**Analyzing the Function of the NucS Gene in Mycobacterium Smegmatis**

**Grant Proposal**

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**Author's Note**

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**Abstract (RQ) or Executive Summary (Eng)**

Antimicrobials have become critical to the treatment of bacterial infections and the preservation of global health. However, antimicrobial resistance in bacteria continues to increase, while development of new drugs decreases (Davies & Davies, 2010). In 2019, the World Health Organization (WHO) declared antibiotic and antimicrobial resistance among the ten most critical threats to public health in the 21st century (Ten Health Issues WHO Will Tackle This Year, n.d.). Many instances of resistance are caused by gene mutations. Of the 700,000 deaths caused by antimicrobial resistant bacteria in 2019, almost a third were caused by multidrug-resistant *Mycobacterium tuberculosis* (New Report Calls for Urgent Action to Avert Antimicrobial Resistance Crisis, n.d.). *M. smegmatis* is a common substitute for *M. tuberculosis* in labs due to their similar genomes and the shorter growth period necessary for *M. smegmatis* cultures (K.C. Murphy, personal communication, October 18, 2022). Both *M. smegmatis* and *M.* tuberculosis share an endonuclease called NucS, also known as EndoMS, which detects and cleaves mismatches in the chromosome. Past studies have suggested that NucS plays a key role in preventing gene mutations, along with proteins such as DnaN. However, its precise mechanisms, as well as those of accompanying proteins that help it prevent mutations, are still unclear (K.C. Murphy, personal communication, October 26, 2022).

*Keywords:* antimicrobial resistance; EndoMS; gene mutation; Mycobacterium smegmatis; Mycobacterium tuberculosis; NucS

**Antimicrobial resistance in the world**

The discovery of antimicrobials from various plants in the 20th century, especially in the years 1950 to 1970, revolutionized the healthcare industry (Singh, 2014). Antimicrobials have since become widespread in the treatment of bacterial infections and thus in the preservation of global health. However, after the initial surge of antimicrobial discoveries, drug development slowed and many bacteria developed antimicrobial resistance (AMR) due to selective pressures from current antibiotics and misuse by humans (Davies & Davies, 2010). Antimicrobial resistance can be conferred in many ways, though the most common method involves mutations enabling the bacteria to reduce its sensitivity through reducing uptake of, extruding, modifying, or otherwise inhibiting the drug with proteins (Annunziato, 2019). The increase in bacterial resistance and lack of new, effective antimicrobial drugs poses a major risk to global health: in 2019 alone, multi-drug resistant diseases were the cause of death of at least 700,000 people worldwide. (WHO, 2019). Also in 2019, the World Health Organization (WHO) declared antibiotic and antimicrobial resistance to be among the ten most critical threats to public health in the 21st century (WHO, 2019b). Figure 1, by Murray et al., indicates deaths per 100,000 people related to antimicrobial resistance in 2019. Antimicrobial resistance causes deaths worldwide, in regions from Western sub-Saharan Africa to East Asia and Tropical Latin America. Its impact is continues to increase, with death rates caused by antimicrobial resistance predicted to skyrocket to 10 million deaths annually within the next three decades (Stéphane Dujarric, 2019).

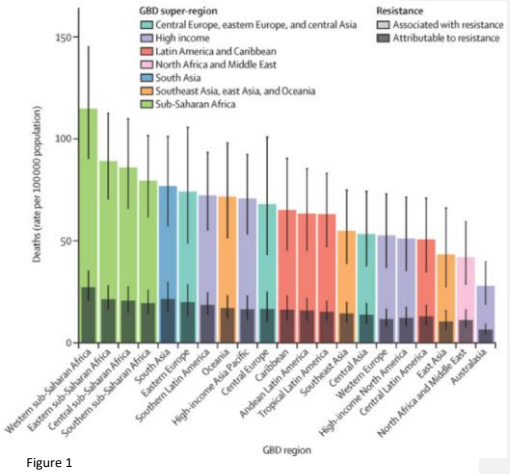


Figure 1

Deaths in 2019 due to and associated with antimicrobial resistance per 100,000 people in various regions of the world. (Murray et al., 2022).

***Mycobacterium tuberculosis* and antimicrobial resistant *Mycobacterium tuberculosis***

*Mycobacterium tuberculosis* causes tuberculosis (TB), an infectious disease which, though often fatal, can be treated. The first-line drugs ethambutol, pyrazinamide, isoniazid, and rifampicin (also called rifampin) are

typically used to treat TB, with isoniazid and rifampicin being the most effective (NIH, n.d.). Unfortunately, due to misuse and mismanagement of TB drugs, strains of TB resistant to the first-line drugs have evolved. Multidrug-resistant TB (MDR TB) are bacteria that are resistant to both isoniazid and rifampicin (Tuberculosis Drugs and Mechanisms of Action | NIH, n.d.). Though MDR TB is rare in the United States, it still causes up to 10 million to fall ill and 1.6 million to die annually. Considering even normal TB infections come at a huge cost, averaging $20,000 to treat, treatment of MDR TB can cost upwards of $568,000, making it a pressing global health and economic issue (CDCTB, 2022).

**Mycobacterium smegmatis**

*M. smegmatis* is a species of bacteria with genes and cell regulatory mechanisms similar to those in the rest of *Mycobacteria*, a genus which includes in *M. tuberculosis*. Unlike *M. tuberculosis*, *M. smegmatis* does not cause fatal infections in humans and can be studied in biosafety level 1 and 2 laboratories. Cultures of *M. smegmatis* also grow faster than cultures of *M. tuberculosis*, taking only about three days to form colonies whereas *M. tuberculosis* can take up to three weeks. Thus, *M. smegmatis* will be used to model *M. tuberculosis*

**MutS and EndoMS/NucS in mismatch repair systems**

Mismatch repair (MMR) systems prevent mutations in bacteria by recognizing, cleaving, and resynthesizing mismatched DNA (Cebrián-Sastre et al., 2021). NucS, also known as EndoMS, is an enzyme that is a part of MMR systems in some organisms such as those belonging to the phylum Actinobacteria. The preventative role of NucS has been investigated by several studies, for example by inhibiting the *endoMS*/*nucS* genes of *Streptomyces coelicolor* and *Mycobacterium smegmatis*. After the gene inhibition, the bacteria exhibited increased frequencies of mutation, results which supported the function of EndoMS/NucS as enzymes key to DNA repair (Takemoto et al., 2018). Due to the central role genetic mutations play in antimicrobial resistance, elucidating the mechanism and interactions of NucS, a key preventer of such mutations, is an important step towards understanding the global issue of drug resistance.

**Knowledge gaps and investigation**

NucS, though identified by Ren et al in 2009, only became a protein of interest in many studies researching mismatch repair after a study by Ishino et al. in 2016 described its role in binding to and cutting mismatches (K.C Murphy, professional communication, 11/25/22). Since then, multiple studies have described its fundamental role in preventing mutations in *Mycobacteria*. However, more understanding of its precise function may elucidate further directions for protecting and promoting DNA repair in Mycobacteria. Identifying the interactions of NucS with other proteins in the repair pathway is one such inquiry that would shed light on its function (Cebrián-Sastre et al., 2021). Such interactions may be observed by the tagging of NucS.

**Section II: Specific Aims**

The mismatch repair (MMR) system in Mycobacteria, involving NucS, works to prevent mutations through cleaving mismatched nucleotides. However, though NucS has been shown to efficiently cleave and thus prevent mismatches in DNA, there are still many aspects of its function that remain unknown. Thus, the long-term goal of this project is to determine one such element of NucS function, the interaction of NucS with the replisome.

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Past studies have indicated that NucS recognizes and cleaves mismatched DNA through interactions with the sliding clamp of the replisome, DnaN (Cebrián-Sastre et al., 2021). Due to the interaction of NucS with a protein known to function in the replisome, we hypothesize that the NucS protein is located at the replisome during MMR. When a sequence of five amino acids at the C terminal of NucS was not available for binding to due to a DAS+4 tag bound to the C-terminal of NucS, mutation rates increased, leading to the hypothesis that this sequence of amino acids, or "replisome tag", is required for the interaction between NucS and DnaN (K.C Murphy, professional communication, 11/25/22).

**Specific aim 1: To construct a chromosomal NucS-mCherry fusion protein.**

**Specific aim 2: To determine if NucS is still active when fused to mCherry.**

**Specific aim 3: To determine if NucS can be identified with fluorescent microscopy and to determine if it interacts with the DnaN protein at the replisome.**

## Section III: Project Goals and Methodology

### Relevance/Significance

This project aims to investigate the mechanisms of NucS, a protein key to the repair of mismatches in Mycobacteria. Such an investigation will improve understanding of mismatch repair functions in Mycobacteria, which may contribute to efforts to prevent potentially deadly mutations.

The specific aims of this project are to observe NucS tagged with mCherry under a fluorescent microscope, to confirm that the fusion with mCherry does not interfere with NucS function, and to observe the interaction of NucS with the DnaN protein at the replisome. This project's intended outcomes are to observe the mCherry-tagged NucS protein tangent to the GFP-tagged DnaN protein, which would support the hypothesis that NucS interacts with DnaN in the replisome to repair DNA mismatches.

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If we observe the expected outcome of this project, we will be able to use the same methods to tag NucS and observe it during various stages of the cell. For example, we may investigate its location and interactions during mutations which may lead to resistance. Additionally, we may perform experiments such as inserting a stronger promoter before NucS to investigate the effect that elevated numbers of NucS has on the cell.

### Innovation

If successful, this project will generate a plasmid for tracking the location of NucS in the cell, as well as a method for creating other tags for NucS. Such plasmids may be used in later experiments to investigate the location, interactions, and impact of NucS during MMR and other cell processes. In this project, the mCherry tag will be used to determine the interaction of NucS with DnaN. Tagging NucS will also allow us to investigate the function of the five amino acids at the C terminal of NucS, as well as enable us to determine if NucS interacts with DnaN during mismatch repair.

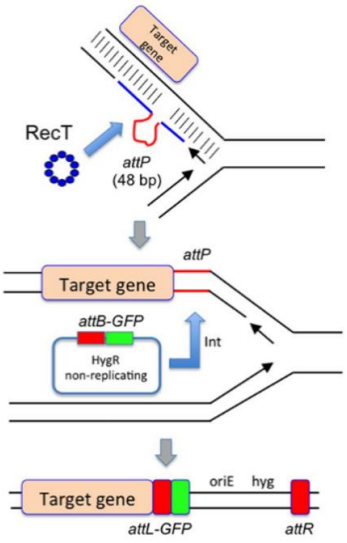
### Methodology

We will first produce a plasmid containing an *attB* site, the mCherry tag, oriE, chloramphenicol resistance, and hygromycin resistance. This plasmid will be electroporated into *E. coli*, where it can replicate due to the oriE

site. *E. coli* with the plasmid will be selected for with chloramphenicol. After purifying out the plasmid from a colony with the plasmid, we will incorporate it into the genome of *M. smegmatis* with DnaN tagged with GFP. Hygromycin will be used to select strains in which the plasmid was successfully incorporated. The strain with the tagged NucS will be tested for normal MMR function then studied under a fluorescence microscope to observe NucS.

***Specific Aim #1:*** **To construct a chromosomal NucS-mCherry fusion protein.**

This aim will generate a plasmid containing mCherry and *attB* from a previously synthesized plasmid containing *attB* and a previously synthesized plasmid containing *mCherry*. There are five stages to this aim: the first stage is to construct a plasmid encoding mCherry, with the last five amino acid sequences of NucS encoded at the C-terminal of mCherry. The five amino acids, or replisome tag, must be integrated because, in a previous unpublished experiment, tagging NucS with Das+4 interfered with its ability to repair mutations (K.C. Murphy, professional communication, 11/25/22). It is believed that this is due to Das+4 inhibiting the replisome tag from binding to DnaN, which we aim to address in this investigation by joining the replisome tag to the C terminal of mCherry instead. The second step is to replicate this plasmid in E. coli. After confirming that the plasmid contains mCherry, we will purify the plasmids from E. coli. Fourth, to integrate the plasmid into the gene, we will follow the ORBIT method of recombineering, described in *ORBIT* by Murphy et al. in 2018. Finally, bacteria successfully tagged with mCherry will be determined using hygromycin.




**Justification and Feasibility:**

This methodology will generate a plasmid for tagging NucS with mCherry. The mCherry tag will be used to observe the interactions and location of NucS in the cell, and functionality of NucS with the tag will help determine the role of the five amino acids at the C-terminal of NucS. If, when grown on rifampicin-containing plates, both the strain with NucS tagged with mCherry and the untagged strain have the same rate of mutation, that would suggest that fusion to mCherry does not affect NucS function.

Figure 2

The process of ORBIT. RecT transports the targeting oligo to the chromosome, after which Bxb1 integrase allows the payload plasmid to be incorporated into the chromosome at the site of the oligo (Murphy et al., 2018).

**Summary of Preliminary Data.**

This method of tagging proteins by integrating plasmids into the bacteria's chromosome was described in *ORBIT* by Murphy et al. Figure 2 provides a visual of the details of the integration of the plasmid. The ORBIT method allows the integration of plasmids at specific sites, which can be determined with oligonucleotides.

**Expected Outcomes.** The expected outcome of this aim is to generate a plasmid containing an *attB* site and encoding mCherry that can be used to fuse a mCherry tag to NucS.

**Potential Pitfalls and Alternative Strategies.** Potential pitfalls may involve failure to generate a mCherry plasmid with the intended sequence. Alternative strategies include use of a different tag or generating a tag for the N-terminal of NucS instead.

***Specific Aim #2:*** **To determine if NucS is still active when fused to mCherry.**

**Justification and Feasibility**

Our approach is to grow the bacteria with mCherry-tagged NucS on a plate containing rifampicin. Our rationale for this approach is that, as the antimicrobial will kill the bacteria unless they mutate, only bacteria that mutate will survive to form colonies. As NucS prevents mutations, if bacteria grown with and without the tag demonstrate the same frequency of mutations, it would suggest that tagging with mCherry does not affect NucS function.

**Expected Outcomes.** The expected outcome of this aim is to produce *M. smegmatis* strains containing NucS tagged with mCherry. The tag should not significantly affect the function of NucS. This outcome may be used for observing how NucS interacts with the replisome and for further studies in which NucS must be tagged.

**Potential Pitfalls and Alternative Strategies**. A potential pitfall is that the NucS protein may not function normally when fused to mCherry. A change to NucS function is indicated by more colonies on the plate of bacteria with mCherry, indicating mCherry interfered with the ability of NucS to repair mismatches. We predict that incorporating the five amino acids at the C-terminal of NucS will preserve the normal function of NucS. However, if it is observed that the tag affects the MMR abilities of NucS, it is possible to incorporate the tag at the N-terminal of NucS, instead.

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***Specific Aim #3:*** **To determine if NucS can be identified with fluorescent microscopy and to determine if it interacts with the DnaN protein at the replisome.** Our approach is to tag NucS and DnaN with mCherry and GFP, respectively. Our rationale for this approach is that the tags will allow us to observe the bacteria with florescent microscopy.

With successful fusion of mCherry to NucS, NucS will appear red under a fluorescence microscope. Similarly, with successful fusion of GFP to DnaN, DnaN will appear green under a fluorescence microscope. If NucS and DnaN do interact, as hypothesized, we expect to see the red NucS tangent to the green DnaN. As we know that DnaN functions in the replisome, the presence of NucS tangent to DnaN would indicate that the two proteins interact, as well as also support that NucS is active in the replisome.

The expected outcome of this work is to see NucS tangent to DnaN when NucS is tagged with mCherry and DnaN is tagged with GFP.

**Justification and Feasibility.**

Similar methods have been utilized in past studies to observe the location of other proteins in *M. smegmatis*. Figure 3 shows imaging of several proteins in *M. smegmatis*, including of DnaN tagged with GFP.

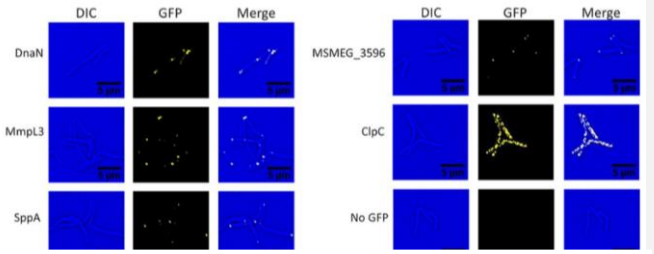


Figure 3

Imaging of proteins in *M. Smegmatis* tagged with GFP, using ORBIT, in a study by Murphy et al. ORBIT effectively tags target proteins for observation. DnaN is among the proteins for which a GFP tagging plasmid has already been synthesized. (Murphy et al., 2018)

**Expected Outcomes.** The expected outcome of this aim is to observe NucS tagged with mCherry next to DnaN tagged with GFP under a microscope. This knowledge will support our hypothesis that NucS functions in the replisome and that it interacts with DnaN.

**Potential Pitfalls and Alternative Strategies.** Potential pitfalls include not seeing the tagged NucS or the tagged DnaN, which may indicate unsuccessful integration of the tags into the bacterial chromosome. To prevent replication of bacteria without the tags, we will test for the expected PCR junctions that are present in the plasmid containing the tag, as well as select for bacteria with resistances encoded in the plasmid. Another potential pitfall is observing that NucS is not present at the replisome, which would warrant further research into the five amino acids at the C-terminal and tag's effects on the binding of NucS to the replisome. Additionally, we may not see mCherry under a fluorescent microscope. This may be due to not enough mCherry proteins being produced when coded after the NucS promoter. To address this, a plasmid encoding a strong promoter, NucS, mCherry, and oriM may be used to produce NucS and mCherry independent of the bacteria's chromosome in *M. smegmatis*.

## Section III: Resources/Equipment

### Materials List:

- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)
- Agar (Solarbio, cat. no. A8190)

- Petri dishes & culture tubes
- Spreaders
- Autoclaved ddH2O kept at room temperature
- Chloramphenicol (Sigma-Aldrich, cat. no. C1919)
- Ampicillin sodium salt (Sigma-Aldrich, cat. no. A9518)
- Kanamycin sulfate (Sigma-Aldrich, cat. no. K4000)
- 70% (vol/vol) ethanol
- Sucrose
- Glycerol
- kb DNA ladder (New England BioLabs, cat. no. N3232)
- 10× Tris/boric acid/EDTA (TBE) buffer (Bio-Rad, cat. no. 161-070)
- Ethidium bromide solution (4 ug mL-1)
- Agarose

**Equipment:**

- 37o incubators
- Shaker and rollers for growing cultures
- Cold room
- Electroporator (Bio-Rad)
- Electrophoresis chamber
- Power source for electrophoresis

**Facilities at UMass:**

- Mass Spectrometry
- Scope Imaging Center
- Dish washing

## Section V: Ethical Considerations

This project will not involve human testing. However, bacteria and biohazardous chemicals will be used. To use such materials, proper procedures for use and disposal must be followed. Additionally, this project involves mutating bacteria, such as by integrating in mutations for drug resistance. Such mutations may only be made after ensuring the mutated bacteria will still have sensitivity to other drugs. The effect of mutations must be carefully considered before implementing them.

## Section VI: Timeline

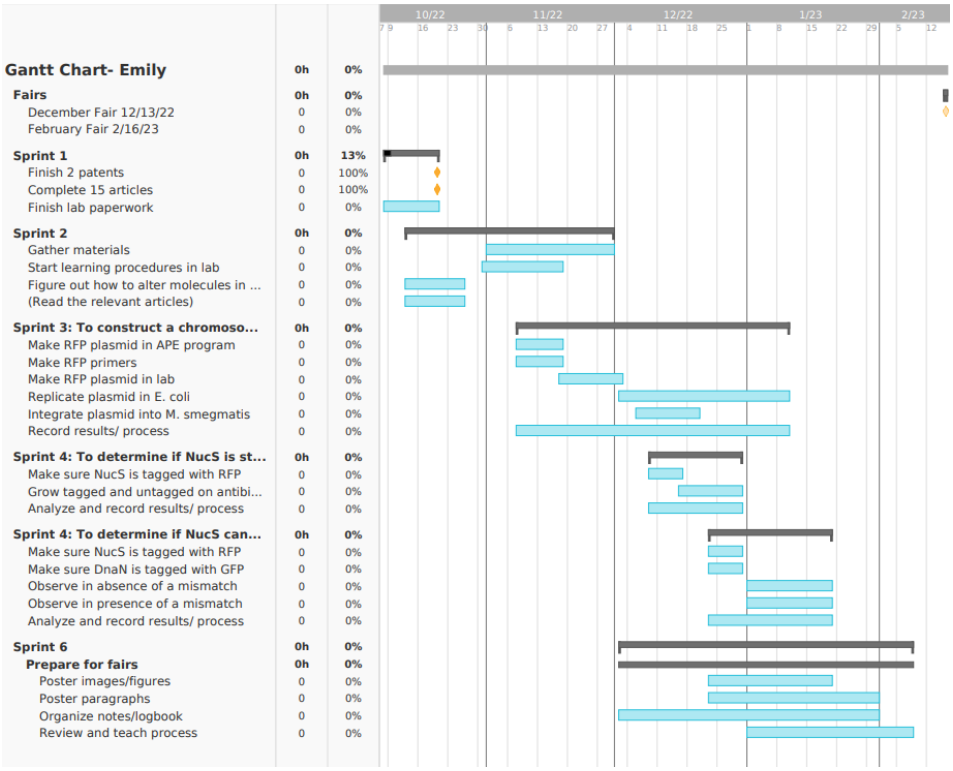


Figure 4: A rough timeline of the project.

**Section VII: Appendix**

**Commented [A4]:** What is this? Where to find a grant?

**Section VIII: References**

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