$ ,  $\sigma = 0.056$ ) vs. *Cgl* $\Delta$ *nucS* ( $\mu = 3.64$ ,  $\sigma = 0.24$ ), p-value = 0.0017. **Panel B:** Comparison of RecA-mVenus foci between *Cgl*WT and *Cgl* $\Delta$ *nucS* after 24 hours of growth. *Cgl*WT ( $\mu = 1.75$ ,  $\sigma = 0.17$ ) vs. *Cgl* $\Delta$ *nucS* ( $\mu = 2.37$ ,  $\sigma = 0.23$ ), p-value = 0.038. **Panel C:** Comparison of RecA-mVenus foci in *Cgl*WT between 5 hours and 24 hours of growth. *Cgl*WT 5 hours ( $\mu = 2.34$ ,  $\sigma = 0.056$ ) vs. *Cgl*WT 24 hours ( $\mu = 1.75$ ,  $\sigma = 0.17$ ), p-value = 0.0099. **Panel D:** Comparison of RecA-mVenus foci in *Cgl* $\Delta$ *nucS* between 5 hours and 24 hours of growth. *Cgl* $\Delta$ *nucS* 5 hours ( $\mu = 3.64$ ,  $\sigma = 0.24$ ) vs. *Cgl* $\Delta$ *nucS* 24 hours ( $\mu = 2.37$ ,  $\sigma = 0.23$ ), p-value = 0.0056. Each condition was tested in triplicate (n = 3) and the statistical test performed was t-student.

### B. 3 Number of RecA-mVenus foci formed in the starvation condition in either *Cgl*WT or *Cgl* $\Delta$ *nucS*

As with the previous experiment, we implemented a condition of nutrient deprivation. Unlike the previous experimentation with the Gam fusion, the hypothesis using the RecA-mVenus under starvation is different. The formation of DSBs or RecA foci is

positively correlated with the activation of the HR. However, there is no positive correlation between HR activation during the stationary phase and the presence of DSBs.

During starvation, HR is unlikely because it relies on the presence of homologous sequences to repair the DSBs. In a haploid genome like *C. glutamicum*, if the cell no longer divides, the genome exists in a single copy, making HR unfeasible. This cell cycle phase is described in the literature as favouring other DNA repair mechanisms like NHEJ or MMEJ. This is particularly noteworthy given the number of actors required for the HR and the energy resources mobilised for this pathway. In light of these factors, even if the number of DSBs increases under starvation conditions, we expect to see a reduction in the number of foci and bundles formed by RecA compared to the exponential growth phase, indicating less activation of the HR.

After 24 hours of culture in the stationary phase, the number of foci in *Cgl*/WT revealed a slight decrease of 24 % from 2,3 % to 1,8 % ( $0.0175 \pm 0.0017$ ), as shown in **Figure 29B**. We recorded a higher decrease in the number of foci in the *Cgl* $\Delta$ *nucS* bacterial population, with the number of foci in the population dropping from 3,6 % ( $0,0364 \pm 0,0024$ ) to 2,4 % ( $0.0237 \pm 0.0023$ ), representing a variation of approximately 33 % between normal growth conditions and starvation. This diminution in *Cgl* $\Delta$ *nucS* of RecA-mVenus foci is likely due to diminished cellular activity and DNA replication.

Regarding the two genetic backgrounds, the *Cgl* $\Delta$ *nucS* bacterial population harboured 27,78 % more foci than *Cgl*/WT under starvation. This variation in the number of foci of the two genetic backgrounds aligns with the data obtained from the Gam-meGFP system, suggesting a consistent pattern of DBSR activity across the different experimental conditions tested.

The analysis of these results suggests that NucS may exhibit a direct or indirect anti-recombination activity, as evidenced by increased foci observed with both Gam-mVenus and RecA-meGFP in *Cgl* $\Delta$ *nucS* bacterial population. This increase could imply that without NucS, cells might struggle with efficient DSBR, leading to DSBs being unrepaired for extended periods. Notably, the number of foci counted between the two genetic backgrounds varies according to the cell growth phase, highlighting how physiological conditions influence DNA repair mechanisms. This result could point towards an alternative repair pathway like MMEJ, which is less dependent on RecA.

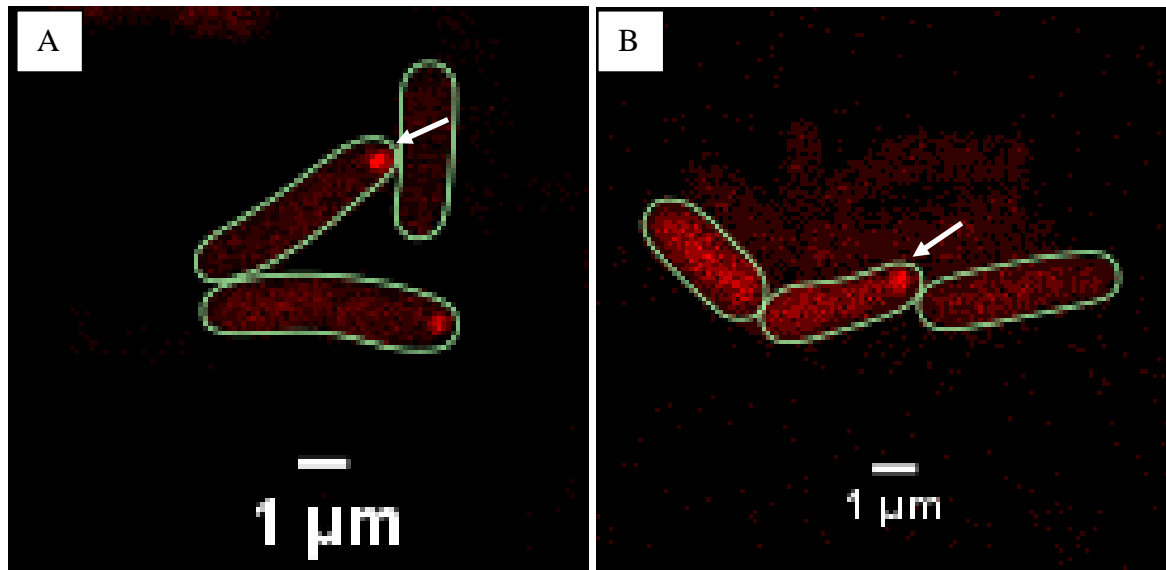
These findings underscore NucS's crucial role in stabilising the genome under stress by potentially inhibiting harmful HR events or promoting alternative ways to repair DSBs. Thus, NucS maintains genomic integrity by reducing episodes of genetic mobility. This suggests that NucS could help manage the balance between different DNA damage repair pathways, prioritising quicker and more error-prone mechanisms such as MMEJ.

## **C. Study of the localisation and conditions of recruitment of NucS in living cell**

### **C. 1 Study of the localisation of a fluorescent construction of NucS**

Despite the absence in the literature of prior observations of NucS either in living or in fixed cells, we made progress in our study of this enzyme by developing a genetic construct to facilitate direct visualisation within cells, thereby enhancing our understanding of its cellular localisation and behaviour under various stress conditions. The mScarlet1-NucSD144A fusion was explicitly designed to associate with chromosomes and form foci. The D144A mutation in the catalytic site of NucS renders it inactive, ensuring a prolonged interaction of NucS with DNA without engaging in enzymatic activity. We used the same plasmid previously described, which allows for the expression of this *mscarlet1-nucSD144A* fusion under the control of the IPTG-inducible promoter. Importantly, this sequence was not integrated into the bacterial genome, allowing us to retain an active copy of *nucS* to accurately recruit the protein fusion to the intended cellular localisation.

We designed a flexible linker for this construction, which is as follows: GGGGSGGGAA. The chosen fluorescent marker, mScarlet1, is a monomeric FP renowned for its high fluorescence emission, significantly enhancing the visibility of tagged molecules under microscopy. This protein was also selected for its rapid maturation, facilitating faster experimental observations. The sequence of this FP was optimised for its expression in *C. glutamicum* using this organism's codon library. Knowing that *C. glutamicum*'s autofluorescence predominantly occurs at green wavelengths and not in red, we effectively positioned our experiment in an emission range devoid of autofluorescence, as no detectable signal was observed in the cells in the absence of IPTG-induced *mscarlet1-nucSD144A* expression. A cytoplasmic signal only becomes apparent upon induction. Under these conditions, fluorescence associated with mScarlet1-NucSD144A was observable in most cells and notably, foci of mScarlet1-NucSD144A were distinctly visible against the cellular background at polar localisation, see **Figure 30**.



**Figure 30:** Confocal image of *Cg/WT* expressing the mScarlet1-NucSD144A construct after 5 hours of IPTG induction and treated with CIP (1  $\mu\text{g}/\text{mL}$ ) **A.** for 30 minutes **B.** or without treatment. The arrows indicate the foci formed on the damaged DNA caused by the antibiotic and detected by the mScarlet1-NucSD144A construct at the pole of the cell.

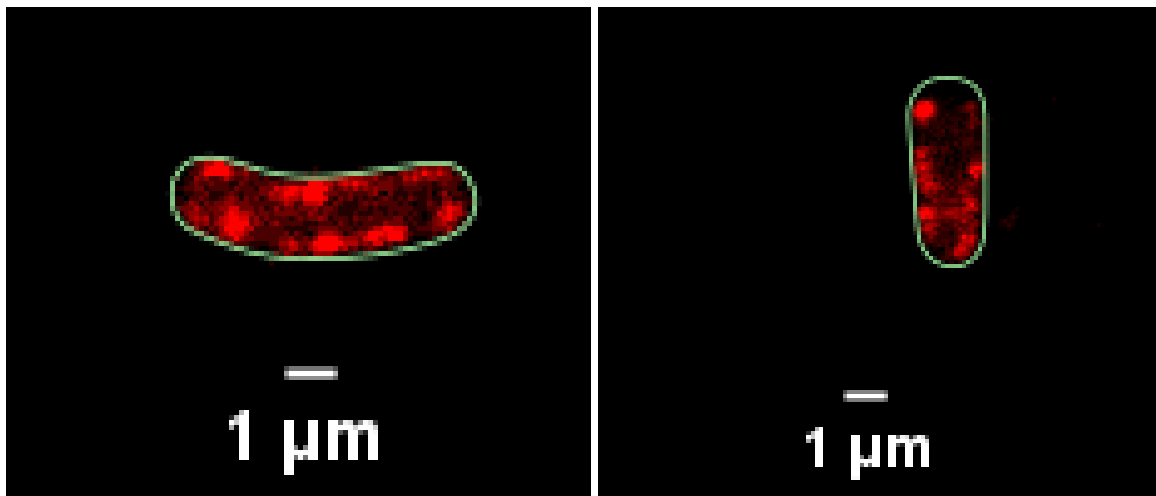
Based on studies conducted on MutS/L and other proteins involved in DNA repair, these proteins often exhibit two types of cellular localisation. Firstly, they can be found in a diffuse state throughout the cell, either freely in the cytoplasm or sliding to the DNA in a non-specific manner. Secondly, these proteins can localise to specific sites of DNA damage, forming foci at DNA where they actively participate in the repair process.

For instance, Msh2-Msh6 can be found sliding along the DNA. Upon detecting DNA mismatches, foci are formed at the damage sites to initiate the repair process (Gorman *et al.*, 2007; Cho *et al.*, 2012). Similarly, the RecA protein exhibits a diffuse cytoplasmic distribution but forms nucleoprotein filaments at DSBs, creating visible foci that mark the repair sites (Bell *et al.*, 2012).

These patterns of localisation are crucial for the functionality of DNA repair proteins. The diffuse presence allows these proteins to survey the genome efficiently. At the same time, forming foci at damage sites facilitates the recruitment and assembly of additional repair factors necessary to resolve the damage. In the case of NucS, the formation of polar foci could signify a targeted response of NucS to DNA damage, where it could regulate or repair specific DNA lesions. This dual localisation strategy ensures that the cell can respond promptly and effectively to DNA damage, maintaining genomic stability (Iyer *et al.*, 2006; Tkach *et al.*, 2012).

It is important to note that the bacterial chromosome, which is not contained within a nucleus, extends throughout the cell, forming filaments with areas of varying density influenced by centres of molecular activity such as transcription or replication. Consequently in bacterial cells, the DNA conformation complicates distinguishing whether a protein is localised on the bacterial chromosome or freely in the cytoplasm, as the nucleoid extends across the entire cellular space.

The presence of NucS as foci at bacterial poles may indicate an active DNA repair environment recruited at these specific localisations (Wang *et al.*, 2004). Unlike MutS/L, which are not typically localised at cell poles, the localisation of NucS resembles that of RecA. The literature suggests such localisation necessitates specific machinery for the recruitment and transport of RecA within the cell, directing it to areas where its activity is essential. If NucS is involved in DSBR, it is reasonable to hypothesise that this protein could be similarly recruited at specific cellular sites that gather all the necessary components for this repair mechanism (Kidane *et al.*, 2005; Kramer *et al.*, 2007).



**Figure 31:** Confocal image of *Cg/WT* expressing the mScarlet1-NucSD144A constructs after 24 hours of treatment with CIP (1 µg/mL).

After 24 hours of exposure to genotoxic agents such as CIP or Phleo, we noted a distinct redistribution of mScarlet1-NucSD144A bundles within less than 1 % of the cells, see **Figure 31**. In these bacteria, the previously diffuse signal became more confined.

These observations support the hypothesis that, in the absence of DNA damage, mScarlet1-NucSD144A is primarily localised on the chromosome sliding or freely in the cytoplasm and, in response to damage, attaches to DNA breaks to form foci. Following a highly concentrated

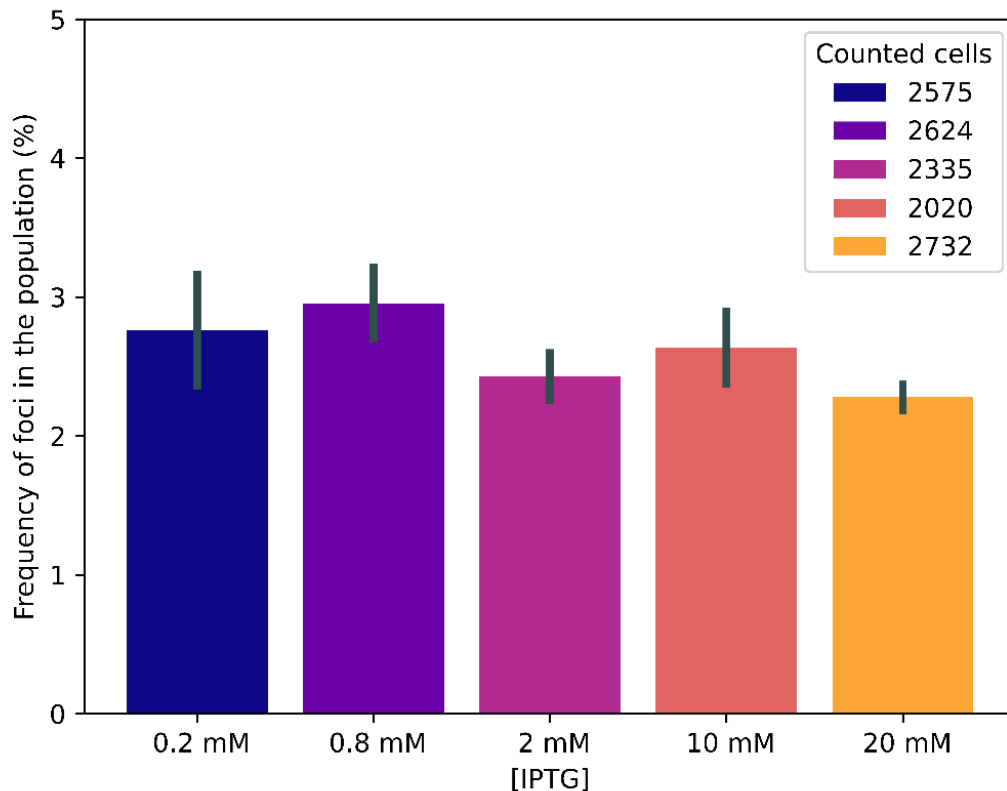
genotoxic treatment, the chromosome should be fragmented and condensed, which is a phenomenon consistent with findings from the literature that describe similar outcomes during treatments with agents like Chloramphenicol (White *et al.*, 2020).

These observations, made with a catalytically inactive protein, suggest that the fusion of NucS to mScarlet1 does not impact its recruitment and localisation in response to the cell's physiological state and biological processes. This fusion thus facilitates the observation of NucS's physiological responses and recruitment to DNA damage sites under various conditions. To go further on these experiments, we aimed to determine if the localisation of NucS as foci at the cell poles could be affected by different environmental factors, such as heat shock, starvation, or exposure to genotoxic antibiotics.

## **C. 2 Induction of mScarlet1-NucSD144A over 4h without external stress**

According to the observations reported in the literature regarding the activity of NucS on mismatched substrates, one would expect proportionally to the expression of mScarlet1-NucSD144A an increase in the number of foci. However, we want to remember that the construct used here is inactive and, therefore, unable to perform any enzymatic activity. We thus consider that if mScarlet1-NucSD144A forms foci, it would be in response to DNA damage and its recruitment at this site, not related to the activation of its endonuclease function. To further investigate the nature of the polar foci observed from our engineered mScarlet1-NucSD144A fusion, we assessed whether these foci could be unfolded protein aggregates segregated at the pole or proteic bundles triggered by biological events like genomic breaks.

Thus, we wondered whether these foci depended on the concentration of mScarlet1-NucSD144A molecules within cells or whether they were responsive to biological processes. Assuming that our construction does not provide an enzymatic activity, an increase in foci under conditions involving overexpression of the mScarlet1-NucS supports the aggregation hypothesis. Conversely, if the number of foci is responsive to exogenous stresses, our construct is responsive to physiological stimuli and, therefore, relevant for further investigation.



**Figure 32: Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg*/WT population over 5 hours after induction of construct expression with 0.2, 0.8, 2, 10, 20 mM IPTG.** 0.2 mM IPTG ( $\mu = 2.76$ ,  $\sigma = 0.43$ ) 0.8 mM IPTG ( $\mu = 2.95$ ,  $\sigma = 0.284$ ) 2 mM IPTG ( $\mu = 2.43$ ,  $\sigma = 0.20$ ) 10 mM IPTG ( $\mu = 2.64$ ,  $\sigma = 0.29$ ) 20 mM IPTG ( $\mu = 2.28$ ,  $\sigma = 0.12$ ) Control: 0 foci for 0 mM IPTG. t-student analysis 0.2 mM and 0.8 mM IPTG: p-value = 0.62; 0.2 mM and 2 mM IPTG: p-value = 0.37; 0.2 mM and 10 mM IPTG: p-value = 0.75; 0.2 mM and 20 mM IPTG: p-value = 0.20. At 0 IPTG, no signals were detectable. Each condition was tested in triplicate.

Our experiments, with different amounts of induction of *mScarlet1-nucSD144A* in the cells, from a minimal 0.2 mM of IPTG, below which no fluorescence is visible up to 20 mM a hundredfold increase in concentration showed that the percentage of cells bearing foci in the population of imaged bacteria remained comprised between 2,95 % ( $0,0295 \pm 0,0028$ ) and 2,28 % ( $0,028 \pm 0,001$ ), see **Figure 32**. This foci frequency is in the same range as the one we observed with the RecA-mVenus construction (1,8 %).

We analysed this result as follows: the mScarlet1-NucSD144A foci formation is not directed by the concentration of this construct in the cells. However, the consistency in the number of the foci counted suggests that these structures most likely respond to specific biological signals rather than being a simple artefact due to overexpression of proteins. This analysis is



backed by research that demonstrates proteins involved in DNA repair pathways, particularly those in HR, typically aggregate into specific foci in response to DNA damage. This recruitment process occurs regardless of the proteins' concentrations within the cell. This is so for the RecA and Rad51 proteins, as shown in Kowalczykowski, 2000 and Liu *et al.*, 2017.

We saw a decreasing trend for the number of foci with increased mScarlet1-NucSD144A induction. NucS forms a homodimer, which is a mixture of the fused protein and normal, endogenous NucS in the cell. Endogenous homodimers recruited at the DNA damage site might be needed to recruit the fluorescent construction. Overexpression of our fused construct might result in its overabundance. The endogenous NucS homodimer required for correct localisation could be out-competed.

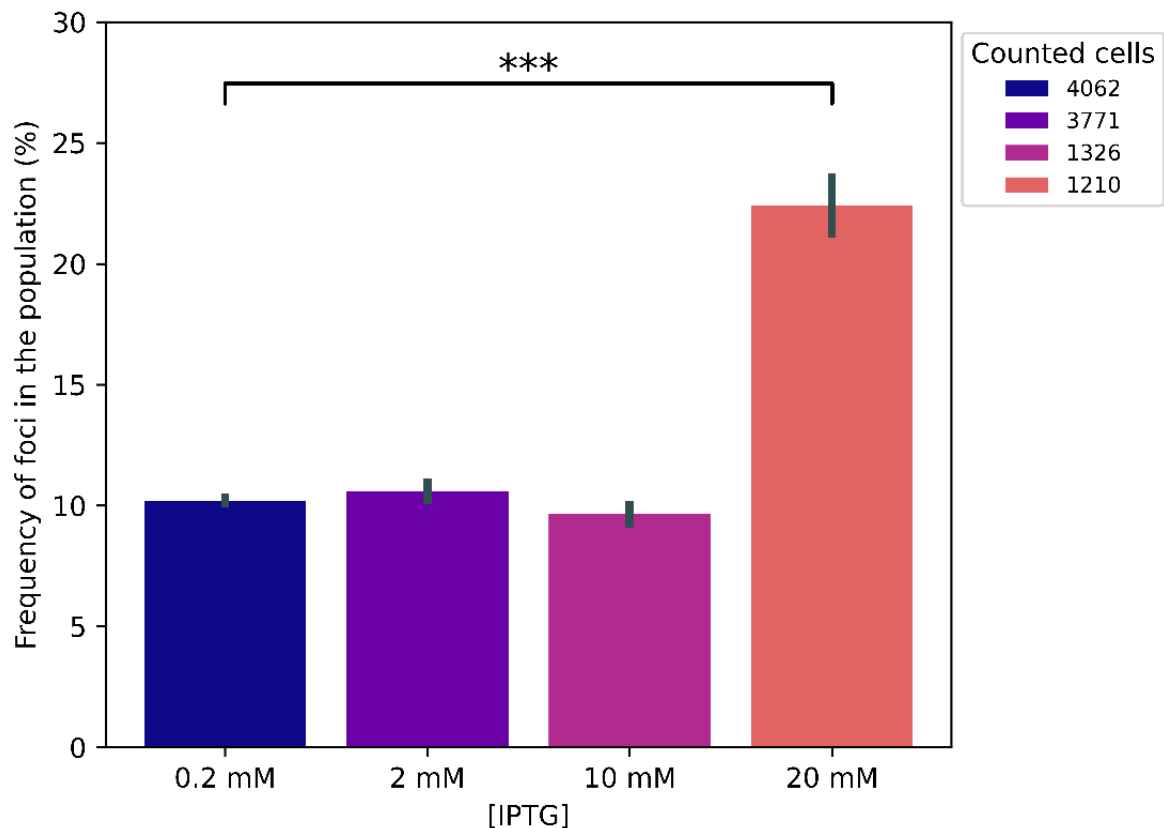
Given our observation that the basal number of foci during the exponential growth phase remains stable regardless of the level of expression of our engineered fusion, we hypothesise that mScarlet1-NucSD144A's foci could be formed in response to a DNA repair environment at the pole of the cell and should vary regarding the appropriate introduction of biological signals. Consequently, following our previous results with the Gam-meGFP construction, we anticipate an increase in both DNA damages and the corresponding number of mScarlet1-NucSD144A foci during the stationary phase. We hypothesise that NucS plays a dynamic role in responding to the cell's metabolic state and the DNA's integrity, especially under stress or nutrient scarcity conditions.

### **C. 3 Induction of mScarlet1-NucSD144A over 24h without external stress**

After 24 hours of inducing the expression of *mScarlet1-nucSD144A*, the number of foci in starving cells in the population of *CgIWT* was 10 %. This percentage is comparable to the one noted under the same condition with the Gam-meGFP construction, approximately 16 %. This similarity could correspond to the activation frequency of a DSBR pathway in the cell.

After 24 hours of culture without medium renewal, the number of mScarlet1-NucSD144A foci showed a 285 % increase compared with the number of foci counted after 5 hours of induction. This difference is similar to that noted for the formation of Gam-meGFP foci, which increased from 4.4 % to 16.1 %, a rise of 266 %. These ratios indicate that NucS is involved in a DNA repair system that is active both during the exponential growth and the

stationary phase and is responsive to the increase of damage incorporated in the DNA molecule during the stationary phase. After 24 hours of growth, regarding induction levels with IPTG ranging from 0.2 to 10mM, we noted that the number of mScarlet1-NucSD144A foci is not influenced by the variation of the induction, remaining at 10 %, as represented in **Figure 33**.



**Figure 33:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population over 24 hours after induction of construct expression with 0.2, 0.8, 2, 10, 20 mM IPTG. 0.2 mM IPTG ( $\mu = 10.21$ ,  $\sigma = 0.29$ ) 2 mM IPTG ( $\mu = 10.60$ ,  $\sigma = 0.53$ ) 10 mM IPTG ( $\mu = 9.65$ ,  $\sigma = 0.56$ ) 20 mM IPTG ( $\mu = 22.42$ ,  $\sigma = 1.33$ ) t-student analysis: 0.2 mM and 2 mM IPTG: p-value = 0.41 0.2 mM and 10 mM IPTG: p-value = 0.28 0.2 mM and 20 mM IPTG: p-value = 0.0002 Each condition was tested in triplicate.

However, at a very high induction level of *mScarlet1-nucSD144A*, 20Mm of IPTG, the foci counted in the bacterial population increase to 23 %. It is plausible that this elevated expression level is perturbative and disrupts interactions between DNA repair actors. This disruption could lead to increased errors and disturbances in the balance between the DNA repair pathways.

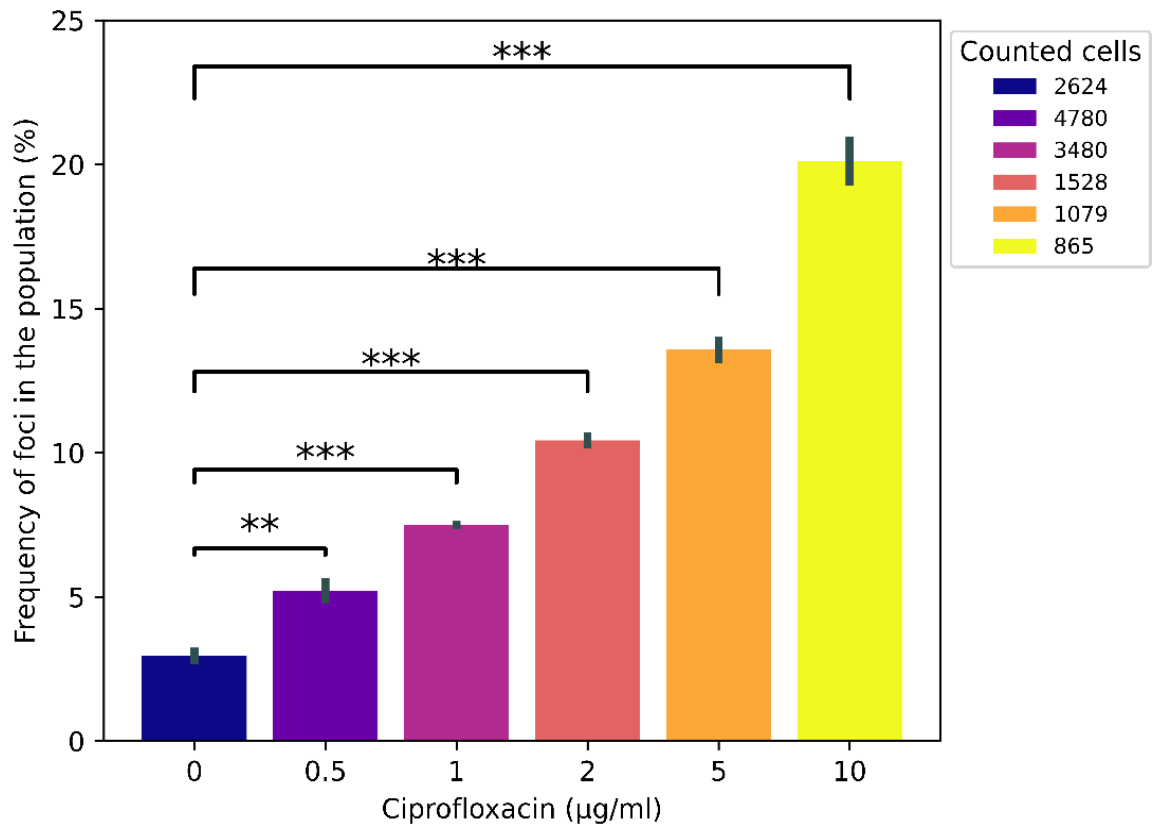
## C. 4 Physiological response of mScarlet1-NucSD144A to short treatment with genotoxic antibiotics

Given that previous findings indicate that mScarlet1-NucS foci formation is driven by biological processes rather than the accumulation of non functional protein aggregates, we aimed to explore whether these sites are active environments for DNA repair, especially concerning the induction of DSBs. To this end, we examined the physiological response of mScarlet1-NucSD144A in the foci formation when exposed to genotoxic agents such as CIP, Phleo and MMC. Concurrently, we investigated the cellular localisation of NucS foci under these varied stress conditions to better understand their role within the cell.

During these experiments, we observed that 30 minutes after introducing a high antibiotic concentration in the medium, more than one foci could form within the cells. There was a clear positive correlation between the number of foci per cell and the antibiotic dosage administered. Up to four foci have been identified in *Cg/WT* cells. Notably, in nearly 99 % of instances, the mScarlet1-NucSD144A foci were located at one of the cell poles. We also noted that when multiple foci were present, they were not symmetrically positioned at opposite poles. Instead, foci were distributed along the periphery of the membrane and throughout the cell rather than mirroring each other at the poles of the cell.

To assess the physiological response of mScarlet1-NucSD144A to genotoxic stress, we administered a range of concentrations of the previously mentioned genotoxic antibiotics. Starting from the lowest concentration, where the number of foci aligns with that observed in untreated cells in the exponential phase, we increased the dosage by a hundredfold. We took specifically care that the concentrations remained below the MIC, at which point cells became unobservable under the microscope.

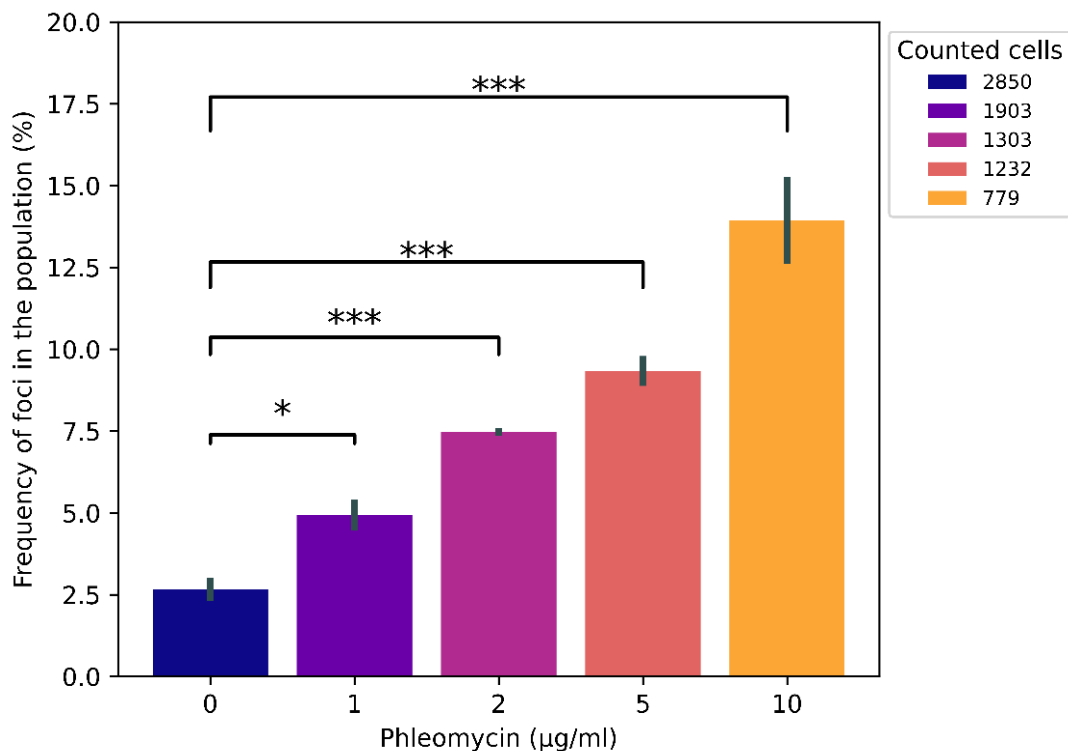
The results in **Figure 35** depict a clear correlation between the number of foci within a population of *Cg/WT* bacteria and the dosage of antibiotics introduced in the environment, especially after 30 minutes of genotoxic stress on the culture in the exponential phase. Specifically, the higher the antibiotic concentration is, the more pronounced the increase in the number of foci in the bacterial population is, going from 2,95 % ( $0,0295 \pm 0,0028$ ) without genotoxic treatment to 20,1 % ( $0,201 \pm 0,009$ ). A series of six concentrations demonstrated this positive correlation for the CIP.



**Figure 35:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population over 5 hours after induction with 2 mM IPTG and a 30-minute treatment with varying concentrations of CIP (0, 0.05, 0.1, 0.2, 0.5, 1 µg/mL). 0 µg/mL CIP ( $\mu = 2.95$ ,  $\sigma = 0.28$ ) 0.05 µg/mL CIP ( $\mu = 5.22$ ,  $\sigma = 0.435$ ) 0.1 µg/mL CIP ( $\mu = 7.49$ ,  $\sigma = 0.13$ ) 0.2 µg/mL CIP ( $\mu = 10.43$ ,  $\sigma = 0.26$ ) 0.5 µg/mL CIP ( $\mu = 13.57$ ,  $\sigma = 0.45$ ) 1 µg/mL CIP ( $\mu = 20.12$ ,  $\sigma = 0.86$ ). T-student analysis: 0 µg/mL and 0.05 µg/mL CIP p-value = 0.0035; 0 µg/mL and 0.1 µg/mL CIP p-value =  $3.35e-5$ , 0 µg/mL and 0.2 µg/mL CIP p-value =  $1.06e-5$ , 0 µg/mL and 0.5 µg/mL CIP p-value =  $9.27e-60$  µg/mL and 1 µg/mL CIP p-value =  $1.14e-5$ . Each condition was tested in triplicate.

To delve deeper, we investigated the physiological response of mScarlet1-NucSD144A to additional genotoxic agents that cause genomic breaks, specifically Phleo and MMC. We observed similar trends in the presence of both.

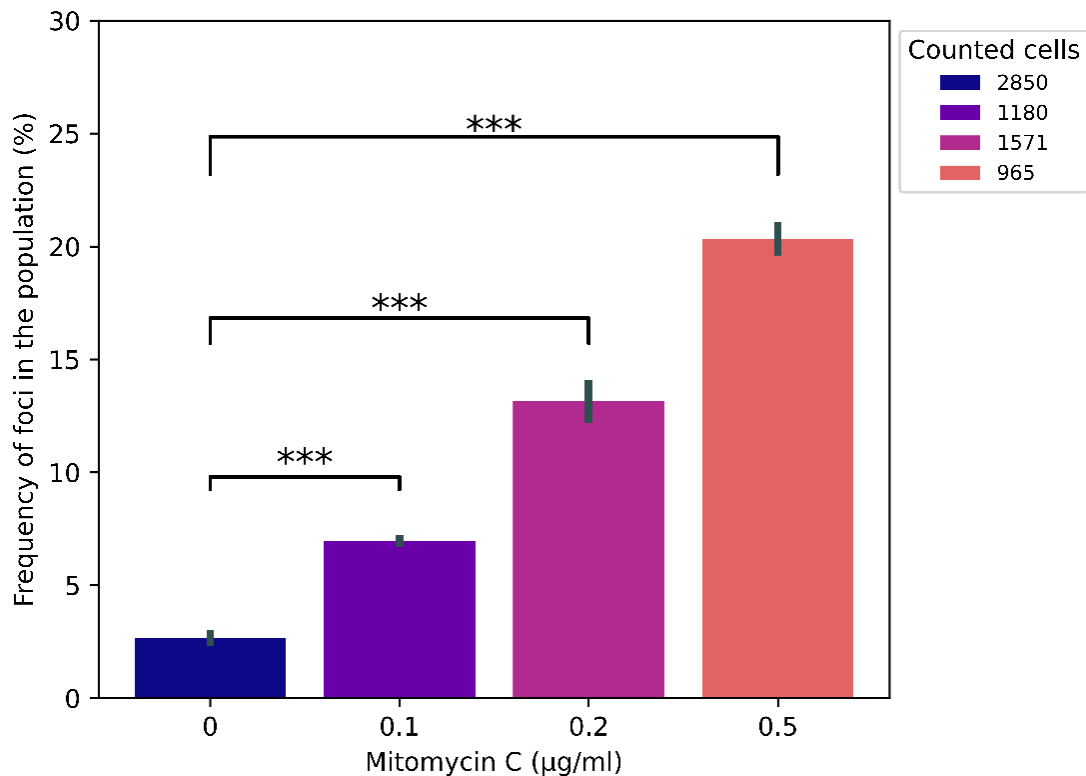
We tested five concentrations of Phleo, showing a similar variation in the number of foci, from 2.7 % ( $0.0266 \pm 0.0035$ ) without Phleo up to 14 % ( $0.149 \pm 0.00132$ ). We also tested four concentrations for the MMC from the same basal number of foci, going up to 20 % ( $0.235 \pm 0.0075$ ) affirming the role of these agents in inducing the formation of mScarlet1-NucSD144A foci at the poles of the cells, see **Figures 35** and **36** for the detail of the T-student tests.



**Figure 35:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population over 5 hours after induction with 2 mM of IPTG and a 30-minute treatment with 0, 1, 2, 5, 10 µg/mL of Phleo. 0 µg/mL Phleo ( $\mu = 2.66$ ,  $\sigma = 0.35$ ) 1 µg/mL Phleo ( $\mu = 4.94$ ,  $\sigma = 0.47$ ) 2 µg/mL Phleo ( $\mu = 7.47$ ,  $\sigma = 0.12$ ) 5 µg/mL Phleo ( $\mu = 9.33$ ,  $\sigma = 0.46$ ) 10 µg/mL Phleo ( $\mu = 13.94$ ,  $\sigma = 1.32$ ) t-student analysis: 0 µg/mL Phleo vs. 1 µg/mL Phleo: p-value = 0.005 0 µg/mL Phleo vs. 2 µg/mL Phleo: p-value = 5.04e-05 0 µg/mL Phleo vs. 5 µg/mL Phleo: p-value = 7.93e-05 0 µg/mL Phleo vs. 10 µg/mL Phleo: p-value = 0.00031 Each condition was tested in triplicate.

The variation in the number of foci in the cells in exponential growth increased in a dose-dependent manner relative to the concentration of these antibiotics. Throughout these experiments, it was uncommon for the cells to display more than one foci and the polar localisation of the foci remained consistent under all the conditions. In less than 1 % of the cell population, we observed cells that appeared more swollen, with the signal of the mScarlet1-NucSD144A fusion distributed more peripherally and in several dense areas, as presented in **Figure 31**. We interpreted these cells as being in an apoptosis-like phase, unable to further divide and destined for cell death.

The consistency of these results across different antibiotics, despite their varied targets but similar induction of cellular damage, supports the hypothesis that NucS is actively involved in regulating or directly participating in a pathway for repairing DSBs. Moreover, these results suggest for the first time, to our knowledge that NucS is specifically recruited to a confined area at the poles in response to the introduction of DSBs into the cells.



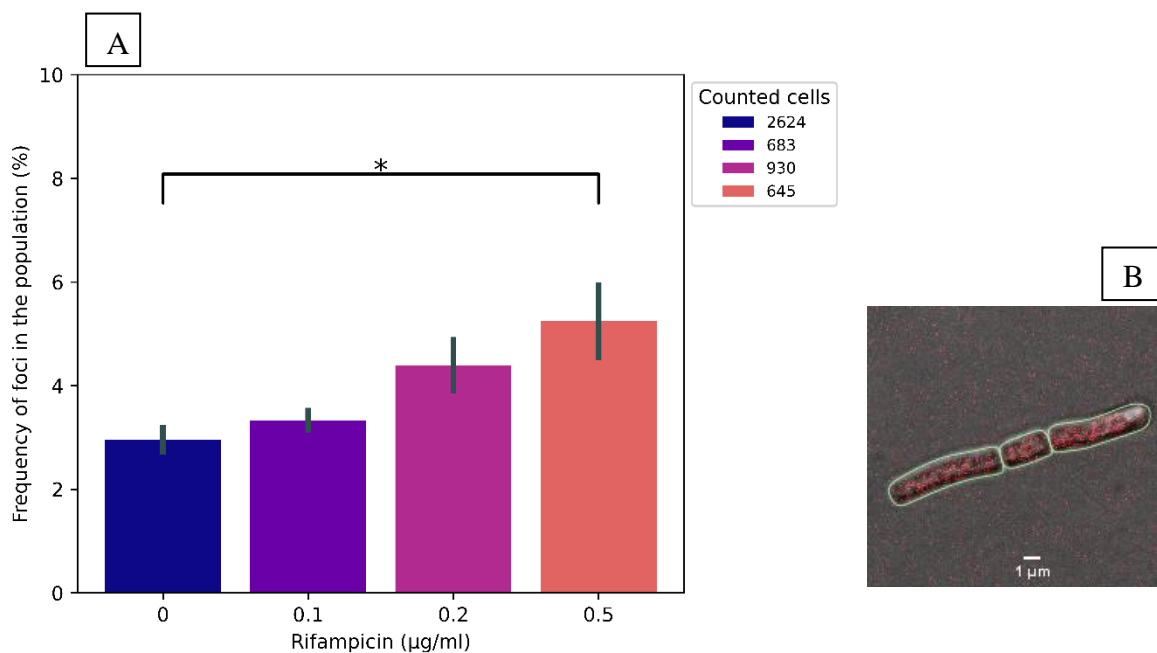
**Figure 36:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population over 5 hours after induction with 2 mM of IPTG and a 30-minute treatment with 0, 0.1, 0.2, 0.5 µg/mL of MMC. 0 µg/mL MMC ( $\mu = 2.66$ ,  $\sigma = 0.35$ ) 0.1 µg/mL MMC ( $\mu = 6.96$ ,  $\sigma = 0.28$ ) 0.2 µg/mL MMC ( $\mu = 13.15$ ,  $\sigma = 0.95$ ) 0.5 µg/mL MMC ( $\mu = 20.35$ ,  $\sigma = 0.75$ ) t-student analysis: 0 µg/mL MMC vs. 0.1 µg/mL MMC: p-value = 0.00017 0 µg/mL MMC vs. 0.2 µg/mL MMC: p-value = 0.00013 0 µg/mL MMC vs. 0.5 µg/mL MMC: p-value = 7.12e-06 Each condition was tested in triplicate.

These experiments revealed variations in size and shape within the bacterial population, including elongation phenotypes (without filamentation as seen in *E. coli*) and occasional defects in cell division, resulting in bacteria arranged in "flower" shapes.

We also observed that, beyond a specific antibiotic dosage, even though *C. glutamicum* is not documented in the literature as a spore-forming species, cells tended to become more compact and spherical. This morphological response suggests a stress-induced morphological adaptation in *C. glutamicum*, which may reflect a survival strategy in the presence of antibiotic stress. Such adaptations can occur as part of the bacterial defence mechanism against harmful conditions, potentially involving changes in the cell wall composition, cytoskeletal rearrangements, or alterations in the regulation of cell division and growth processes. The influence of NucS in these rearrangements could be interesting to assess (Ciano-Oliveira *et al.*, 2006).

Our primary objective was to determine whether the observed foci were exclusively formed in response to the introduction of breaks in the genome or if they could also result from mismatches introduced into the bacterial chromosome. To explore this, we conducted parallel tests using Rif. Unlike previous experiments where the observation of the bacteria was conducted 30 minutes to 1 hour after the introduction of the antibiotic, the observation with Rif was made after extended periods of 3 hours of incubation, reflecting the time required to observe the effects of Rif on nucleotide incorporation during the replication. Based on the literature on NucS, we anticipated variations in mScarlet1-NucSD144A foci corresponding to the incorporation of incorrect base pairs induced by Rif.

Surprisingly, even with a high concentration of Rif and an incubation time ranging from 3 to 24 hours, no specific variations in the number of foci formed by the fusion were noted. Only 5.3 % ( $0,0524 \pm 0,0075$ ) of the bacteria in the population imaged were positive for a foci at the highest antibiotic concentration tested, permitting cells to remain alive, see **Figure 37A**. Compared to the previous treatment with the CIP, Phleo or MMC, this experiment is less effective in recruiting the mScarlet1-NucSD144A.



**Figure 37:** Treatment of *Cg/WT* with Rif **A**. Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population over 5 hours after induction with 2 mM of IPTG and a 3-hour treatment with 0, 0.1, 0.2, 0.5 µg/mL of Rif. 0 µg/mL Rif ( $\mu = 2.95$ ,  $\sigma = 0.28$ ) 0.1 µg/mL Rif ( $\mu = 3.33$ ,  $\sigma = 0.24$ ) 0.2 µg/mL Rif ( $\mu = 4.40$ ,  $\sigma = 0.54$ ) 0.5 µg/mL Rif ( $\mu = 5.24$ ,  $\sigma = 0.75$ ) t-student analysis: 0 µg/mL Rif vs. 0.1 µg/mL Rif: p-value = 0.32 0 µg/mL Rif vs. 0.2 µg/mL Rif: p-value = 0.057 0 µg/mL Rif vs. 0.5 µg/mL Rif: p-value = 0.0336 Each condition was tested in triplicate. **B**. Confocal image of *Cg/WT* expressing the mScarlet1-NucSD144A constructs after 24 hours of treatment with Rif (0.2 µg/mL). There is abnormal division and size of the cells.

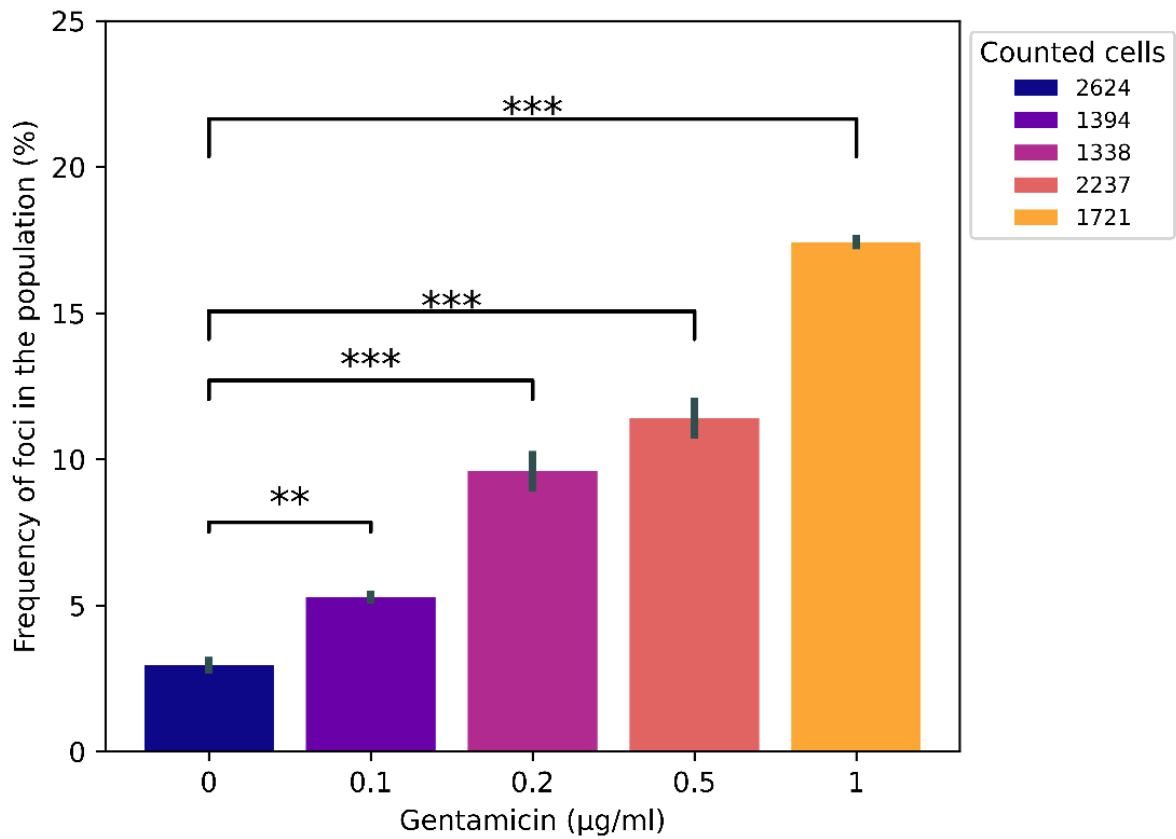
These results indicate that although Rif triggers stress responses in the cell, evident through changes in cell morphology, as presented in **Figure 37B**, it does not lead to the formation of mScarlet1-NucSD144A foci, as previously observed with genotoxic agents introducing DNA breaks. Instead, the stress response we observed seems more connected to disruptions of normal replication processes than to the repair of direct DNA damage, as the cells were longer. This suggests that the formation of mScarlet1-NucSD144A foci is specifically linked to DNA strand breaks rather than to other forms of DNA damage or replication stress.

We extended our experimentations to include other antibiotics to further complement these findings, which underscore the specificity of physiological contexts required for forming mScarlet1-NucSD144A foci.

Similar results to Rif were obtained with Erythromycin and Ampicillin. Neither of these antibiotics led to any critical change in the number of foci across the bacterial population. Indeed, only about 5 % of the observed cells displayed foci at maximum antibiotic concentrations with these treatments. This modest increase, much lower than what was measured with agents directly inducing DNA breaks, suggests that this variation in the number of foci is likely due to an indirect phenomenon related to the action of the antibiotics, such as the accumulation of errors conducing to the introduction of a DSB in the genome.

This study sought to investigate the response of mScarlet1-NucSD144A foci formation to other stressors, including thermal shock at 42 °C, UV exposure and oxidative stress simulated by H<sub>2</sub>O<sub>2</sub>, mimicking the effects of Reactive Oxygen Species (ROS) in the environment. Across all these experimental conditions, no variation was observed in the number of foci formed by mScarlet1-NucSD144A. The data from thermic stress experiments proved more complex to analyse due to reproducibility issues. It appears that no particular response could be reported at a thermal shock of 37 °C; similarly, at 42 °C. However, at 42 °C and in the presence of low concentrations of genotoxic agents, there seems to be a trend towards increasing foci. Prolonged heating appears to lead to cell death, with cells generally exhibiting a phenotype where the NucS fusion aggregates around the cell's periphery.

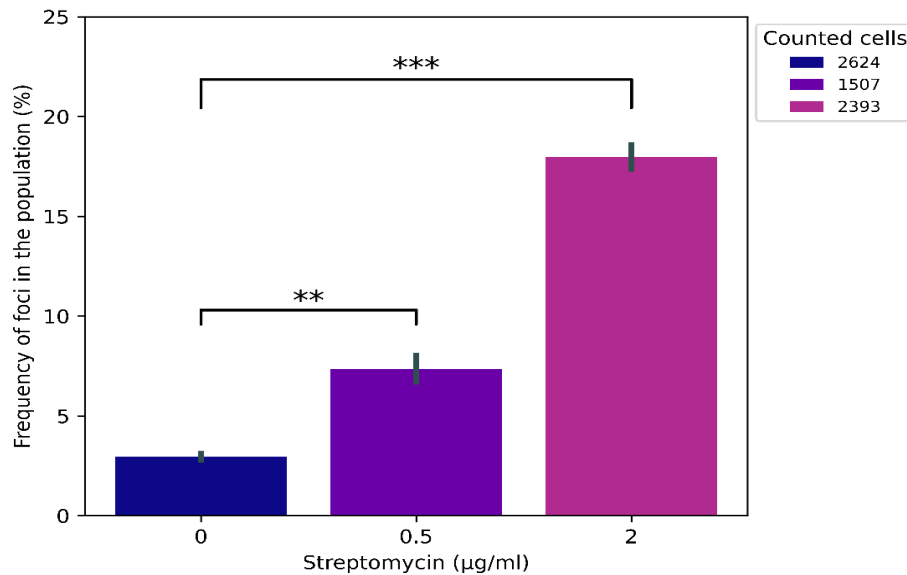




**Figure 38:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg*/WT population over 5 hours after induction with 2 mM of IPTG and a 30-minute treatment with 0, 0.1, 0.2, 0.5, 1 µg/mL of Gen. 0 µg/mL Gen ( $\mu = 2.95$ ,  $\sigma = 0.28$ ) 0.1 µg/mL Gen ( $\mu = 5.297$ ,  $\sigma = 0.22$ ) 0.2 µg/mL Gen ( $\mu = 9.59$ ,  $\sigma = 0.70$ ) 0.5 µg/mL Gen ( $\mu = 11.41$ ,  $\sigma = 0.71$ ) 1 µg/mL Gen ( $\mu = 17.43$ ,  $\sigma = 0.24$ ) t-student analysis: 0 µg/mL Gen vs. 0.2 µg/mL Gen: p-value = 0.00078 0 µg/mL Gen vs. 0.5 µg/mL Gen: p-value = 0.00024 0 µg/mL Gen vs. 1 µg/mL Gen: p-value = 9.71e-05 Each condition was tested in triplicate.

Most tested conditions that do not directly cause DSBs in the genome showed no variation in the formation of mScarlet1-NucSD144A foci within the bacterial population, highlighting a specificity in the events and signalling needed to recruit NucS to the cellular poles. An unexpected observation was made regarding treatment with Gen. In this condition, we observed that, as presented in **Figure 38**, increased concentrations of Gen in the cellular environment induced foci formation, similar in appearance, localisation and number in the population of bacteria to those produced by treatments known to cause DSBs, like with CIP or Phleo. Almost 17 % ( $0,1743 \pm 0,0024$ ) of the bacteria observed presented polar foci after 30 minutes of treatment with 1 µg/mL of Gen.

Existing literature does not suggest that Gen should have this effect, making this an intriguing finding. Interestingly, we noted the same variation in the formation of foci in the presence of Streptomycin, another Aminoglycoside, where almost 18 % ( $0,180 \pm 0,007$ ) of the cells presented foci at the highest concentration tested, as presented in **Figure 39**, suggesting an impact of this family of antibiotics on the recruitment of NucS in the cell.



**Figure 39:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg*/WT population after 5 hours of treatment with 0, 0.5 and 2 µg/mL of Streptomycin. 0 µg/mL Streptomycin:  $\mu = 2.95$ ,  $\sigma = 0.28$  0.5 µg/mL Streptomycin:  $\mu = 7.36$ ,  $\sigma = 0.79$ , 2 µg/mL Streptomycin:  $\mu = 17.97$ ,  $\sigma = 0.73$ , T-Student analysis: 0 µg/mL vs. 0.5 µg/mL Streptomycin: p-value = 0.00175, 0 µg/mL vs. 2 µg/mL Streptomycin p-value = 1.104e-05) Each condition was tested in triplicate.

We can consider several explanations for this unexpected phenomenon. Indeed, while Gen is primarily known for inhibiting protein synthesis by binding to the ribosome, the presence of this antibiotic can also lead to the production of ROS. This reactive oxygen species can cause various types of cellular damage, including DNA breaks.

Although the oxidative stress induced by Gen might not directly break the DNA strands, it can create conditions that lead to the formation of DSBs as secondary damage. Additionally, by inhibiting protein production, Gen can affect replication and generate secondary stress due to the lack of key replication proteins, such as topoisomerases. The stalling or collapse of replication forks due to the absence of translated necessary proteins might result in the formation of DSBs, which could be a required signal to recruit NucS/EndoMS to the damaged sites.

Finally, by blocking protein synthesis, Gen can also lead to the accumulation of misfolded proteins, which can indirectly cause DNA breaks.

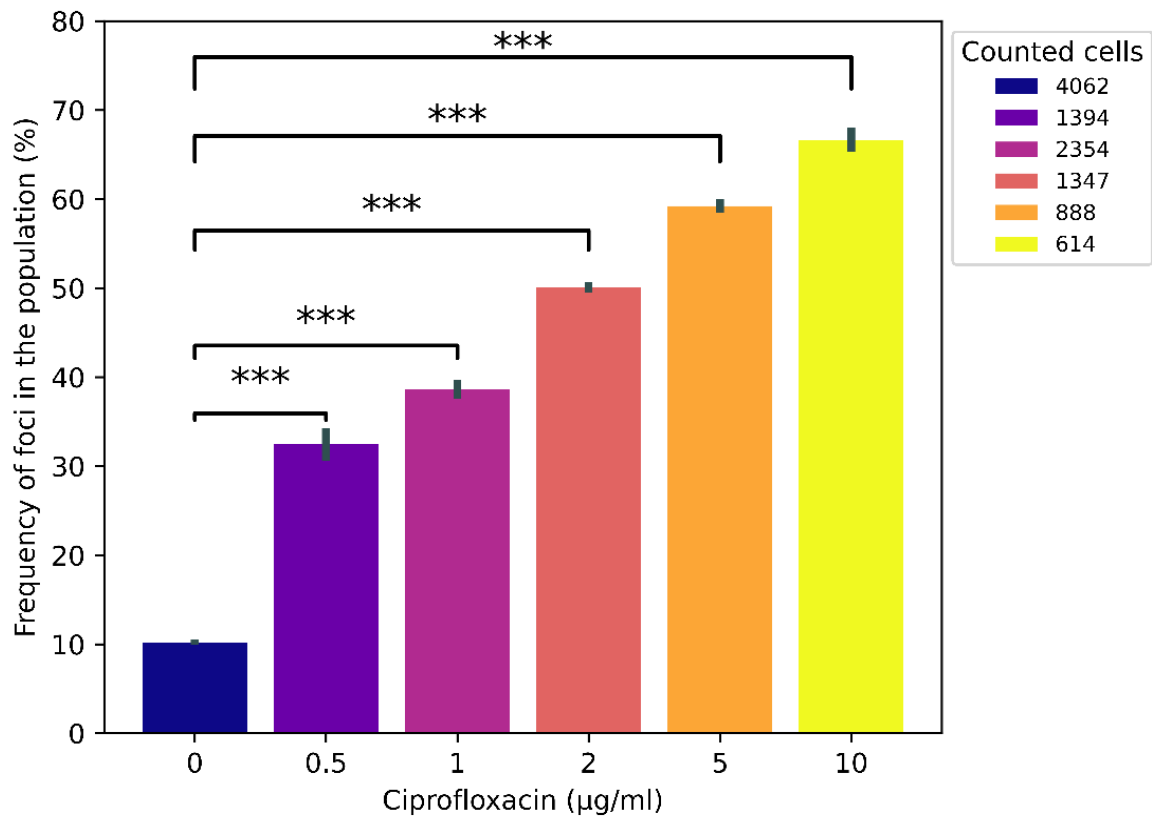
In contrast, Ampicillin targets cell wall synthesis, Rif inhibits RNA synthesis by targeting RNA polymerase and H<sub>2</sub>O<sub>2</sub>, while inducing oxidative stress, might not produce the same extent or type of oxidative damage leading to DSBs under the conditions tested. Thus, these agents do not show the same effect on the variation of mScarlet1-NucSD144A foci formation as Gen does.

### **C. 5 Physiological response of mScarlet1-NucSD144A to 24h of treatment with genotoxic antibiotics**

In our quest to deepen our understanding of NucS recruitment to the extremities of the cells and its physiological response to DSBs, we introduced an additional condition where bacteria were observed after 24 hours of CIP treatment. Our objective was to determine whether prolonged exposure influences the number of foci per cell, their localisation and the number of foci in the population. According to studies on CIP's impact on bacteria, we deduced that at CIP concentrations close to the MIC, approximately 50 to 70 % of bacterial cells should exhibit at least one DSB after 24 hours of treatment. (Drlica *et al.*, 1997; Malik *et al.*, 2007; Cirz *et al.*, 2005; Chow *et al.*, 1988). Additionally, we expect this number to vary with the antibiotic concentration applied.

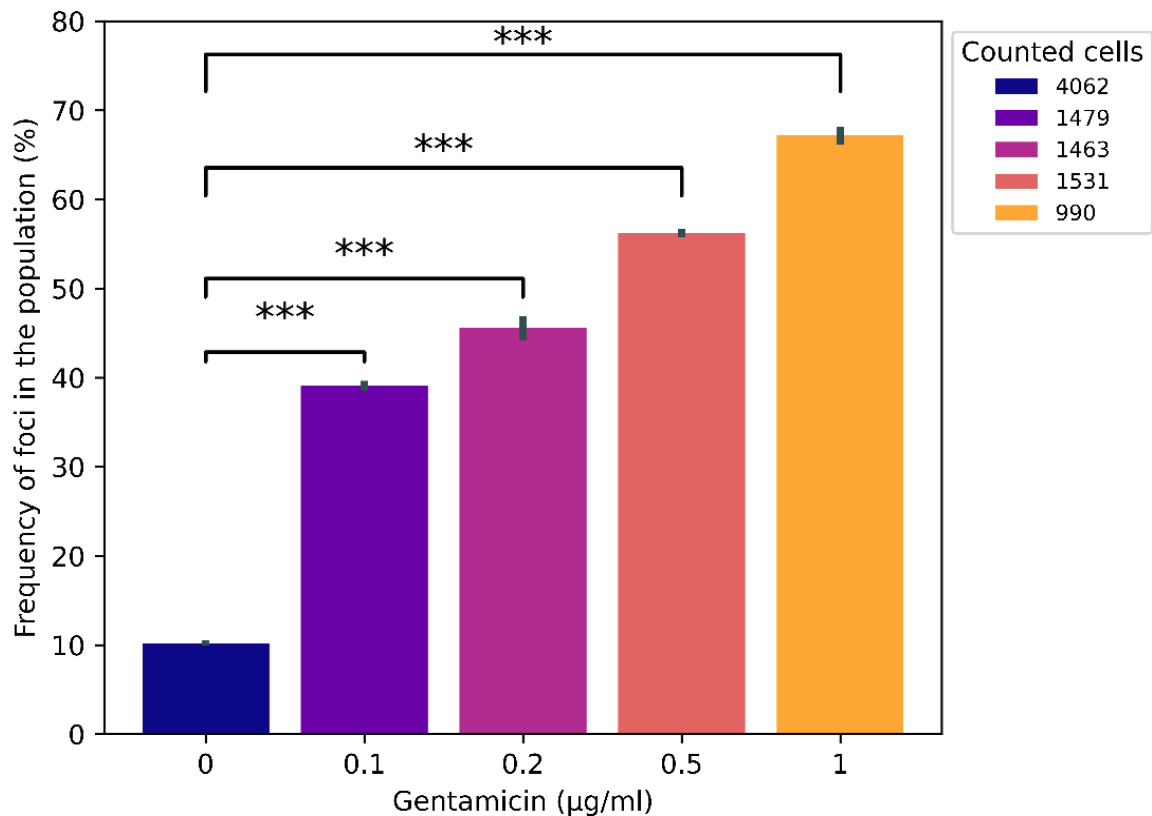
Previous observations indicated that the number of DSBs in the cells at 24 hours was around 16 %, a value comparable to the number of mScarlet1-NucSD144A foci noted at 24 hours, 10 % ( $0,0102 \pm 0,0029$ ). From this dataset, we determined that only a significantly higher number of foci exceeding 16 % after a 24-hour treatment would indicate a biological response to the prolonged treatment.

Upon further examination, we observed a dose-dependent formation of mScarlet1-NucSD144A foci at 24 hours in response to increasing doses of CIP, with the percentage of positive cells presenting foci in the population ranging from approximately 32 % ( $0,325 \pm 0,018$ ) to nearly 67 % ( $0,667 \pm 0,014$ ) as presented in **Figure 40**. These findings align with our predictions based on the literature regarding CIP's impact on bacteria, demonstrating consistent ratios of cells exhibiting at least one foci under these conditions.



**Figure 40:** Evolution of the frequency of mScarlet1-NucSD144A foci in a Cg/WT population after 24 hours of treatment with 0, 0.05, 0.1, 0.2, 0.5 and 1 µg/mL of CIP. 0 µg/mL CIP:  $\mu = 10.21$ ,  $\sigma = 0.29$ , 0.05 µg/mL CIP:  $\mu = 32.46$ ,  $\sigma = 1.82$ , 0.1 µg/mL CIP:  $\mu = 38.62$ ,  $\sigma = 1.02$ , 0.2 µg/mL CIP:  $\mu = 50.06$ ,  $\sigma = 0.62$ , 0.5 µg/mL CIP:  $\mu = 59.22$ ,  $\sigma = 0.75$ , 1 µg/mL CIP:  $\mu = 66.66$ ,  $\sigma = 1.37$ , T-Student analysis 0 µg/mL vs. 0.05 µg/mL CIP: p-value =  $6.92e-05$ , 0 µg/mL vs. 0.1 µg/mL CIP: p-value =  $2.94e-06$ , 0 µg/mL vs. 0.2 µg/mL CIP: p-value =  $1.32e-07$ , 0 µg/mL vs. 0.5 µg/mL CIP: p-value =  $1.065e-07$ , 0 µg/mL vs. 1 µg/mL CIP: p-value =  $5.62e-07$  Each condition was tested in triplicate.

A similar dose-dependent increase in the number of foci was noted after a treatment of 24 hours with Gen, ranging from 39 % ( $0.039 \pm 0.055$ ) to 67 % ( $0.672 \pm 0.099$ ) of the cells in the population harbouring at least one foci, see **Figure 41**. Additionally, upon analysing our images, we observed that most positive cells exhibited only a single foci that harboured a greater size and luminosity than those seen with a 30-minute CIP treatment. This variation might result from the accumulation of cellular damage over time, leading to an enlargement of the repair site.



**Figure 41:** Evolution of the frequency of mScarlet1-NucSD144A foci in a *Cg/WT* population after 24 hours of treatment with 0, 0.1, 0.2, 0.5 and 1 µg/mL of Gen. 0 µg/mL Gen:  $\mu = 10.21$ ,  $\sigma = 0.29$ , 0.1 µg/mL Gen:  $\mu = 39.08$ ,  $\sigma = 0.55$ , 0.2 µg/mL Gen:  $\mu = 45.57$ ,  $\sigma = 1.35$ , 0.5 µg/mL Gen:  $\mu = 56.22$ ,  $\sigma = 0.50$ , 1 µg/mL Gen:  $\mu = 67.17$ ,  $\sigma = 0.99$ , T-Student analysis: 0 µg/mL vs. 0.1 µg/mL Gen: p-value =  $3.24e-07$ , 0 µg/mL vs. 0.2 µg/mL Gen: p-value =  $3.45e-06$ , 0 µg/mL vs. 0.5 µg/mL Gen: p-value =  $3.79e-08$ , 0 µg/mL vs. 1 µg/mL Gen: p-value =  $1.60e-07$ . Each condition was tested in triplicate.

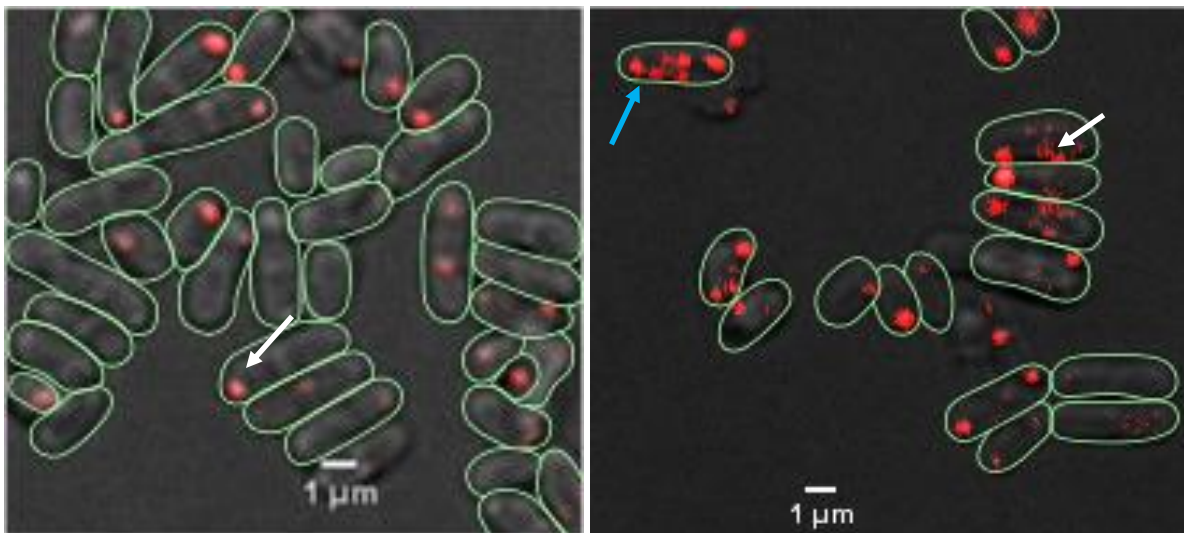
### C. 6 Complex recruitment of mScarlet1-NucSD144A after 24h of treatment with genotoxic antibiotics

It is important to note that after 24 hours of CIP treatment, many cells were dead and lysed, with visible aggregates forming among cellular debris. Despite the foci within these clusters, we did not analyse these formations further due to the complexity of observing their 3D organisation.

These observations highlight the severe impact of prolonged genotoxic stress during 24 hours. Despite these challenges, observing these clusters suggests that mScarlet1-NucSD144A molecules may remain associated with DNA even in non-viable cells. Foci counts were only performed on isolated cells in 2D on the agar pad. Except for the seemingly

resistant cells, which were round with centrally located foci, the signal of mScarlet1-NucSD144A foci in the remaining rod-shaped bacteria was consistently positioned at the poles, as illustrated in **Figure 42**.

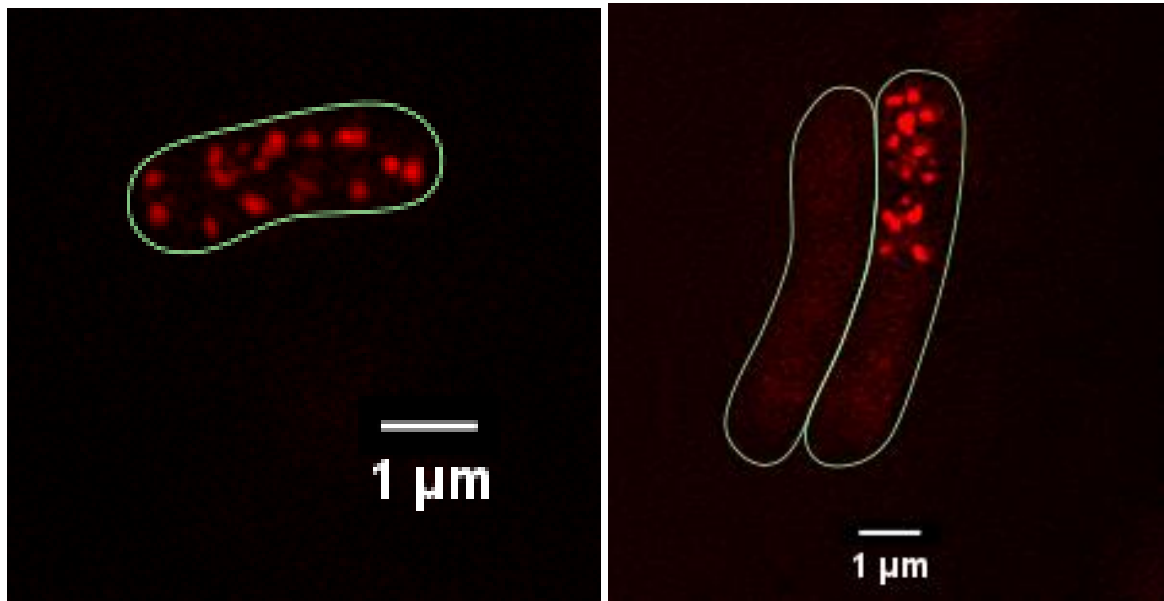
This polar localisation conserved at 24 hours suggests specialised regions within the cell where DNA repair actors are concentrated, possibly due to spatial constraints or the organisation of the bacterial chromosome. This distinct localisation pattern underscores the potential for polar regions to serve as hubs for critical DNA repair processes, reflecting an organised cellular response to DNA damage.



**Figure 42:** Confocal image of *Cg*/WT expressing the mScarlet1-NucSD144A construct after 24 hours of treatment with CIP (1 µg/mL). The white arrows indicate complex recruitment of the mScarlet1-NucSD144A that forms foci at the polar localisation of the cell; the blue arrows indicate complex structures in the cell's periphery.

As discussed in paragraph C.1 about mScarlet1-NucSD144A localisation on the bacterial chromosome, a small proportion of cells, around 1 %, exhibited complex fluorescent clusters organised peripherally within the cells. Multiple foci were even more apparent in bacterial populations treated with high concentrations of genotoxic antibiotics for 24 hours.

However, the limitations in resolution associated with using a confocal microscope do not allow for the precise resolution of the independence of these molecular complexes or, conversely, their binding. We replicated the 24-hour CIP treatment and observed these cells using a 3D SIM microscope.

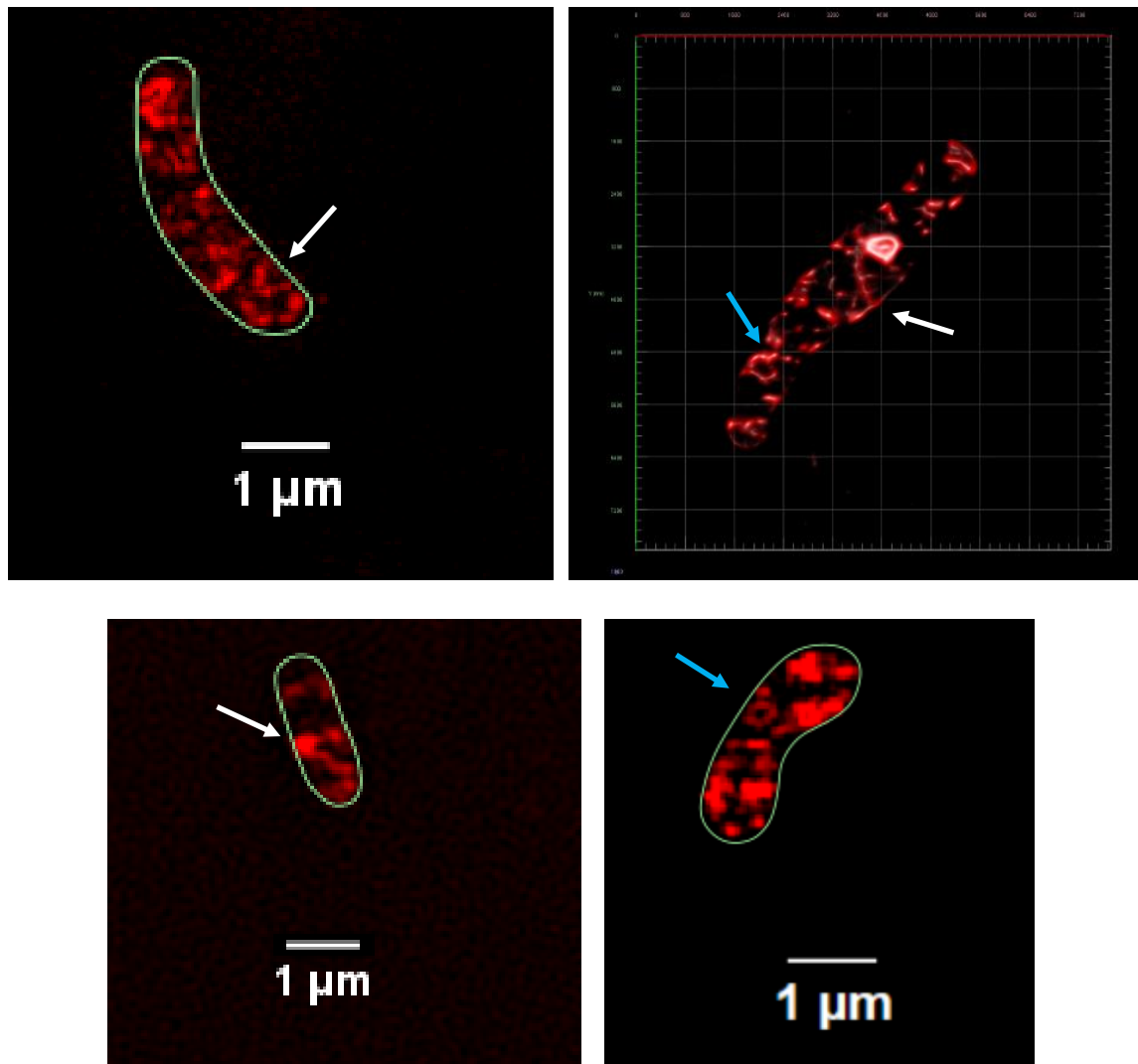


**Figure 43:** 3D SIM of *Cg/WT* expressing the mScarlet1-NucSD144A construct after 24 hours of treatment with CIP (1 µg/mL). Multiple foci distributed in the cytoplasm are visible.

In a portion of the cells identified with this specific distribution pattern of the mScarlet1-NucSD144A fusion, 3D SIM pictures enabled the differentiation of more than ten foci differentiable within the cell, organised around the periphery and in the cytoplasm of the bacteria. We also observed cells with division defects having foci grouped around one portion of the cell, suggesting issues with chromosomal segregation, see **Figure 43**.

In eukaryotes, similar phenotypes are seen when cells experience high levels of DNA damage during mitosis, leading to chromosomal fragments and missegregation (Novais-Cruz *et al.*, 2022; Fennech, 2000). In bacteria, improper segregation of damaged chromosomes might lead to asymmetric distribution of repair proteins, with mScarlet1-NucSD144A foci accumulating where the breaks are most concentrated.

Although these occurrences were marginal, these results suggest a physiological response of NucS to cell death and genome lysis in bacteria undergoing apoptosis-like processes (Bayles, 2014). This pattern of foci distribution suggests a role of NucS in managing DNA damage under extreme stress conditions, possibly by localising and stabilising broken DNA ends.

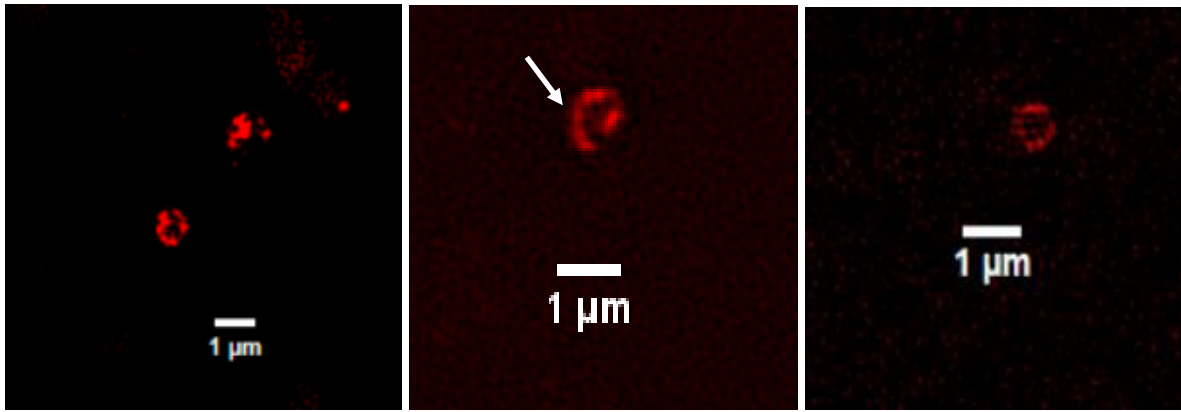


**Figure 45:** 3DSIM images of *Cg/WT* expressing the mScarlet1-NucSD144A construct after 24 hours of treatment with CIP (1  $\mu\text{g}/\text{mL}$ ). The white arrows indicate complex recruitment of the mScarlet1-NucSD144A that forms networks of filament-like structures in the cell; the blue arrows indicate ring-like structures observed in the cell.

More intriguingly, as observed in **Figure 45**, the enhanced resolution of images taken of cells with complex organisations of bundles formed by mScarlet1-NucSD144A, enabled by 3D SIM microscopy, allowed us to identify what appear to be filaments and networks of foci. These structures seem interconnected, providing new insights into the spatial organisation of mScarlet1-NucSD144A within cells under high genotoxic stress, drawing a parallel to the filaments of RecA-mVenus on DNA.

Similarly, the presence of ring-like structures, as pointed out by the blue arrows in **Figure 44** and further observed in **Figure 45**, suggests that NucS might play a role analogous to that of  $\gamma\text{-H2AX}$  in DNA repair and apoptosis. These ring-like structures were observed exclusively after prolonged treatments with genotoxic antibiotics.





**Figure 45:** 3DSIM images of ring-like filamentous assembling of mScarlet1-NucSD144A construct after 24 hours of treatment with CIP (1  $\mu\text{g}/\text{mL}$ ) indicated by the white arrows.

This observation aligns with phenomena described in eukaryotes during apoptosis, particularly the formation of nuclear  $\gamma\text{-H2AX}$  apoptotic rings. A notable structure associated with DNA damage response that is crucial for maintaining genomic integrity during the apoptotic process. The formation of such rings highlights the complex spatial organisation of proteins in response to DNA damage and cell death signals.

Despite the severe genotoxic stress, mScarlet1-NucSD144A molecules remained associated with DNA even in non-viable cells, indicating a persistent interaction with damaged DNA. Foci of mScarlet1-NucSD144A were observed predominantly at the poles of rod-shaped bacteria, suggesting these regions might serve as hubs for DNA repair processes due to spatial constraints or chromosomal organisation. This study used advanced 3D SIM microscopy to identify complex fluorescent clusters within a small proportion of cells, revealing more than ten distinct foci, sometimes organised in a network. The formation of these NucS filaments and ring-like structures reinforces the hypothesis of its role in DNA damage response.

These results provide novel perspectives on NucS's involvement in DNA repair and the structural reorganisation of cellular components during apoptosis. These findings support the hypothesis that NucS is involved in maintaining genomic stability under stressful conditions, warranting further investigation into its precise molecular interactions and functional implications.

## Discussion

In this section of our study, we aimed to investigate whether NucS has a role outside MMR in DNA repair pathways. We hypothesised that given its activity on Y-shaped and fork substrates, which are typical of HR intermediates and its double-strand cleavage activity, NucS could be especially questioned as an actor in DSBR.

To test this hypothesis, we used Gam-meGFP as a DSBs reporter system to visualise DSBs in *Cgl*WT and *Cgl* $\Delta$ *nucS* living bacteria. We completed this approach by designing a system reporter of the activation of the HR based on a fusion between RecA and mVenus observed in the same two genetic backgrounds. Additionally, for the first time, we observed NucS within the cell to gather information on its localisation and physiological response to DSBs in the bacterial genome.

These findings allowed us to identify NucS's role in processing and regulating DSBR without stress. On the one hand, upon visualisation of an increased number of Gam-meGFP foci in the *Cgl* $\Delta$ *nucS* bacterial population, our findings indicated an increase in the unrepaired DSBs of these cells. This phenomenon could be related to a longer persistence of the breaks in this genetic background, pointing to some imbalance of the DSBR absence of NucS.

Moreover, an increased number of RecA-mVenus foci was also detected in *Cgl* $\Delta$ *nucS*. These results further reinforce the hypothesis about NucS' involvement in DSBR related to HR, a pathway that NucS could inhibit or compete with. While it is known that RecA is essential for strand invasion and exchange during HR, its over-recruitment in the absence of NucS might indicate a role of NucS in regulating the extent of HR to prevent genomic instability.

In contrast to the results obtained with the RecA-mVenus1 construct, for which the number of foci decreases when the bacteria enter the stationary phase, reflecting a reduced replication of the haploid genome, the experiments with mScarlet1-NucSD144A showed a rise in the number of foci in this phase. This variation is similar to what was seen with the Gam-meGFP construct. These findings suggest that mScarlet1-NucSD144A responds physiologically to the stress induced by the stationary phase. This indicates that mScarlet1-NucSD144A recruitment is not dependent on multiple genome copies, as it is required for HR activation. Therefore, NucS might be involved in an alternative DNA repair pathway.

In light of these results, two hypotheses could be addressed: NucS could directly inhibit the HR, as demonstrated in the case of MutS/L, or NucS could bind to the free ends generated by the break and recruit an alternate DNA repair machinery. This function could modify the balance of the DSBR by favouring another pathway other than HR.

Following these statements, our results suggest that NucS could favour a more time-effective DSBR pathway than HR. This could explain the more significant number of Gam-meGFP foci observed in the *CglΔnucS* bacterial population. The MMEJ is the pathway we consider more consistent with this hypothesis since specific genes essential for the NHEJ are not present in the genome of *C. glutamicum*.

Moreover, competition essays between *Cgl*/WT and *CglΔnucS* genetic backgrounds showed a competitive advantage of the *CglΔnucS* bacteria over the *Cgl*/WT in the presence of genotoxic stress for 6 and 24 hours. We hypothesise that NucS intervenes in an alternative system of DSBR like the MMEJ and induces an anti-recombination phenomenon. Hence, this advantage in the *CglΔnucS* could be due to the repair of the genome in a less mutagenic way since the HR is a remarkably accurate DNAR mechanism. We do not exclude that in *CglΔnucS* bacteria, the metabolism and energy allocation in the presence of a genotoxic agent could also be modified.

We have also explored in this chapter how mScarlet1-NucSD144A is recruited into the cell upon the occurrence of different events. First, our results show that mScarlet1-NucSD144A has a dual localisation: its signal can be sensed diffused in the cytosol or aggregated in polar foci. The visualisation of our samples with a 3D SIM microscope enabled us to detect NucS that remained bound to DNA during cell death. Additionally, we were surprised that multiple foci could be formed within a cell under heavy stress, as in the 24-hour incubation of the bacteria with high concentrations of genotoxic antibiotics. Some intriguing structures resembling filaments and even rings could be identified from images of apoptosis-like bacteria.

The results from our study suggest NucS's role in DSBR, which is marked by its recruitment at the poles of the bacteria, specifically in response to DNA-damaging agents. Its behaviour was compared with well-known DNA repair proteins, such as MutS/L and RecA. While MutS/L proteins have been shown to contact DNA transiently, NucS forms more static foci resembling RecA's filamentous structures during HR (Chen *et al.*, 2008). These results indicate the spatially and temporally regulated recruitment of NucS at specialised repair centres at the pole.

The gradually increasing concentrations of CIP, Phleo and MMC induced dose-dependent formation of mScarlet1-NucSD144A foci within 30 minutes after treatment, indicating that the recruitment of NucS is proportional to the levels of DNA damage. This dynamic interaction is reminiscent of the situation observed with eukaryotic DNA repair pathways, in which multiple proteins are differentially recruited depending on the timing and context of damage and repair needed. This could be interpreted as representative of proteins involved in the early recognition and response to DNA breaks. Similar to Ku or Rad51, which are among the first actors recruited to DSBs to stabilise and process broken DNA ends. Parallels could also be drawn with eukaryotic proteins like  $\gamma$ H2AX and P53BP1 which form multiple foci in response to extensive DNA damage, often marking multiple DSBs (Pignataro *et al.*, 2017; Otsuka *et al.*, 2018). These foci increase with the severity of DNA damage and have been associated with various DNA repair systems, such as the BER or the HR (Galick *et al.*, 2017; Tsvetkova *et al.*, 2017).

We found that in the literature, Rad51 foci formation following DSB induction parallels NucS foci formation, indicating that NucS could act in a similar repair pathway and might represent an initial sensor for damage (Harper *et al.*, 2010). Rad51 is essential for HR; it creates nucleoprotein filaments at sites of DNA damage. While that might not be the case, NucS foci could still form as an early step in the DNA damage response pathway, stabilising breaks specifically for subsequent repair processes. Compared with the more general damage recognition spectrum of the UvrABC system, the role of NucS seems quite specific to DSBR. This sets a basis for the potential of NucS acting in specific damage detection and repair mechanisms. Like the Ku protein, NucS could participate in DSBR by recognising, stabilising and processing breaks early in the repair process. While Ku proteins directly facilitate the NHEJ, NucS might facilitate MMEJ.

NucS likely plays a pivotal role in balancing the activation of HR and alternative DSBR pathways like the MMEJ. In the process, NucS might modulate the accessibility of free ends for these repair pathways to favour one pathway over the other, ensuring efficient and correct processing that avoids excess HR events for genomic stability. By drawing parallels between NucS and eukaryotic DNA repair proteins, these findings offer new insights into bacterial DNA repair strategies and their regulatory mechanisms. Further research will be essential to delineate the exact molecular interactions and recruitment mechanisms of NucS, potentially revealing novel aspects of DNA repair pathways in bacteria.

## Material and Methods

### Bacterial strains, plasmids and growth

The bacterial strains and plasmids used in this study are listed in **Table 3**. To ensure the proper folding of fused proteins, we selected and designed flexible linkers from the literature known for their efficacy in functional fusion between FPs and other proteins for Gam-meGFP GPGLSGLGGGGGSLG for RecA-mVenus GGCCCAGGCCTGTCCGGCCTGGGCGGCG GCGGCGGCTCCCTGGGC for mScarlet1-NucSD144A,GGCGGCGGCGGCTCCGGCGG CGGCGCGGCTCC. Constructs with flexible linkers were inserted into the plasmid pGKJE8, specific for gene expression in *C. glutamicum*. This plasmid contains a Chloramphenicol resistance gene and an origin of replication. FPs were optimised based on codon prevalence for *C. glutamicum* and checked for termination codons and restriction sites (Chen *et al.*, 2004; Kidane *et al.*, 2012; Serrano *et al.*, 2018; Carrasco *et al.*, 2019). Unless otherwise stated, cells were grown at 30 °C in a BHI medium before imaging. Supplements were used at the following concentrations: Ampicillin (Amp) 100 µg/mL, Chloramphenicol (Cm) 20 µg/mL, Kn 40 µg/mL.

### Construction of NucS D144A Mutant

The NucS D144A mutant was constructed using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). The mutation was introduced with the oligonucleotide SK171 and the plasmid pHM449 as a template. The amplification product, treated with the DpnI enzyme, was introduced by transformation into competent XL 10-Gold strain cells following the manufacturer's instructions. Bacteria that recovered the plasmid were selected on an LB agar medium supplemented with ampicillin at 100 µg/mL. Six transformants were isolated on the same medium and the plasmids were purified and verified by sequencing with the pQE80-F/pQE80-R primer pair. A plasmid carrying the desired mutation was named pHM483.

### Transformation and preparation of competent cells

Two transformation methods were tested: heat shock and electroporation, with electroporation yielding the best results. A 20 mL pre-culture (LB for *E. coli*, BHI for *C. glutamicum*) was incubated overnight at 37 °C (for *E. coli*) or 30 °C (for *C. glutamicum*) with agitation at 180 rpm. The next day, 1 litre of LB or BHI was inoculated to an OD600 of 0.01 and incubated with agitation until an OD600 of 0.5-0.6 for *E. coli* or 1.2-1.5 for *C. glutamicum*. The cells were cooled to 4 °C for at least 15 minutes and centrifuged four

times for 15 minutes at 5000 g at 4 °C, with the first three washes in cold Milli-Q water and the last one in 24 mL of cold 10 % glycerol. Cells were collected by centrifugation at 4000 x g for 20 minutes, washed once with 500 mL of chilled 10 % glycerol and thrice with 100 mL of chilled 10 % glycerol. Cell density was adjusted to an OD600 of 20 before electroporation.

### Electroporation of DNA

Approximately 10ng of DNA was mixed with 100 µL of electrocompetent cells in a 1 mm electroporation cuvette (Genesee Scientific). The cells were then electroporated at 2.5 kV using a MicroPulser electroporator (Bio-Rad). The cells were recovered in 2 mL of BHI or LB for 1 hour with *E. coli* or 2 hours for *C. glutamicum* at 30 °C and 150 rpm before plating. The cells were then grown at 30 °C for 2 to 3 days. Before microscopy observation, bacteria were cultured in BHI for an overnight pre-culture, then diluted to an OD600 of 0.05 and incubated for 5 hours at 30 °C until reaching the exponential growth phase. At this stage, appropriate concentrations of antibiotics were added. The cells were then reincubated for 30 minutes with the antibiotic before being deposited on the agar pad. For 24-hour observations, the same protocol was followed and cells were imaged 24 hours post-antibiotic treatment. Specifically for Rif, cells were imaged 3 hours after its introduction into the culture medium.

Antibiotic	Range of antibiotic concentration
Ciprofloxacin	1 µg/mL 0,5 µg/mL 0,2 µg/mL 0,1 µg/mL 0,05 µg/mL
Phleomycin	10 µg/mL 5 µg/mL 2 µg/mL 1 µg/mL
Mitomycin C	0,5 µg/mL 0,2 µg/mL 0,1 µg/mL
Gentamicin	1 µg/mL 0,5 µg/mL 0,2 µg/mL 0,1 µg/mL
Streptomycin	2 µg/mL 0,5 µg/mL
Rifampicin	0,5 µg/mL 0,2 µg/mL 0,1 µg/mL
Ampicillin	2 µg/mL

**Table 3:** Range of concentration for the antibiotics used for the foci induction tests; values in blue were chosen for treating the bacteria prior to the microscopy picture shared.

### Live-cell microscopy experiments

For live-cell microscopy experiments, the overnight culture of cells was diluted to an OD600 of 0.01 and grown further in shaking tubes at 30 °C to an OD600 of 0.5. Cell cultures were treated as described before imaging. Samples for microscopy were collected at indicated time

points. 2  $\mu$ L of culture was spotted on a 5 % agar pad and imaged on a confocal microscope in an environment heated to 30 °C.

### **Image acquisition**

Conventional wide-field fluorescence microscopy imaging was carried out on a TCS SP8 confocal microscope (Leica) equipped with an environmental chamber (Life Imaging Services) at 30 °C using an HC PL APO 60x/ 1.40 Oil W CS2 objective (Leica). Additionally, super-resolution imaging was achieved using two different 3D Structured Illumination Microscopes (3D SIM): one from Zeiss Elyra and a ZEISS Lattice SIM 5 with a Plan-Apochromat 63x/1.40 Oil DIC and an sCMOS camera (Hamamatsu ORCA-Fusion BT) that permitted the 3D reconstructions presented.

### **Image analysis**

Microscopy images were opened and visualised using the open-source software ImageJ/Fiji. Quantitative image analysis was performed using ImageJ/Fiji and the free MicrobeJ plugin. Omnipose was used to detect the number of bacteria. The foci formed by mScarlet1-NucSD144A, Gam-meGFP and RecA-mVenus were assessed using the Editing tool of the Feature detection of the MicrobeJ plugin. All data presented are cumulative from at least three independent experiments, each yielding similar results. Statistical analyses were performed in Python.

### **Preparation and use of agar pads**

Five microscope slides were stacked on a levelled surface to prepare agar pads. The molten agar solution was poured onto the slides and mixed with BHI and Noble Agar (5 %). The mixture was covered with another slide, weighted down and solidified for 30 minutes at room temperature. Excess agar was removed with a razor blade, leaving the top and bottom slides for easy storage and handling. A 1  $\times$  1 cm agar pad was excised with a razor blade for imaging. A 2  $\mu$ L bacterial suspension (OD600 0.5-1) was pipetted onto the centre of a 24  $\times$  50 mm coverslip. The agar pad was then gently placed on top of the cell suspension droplet using a razor blade and the pad was covered with a 22  $\times$  22 mm coverslip.

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**Chapter 3: Bioinformatic Prediction of NucS Activity, Partners and  
Phylogenetic History**



## Abstract

In this study, bioinformatics analyses were conducted to complement our experiments on living cells. Modern bioinformatics analyses provide structural protein databases, enabling researchers to determine phylogenetic relationships and predict enzymatic function on different substrates and interactions between proteins. This provides deeper insights into mechanisms, interactions and evolutionary pathways. Following our previous analysis, we also hypothesised in this section that NucS is involved in DSBR.

### 1. Molecular Dynamics Simulations:

- **NucS Interaction with DNA:** We utilised CHARMM to simulate NucS interactions with different DNA substrates. Our results suggest that NucS could stabilise DNA at DSB sites and prevent extremities' degradation. These simulations suggest that NucS could compete with HR proteins for the free extremities generated at the site of a DSB and favour alternative repair pathways such as MMEJ.

### 2. Interaction Predictions:

- **Protein-Protein Interactions:** Studies of the interactome of NucS in bacteria and archaea by the STRING database and in vitro pull-down assays revealed several potential interactions with helicases and DNA repair proteins, including RadA, RecA, Mre11, FEN1, ERCC4/XPF. These interactions suggest NucS's involvement in multiple DNA repair pathways.
- **Gene Neighborhood Analysis:** Genomic neighbourhood analysis of NucS in different organisms identified genes involved in DNA repair, metabolic functions and energy production that support NucS's multifaceted role in genomic stability.

### 3. Phylogenetic Analysis:

- **Evolutionary Origin:** Phylogenetic trees suggested an archaeal origin for NucS, with potential horizontal gene transfer to extremophile Actinobacteria.

NucS seems to play a role in DNA repair, particularly in stabilising DSBs and regulating repair pathway activation. Together, bioinformatic predictions and simulations, supported by interaction and gene neighbourhood analyses, indicate that NucS could favour MMEJ over HR.

## Introduction

In addition to the biochemical and living-cell analyses conducted to elucidate the physiological role of NucS in bacterial cells, we aimed to enhance our findings' robustness through bioinformatics tools.

Traditionally, the classification of living organisms has relied on their morphological characteristics, as demonstrated by Darwin's work on evolutionary relationships inferred from common ancestors preserved in fossil records (Woese *et al.*, 2006). However, this method falters in cases of convergent evolution and has been unable to define the evolutionary proximity of organisms. For example, it cannot determine whether the fungi are evolutionary relative to plants or animals nor classify prokaryotes. Thus, microbiologists have attempted to classify prokaryotes based on biochemical and nutritional criteria. Still, this method has limitations due to the diversity in biochemical behaviours that exist for these organisms.

Modern bioinformatic analyses have demonstrated that protein structures within a family are often more conserved than their amino acid sequences. Under evolutionary pressure, these DNA sequences may diverge significantly, necessitating comparisons of protein folding and 3D conformations to determine familial relationships. Research has shown that proteins with over 24 % sequence identity frequently share a common ancestor and exhibit similar overall structures (Chothia *et al.*, 1986; Jones *et al.*, 2012; Moulton *et al.*, 2016; Senior *et al.*, 2020). Remarkably, despite only 24 % amino acid sequence identity, the backbone atoms in protein domains can maintain a common fold with high precision. This structural similarity is often measured by the root-mean-square deviation (RMSD) of backbone atom positions and typically falls within 0.2 nanometers (2 angstroms), underscoring the robustness of structural motifs across different proteins (Moulton, 2004; Levitt *et al.*, 1976).

In bioinformatics, dendrograms visualise the classification of proteins into families based on sequence and structural similarities. These trees represent the degree of similarity between proteins, with greater distances indicating higher divergence. Beyond the folding and conservation of the total protein sequence, bioinformatics analyses can focus on the functional sites of the protein. Sites interacting with other molecules, such as proteins, RNA, or DNA, will most likely be conserved over time (Woese, 1987; Sali *et al.*, 2015).

Since the early 2000s, advancements in techniques such as crystallography, cryo-EM and insights into protein domain interactions have enabled the prediction of molecular interactions. This interdisciplinary field, at the intersection of biology, chemistry, computer science and physics, allows researchers to establish connections that might be overlooked through experimental results alone, thereby soliciting new perspectives and hypotheses (Jones *et al.*, 1992; Sali *et al.*, 1993; Chothia *et al.*, 2006; Sali *et al.*, 2015; Jumper *et al.*, 2021). These techniques provide valuable information on the conformations and 3D shapes of up to 100,000 proteins. A limited number of possible folds associated with these sequences have been identified by combining analyses of amino acid sequences and their folding into secondary and tertiary structures. This has paved the way for predictive protein folding and 3D modelling tools, such as AlphaFold and CHARMM (Brooks *et al.*, 1983; Jumper *et al.*, 2021; Pereira-Leal *et al.*, 2008).

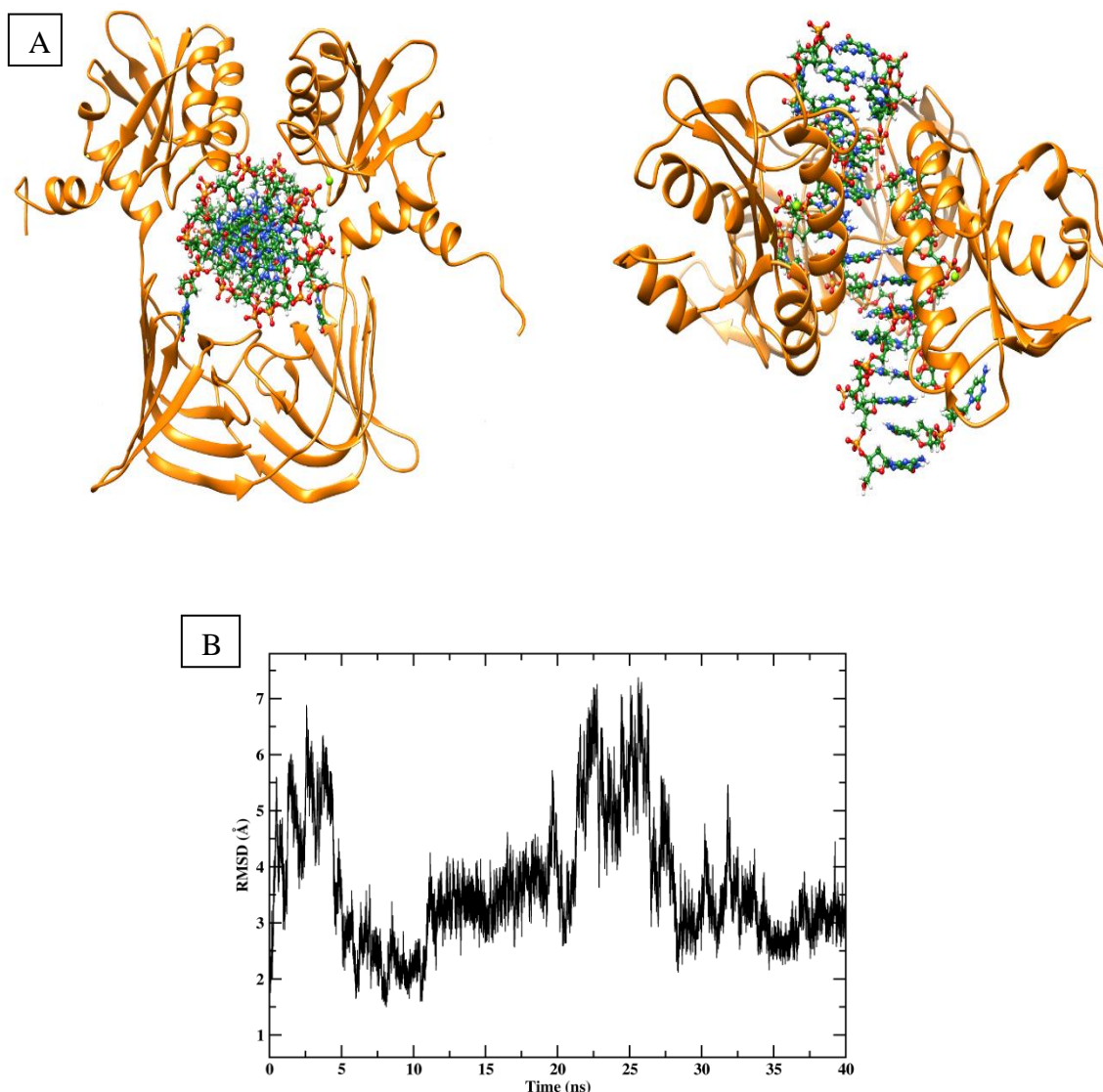
Comparing similar protein structures or proteins within the same families allows for formulating hypotheses about their related functions. This approach can save experimental time by refining the initial approach before starting time-consuming experiments. It identifies previously resolved proteins with similar amino acid sequences or structures and draws parallel (Moult *et al.*, 1999; Zhang *et al.*, 2005). Genome analysis offers a straightforward, direct and robust method to determine evolutionary relationships and partnerships between proteins. The differences between DNA sequences provide a quantitative measure of evolutionary distance (Doolittle, 1999; Eisen *et al.*, 2010). Additionally, analysing the genomic neighbourhood of a gene of interest can indicate co-regulated genes involved in parallel or partner pathways.

By leveraging these bioinformatic tools and techniques, we aimed to gain deeper insights into NucS's role and interactions, thereby providing a more comprehensive understanding of its function in bacterial cells.

## Results

### A. Bioinformatical simulation of NucS bound onto various damaged DNA substrates

To understand more precisely the activity of NucS on DNA, we utilised the software CHARMM to model and predict the interaction of *Mtb*NucS and several DNA structures. Our initial predictions involved a 15-nucleotide base pair sequence of DNA containing a T-G mismatch at the 8<sup>th</sup> position.

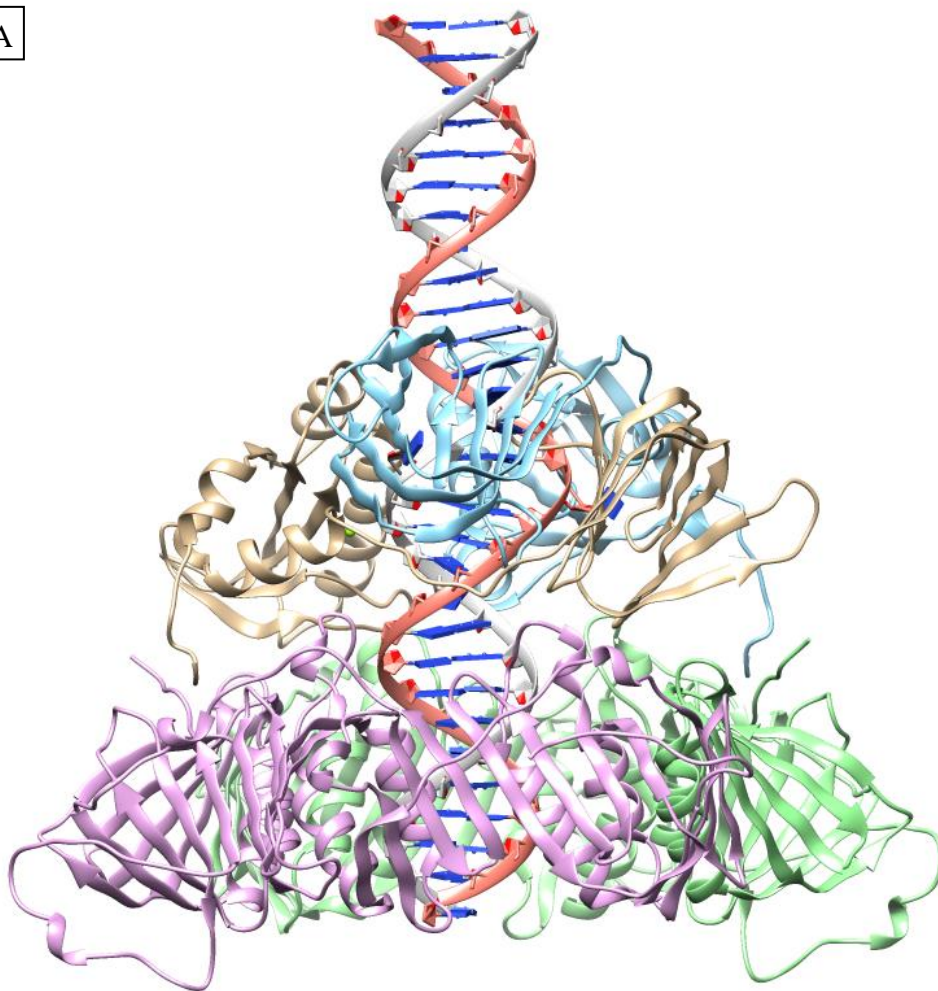


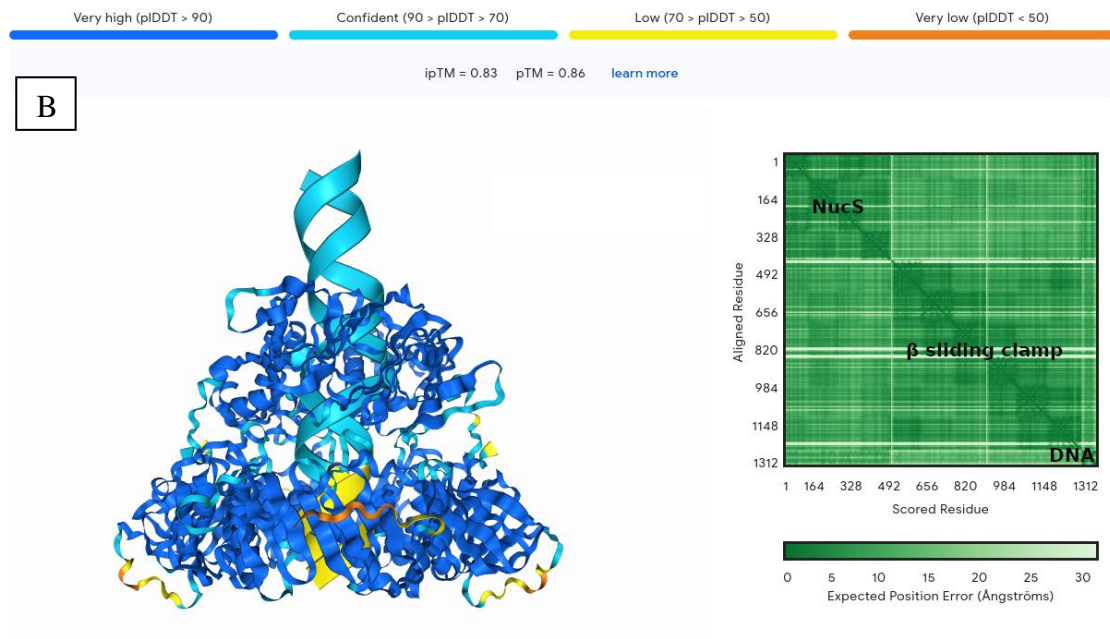
**Figure 46:** A. 3D representation of *Mtb*NucS bound to a Mismatch generated with CHARMM. B. RMSD calculation. The RMSD is calculated by optimally superimposing the simulated structure onto the structure at  $t=0$ , focusing on the alpha-carbon atoms of amino acids 1 to 202 from the NucS polypeptide chains. The C-terminal region (residues 203 to 223) and the DNA are excluded from the RMSD calculation due to their high flexibility.

For this simulation, we utilised the shared 3D structure predicted of NucS in orange and a DNA sequence containing a mismatch, in blue, available on AlphaFold. As illustrated in **Figure 46**, we observed the destabilisation of one nucleotide, six bases after the mismatch, within the first picosecond of the prediction. This finding led us to investigate whether any specific sequence motifs or structural features in the DNA sequence could have led to this destabilisation. However, our analysis revealed no repetitive sequences or secondary structures that could have contributed to the nucleotide destabilisation.

It appears that *Mtb*NucS induces a conformational change in DNA containing a mismatch, such as twisting or unwinding the DNA helix. These structural changes can locally disrupt DNA stability, weakening the hydrogen bonds between nucleotides. In our scenario, a nucleotide adjacent to the NucS binding site may become energetically unfavourable and detach. This detachment prompts the DNA structure to reorganise to accommodate the changes caused by the protein binding.

A

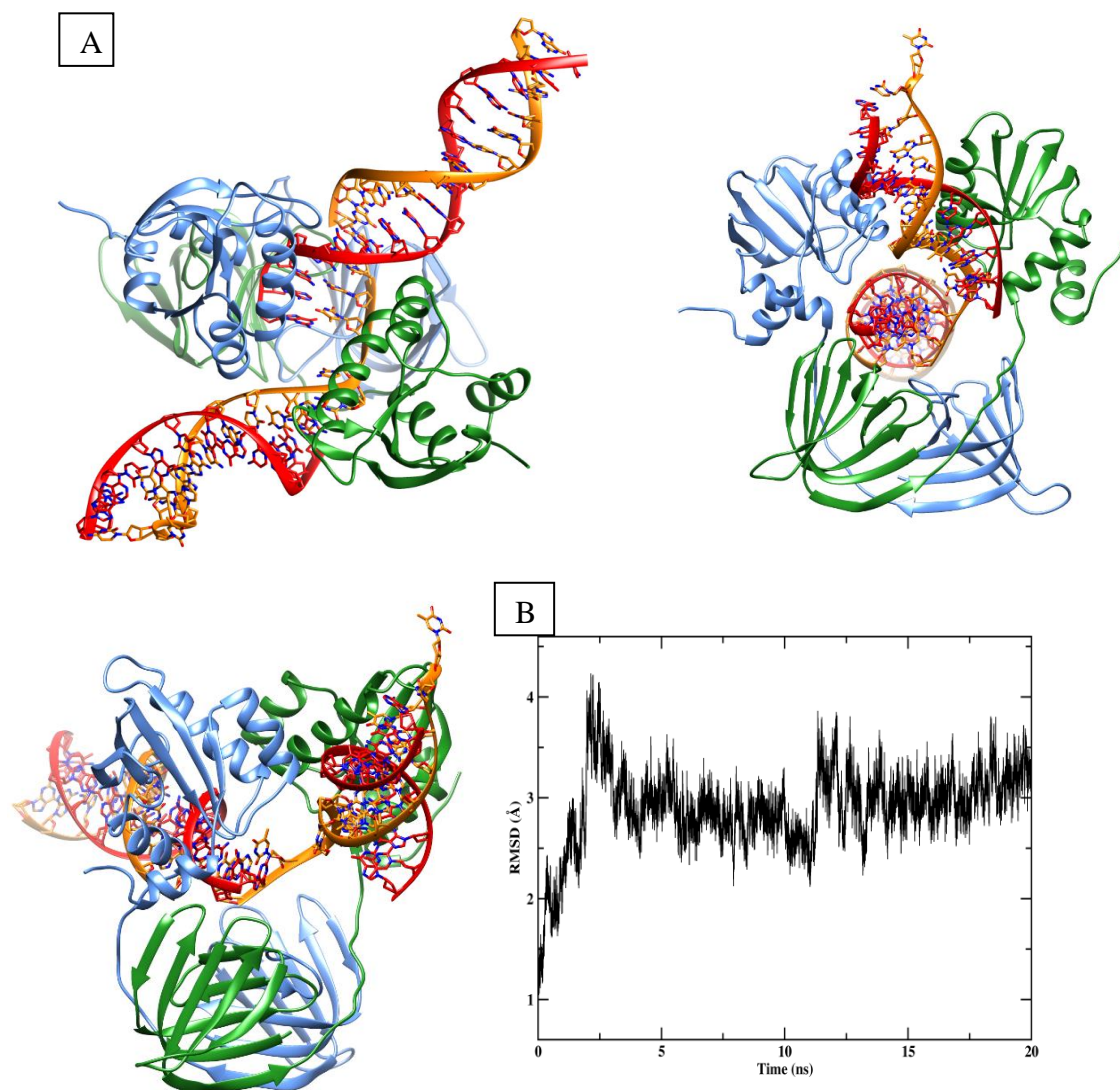




**Figure 47: A.** Simulation on AlphaFold of the interaction of NucS with PCNA loaded on a DNA substrate. **B.** The prediction obtained using AlphaFold3 reveals a structure predominantly colored in blue or cyan, indicating a high level of confidence in the predictive calculations performed by the algorithm.

During our simulations, we also sought to test the impact of the interaction between NucS and DnaN on the processivity of NucS on DNA and its activity on mismatches. Initially, we were able to confirm the presence of an interaction site between NucS and DnaN, as shown in **Figure 47**. Subsequently, the analysis of the simulation allows us to hypothesize that the binding of DnaN and NucS to DNA seems to modify its double helix structure. The constraints imposed by the two molecules on the DNA could lead to the formation of flatter zones that might facilitate their movement along the DNA. This result synergizes with the activity report of NucS in the presence of DnaN, biochemical tests revealing an improvement in the processivity of NucS in the presence of DnaN, a phenomenon that could be due to this facilitated movement on the DNA.





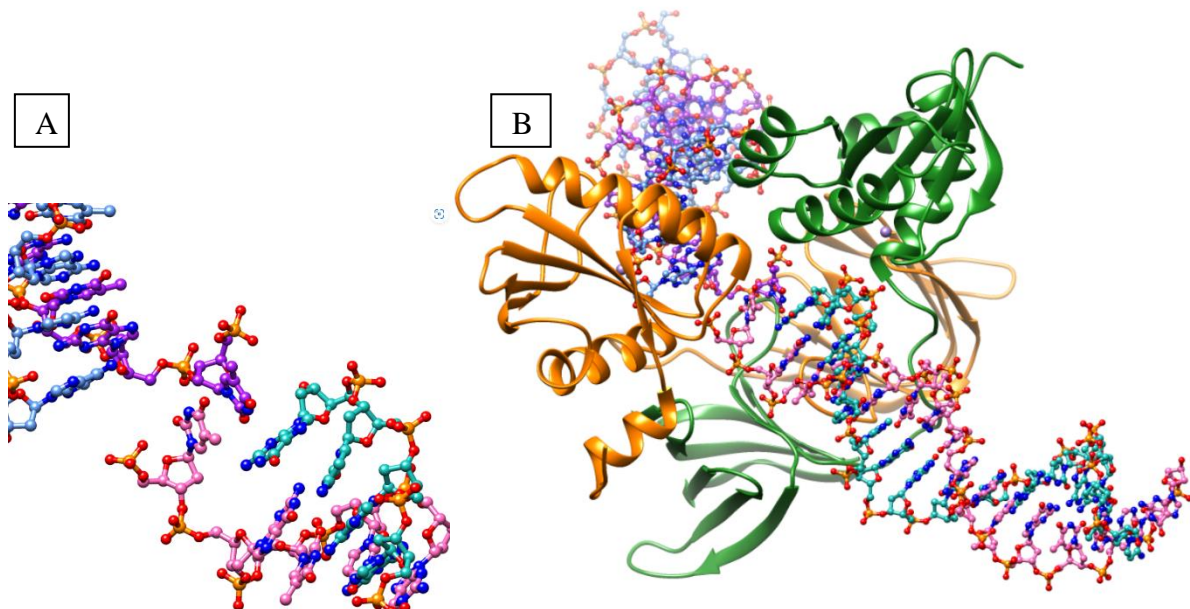
**Figure 48:** **A.** 3D representation of *MtbNucS* loaded onto DNA featuring two cleavages with CHARMM. **B.** RMSD calculation The RMSD is calculated by optimally superimposing the simulated structure onto the structure at  $t=0$   $t=0$ , focusing on the alpha-carbon atoms of amino acids 1 to 202 from the NucS polypeptide chains. The C-terminal region (residues 203 to 223) and the DNA are excluded from the RMSD calculation due to their high flexibility.

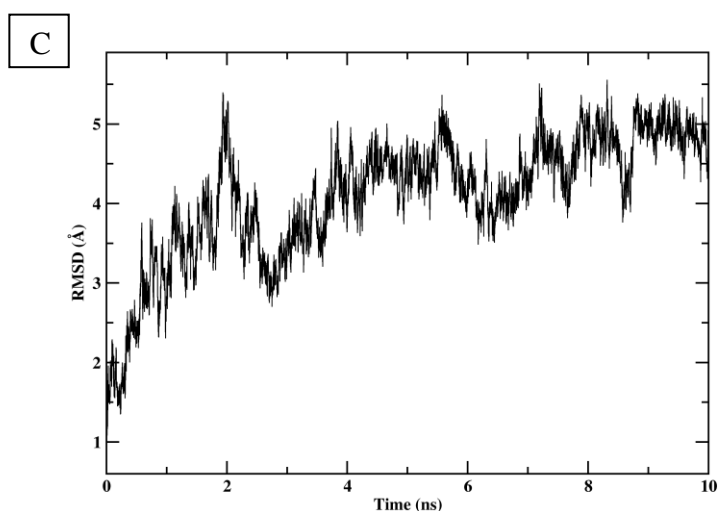
In synergy with experimental observations suggesting NucS's involvement in DSBR, we further examined the behaviour of this enzyme on a substrate presenting a DSB with 5' protruding ends.

In the upper panel of **Figure 48**, we observe the interaction between NucS and a DNA substrate featuring a DSB. The DNA is depicted in a helical structure with a break in red and orange and NucS in blue and green is bound to this region. The break in the DNA is indicated, with two separated strands and NucS loading seems to be stabilising these ends.

The leftmost panel of **Figure 48** shows a more complex interaction observed after a 128ps prediction. A portion of the DNA appears to be stabilised at the NucS binding site, highlighted by the absence of significant movement in the DNA backbone. The other portion of the DNA presents a twist in the DNA backbone at almost a 90° angle, even if the DNA structure retains its integrity. This twist indicates a conformational change of the DNA induced by NucS, which could be critical for its role in DNA repair processes. The rightmost panel provides a 2<sup>nd</sup> view of this prediction at 128 ps. NucS appears to clamp onto the DNA, providing structural support that prevents the typical destabilisation seen with broken DNA strands. The stabilisation suggests that NucS binding might prevent further unwinding or separating of the DNA strands, a crucial step in the initial stages of DSBR, as the availability of the free ends is fundamental for HR.

The figures collectively demonstrate the potential stabilising function of NucS in DNA repair, particularly at sites of DSBs. The visualisations indicate that NucS binds to damaged DNA and induces conformational changes to stabilise the DNA structure. This stabilisation could be critical in preventing further degradation and maintaining the DNA's integrity for repair. These bioinformatic predictions provide a foundation for understanding the molecular mechanics of NucS in DNA repair pathways and suggest avenues for further experimental validation.





**Figure 49:** **A.** Molecular structure of the DNA containing a DSB and a mismatch after predicting the fixation of *MtbNucS*. **B.** 3D representation of *MtbNucS* bound to a DSB containing a mismatch generated with CHARMM. **C.** RMSD calculation. The RMSD is calculated by optimally superimposing the simulated structure onto the structure at  $t=0$ , focusing on the alpha-carbon atoms of amino acids 1 to 202 from the NucS polypeptide chains. The C-terminal region (residues 203 to 223) and the DNA are excluded from the RMSD calculation due to their high flexibility.

Encouraged by these results, we hypothesised that NucS could be involved in a DSB repair pathway, such as MMEJ. To test this hypothesis, we modified the DNA substrate to include a mismatch in the complementary sequences of the protruding ends. As represented in **Figure 49**, we observed the binding interface of NucS with the DNA. This enzyme seems to make several contacts with the DNA backbone and bases, indicating potential interaction sites by which the binding of NucS in its closed form could stabilise the DNA structure, with minimal movement observed during the simulation. These results reinforce our previous observations, indicating that NucS stabilises free ends generated by a DSB, preventing their availability for other enzymes like RecA and holding them nearby in space. By stabilising these ends, NucS. As shown in **Annexe 4**, preliminary tests with purified *CgNucS*, *CgPCNA*, and looped DNA substrates suggest NucS activity on this substrate, aligning with previous hypotheses, though further validation is needed.

In conclusion, the bioinformatic predictions and molecular dynamics simulations presented here provide a deeper insight into the potential roles of NucS in DNA repair mechanisms. Our findings suggest that NucS interacts with mismatched DNA and plays a crucial role in the early stabilisation of DSBs, thereby influencing the DNA repair pathways in bacterial cells. Further validations are necessary to confirm these predictions and to elucidate the detailed mechanisms of NucS's involvement in DNA repair.

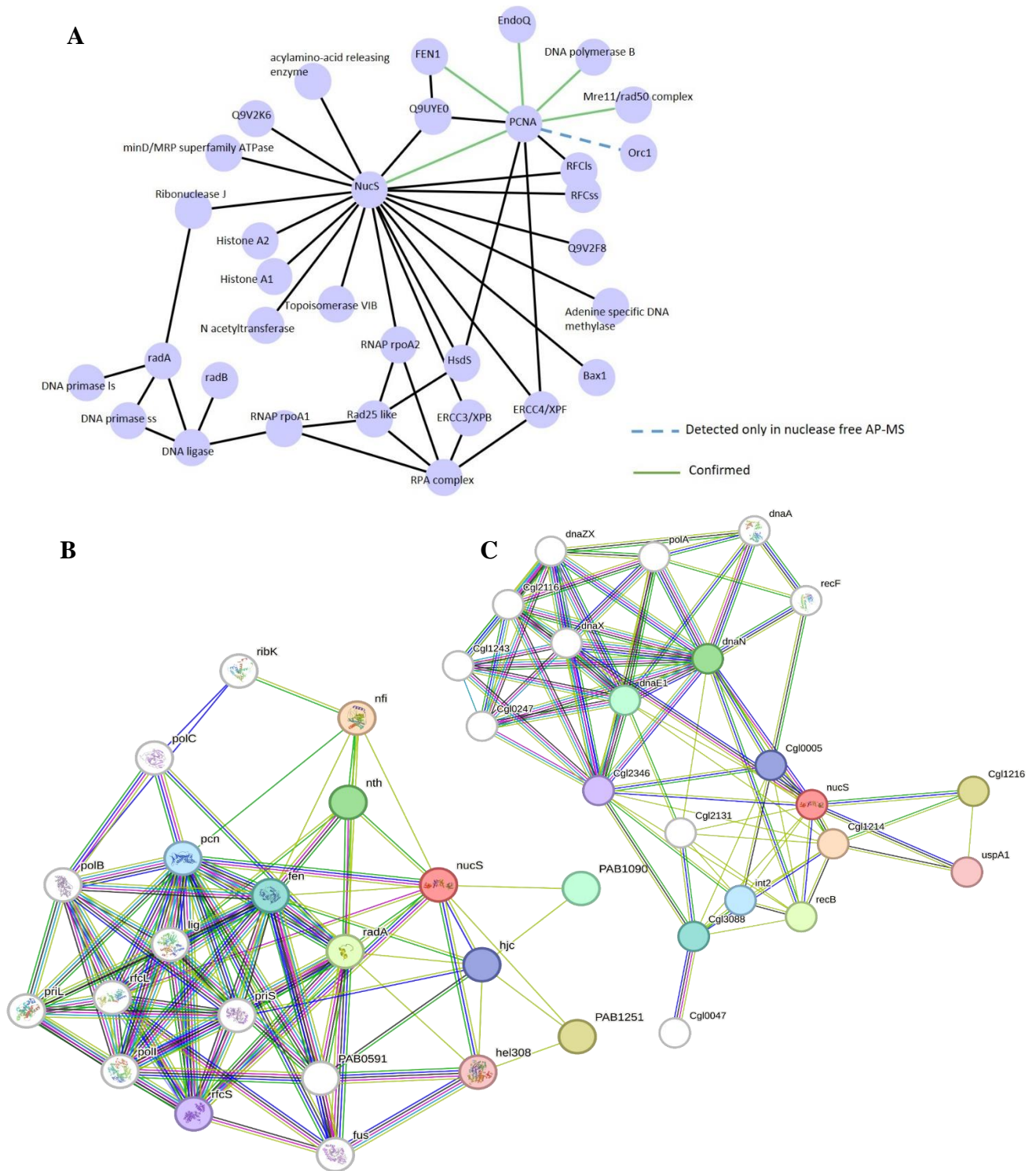
## B. Partners prediction of NucS/EndoMS

Although pull-down assays have been established using the *Cgl*/NucS protein, the literature has reported only a few candidates interacting with NucS. The beta-clamp has been identified for facilitating NucS activity with mismatched DNA in bacteria. This interaction has led to hypotheses suggesting a direct relationship between NucS and the DNA replication process, where NucS may be loaded onto the DNA following the beta-clamp's action. This potential mechanistic link underscores the importance of beta-clamp in directing repair proteins to DNA synthesis and repair sites.

More partner candidates have been identified in archaea, including XPF, PCNA and RFCs. These proteins are involved in replication and various DNA repair pathways. XPF, a structure-specific endonuclease, plays a role in NER, PCNA and RFCs are both crucial for DNA replication and repair, indicating a potential multifaceted role for NucS in maintaining genomic integrity. Several bioinformatics tools can predict protein-protein interactions, STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) being a notable example. STRING compiles known interactions from various sources, including experimental data, curated databases and predicted interactions based on gene neighbourhood, gene fusions and gene co-occurrence. These data are sourced from experimental repositories (such as BioGRID, DIP and MINT), scientific literature text mining, computational prediction methods and pathway databases like KEGG and Reactome.

STRING also incorporates systematic co-expression and co-occurrence analyses. By integrating data on both direct interaction predictions (physical contacts) and indirect interactions (functional associations within protein clusters), STRING provides interactive graphical representations of protein networks. These visualisations allow researchers to explore the complex web of protein interactions, with each interaction assigned a confidence score based on the co-occurrence of events across different data sources.

To deepen our understanding of NucS's potential indirect partners and gain a clearer view of the mechanisms in which this enzyme might be involved, we integrated high-confidence interaction candidates for NucS into these graphical representations. This virtual analysis of the NucS interactome in *C. glutamicum* and *P. abyssi* highlighted several intriguing candidates. Additionally, we conducted in vitro pull-down assays to investigate NucS/EndoMS protein-protein interactions in *P. abyssi*, revealing a list of promising candidates and their associated functional hypotheses, as in **Figure 50**.



**Figure 50:** A Protein-Protein Interaction Network for NucS with other significant genes and their gene products generated by the STRING database. **A.** Systematic in vitro pulldown analyses of archaeal protein-protein interactions network analyses of NucS in *P. abyssi* **B.** Protein-Protein Interaction Network for NucS from STRING in *P. abyssi*, **C.** Protein-Protein Interaction Network for NucS from STRING in *C. glutamicum*. The network nodes represent proteins (all the proteins produced by a single protein-coding gene locus) and edges represent protein-protein associations - known (from curated databases and experimental data), predicted (gene neighbourhood, gene fusions, gene co-occurrence) and others (like text mining, co-expression, protein homology).

Our study identified several helicases interacting with NucS from both *P. abyssi* virtual pulldown data and *C. glutamicum*. These helicases include Rad24-like (XPD/ERCC2), ERCC3/XPB, Hel308, RecF and DnaA. Understanding the functions of these helicases and their potential interaction with NucS provides valuable insights into the cellular mechanisms involving DNA repair and replication, as summarised in **Table 4**.

The interaction with these helicases indicates that NucS might participate in DNA repair processes. For example, Rad24-like (XPD/ERCC2) and ERCC3/XPB are involved in NER, a critical process for removing bulky DNA lesions. These interactions suggest that NucS may play a role in coordinating complex repair processes.

Helicase	Function	Archaea/ Bacteria
Rad25-like (XPD/ERCC2)	Part of the TFIIH complex, crucial in NER, for UV-induced DNA damage.	Archaea
ERCC3/XPB	Another helicase in TFIIH aids in unwinding DNA at damaged sites, crucial for NER.	Archaea
Hel308	Involved in DNA repair and HR, unwinds DNA strands to facilitate repair processes.	Archaea (Bacteria)
RecF	Part of the RecF pathway, essential for processing DNA gaps and DSBs.	Bacteria
DnaA	Involved in initiating DNA replication, unwinds DNA at the origin of replication.	Bacteria

**Table 4:** List of the helicases and their function in predictions of the interactome of NucS in *P. abyssi* or *C. glutamicum*.

Furthermore, Hel308 is involved in DNA repair and HR, specifically unwinding DNA strands to facilitate repair. This, along with RecF's involvement in processing DNA gaps and DSBs, suggests that NucS might participate in DSBR. RecF's interaction with proteins like RecO and RecR, which help load RecA onto ssDNA gaps, supports the idea that NucS might be involved in early DNA damage recognition and repair stages. The interaction with DnaA, primarily involved in initiating DNA replication, suggests a possible role for NucS in replication-associated repair processes. DnaA interacts with initiation factors like DnaB and DnaC to form the replication fork, which could be crucial for NucS recruitment to replication-related damage sites.

Given these interactions, NucS might slide on the DNA with these helicases. The sliding of NucS on the DNA could facilitate its role in recognising and processing DNA damage. Helicases could also help recruit NucS to sites of DNA damage. For instance, as helicases unwind DNA to reveal damaged sites, NucS could be recruited to these sites to perform its repair functions. This recruitment mechanism could be similar to how other repair proteins like Ku and Rad51 are recruited to DSBs. NucS may function at different stages of DNA repair, depending on the helicase involved. This is significant because helicases are essential for unwinding DNA, enabling repair proteins to access and process damaged DNA.

The STRING database has revealed significant insights into NucS's interactions with various proteins involved in DNA repair processes, particularly those associated with HR and other alternative DNA repair mechanisms. This discovery broadens our understanding of NucS's potential roles in bacterial DNA repair pathways.

Protein	Function	Archaea/ Bacteria
FEN1	Flap endonuclease processes DNA flaps	Archaea
ERCC4/XPF	DNA end processing	Archaea
Bax1	DNA damage response, pro-apoptotic	Archaea
RadA	HR	Archaea
Mre11/rad50	Repair of DSBs via HR	Archaea (Bacteria)
PCNA	Sliding clamp of the DNA polymerase	Archaea (Bacteria)
DNA ligase	Sealing nicks in the DNA backbone Crucial for various repair pathways	Archaea/ Bacteria
Nth	Base excision repair by removing oxidised pyrimidines	Archeae
RecB and RecF	DNA strand gaps repair	Bacteria
UspA1	Universal stress protein and related nucleotide-binding protein	Bacteria

**Table 5:** Summary of the proteins implicated in the DNA repair in the predicted interactome of NucS in *P. abyssi* or *C. glutamicum* (prediction from STRING).

One notable interaction is with ERCC4/XPF, which forms a complex with ERCC1, a protein involved in NER and interstrand cross-link repair. Interestingly, FEN1, involved in processing DNA flaps during replication and which plays a role in DNA end processing

during MMEJ, was present in the prediction in Archaea (Huang *et al.*, 2011; Harrington & Lieber, 1994).

Bax1, known for its role in the DNA damage response primarily as a pro-apoptotic protein, was also found, as well as Nth, which participates in BER by removing oxidised pyrimidines from DNA (Wilson *et al.*, 2000). The Mre11/rad50 complex, essential for repairing DSBs through HR and potentially MMEJ, appears in the archaeal interactomes (Williams *et al.*, 2010). As expected, PCNA was found in all predictions. This protein acts as a sliding clamp for DNA polymerase during DNA replication and repair, which could also include roles in BER and MMEJ, as presented in **Table 5** (Moldovan *et al.*, 2007). Ogt was also found in *M. tuberculosis*; this protein is involved in repairing the mutagenic and cytotoxic lesion O-6-methylguanine. We also identified several additional proteins linked with DNA repair pathways. These include endonuclease V (Nfi), involved in repairing deaminated bases and mismatches (Morita *et al.*, 2010); DNA Polymerase III (PolC), which is essential for bacterial DNA replication and repair (Kornberg & Baker, 2005); and DNA Polymerase II (PolB), which participates in the SOS response and various repair mechanisms, including MMEJ (Napolitano *et al.*, 2000).

The interaction of NucS with these helicases and DNA repair proteins suggests multiple potential roles. NucS may act similarly to repair proteins like Ku or Rad51, recognising and processing DNA breaks (Sung, 1994; Walker *et al.*, 2001). This interaction indicates that NucS might help balance the use of different DNA repair pathways, such as HR and MMEJ, thereby maintaining genomic stability.

While the STRING database is invaluable for studying protein-protein interactions and functional associations, it has limitations. These include potential errors due to data unavailability, limited predictive capabilities for less-characterized species, possible outdated data and uncertainties in functional annotations. Recognising these limitations allows researchers to interpret the database's findings rationally and make more informed decisions. By integrating these insights, we can hypothesise that NucS plays a significant role in DNA repair pathways, potentially coordinating with various helicases and other repair proteins to maintain genomic stability. This comprehensive understanding of NucS's interactions and functions provides a solid foundation for further experimental validation and exploration.



### C. Gene neighbourhood and conserved domain analysis of NucS/EndoMS

Gene neighbourhood analysis is a bioinformatic approach that involves identifying genes' physical arrangement and position within the genome to gain insights into their functional associations and evolutionary history. In our study, we utilised the Enzyme Function Initiative—Genome Neighborhood Tool (EFI-GNT) and the webFlaGs tool by The Atkinson Lab to identify the flanking genes of *nucS* in *P. abyssi*, *C. glutamicum* and *M. tuberculosis*.

We aimed to determine new plausible associations and interactions with NucS/EndoMS. The data obtained for *C. glutamicum*, *M.tuberculosis* and *P. abyssi* are disclosed in respectively Annexes 1, Annexe 2 and Annexe 3. Their analysis highlights several critical genes coding for proteins involved in diverse cellular functions, from DNA repair to metabolic processes, as summarised in **Table 6**.

We observed that *nucS* is located near various metabolic genes, such as those coding for adenylate/guanylate cyclase domain-containing proteins, methyl malonyl-CoA epimerase and acetyl-CoA C-acetyltransferase. Moreover, proteins such as S-methyl-5'-thioadenosine phosphorylase, involved in metabolic processes and ATP-binding proteins, critical for energy transfer, were also found. For instance, methylmalonyl-CoA epimerase is involved in the propionate metabolism pathway, which is essential for energy production and carbon flow within the cell. Additionally, the presence in the neighbourhood of *nucS* of energy-coupling factor, transmembrane transporter and DMT family transporters was also confirmed. This result highlights NucS's involvement in cellular processes requiring efficient transport and utilisation of molecules and ions. These transporters connect DNA repair to the cell's metabolic state and transport systems.

Transcriptional regulation and protein interaction partners, such as the LysR family transcriptional regulator and tetratricopeptide repeat proteins, suggest that *nucS* may be under regulatory control or part of regulatory pathways that respond to environmental changes. LysR family regulators often respond to oxidative stress, which frequently induces DNA damage and necessitates repair mechanisms (Maddocks & Oyston, 2008).

These proteins also include DUF473 family proteins, proteasome assembly chaperone family proteins, S-methyl-5'-thioadenosine phosphorylase, ion channels, NAD-binding proteins, DNA-binding proteins, ORC1-type DNA replication proteins, RadA, DNA-directed DNA polymerase II small subunits and various other hypothetical proteins.

The presence of RadA, a protein homologous to bacterial RecA, which is described for its role in DNA repair and HR, suggests an emphasis on HR in the DNA repair mechanisms associated with NucS. RadA's repeated presence between our analyses emphasises its critical role in maintaining genomic stability through repairing DSBs, which aligns with our hypothesis of NucS involvement in DSBR (Aravind *et al.*, 2000; Seitz *et al.*, 2001). Additionally, the proximity of *nucS* to DNA repair-related genes such as *dna-3-methyladenine glycosylase 2* and *hnh endonuclease signature motif-containing proteins* further supports its role in DNA repair. These genes are known to be involved in detecting and processing DNA damage.

Organism	Flanking Genes	Function/Association
<i>P. abyssi</i>	<i>cdc6</i> (ORC1-type DNA replication)	Regulation of DNA replication, binding to oriC region
<i>P. abyssi</i>	<i>polB, polC</i> (DNA polymerase II small and large subunits)	DNA synthesis and exonuclease activity, degrading SSD
<i>P. abyssi</i>	<i>radA, radB</i>	HR, repair of branched substrates, interactions with DNA Pol we and hjc
<i>C. glutamicum</i>	<i>Adenylate/ Guanylate cyclase domain-containing</i>	Metabolic functions and energy production
<i>M. tuberculosis</i> / <i>C. glutamicum</i>	<i>methylmalonyl-CoA epimerase</i>	Propionate metabolism, energy production
<i>C. glutamicum</i>	<i>Acetyl-CoA C-acetyltransferase</i>	Intermediary metabolism and energy production
<i>C. glutamicum</i>	<i>DNA-3 methyladenine glycosylase 2</i>	Detection and processing of DNA damage
<i>C. glutamicum</i>	<i>HNH endonuclease signature motif containing</i>	Detection and processing of DNA damage
<i>C. glutamicum</i>	LysR family transcriptional regulation	Responding to oxidative stress and environmental changes
<i>C. glutamicum</i> / <i>M. tuberculosis</i>	<i>Tetratricopeptide repeat protein</i>	Regulatory control and response to environmental changes

<i>C. glutamicum</i>	<i>ATP synthase subunits epsilon, beta, gamma</i>	Energy transfer and metabolic functions
<i>M. tuberculosis</i>	<i>RNA-guided endonuclease TnpB family protein</i>	Associated with the CRISP-Cas system, use RNA guides to identify and cleave DNA
<i>M. tuberculosis</i>	<i>MFS transporters</i>	Membrane transport proteins

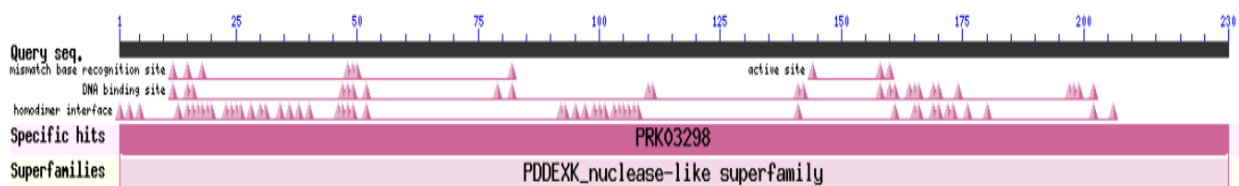
**Table 6:** Genes and their function found in the neighbourhood of *nucS* in either *C. glutamicum* or *M. tuberculosis* for the bacteria or *P. abyssi* for the archaea (Kumar Saha *et al.*, 2021).

ORC1-type DNA replication proteins are essential for initiating DNA replication, indicating that NucS might interact with proteins involved in the early stages of DNA replication. This is also coherent with the protein DnaA found in the interactome of *C. glutamicum*. This connection suggests that NucS could play a role in preparing the DNA for replication or stabilising replication forks, which is crucial for accurate DNA replication and repair processes (Bell & Botchan, 2013).

Including DNA-directed DNA polymerase II small subunits further supports the involvement of NucS in DNA replication and repair pathways. DNA polymerase II is known for its role in DNA synthesis during replication and repair (Kornberg, 1988). The genes *polB* and *polC*, also found in the neighbourhood of *nucS*, are coding for DNA polymerase II small subunit and large subunit, respectively and are involved in DNA synthesis as polymerase with template-primer preference and exonuclease activity to degrade single-stranded DNA in the 3' to 5' direction.

Given the proximity of genes related to both DNA repair, energy production and metabolic functions near *nucS*, it is plausible that *nucS* expression is regulated in response to the cell's metabolic state and energy availability. This regulation might ensure that DNA repair is coordinated with the cell's ability to support the energetically demanding repair and replication processes. For instance, the presence of ATP synthase subunits and other metabolic enzymes suggests a tight regulation linking NucS activity with cellular ATP levels, which are crucial for repair processes. This linkage between DNA repair and metabolic functions is well-documented in bacterial systems, where the metabolic state directly influences DNA repair mechanisms. For example, the SOS response, triggered during nutrient starvation, links metabolic stress to increased DNA repair activity (Foster, 2007).

The conserved domain analysis was performed for the blastp results of the *MtbNucS* using the Conserved Domains search tool (CD-search or batch CD-search) by NCBI. We observed four major domains conserved in the homologous protein sequences of NucS in *M. tuberculosis*, as presented in **Figure 51**: Mismatch base recognition site, DNA binding site, Active site and the homodimer interface, essential for DNA repair and HR activity, belonging to the PDDEXK nuclease-like superfamily and NucS. Superfamily of PDDEXK nucleases including very short patch repair (Vsr) endonucleases, archaeal Holliday junction resolvases, MutH methyl-directed DNA mismatch-repair endonucleases and catalytic domains of many restriction endonucleases, such as EcoRI, BamHI and FokI.



**Figure 51:** Conserved Domain Analysis. A) Structure of *MtbNucS* (Uniprot ID -P9WIY5) [21, 22], B) Conserved Domain analysis for the blastp results (homologous sequences) of the *MtbNucS*.

#### D. Phylogenetic history of NucS/EndoMS

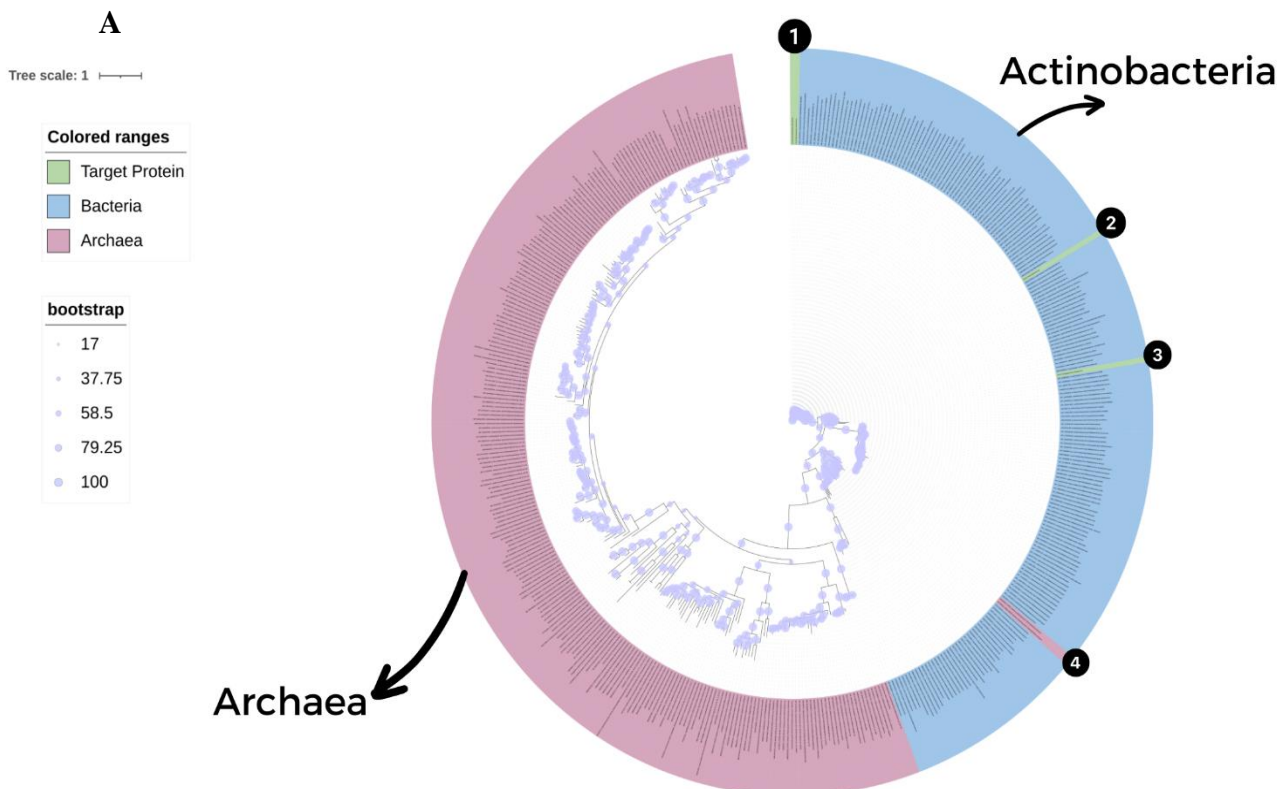
Two types of phylogenetic trees—the circular gene tree and the unrooted gene tree—can be used to examine the evolution of NucS proteins in archaea and Actinobacteria. These trees visualise the taxonomic distribution of NucS protein sequences, with target sequences from *M. tuberculosis* and *C. glutamicum* shown in green, archaeal sequences in red and Actinobacterial sequences in blue. Bootstrap values for these trees range from 17 to 100, with a median bootstrap value greater than 80, indicating the reliability of the branching patterns.

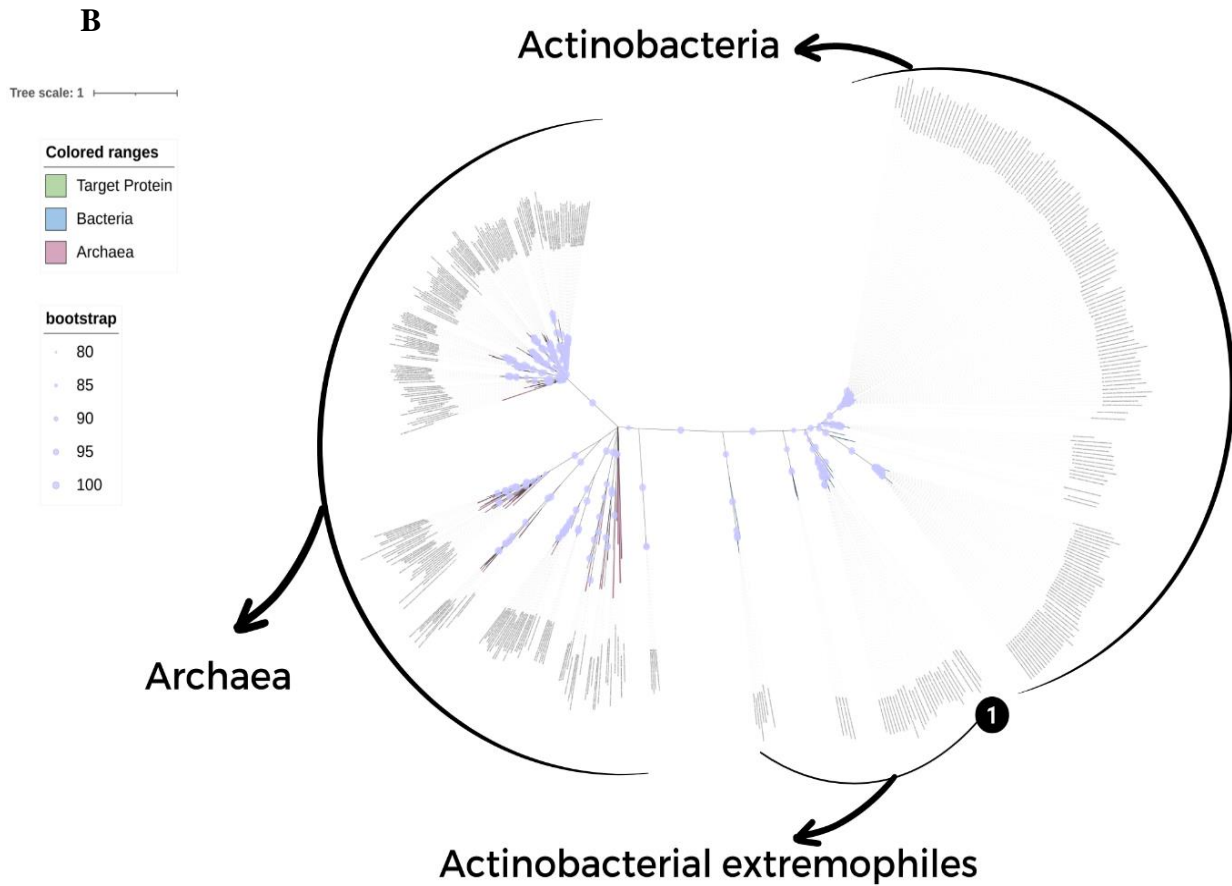
The circular gene tree in **Figure 52A** illustrates the taxonomic distribution of homologous NucS sequences across archaea and Actinobacteria. The scattered pattern indicates the presence of NucS proteins in both groups across various species that are not closely related evolutionarily. The clustering of target protein sequences within the actinobacterial taxon suggests that these sequences are more closely related to each other than to archaeal sequences. The presence of two archaeal sequences among the actinobacterial taxon suggests a high likelihood of horizontal gene transfer of the NucS gene between these lineages.

The unrooted tree of **Figure 52B** represents the likely evolutionary pathway of the NucS gene, showing divergent evolution through distinct clusters of archaea and Actinobacteria. The high density of actinobacterial clusters suggests significant similarity among these

homologous sequences compared to the target NucS sequences from Actinobacteria. Conversely, the scattered clusters of archaea may result from HR, mutations, ecological variations and other factors, leading to reduced identity of homologous NucS sequences over time.

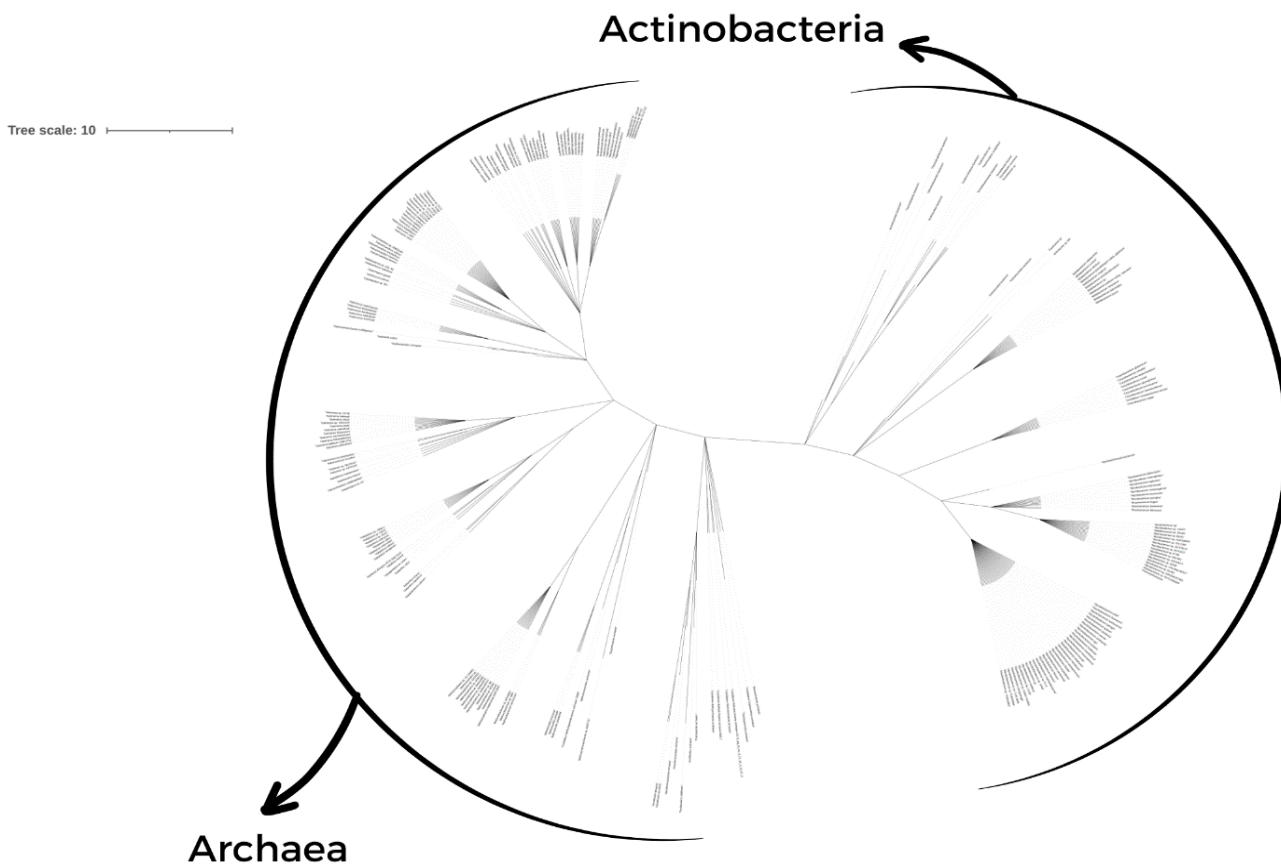
This pattern suggests that *nucS/endoMS* might have originated in archaea, with vertical gene transfer occurring within its taxa. Additionally, the presence of clusters of archaea closely associated with actinobacterial extremophiles, including two thermophilic archaea among actinobacterial extremophiles, indicates a high possibility of horizontal gene transfer from archaea to extremophiles Actinobacteria due to ecological and functional promiscuity, followed by vertical gene transfer to other members of the taxon. This gene transfer is crucial for DNA repair mechanisms, especially in the absence of MutL-MutS MMR pathways, to face increased mutation rates and HR due to extreme environmental conditions. High bootstrap values in phylogenetic trees provide statistical support for the branching patterns, indicating greater confidence and reliable associations in the tree (Felsenstein, 1985).





**Figure 52:** Phylogenetic prediction of *nucS* in archaea and bacteria **A.** model depiction of *NucS* evolution between Archaea and Actinobacteria. A) Unrooted Gene Tree of *NucS*, Circular Gene Tree of *NucS*. 1, 2, 3) Target Proteins – *M. tuberculosis* (*NucS*, *NucS1*, *NucS2*) and *CglNucS*. 4) 2 Archaeal sequences **B.** Unrooted Gene Tree with median bootstrap data value > 80.

A species tree represents the divergence and evolutionary relationships among various species over time, depicted in the circular and unrooted trees **Figure 53**. The nodes represent speciation events, where a new species arises from a common ancestor and the branches represent different species. The species tree was constructed to reconcile the gene tree, aligning the gene tree with the known species tree to reveal the evolution of the gene family by the duplication-loss model proposed by Goodman (1979).



**Figure 53:** Predicted Unrooted Species Tree for phylogenetic reconciliation analysis of NucS among archaea and bacteria.

In our study, *nucS* gene tree was reconciled with the species tree, which was constructed using all species found in the *nucS* gene tree. This approach helps uncover evolutionary events such as horizontal gene transfers, gene duplications, insertions and deletions that have influenced the sequences of these taxa.

Preliminary reconciliation analysis corroborates the hypothesis of potential horizontal gene transfers of *nucS* from archaea to extremophiles Actinobacteria due to the close association of specific clusters. It also suggested a higher likelihood of NucS originating from archaea rather than Actinobacteria, given the presence of scattered clusters and low identity of NucS homologs in archaea, which could result from various gene duplications, insertions and deletions over time.

Despite these preliminary results and high bootstrap values supporting our hypothesis, further analysis and reconciliation are needed. Potential limitations to our hypothesis include incomplete data for specific taxa, possible errors during the construction of phylogenetic trees and reconciliation analysis and shared similarities between specific motifs and conserved regions with the NucS protein. An alternative hypothesis could be that the fusion of the two domains of NucS has occurred twice independently in archaea and bacteria.



## Discussion

Molecular dynamics simulations and bioinformatics analyses are powerful tools in the study and characterisation of protein functions. By using existing databases, it helps give a head start for any researcher and saves valuable time by refining hypotheses, consolidating experimental observations and guiding the design of future experiments.

In the study of NucS, our hypothesis positions this enzyme within a DNA repair system that responds to the introduction of DSBs in the genome. We have developed several bioinformatics approaches, such as observing predictions of NucS interactions with other proteins. The analysis picked up five helicases within the NucS interactome, including those involved in NER, as represented by ERCC3/XPB, as well as FEN1 or RecF, which are proteins mobilised for the processing of DSBs. Their identification among NucS-interacting proteins suggests that NucS is directly involved in DNA repair pathways or through substrate competition.

In addition, numerous other proteins associated with DNA repair were detected, particularly in BER and long-patch base excision repair, as well as several enzymes involved in HR that have been mentioned previously, such as RadA, RecA, Mre11 and FEN1. The presence of the ligases further confirmed our suggestion of the putative connection of NucS to DNA breaks, possibly at an early stage after their introduction.

An analysis of *nucS* neighbourhood was conducted in *C. glutamicum*, *M. tuberculosis* and *P. abyssi* to complement this approach. This approach gives a broader view of NucS included in a more complex environment and lists genes coding for proteins consistent with our hypotheses. Identification of genes encoding proteins involved in DNA damage detection and processing included an RNA-guided endonuclease TnpB, the hnh endonuclease, DNA-3 methyladenine glycosylase 2, PolB and PolC (DNA polymerase II small and large subunits) that is implicated in creating mismatches during DNA replication/repair. RadA and RadB, which are directly implicated in HR, were found, reinforcing the suggestion of NucS intervention in DSBR.

Interestingly, most genes surrounding *nucS* are implicated in managing metabolic pathways, like the propionate pathway or energy production/transfer within the cell. These genes remind us that DNA repair is an energy-consuming process and thus involves adjusting cellular

energy production pathways. They also highlight the importance of extrinsic parameters for bacterial survival, such as the detection of oxidative stress and other environmental changes—evidenced by LyxR being a neighbour of NucS. The interplay between metabolism and DNA repair is critical, as seen in other systems where metabolic stress can trigger DNA repair responses (Foster, 2007; Maddocks & Oyston, 2008). This subset of our analyses complicates our understanding of NucS. Nevertheless, it further cements our prediction for its role in DNA repair, likely through a pathway that interplays or competes with HR—like the MMEJ, an intrinsically faster but less accurate way to perform DSBR compared to HR (Sfeir & Symington, 2015).

We chose to question our results on NucS' involvement in the DSBR by designing a simulation with CHARMM to observe what kind of behaviour NucS would take on a DNA substrate presenting this kind of damage. We wanted to observe the dynamic of NucS bound to that kind of damage without a partner. Based on the results obtained, we suggest that the binding of *Mtb*NucS to a DNA substrate containing a DSB would restrict the mobility of the free DNA ends. NucS seems to hold and stabilise the DNA extremities during such events. This interaction could protect the exposed DNA ends from exonuclease activity.

These observations reinforce the hypothesis of an early recruitment of NucS to the sites of DSB. Moreover, it seems that the fixation of NucS could also favour one DSBS pathway over another, as the HR requires the availability and mobility of the DNA-free ends. This pathway could be the MMEJ. Reproducing this prediction with a DNA sequence presenting a DSB and a mismatch in the middle confirmed a stabilisation of the DNA structure with contact points between the ends and NucS, rendering them unavailable to other enzymes.

Finally, we investigated the phylogenetic history of NucS. Our results support the findings of Castaneda *et al.*, 2016. Our analyses suggest an archaeal origin for NucS, which might have been transmitted to extremophile Actinobacteria through a horizontal event.

Future research should focus on several key areas to advance our understanding of NucS and its role in DNA repair. First, the bioinformatics predictions will have to be experimentally proven. Second, characterisation of the physical contacts between NucS and predicted partners with techniques such as EMSA or Plasmon Resonance Spectroscopy could confirm the existence and nature of these interactions, thus providing direct evidence for NucS's engagement in various DNA repair pathways.

## Material and Methods

### Data Acquisition

The protein sequences of NucS from the target bacteria – *C. glutamicum* (Uniprot ID - Q8NR49) and *M. tuberculosis* (Uniprot ID - P9WIY5, P9WIY4) were acquired from the AlphaFold Protein Structure Database, developed by DeepMind and EMBL-EBI (<https://alphafold.ebi.ac.uk/>). These sequences were subject to local alignment using the Protein Basic Local Alignment Search Tool (blastp) hosted at the National Center for Biotechnology Information (NCBI; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to collect protein sequences of the organisms - archaea and Actinobacteria which are identical to that of the target proteins (Homologous) from the non-redundant protein sequence database (nr). 450 protein sequences from various archaea and Actinobacteria were collected from the blastp results with a percentage of identity > 30 % to assemble the protein sequence dataset for the gene tree construction.

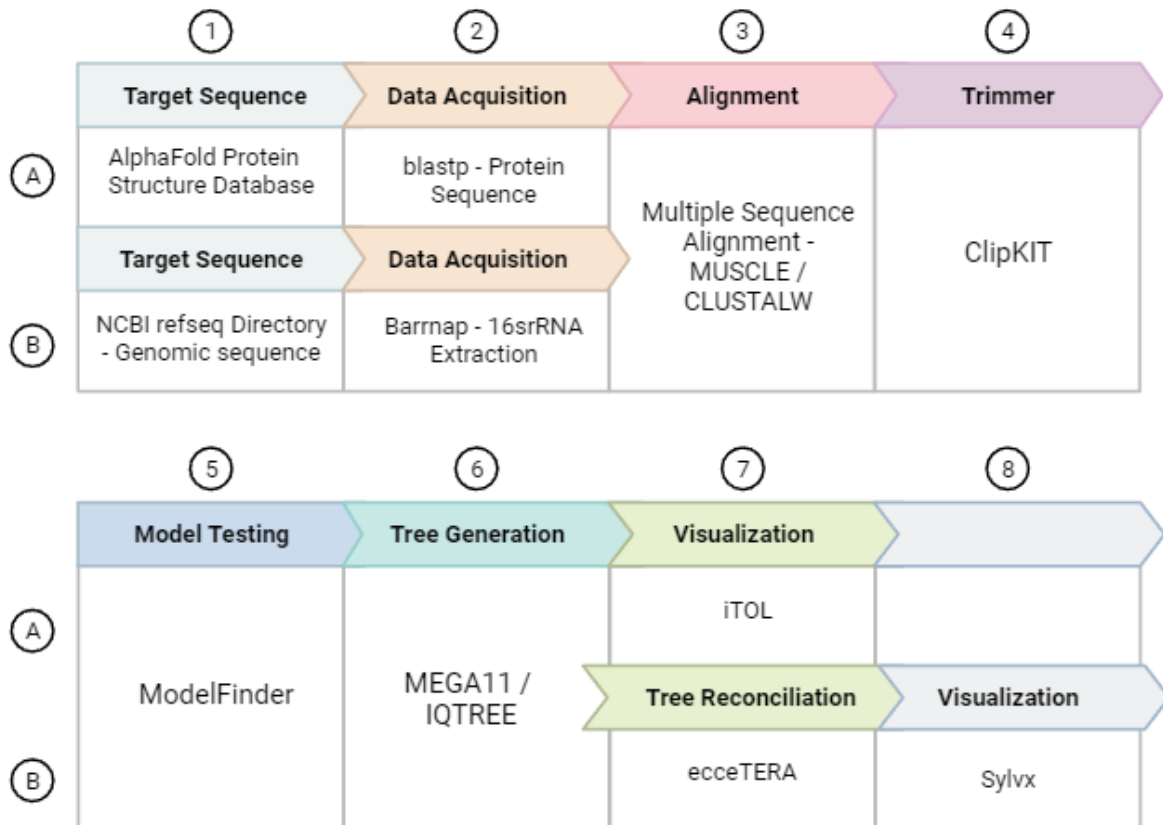
### Tree construction workflow

The assembled dataset is subject to Multiple Sequence Alignment (MSA) using Multiple Sequence Comparison by Log-Expectation (MUSCLE) and Clustal Omega (CLUSTALW) in the Molecular Evolutionary Genetics Assembly 11 (MEGA11, <https://www.megasoftware.net/>) software. The aligned protein sequences are then trimmed by a multiple sequence alignment trimming software - ClipKIT (<https://github.com/JLSteenwyk/ClipKIT>) for accurate phylogenomic inference. The output data is used for the gene tree construction by Maximum likelihood software - MEGA 11 and IQTREE (<http://iqtree.cibiv.univie.ac.at/>) using the Best-fit model - LG+F+I+G4 determined by the ModelFinder, according to Bayesian Information Criterion (BIC). Interactive Tree Of Life (iTOL, <https://itol.embl.de>) was later used to visualise and enhance the constructed gene tree.

### Species Tree construction

Complete genomic sequences of archaeal and bacterial species corresponding to the gene tree dataset were collected from the NCBI RefSeq directory (<https://ftp.ncbi.nlm.nih.gov/genomes/refseq>) using File transfer protocol software - FileZilla (<https://filezilla-project.org/>).

The extraction of 16S rRNA from the genomic sequences is performed using Barrnap V.0.9 (<https://github.com/tseemann/barrnap>) - a location prediction tool for ribosomal RNA genes in genomes. The extracted 16S rRNA sequences corresponding to the gene tree dataset are collected to form an input dataset for the construction of the species tree, using the same workflow used for the construction of the Gene Tree (MSA => ClipKIT => IQTREE => iTOL).



**Figure 52:** Phylogenetic Tree Construction Workflow. A. Gene Tree construction, B. Species Tree and Phylogenetic reconciliation Tree construction.

### Reconciliation analysis

The reconciliation analysis of the constructed gene tree with the species tree is performed using ecceTERA - a comprehensive phylogenetic tool for parsimonious reconciliation of the gene tree - species tree using Duplication-Transfer-Loss (DTL) model and amalgamation (<https://mbb.univ-montp2.fr/MBB/subsection/softExec.php?soft=ecceTERA>). The visualisation of the phylogenetic reconciliation tree is viewed using SylvX software (<http://www.sylvx.org/>).

## **Protein Interaction Networks**

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, <https://string-db.org/>) database was used to retrieve the protein-protein interaction data to understand better the plausible interactions and functional associations of NucS protein with other proteins or gene products in the target bacteria, *C. glutamicum* and *M. tuberculosis* and in archaea, *P. abyssi*.

## **Neighbouring genes and conserved domains analysis**

Identifying the genomic neighbourhoods of *nucS* can help unveil its functional and evolutionary relationship in the target organisms, such as if the proteins or enzymes function has been conserved over the evolution of the organism or has diverged from its ancestral function to a new specialised function. The Enzyme Function Initiative-Genome Neighborhood Tool (EFI-GNT, <https://efi.igb.illinois.edu/efi-gnt/>) and webFlaGs tool by The Atkinson Lab (<https://server.atkinson-lab.com/webflags>) was used to determine the flanking genes of *nucS* in *P. abyssi*, *C. glutamicum* and *M. tuberculosis*, also provide valuable insights into the functions of protein within genomes and their evolutionary relationships. A maximum of 30 Blastp hits were used in homologue searching. The E-value cutoff for Blastp searching was  $1e^{-3}$  (Kumar Saha *et al.*, 2021).

The Conserved Domains search tool (CD-search or batch CD-search) by NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) was used to recognise the different domains conserved in the homologous protein sequences of NucS among various organisms from the blastp results of the gene tree construction.

## **CHARMM Simulations**

### **Model Construction**

As no experimental structure of the NucS protein from *M. tuberculosis* exists, we used the theoretical structure AF-A0A0E9ARW8-F1-model\_v4.pdb determined by the AlphaFold program. This structure is in its apo form and is divided into an N-terminal part (amino acids 1-97) and a C-terminal part (amino acids 98-223). Using the PDB structure 5gke (NucS protein from *C. glutamicum* in the presence of DNA) as a template, the two parts of NucS were superimposed using the Chimera program. This procedure was repeated for the second polypeptide chain to form the homodimeric structure of the NucS protein from *M. tuberculosis*. A 30-base pair double-stranded DNA molecule (constructed using the website

<http://www.scfbio-iitd.res.in/software/drugdesign/bdna.jsp>) was inserted and two manganese ions were added. Water molecules present in the PDB 5gke structure were retained. Sometimes, a G-T mismatch and/or strand breaks were introduced into the DNA.

## **Simulations**

The coordinates of the hydrogen atoms absent from the model were generated using the CHARMM molecular modelling program. The protonation states of histidine amino acids were visually determined based on their local environment. After short energy minimisation, a cubic box of water molecules was added around the model atoms, along with potassium and chloride ions at a concentration of 0.15 M. The number of positive and negative ions was adjusted to achieve an overall neutral charge for the molecular system. With periodic boundary conditions, the model was placed in a solvated environment similar to experimental conditions.

The covalent bond length between a hydrogen atom and another atom was kept constant, allowing the use of a 2 fs integration step for the atomic motion equations. The CHARMM version 36 force field was used. The range of non-bonded interactions was gradually nullified between 10 Å and 12 Å and electrostatic interactions were calculated using the PME (Particle Mesh Ewald) method with a grid step of 1 Å. The system's temperature and pressure were maintained constant (NPT ensemble) using Langevin and Nosé-Hoover algorithms, respectively.

After energy minimisation, where only hydrogen atoms were allowed to move, the model was gradually heated from 60 K to 310 K in 10 steps of 24 K. This was followed by an equilibration phase, where the velocities of all atoms were periodically recalculated at 310 K. Constraints were applied to the alpha carbon atoms of the polypeptide chains and the phosphate groups of the DNA, which were gradually relaxed to allow all atoms in the model to move freely finally. All calculations were performed using the NAMD program, which efficiently utilises GPU properties for faster computation.

During a simulation lasting from 50 ns to 500 ns, the coordinates of the atoms were saved every 10 ps.

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## **Chapter 4: Archeal DNA repair**

# Réparation archéenne de l'ADN

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## 2.1. Introduction

Tous les organismes cellulaires, y compris les archées, ont adopté l'acide désoxyribonucléique comme moyen de stocker l'information génétique. Ce choix est à double tranchant. Tandis que la fidélité de la réplication de l'information génétique est nécessaire pour maintenir la stabilité génétique entre les générations, l'introduction de modifications stables des séquences d'ADN (mutations) permettent, d'autre part, l'évolution des populations et soutiennent l'adaptation microbienne, par exemple, face à des conditions environnementales changeantes de manière rapide. Des modifications fréquentes de la séquence ou de la structure chimique de l'ADN peuvent, dans certains cas, modifier l'information stockée dans l'ADN double brin (Lindahl 1993). Heureusement, des machineries dédiées à la réparation de l'ADN réparent la vaste majorité de cette information erronée, limitant ainsi son passage aux générations suivantes. L'objectif spécifique de ce chapitre sur la réparation archéenne de l'ADN est de souligner comment l'évolution a conduit à la sélection de stratégies de voies de réparation de l'ADN pour détecter et corriger différents types de dommages infligés à l'ADN. Les dommages à l'ADN peuvent affecter soit un seul brin, soit les deux brins de l'ADN double brin.

Au cours du processus direct de réparation de l'ADN, des modifications chimiques de cette molécule sont résolues sans couper la chaîne principale de l'ADN. C'est, par exemple, le cas de la réparation des photoproduits causés par les radiations UV, qui sont directement réparés par l'utilisation de l'enzyme photolyase. Chez les archées, l'information génétique erronée est fréquemment réparée par une des voies de réparation reposant sur différentes excisions, ce qui inclue les voies de réparation des mésappariements (MMR), de réparation par excision de base (BER), réparation par excision de nucléotide (NER), et de réparation par excision de ribonucléotide (RER). Les cassures double brin (DSB) peuvent être réparées par la recombinaison homologue (HR) qui utilise le brin d'ADN intact comme sauvegarde de

l'information génétique correcte (pour des analyses détaillées de la réparation archéenne de l'ADN, voir, par exemple (White et Allers 2018)).

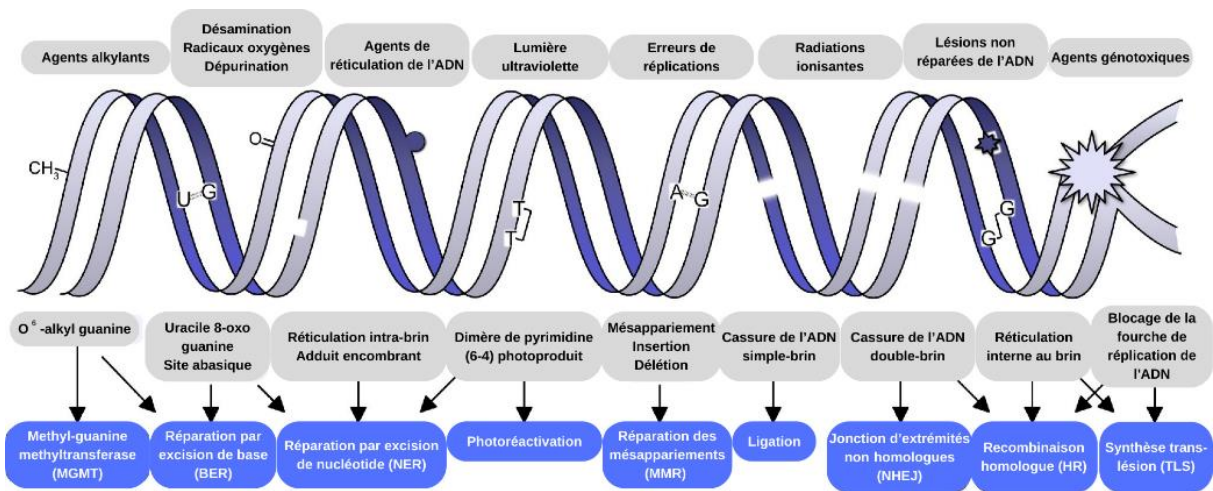


Figure 2.1. Les différents types de sites anormaux, y compris les erreurs de réplification (mésappariements) et de sites de dommage chimique à l'ADN, sont indiqués dans la molécule d'ADN double brin. Ces sites sont soit réparés efficacement par des machineries moléculaires hautement spécifiques, soit, si elles ne sont pas réparées, peuvent entraîner la formation de mutations, qui sont transmises de génération en génération.

Chez les humains, l'importance cruciale de la réparation de l'ADN pour la physiologie cellulaire est soulignée par le fait qu'elle est souvent liée à des maladies et/ou requise pour la progression optimale des cellules dans le cycle cellulaire. Dans le cas des archées, la nécessité de maintenir l'information génétique est une évidence si l'on considère qu'une portion conséquente des *clusters* de gènes orthologues (COG) ancestraux prédite est largement distribuée et code pour les protéines nécessaires à la réplification de l'ADN, à la réparation de l'ADN, à la transcription et à la traduction (figure 2.2). Ainsi, une portion assez considérable du génome de l'ancêtre archéen pourrait coder pour des fonctions de réplification de l'ADN et/ou de réparation, ce qui indique donc que les systèmes de réplification et les voies de réparation sophistiqués de l'ADN assurant une réplification hautement fidèle de l'ADN étaient déjà présents chez le dernier ancêtre commun archéen (LACA). Au regard de la réplification de l'ADN, des taux d'erreurs plus élevés sont tolérés pour l'ARN et la synthèse de protéines, ce qui relève du fait que l'information portée par le dernier groupe de polymères biologiques n'est pas transmise aux générations futures.

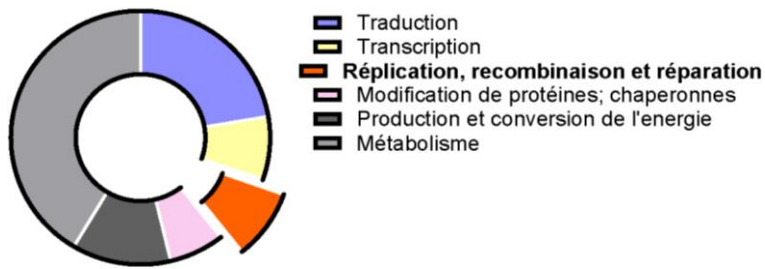


Figure 2.2. Les premières analyses génomiques évolutives de génomes archéens indiquaient que jusqu'à 10 % des gènes archéens ancestraux pourraient participer à la réplication et à la réparation du génome chez LACA. Cette figure s'appuie sur l'analyse de 41 génomes archéens, contenant un total de 681 clusters de gènes, comme indiqué par (Makarova et al., 2007).

## 2.2. Production de plusieurs types de dommage à l'ADN chez les archées

L'information contenue dans les génomes archéens est constamment mise en danger et attaquée par un large éventail de causes internes et externes qui seront résumées dans cette section.

### 2.2.1. Le réplisome archéen comme source intrinsèque d'erreurs de réplication et de dommage de l'ADN

Le réplisome archéen est une machinerie moléculaire complexe qui copie fidèlement l'information génétique portée par l'ADN, permettant ainsi la transmission de l'information biologique de génération en génération. L'exactitude du réplisome, et en particulier de l'ADN polymérase, est facilitée par plusieurs mécanismes distincts. Pendant la réplication, l'incorporation du nucléotide correct est garantie par la sélection de la bonne base et par les activités de relecture des ADN polymérases pendant la polymérisation de l'ADN. Chez les archées, la majorité des études s'est concentrée sur la caractérisation biochimique des ADN polymérases des familles B et D (analysée en (Cooper 2018), la dernière ayant été découverte par le laboratoire d'Ishino et se trouvant seulement chez les archées (Cann *et al.*, 1998)). Ces polymérases font la différence entre les paires de bases correctes et incorrectes grâce à leur liaison différentielle au site actif. Cet événement, initial et hautement discriminant de liaison, est suivi par une étape catalytique qui garantit la formation de la liaison phosphodiester, et donc la continuité du brin d'ADN. Cette sélectivité de reconnaissance de nucléotides est le résultat de plusieurs facteurs, tels que les liaisons hydrogènes entre les bases

complémentaires et l'arrivée de dNTP grâce à l'exclusion des interactions avec les molécules d'eau liées. La complémentarité de taille et de forme entre les paires de bases naissantes et la cavité du site actif jouent aussi un rôle important. Les paires de bases Watson-Crick A-T et G-C sont formées par l'appariement d'une purine et d'une pyrimidine. Par conséquent, ces paires de bases canoniques sont hautement similaires en termes de taille et de forme, ce qui facilite largement la reconnaissance par rapport aux bases mésappariées. Néanmoins, les ADN polymérase commettent occasionnellement des erreurs au cours de la sélection de bases, qui conduisent à l'incorporation de mauvais nucléotides dans la chaîne d'ADN en croissance. Pour faire face à ces potentielles erreurs de réplication de l'ADN, les ADN polymérase archéennes répliquatives possèdent une activité exonucléase 3'-5' (de relecture) pour retirer les nucléotides mésappariés avant la poursuite de l'élongation de la chaîne, augmentant encore l'exactitude de la synthèse de l'ADN.

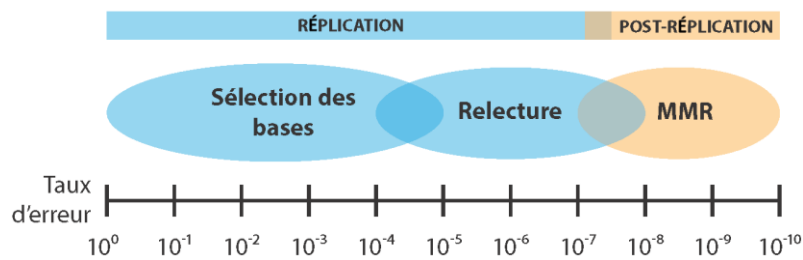


Figure 2.3. Les différents mécanismes assurant une réplication de l'ADN hautement fidèle chez l'archée (présentés à l'aide d'une échelle logarithmique). La sélection de base et la relecture sont effectuées pendant la réplication par les ADN polymérase, tandis que la MMR se produit de manière post-répliquative par des complexes protéiques spécifiques.

Malgré ces mécanismes de fidélité intégrés et sophistiqués, il arrive occasionnellement que les ADN polymérase archéennes créent des erreurs de réplication et des dommages dans l'ADN de la cellule. Un taux d'erreur caractéristique des ADN polymérase est de  $\approx 10^{-4} - 10^{-5}$ . La fidélité générale de la synthèse d'ADN peut être améliorée d'un facteur approximatif de  $10^2 - 10^3$  par l'activité de relecture des ADN polymérase. On suppose donc qu'un mauvais désoxyribonucléotide est incorporé approximativement une fois tous les  $10^6 - 10^8$  nucléotides. Étonnamment, les ADN polymérase incorporent aussi fréquemment des ribonucléotides (rNTP) dans l'ADN chromosomique, avec un taux d'erreur de  $\approx 10^{-3}$ . Ceci est assez surprenant, puisque les ADN polymérase portent des dispositifs structuraux

spécifiques qui bloquent la liaison des rNTP avec leur site actif. Cependant, les concentrations intracellulaires en rNTP sont sensiblement supérieures à celles des dNTP dans les cellules archéennes, ce qui contribue à une haute fréquence de ribonucléotides incorporés dans l'ADN double brin, jusqu'à un par kilobase. Ces événements contribuent à la diminution de la stabilité chimique des génomes archéens, remettant en cause l'exactitude de l'information génétique. Les cellules archéennes doivent utiliser des stratégies spécifiques pour retirer de l'ADN les bases mal incorporées avant que l'information génétique ne soit transmise à la génération suivante.

### ***2.2.2. Des facteurs chimiques et physiques menaçant l'intégrité des génomes archéens***

La molécule d'ADN est un complexe chimique organique qui est, en plus des erreurs de réplication, susceptible de subir des modifications dues à des réactions chimiques intracellulaires ayant pour résultat la dégradation de l'information génétique. Les défis chimiques majeurs qui attaquent l'intégrité chimique de l'ADN à l'intérieur des cellules sont les attaques hydrolytiques et les dommages oxydatifs à l'ADN (figure 2.1). La molécule d'ADN est aussi fréquemment modifiée chimiquement par des donneurs de carbones intracellulaires activés, comme la S-adénosylméthionine, ce qui a pour conséquence l'alkylation (méthylation) de l'ADN (Lindahl 1993).

Les réactions hydrolytiques concernent les réactions chimiques au cours desquelles des molécules d'eau agissent comme des catalyseurs pour briser des liens chimiques, conduisant à la division de larges molécules, telles que l'ADN, en de plus petits composants. L'occurrence des réactions hydrolytiques qui attaquent l'ADN est amplifiée par une très haute concentration intracellulaire en eau, qui peut approcher 55M. L'omniprésence de l'eau favorise les attaques hydrolytiques des liens chimiques qui relient les bases de l'ADN aux sucres (figure 2.1), ce qui a donc pour conséquence le retrait des bases purines (dépurination) ou des bases pyrimidines (dépyrimidination) de l'ADN double brin. Les réactions hydrolytiques peuvent aussi rapidement exciser un groupe amine d'une base cytosine, en particulier sur une molécule d'ADN simple brin, ce qui a pour résultat la désamination de la cytosine. Les espèces réactives de l'oxygène (dommage oxydatif de l'ADN) ont aussi un grand potentiel de modification chimique de la structure de l'ADN, leur présence peut impliquer, par exemple, la fragmentation de l'ADN et la formation de 8-Oxoguanine, une des lésions de l'ADN les plus communes. En plus de ces facteurs chimiques, les radiations

ultraviolettes et gamma peuvent drastiquement modifier la structure de l'ADN. Les UV peuvent provoquer la formation de dimères de thymidines interbrin (photoproduits) et potentiellement arrêter la réplication de l'ADN. Les radiations gamma peuvent aussi introduire des cassures de l'ADN simple brin ou double brin nécessitant une action de réparation. Au laboratoire, plusieurs conditions expérimentales différentes peuvent être utilisées pour imiter les différents dommages de l'ADN.

Les archées prospèrent fréquemment dans des conditions extrêmes, par exemple à haute température, niveau de sel, pression, ou encore lorsqu'elles sont exposées à des radiations ultraviolettes ou gamma. Toutes ces conditions environnementales peuvent potentiellement accélérer la formation des différentes lésions de l'ADN indiquées en figure 2.1. Par exemple, de hautes températures peuvent accélérer la dégradation de l'ADN et la désamination de la cytosine par un facteur de 3 000 entre 37 et 100 degrés Celsius. Néanmoins, le taux de mutation des archées est similaire à celui des bactéries mésophiles, ce qui indique que des voies de réparation de l'ADN très efficaces doivent exister chez les archées.

<b>Composé/conditions</b>	<b>Principaux dommages de l'ADN induits</b>	<b>Voies de réparation de l'ADN impliquées</b>
<b>UV</b>	Dimères de cyclobutane-pyrimidine (CPD) et adduits de pyrimidine (6-4PP)	Photolyase (réparation directe) ou réparation par excision de nucléotide (NER)
<b>Radiation gamma Phléomycine</b>	Cassures de l'ADN double brin (DSB)	Recombinaison homologue
<b>Agents alkylants (peroxyde d'hydrogène, méthanesulfonate de méthyle)</b>	Bases oxydées et méthylées	Réparation par excision de Base (BER)
<b>Mitomycine C</b>	Réticulations intra et interbrins (ICL)	Réparation ICL

*Tableau 2.1. Conditions expérimentales utilisées en laboratoire pour imiter différents dommages à l'ADN se produisant dans la nature*



## 2.3. Les différentes voies archéennes de réparation de l'ADN

Dans ce paragraphe, les voies clés de réparation de l'ADN se produisant chez les archées seront décrites.

### 2.3.1. *Les mésappariements menaçant l'intégrité du génome dans tous les domaines du vivant*

Les erreurs de réplication entraînent souvent la formation de mésappariements (MM) qui sont formés par l'appariement entre des bases de formes chimiques spécifiques (tautomères). Le mésappariement G : T imite très bien l'association de paires de bases de Watson-Crick se produisant naturellement, les ADN polymérases ne les identifient et n'éliminent pas immédiatement en tant qu'erreurs au cours de la sélection de bases ou de la relecture (Kunkel et Erie 2015). Cependant, ces mésappariements sont efficacement retirés par la voie de réparation des mésappariements (MMR), comme décrit ci-dessous. L'absence de fonctionnement de la voie MMR mène à une multiplication des taux de mutation par un facteur allant de 10 à 1 000, soulignant l'importance de la voie MMR post-réplivative. L'action de mutagènes chimiques, de radiations ionisantes ou la désamination spontanée des bases peuvent aussi former des mésappariements, indépendamment de la réplication de l'ADN. Des études génétiques soulignent l'interdépendance des voies de réparation de l'ADN, ainsi que l'importance de leur régulation, et indiquent que la voie MMR, au moins chez certaines bactéries, inhibe aussi la recombinaison de séquences très proches, mais non identiques, agissant ainsi comme une anti-recombinase. Cela implique que les mésappariements entre deux bases de l'ADN réparés par la MMR peuvent aussi se produire au cours de la formation de l'ADN en D-loop pendant la recombinaison. Dans certains cas, la MMR participe aussi à la réparation d'insertions/délétions, en plus de celle des mésappariements de base.

L'évolution a permis de sélectionner deux solutions indépendantes pour détecter et corriger ces mésappariements (figure 2.4 (Kunkel et Erie 2015)). Le système MMR canonique se compose de MutS et de MutL, des protéines qui reconnaissent et excisent respectivement les mésappariements au cours du processus de MMR archéen (Minobe *et al.*, 2019). La liaison de la protéine MutS sans nucléotide (ni lien ADP) aux mésappariements initie la MMR. En présence d'ATP, on pense que MutS se courbe, promouvant ainsi la formation du complexe nucléase MutSL nécessaire à l'excision du/des nucléotide(s)

mésapparié(s). Le processus complexe de réparation de l'ADN est ensuite poursuivi par la resynthèse du brin d'ADN excisé en fonction de l'ADN parental. Les MMR canoniques post-répliquatives distinguent l'information génétique incorrecte (ADN néosynthétisé) de l'information génétique correcte (ADN modèle). Ce phénomène s'explique par chargement asymétrique de l'endonucléase MutL, qui est facilité par le facteur de processivité de la polymérase PCNA. La spécificité du signal du brin néosynthétisé correspond probablement à des discontinuités dans le brin d'ADN (« entailles ») créées par le traitement des fragments d'Okazaki et/ou à l'élimination de ribonucléotides incorporés par erreur. D'autre part, *Escherichia coli* et les gamma protéo-bactéries de la même famille utilisent un système MMR dirigé par les méthyles, ce dernier est plus complexe. Il est facilité par la protéine MutH permettant d'effectuer des réparations de l'ADN spécifiques au brin néosynthétisé (non montré dans la figure 2.4).

Malgré le rôle fonctionnel crucial de la MMR, les premières études en bio-informatique n'ont étonnamment pas permis d'identifier la présence du système canonique MutSL dans de nombreuses archées et actinobactéries. Cependant, une combinaison d'études génétiques et de biochimiques ont mené à la découverte d'une endonucléase non canonique, NucS, spécifique aux mésappariements (aussi appelée EndoMS) qui se trouve chez de nombreuses archées et actinobactéries (Nakae *et al.*, 2016). Cette protéine ne présente pas de similarité de séquence ou de structure avec les protéines du MMR caractérisées précédemment. L'inactivation génétique de NucS multiplie le taux de mutations spontanées par  $\approx 200$  (Castaneda-Garcia *et al.*, 2017 ; Ishino *et al.*, 2018). Cette augmentation du taux de mutation est similaire à celle qui a été observée, par exemple, chez des *E. coli*, présentant une déficience de MMR. Ces résultats indiquent que l'efficacité *in vivo* des deux systèmes MMR est comparable. De récentes études ont établi que l'endonucléase NucS fonctionne très différemment des complexes MutS/L, puisque i) le complexe PCNA- NucS seul reconnaît et clive les mésappariements (préférentiellement G/T, T/T, G/G, dans cet ordre) ; ii) l'activation par l'ATP de NucS n'est pas nécessaire pour la réparation de l'ADN ; iii) NucS retire les mésappariements en créant une cassure de l'ADN double brin (DSB) hautement recombinogène, avec une extrémité cohésive centrée au niveau du mésappariement.

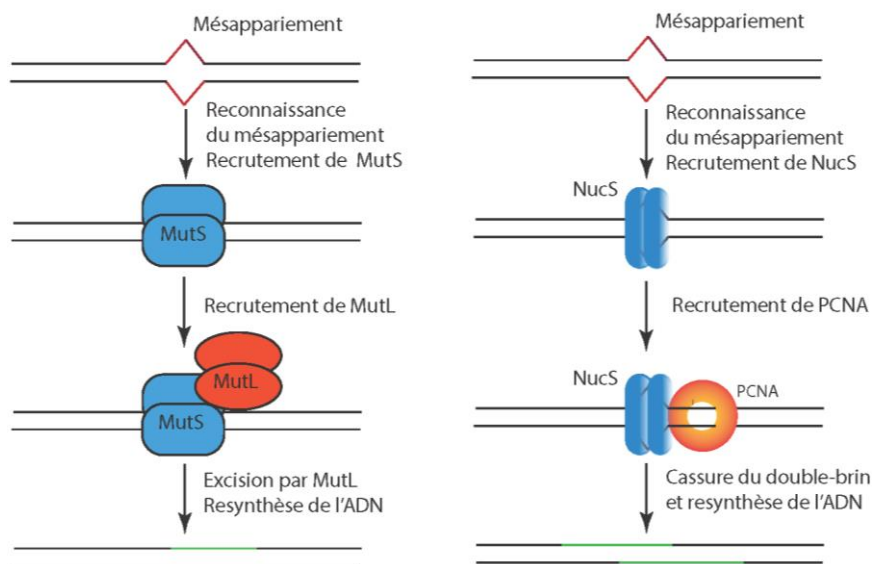


Figure 2.4. Les deux différentes stratégies de réparation des mésappariements qui se produisent au cours de la réplication de l'ADN. Le mésappariement est reconnu soit par la protéine MutS, soit par la protéine NucS/EndoMS. Tandis que MutS recrute une endonucléase MutL additionnelle au niveau du mésappariement, NucS/EndoMS peut aussi retirer le mésappariement avec l'aide de PCNA, en utilisant son activité nucléase.

De plus, la formation du complexe PCNA et NucS est nécessaire pour éviter les mutations. Ce qui suggère que l'interaction physique entre NucS et PCNA, le facteur de processivité de la polymérase, est médiée par un motif peptidique conservé au cours de l'évolution (« peptide d'interaction avec PCNA ») localisé à l'extrémité C-terminale des orthologues de NucS (Ishino *et al.* 2018). Il est probable que le système NucS ne nécessite pas d'identification du brin d'ADN modèle néosynthétisé, puisque la réparation est probablement effectuée par l'arrivée d'ADN double brin *via* HR (ou par NHEJ, plus enclin à l'erreur, ou par réparation dépendante de micro-homologie). Des études génétiques ont aussi indiqué que similairement au système MutS/L, NucS agirait aussi comme une anti-recombinase.

Les espèces archéennes présentent des protéines homologues de MutS et MutL sont généralement des halophiles ou des méthanogènes qui pourraient avoir récupéré ces séquences par transfert de gènes à partir de bactéries. Il a récemment été proposé que les protéines homologues de MutS/L, retrouvées chez les eucaryotes, puissent être originaires des archées Asgard, tandis que celles qui fonctionnent dans les mitochondries ou les chloroplastes auraient été transférées aux eucaryotes à partir d'alpha-protéo-bactéries ou de

cyanobactéries, respectivement. Par ailleurs, les homologues de NucS/EndoMS se retrouvent fréquemment chez des archées thermophiles.

### **2.3.2. Réparation par excision de nucléotide archéenne (NER)**

La voie de réparation par excision de nucléotide (NER) répare de nombreux types de dommages de l'ADN qui déforment l'hélice en déstabilisant localement l'ADN double brin. Des exemples de ces types de dommages incluent la « réparation obscure » des photoproduits de l'ADN (lorsqu'ils ne sont pas réparés directement par des photolyases) et des modifications de bases créées par le stress oxydatif ou par des agents chimiques exogènes. Ces différentes modifications de l'ADN ont un grand potentiel, celui de mettre en arrêt des processus essentiels concernant l'ADN dans la cellule, telles la réplication et la transcription de l'ADN. Par conséquent, une réparation efficace des dommages de l'ADN déformant l'hélice est cruciale pour la conservation de l'information génétique archéenne (Rouillon et White 2011).

Deux différentes variantes des mécanismes NER généraux ont été décrites. Lors du NER réparant les lésions sur l'ensemble du génome (GG-NER), plusieurs enzymes reconnaissent les déformations de l'ADN, entraînant une excision spécifique sur le brin porteur du dommage à l'ADN (figure 2.5). L'élimination de l'information génétique endommagée est suivie par la resynthèse de l'ADN en utilisant le brin intact comme modèle. La NER couplée à la transcription (TCR-NER) retire les lésions de l'ADN qui font barrage à l'ARN polymérase. Par conséquent, la TCR-NER est spécifique au brin matrice de l'ADN qui est activement transcrit par l'ARN polymérase. Dans le cas de la TCR-NER, le retardement de l'ARN polymérase fournit un signal qui initie la TCR-NER *via* le recrutement des protéines de la NER, ce qui est facilité par les facteurs d'assemblage de la transcription et de réparation CSB ou Mfd chez les archées. Ainsi, la protéine ETA, qui partage des caractéristiques similaires avec Mfd, a récemment été décrite chez les Thermococcales et pourrait fonctionner, au cours du TCR-NER, chez certaines archées (Walker *et al.* 2017).

Le système NER le mieux caractérisé chez les archées est sans doute celui de *Haloferax volcanii* (pour les étapes enzymatiques, voir figure 2.5). Cette archée et d'autres halophiles utilisent essentiellement les protéines bactériennes UvrABC que l'on retrouve chez une minorité d'espèces archéennes (Perez-Arnaiz *et al.* 2020). Ces protéines reconnaissent les dommages à l'ADN (UvrA) et ouvrent localement les brins d'ADN double brin autour de ce

site d'ADN (UvrB). L'ADN dénaturé autour du dommage à l'ADN facilite ensuite l'excision de l'adduit endommagé par UvrC, suivi par une synthèse d'ADN et une ligation pour assurer la continuité du brin d'ADN excisé. La délétion de ces gènes chez les halophiles provoque une nette sensibilité aux UV. Cette observation souligne le rôle central de ces gènes dans la NER haloarchéen. Puisque ces espèces sont fréquemment exposées aux radiations UV dans leur environnement naturel, ce constat est probablement hautement significatif au niveau physiologique. L'analyse phylogénétique des protéines de la NER a révélé que des homologues des protéines bactériennes UvrABC se retrouvent seulement chez certaines archées mésophiles, qui les ont probablement acquises par transfert horizontal de gènes à partir de bactéries.

En comparaison avec les bactéries ou les halophiles, la NER eucaryote utilise une machinerie beaucoup plus complexe pour réparer les mêmes lésions. Chez la plupart des archées, certaines nucléases de la NER (XPF et Fen-1/XPG) et des homologues d'hélicases (XPB et XPD) eucaryotes, ainsi qu'une nucléase spécifique aux archées, Bax, ont été identifiés, ce qui suggère l'existence d'une voie, au moins partielle, de type eucaryote chez les archées. Cependant, un système XP complet de type eucaryote n'a pas encore été décrit chez les espèces archéennes. De plus, malgré des études approfondies sur la biochimie, la structure et la génétique des protéines archéennes de type XP, leur rôle direct dans la NER archéenne n'est pas évident. En particulier, certaines protéines de reconnaissance ou de vérification des dommages, y compris des homologues de XPA et XPC, semblent absentes chez les archées. Étonnamment, XPF a été impliquée dans le redémarrage des fourches de réplication archéennes. Par conséquent, l'identité d'une voie NER analogue à celle des eucaryotes, si elle existe, n'a pas encore été complètement établie. Il reste possible que l'absence de voie NER complète ait été compensée par les autres voies de réparation de l'ADN fonctionnelles chez la majorité des espèces archéennes.

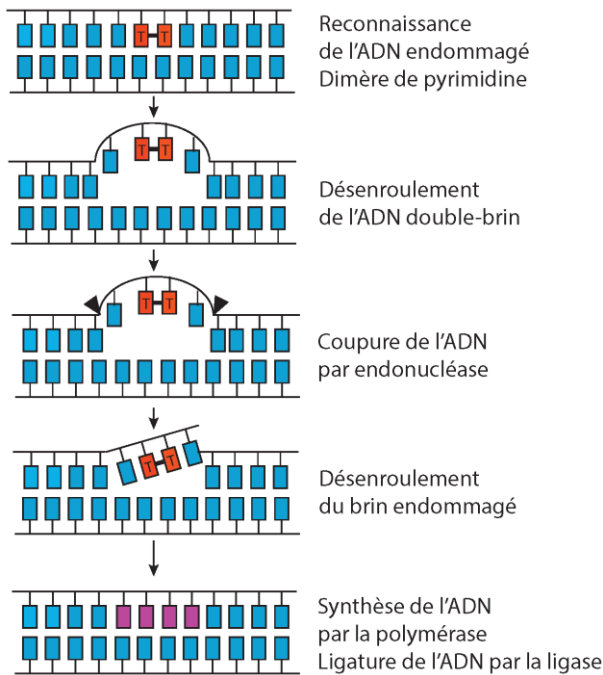


Figure 2.5. Vue d'ensemble d'une génomique NER (GG-NER) globale, où diverses enzymes peuvent reconnaître des déformations, comme des photoproduits (dimères de pyrimidine). Cela initie le déroulement de l'ADN double brin autour du site endommagé et facilite l'excision brin-spécifique du dommage à l'ADN pour retirer l'information génétique erronée/manquante. Les ADN polymérases et les ligases sont ensuite utilisées pour rétablir la continuité du brin d'ADN endommagé.

### 2.3.3. Réparation par excision de base (BER) chez les archées

La réparation par excision de base (BER) est principalement impliquée dans la réparation de bases endommagées de l'ADN qui n'affectent pas fortement la structure de la double hélice de l'ADN. De plus, les bases uraciles trouvées dans l'ADN, provenant, par exemple, de la désamination de cytosines, sont également réparées par cette voie. Il est possible de prendre pour exemple les bases oxydées et alkylées qui sont soumises à la BER. Par conséquent, on peut s'attendre à ce que les lignées archéennes dont la BER est inactivée soient sensibles au stress oxydatif et aux agents alkylants. Notons que les conditions extrêmes dans lesquelles prospèrent souvent les archées peuvent considérablement accélérer les modifications chimiques des bases de l'ADN.

La BER canonique archéenne (figure 2.6 (Grasso et Tell 2014)) est initiée par les glycosylases qui reconnaissent spécifiquement les bases oxydées (OGG), alkylées (AlkA) ou

désaminées, comme l'uracile (Udg). Deux types de glycosylase ont été décrits. Les enzymes monofonctionnelles clivent le lien glycosidique entre la base et la chaîne phosphodiester, qui résulte en la formation d'un site apurinique/apyrimidinique (AP). Ce site AP est ensuite traité plus en profondeur par des lyases/endonucléases AP-spécifiques, ce qui permet la résolution des bases endommagées. Un procédé en plusieurs étapes permet ensuite de discriminer l'information génétique correcte à l'aide de l'activité d'ADN polymérase et de ligases. Une des glycosylases archéennes les mieux caractérisées appartient à la famille des protéines Ogg qui agit comme une enzyme de la BER bifonctionnelle (Gehring *et al.* 2020). Cela signifie qu'elles possèdent à la fois une activité glycosylase et une activité lyase AP pour l'ablation de la base endommagée et le clivage de la chaîne de phosphodiester. L'activité lyase AP crée une cassure simple brin qui peut être traitée soit par un simple échange de nucléotide (BER à *patch* court), soit par la synthèse de 2-10 nucléotides (BER à *patch* long). L'extrémité 5' sortante générée au cours du BER à *patch* long est clivée par l'endonucléase Fen1.

Chez certaines archées, des endonucléases alternatives, très différentes des glycosylases canoniques, peuvent initier la BER. L'endonucléase Q (EndoQ) et l'endonucléase V (EndoV) coupent des bases endommagées 5' et 3' (Ishino *et al.* 2015). Parmi ces enzymes, EndoQ présente une spécificité pour l'uracile (qui résulte de la désamination de la cytosine), l'hypoxanthine (désamination oxydative de l'adénine) et les sites abasiques. Au sein des archées, EndoQ se trouve principalement chez les Euryarcheota, en particulier chez les hyperthermophiles et certaines méthanogènes. Beaucoup d'enzymes de la voie BER sont conservées dans les trois domaines du vivant, ce qui indique l'importance fondamentale et générale de cette voie de réparation de l'ADN.

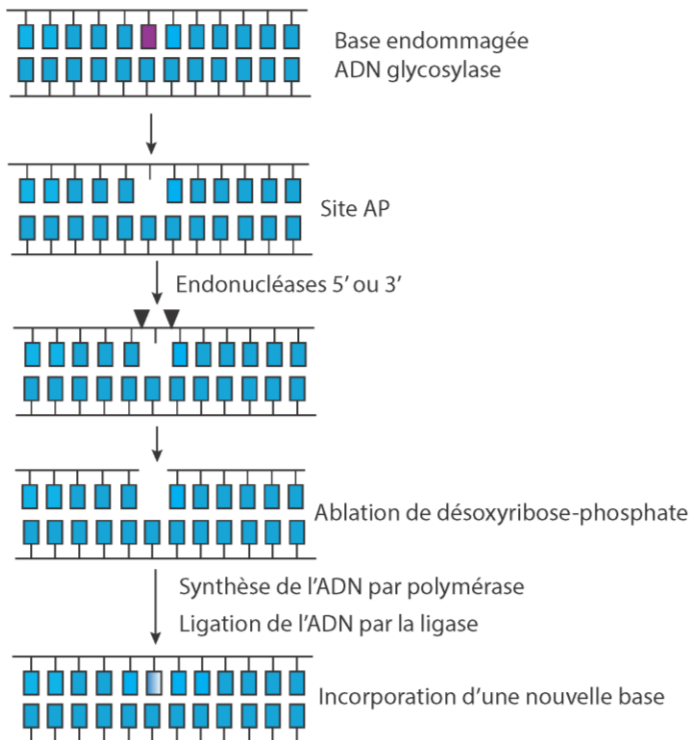


Figure 2.6. Au cours de la BER à patch court, une ADN glycosylase monofonctionnelle clive le lien glycosidique entre la base et la chaîne phosphodiester, ce qui entraîne la formation d'un site apurinique/aprimidinique (AP). Ce site AP est ensuite traité par des lyases/endonucléases AP-spécifique, provoquant la formation d'une coupure dans l'ADN. Les activités ADN polymérase et ligase sont utilisées pour déterminer l'information génétique correcte.

#### 2.3.4. Réparation par excision de ribonucléotide (RER) chez les archées

Comme nous l'avons décrit dans l'introduction, les ADN polymérases archéennes incorporent dans l'ADN génomique des ribonucléotides par erreur, au lieu de désoxyribonucléotides, étonnamment fréquemment ( $\approx 1$  rNTP chaque 1 000 bases). Si ces ribonucléotides restaient dans l'ADN, ils pourraient potentiellement déformer la structure et affecter la stabilité de l'hélice de l'ADN. Pour empêcher une éventuelle augmentation du taux de mutation et une baisse de stabilité du génome, ces ribonucléotides incorporés par erreur sont retirés par la voie de réparation par excision de ribonucléotide (RER). La RER est initiée par la Rnase H2 qui coupe l'ADN du côté 5' par rapport au rNMP inséré dans l'ADN génomique. Parmi les autres enzymes participant à la RER archéenne, on trouve l'ADN polymérase (déplacement de l'extrémité 3'), Fen1 (clivage de l'extrémité simple brin formée pendant le processus) et l'ADN ligase qui scelle la brèche restante dans l'ADN (Heider *et al.*



2017). Le rôle de ces enzymes dans la RER et le traitement des fragments d'Okazaki ont été montrés par des études de reconstruction biochimique basées sur des protéines archéennes.

### ***2.3.5. Réparation des cassures double brin (DSBR) chez les archées***

Les cassures double brin (DSB) sont des lésions de l'ADN particulièrement dangereuses, toxiques et une menace majeure pour la stabilité génomique. En coupant simultanément les deux brins de l'ADN, elles bloquent la réplication et la transcription de l'ADN. L'échec de la réparation des DSB peut mener à une perte chromosomique, un réarrangement chromosomique, la mort de la cellule ou à une prolifération anormale. Par conséquent, les organismes ont développé plusieurs voies de réparation des DSB. Les réparations de DSB archéennes incluent trois voies majoritaires : la réparation par recombinaison homologue (HR), la jonction d'extrémité non homologue (NHEJ) et la jonction d'extrémités par micro-homologie (MMEJ) (revue en White et Allers 2018). La réparation par HR inclut l'invasion d'une molécule d'ADN non endommagée par une molécule endommagée présentant une séquence identique. La synthèse de la région endommagée est effectuée en utilisant la molécule non endommagée comme modèle. La réparation par HR ne peut se produire que dans des cellules avec au moins deux copies chromosomiques (par exemple, après réplication chez les espèces haploïdes ou chez les espèces polyploïdes). Puisqu'un brin non endommagé est disponible comme modèle pour la réparation, la HR est considérée comme étant sans erreur. Deux autres voies, s'appuyant sur la jonction des deux extrémités cassées existantes, ont été majoritairement étudiées chez les eucaryotes. La jonction d'extrémités non homologues (NHEJ) joint directement les deux extrémités de la cassure double brin et est relativement fiable, bien que des insertions ou des délétions puissent se produire. La jonction d'extrémités par micro-homologie (MMEJ) utilise des séquences micro-homologues pour aligner les brins cassés. La MMEJ est associée avec des délétions et des insertions au niveau du site originel de la cassure, ainsi qu'avec des translocations de chromosomes.

La recombinaison homologue peut être divisée en trois étapes : pré-synapse, synapse et post-synapse. Au cours de la pré-synapse, les extrémités DSB sont reconnues et traitées pour produire des extrémités simple brin 3' sortantes. Le modèle archéen actuel suppose que les DSB sont reconnues par le complexe Mre11/Rad50 qui initie la dégradation de l'ADN à l'aide de son activité exonucléase 3'-5'. Cet événement est suivi par le recrutement du complexe HerA-NurA, responsable de la formation des extrémités 3' sortantes grâce au

couplage de l'activité de translocation de HerA, une hélicase bipolaire, à la dégradation de l'ADN simple brin avec une polarité 5' vers 3' par la nucléase NurA. Chez les archées hyperthermophiles, les gènes Mre11/Rad50 sont généralement codés dans des opérons qui contiennent à la fois HerA et NurA, ce qui implique un lien fonctionnel précoce dans la résection archéenne des extrémités de l'ADN. Au cours de la synapse, les extrémités d'ADN simple brin 3' sortantes sont reconnues par la recombinase conservée RadA (RecA chez les bactéries, rad51 chez les eucaryotes) qui présente une activité de polymérisation le long de la région d'ADN simple brin. Le filament de nucléoprotéine dynamique de RadA cherche ensuite une séquence homologe, envahissant les molécules d'ADN double brin. La liaison du filament RadA à sa séquence homologe dans la molécule envahie entraîne la formation d'une structure intermédiaire que l'on appelle la « boucle D ». Par la suite, pour l'étape post-synapse, il existe deux voies. Le brin envahissant dans la boucle D, après l'amorce de la synthèse de l'ADN, peut être rejeté de la boucle d'ADN envahie et hybridé avec la deuxième extrémité. Ce processus s'appelle « synthèse dépendante de l'hybridation de brin » (SDSA). Le déroulement du brin néosynthétisé est facilité par l'hélicase Hel308. Il mène à une conversion localisée sans *cross-over*. Sinon, la capture de la deuxième extrémité mène à la formation d'une structure d'ADN qui se ramifie en quatre branches, aussi appelée jonction de Holliday (HJ), qui doit être résolue pour achever la réparation. Une résolvasse archéenne reconnaît les jonctions à quatre voies de l'ADN (jonction de Holliday Hjc) et active sa fonction nucléase pour résoudre la jonction. La résolution des HJ peut produire soit des produits ADN double brin sans *cross-over*, soit des produits *cross-overs* d'ADN double brin, résultant d'un échange de matériel génétique entre la molécule d'ADN cassée et la molécule envahie. Les modifications post-traductionnelles des composants de réparation de DSB, qui incluent la méthylation de Mre11/Rad50 ou la phosphorylation de Hjc, contribuent probablement au taux d'efficacité des différentes voies de HR.

La jonction d'extrémités par micro-homologie (MMEJ) a été décrite chez les halophiles et les *Sulfolobales*. Après les DSB, les micro-homologies sont révélées par des exonucléases cellulaires, permettant l'hybridation des séquences complémentaires. Cela produit une structure branchée. Les extrémités simple brin sont probablement clivées par l'endonucléase Fen1 ou par la nucléase associée à RecJ/GINS (GAN). Ensuite, les extrémités sont associées par l'ADN ligase pour achever la réparation de l'ADN. Dans le cas où les micro-homologies sont loin du site endommagé, les séquences intervenantes sont souvent perdues au cours du processus de réparation. La jonction d'extrémités non homologue ne nécessite pas de région

d'homologie. Les extrémités cassées sont plutôt réunies par un complexe protéique médiateur. Bien que les détails moléculaires du NHEJ archéen n'aient pas été déterminés, il est probable que la protéine Ku, décrite pour sa liaison aux extrémités de l'ADN, conservée chez certaines espèces archéennes, lie également les extrémités de l'ADN et permette un traitement ultérieur des extrémités de l'ADN menant au remplissage de la brèche et la ligation.

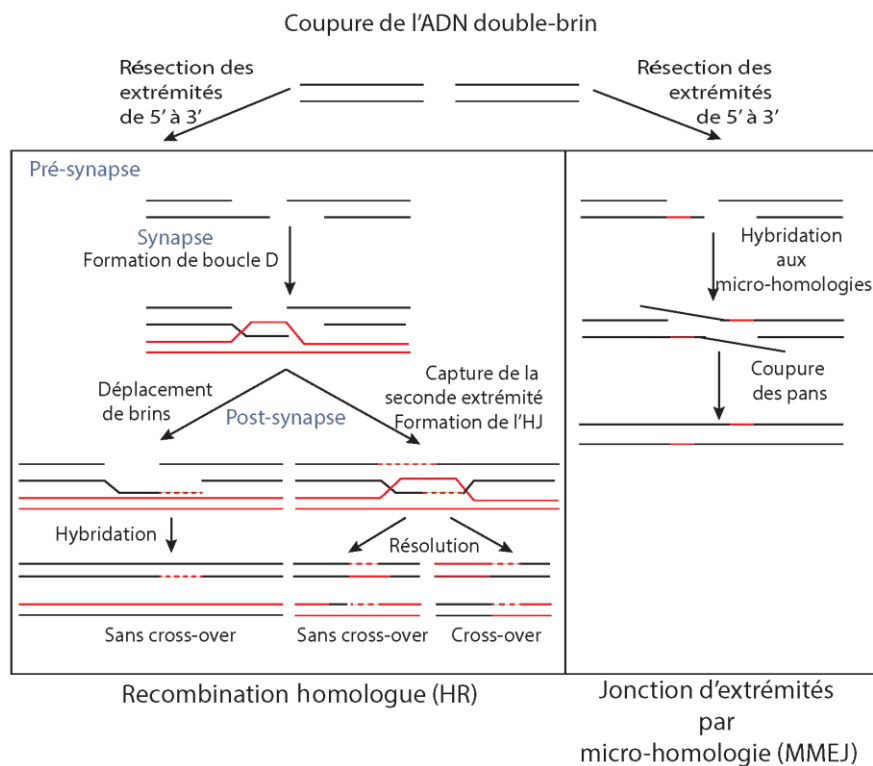


Figure 2.7. Chez les archées, les deux voies majeures de réparation des DSB sont la recombinaison homologue (HR) et jonction d'extrémités par micro-homologie (MMEJ). Pour une discussion détaillée, voir le texte

#### 2.4. Coordination des différentes voies par le facteur de processivité de la polymérase

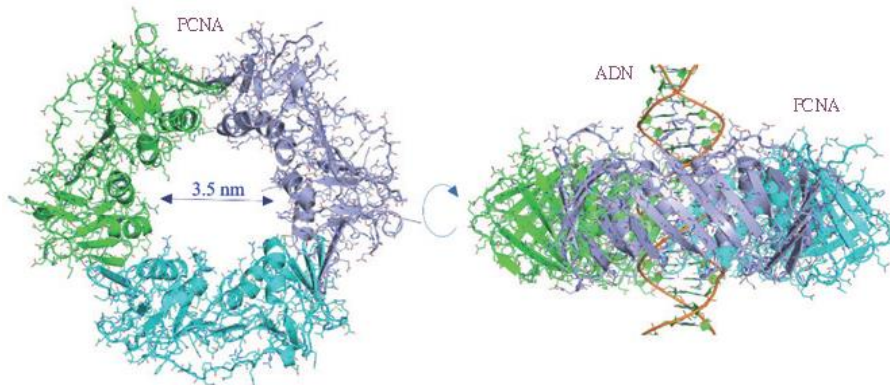
Différents types de dommage à l'ADN proviennent d'erreur de réplication de l'ADN ou d'arrêts du replisome. Certaines protéines de réparation de l'ADN archéennes comme Fen1 et potentiellement l'ADN polymérase PolB sont aussi multitâches entre réplication et réparation de l'ADN. Ces observations supportent l'hypothèse de l'existence de mécanismes efficaces et spécifiques permettant la coordination entre la réplication de l'ADN et les divers mécanismes de réparation de l'ADN, tel qu'exposé plus haut.

Dans tous les domaines du vivant, le facteur de processivité (PCNA) agit comme conducteur majeur des différentes transactions de l'ADN. Chez les archées, des facteurs de processivité, tant homo- qu'hétéro-trimère, avec une structure en trois dimensions en forme d'anneau ont été décrits (MacNeill 2016). Ces protéines étaient à l'origine nommées « antigène nucléaire de prolifération cellulaire (PCNA), puisque leur orthologue humain avait originellement été identifié en tant qu'antigène présent dans les noyaux des cellules pendant leur division, réagissant avec un auto-anticorps dans le sérum des patients souffrant de lupus érythémateux. Plus tard, il a été démontré que le PCNA humain entoure l'ADN et agit comme facteur de processivité des ADN polymérase répliquatives eucaryotes.

Les structures de PCNA de certaines espèces archéennes ont été décrites. Elles incluent des homotrimères des espèces euryarchaeota thermophiles *Pyrococcus furiosus* et *Archaeoglobus fulgidus*, ainsi que l'haloarchée *Haloferax volcanii*. Les espèces *Sulfolobus* et d'autres crénarchéotes utilisent l'hétérotrimère de PCNA (Pan *et al.* 2011). Les structures en trois dimensions du PCNA archéen sont pratiquement identiques à celles de leurs équivalents humains. Le diamètre intérieur de l'anneau de PCNA est d'approximativement 3,5 nm, ce qui est assez grand pour contenir l'hélice de l'ADN (forme B), qui a un diamètre de 2 nm (figure 2.8). L'une des faces de l'anneau de PCNA interagit avec de nombreuses protéines de la réplication et de la réparation de l'ADN, tandis que l'autre face n'est impliquée dans aucune interaction protéine-protéine directe. La surface extérieure du trimère de PCNA en forme d'anneau porte des charges négatives, tandis que la cavité centrale, qui contient l'ADN, est chargée positivement. Un monomère de PCNA se compose de deux domaines reliés par une boucle de connexion des domaines. Cette boucle s'est conservée au cours de l'évolution et est l'intermédiaire d'interactions spécifiques de PCNA avec ses partenaires.

Pour être fonctionnellement actives, les différentes protéines PCNA interagissent en s'associant et en se dissociant de manière ordonnée dans le temps. Il serait assez surprenant que les motifs des domaines peptidiques identifiés sur les protéines PCNA interagissant (motifs PIP) archéennes soient suffisamment similaires et aient des sites de liaison se recoupant (tableau 2.1). Par exemple, le motif de séquence Q-x(2)-(h)-x(2)-(a)-(a) (h, résidu modérément hydrophobe ; a, un résidu hautement hydrophobe) est connu pour être l'intermédiaire d'interactions des PCNA archéennes et humaines avec de nombreux partenaires (voir, par exemple (Meslet-Cladiere *et al.* 2007)). Les variations dans les séquences des motifs de liaison archéens avec PCNA peuvent soutenir l'hypothèse d'une

liaison différentielle des différents partenaires protéiques de PCNA, facilitant ainsi la coordination des différentes forces de transactions de l'ADN.



*Figure 2.8. Deux vues orthogonales du PCNA archéen formant un complexe avec l'ADN. Les monomères de la structure homotrimère de PCNA sont colorés en cyan, en vert et en violet. Le pore dans PCNA est indiqué par une flèche*

De nombreux exemples d'interactions fonctionnelles entre PCNA et d'autres protéines ont été décrits dans la littérature. Par exemple, PCNA stimule sensiblement la récessivité des ADN polymérases, ADN ligases et endonucléases archéennes. De plus, le fait que l'interaction entre PCNA et UDG participe dans la BER archéenne a été reporté tant chez les crenarchaeota que les euryarchaeota, et il a été proposé qu'une telle interaction puisse permettre aux UDG d'être recrutées au niveau de la fourche de réplication une fois que l'ADN polymérase est bloquée au niveau de l'uracile. Par conséquent, la PCNA archéenne fonctionne probablement comme une plateforme qui permet la coordination des différentes voies de réparation de l'ADN. Le tableau 2.2 indique des exemples additionnels des interactions de la PCNA archéenne.

Protéine archéenne	Effet de la PCNA	Voie de réparation de l'ADN	Organismes (exemples)
<b>Fen1</b>	Stimulation	BER, RER	<i>P. abyssi</i> , <i>S. solfataricus</i>
<b>RnaseH2</b>	Recrutement ?	RER	<i>P. abyssi</i> , <i>A. fulgidus</i>
<b>PolB</b>	Augmentation de la processivité	Nombreuses	<i>P. furiosus</i> , <i>S. solfataricus</i>
<b>UDG</b>	Stimulation	BER	<i>P. aerophilum</i> , <i>P. furiosus</i>
<b>ADN ligase</b>	Recrutement	Nombreuses	<i>P. abyssi</i>
<b>AP endonucléase</b>	Stimulation	BER	<i>P. furiosus</i>
<b>XPF</b>	Stimulation	NER ?	<i>S. solfataricus</i>
<b>NucS/EndoMS</b>	Stimulation	MMR	<i>Thermococcales</i>
<b>Hjc</b>	Stimulation (nucléase)	HR	<i>S. solfataricus</i>
<b>Hjm</b>	Stimulation (hélicase)	HR	<i>P. furiosus</i>
<b>Mre11-Rad50</b>	Modulation de la spécificité de la nucléase	HR	<i>P. furiosus</i>

Tableau 2.2. Exemples de modulations fonctionnelles des enzymes archéennes de réparation de l'ADN par le facteur de processivité de la polymérase PCNA

## 2.5. Résumé et conclusion

Les efforts intensifs en génomiques comparatives, en génétique, en biochimie, et en biologie structurale ont, au cours des dernières années, révélé que les archées utilisent beaucoup de voies parallèles pour garantir l'entretien de l'information génétique, parfois dans des conditions extrêmes. Le but de ce paragraphe sur la réparation archéenne de l'ADN était de souligner comment des facteurs endogènes et exogènes causent des dommages à l'ADN et comment ces événements peuvent être amplifiés en lien avec des facteurs environnementaux, tels que de hautes températures. Nous avons aussi souligné la diversité phylogénétique (tableau 2.3) et mécanistique, avec laquelle l'ADN endommagée est efficacement réparée par

de multiples voies de réparation de l'ADN. Il est frappant que les archées puissent apparemment utiliser non seulement des enzymes uniques de réparation de l'ADN, mais également leurs équivalents « bactériens » ou « eucaryotes ». Il est probable que pendant les années à suivre, nous continuerons à découvrir de nouvelles enzymes archéennes de réparation de l'ADN dans les nouveaux groupes phylogénétiques encore sous-étudiés, venant seulement d'être découverts.

Groupe	RPA	EndoMS (NucS)	Uvr ABC	Mut SL	XPF euk/short	XPBB	XPB	Bax1
<b>Filarchaeota :</b>								
<i>Sulfolobus</i>								
<i>Cenarchaeum</i>								
<i>Thorarchaeum</i>								
<i>Lokiarchaeum</i>								
<b>Euryarchaeota:</b>								
<i>Thermococcus</i>								
<i>Methanosarcina</i>								
<i>Haloferax</i>								
<i>Thermoplasmama</i>								
<i>Nanoarchaeum</i>								

Tableau 2.3. Représentation simplifiée de la distribution phylogénétique des principaux gènes de réparation de l'ADN chez des espèces archéennes clés, des lignées Filarchaeota Eteuryarchaeota.

## 2.6. Bibliographie

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## Conclusion and Perspectives

### Scientific context

Examining genomic integrity has led to numerous insights into our understanding of species diversification and offspring survival over generations (Kang *et al.*, 2006). All living organisms need DNA repair systems to correct lesions and avoid mutations. MMR is essential for genomic maintenance. It repairs mismatches that occur during DNA replication and persist despite the proofreading activity of DNA polymerases, thereby contributing to genomic stability (Friedberg *et al.*, 2006). MMR also regulates HR in bacteria to suppress recombination events between dissimilar DNA sequences (Spies *et al.*, 2015; Zahrt *et al.*, 1997). The DNA repair mechanisms become even more important when antibiotic-resistant strains emerge. Antibiotic-resistant strains, such as *M. tuberculosis* and *M. leprae* (Gygli *et al.*, 2017), pose health challenges and underscore the need to understand DNA repair pathways and develop new treatments to face these threats. Dysregulation of the DNA repair pathways, particularly MMR, greatly influences antibiotic resistance and disease emergence (Evans *et al.*, 2000; Tham *et al.*, 2016). Furthermore, substantial crosstalk exists between DNA repair systems (Hakem R, 2008). For example, a tight association between MMR and HR implies that the suppression of one system is very likely to affect the activity of another (Spies *et al.*, 2015).

While some archaea and bacteria, such as members of the Actinobacteria phylum, do not encode canonical MMR enzymes MutS/L, they do not suffer a higher mutation rate (Sachadyn P, 2010). Research has revealed that some of these organisms possess NucS, an endonuclease shown to be involved in the repair of mismatches in a non-canonical way (Castañeda-García *et al.*, 2017), implying that NucS could complement the absence of the MMR. Regulation and competition between DNA damage repair pathways and their links to cell growth and division are only beginning to be understood in Corynebacteria, let alone in the broader phylum of Actinobacteria.

Some species, such as *M. tuberculosis*, show various degrees of virulence and pathogenicity influenced by the expression of proteins implicated in DNA repair mechanisms, such as NucS (Gonzalez *et al.*, 2012; Covacci *et al.*, 1997). While significant evidence has been provided for the role of NucS in a non-canonical MMR, this endonuclease's additional roles in DNA repair pathways beyond the MMR remain uncharacterised. NucS is proposed to act as a

multi-enzyme, with its activity on branched subtracted or deaminated bases possibly correlated with interaction with replication machinery through an association with DnaN (Ren *et al.*, 2018; Zhang *et al.*, 2019; Zhang *et al.*, 2020).

Given the activity of NucS, as a DSB endonuclease demonstrated in the literature and our suspicion that NucS carries out an activity involving several DNA repair pathways through its multi-enzyme function, we were interested in determining whether NucS interacts with DSB and plays a role in their repair. DSBs are among the most deleterious forms of DNA damage and represent a severe threat to cellular integrity and cell viability (Kong *et al.*, 2021; Mladenova *et al.*, 2022; Oster *et al.*, 2020). Complete breaks of both strands in the DNA helix interrupt the continuity of the genetic information. Unless quickly and properly repaired, it could cause chromosomal aberrations, loss of genetic material, or cell death (Pfeiffer *et al.*, 2000; Richardson *et al.*, 2000). The loss of timely repair can lead to the formation of chromosomal rearrangements, a hallmark of cancer cells (Liu *et al.*, 2017). While DSBR plays a broader role in cellular survival, the importance of DSB has been exploited for therapeutic purposes, especially in cancer treatment, as the induction of DSB would ultimately lead to cell death. Thus, it is a critical process that is targeted by therapeutic strategies (Samadder *et al.*, 2016; Schrepf *et al.*, 2020; Trenner *et al.*, 2019).

After the induction of a DSB, cells can use several repair pathways, each characterised by its own mechanisms, fidelity and energy requirements. Among these, the majors are HR and NHEJ (Her *et al.*, 2018). The first cited, HR, utilises an identical sequence, usually the sister chromatid, to that of the original segment of DNA as a template for fixing the break to restore the break faithfully (Carr *et al.*, 2013). It is considered a high-fidelity repair mechanism since it depends on an identical sequence guaranteeing proper repair. However, HR is a very energy-demanding process as it requires a significant number of partners, the stabilisation of specific DNA conformation and the complete synthesis of new DNA strands (Wright *et al.*, 2018; Savir *et al.*, 2010; Clark *et al.*, 1994). Moreover, this pathway is mainly active during the S and G2 phases of the eukaryotic cell and the exponential growth phase of the bacteria, when the sister chromatid is available as a template (Arnoult *et al.*, 2017).

NHEJ directly ligates the DNA ends without requiring a homologous template. As a result, this pathway is more error-prone than HR and can, thus, lead to small insertions or deletions at the repair site. However, NHEJ is less energy-consuming and faster than HR (Mao *et al.*,

2008). This pathway is often activated during the G0/G1 or stationary phase for bacteria when the cell is not actively dividing (Mao *et al.*, 2018; Frank-Vaillant *et al.*, 2002; Karanam *et al.*, 2012).

MMEJ repairs DSBs by aligning microhomologous sequences at break sites, leading to the deletions of the DNA flanking region at the DSB site (McVey *et al.*, 2008). This is one of the higher error-prone mechanisms, often resulting in the deletion of sequences or the introduction of other mutations in the genome (Jiang Y, 2022). The growth state of the cell and the availability of repair templates are not required for this pathway (Sfeir *et al.*, 2024). Hence, these different mechanisms compete and are influenced by the cell's growth state, availability of repair templates and the specific proteins and complexes involved in the DNA damage response (Hartlerode *et al.*, 2009; Meyer *et al.*, 2015).

### **Results and perspectives**

Our bioinformatics analysis supports the hypothesis that NucS may interact with DSBR and affect HR and MMEJ. The interaction of NucS with other proteins was predicted to involve five helicases participating in NER and other repair pathways. These results tend to reinforce the hypothesis of NucS' role in an early stage of DNA repair due to a possible direct intervention at the damaged site of the DNA. The presence of genes directly involved in the replication and DNA repair, such as RadA, also conveyed the idea that NucS might be recruited to sites of damage or stalled replication forks and play a role in genomic stability maintenance.

Interestingly, our analysis of *nucS*' neighbouring genes indicated the presence of genes related to energy production and metabolic pathways. The fact that such genes may be associated with NucS could emphasise that DNA repair activities are energetically demanding and must be regulated appropriately based on the cell's metabolic state (Foster, 2007; Maddocks *et al.*, 2008). This gene neighbourhood suggests a regulatory mechanism at play whereby NucS gets regulated in response to ATP levels, allowing the cell to commit the resources to DNA repair. Together, these results have enhanced our understanding of NucS activity *per se* and its links with replication and cellular metabolism in an interconnection that brings into focus a bigger picture of how cells maintain stability in response to damage.

Future research should experimentally explore these bioinformatics predictions by investigating the regulation of *nucS* by the metabolic state of the cell and DNA damage levels, as well as the response of NucS to long-term stress exposition.

Phylogenetic studies supported NucS as an archaeal protein transferred horizontally to extremophilic Actinobacteria (Castaneda-Garcia *et al.*, 2017). This feature might well mirror its role in adaptation to extreme environments and its significance in the DNA repair process. This evolutionary view expands the understanding of NucS's role in maintaining genomic stability under stress conditions. The DDR could be particularly significant for extremophile organisms thriving in harsh conditions, including high temperatures, radiation and extreme pH levels (Ishino *et al.*, 2015). These organisms frequently experience high levels of DNA damage due to their extreme environments. The presence of NucS in extremophile organisms suggests an evolutionary adaptation that enhances their DNA repair capabilities, allowing them to survive and proliferate under extreme conditions. Consequently, the activity of this enzyme could be crucial for extremophiles, such as *P. abyssi* and *T. kodakarensis*, which must repair frequent DNA damage induced by their environments to maintain cellular functions and integrity (Chen *et al.*, 2022). Broader species comparisons of the *nucS* sequence will increase our insight into its evolution and functional diversification. This approach would highlight how its role in DNA repair may have evolved in response to specific ecological niches and challenges.

Bioinformatic simulations have revealed NucS's structural and functional properties. This protein's ability to bind and cleave mismatched DNA suggests a role in DNA repair beyond conventional MMR. Our *in silico* modelling studies of *MtbNucS* bound on a DSB have revealed a DNA conformation that could favour the MMEJ. The ability of NucS to stabilise free DNA ends generated by a DSB that contains a mismatch could also play a part in the alignment of microhomologous sequences. Hence, NucS could be a candidate to participate in the initial steps of MMEJ, where microhomologies must be identified and matched.

Further structural studies of NucS, especially in complexes with DNA substrates, would help elucidate the molecular basis of its interactions and enzymatic activities. Resolution in the atomic range could provide critical information from techniques such as cryo-electron microscopy (cryo-EM) and X-ray crystallography. Comparative genomics across a wider diversity of species will shed more light on the evolution of NucS and the functional diversification of these nucleases.

To gain new insight into the cellular functions of NucS, especially in DNA damage repair and cell cycle, we described phenotypes associated with a genetic background deleted for *nucS* in *C. glutamicum*. Initial morphological and growth tests did not reveal any striking differences; however, a valuable data set was obtained from a competitive growth test carried out under treatment with genotoxic stress such as CIP, Phleo or MMC. From our results, the deletion of *nucS* seems highly beneficial to the bacterial population under conditions of genotoxic stress, particularly when exposed to agents known to result in DSBs introduction. Notably, this advantage could be linked to the ability of the bacteria to repair complex DNA damage rather than a general growth superiority. An absence of competitive edge under non-DSB-inducing conditions like Gen exposure was noted. This advantage is hypothesised to stem from an altered balance between DSBR mechanisms. The absence of NucS may dysregulate and alter the DSBR by affecting the repair of mismatches close to DSBs and influencing an alternative mechanism involved in the DSBR. Suppressing NucS's inhibitory activity on HR may lead to an elevated HR rate. This hypothesis may also implicate changes in the cell's energy consumption and allocation depending on the pathway selected for DSBR, which would be altered in the absence of NucS.

Comparing DSBR pathways reveals varying energy consumption levels, actor requirements and repair fidelity (Kochan *et al.*, 2017; Ahrabi *et al.*, 2016) . If NucS promotes MMEJ over HR, the  $\Delta nucS$  genetic background may favour a more energy-efficient repair process, potentially leading to increased genetic mobility and rearrangement. An enhanced capacity for HR in such a  $\Delta nucS$  strain would then augment the accuracy of the repair of DNA damage induced by antibiotics like Phleo and CIP, allowing them to adapt swiftly to hostile environments by accelerating genetic reshuffling.

Further experiments should include tracking growth change between these two bacterial populations by expressing fluorophores such as GFP and mScarlet and introducing different genotoxic agents. It would give us a better understanding of the specificity of NucS. Furthermore, it would be interesting to pre-stress the cells and look at the competition after removing the stressing factor. This last condition may give insight into the survival mechanisms under the stationary phase.

In this work, we aimed to test the function of NucS in DNA repair pathways other than MMR. Observations of DSBR dynamics in *CglWT* and *CglΔnucS* cells using the Gam-meGFP reporter system revealed that the repair dynamics of DSBs in *CglΔnucS* differed from *CglWT* cells. The deleted genetic background showed more Gam-meGFP foci, possibly due to an increased number of unrepaired DSBs, eventually linked with an upper time remanence in the cell. We also observed more RecA-mVenus foci in *CglΔnucS*, revealing a higher activation of HR, confirming the involvement of NucS in DSBR pathways. Two hypotheses arise about the previous observations: that NucS directly inhibits HR or interacts with free DNA ends to promote a repair pathway alternative to HR. The latter suggests NucS favours a faster repair pathway like MMEJ over HR, reducing the remanence time of DSBs in the cell.

By analysing NucS recruitment in response to different stress conditions, we observed a dual localisation of mScarlet1–NucSD144A: the construction is either present diffusely in the cell or aggregated in polar foci. The localisation of the construct at the pole follows 30 minutes after a treatment with a genotoxic agent. Advanced microscopy techniques revealed that NucS remains attached to DNA during cell death. Moreover, extreme stress induced multiple foci organised in a complex network. These findings suggest placing NucS as proteins, like Rad51 and Ku, in a process that occurs right after the recognition of DNA damage. Comparison of NucS to RecA, which is involved in the HR process, suggests that our protein of interest may also stabilise the DNA ends before repair, maybe through MMEJ. This aligns with our bioinformatic's predictions that NucS could bind to DNA substrates with DSBs and stabilise the DNA structure. This activity would prevent exonuclease activity and favour one repair.

Future studies should incorporate advanced techniques such as microfluidic devices for live-cell imaging and single-molecule studies (Sipos *et al.*, 2021). These advanced techniques would help understand the activity and dynamic of NucS recruitment at the site of damage, providing high-resolution images. Most importantly, co-localization studies should be performed with NucS and other DNA repair proteins, such as RecA and DnaN. Finally, the impact on the recruitment and the physiological response of NucS in a strain deleted of the HR could offer another approach.

## Discussion and broader directions

### Post-translational modifications and additional factors in the regulation of NucS expression

Understanding the regulation of *nucS* expression will be critical in figuring out how it fits into DNA repair. Further research should examine how its expression is controlled at the transcriptional and post-transcriptional level, what proteins or RNAs may regulate and modify its expression according to DNA damage or other stress signals. Post-translational modifications or cofactors could significantly influence NucS activity. This might be important in the DNA-binding ability of NucS or its nuclease activity. Identifying these modifications might provide insight into NucS activity regulation under a different cellular setting. Identifying cofactors interacting with NucS could provide information about the conditions at which NucS is most active and potent (Shrivastav *et al.*, 2009).

It will further delineate, for example, whether NucS expression is linked to specific cell cycle phases, how its activity is coordinated with DNA replication, or in response to DNA damage—hence providing a better picture of its functional role in maintaining genomic integrity. It would be interesting to know if NucS expression and activity respond to environmental factors, including nutrient availability or oxidative stress, thus linking DNA repair to the cell's metabolic state and general health.

### Elucidating complete repair pathways

Future research should aim to elucidate the complete repair pathways following the initial cleavage of the DNA by NucS. This would involve unveiling the roles of other enzymes in processing cleaved DNA and reconstituting genomic integrity. Understanding how NucS interacts with the replication machinery could reveal additional details of the functional interplay between DNA replication and repair. For example, pull-down experiments and SPR can be conducted to uncover the proteins interacting with NucS and, therefore, its complete repair pathway. The elucidation of mutants could aid in defining the role of different domains and residues in NucS activity (Wang *et al.*, 2021). Such studies would consequently provide more insights into how NucS works in collaboration with other components of the DNA repair machinery towards the maintenance of genomic stability.



### **Biotechnological applications and therapeutic strategies**

The knowledge derived from the study of NucS could be used to develop biotechnological applications that function under extreme conditions. NucS's capability of DNA cleavage is similar to restriction endonucleases and its activity at high temperatures within extremophile archaea opens ways for its application in molecular biology and biotechnology. In addition, NucS could be used in new therapeutic interventions against mycobacterial diseases such as tuberculosis (Cardoso *et al.*, 2022). Using NucS under such circumstances might provide novel ways of combating antibiotic resistance and increasing treatment efficacy.

The study of NucS could also represent significant edges for understanding antibiotic resistance. Antibiotics that induce DNA damage, such as quinolones, can create selective pressure for bacteria to select enhanced DNA repair mechanisms (Merschundermann *et al.*, 1994; Thomas *et al.*, 1990). NucS, by facilitating the repair of antibiotic-induced DNA damage, could contribute to the survival of bacteria under antibiotic stress. This repair capability may enable bacteria to maintain genomic integrity and avoid a lethal accumulation of mutations, eventually contributing to the development of antibiotic resistance. Knowledge about the relationship of NucS activity with antibiotic resistance could inform the development of novel therapeutic strategies that target DNA repair pathways with new means of potentiation of the efficacy of antibiotics. This could include the design of NucS-targeted inhibitors or interacting partners, thereby sensitising bacteria to DNA-damaging antibiotics and reducing the emergence of resistant strains.

### **Polar localization and DNA uptake**

One remarkable feature of NucS is its polar localisation within bacterial cells. This localisation might be linked to the mode of DNA uptake, which is also, in many bacteria, carried out preferentially at the cell poles (Hahn *et al.*, 2005). Therefore, it is reasonable to consider that the polar localisation of NucS is critical to regulating the incorporation of exogenous DNA fragments. NucS may be positioned strategically to process and stabilise those incoming DNA fragments during horizontal gene transfer events, such as the transformation, during which bacteria take up DNA from their environment. Such action may avert unwanted HR or deleterious genetic material integration and maintain genomic integrity. This hypothesis suggests a dual role for NucS in both endogenous DNA repair and regulating the integration of exogenous DNA, adding another layer of complexity to its function in the cell.

The complex filamentous-like localisation of NucS observed in our study could be similar to the cellular response to persistent DNA DSBs where Rad51 spreads chromosome-wide from the DSB site and the damaged chromosome is eventually fixed to the nuclear periphery, involving the histone variant H2A.Z and its SUMOylation (Chen *et al.*, 2021; Hendriks *et al.*, 2015). NucS could thus be localised to foci at cellular poles to promote an ordered repair reaction analogous to the clustering of DSBs at the nuclear periphery. The potential interactions between NucS and other repair proteins in DNA damage recognition and stabilisation could mirror the coordinated actions of Rad51 and H2A.Z in response to DNA breaks (Sung *et al.*, 1995; Haas *et al.*, 2018). This suggests that the polar localisation of NucS may be an evolutionarily conserved strategy to increase the efficiency and accuracy of DNA repair, hence ensuring cell survival under genomic stress.

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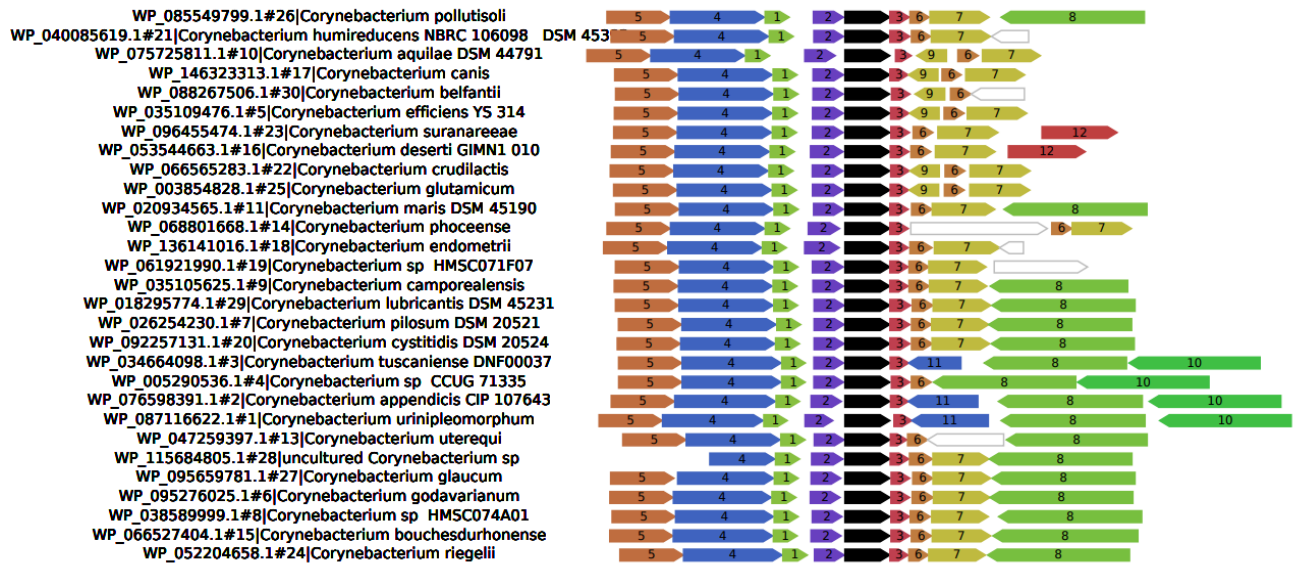
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## **Appendix**

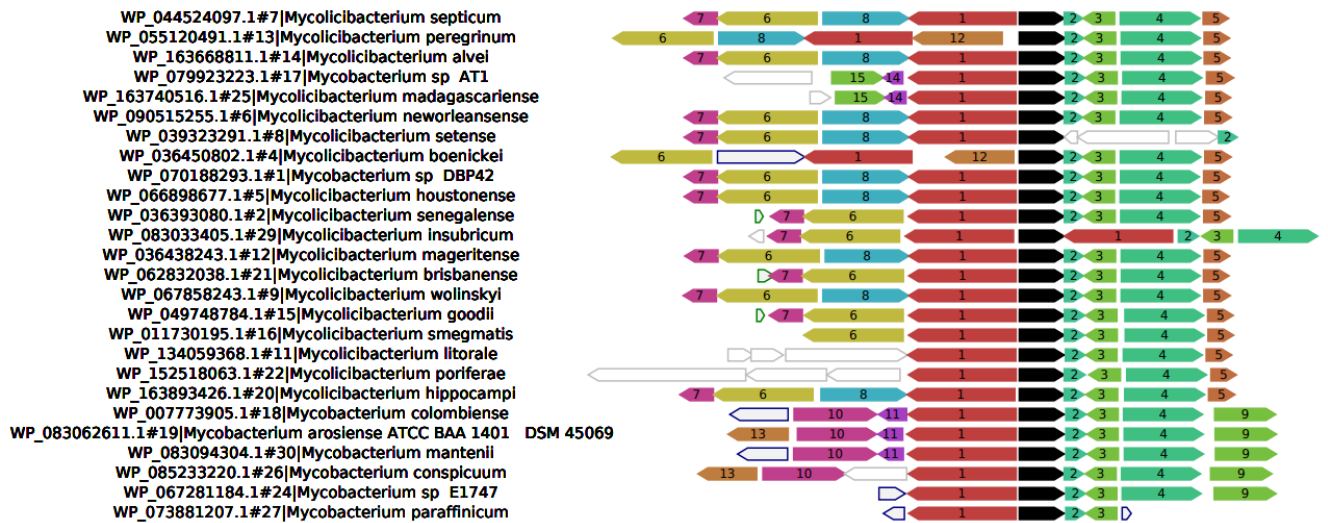


**Annexe 1** Prediction of protein functional association by conservation of the genomic neighbourhood surrounding *CglnucS* in Corynebacteria.



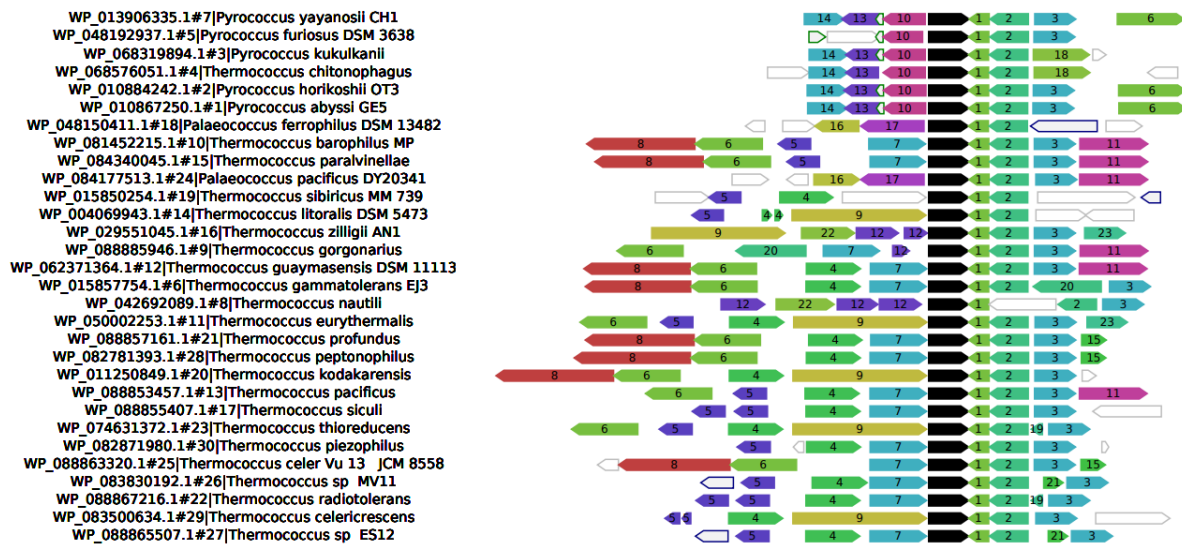
1. ATP synthase subunit epsilon
2. DUF2450 domain-containing protein
3. hypothetical protein
4. F0F1 ATP synthase subunit beta
5. F0F1 ATP synthase subunit gamma
6. thiamine-binding protein
7. tetratricopeptide repeat protein
8. 1,4-alpha-glucan branching protein GlgB
9. methylmalonyl-CoA epimerase
10. maltotransferase domain-containing protein & DUF3416 domain-containing protein
11. HNH endonuclease signature motif containing protein
12. LLM class flavin-dependent oxidoreductase

**Annexe 2** Prediction of protein functional association by conservation of the genomic neighbourhood surrounding *MtbnuoS* in Mycobacteria.



1. adenylate/guanylate cyclase domain-containing protein
2. hypothetical protein
3. methylmalonyl-CoA epimerase
4. acetyl-CoA C-acetyltransferase
5. DUF3817 domain-containing protein
6. DNA-3-methyladenine glycosylase 2 family protein
7. methylated-DNA—
8. glutaminase A
9. tetratricopeptide repeat protein & co-chaperone YbbN
10. MFS transporter
11. hypothetical protein
12. RNA-guided endonuclease TnpB family protein & helix-turn-helix domain-containing protein
13. LysR family transcriptional regulator
14. hypothetical protein
15. hypothetical protein

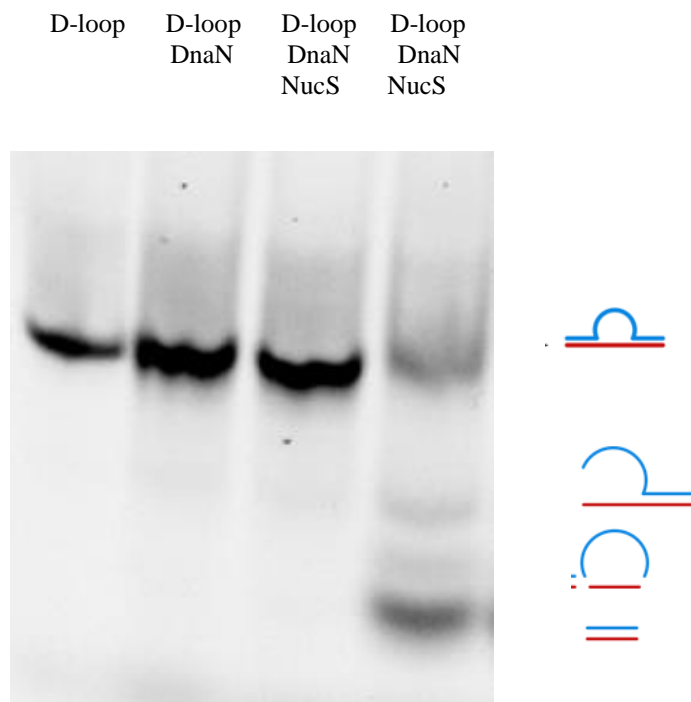
**Annexe 3** Prediction of protein functional association by conservation of the genomic neighbourhood surrounding *PabnuoS* in Pyrococcus.



1. DUF473 family protein
2. proteasome assembly chaperone family protein
3. S-methyl-5'-thioadenosine phosphorylase
4. ion channel & NAD-binding protein
5. DNA-binding protein
6. ORC1-type DNA replication protein
7. DNA repair and recombination protein RadA
8. DNA-directed DNA polymerase II small subunit
9. DNA repair and recombination protein RadA
10. DUF63 family protein
11. amidohydrolase family protein
12. hypothetical protein
13. hypothetical protein
14. energy-coupling factor transporter transmembrane
15. thermonuclease family protein
16. DMT family transporter
17. glycine cleavage system aminomethyltransferase GcvT
18. ATP-binding protein
19. hypothetical protein
20. hypothetical protein
21. hypothetical protein
22. hypothetical protein
23. halocin C8-like domain-containing protein & hypothetical protein

#### Annexe 4 Biochemical study of *Cg*/NucS purified on intermediates of recombination

We purified *Cg*/DnaN and *Cg*/NucS using the same protocol as in Ren *et al.*, 2016. We used in addition the plasmid PGKJE8, coding for chaperon proteins (GroEL), to stabilize NucS and reduce the precipitation of the protein during the purification. Analysis of the samples by DLS has permitted us to confirm the quality of the protein. Activity gels with urea have also been conducted to confirm the activity of NucS on mismatch as published in the literature. Different templates of DNA have been chosen regarding previous experiments published in the literature. To begin with, we assembled a simple construction based on two strands of DNA forming a Loop, representing early DNA structure during replication. Only the top segment of DNA containing the nucleotides of the loop was marked by Cy5. Experimentation with a version of NucS that we mutated in its catalytic site (D144A) and purified with the same protocol has permitted us to prove that this activity is specific as only visible when NucS is active. With this DNA construction, we observe what seems to be a double-strand break activity of NucS on the loop.



Cleavage of loop DNA by *Cg*/NucS-WT with *Cg*/β-clamp (DnaN). The 5'-Cy5-labeled DNA substrates (5 pM) containing the loop were incubated with the proteins. The products were analyzed by denaturing 15 % Urea gel followed by laser scanning, cropped gel image (20 nM (3- 20 nM of NucS, 7nM of DnaN) Image taken with a Chemi Doc MP Imaging System.

### **Protocol Electromobility shift assay (EMSA)**

Fluorescently labeled oligonucleotide substrates (D-loop) were prepared by annealing the 90bp 5' - 3' fluorescent-labeled (CY3) D-looptop strand with the complementary 90bp D-loopbot strand in a buffer containing 10mM Tris, 150mM NaCL, 1mM EDTA and pH 8.0 by heating at 99 °C for 5 mins (Denaturation) and slowly cooling down to the room temperature (Annealing and Renaturation). Different concentrations of the purified NucS (0.1 μM, 0.3 μM, 0.5 μM, 1 μM, 2 μM) were tested with the 18 μL master mix (containing 2 μL of the annealed DNA substrate (200-fold dilution) + 2 μL of 10x TBE buffer (pH 6.4) + 2 μL of 20 μM DDT + 2 μL of 50 % glycerol + 8 μL of H<sub>2</sub>O + 1 μL of MgCl<sub>2</sub> + 1 μL of 0.1 μM DnaN protein), which were placed in an incubator at 30 °C for 30 mins before the addition of 3 μL 6x orange loading dye.

This mix was loaded onto the 12 % pre-run EMSA gel (pre-run at 150V, 4 °C for 30 mins) and was run at 210V, 4 °C for about 6 hours. The gel was later visualised using the ChemiDoc MP Imaging System.

## Résumé en français

La précision de la réplication de l'ADN est essentielle pour la transmission correcte de l'information génétique chez tous les êtres vivants. Lors de ce processus, chaque cellule doit copier son ADN avec fidélité pour garantir que les informations génétiques soient correctement transmises à la génération suivante. Cependant, l'introduction de dommages à l'ADN peut perturber cette précision. Ces dommages peuvent être causés par des facteurs externes tels que les radiations ionisantes, qui induisent des cassures dans l'ADN, ou les antibiotiques, pouvant également cibler et dégrader directement la molécule d'ADN. En outre, des événements internes, tels que des erreurs spontanées lors de la réplication de l'ADN ou des défauts dans les mécanismes de réparation de l'ADN, peuvent également compromettre l'intégrité génomique. Bien que les mutations augmentent la diversité génétique et contribuent à l'évolution des populations microbiennes, il est crucial pour la survie de ces organismes de prévenir la fixation mutations nocives. Ces dernières peuvent entraîner des dysfonctionnements cellulaires, mener à la mort cellulaire ou favoriser l'apparition de résistances aux antibiotiques. Ainsi, les systèmes de réparation de l'ADN jouent un rôle crucial en corrigeant les erreurs de réplication et en maintenant la stabilité génomique, assurant la survie des organismes.

Le système canonique de réparation des mésappariements (MMR), impliquant des protéines telles que MutS et MutL, est décrit en finesse dans la littérature. Ces protéines jouent un rôle crucial dans la correction des erreurs de réplication de l'ADN en identifiant et en réparant les mésappariements. Cependant, ces protéines sont absentes chez les Actinobactéries, telles que *Mycobacterium tuberculosis* et *Corynebacterium glutamicum*, ainsi que chez certaines archées, ce qui a conduit à la découverte d'une voie de MMR alternative impliquant la protéine NucS (EndoMS).

NucS présente une activité endonucléasique au niveau des mésappariements, en causant des cassures double-brin (DSBs). Plusieurs points de comparaison avec MutS et MutL sont soulignés dans la littérature tels que l'interaction avec DnaN, qui intervient dans la processivité de NucS ainsi que la coordination de son activité. Son indépendance de l'ATP marque cependant l'un de principal point de contraste avec le système canonique de MMR. Malgré des recherches approfondies sur l'activité de NucS en laboratoire, sa fonction *in vivo* demeure en grande partie inconnue, de même que son rôle potentiel dans d'autres systèmes de réparation des dommages à l'ADN (DDR).

Au cours de ce doctorat, l'accent a été mis sur la caractérisation de la fonction de NucS en présence d'un substrat différent aux mésappariements, les cassures double-brin (DSBs). Par ailleurs, cette étude s'inscrit dans un cadre plus large visant à comprendre les interactions entre les acteurs moléculaires impliqués dans l'activation et la régulation des voies de réparation des dommages de l'ADN, lesquels pourraient représenter des cibles thérapeutiques potentiels.

Pour ce faire, nous avons concentré notre attention sur la caractérisation d'un fond génétique de *C. glutamicum* délété pour *nucS*, dans le but de vérifier l'hypothèse de l'intervention de NucS dans la croissance cellulaire, notamment soutenue par son interaction avec DnaN.

En accord avec les résultats obtenus dans cette première section nous avons poursuivi notre analyse en utilisant l'imagerie confocale et la microscopie 3D SIM pour explorer le rôle de NucS dans la réparation des DSBs et l'activation de la recombinaison homologue (HR). Lors de ce volet de nos recherches, nous avons également étudié la localisation de NucS, observée pour la première fois dans des cellules vivantes, ainsi que sa réponse physiologique à l'introduction de stress genotoxiques, particulièrement ceux causant des DSBs.

Enfin, nous avons développé une analyse bioinformatique de l'interactome de NucS et du voisinage génétique du gène codant pour cette protéine. L'analyse de la modélisation de l'activité de NucS sur les DSBs, ainsi qu'une nouvelle prédiction de la phylogénie de *nucS* ont apporté plus de robustesse aux approches expérimentales et ouvert vers la formulation de nouvelles hypothèses. Ces approches combinées ont permis de mieux comprendre le rôle de NucS dans le maintien de l'intégrité génomique et ses interactions potentielles dans des voies de réparation de l'ADN autres que celle des mésappariements.

## **Chapitre 1**

Comprendre la survie et la diversification des espèces implique d'étudier les mécanismes à l'origine du maintien de l'intégrité génétique. Les processus de réparation de l'ADN sont essentiels pour que les cellules restent viables et évitent les mutations. Inspiré par les résultats de récentes études sur NucS chez les archées ainsi que son interaction démontrée chez les bactéries avec DnaN, nous avons considéré l'hypothèse que NucS soit impliqué dans la croissance cellulaire et puisse intervenir dans plusieurs mécanismes de réparation de l'ADN. Par cette dernière hypothèse, nous avons choisi de mettre l'accent sur la réparation des DSBs, et l'exploration de l'implication de NucS dans la régulation de voies recrutées pour

la réparation ce de type de dommages telles que la HR ou encore la jonction des extrémités par microhomologie (MMEJ). Dans le but de vérifier ces hypothèses, nous avons comparé les mesures de croissance de *Corynebacterium glutamicum* de type sauvage (*Cgl*WT) avec celles d'un mutant dépourvu de *nucS* (*Cgl* $\Delta$ *nucS*). Nos résultats montrent qu'en l'absence de stress, il n'y a pas de différences significatives en termes de temps de génération ou de profils de courbes de croissance entre les deux fonds génétiques, suggérant que NucS n'est pas essentiel pour la croissance cellulaire dans des conditions normales. De plus le test de culture en compétition réalisé dans des conditions normales indique que *Cgl* $\Delta$ *nucS* n'affiche pas d'avantage compétitif dans une co-culture de 24 heures avec *Cgl*WT. Cependant, en réaction à l'introduction d'agents génotoxiques, tels que la phléomycine, la mitomycine C ou la ciprofloxacine dans la co-culture, les bactéries *Cgl* $\Delta$ *nucS* présentent un avantage compétitif par rapport au fond génétique sauvage. Nous avons noté une absence de cet avantage compétitif en présence de la gentamicine qui n'induit pas la formation de DSBs. Ces analyses soulignent l'importance de NucS dans les réponses cellulaires aux dommages de l'ADN et suggèrent que NucS pourrait être un acteur clef de la réparation des DSBs.

## Chapitre 2

Les mécanismes de réparation de l'ADN jouent un rôle crucial dans la correction des dommages génomiques, qu'ils soient endogènes ou exogènes, ce qui est particulièrement le cas pour les DSBs qui sont hautement toxiques. La réparation fidèle et prompte des DSBs est donc indispensable pour préserver l'intégrité génomique et assurer la survie des cellules.

Ce chapitre, en synergie avec les analyses issues du premier volet de la thèse, explore l'hypothèse de l'intervention de NucS dans la réparation des DSBs par la régulation de la HR et la favorisation de voies alternatives de réparation telle que la MMEJ. L'utilisation d'un système reporter Gam-meGFP décrit dans la littérature pour son affinité pour les DSBs a révélé par l'utilisation de la microscopie confocale que les cellules vivantes dépourvues de *nucS* présentent plus de foci et donc de DSBs que les bactéries sauvages. Ces données suggèrent que NucS pourrait faciliter une voie de réparation des DSBs qui réduit leur temps de rémanence dans la cellule, par un mécanisme de réparation plus rapide que celui favorisé en son absence. De plus, nous avons observé que les cellules *Cgl* $\Delta$ *nucS* montrent également un recrutement accru de RecA-mVenus, ce qui suggère que NucS pourrait jouer un rôle dans la régulation de la HR, soit par son inhibition directe, soit par compétition pour le substrat DSB en favorisant l'activation une autre voie de réparation des DSBs.



Pour la première fois, la localisation de NucS dans un organisme vivant a pu être observée, par l'utilisation de la fusion mScarlet1-NucSD144A, une construction avec une mutation dans le site catalytique de NucS rendant cette molécule inactive. Il a pu être constaté que proportionnellement à l'introduction de dommages dans la molécule d'ADN, et plus spécifiquement à la création de DSBs, un nombre croissant de foci mScarlet1-NucSD144A se forme au niveau de l'un des pôles de la cellule dans les 30 minutes suivant la mise en présence avec l'antibiotique. Le nombre de foci recensé dans la population bactérienne augmente également en fonction des conditions de croissance, notamment en réponse à une diminution des ressources nutritives ou à l'introduction de stress génotoxiques sur une durée prolongée de 24h. Des réseaux filamenteux complexes de foci ont été observés dans des bactéries semblant en apoptose, ainsi que la formation d'anneaux de mScarlet1-NucSD144A. Les aminosides apparaissent également déclencher de telles réponses physiologiques.

Nos données suggèrent que NucS pourrait être crucial pour réguler les voies de réparation des DSBs, en favorisant potentiellement la MMEJ par rapport à la HR, et en jouant un rôle important dans les premières phases nécessaires au maintien de la stabilité d'un génome soumis à un stress. Ces résultats semblent également indiquer que NucS puisse favoriser une voie de réparation active en phase stationnaire, donc non tributaire de la présence de séquences d'ADN homologues pour réparer les DSBs.

### **Chapitre 3**

Afin de diversifier les approches utilisées et apporter plus de robustesses aux discussions menées dans les deux chapitres précédents, des analyses bio-informatiques ont été réalisées. Les bases de données modernes sur les conformations de protéines, permettent aux chercheurs de déterminer leurs relations phylogénétiques, de modéliser leurs interactions et leur activité en présence de différents substrats ou protéines. En accord avec la démarche développée dans les chapitres précédents, nous avons émis l'hypothèse que NucS soit impliqué dans la réparation des cassures double-brin (DSBR).

Nous avons utilisé CHARMM pour simuler les interactions de NucS avec différents substrats d'ADN, notamment des DSBs. Nos résultats suggèrent que NucS stabilise l'ADN au niveau des extrémités libres générées par les DSBs et prévienne leur dégradation. Cette interaction de NucS avec l'ADN pourrait concourir à une compétition avec les protéines de la HR et favoriser des voies de réparation des DSBs alternatives telles que la MMEJ.

Nos analyses de l'interactome de NucS chez les bactéries et les archées, via la base de données STRING et des essais de pull-down in vitro, ont révélé plusieurs interactions potentielles de NucS avec des hélicases et des protéines de réparation de l'ADN, notamment RadA, RecA, Mre11, FEN1 et ERCC4/XPF, suggérant l'implication de NucS dans plusieurs voies de réparation de l'ADN.

L'analyse du voisinage génomique de NucS dans différents organismes a également permis d'identifier des gènes impliqués dans la réparation de l'ADN ainsi que d'autres gènes ayant des fonctions dans des voies de régulation métaboliques ou encore la production d'énergie. Ces données soutiennent l'hypothèse d'un rôle aux multiples facettes de NucS et son implication dans le maintien de la stabilité génomique. Les arbres phylogénétiques réalisés suggèrent quant à eux une origine archéenne de *nucS*, avec un transfert horizontal de ce gène vers les Actinobactéries extrémophiles, indiquant que NucS pourrait jouer un rôle dans des contextes de stress sévères.

En considérant l'ensemble des modélisations bio-informatiques ainsi que les analyses d'interactions et de voisinage génétique, l'hypothèse selon laquelle NucS pourrait favoriser la MMEJ en dépit de la HR est renforcée, ainsi que celle de son intervention dans d'autres voies de réparation de l'ADN que la MMR.

## Conclusion

Les résultats de cette étude suggèrent que NucS pourrait jouer un rôle crucial dans la réparation des dommages à l'ADN, et particulièrement les DSBs, d'une façon similaire aux protéines telles que Rad51 ou Ku. En stabilisant les extrémités libres de l'ADN avant sa réparation, possiblement via la MMEJ, NucS empêche l'action des exonucléases et pourrait favoriser une voie de réparation rapide bien que mutagène en comparaison avec la HR.

De futures études devraient intégrer des techniques d'étude avancées, telles que les dispositifs microfluidiques pour l'imagerie des cellules vivantes et les études sur molécule unique, afin de mieux comprendre l'activité et la dynamique du recrutement de NucS au niveau des sites de dommages. Comprendre la régulation de l'expression de *nucS* aux niveaux transcriptionnel et post-transcriptionnel sera un axe primordial pour déterminer son rôle exact dans la réparation de l'ADN. Des modifications post-traductionnelles et des cofacteurs pourraient également jouer un rôle prépondérant dans la régulation de l'activité de NucS, affectant sa capacité de liaison à l'ADN et son activité endonucléasique.

Les connaissances issues de l'étude de NucS pourraient être exploitées dans le cadre d'applications biotechnologiques à vocation d'utilisation dans des conditions extrêmes, notamment grâce à la capacité de NucS à cliver l'ADN à haute température. Par ailleurs, nos résultats encouragent la poursuite de la caractérisation de la fonction de NucS dans la réparation de l'ADN dans la perspective de développer de nouvelles stratégies thérapeutiques contre les maladies mycobactériennes telle que la tuberculose.

Enfin, la localisation polaire de NucS au sein des cellules bactériennes, pourrait être associée à l'absorption préférentielle de l'ADN au niveau des pôles cellulaires, ce qui pourrait suggérer un rôle de NucS dans la régulation de l'incorporation de fragments d'ADN exogènes. Cette localisation stratégique pourrait permettre à NucS de traiter et stabiliser ces fragments lors des événements de transfert horizontaux de gènes, en association avec un microenvironnement protéique adéquat.



**Titre :** Etude de la Réponse Physiologique de NucS à des Stress Génotoxiques chez les Actinobactéries

**Mots clés :** NucS, Réparation de l'ADN, Intégrité génomique, Recombinaison, Cassures double-brin, Imageries confocale sur cellules vivantes/living cell

**Résumé :** La précision de la réplication de l'ADN assure la fidélité de la transmission génétique. Les dommages causés par des facteurs externes ou internes menacent l'intégrité génomique. Les Actinobactéries, dépourvues des protéines majeure de la Réparation des Mésappariements (MMR) canonique, possèdent NucS (EndoMS), une enzyme qui répare ces erreurs indépendamment de l'ATP. Bien que l'activité de NucS dans ce mécanisme soit étudiée, sa fonction *in vivo* et dans d'autres systèmes de réparation de l'ADN conservent des zones d'ombre.

Cette étude vise à caractériser la fonction de NucS dans la réparation des cassures double brin (DSB). Nos résultats montrent que mScarlet1-NucSD144A forme des foyers polaires en réponse aux dommages de l'ADN, particulièrement les DSB. Chez *Corynebacterium glutamicum*, les cellules *CglΔnucS* présentent une activation plus élevée de la Recombinaison Homologue (HR) et un nombre accru de DSB par rapport aux *Cgl/WT*, indiquant un rôle de NucS

dans l'efficacité et la sélectivité de la réparation des DSBs. Les bactéries *CglΔnucS* présentent un avantage de croissance en présence de stress génotoxiques, probablement en raison de mécanismes de réparation des DSB altérés. Nos analyses bioinformatiques prédisent l'interaction de NucS avec des enzymes clés de la HR et d'autres voies de réparation de l'ADN, ainsi que des gènes impliqués dans la réparation des dommages, le métabolisme et la régulation énergétique.

NucS pourrait stabiliser les extrémités libres de l'ADN générées par les DSBs rapidement après leur formation, favorisant leur réparation par une voie alternative telle que la Jonction des Extrémités par Microhomologie (MMEJ). Les études futures devraient explorer les modifications post-traductionnelles et les conditions métaboliques régulant l'activité de NucS et son activité *in vitro* sur les DSB et les intermédiaires de HR.

**Title :** Study of the Physiological Response of NucS to Genotoxic Stress in Actinobacteria

**Keywords :** de NucS, DNA Damage Repair, Genomic integrity, Recombination, Double-strand breaks, Confocal imaging on living cells

**Abstract :** DNA replication accuracy ensures proper genetic transmission. Damage from external factors or internal events threatens genomic integrity. Actinobacteria, lacking canonical MMR proteins, possess NucS (EndoMS), an ATP-independent enzyme involved in a non-canonical mismatch repair pathway. While NucS's activity on mismatches is documented, its *in vivo* role and implications in DNA Damage Repair systems require further understanding.

This study aims to characterise NucS's role in Double-Strand Break Repair (DSBR). Our findings show that mScarlet1-NucSD144A forms polar foci in response to DNA damage, especially DSBs and complex recruitment in apoptosis-like cells.

*Corynebacterium glutamicum*, *CglΔnucS* bacteria exhibits higher homologous recombination (HR) activation and increased DSBs compared to *Cgl/WT*, indicating NucS's role in DSBR efficiency and regulation. *CglΔnucS* bacteria have a growth advantage under genotoxic stress, likely due to altered DSBR mechanisms. Bioinformatic analyses predict NucS interactions with key enzymes of HR and other DNA repair pathways and metabolism and energy regulation.

NucS may bind and stabilise free DNA ends generated by DSBs, balancing HR and participating in DSB repair through microhomology-mediated end joining (MMEJ). Future studies should explore post-translational modifications and metabolic conditions regulating NucS response and its *in vitro* activity on DSBs and HR intermediates.