Identification and Characterization of Genes Responsible for the Resistance of Gram-Negative Bacteria towards Nodule-Specific Cysteine Rich Peptides

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled '**Identification and Characterization of Genes Responsible for the Resistance of Gram-Negative Bacteria towards Nodule-Specific Cysteine Rich Peptides'** towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents work carried out by Shruti Patil at Stowers Institute for Medical Research, USA under the supervision of **Dr. Siva Sankari**, Assistant Investigator, during the academic year 2023-2024.

04/08/2024

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This thesis is dedicated to Siva Sankari and Vandana Patil.

Declaration

I hereby declare that the matter embodied in the report entitled "**Identification and Characterization of Genes Responsible for the Resistance of Gram-Negative Bacteria towards Nodule-Specific Cysteine Rich Peptides**" are the results of the work carried out by me at the Stowers Institute for Medical Research, USA under the supervision of **Dr. Siva Sankari**, Assistant Investigator, during the academic year 2023-2024. and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgment of collaborative research and discussions.

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Table of Contents

Abbreviations

AMP	Antimicrobial Peptides
lRLC	Inverted Repeat Lacking Clade
TBD	Terminal Bacteroid Differentiation
NCR	Nodule-specific Cysteine-Rich
AMR	Antimicrobial resistance

List of Tables

List of Figures

Abstract

Antibiotic resistance poses a significant threat to public health, necessitating the development of alternative antimicrobial agents. Antimicrobial peptides (AMPs) have emerged as promising candidates due to their broad-spectrum activity and unique mechanisms of action. Among antimicrobial peptides, Nodule-specific Cysteine-Rich (NCR) peptides from leguminous plants exhibit potent antimicrobial properties and orchestrate Terminal Bacteroid Differentiation (TBD) in symbiotic nitrogen-fixing bacteria such as *Sinorhizobium meliloti*. However, the mechanisms underlying bacterial resistance to NCR peptides remain poorly understood. Here, we investigate the genetic basis of resistance to NCR247 peptide in *Bradyrhizobium japonicum* and *Escherichia coli*. We developed a transposon library in *B. japonicum*, enabling future exploration of cellular pathways mediating resistance to AMPs treatment. Using modern techniques like BARseq and Tn-seq, our study provides a robust framework for understanding the molecular mechanisms governing symbiotic interactions and bacterial resistance to AMPs. We also attempted to identify genes involved in polyploidy in *Sinorhizobium meliloti* upon NCR247 peptide treatment These findings offer insights into developing novel antimicrobial strategies and highlight the importance of investigating bacterial defense mechanisms in symbiosis.

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Contributions

Contributor name	Contributor role
Dr. Siva Sankari	Conceptualization of Ideas
Dr. Siva Sankari, Shruti Patil	Methodology
Cytometry and Computational Biology Cores, Stowers Institute	Software
Shruti Patil, Dr. Siva Sankari	Validation
Shruti Patil	Formal analysis
Shruti Patil, Sankari lab	Investigation
Sankari lab	Resources
-	Data Curation
Shruti Patil	Writing - original draft preparation
Shruti Patil, Dr. Siva Sankari	Writing - review and editing
Shruti Patil	Visualization
Dr. Siva Sankari	Supervision
Dr. Siva Sankari	Project administration
Stowers Institute for Medical Research	Funding acquisition

Chapter 1

Introduction

Antibiotics have revolutionized modern medicine, offering effective treatments against bacterial infections that were once life-threatening. These medications are designed to kill bacteria (bactericidal) or inhibit their growth (bacteriostatic), allowing the body's immune system to combat the infection effectively. However, antibiotic resistance has emerged due to the widespread and often use of antibiotics. (Sun et al., 2024a). It has become a significant health issue worldwide, creating major barriers to efficiently managing bacterial diseases. Consequently, several widely prescribed antibiotics are losing their efficacy due to emerging bacterial resistance mechanisms while the development rate of new antibiotic classes continues to lag. Antimicrobial peptides (AMPs) are an emerging class of therapeutic agents with many clinical studies (Epand, 2016; Mahlapuu et al., 2016). The production of natural antibiotic peptides is part of the host's innate immune response in plants and animals and is effective against a broad spectrum of bacteria and fungi (Diamond et al., n.d.; Lei et al., 2019). Although many antimicrobial peptides have been identified from various eukaryotic hosts, the detailed mechanism of action is only known for a few peptides (Kumar et al., 2018). Examples of AMPs include Defensins, Protegrins, and Cathelicidins. Defensins are a family of cationic antimicrobial peptides that are effective against many pathogenic microorganisms, such as bacteria, fungi, and viruses. They also serve as crucial innate effectors and immune modulators in the immunological control of microbial infection. It is essential to understand the detailed mechanism of action of AMPs for developing them into potential drugs.

As mentioned before, antibiotics are an important class of medicine that has continued to save lives since their invention. However, later, we started discovering bacterial infections that do not respond to antibiotics, highlighting this new phenomenon: Antimicrobial resistance. Antimicrobial resistance (AMR) arises from the overuse and misuse of antibiotics in healthcare, agriculture, and veterinary medicine, creating selection pressure on bacteria (Sun et al., 2024b). Poor infection control practices in healthcare settings facilitate transmission, while antibiotic use in agriculture spreads resistance through the food chain. Globalization aids the global spread of resistant

bacteria and genes, complicating containment efforts. Inadequate surveillance and monitoring hinder timely detection, while the slow pace of new antibiotic development exacerbates the problem. Even after all these studies, evolution as a biological phenomenon is poorly understood. Hence, as we understand, AMR might be due to evolutionary selection pressure; the molecular mechanism that enables the bacteria to resist antibiotics is also poorly understood. Therefore, it is essential to understand the mechanisms of AMR in bacteria to find ways to evade the phenomena and develop new classes of AMPs with better efficacy.

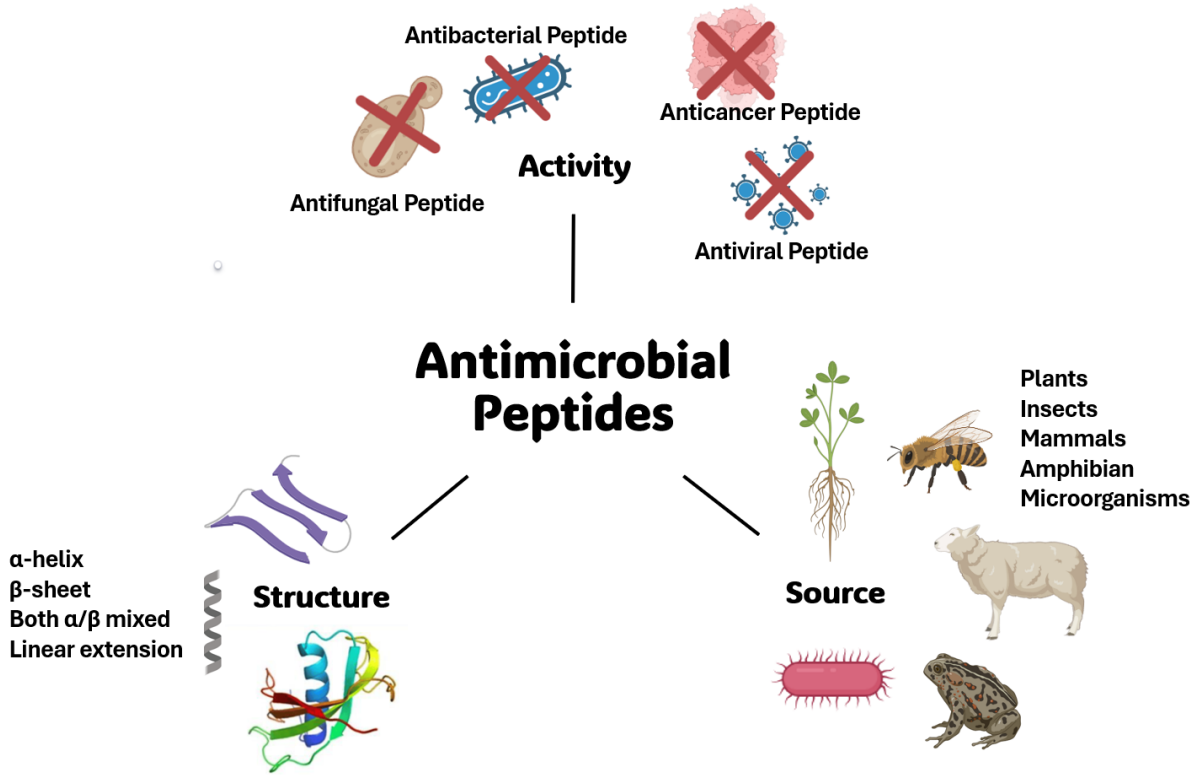

Fig 1: Figure depicting the different facets of Antimicrobial Peptides.

1.1 Antimicrobial Plant Peptides

Plants have a barrier defense mechanism that includes antimicrobial peptides from plants. A single species might contain a multitude of AMPs. They can be isolated from different parts of plants of a wide variety of species and have pathogenic activities to humans, bacteria, fungi, and viruses (Montesinos, 2007; Nawrot et al., 2014). Plant AMPs have diverse functions, expression patterns, and structures, which makes them

more complex and difficult to classify. They can be classified based on the charge and their sequence similarity.

Plant defensins are a broad superfamily of antimicrobial peptides within the plant kingdom. Plant defensins are well-known and might be the largest family of all membrane-soluble plant AMPs (Graham et al., 2008). It is an essential class of AMPs and, therefore, found in almost all plants. They have highly conserved structural scaffolds (Taylor et al., 2008). Even after having a highly conserved structure, they have varying amino acid sequences, except for the cysteine residues that make stable disulfide bonds (Parisi et al., 2019). They exhibit diverse biological functions, including the inhibition of microbial growth, modulation of self-incompatibility, and mediation of abiotic stress responses (FUJIMURA et al., 2003; Gao et al., 2000) (Li et al., 2021).

1.1.1 Plant Microbe Symbiosis

Symbiosis, the mutually beneficial relationship between two organisms, is a feature of many living systems. A popular example of symbiosis is the relationship between nitrogen-fixing rhizobial bacteria and leguminous plants.

Most plants cannot utilize atmospheric nitrogen; therefore, they form symbiotic relationships with these nitrogen-fixing bacteria called rhizobia. This has evolved as a remarkable solution for leguminous plants. This interaction leads to the development of specialized root organs called nodules, where the rhizobia bacteria reside and convert atmospheric nitrogen into ammonia, which helps in plant growth. Rhizobia enters roots via plant-made infection threads, which allow the rhizobia to grow inside the root (Haag et al., 2012).

Rhizobia are diazotrophic bacteria whose one of the most remarkable aspects is their ability to fix atmospheric nitrogen into ammonia, a form of nitrogen that plants can utilize for growth. The enzyme nitrogenase facilitates this process, known as nitrogen fixation. Nitrogenase comprises various protein components, notably the nitrogenase reductase and the nitrogenase iron protein (Lindström & Mousavi, 2020). These proteins collaborate to facilitate the conversion of atmospheric nitrogen into ammonia via a sequence of enzymatic processes.

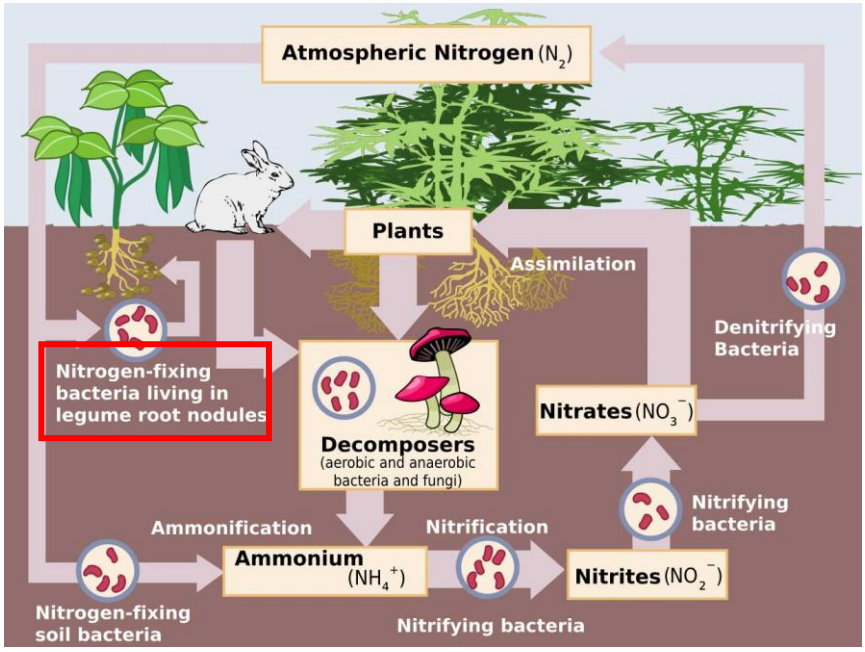

Fig 2: Image showing the flow of nitrogen throughout the ecosystem. Bacteria are essential elements of Nitrogen Fixation (Wikimedia commons, n.d.).

The Legume-rhizobial system is an excellent model for studying host-microbial interaction for the following reasons. Many bacteria that form intracellular infections (like closely related Brucella, Rickettsia, and Wolbachia) are difficult to grow outside their hosts. However, in the legume-rhizobium system, the bacteria's culture-grown, free-living form and the host-associated form from the root nodules can be comparatively studied under standard laboratory conditions. This 1:1 association between host and microbe in nodules, without interference from other microbes, is advantageous in checking the molecular basis of this relationship in detail.

1.1.2 Defensin-like Antimicrobial Peptides in Symbiosis

Legumes can be classified into Inverted Repeat Lacking Clade (IRLC) and Non-Inverted Repeat Lacking Clade (Non-IRLC). IRLC is a clade belonging to the Fabaceae family. They primarily include most agriculturally used legumes and are characterized by losing one of the two 25-kb inverted repeats in the plastid genome found in most land plants (Mergaert et al., 2003) (Lavin et al., 1990). The presence and absence of the Inverted repeats provide essential distinctions in their symbiosis with rhizobia. Legumes that belong to the IRLC, like *Medicago sp.*, produce defensins-like NCR peptides (Nodule-specific Cysteine-Rich) in the nodule. These peptides

guide the bacteria in the nodules and are called bacteroids to undergo a process called Terminal Bacteroid Differentiation (TBD) (Smith et al., 2024; Tiricz et al., 2013). Bacteroids that undergo TBD lose their ability to survive outside the host due to irreversible loss of cell division ability, cell elongation, and changes in the cell wall. On the other hand, legumes that belong to the non-IRLC do not produce NCR peptides. Hence, their bacteroids do not undergo differentiation during nitrogen fixation and resemble free-living bacteria. Due to the lack of NCR peptide, bacteria involved in symbiosis in non-IRLC plants do not undergo any phenotypic changes that disable them from living freely without a host.

1.2 Nodule-specific Cysteine-Rich (NCR peptides)

NCR peptides are secreted in the nodules of the IRLC clade of the Leguminosae family, like peas and *Medicago* (Lima et al., 2020). They are responsible for orchestrating Terminal Bacteroid Differentiation (TBD) (Van de Velde et al., 2010). Terminally differentiated nitrogen-fixing bacteroids show certain characteristic features such as chromosomal endoreduplication (polyploid) (Maróti & Kondorosi, 2014), cell enlargement (Oono & Denison, 2010), cell arrest, change in the cell wall, more efficient nitrogen fixation, and membrane permeability also affect the pattern of gene expression (Alunni & Gourion, 2016; Batut et al., 2011; Haag et al., 2013; Kereszt et al., 2011; Kondorosi et al., 2013; Mergaert et al., 2003; Montiel et al., 2017).

Medicago truncatula, the model legume, produces about 700 peptides; only a few are being investigated. NCR peptides form an extremely large family of peptides, and they are highly diverse in their physio-chemical properties and categorized into anionic, cationic, and neutral peptides (Farkas et al., 2018; Jenei et al., 2020). The NCR peptide coding genes normally consist of 2 exons; the first codes for a relatively conserved signal peptide, and the other is for a highly diverse mature peptide (Jenei et al., 2020). This mature peptide contains two or three pairs of cysteine residues at a conserved position. Peptides are also known to enter the bacteroids, inducing the changes associated with the terminal differentiation (Mergaert et al., 2006; Penterman et al., 2014; Tiricz et al., 2013; Van de Velde et al., 2010).

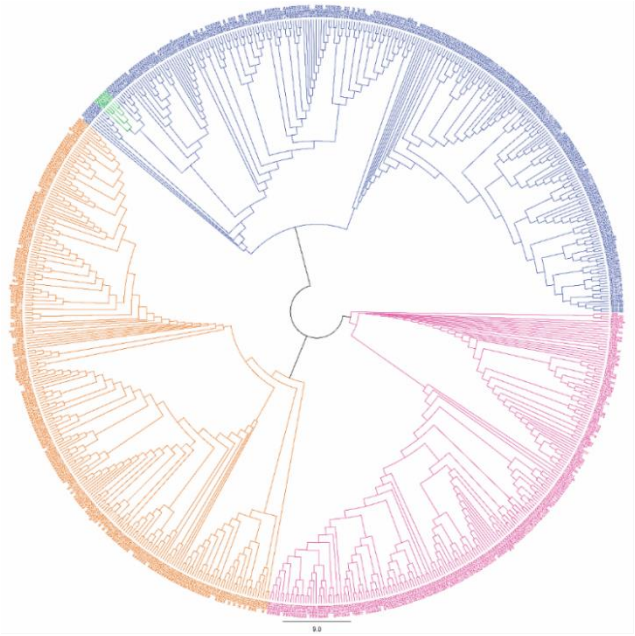

Fig 3: This figure shows the Sequence-dependent classification of NCR peptides in *Medicago truncatula*.

Early studies have shown that cysteine is essential for symbiosis, as replacing one cysteine with serine resulted in the inactivation of the *Medicago*-specific NCR peptide (Lima et al., 2020). Creating disulfide bonds between the conserved cysteines is crucial to NCR peptides' structure and function. These bonds typically form within the endoplasmic reticulum, where enzymes responsible for oxidizing cysteines to form disulfide bonds, such as protein disulfide isomerase (Mergaert et al., 2003) (Roux et al., 2014).

Cationic NCR peptides exhibit potent antimicrobial activity against clinically relevant bacteria such as *S. Typhimurium and S. aureus* (Tiricz et al., 2013) and fungi, including *Candida albicans* (Ördögh et al., 2014). Their broad-spectrum activity and unique mechanisms of action make them good candidates for developing novel antimicrobial therapeutics and enhancing existing treatment strategies.

1.2.1 NCR247 Peptide

The significant variation observed in the NCRs in terms of amino acid composition, charge, and sequence means that it is likely that the peptide has different ways of action and bacterial targets. Previous studies have seen that artificially synthesized cationic NCR peptides exhibit antibacterial effects *in vitro*, enhancing the permeability of bacterial membranes and leading to cell death (FUJIMURA et al., 2003; Kumar et

al., 2018; Mergaert et al., 2006). For this thesis, we focus on one of the cationic peptides, NCR247. It comprises of 24 amino acids; four of them are cysteines, which are thought to create intramolecular bridges in the secretory systems.

Previous studies have shown that this cationic peptide has self-penetrating abilities. It can go into the bacterial cytosol, has high protein binding ability, and interacts with bacterial proteins (Farkas et al., 2014). It also interacts with a few ribosomal proteins, preventing translation, and transcriptionally downregulates the ribosomal genes (Farkas et al., 2014).

When a higher concentration of NCR247 is treated on S. *meliloti in vitro*, it demonstrates antimicrobial activity (Penterman et al., 2014; Van de Velde et al., 2010). It acts on the cell membrane and depolarizes it in *S. meliloti* (Tiricz et al., 2013). NCR247 can act as an antimicrobial against multiple species of bacteria (Jenei et al., 2020). It has also been shown that low concentrations of NCR247 peptide have significant effects on the *S. meliloti* transcriptome *in vitro* and induce TBD (Arnold et al., 2017; Farkas et al., 2014; Penterman et al., 2014; Tiricz et al., 2013; Van de Velde et al., 2010).

Like many other NCR peptides, the NCR247 peptide also possesses defensin-like antimicrobial properties. Despite recent developments, a large spectrum of functions associated with this peptide is yet to be found. Moreover, the mechanism is not clearly understood, specifically in the context of different bacterial strains (Jenei et al., 2020; Tiricz et al., 2013). Although there is ample evidence of the property itself or its associated functional significance, it is yet to be characterized.

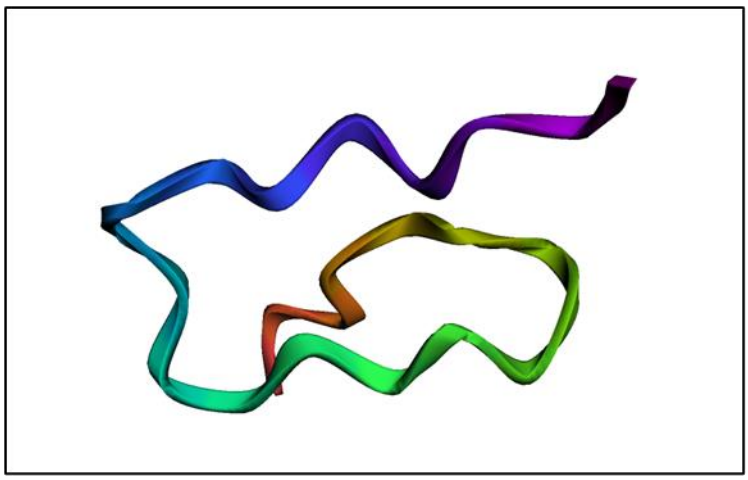

Fig 4: This figure represents predicted structure of NCR247 peptide using Alphafold.ipynb

Model organisms used in my study are *Sinorhizobium meliloti, Bradyrhizobium japonicum,* and *Escherichia coli*. *S. meliloti* and *B. japonicum* are Alphaproteobacteria. The Alphaproteobacteria class includes various organisms with diverse metabolic capabilities and ecological roles. This group encompasses most phototrophic genera, along with several genera capable of metabolizing C1-compounds (e.g., *Methylobacterium spp*.), symbiotic partners of plants (e.g., *Rhizobium spp*.) and animals, as well as a group of pathogens known as the Rickettsiaceae. Additionally, it's believed that the ancestors of eukaryotic mitochondria originated from bacteria related to *Rickettsia spp* (Young & Haukka, 1996).

On the other hand, the gram-negative *E. coli* belongs to the Gamma proteobacteria class of Proteobacteria.

E. coli is employed as a model organism due to its well-established genetics that allows easy manipulation. It is often employed in antimicrobial studies due to its status as a gram-negative bacterium, similar to the target pathogens of interest in the studies. It is also a totally different class of bacteria compared to *S. meliloti* and *B. japonicum*. Therefore, results spanning these three bacterial strains will provide a complete picture to elucidate gene functions.

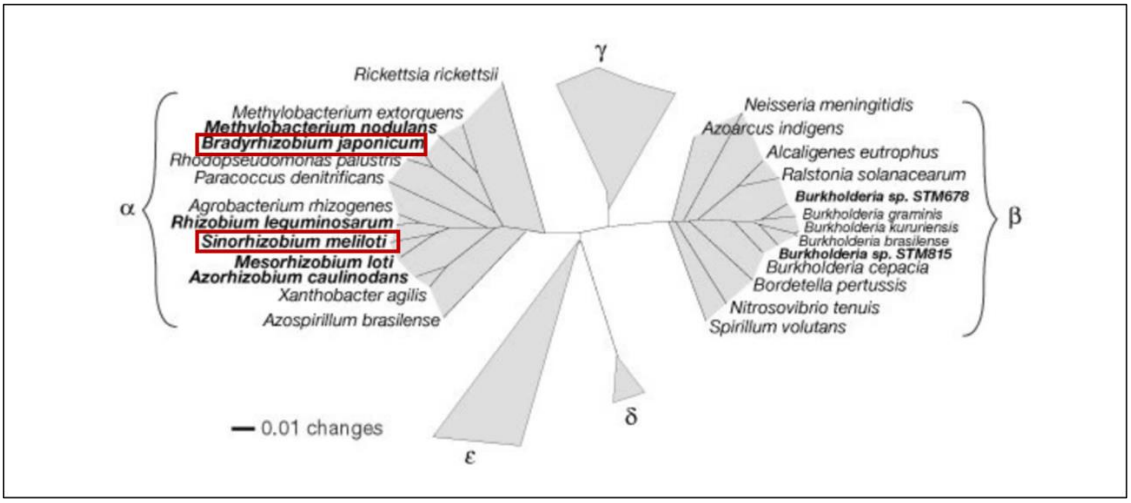

Fig 5: This figure shows phylogenetic relationships between the different rhizobial genera. Represents the various subdivisions of the Proteobacteria (Moulin et al., 2001).

As mentioned, *S. meliloti* is susceptible to the NCR247 peptide antimicrobial activity. A non-rhizobial bacteria, *E. coli,* and a rhizobia bacterium, *B. japonicum*, are resistant

to peptide treatment (Shown in results). This is despite *S. meliloti* and *B. japonicum* possessing homologous peptide import protein (Barrière et al., 2017).

The factors that make these bacteria resist the peptide treatment are yet to be discovered. We already know that NCR247 peptide causes significant effects on *S. meliloti* upon *in vitro* treatment, induces TBD, and exhibits antimicrobial properties at higher concentrations. Previous studies have demonstrated the genes responsible for *S. meliloti*, whose interruption alters resistance to NCR247 peptide (Arnold et al., 2017). However, the mechanism of TBD in *S. meliloti* is not known.

In this project, we have investigated the unknown factors that help *E. coli* and *B. japonicum* resist the peptide treatment. For *E. coli,* this project takes advantage of the Keio collection of *E. coli*. The *E. coli* Keio collection is a systematic collection of single-gene knockout of all nonessential genes from *E. coli* K-12 strains, BW25113. There are two independent deletion strains for each of the 3985 genes, for a total of 7970 strains. By using “one-step inactivation,” each deleted gene is replaced with a kanamycin resistance cassette that can be excised by an FLP recognition target (FRT) (Baba et al., 2006; Datsenko & Wanner, 2000). While making the Keio collection, they designed primers to amplify a kanamycin resistance gene flanked by FRT (Flippase Recognition Target) sites. The kanamycin gene flanked by FRT sites is amplified. Insertion of the kanamycin resistance gene flanked by FRT sites into the target gene achieved by a one-step inactivation method using λ Red recombination system. The kanamycin cassette is inserted into the target gene via homologous recombination, disrupting the target gene. The kanamycin-resistant colonies are screened to confirm the disruption of the target gene.

The *E. coli* K-12 Keio collection is an efficient tool for reverse genetics studies. The Keio collection's high-throughput screening capabilities expedite the discovery process by allowing rapid analyses of many mutant strains. This accelerates the identification of genes involved in bacterial susceptibility or resistance to antimicrobial peptides, paving the way for comprehensive and systematic analyses of the genetic determinants underlying these responses.

Exploitive studies were performed on *E. coli* to establish a comparative basis as it is a proven model organism with existing genetics screening tools like Keio Collection in K-12 (Baba et al., 2006).

For identifying resistant genes in *B. japonicum*, we created a genetic screening tool (Tn library) in *B. japonicum* LO to enable future detection of cellular pathways that allow them to resist NCR247 treatment. We used Barcode Sequencing, which is a high-throughput genetic screening technique that uses unique DNA barcodes to look the fitness of individual mutants within a pooled population. This allows for comprehensive analysis of gene function and phenotypic traits in diverse conditions (Yang et al., 2024). This library can also be used for different applications under different conditions. Whole genome sequencing was also performed on *B. japonicum* LO, serving as a novel database for this established model strain. The study aims to provide a broad overview that will serve as a basic framework and provide insight for further analysis in this field.

Additionally, we have standardized a cell sorting assay, to sort *S. meliloti* that has not undergone polyploidy, an important feature of differentiation. We have used a previously available transposon library from S. meliloti (Arnold et al., 2017) to identify genes responsible for polyploidy induction in *S. meliloti.*

Chapter 2

2.1 Materials

All the bacterial media LB, GSY(glutamate-salts-yeast), LB agar plates, GSY agar plates, 0.5M $CaCl_2$, 1M $MgSO_4$, 3M NaOAc (Lot No: 81986), TES buffer(Lot No: 82004), 10% SDS (Lot No: 81480), 1% PBS(Lot No: 82710), 50% Glycerol(Lot No: 82330), 0.85% Saline (Lot No: 82620), 10X TEN buffer(Lot No: 82002), 10X TE(Lot No: 81015), 50X TAE (Lot No: 81285), MOPS-GS (Lot No: 82089), and MOPS-GS+1% Casamino acids (Lot No: 82090) were purchased from the Stowers Institute, Media prep. NCR247 peptide was purchased from Genscript, and the DNA extraction kit (Cat No: A2920) and Proteinase K (Lot No: 0000545273) were purchased from Promega. Trimethoprim (Cat No: T7883), Diaminopimelic Acid (CAS No: 583-93-7), Phenol (CAS No: 108-95-2), Ethanol (CAS No: 64-17-5), Methanol (CAS No: 67-56-1), PVDF membrane filter (Lot No: 0000222708), and Swinnex (Cat No: SX0002500) was purchased from Sigma. Tween (CAS No: 9005-65-6), Isopropanol (CAS No: 67-63-6), and Agarose (CAS No: 9012-36-6) were purchased from VWR. Chloroform (CAT No: 194002) was purchased from Thermo Scientific. *Taq* DNA Polymerase with ThermoPol buffer (Cat No: M0267L), Quick-Load Purple 1 kb Plus DNA ladder (Cat No: N0469S), and Deoxynucleotide Solution Mix (Cat No: N0447L) were purchased from NEB. BacLight™ Green Bacterial Stain (Cat No: B35000) and SYTOX™ Green Nucleic Acid Stain (Cat No: S7020) were purchased from Thermo Fisher Scientific.

The following Antibiotics were used at the indicated concentration: Kanamycin (kan) 25 µg/ml, Gentamincyin (genta) 50 µg/ml, and Diaminopimelic Acid (DAP) 300 µM.

2.2 Growth Conditions and Bacterial Strains

The bacterial strains used in this study are mentioned in the table below.

Strains	Description	Media	Growth Condition
Escherichia coli			
HB101	HB101:pRK2013: mobilization helper plasmid	LB	37°C
APA_752	Mariner vector library pKMW3 in WM3064	LB supplemented with 50 µg/ml kanamycin and DAP	37°C
MG1655	Wild type	LB	37°C
BW25113	derivative of *E. coli* K-12, parent strain for Keio collection	LB	37°C
Sinorhizobium meliloti			
S. meliloti 1021	Wild type, derivative of SU47	LB supplemented with 2.5mM $MgSO_4$ and $CaCl_2$(LBMC)	30°C
Tn 1021	HIMAR1 transposon library pSAM_DGm in Sm10λpir	LBMC with gentamicin 50 µg/ml	30°C
Bradyrhizobium japonicum			
B. japonicum LO	Wild type, derivative of USDA 122	GSY (glutamate-salts-yeast) supplemented with MgSO4.7H2O and CaCl2. 2H2O	30°C
APA_752_LO	Mariner vector library pKMW3 in LO	GSY supplemented with MgSO4.7H2O, CaCl2. 2H2O, 75 µg/ml kanamycin and trimethoprim	30°C

Table 1: Showing different bacteria with their media and growth condition.

2.3 Methods

2.3.1 Growth Curve assay for Keio collection with the treatment of NCR peptide

Growth Curve assay is used to study the growth and proliferation of bacteria populations over time. This assay provides insight into the different phases of bacterial growth and its physiology and phenotypic condition.

Preparation for growth curve assay 96 well plates were made using 200 µl of LB in each well. 25 mg/ml kanamycin was used. This plate was inoculated from glycerol stock of Keio collection and incubated overnight at 37°C to be grown as primary culture.

The next day, another 96-well plate was made with 100 µl of LB, 25mg/ml kanamycin with 10 µM of NCR247 peptide. 2 µl of liquid culture from each well of the primary culture was added to this newly made plate. The optical density of each of these wells was measured at 600 nm wavelength at 30-minutes intervals for 16 hours at a constant temperature of 37°C. All growth curve experiments were performed in a Tecan SPARK plate reader using a polystyrene flat-bottomed, non-treated, sterile 96-well plate.

2.3.2 Agarose Gel Electrophoresis

Isolated DNA from the bacterial strains was validated using an agarose gel. Genomic DNA was run on a 1% agarose TBE gel, and 4 µl of Ethidium bromide (EtBr) was added to visualize DNA. 2 µl of DNA, and 2 µl of sample loading dye were mixed with 8 µl of water to make up the volume up to 12 µl. It was run for 1 hour on 100 volts and visualized under the gel doc.

2.3.3 Spot Assay

Spot Assay is also known as drop assay. This test checks the growth rate of bacteria or viability of microbial colonies under different experimental conditions. This assay is particularly useful for examining the effect of various treatments.

The cells were grown in LB supplemented with Kanamycin and 10 µM NCR247 Peptide for 16 hours. Then, serial diluted from 20^{-1} to $20^{-6,}$ and 7 µl of the sample was spotted on the LB plates containing Kanamycin at 37^{o}C and incubated for 20 hours.

2.3.4 Genomic DNA Isolation

Genomic DNA was isolated from the *B. japonicum* LO strain for genome sequencing. A 20 ml culture of OD_{540} 0.3-0.5 had been centrifuged at 9000 rpm for 5 minutes. The supernatant had been decanted, and the cells had been washed with TES, repeating the centrifuge step. After that, the cells were resuspended in 7.5 ml TEN buffer, 37.5 µl of 10 mg/ml RNase, and 37.5 µl of 10 mg/ml Proteinase K were added, along with 0.750 mL of 10% SDS, and incubated at 37°C for 2 hours. Extraction was performed with 7.5ml Phenol (TE-saturated, pH 7.8), slowly rotating at room temperature for 5 minutes and centrifuging at 2500 x g for 15 minutes to separate the phases. The top layer had been removed from the tube without disturbing the interphase. The repetitive extraction was performed with an equal volume of 1:1 phenol: chloroform. Final extraction was performed with an equal volume of chloroform. 2x Volume of 100% EtOH (cold) and 0.1x Volume of 3M NaOAc had been added and mixed carefully and properly and stored at -20°C for 30 minutes. The genomic DNA was transferred into a 1.5 ml centrifuge tube and centrifuged at maximum speed (usually 13,600 rpm) at 4°C for 5 minutes to pellet the gDNA. The gDNA was washed with Ethanol two times, and then ethanol was evaporated. The genomic DNA was resuspended in 200 µl TE, incubated at 68°C for about 2 hours, and then mixed gently. DNA concentration and purity were quantified using a Nanodrop machine, and the integrity of gDNA was checked by running it on an agarose gel.

2.3.5 Transposon Insertion Library preparation

2.3.5.1 Triparental Mating

Triparental Mating is bacterial conjugation; here, a conjugative plasmid is present in one bacterial strain and assists the transfer of a mobilizable plasmid present in a second bacterial strain into a third strain. Wild type *B. japonicum* LO (recipient), *E. coli* HB101 (helper), and *E. coli* APA_752 (mariner transposon vector library with ~3 million unique 20mer DNA barcodes flanked by common PCR priming sites derived from pKMW2) carrying pKMW3 (donor) were grown to an optical density $O.D_{600}$ 0.5 for *E. coli* strains and *B. japonicum* $O.D_{540}$ 0.4-0.6. Then, the *B. japonicum* cell is harvested at 8000 rpm for 10 minutes and wash the pellet with TES. Collect cells by spinning at 8000 rpm for 10 minutes and wash the pellet twice with TE to eliminate salts. Resuspend the pellet in LB Broth and measure the $O.D_{540.}$ Mix $5x10^8$ cells of *B. japonicum* with 108 cells of each *E. coli* strain. Using the syringe, filter cell suspension through sterilized filter paper (PVDF Membrane Filter, 0.45 µm Pore Size). Transfer

the filter to 0.2% YE plate Bug Side-up using sterile forceps and grow at 29°C. After 3 days, the cell grows into a lawn over the filter; transfer the filter to the 50 ml falcon and add 6 ml 0.01%tween-80 vortex for a minute. Plate 200 µl cell suspension on selection plate: GSY with 100µg/ml trimethoprim, kan 75 µg/ml. Incubate at 29°C until Colonies appear (approximately 7-10 days takes). After colonies were applied, cells were harvested from all the plates. Adding 5-6 ml of GSY media with 100µg/ml trimethoprim, kan 50 µg/ml on each plate. Scrap the colonies and pipetting into the empty autoclaved flask and dilute the resultant culture to target OD600, which should be 0.1. This cell mixture was grown at 30°C with shacking to get the final 0.6 O. D point. Then, the cell was centrifuged at 8000xg for 10 minutes and resuspended the cells in 20 ml of GSY media with 100µg/ml trimethoprim, kan 50 µg/ml, containing 15% glycerol, and stored as 1ml of aliquots at -80°C.

2.3.5.2 Colony PCR for confirmation of insertion

Colony PCR was performed to confirm the successful insertion of pKMW3 in the *B. japonicum* strain. Random colonies were picked partially from different plates using tips and resuspended in the 75 µl sterile water. Vortex well and incubate at 100°C for 5 minutes. PCR was set up by using oAD493 fwd (5'-ACA CTG GCA GAG CAT TAC GCC CT-3') and Inverted repeat rev (5'-ACA GGT TGG CTG ATA AGT CCC C -3').

PCR Step	Temperature °C	Duration
Denature template	95°C	5 minutes
35 cycles	95°C 55°C 72°C	30 seconds 30 seconds 2 minutes
Extension	72°C	2 minutes
Hold	12°C	

Table 2: The table represents the thermocycling condition for a colony PCR.

2.3.5.3 Sample preparation for Tn-seq

Genomic DNA isolation for the Transposon Library using Phenol Chloroform Extraction method. A Qubit assay was formed to check concentration, and the sample was submitted to the Sequencing core for Illumina sequencing, and the bioinformatics core analysed sequencing data.

2.3.6 Polyploidy and cell size sorting using FACS assay

The *S. meliloti* transposon mutant library (Tn1021) was inoculated into 500 ml of LBMC with Gent and incubated at 30^{o}C with continuous shaking until the culture reached an OD_{600} between 0.1 and 0.2. That 500 ml culture was divided into two 250 ml cultures. One was used as a control, and the other was treated with NCR247 peptide. The culture was spun down at this point, and the pellet was washed with 0.85% saline twice. And resuspended in 25 ml of prewarmed 3-(N-morpholino) propane sulfonic acid (MOPS)-buffered minimal medium (50 mM MOPS, 1 mM MgSO4, 0.25 mM CaCl2, 19 mM glutamic acid, 4 mM biotin [pH 7.0]), and incubate for 4.5 hours, in that time ~95% of cell will have one copy of their genome (1n). And resuspended the arrested cell pellet in 25ml of prewarmed 3-(N-morpholino) propane sulfonic acid (MOPS)-buffered minimal medium supplemented with 1% Casamino Acids (MOPS-GS Cas). Then, 4µM NCR247 peptide was added to the culture. The other one was kept as an untreated control. The addition of Peptide was considered as the initial time point (t =0), and 4ml of culture were collected at 0,70,100,130,160 and 190 minutes. The cultures were kept at 30°C with continuous shaking between the time points. The collected samples were spun down and resuspended in 1ml of 1%PBS and Stored at 4^{o}C overnight. The samples were stained with BacLight™ Green Bacterial Stain. The BacLight™ Green bacterial stains are fluorescent, non–nucleic acid labeling reagents for detecting and monitoring bacteria. Bacteria were stained with 10 µM concentration of the stain and run on the S6 sorter. The cells were sorted based on size and collected in culture media for further downstream assays.

2.3.7 Genomic DNA Isolation from *S. meliloti*

Genomic DNA from the Sorted cells from the FACS (*S. meliloti* transposon mutant library) was isolated using a Promega DNA Extraction kit. Sorted cells from FACS were grown in LB supplemented with gentamicin for 24 hours, collected, and genomic DNA was isolated using the protocol provided by the manufacturer. DNA concentration and purity were quantified using a nanodrop machine and validated using Agarose TBE gel.

2.3.8 Analysis and Plotting

All growth curve assay results were analysed using MS Excel, and graphs were plotted using Origin 2022 and GraphPad Prism 9.0.1. FACS results were analysed using using FlowJo™ v10.8 Software (BD Life Sciences).

Chapter 3
Results

3.1 Optimization of the Growth Curve of Bacteria in different media.

To study the impact of NCR247 peptide on the growth of different gram-negative bacteria, we performed a growth curve assay. The study was performed on three vastly different strains of bacteria, namely *E. coli*, *S. meliloti*, and *B. japonicum*. Each of these strains was treated with NCR247 peptide at different concentrations and compared to their respective untreated groups. It was found that *S. meliloti* and *B. japonicum* are slow-growing strains compared to *E. coli* and reach the saturation phase way later than *E. coli*.

Bacteria strain	Growth Media	Concentration of peptide	Duration
E. coli	LB	10 μM	16 hours
S. meliloti	LB	10 μM	32 hours
B. japonicum	GSY	10 μM	72 hours

Table 3: This table shows the optimized media, sublethal concentration of NCR247 peptide, and duration of growth curve assay.

A growth curve assay was performed with various concentrations of NCR247 peptide, and we noticed that at 10 μM where *S. meliloti*, after certain hours of growth, started showing growth defects. But at higher concentrations (15 μM and 20 μM), *S. meliloti* showed very little growth. Interestingly, *E. coli* and *B. japonicum* grew normally in higher concentrations. So, we decided to use 10 μM of NCR247 to assess growth in all further experiments. Each strain was grown till the saturation phase to see the effect of the NCR247 peptide. And then, the final duration for the growth curve assay was decided, as mentioned above in Table 3. The growth media respective to each strain is mentioned in Table 3.

As mentioned in Table 1, for *S. meliloti* and *B. japonicum*, their respective media are supplemented with $MgSO_4$ and $CaCl_2$. However, during all growth curves assay, they are not supplemented with $MgSO_4$ and $CaCl_2$; it has been seen in the previous study that in typical growth medium, the majority of cationic peptides are unable to cause

damage to the electronegative bacterial membranes. In particular, when Mg^{2+} and Ca^{2+} are present, they interact with negatively charged lipid moieties to prevent them from reaching the bacterial membranes. (Hancock & Sahl, 2006) (Jenei et al., 2020).

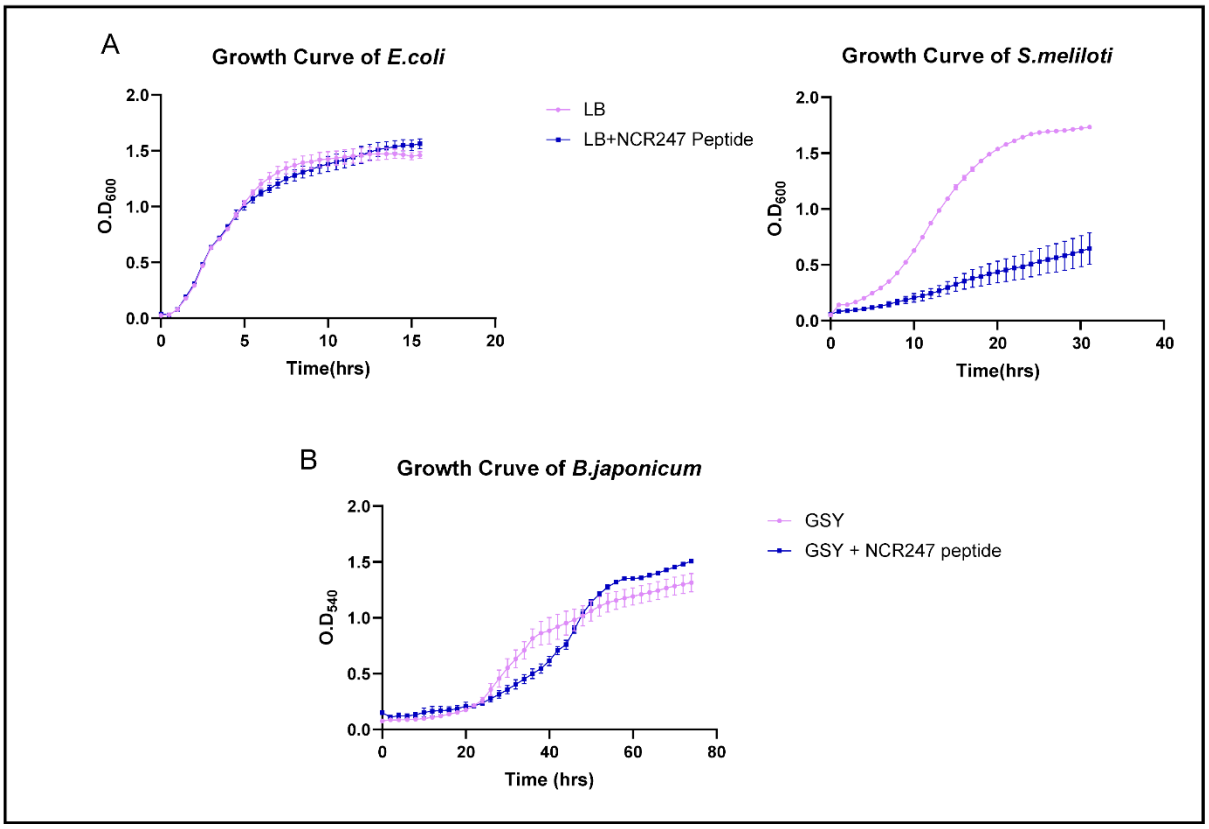

Fig 6: This figure denotes the growth curve assay of three different bacteria.
(A) Growth curve of *E. coli* and *S. meliloti.* In the graph, the X-axis represents the time in hours, and the Y-axis represents the optical density at 600nm.
(B) Growth Curve of *B. japonicum.* In the graph, the X-axis represents the time in hours, and the Y-axis represents the optical density at 540 nm. Pink indicates the untreated sample, and blue indicates the treated sample with 10 μM of NCR247 peptide. Each graph represents three technical replicates, and their mean value is plotted with SEM as an error bar.

The growth curve assay was performed to see how these three distant bacteria show phenotype on the treatment of NCR247 peptide. In (Fig 6A), *E. coli* WT MG1655 did not exhibit any defect and grew normally compared to untreated. Meanwhile, the *S. meliloti* growth curve shows that upon treatment of NCR247 peptide, *S. meliloti* WT exhibits strong growth reduction, signifying defects in their growth. However, in (Fig 6B), upon treatment of NCR247 peptide, *B. japonicum* LO did show a slight lag in the

initial growth phase in the treated condition compared to untreated, but it reached the same final O.D as untreated.

We conclude that upon treatment with the NCR247 peptide, *E. coli* and *B. japonicum* do not show any defect in their growth. *S. meliloti* shows growth defects upon the NCR247 peptide treatment, which validated previously known data.

3.2 Investigating the genes involved in resistance towards NCR247 Peptide in *E. coli*

As observed from (Fig: 6), *E. coli* resists NCR247 peptide treatment. The Keio Collection is a great and efficient genetic screening tool for addressing this question. All Keio mutants were grown in LB supplemented with 25 µg/ml kanamycin overnight. Then, the fresh plate was inoculated using an overnight culture made with LB supplemented with 25 µg/ml kanamycin and treated with 10 µM NCR247 peptide. The treated plate was run in a plate reader for 16 hours., and optical density was measured at 600nm after every 30 minutes.

There are two copies of each mutant in the Keio collection for each of the 3985 genes. This makes the total number of 7970 mutant strains.

After completing the first round of screening for all mutants, we got 188 mutants, of which only one single copy showed a defect in the growth curve, and 62 mutants, of which both copies showed a defect. Mutants from the first screening were further assayed with normal growth media without any treatment of NCR247 peptide. It was found that few of those mutants have growth defects irrespective of NCR247 peptide treatment. All these mutants having growth defects irrespective of NCR247 treatment were eliminated, and the remaining mutants were considered. After multiple screenings, only 10 mutants showed growth defects consistently when treated with NCR247 peptide.

From (Fig: 7), it could be concluded that the loss of these genes knockout made the bacteria susceptible to NCR247 peptide through the demonstrated phenotypic growth defects.

To check the dose-dependence of NCR247 peptide on bacteria, we consecutively increased the peptide concertation of peptide treatment on bacteria to 12 µM and 15 µM. It was found that *ΔsurA* and *ΔtopB* showed more prominent growth defects on 15 µM treatment.

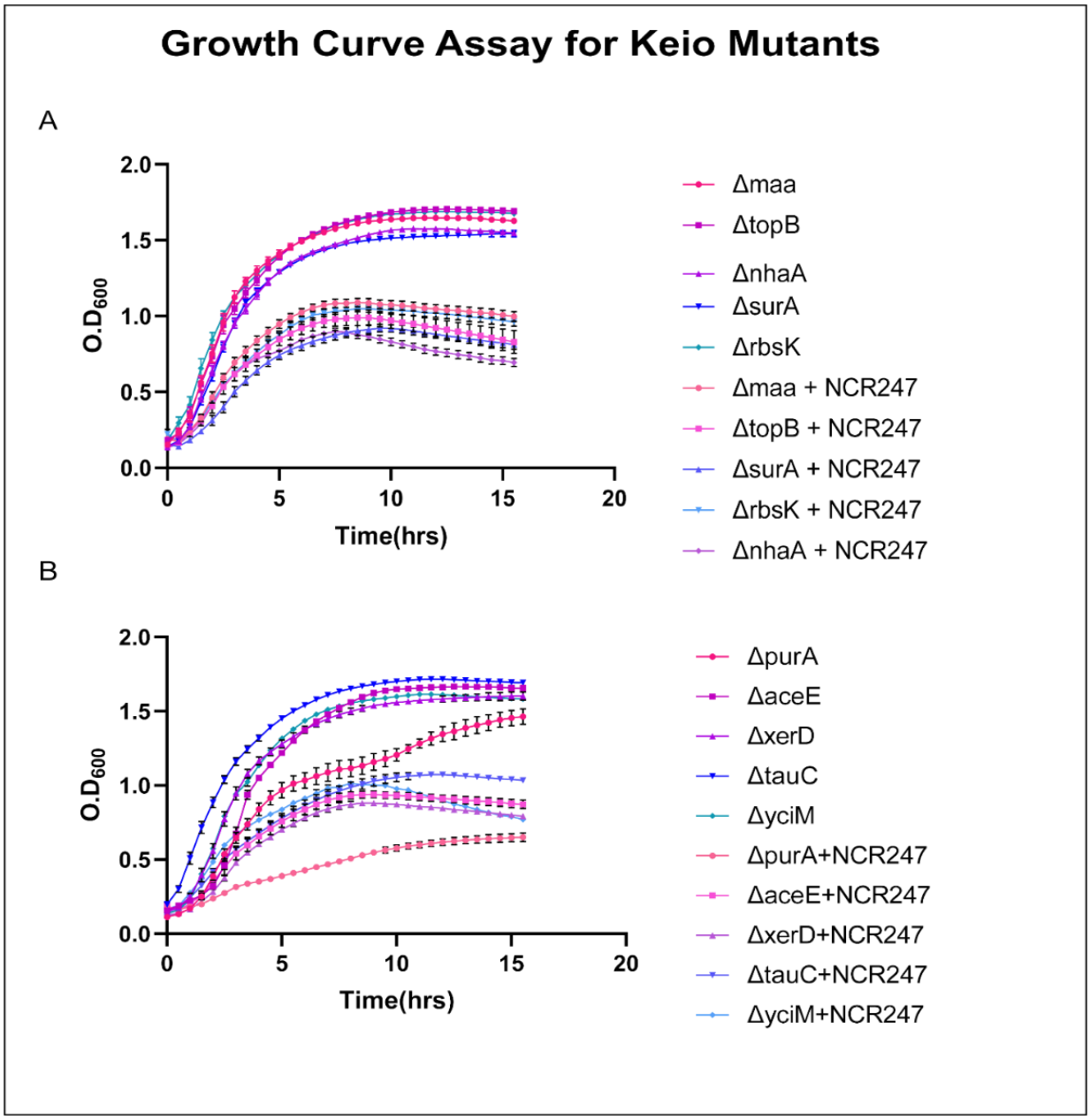

Fig 7: The figure represents the Growth curve assay for Keio Mutants.

(A) Represents the first five mutants with and without NCR247 peptide treatment.

(B) Represents the remaining five mutants with NCR247 peptide treatment and without treatment. In the graphs, the X-axis represents the time in hours, and the Y-axis represents the optical density at 600nm. Each graph represents three biological replicates, and their mean value is plotted with SEM as an error bar. All the treatments were done with 10 μM of NCR247 peptide.

3.2.1 Determination of function of identified genes.

Gene	Function
ΔpurA	Involved in the de novo purine biosynthesis pathway and encodes for adenylosuccinate synthetase.
ΔaceE	Involved in converting pyruvate to acetyl-CoA in the tricarboxylic acid (TCA) cycle and encoding for pyruvate dehydrogenase.
ΔxerD	Involved in the site-specific recombination and chromosome segregation and encodes for site-specific recombinase.
ΔtauC	Involved in the uptake of taurine and encodes for taurine transport protein.
ΔyciM	Involved in the assembly of lipopolysaccharides.
Δmaa	Encodes for maltose O-acetyltransferase.
ΔtopB	Involved in the replication fork and plays a role in chromosome decatenation and encodes for DNA topoisomerase III.
ΔnhaA	Involved in the uptake of protons in exchange for cytoplasmic sodium ion, alkaline pH homeostasis, and encodes a sodium-proton antiporter.
ΔsurA	Involved in the proteins folding and encodes for periplasmic chaperone
Δrbsk	Involved in the catabolism of ribose, encodes for ribokinase.

Table 4: The table represents the name of the 10 mutants and their functions.

To check the molecular functions where this mutant can act and give susceptibility to *E. coli.* We manually checked the gene functions and found that out of 10 mutants, 4 mutants encode for membrane protein. Among them, three are localized in the inner membrane and one in the periplasmic space. The remaining 6 mutant genes are involved in different pathways in *E. coli.*

3.2.2 Validation of candidate genes with Spot assay.

After getting 10 candidates from the growth curve assay, we wanted to check if the NCR247 peptide is bactericidal or bacteriostatic. We did a spot assay on those 10

mutants by treating them with 15 µM NCR247 peptide based on the data from the growth curve assay.

After treatment with 15 µM of NCR247 peptide, all the mutants were grown in solid medium LB agar supplemented with kanamycin.

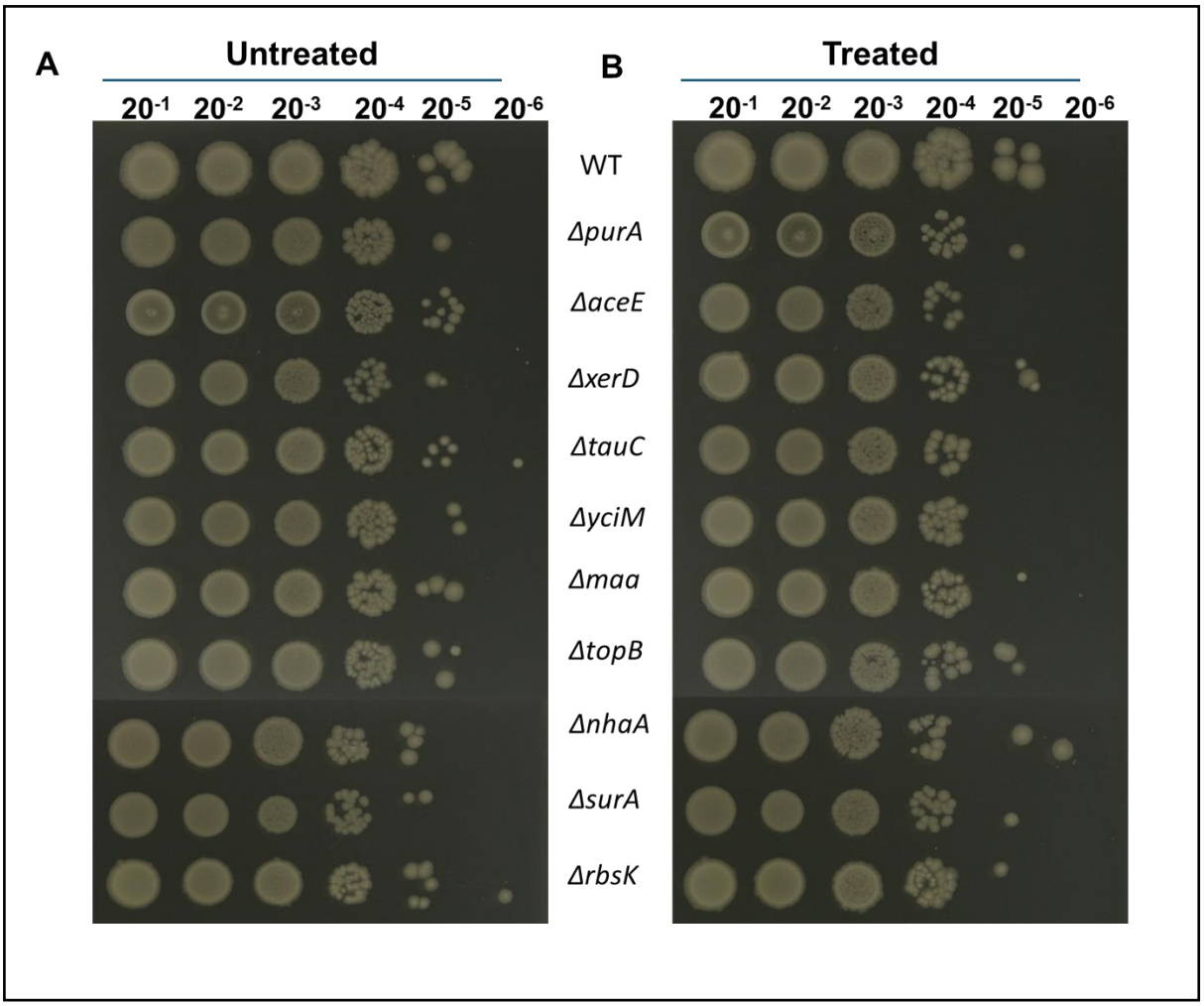

Fig 8: The figure represents the spot assay. (A) Represents wild type and 10 mutants without NCR247 peptide treatment as a control group. (B) Represents wild type, and 10 mutants were treated with 15 µm of NCR247 peptide. Both were serial diluted 20^{-1} to 20^{-6}- fold and 7 µl was spotted on plates containing LB agar supplemented with kanamycin.

In both treated and untreated conditions, the wild type showed proper growth and started showing single colonies from the 5th dilution. Also, all mutants in untreated conditions were similar to wild type. However, mutants in treated conditions showed lesser growth in general. *ΔaceE, ΔtauC*, and *ΔyciM* mutant showed no growth in the 5th dilution (Fig: 8B). Compared to untreated, we started getting lesser isolated colonies from the 4th dilution (Fig: 8A & B). Compared to untreated, the other 6

mutants showed lesser colonies and started showing isolated colonies from the 4th dilution itself (Fig: 8A & B). This also agreed with what we saw in the growth curve assay.

3.3 Successful preparation of Transposon Mutant Library in *B. japonicum* LO for Barcode Analysis by Sequencing (BARseq)

To identify the gene involved in the resistance of NCR247 peptide in the *B. japonicum*. The transposon mutant library was created in the *B. japonicum* LO strain. To create the transposon mutant library, we selected BARseq over Tn-seq because BARseq has a higher resolution. It allows for the simultaneous screening of thousands of mutants in a single experiment. BARseq precisely quantifies the prevalence of mutants in mixed populations by tagging individual mutants with distinct DNA barcodes. The relative fitness differences between mutants under different environments can be understood using this quantitative data.

We used the APA_752 strain of *E. coli* to create the transposon mutant library, which has *mariner* transposon delivery with vector pKMW3, a pHIMAR derivative (Bouhenni et al., 2005; Wetmore et al., 2015). We used triparental mating for creating the transposon mutant library. After the successful preparation of the library, genomic DNA was isolated from the library and sent for Tn-Seq library preparation and sequencing.

However, sequencing requires a previously annotated genome to which it could be aligned. The genome of *B. japonicum* LO was not sequenced. Therefore, we went ahead and isolated the Genomic DNA from the wild type of LO strain and submitted it to PacBio for whole genome sequencing.

3.3.1 Summary of Whole Genome Sequencing for *B. japonicum* LO

The complete genome of the symbiotic bacterium *Bradyrhizobium japonicum LO* was sequenced. *B. japonicum* strain LO is a derivation of the agriculturally important strain USDA122. This strain was modified with the treatment of the antibiotic Nalidixic acid (Nal^r) and is being used as a wild type of strain nowadays (Maier et al., 1978; O'brian et al., 1987). *B. japonicum* LO genome comprises of a single circular chromosome 9,165,923 bp in length with an average GC content of 64.97%. No plasmids were

detected after sequencing. To do the comparative study in different standard *B. japonicum* strains, *B. japonicum* USDA110 was selected as the most used wild type of strain in the laboratory and had an already sequenced whole genomic database. In the comparison between *B. japonicum* USDA110 and its parental strain, *B. diazoefficiens* USDA122, it was observed that *B. japonicum* LO exhibits a longer genome length. The *B. japonicum* LO genomic assembly was aligned with the other two strains to do more analysis and look at the genomic disparities. It was revealed that *B. japonicum* LO had more similarities to USDA122 than *B. japonicum* USDA110.

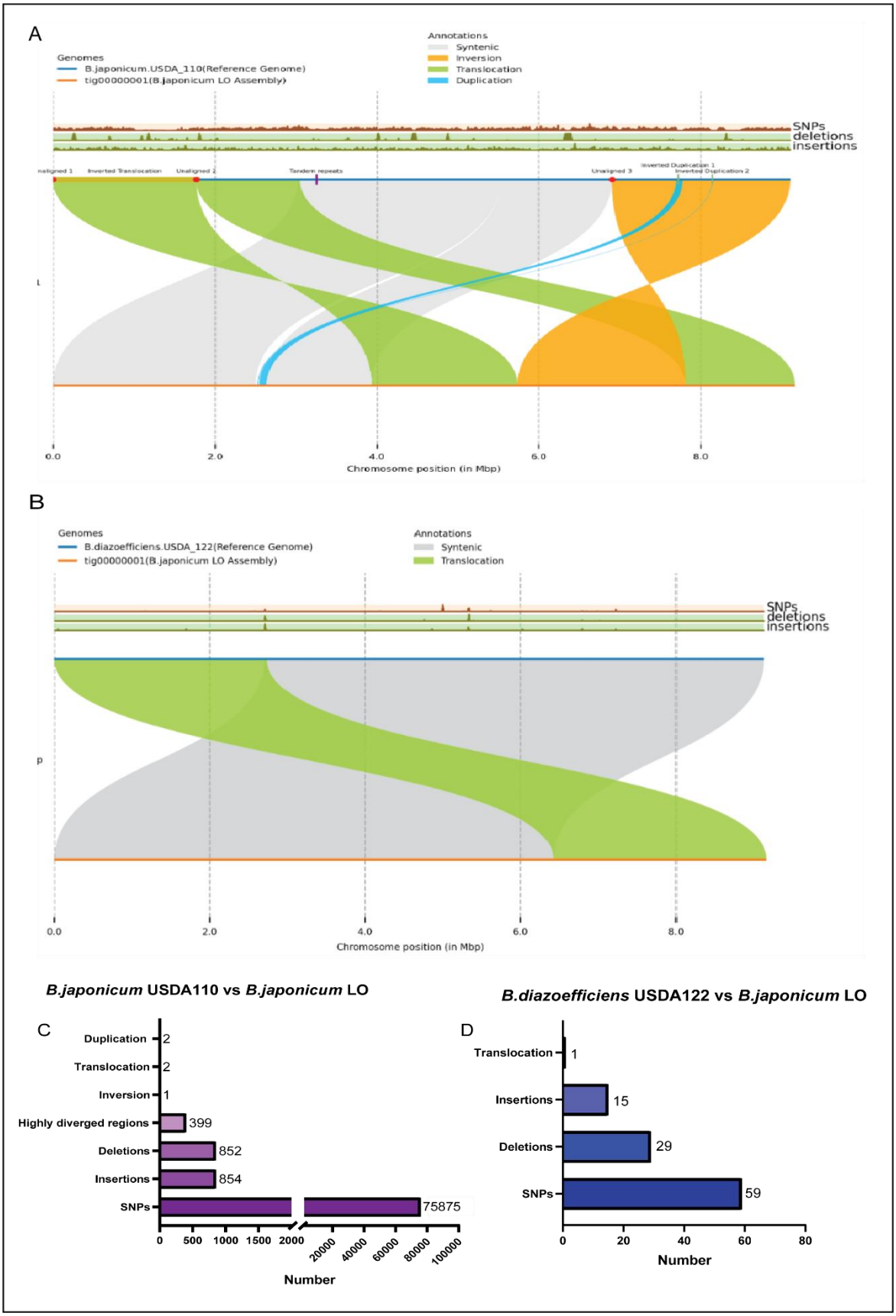

Fig 9: This figure represents the Genomic data of *B. japonicum* LO. (A) Represents the synteny plots of the chromosome of *B. japonicum* USDA110 and *B. japonicum*

LO. (B) Represents the synteny plot of the chromosome of *B. diazoefficiens* USDA122 and *B. japonicum* LO. (C, D) Represents the distribution of variations.

When it was aligned with *B. japonicum* USDA110, it was found there were 75,875 Single nucleotides polymorphism (SNP), 854 Insertions, 852 Deletions, 399 highly diverged regions, 1 Inversion, 2 Translocations from that one inverted translocation and two duplications in the *B. japonicum* LO assembly. Furthermore, compared with the parental strain of *B. japonicum* LO, USDA122. It was observed that there are 59 SNPs, 29 deletions, 15 Insertions, and 1 Translocation in the *B. japonicum* LO assembly.

3.4 Investigating the genes involved in TBD upon treatment of NCR247 Peptide in *S. meliloti*

S. meliloti, upon *in vitro* treatment of NCR247 peptide, induces cell elongation and polyploidy. To investigate the genes involved in these phenotypes, we used the high-quality, pooled transposon mutants library in *S. meliloti* (Arnold et al., 2017). The pooled transposon mutants were synchronized to get all bacteria in the same cell cycle phase treated with 4μM of NCR247, a sub-lethal dose, and sorted based on the intensity of the BacLight™ Green Bacterial Stain. BacLight™ Green Bacterial Stain is a fluorescent dye used for staining bacterial cells. It interacts with intact cell membranes and gives a green fluorescent signal. Thus, the intensity of BacLight™ Green can denote the cell size. More intensity of BacLight™ Green denotes a bigger cell size.

We did an optimization experiment with a wild type of *S. meliloti*, Rm1021. Cell synchronization assays were performed on the *S. meliloti*, Rm1021, and treated with 4 μM of NCR247 peptide. Starting with 70 minutes of treatment, we observed a significant shift in the intensity of backlight Green in the cells in the treated condition when compared to the untreated. As seen in (Fig: 10), the cell shifts to higher intensity became prominent with time.

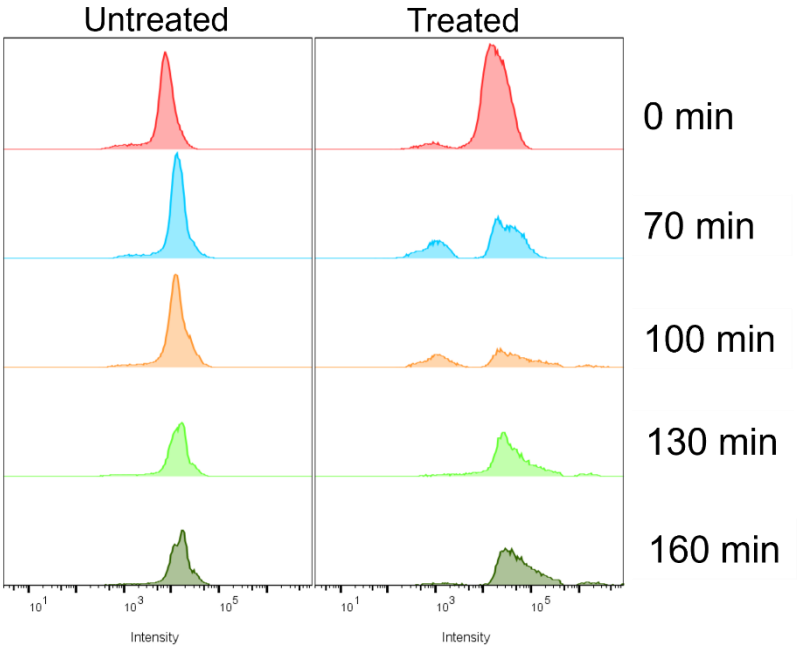

Fig 10: This histogram denotes the distribution of wild type *S. meliloti*, Rm1021 after NCR247 peptide treatment. The left panel denotes untreated condition, and the right denotes treated condition at different time points.

The cell shift in the treated condition signifies that the cell is undergoing the TBD. TBD has a characteristic of cell enlargement. With increased cell size, the intensity of BacLight™ Green Bacterial Stain also increases.

After confirming all the conditions and validating our experimental setup in the wild type of *S. meliloti*, Rm1021, we proceeded with the *S. meliloti* transposon mutants library per the pre-optimized conditions. Cell Synchronization assays were performed on the *S. meliloti* transposon mutants and treated with 4 µM of NCR247 peptide, and they were run on the S6 sorter with the same setting. We observed the characteristics of a significant shift starting from 70 minutes post-treatment. However, at later time points, we can observe peaks at two different intensities of BacLight™ Green Bacterial Stain in bacteria populations (Fig:11). The peak at lower intensity denotes those mutant populations that could not achieve the TBD. Hence, those populations were sorted in growth media and grown overnight. The populations that achieved TBD (peak at higher intensity) were also sorted in growth media and grown overnight. Genomic DNA was isolated from both populations using the Promega Genomic DNA isolation kit and sent for Tn-Seq library preparation and sequencing. Mutants that fail to differentiate will be identified through sequencing.

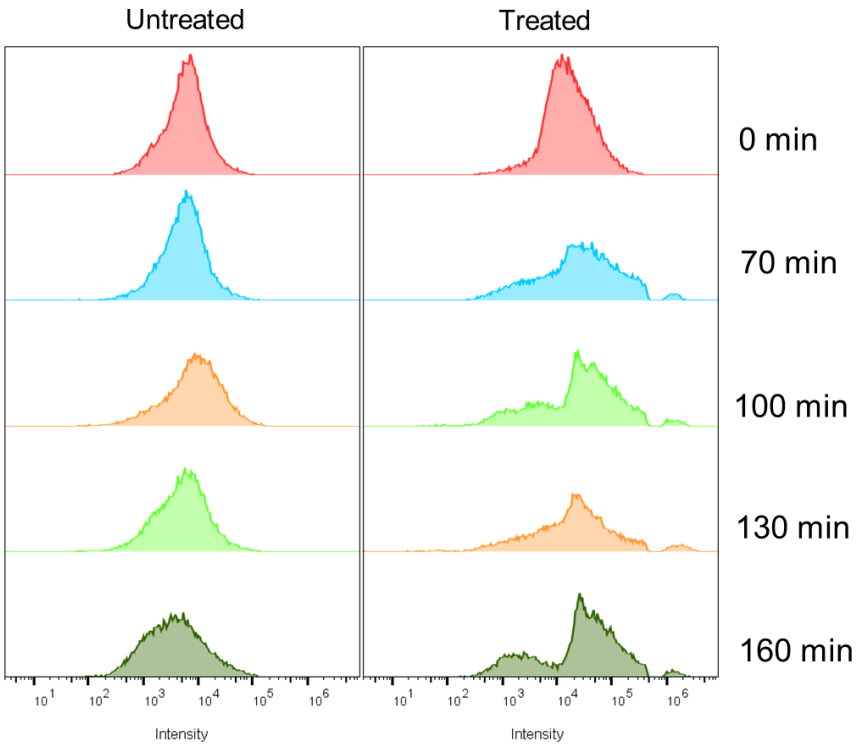

Fig 11: This histogram denotes the *S. meliloti* transposon mutant library distribution after NCR247 peptide treatment. The left panel denotes untreated condition, and the right panel denotes treated condition at different time points.

Discussion

In this study, we showed that *E. coli* and *B. japonicum*, upon treatment with the NCR247 peptide, do not show any growth defect and are resistant to the NCR247 peptide. We started with a Growth curve assay on Keio collection mutants and found 10 mutants, which are potential candidates for further study. We also did the spot assay to determine if the NCR247 peptide is bactericidal or bacteriostatic. The preliminary results showed that the NCR247 peptide is bactericidal in those 10 mutants. Many cationic peptides act on membrane proteins. Among 10 of the Keio mutants, 4 are associated with membrane proteins. It suggests that these membrane proteins provide stability and resistance to NCR247's action on the membrane of NCR247 in *E. coli, which* might follow a similar pattern to that of *S. meliloti*. However, to validate this claim, we will have to make gene knockouts in a clean wild type background and verify the membrane stability and morphological changes upon peptide treatment by using scanning electron microscopy. Membrane permeability of these mutants can also be verified by propidium iodine uptake and subsequent staining of the bacteria. We can also perform some transcriptomic analysis, showing the changes in gene expression patterns in response to NCR247 treatment and giving information about affected cellular processes and pathways associated with peptide treatment. To identify the proteins interacting with NCR247, a pull-down assay using biotinylated peptide can be performed, and the targets can be identified by mass spectrometry. Correlating the transcriptomic and proteomic analysis with the growth mutants that we have identified will give us a broad picture of pathways in *E. coli* that provide resistance to NCR247.

We did the gene ontology to check the correlation between the genes but did not get a significant enrichment from it. The reason for not getting significant enrichment in the gene ontology might be the small number of entries due to the limited sample size and insufficient statistical significance. We might have to do different analyses to know the correlation between these 10 genes, as mentioned above.

We investigated 10 genes manually and got the correlation between them, as shown in (Fig:12). *tauC* and *nhaA* are involved in transmembrane transport; therefore, their absence might have altered the membrane permeability and allowed the peptide to penetrate into the cell easily, affecting the growth of the bacteria.

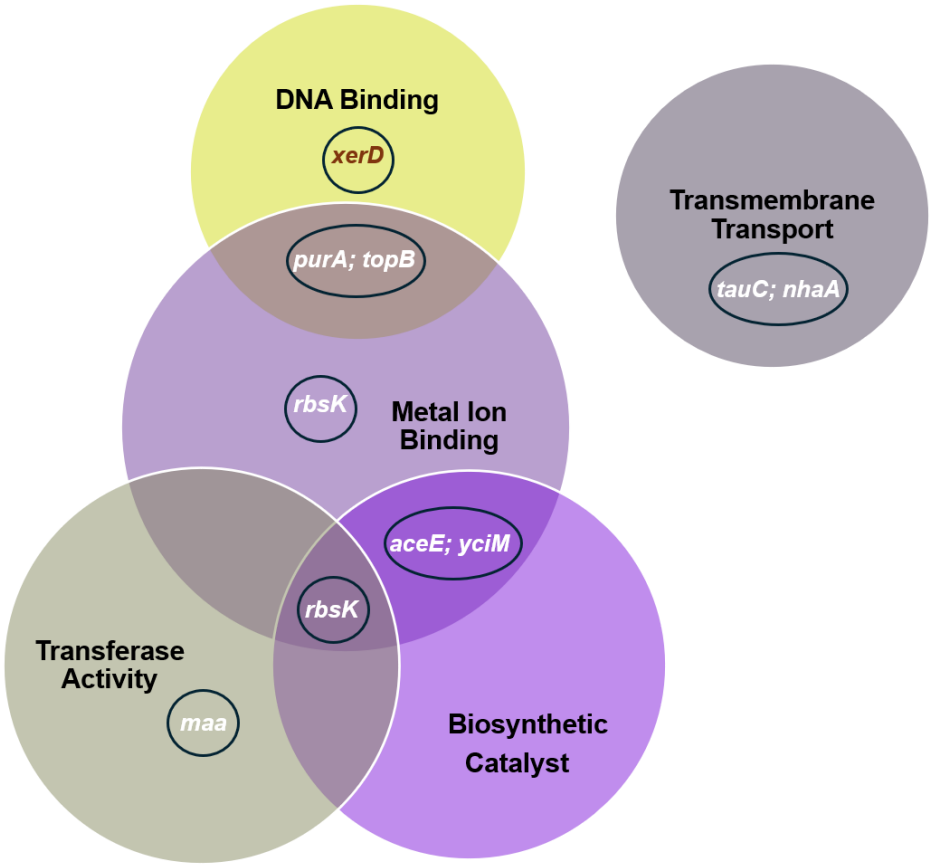

Fig 12: The image represents a Venn diagram showing the correlation between the 10 Keio mutants gene.

xerD, *purA*, and *topB* are involved in DNA binding; among them, *purA* and *topB* have metal binding activities. *rbsK*, *aceE*, and *yciM* also have metal binding activities. Moreover, *rbsK*, *aceE*, and *yciM* are involved in biosynthetic catalyst activities. Metal ion binding proteins might bind to the cationic peptide and neutralize them. However, in their absence, the peptide might be freely available, causing defects in the growth. *maa* and *rbsK* are involved in transferase activities. Transferase enzymes are essential for activating or deactivating different protein functions and cellular pathways. With peptide treatment, this protein might have enzymatically inactivated the peptide. But, in the absence of this protein, this might not have been possible, leading to bacterial cell death.

surA is a very specific case involved in protein folding of outer membrane proteins. In the absence of *surA*, the peptide might have misfolded several proteins affecting

bacterial growth. This is a very preliminary prediction of the mode of action for NCR247, which needs to be validated with further indeed analysis.

To valid whether this is a global phenomenon and not strain-specific, deletion of those specific 10 genes could be made in the different strains of *E. coli* and check for a similar phenotype.

The Keio collection did not have mutants for all essential genes in *E. coli*, but it would be fascinating to understand if those crucial genes are also involved in the resistance of NCR247 in *E. coli*.

The Transposon library is made in the *B. japonicum* LO strain to investigate the genes involved in resistance towards NCR247 Peptide. We could perform the growth competitive assay on the mutant library, treating them with NCR247 peptide. This assay will get the mutants that are sensitive to the NCR247 peptide. After getting the mutants, we can isolate the genomic DNA and send them for BARseq. The BARseq data will reveal the genes that made the *B. japonicum* resistant to the NCR247 peptide. The same assays described for *E. coli* mutants can be performed on *B. japonicum* to arrive at the pathways contributing to resistance in *B. japonicum.*

To investigate the genes involved in TBD upon treatment of NCR247 peptide in *S. meliloti*, we isolated the genomic DNA from the sorted population that did not undergo TBD. We sent it to a sequencing facility for creating a Tn-seq library and subsequent sequencing analysis.

As TBD has a characteristic feature of increasing ploidy (endoreduplication), we tried to do an analysis based on the DNA content. We used SYTOX™ Green Nucleic Acid Stain, which binds to fixed cell nucleic acid. However, we encountered a problem while sorting the fixed cell. We were not able to count the cell pellets after sorting. This might have happened due to the busting of cells during the process; hence, we could not isolate DNA. Therefore, we proceeded with BacLight™ Green Bacterial Staining and performed subsequent sorting using cell size.

The Tn-seq data will reveal the mutants that did not undergo TBD and the genes involved in cell elongation and polyploidy upon peptide treatment in *S. meliloti.* Individual gene knockouts in the wild type could be made to validate that data. The *S. meliloti* knockout strains could be treated *in vitro* with NCR247 peptide, and phenotypic changes can be verified.

This analysis will help us to identify molecular mechanisms and cellular pathways. That enables *S. meliloti* to undergo TBD and *E. coli* and *B. japonicum* to develop resistance upon treatment with the same peptide. A comparison of the resistance genes from *E. coli* and *B. japonicum* might also indicate the evolutionary conservation of related functions across species. This will help us design drugs as novel antibiotics or as adjuncts to the already available ones. Moreover, we might be able to therapeutically target antimicrobial resistance or multi-resistant bacteria. Deciphering the molecular pathway behind TBD might provide insight into what makes *S. meliloti* unique in undergoing differentiation.

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