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Characterization of Antigenic Properties of Two Immunogenic Proteins of *Streptococcus pneumoniae*

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Table of content

Table of Figures

List of Tables

Table of Abbreviations

ChoP	Pneumococcal Phosphocoline
CbpA	Choline-Binding Protein A
CPS	Capsular Polysaccharide
DC	Dendritic Cell
GFP	Green Fluorescent Protein
IPD	Invasive Pneumococcal Disease
LR	Laminin Receptor
MV	Membrane Vesicle
NVT	Non-Vaccine Type
OMV	Outer Membrane Vesicles
PLY	Pneumolysin
PspC	Pneumococcal Surface Protein C
PAFR	Platelet Activating Factor Receptor
pIgR	Polymeric Immunoglobulin Receptor
PCR	Polymerase Chain Reaction

PPIase	Peptidyl-Prolyl Cis-Trans Isomerase
PCV	Pneumococcal Conjugate Vaccine
S. pneumoniae	*Streptococcus pneumoniae*
SP	Signal Peptide
TLR	Toll-Like Receptor
URT	Upper Respiratory Tract
VT	Vaccine Type

Abstract

The bacterium *Streptococcus pneumoniae* (pneumococcus), is considered to be a leading cause of morbidity and mortality globally, particularly in infants and the elderly. It is one of the most frequent causes of respiratory tract infections, which sporadically have the potential to develop into serious invasive symptoms including sepsis and meningitis.
The development of effective vaccination against this pathogen is essential for reducing the morbidity and mortality it causes since the currently available vaccines can protect against only a limited number of the 100 pneumococci serotypes which target the polysaccharidic capsule of the bacterium.

The potential use of conserved protein antigens could provide a defense to a wider range of serotypes and clonal types. The immunogenic properties of the proteins MalX and PrsA as well as their role in vital biological functions of *S. pneumoniae* have made them stand out as potential targets. MalX is a crucial membrane protein involved in the metabolism of maltose, whereas PrsA is a chaperone-like protein that is connected to the cell envelope. To understand these proteins' potential as vaccine candidates, it is essential to understand their immunogenic characteristics and physiological roles.

In this project, we tried to characterize the two antigens to determine the functional significance of different regions and domains in antigen recognition and their expression dynamics in bacterial host. A better understanding of the antigenic properties of the PrsA and MalX proteins will drive the construction of improved versions of antigens for vaccine prototypes. Some approaches were used to clarify the structural characteristics and antigenic determinants associated with these proteins including, protein expression, purification, and structural characterization. Additionally, their expression in *E. coli* was examined using immunological

assays including ELISA and Western blot. The identification of antigenic regions of these proteins also provides insight into how to develop epitope-based vaccinations that specifically target *S. pneumoniae.*

This project discusses the possibility of using membrane vesicles (MVs) as a platform for vaccination. Membrane vesicles made from bacterial cells have innate immunogenic qualities that expose the immune system to a wide variety of antigens. Incorporating MalX and PrsA into such vesicles can improve the vaccine candidate's overall immunogenicity and effectiveness and trigger a stronger immune response against *S. pneumoniae*.

Popular science summary

Pneumococcus bacterium is known to cause respiratory infections like pneumonia and has the potential to cause life-threatening complications like meningitis, bacterial pneumonia, and other invasive diseases, so it is considered to be a leading cause of mortality and morbidity globally especially among young children and elderly.

Our group has been able to identify the antigenic characteristics of two proteins found in *S. pneumoniae*, PrsA and MalX through careful studies, shedding light on their potential as vaccine candidates against *S. pneumoniae* infections. Antigens are substances that elicit an immune response from the body, and they are essential for developing efficient vaccinations. The study of the antigenic characteristics of these two immunogenic proteins has progressed in an exciting way.

PrsA and MalX had been shown to be extremely immunogenic, meaning they are able to strongly stimulate the host's immune system. The specific regions, or epitopes, on these proteins that activate the immune system, were identified and validated. PrsA and MalX are considered to be appealing targets for vaccines since these epitopes were discovered to be highly conserved, which means they are similar across many strains of *S. pneumoniae*.

Furthermore, studies have shown that PrsA and MalX can activate cellular and humoral immune responses. Cellular immunity involves the activation of immune cells like T cells, whereas humoral immunity involves the activation of antibodies. Due to their complementary immune responses, PrsA and MalX may be able to offer complete defense against *S. pneumoniae* infections. Additionally, researchers discovered that PrsA and MalX share little in common with human proteins in terms of sequence, which lowers the possibility of autoimmune reactions in those who have received vaccinations. This is a crucial safety factor for the development of vaccines.

Overall, the identification of PrsA and MalX proteins in *S. pneumoniae* represents a significant advancement in our knowledge of the potential of these proteins as vaccine candidates. To properly assess their efficiency in reducing *S. pneumoniae* infections and the consequences they are connected with, more analysis and clinical trials are required. However, these discoveries provide promise for the development of fresh and enhanced vaccinations against this widespread and sometimes fatal pathogen.

Keywords

Streptococcus pneumoniae, Antigenic properties, Immunogenic proteins, PrsA, MalX, Characterization, Antigenicity, Protein properties, Membrane vesicles, Immune response, *Streptococcus pneumoniae* virulence factors.

Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is an encapsulated gram-positive bacterium that is frequently present as part of the natural flora in the nasopharynx of healthy children and adults. It belongs to the mitis group of streptococci which makes up the majority of the commensal flora in the upper respiratory tract (URT) and is considered to be the main cause of acute otitis media, pneumonia, and in its severe (invasive) form, causing meningitis and bacteremia in children, resulting in high morbidity and mortality, especially in developing countries [1].

The World Health Organization (WHO) reports that pneumococcal disease is among diseases that can be prevented by vaccination. However, it continues to be the leading cause of mortality with 500,000 cases of pneumonia and 7 million cases of otitis media in the United States each year [2]. Recently, it was discovered that pneumococci are also capable of causing microlesions in the myocardium, which may be a contributor to the cardiovascular events that frequently follow an Invasive Pneumococcal Disease (IPD), especially in the elderly [3].

Pneumococci are encapsulated bacteria consisting of polysaccharide capsule that surrounds the cell wall. There are currently about 100 distinct capsular serotypes identified and the capsule is considered to be a key virulence factor [3]. A variety of virulence factors and host-interaction pathways are involved in the mechanisms by which the pneumococcus spreads via circulation from the nasopharynx into other organs such as lungs and meninges. The pneumococcus can translocate through the epithelium and endothelium by utilizing receptor recycling routes. The Pneumococcal Phosphocholine (ChoP) binds to the Patelet-Activating Factor Receptor (PAFR) and Pneumococcal Surface Protein C (PspC) to the Polymeric Immunoglobulin Receptor (pIgR) in order for the bacteria to move from the apical to the basal membranes. It may also break down the extracellular matrix via hyaluronan lyase to facilitate entry to the tissue more deeply [4].

The thick capsule not only protects the bacterium from opsonization and prevents the pneumococcus from being destroyed by complement, but also increase the ability of the bacteria to survive in the bloodstream. The immune system's innate and adaptive components are both implicated in host-mediated killing at the infection site. The involvement of the innate immune system includes Toll-Like Receptor (TLR) signaling, particularly TLR2 detection of TA/lipoproteins and subsequent immune activation, while the adaptive immune response will produce anti-capsular antibodies that mark the bacteria for phagocytosis in immunocompetent hosts [5].

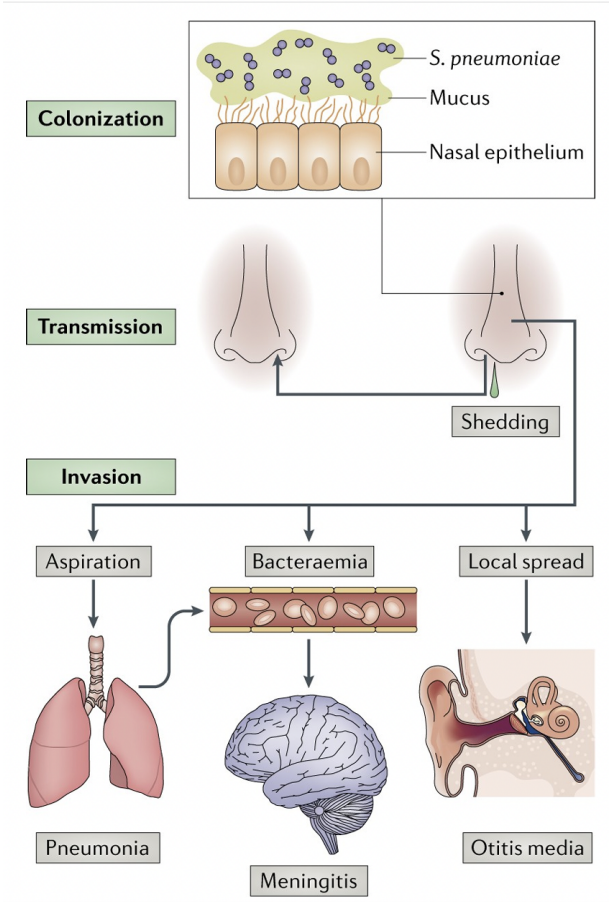

Figure 1. A schematic illustration of the spread and progression of pneumococcal disease [23].

Additionally, it has been suggested that Pneumolysin (Ply) and PspC help the pneumococcus evade opsonophagocytosis. Once the bacteria enter the bloodstream, they can spread to many organs, including the brain, where they can cause meningitis. less frequently, *S. pneumoniae* can cause ply-mediated microlesions in the heart by translocating into myocardial tissue through Choline-Binding Protein A (CbpA) that binds to the Laminin Receptor (LR) and PAFR on the cardiac vasculature. Although pneumococcus is often an external pathogen, it can occasionally

survive and even multiply inside cells. The spleen is the organ responsible for clearing pneumococci from the blood. Contrary to popular belief, *S. pneumoniae* has been found to be able to proliferate within splenic macrophages, suggesting that the spleen may also operate as a reservoir feeding bacteremia [6].

The production of capsules is essential for pneumococcal pathogenicity and has a strong anti-phagocytic effect in non-immune hosts. The thickness of the capsule of a specific strain and serotype of *S. pneumoniae* is correlated with its pathogenicity. However, the ability of pneumococci from various Capsular Polysaccharide (CPS) serotypes to induce disease varies significantly. This most likely reflects differences in their ability to induce a humoral immune response as well as variances in their relative capacity to resist phagocytosis [7].

The potential for cell-surface proteins to act as vaccine antigens and trigger the development of opsonic antibodies has acquired a lot of attention. Choline-binding proteins, lipoproteins, and proteins that are covalently attached to the bacterial cell wall by a carboxy (C)-terminal sortase (LPXTG; where X signifies any amino acid) motif are three important categories of pneumococcal cell-surface proteins that have been discovered [7].

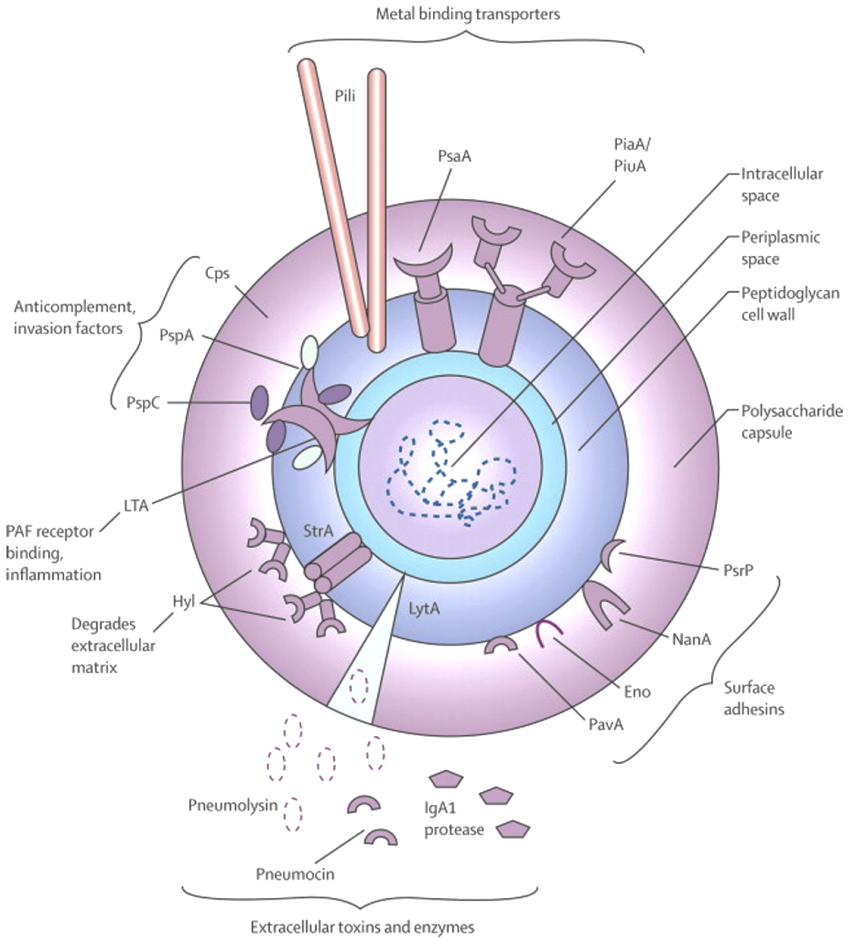

Figure 2. Graphical summary of the structure of a pneumococcal cell and virulence factors involved in colonization and pathogenesis [9].

1.2 Pneumococcal vaccines

The rapid increase in bacterial antibiotic resistance, high cost, and limited serotype coverage offered by the currently available vaccines encourage the search for novel vaccination candidates that would be more cost-effective and elicit protection against a wide range of pneumococcal strains [8]. There are two vaccine formulations available to prevent the spread of pneumococcal infection, the polysaccharide vaccine which includes the 23 most common capsular serotypes that cause invasive pneumococcal disease in developed countries. This vaccine generates T-cell-independent B-cell responses, usually the efficacy is limited by poor vaccine responses in elderly people, patients with immunocompromised systems, and infants younger than 2 years of age which are considered to be at the highest risk for severe pneumococcal disease [9].

Pneumococcal Conjugate Vaccines (PCVs), in which the sugars are conjugated to a protein carrier to induce a greater antibody response, were first developed after polysaccharide-based vaccines (PPV23), but due to inadequate protection in the risk categories. The 10-valent (PCV10) and 13-valent (PCV13) vaccines have been used in childhood vaccination programs all around the world since 2010 [8]. This vaccine has significantly decreased the incidence of invasive pneumococcal disease by 82% in children younger than the age of 1 year. The degree of herd immunity induced by the pneumococcal conjugate vaccination has been one of its most remarkable characteristics [9]. The introduction of PCV has resulted in a considerable decline in IPD in immunized children and a decrease in the nasopharyngeal carriage of Vaccine-Type (VT) strains of healthy children. However, since the Non-Vaccine type (NVT) strains have replaced VT in young individuals, pneumococcal carriage rates have remained the same [10].

Our research group investigated the possibility of developing a vaccine based on the nano-sized membrane vesicles that pneumococcal bacteria naturally release from their cell membrane in order to communicate with their environment and affect other cells. Proteins found in these vesicles help the bacteria in avoiding the host immune system.

The vesicles produced during colonization serve as important carriers of immunogens that may result in the development of protective immunity against IPD. Additionally, it can be internalized by host cells such as respiratory epithelium and dendritic cells (DCs), which may activate the DCs and cause the release of proinflammatory cytokines. Also, in *vivo*, experiments have demonstrated that vesicles produced by pneumococcal and administered intramuscularly to mice provide protection against homologous serotype challenges [10].

1.3 Membrane Vesicles

Membrane Vesicles (MVs) are spherical vesicles that are released by many bacteria including *S. pneumoniae*. These small, biologically active vesicles are surrounded by a phospholipid bilayer and range in diameter from 20 to 150 nm, derived directly from the cytoplasmic membrane and contain fatty acids, phospholipids, membrane-associated virulence proteins, cytoplasmic proteins, lipoteichoic acid, peptidoglycan, DNA, and sRNAs as depicted in **Figure 3**. These vesicles play an important role in the transmission of virulence factors, adhesion, niche colonization, host immune response mitigation, and cytotoxicity [11].

Pneumococcal MVs contain a number of antigenic virulence factors, including the pore-forming toxin PLY, pyruvate oxidase SpxB, IgA protease, metalloprotease ZmpB, metal ion, sugar transporters, and host-adhesion proteins which enables the bacteria to generate highly effective and virulent MVs [12]. Additionally, they play an important role in the survival of bacteria under different environmental stress conditions associated with intracellular interactions since it has several advantages for bacteria, including protection against enzymatic degradation, target specificity, sustained toxin delivery, resistance to antibiotics, immunological evasion, bacterial invasion, and adhesion. For instance, some bacteria produce MVs to reduce the destructive effects of antibiotics and other antibacterial treatments. Moreover, MVs can benefit a large community of bacteria through the transfer of virulence and resistance factors during infections [13]. The vesicles produced by bacteria can be also used for antigens display or for further therapy development [14].

Bacterial MVs have been examined for their immunogenicity to be used as possible pneumococcal infection vaccine candidates. It has been demonstrated that *S. pneumoniae* MVs trigger the activation of the complement system. Recent studies have shown that mice immunized with MVs have high IgG titres specific for protein antigens contained in the MVs. These antigens are also recognized by antibodies present in blood sera of convalescent pateints who had a *S. pneumoniae* infection [15]. These results can provide protection from future challenges associated with pneumococcal infections.

Bacterial vesicles are considered to be promising vaccine candidates for pneumococcal and other respiratory diseases since they are less virulent than live organisms and can contain a number of the pathogen's antigenic molecules [10]. Therefore, it has been suggested in the findings of our research group that MVs containing pneumococcal conserved membrane-bound lipoproteins MalX and PrsA could serve as a platform for the development of pneumococcal vaccines for the elderly and immunocompromised individuals.

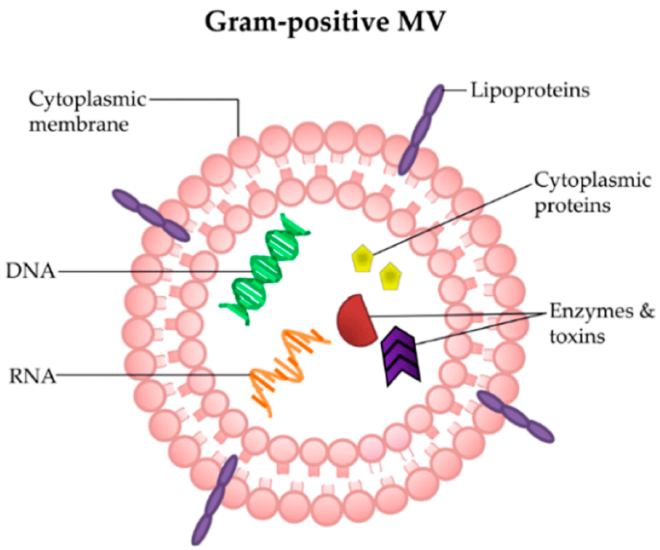

Figure 3. Architecture and composition of bacterial membrane vesicles [6].

1.4 MalX and PrsA lipoproteins in *Streptococcus pneumoniae*

There are two immunogenic proteins that have been found in *S. pneumoniae* and identified as the main antigens responsible for cross-protection known as MalX and PrsA.

PrsA is a lipoprotein found in *S. pneumoniae*, and it is essential for protein folding, secretion, and cell surface anchoring. It serves as a chaperone to aid in the proper maturation and folding of different surface proteins. It allows proper folding of the adhesins and invasins crucial for the pathogenicity of the bacterium. It also helps the bacterial's cell wall to maintain its strength and integrity.

Research studies on the function of PrsA in a variety of Gram-positive bacteria, including *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus* mutants, and *Clostridioides difficile*, have shown that PrsA is a crucial molecular chaperone of proteins involved in cell wall biogenesis, resistance to external stress, antibiotics, and bacterial virulence. These findings imply that PrsA plays a significant role in the pathogenesis of numerous Gram-positive bacteria that cause diseases [16].

PrsA is attached to the cytoplasmic membrane's outer surface. It has a diacylglycerol membrane anchor, a large functionally unidentified N-terminal domain, and a Peptidyl-prolyl cis-trans isomerase (PPIase) domain that resembles the parvulin family of PPIases, followed by a small functionally unidentified C-terminal domain. Although PrsA has PPIase activity, it may potentially operate as a chaperone in *vivo* [17].

MalX is a different lipoprotein that is present in *S. pneumoniae* and is involved in the metabolism of carbohydrates. It is a lipid-anchored solute-binding protein specific to the maltooligosaccharide-specific ATP-Binding Cassette (ABC)-transporter [16]. MalX binds to various sizes of maltooligosaccharides. In a signature mutagenesis screen, MalX was identified as a protein necessary for a lung infection in an intranasal challenge model [20].

MalX is considered to be part of the malolactic fermentation pathway, which turns malate into lactate and enables the bacterium to use malate as a carbon and energy source. The adaptability of *S. pneumoniae* to various host settings, especially the respiratory system, depends mainly on this route.

Aim

The aim of this project is to construct tools for characterizing two immunogenic proteins (PrsA and MalX) found in *Streptococcus pneumoniae,* which were previously identified as the main antigens responsible for cross-protection in a protein-based vaccine prototype. The development of enhanced forms of antigens for vaccines will be driven by a better understanding of the immunogenic characteristics of the two proteins.

Materials and Methods

Table 1. Sequences of the primers used in the project.

Primers	Sequences
FR_5utr-MalX_F	CACATGCATAGTCCATTTAGGAGGAAACGCGTATGTCATCTAAATTTATGAAGAGCGC
FR_5utr-MalXb_F	CACATGCATAGTCCATTTAGGAGGAAACGCGTATGGGAAGCAAAACTGCTGATAAG
FR_MalX-H_R	GTAGAGCTCCTAGTGATGGTGATGGTGATGGAATTCACCAAATTTTTGTTTGATTGTTTC
FR_MalX_c1ab_R	ATGGAATTCACCGTAAACTTTACCATTAGCAGC
FR_MalX_c1b_F	GAAACGCGTATGGGAAGCAAAACTGCTGATAAG
FR_MalX_c2_F	GAAACGCGTATGGCTGCTAATGGTAAA

	GTTTAC
FR_MalX_c2_R	ATGGAATTCCTTAACGGCTTGAGGAATGACCC
FR_MalX_c3_F	GAAACGCGTATGATTCCTCAAGCCGTTAAGAACC
FR_5utr-PrsA_F	CACATGCATGTAACACTTATCTCAAAGGAGTACATATGAAGAAAAAATTATTGGCAGGTGCC
FR_5utr-PrsAb_F	CACATGCATGTAACACTTATCTCAAAGGAGTACATATGTCGAAAGGGTCAGAAGGTGCAG
FR_PrsA-H_R	GTAGAGCTCCTAGTGATGGTGATGGTGATGGAATTCGTTTGATGTACTACTGCTTG
FR_PrsA_c1ab_R	ATGGAATTCATCTGGAGTGTACTCATCAAAGGC
FR_PrsA_c1b_F	GTACATATGTCGAAAGGGTCAGAAGGTGCAG
FR_PrsA_c2_F	GTACATATGACTCCAGATGTAACGGCTCAAATC
FR_PrsA_c2_R	ATGGAATTCATTAGATGATTTTTCTGTTTTCTTAGTG
FR_PrsA_c3_F	GTACATATGATTGATGACTACAAAGAAAAATTAAAAAC

Table 2. Bacterial strains used in the project.

Bacterial strains	**Characteristics**
E. coli XL-10 Gold cells	Tetracycline and chloramphenicol resistant purchased from Agilent company, 200315.
E. coli- PssrA/PrsA-SP	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, full-length PrsA construct lacking SP, termination sequence, and PssrA promoter region.

E. coli- PssrA/PrsA C1b	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, PrsA construct C1b, termination sequence, and PssrA promoter region.
E. coli- PssrA/PrsA C2	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, PrsA construct C2, termination sequence, and PssrA promoter region.
E. coli- PssrA/MalX C1b	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, MalX construct C1b, termination sequence, and PssrA promoter region.
E. coli- PssrA/MalX C2	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, MalX construct C2, termination sequence, and PssrA promoter region.
E. coli- PssrA/MalX C3	Harboring PJET vector plasmid incroporating Erm resistance cassette, bga locus, MalX construct C3, termination sequence, and PssrA promoter region.

Table 3. List of plasmids used in the project.

Plasmids	**Characteristics**
PJET-PssrA-PrsA	PJET vector plasmid incroporating Erm resistance cassette, bga locus, PrsA constructs, termination sequence, and PssrA promoter region.
PJET-PssrA-MalX	PJET vector plasmid incroporating Erm resistance cassette, bga locus, MalX constructs, termination sequence, and PssrA promoter region.

Bacterial growth

A single colony of each *E. coli* with PJET plasmid containing PssrA promoter was inoculated from a frozen stock into 6 mL of LB broth in a sterile culture tube and left in a shaking incubator at 37°C until it reached the desired optical density OD_{600}= 0.5.

The *E. coli* culture was diluted to the appropriate concentration (1:10) using sterile LB broth and 1000 µL of bacterial culture was added to a microcentrifuge tube to be centrifuged for 1 minute, and the supernatant was discarded. Using a sterile pipette, approximately 100 µL of pellet cells were dissolved and plated onto the surface of prepared LB agar plates containing Ampicillin and bacterial culture liquid was spread across the surface of the agar plates and incubated overnight at 37°C.

The Polymerase chain reaction (PCR)

The Polymerase chain reaction (PCR) was performed using Phusion® Flash High-Fidelity (Thermo Scientific™ F548S) Master Mix.

The Phusion Flash PCR Master Mix contained all the reagents required for PCR (Polymerase, dNTPs, and Buffers) except for the DNA template and primers.

All reagents were thawed in an ice including, 2X master mix, primers, and DNA template. Then a 50 µL reaction was set once thawed according to the following:

	The volume required	Final concentration
2X Phusion master mix	25µL	1X
Primer FW**	X µL	0.5 µM
Primer RV**	X µL	0.5 µM
DNA template*	X µL plasmid: 2.5-25ng	DNA: 25-250ng
H_2O	up to 50 µL	

** The primer bought from Eurofins came at a concentration of 100 µM. Primers were diluted 1/10 before use (diluted primers 10 µM)

* The concentration of the template was measured with nanodrop.

Once the reaction was ready. The cycling condition was set in the thermocycler.

Table 4. PCR cycling instructions.

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Initial denaturation	98 °C	10 s	98 °C	10 s	1
Denaturation (see 6.2) Annealing (see 6.3) Extension (see 6.4)	98 °C - 72 °C	0 or 1 s - 15 s/1 kb	98 °C X °C 72 °C	0 or 1 s 5 s 15 s/1 kb	30
Final Extension	72 °C 4 °C	1 min hold	72 °C 4 °C	1 min hold	1

To calculate the annealing temperature, the temperature of primers Fw and Rv were checked and the temperature close to the lowest was chosen. While the extension time was changed according to the length of the PCR product amplified. 1kb was amplified in 15 sec.

Cloning PrsA constructs

Full-length PrsA with His-tag

Initially, a PCR reaction of PrsA (full length) with His-tag was prepared by adding 2.5µL of 5UTR_PrsA forward primer, 2.5µL of PrsA_H reverse primer, 2µL of the template (S.p T4 lysate/gDNA), 20µL of H_2O, and 25µL of 2X master mix into a PCR tube.

PrsA construct 1a

Amount of 2.5µL of 5UTR_PrsA forward primer, 2.5µL of PrsA_C1ab reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/prsA full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

PrsA construct 1b

Amount of 2.5µL of PrsA_C1b forward primer, 2.5µL of PrsA_C1ab reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/prsA full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

PrsA construct 2

Amount of 2.5µL of PrsA_C2 forward primer, 2.5µL of PrsA_C2 reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/prsA full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

PrsA construct 3

Amount of 2.5µL of PrsA_C3 forward primer, 2.5µL of PrsA_H reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/prsA full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

Cloning MalX constructs

Full-length MalX with His-tag

Initially, two PCR reactions of MalX (full length) with His-tag were prepared. One reaction contained 2.5µL of 5UTR_MalX forward primer, 2.5µL of MalX_H reverse primer, 5µL of the template (S.p T4 gDNA), 15µL of H_2O, and 25µL of 2X master mix. Another reaction contained 2.5µL of 5UTR_MalX forward primer, 2.5µL of MalX_H reverse primer, 2µL of the template (lysate), 18µL of H_2O, and 25µL of 2X master mix.

MalX construct 1a

Amount of 2.5µL of 5UTR_MalX forward primer, 2.5µL of MalX_C1ab reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/MalX full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

MalX construct 1b

Amount of 2.5µL of MalX_C1b forward primer, 2.5µL of MalX_C1ab reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/MalX full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

MalX construct 2

Amount of 2.5µL of MalX_C2 forward primer, 2.5µL of MalX_C2 reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/MalX full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

MalX construct 3

Amount of 2.5µL of MalX_C3 forward primer, 2.5µL of MalX_H reverse primer, 2µL of the template (S.p T4 lysate/ gDNA/MalX full-length PCR), 20µL of H_2O, and 25µL of 2X master mix were added into a PCR tube.

The PCR tubes were then collected and placed in the thermocycler to perform a polymerase chain reaction under specific cycling conditions and the resulting PCR reactions were analyzed by agarose gel electrophoresis to confirm the presence of the expected DNA fragment sizes.

Plasmid Isolation

The plasmid-containing bacteria was inoculated into 50mL of liquid media in a 250mL Erlenmeyor flask and then left overnight incubation at 37°C in a rotary shaker at 300 rpm.
From the two overnight cultures, 40mL were transferred to a 50mL screw-cap centrifuge tube and the cells were spined down by centrifugation for about 5 minutes at 5000 rpm and the supernatant was discarded.
A total of 5mL of cell resuspension solution was added to the cell pellet and the cells were vortexed to resuspend the pellet and to make sure the cell pellet is completely resuspended. Then a volume of 5mL of cell lysis was added and mixed very well by inverting the tube 6-8 times (without vortexing). The solution became viscous and somewhat clear. Followed by adding 5mL of neutralization solution to the mix and the tube was caped and mixed by inverting the tube 6-8 times, in this stage the solution became cloudy and developed a flocculant white precipitate.
The tube then was centrifuged for 10 minutes at 8000 rpm, and carefully the supernatant was poured into new tubes.
The Quantum Prep matrix was resuspended by shaking vigorously and 1mL from this matrix was added to the cleared lysate and swirled gently for about 15-30 seconds to mix. To pellet the matrix, the solution was centrifuged for 2 minutes at 8000 rpm and the supernatant was carefully poured off from the pelleted matrix.
A 10mL of the wash buffer was added to the matrix and resuspended by shaking and then centrifuged for 2 minutes at 8000 rpm. The wash buffer was poured from the pelleted matrix, then another 600mL of wash buffer was added to the matrix and resuspended. A spin column was placed inside a 2mL collection tube and the resuspended matrix from the previous step was transferred to the spin column and spun for 30 seconds in a microcentrifuge at 12-1400xg. After that, the spin column was removed from its microcentrifuge tube and the wash buffer at the bottom of the tube was discarded and the filter was replaced in the same tube.
A volume of 500µL of wash buffer was added to the tube and spun for 30 seconds in a microcentrifuge at 12-4000xg and then the spin column was removed and the wash buffer was discarded. The spin column in the tube was replaced and spun for an additional 2 minutes at maximum speed to remove any residual wash buffer.
Lastly, the spin column was transferred to a clean 2mL microcentrifuge tube, and a total of 600µL of water or TE (Nuclease-free water) was added. The tube then was spined for 2 minutes in a microcentrifuge and the spin column containing the matrix was discarded.

Restriction digestion and ligation reactions

After determining the specific locations of the promoter regions of PssrA in the plasmid, the restriction enzyme recognition sites were identified near the promoter region and an initial restriction digestion was performed using NsiI and SacI enzymes to cleave the desired region of the *Super-Folded Green Fluorescent Protein* (sfGFP) gene. The resulting gap was used for subsequent insertion of different constructs of PrsA and MalX proteins.

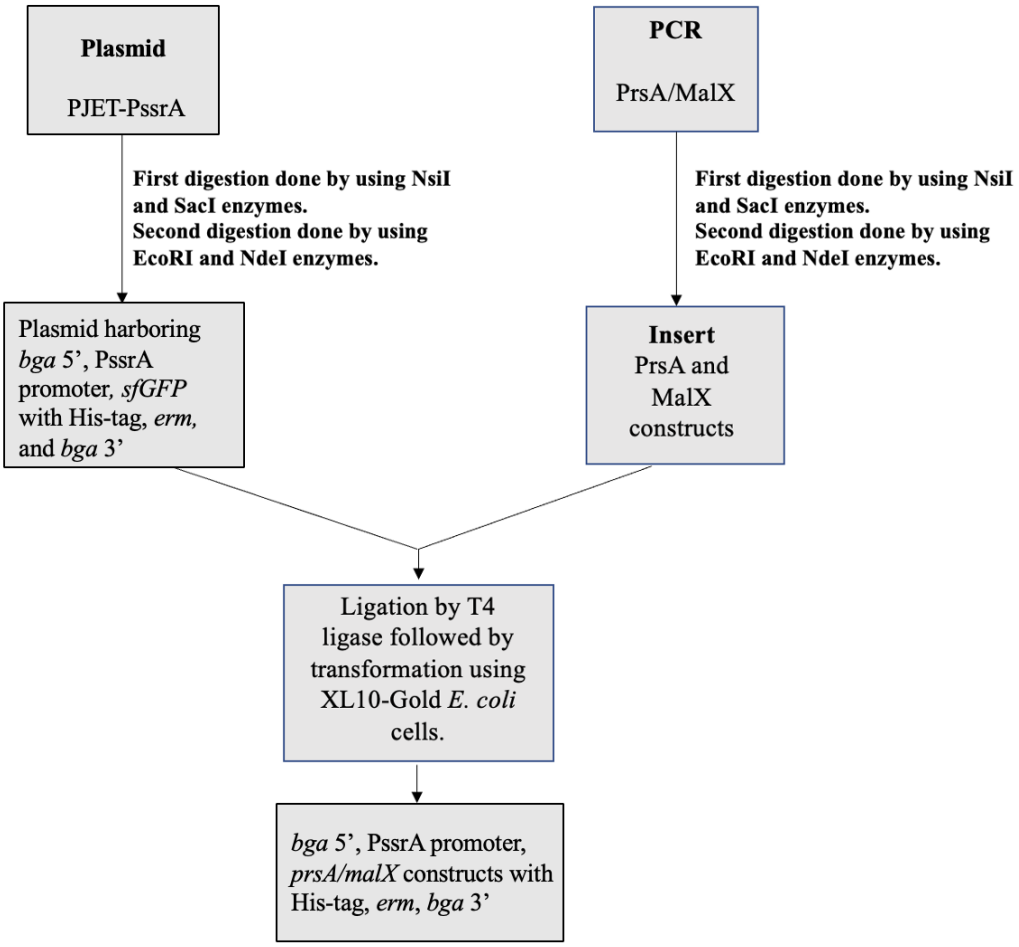

Figure 4. A schematic representation of the restriction digestion and ligation reactions performed in the experiment. Initially, the incorporation of the GFP gene into the vector served as a reporter gene indicating the successful integration of the vector into the bacteria. Subsequently, the GFP reporter gene was removed using restriction digestion enzymes and replaced with different constructs of PrsA and MalX proteins that are ligated within the plasmid using T4 ligase.

The restriction digestion reaction was incubated at 37°C for 2 hours and column purification was performed according to a protocol suggested (Wizard® SV Gel and PCR Clean-Up System protocol, Ref. A9283 and A9284).

A secondary digestion reaction was performed using EcoRI and NdeI enzymes to cleave specific regions of the different constructs of PrsA and MalX proteins. The digestion reaction was incubated for 1 hour at 37°C and the resulting fragments were analyzed by agarose gel electrophoresis.

The resulting cleaved fragments were integrated and ligated into the plasmid using T4 ligase. The ligation reaction was left at room temperature for 3 hours. After the incubation time is over, a bacterial transformation was performed.

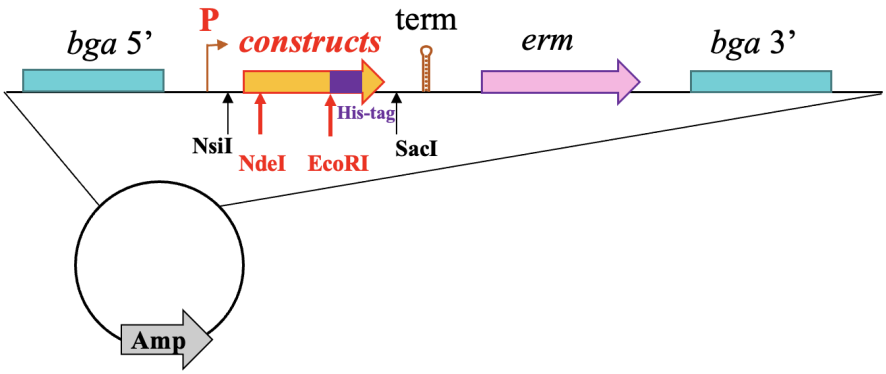

Figure 5. Showing the cloning scheme of *prsA* and *malX* constructs under the control of PssrA promoter (P). The arrows indicate the place of restriction digestion sites targeted by distinct enzymes. Incorporation of Ampicillin (Amp) resistance gene in the plasmid allow for selective growth of the transformed bacterial cells harboring the plasmid.

Bacterial transformation

The competent cells (*E.coli* XL-10 Gold) were thawed on ice to maintain their activity and 7µL of ligation reaction mix was added to 75µL competent cells in a microcentrifuge tube. The competent cells and ligation mix were mixed gently and incubated on ice for 20 minutes.
The mixture of competent cells and ligation reaction were heated in a water bath at 42°C for 30 seconds and the mix is then transferred to the ice for 1 minute.
A 700µL of LB broth was added to the transformation mixture and incubated in the shaking incubator for >1 hour.
The amount of 100µL of the transformation mixture was plated onto a selective agar plate containing Ampicillin and the rest of the mixture was spun at 13000 rpm for 1 minute and the supernatant was discarded. Later, a 100µL of the mixture was resuspended with the pellet and plated on another agar plate containing Ampicillin antibiotic and the plates were left in the incubator at 37°C overnight.

Colony PCR

A colony PCR was performed to help identify bacterial colonies that have successfully integrated the plasmid containing *prsA* and *malX* constructs. A number of colonies (8 or 16 per each transformation) were picked and dissolved in 15µL of 1x PBS, and the colonies were patched on

a new plate containing antibiotics. The newly patched plate was incubated at 37°C. The colonies were boiled in PBS for 15 minutes at 95°C in the thermocycler.

Two reactions were prepared in different microcentrifuge tubes. One reaction contained 117µL of H_2O, 18µL of bga5_seq forward primer, 18µL of PrsA-H reverse primer, and 180µL of Mastermix 2X was added to the tube and an aliquot of 18.5µL of the mixture was added to different PCR tubes and 1.5µL of the boiled colony was added to each tube. Another reaction contained 117µL of H_2O, 18µL of bga5_seq forward primer, 18µL of bga3-seq reverse primer, and 180µL of Mastermix 2X was added to the other tube and an aliquot of 18.5µL of the mixture was added to different PCR tubes and 1.5µL of the boiled colony was added to each tube.

The PCR reaction was set up according to the following cycling conditions:

- Initial denaturation 98°C for 5 minutes
- Denaturation 98°C for 8 seconds
- Annealing 60°C for 10 seconds
- Extension 72°C for 30 seconds
- Final extension 72°C for 3 minutes

After the completion of one cycle, the process begins again for 35 cycles starting with denaturation of newly synthesized DNA strands, followed by annealing and extension)

Stock culture

An overnight culture broth was prepared by adding 5ml of LB+Ampicillin to a falcon tube and by using a sterilized loop, a small amount of bacterial colonies from each construct (PssrA-PrsA C1b, PssrA-PrsA C2, PssrA-MalX C1b, PssrA-MalX C2, and PssrA-MalX C3) were transferred to the fresh media and incubated overnight at 37°C.
After sufficient incubation period for bacterial growth, the purity and quality of the culture were checked first. Otherwise, any contaminants must be discarded.
By using a sterilized syringe, a 500µL of glycerol was added to the tubes containing corresponding constructs. Then, an amount of 500µL of bacterial culture was added to the tubes. Tubes were labeled with the name of the bacterium, type of strain, and date of preparation, and stored at -80°C for long time preservation.

Western blot

The *E. coli* lysates of cultures harvested in an exponential growth rate OD_{600}= 0.5 were washed using 300µL Phosphate-Buffered Saline (PBS) to achieve homogenous cell suspension followed by adding 400µL of 4X protein lysis buffer for the solubilization of cellular proteins.

Amount of 20µL of each sample lysates were loaded equally into 1.0mm X 15 well NuPAGE 4-12% Bis-Tris commercially ready-made gel, obtained from Invitrogen company by Thermo Fisher Scientific (Ref. NP0323BOX). The gel was well submerged in the running buffer and the

prestained protein ladder and Magic Mark ladder were added. After separating proteins by electrophoresis, proteins were transferred from within the gel to a solid support transfer PVDF mini size membrane (7.1x 8.5 cm), obtained from Bio-RAD (Cat.1704272). The member was wet enough to prevent gel damage or disturb the protein bands.

For primary antibody detection, we used Anti-His HRP (mouse) at a dilution of 1:2000 and Anti-PrsA/Anti-MalX (rabbit) at a dilution of 1:20000, both sourced from Bio-RAD company. These primary antibodies were incubated overnight and washed with a 5X transfer buffer (PBST) to remove any unbounded antibodies. Following the primary antibodies incubation, secondary antibodies including Anti-rabbit HRP (for PrsA/MalX antibodies) and Anti-mouse AGG HRP (for Anti-His antibodies) were introduced at a dilution of 1:5000 and was also obtained from the same company.

The membrane was blocked with 25 mL of PBST milk and incubated for 30 minutes at room temperature. Later, the membrane was washed multiple times with PBST to remove the unbound primary antibody and blocking buffer.

The membrane was incubated with a secondary antibodies for 2 hours and the membrane was washed multiple times with PBST to remove the unbound secondary antibody.
For protein visualization, the electrochemiluminescence (ECL™ Select Western Blotting Detection Reagent) system obtained from Cytiva (Ref. RPN2236) was used to capture the image for further analysis.

Results

In this project, we designed distinct constructs that represented different domains of PrsA and MalX proteins based on their structural considerations and accessibility. As shown in **Figure 6**, different constructs of PrsA protein designated as C1a, C1b, C2, and C3 were generated to investigate different regions of the protein. A similar approach was taken for MalX protein, where we designed different constructs covering its four major parts as shown in **Figure 7**. This methodical design of constructs allows us to analyze specific regions within both PrsA and MalX proteins, providing a comprehensive understanding of their functional and structural characteristics.

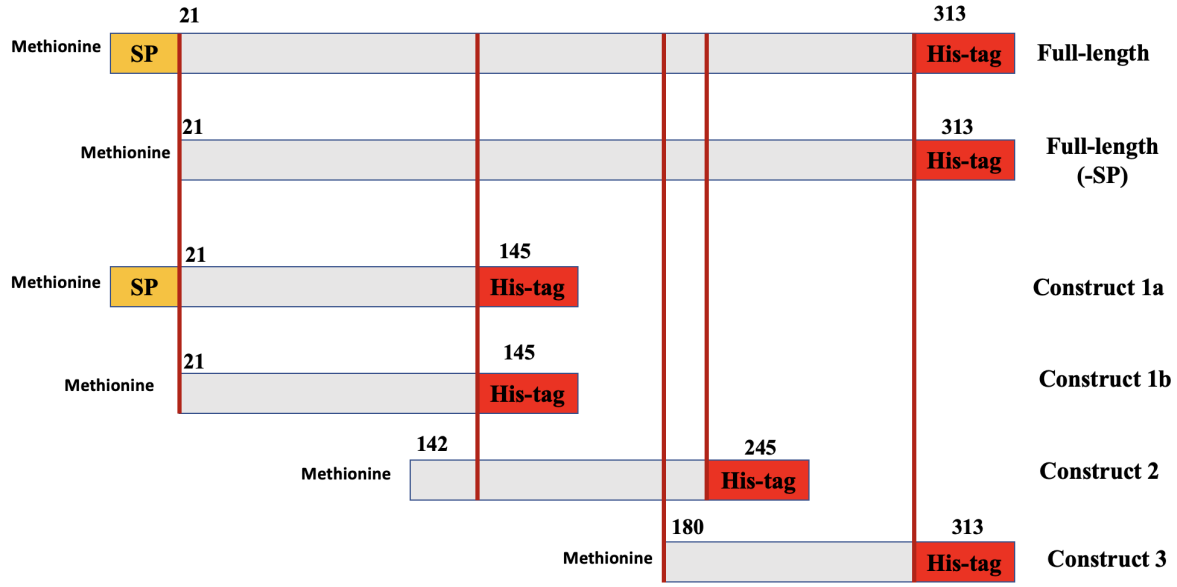

Figure 6. Graphic summary of PrsA protein constructs, revealing the presence of signaling peptide (SP) in both the full-length protein and construct 1a. The initiation codon represented by the methionine at the beginning of protein sequence, served as the start codon for protein synthesis. Numerical annotations on the constructs correspond to the amino acid positions within the full-length wildtype protein sequence, aiding in the precise identification of regions. Additionally, overlapping lines in the graphic serve to highlight the regions where the protein variants share common amino acid sequences.

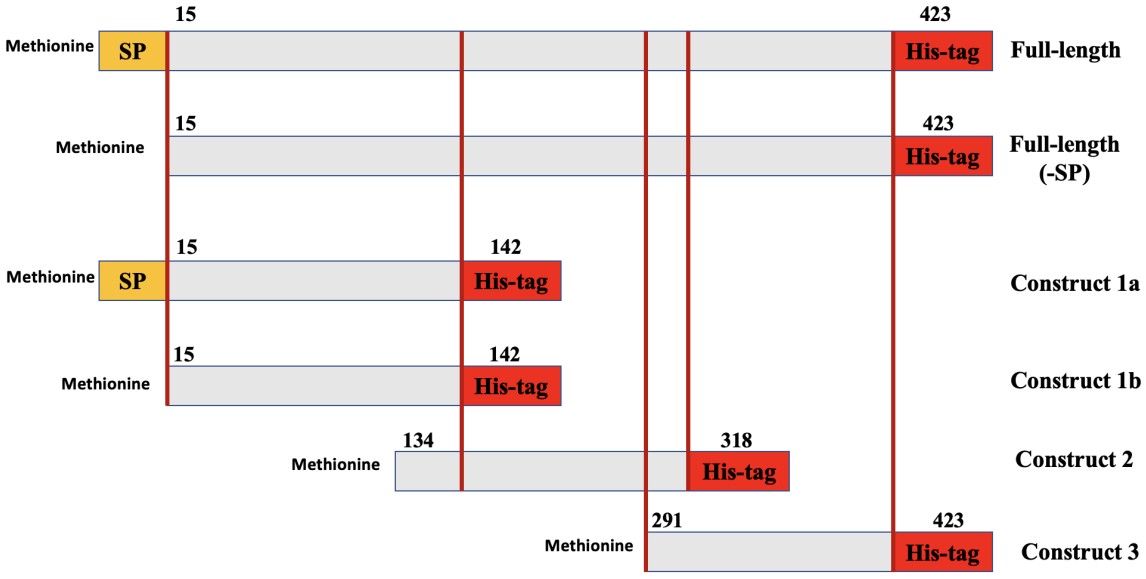

Figure 7. Graphic summary of MalX protein constructs, revealing the presence of signaling peptide (SP) in both the full-length protein and construct 1a. The initiation codon represented by the methionine at the beginning of protein sequence, served as the start codon for protein synthesis. Numerical annotations on the constructs correspond to the amino acid positions within the full-length wildtype protein sequence, aiding in the precise identification of regions.

Additionally, overlapping lines in the graphic serve to highlight the regions where the protein variants share common amino acid sequences.

Through the identification of the immunogenic epitopes of PrsA and MalX proteins, we can determine the specific regions responsible for eliciting an immune response. This knowledge serves as a foundation for designing more effective vaccine candidates that can enhance the protection against different Streptococcus serotypes.

Furthermore, examining the structural and functional features of these proteins and their interactions with the host immune system can significantly advance our understanding of the pathogenesis of *S. penumoniae*. Based on a molecular-level understanding of these interactions, such comprehension may pave the way for the development of efficient therapeutic methods to combat pneumococcal infections.

The structural characterization of PrsA and MalX proteins primarily relied on X-ray crystallography, revealing valuable insights about their three-dimensional structure. Analyzing these protein structures allow further identification of accessible regions, surface loops, and potential antigenic regions, which serve as targets for vaccine development.

Based on the protein structures and epitope analysis, several PrsA and MalX protein fragments are generated to maximize their immunogenicity and accessibility. These strategies encompassed fusion with adjuvants including MV or selective truncation of non-essential regions. Through these efforts, our research group successfully generated protein deletions based on existing knowledge and experimental evidence that has been shown to be relevant for vaccine development and functional characterization of the proteins by targeting specific regions within these proteins that are surface-exposed.

The selection of these regions was guided by the 3D structure of PrsA and MalX proteins as shown in **Figure 8**, and **9**, taking into consideration the identification of functional domains that are more likely to engage in interactions with antibodies and other molecules.

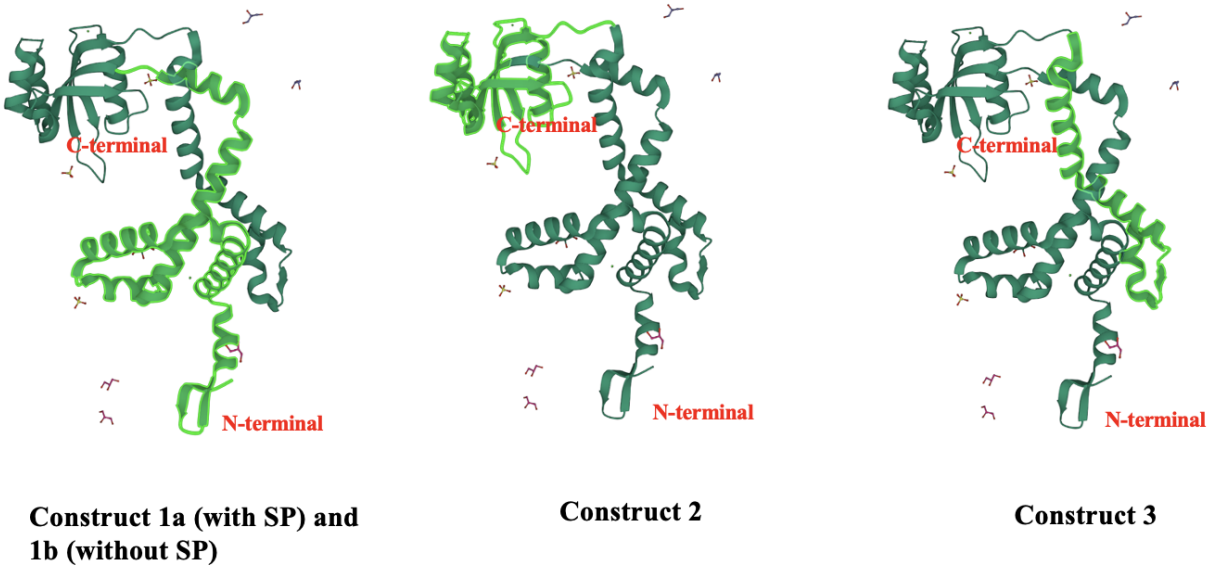

Figure 8. A monomer of the full-length PrsA of *S. pneumoniae* that shows different protein constructs, C1a (with signaling peptide), C1b (without signaling peptide), C2, and C3 based on the crystal structure solved by X-ray diffraction (PDB: 5TVL). The highlighted region, represented by a light green, serves to indicate the different constructs of the protein. Conversely, the remaining regions of the protein are depicted in a darker shade of green.

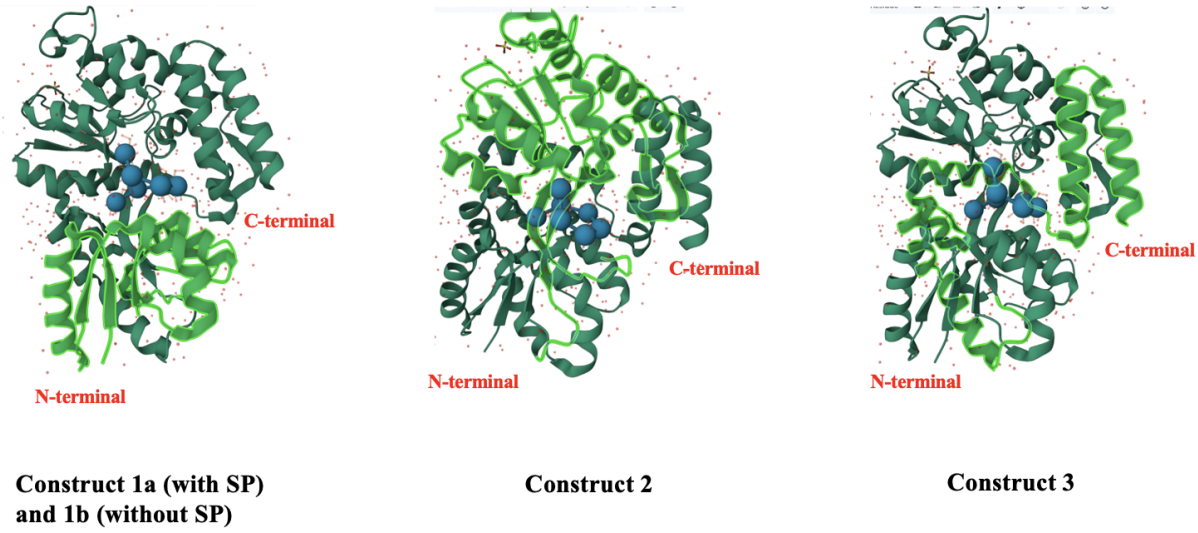

Figure 9. A monomer of the full-length MalX of *S. pneumoniae* that shows different protein constructs, C1a (with signaling peptide), C1b (without signaling peptide), C2, and C3 based on the crystal structure solved by X-ray diffraction (PDB: 2XD3). The highlighted region, represented by a light green, serves to indicate the different constructs of the protein. Conversely, the remaining regions of the protein are depicted in a darker shade of green.

This approach allowed us to develop modified protein variants with specific regions removed, providing valuable knowledge regarding the functional roles and potential antigenic regions of interest within these proteins to determine the role of different regions and domains of the two antigens in their ability to evoke the immune response and antige recognition.

At this point, we wanted to check which parts of each protein constructs has the ability to interact with antibodies generated from mice. Another factor to take into account is the presence and absence of signaling peptide. The signaling peptide influence the presentation and accessibility of the protein regions to the immune system by increasing their ability to interact with antibodies.

Cloning PrsA and MalX constructs

In order to identify antigenic regions within the PrsA and MalX proteins, the full-length *prsA* and *malX* genes were amplified, as shown in **Figure 10** and **11**, respectively. Subsequently, the polymerase chain reaction (PCR) was used to amplify the specific deletions (constructs) of each protein to further investigate the antigenic properties and functional implications of the modified constructs.

The gel electrophoresis results showed the amplicons of *prsA* and *malX* that were successfully expressed. The sizes of these amplicons were determined. In particular, it was confirmed that *prsA* C1a was 450 base pairs (bp) , C1b was 400 bp, C2 was 340 bp, and C3 was 200 bp as shown in **Figure 10**. Whereas for *malX*, we were not able to obtain C1a and C1b from the first time but had successfully expressed C2 and C3 as shown on the left side of **Figure 11**. The standard PCR reaction did not yield the desired expression of C1a and C1b amplicons. Therefore, gradient PCR was used as an alternative approach to identify the annealing temperature necessary for achieving efficient amplification of these constructs as shown on the right side of **Figure 11**.

For *malX* constructs, it was confirmed that the size of C1a was 440 bp, while C1b was 370 bp, C2 was 570 bp, and C3 was about 360 bp in length as shown in the result of gel electrophoresis.

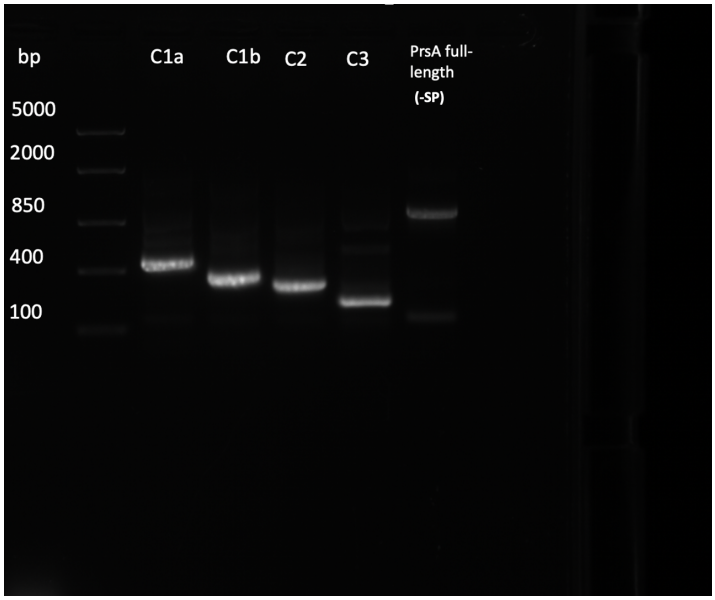

Figure 10. Agarose gel electrophoresis showing successful PCR amplification of the full length and C1a, C1b, C2 and C3 fragments of the *prsA* gene.

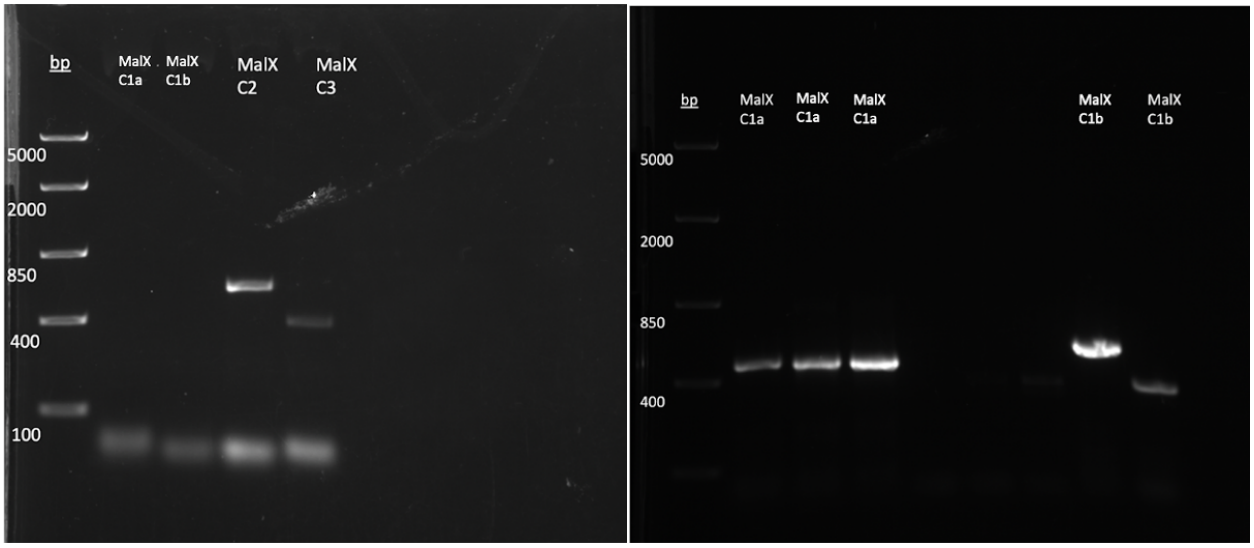

Figure 11. Agarose gel electrophoresis showing successful PCR amplification of C1a, C1b, C2, and C3 fragments of *malX* gene. The gel result depicted on the left side of the figure indicated the absence of C1a and C1b fragment expression. Conversely, prominent expression of C2 and C3 fragments was clearly observed. Whereas, C1a and C1b constructs were obtained through gradient PCR, as shown on the right side of the figure.

Later, we isolated the plasmid harboring the PssrA promoter, specifically the PJET vector plasmid, to further study the expression patterns of the plasmid. This will allow us to determine the optimal expression levels of the PrsA and MalX proteins, ensuring the desired biological effects, including protein stability, functionality, and elicitation of immune response.

Following the precise identification of PssrA promoter region within the plasmid, we used restriction enzymes, including NsiI and SacI to perform site-specific cleavage in close proximity to the promoter region. Subsequently, different fragments of *prsA* and *MalX* genes were ligated into the plasmid. This approach enabled for the incorporation of different fragments under the control of the PssrA promoter.

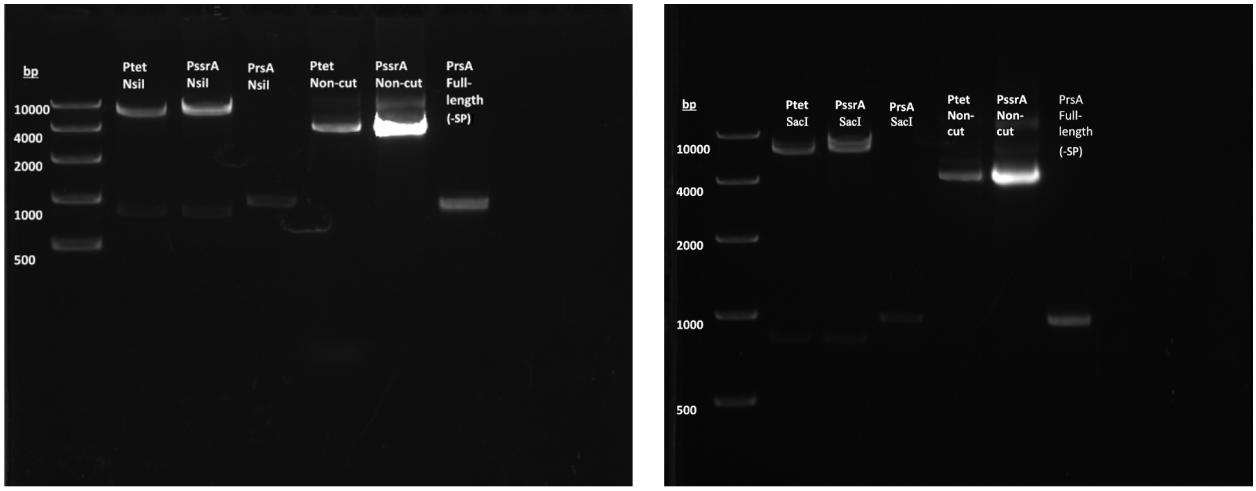

Figure 12. Agarose gel electrophoresis shows successful PCR amplifications of a plasmid containing the PssrA promoter following an enzymatic cleavage using NsiI and Sac1enzymes. Additionally, the non-cut plasmid (lacking signaling peptide) and full-length *prsA* were included as a control for comparison.

In the context of PrsA, we performed a two-step enzymatic digestion process. Initially, the plasmid containing PssrA promoter was subjected to NsiI enzyme cleavage targeting a specific site near the promoter region, followed by another digestion using SacI, resulting in a cleavage at a different site within the plasmid. As shown in **Figure 12**, the plasmid subjected to NsiI and SacI digestion exhibited a size of 10,000 bp, whereas the non-digested plasmid retained its original size of 4,000 bp.

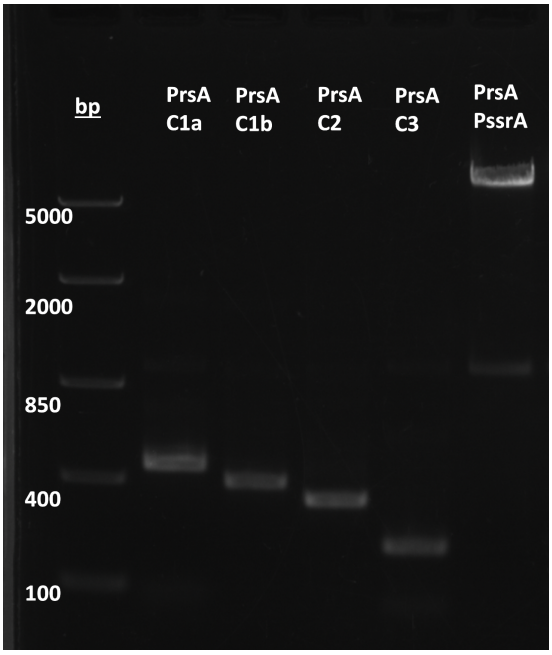

Figure 13. Agarose gel electrophoresis shows successful PCR amplifications of different *prsA* constructs subjected to NdeI, and EcoRI enzymatic cleavage. The full-length *prsA* (lacking signaling peptide) was included as a control to verify the efficacy of the digestion reaction.

Subsequently, the *prsA* constructs subjected to a secondary digestion process using NdeI and EcoRI enzymes as shown in the gel electrophoresis results depicted in **Figure 13**. A similar procedure was conducted for *malX*, allowing for subsequent integration of *prsA* and *malX* fragments.

The final step involved ligating different constructs of PrsA and MalX proteins respectively into the PssrA vector using T4 ligase enzyme. The constructs were transformed into *E. coli,* and the pure cultures of bacteria with correct constructs were used to analyse expression of the desired proteins by western blot.

Table 5. Shows the expected size of the protein domains [kDa].

protein	FL(-SP)	C1b	C2	C3
PrsA	34	15	12	x
MalX	44	13	20	13

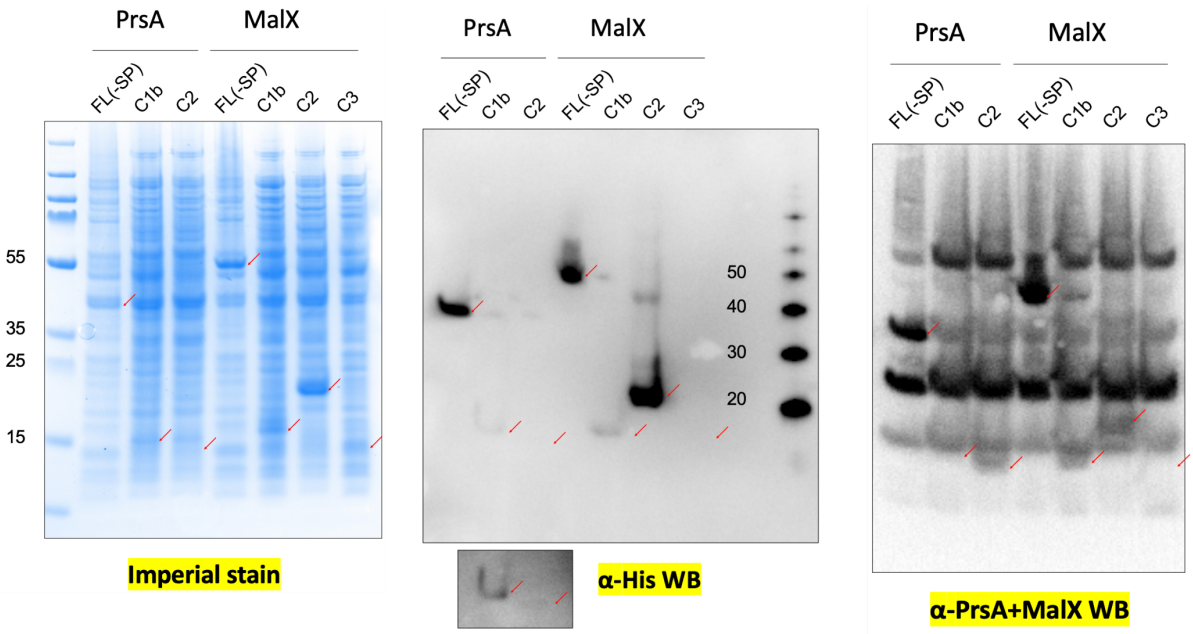

Figure 14. Western blot results show the expression of the proteins in *E. coli.* The first panel on the left is the imperial staining that shows all the proteins and antibodies in bacterial lysates. The second panel shows the western blot results with His antibodies, and the final panel on the right shows the rabbit antibodies for both PrsA and MalX mixed. The red arrows serves as a reference marker, indicating the expected sizes of the proteins as anticipated in our western blot results.

The *E. coli* lysates of cultures harvested in an exponential growth OD_{600}= 0.5 loaded equally in the three panels as shown in **Figure 14**. The first panel on the left is a loading control, staining all proteins from the lysates with imperial staining, the second panel is a western blot with anti-His antibody that is specific for the His-tag sequence, Whereas the third panel showed the western blot in which we used a mix of anti- PrsA and anti-MalX antibodies generated in rabbit from recombinant PrsA.

Discussion

Pneumococcal infections are a significant cause of morbidity and mortality worldwide and the development of effective vaccines against *S. pneumoniae* is essential due to its high disease burden, antibiotic resistance, and its impact on public health. Vaccines play an important role in preventing pneumococcal infections, reducing mortality, and morbidity rates, and limiting the spread of antibiotic resistance. Studying the antigenic properties of PrsA and MalX proteins of *S. pneumoniae* was the main focus of this project to contribute to the development of vaccines with broad serotype coverage and enhance vaccine formulations for greater efficacy by assessing their ability to trigger protective immune responses.

The fragmentation of both PrsA and MalX proteins allows us to study specific domains individually. Different fragments were cloned in a plasmid suitable for expression in *E. coli* as well as integration in *S. pneumoniae* genome. Certain constructs exhibited a lack of expression within the bacterial system, suggesting that the bacteria did not successfully express the target proteins within these constructs. As a result, we chose not to proceed with cloning the full-length PrsA and MalX (includes signaling peptide) and construct 1a assuming one possible explanation is that these constructs may exhibit toxicity towards the bacteria and we opted to pursue using the remaining constructs including C1b, C2, and C3 of PrsA and MalX proteins.

The western blot analysis of our results shown in **Figure 14** revealed a significant amount of C2 in MalX, accompanied by a relatively lower expression of C3. Conversely, C3 of PrsA exhibited no detectable expression in the samples assumed to be degraded by the bacteria. Therefore, it is reasonable to hypothesize that PrsA protein might be non-functional or have impaired functionality. The absence of correct fragment size in the unsuccessful cloning, taking into account the protein sizes shown in **Table 5**, suggests a potential problem with protein synthesis or stability.

On the other hand, the C1b constructs in addition to the full-length of both PrsA and MalX (without signaling peptide) were successfully expressed, and the cloning in *S. pneumoniae* was done only with the full-length PrsA and MalX, but the strains were not analyzed due to the time constraints of the project.

The use of pneumococcal virulence proteins for novel vaccine development is a promising approach compared to conventional vaccination strategies. The majority of these proteins are well-conserved across several serotypes and are surface-displayed and thus accessible to antibodies. Protein antigens have the ability to trigger the production of opsonophagocytic antibodies, which may provide serotype-independent defense against pneumococcal disease. For instance, it has been shown that human antibodies against several pneumococcal proteins decrease pneumococcal adhesion to human lung epithelial cells and murine nasopharyngeal colonization by pneumococcus [21].

Another alternative strategy for the development of vaccines against *S. pneumoniae* involves the utilization of bacterial MVs as these are relatively cheap to produce, safe, and provide high levels of protection. They are adaptable tools for the delivery of a variety of vaccine antigens, including those from bacterial, viral, and even cancer sources, and have the ability to present multiple protein antigens at the same time.

MVs are characterized by their spherical morphology and proteinous surface coating and can represent biomimetic structures resembling bacterial entities. These vesicles stimulate the molecular display observed on the surface of bacteria by presenting protein antigens in their

native orientation and conformation to replicate the manner in which the immune system detects antigens during an actual infection. They also have the potential to enhance the stability of their cargo proteins, providing protection against proteolytic degradation inside the host's body. These characteristics are thought to add advantages over isolated, encapsulated proteins. As an example, the Bexsero vaccine that was developed against meningococcal disease in 2013 employs a formulation that combines conserved and highly immunogenic surface-expressed protein antigens which are found on the surface of certain meningococcal strains, with an Outer Membrane Vesicle (OMV) preparation [22].

Incorporating these different antigens helps to stimulate the immune system to produce a protective response against the targeted strains of *Neisseria meningitidis.* The addition of an OMV increases the immunogenicity of the vaccine by providing a platform that exposes the antigens in a way that is similar to how they appear on the surface of the bacteria during an actual infection. This strategy aids in inducing an effective immune response that is specifically directed against the meningococcal bacteria strains targeted by the vaccine. Similar to our work that focuses on developing vaccine candidates based on bacterial MVs that is believed to have great potential to be used also against other pathogens.

In this project, we could not obtain in time blood sera from mice vaccinated with *S. pneumoniae* MVs. As a consequence we used antibodies from rabbits immunized with recombinant PrsA and MalX. The red arrows in each western blot result shown in **Figure 14** indicated the theoretical bands we should see with the corresponding size while the other bands (darker bands) indicated the unspecific signals expressed for most of the constructs assuming that signals not corresponding to the expected sizes are nonspecific signals.

In the context of future implications for advancing our approach, we can optimize the expression of the domains. The expressed portions of the proteins have to be modulated to incorporate stable domains adjustable for a proper evaluation. By generating strains with mutations that allow the expression of enhanced PrsA and MalX domain combinations with improved immunogenic properties, we can make progress toward optimizing the vaccine. After achieving a robust expression in *S. pneumoniae*, several steps must be taken into account including, the isolation and characterization of vesicles. The optimization of MVs as vaccine delivery systems requires consideration of growth parameters, purification, and size modulation methods such as extrusion to modulate vesicle sizes to increase antigen loading capacity, followed by determining the optimal ratio of MalX and PrsA domains within MVs to ensure a balanced immune response. Further in *vivo* and in *vitro* studies must be performed to assess the immunogenicity and protective efficacy of MV-based vaccines using animal models. In addition to conducting comparative studies evaluating the immunogenicity and efficacy of MV-based vaccines against existing vaccines including polysaccharide and protein-based vaccines.

In conclusion, the development of a novel vaccine against *S. pneumoniae* based on the immunogenic proteins PrsA and MalX as vaccine candidates within MVs hold promising potential. The immunogenic properties of these proteins combined with the advantageous characteristics of MVs such as stability, and antigen presentation offer an appealing strategy for developing an effective vaccine that tackles the problems caused by *S. pneumoniae* and contributes to the prevention and control of pneumococcal infections.

Acknowledgment

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