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EXPRESSION AND PURIFICATION OF THE ANTIMICROBIAL PEPTIDES BIN1B, MP1106, AND BACTERIOCIN ENTEROCIN DD14 USING THE NOVEL PROTEIN TAG SMALLTALK

Por

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Como requisito parcial para obtener el Grado de
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“EXPRESSION AND PURIFICATION OF THE ANTIMICROBIAL PEPTIDES BIN1B, MP1106 AND BACTERIOCIN ENTEROCIN DD14 USING THE NOVEL PROTEIN TAG SMALLTALK”

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DEDICATIONS

To the children of Gaza,
Whose lives were silenced before they could fully begin.
To the dreams they never had the chance to pursue,
and a plea for a world where no child's life is stolen by war.

&

To my father who gave me wings and encouraged me on my journey with the reminder that "eagles fly alone"

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ABSTRACT

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Title of the thesis: *"*EXPRESSION AND PURIFICATION OF THE ANTIMICROBIAL PEPTIDES BIN1B, MP1106 AND BACTERIOCIN ENTEROCIN DD14 USING THE NOVEL PROTEIN TAG SMALLTALK

The thesis is submitted for partially fulfils the requirements for the **DEGREE OF DOCTOR OF PHILOSOPHY IN SCIENCE** with an orientation in Applied Microbiology.

An alarming global public health and economic peril has been the emergence of antibiotic resistance resulting from clinically relevant bacteria pathogens, including *Enterococcus* faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, *Pseudomonas aeruginosa,* and *Enterobacter* species constantly exhibiting intrinsic and extrinsic resistance mechanisms against last-resort antibiotics like gentamycin, ciprofloxacin, tetracycline, colistin, and standard ampicillin prescription in clinical practices. The discovery and applications of antimicrobial peptides (AMPs) with antibacterial properties have been considered and proven as alternative antimicrobial agents to antibiotics. In this study, we have designed, produced, and purified recombinant proteins ST-Bin1b, ST-MP1106 and ST-Enterocin n for the first time via the application of newly designed fusion protein tag, SmallTalk that allow for an enhanced bacterial expression, using BL21(DE3), Lemo21 (DE3) and SHuffle T7(DE3) *E. coli* strains, and purification of the recombinant peptides via immobilized metal affinity chromatography (IMAC). The purified antimicrobial peptides were tested against gram positive and gram negative bacteria to ascertain the antimicrobial potencies while the tag being attached and Minimal Inhibitory Concentrations (MIC) were calculated using microdilution assays. The newly designed fusion tag helped to solubilize the beta-sheets incorporated Bin1b and MP1106 AMPs more than previously tested fusion tags. Enterocin DD14 also shown to exhibit comparable antimicrobial activity with the tag, establishing the significance of SmallTalk as a fusion tag shown not to alter the bioactivity of protein of interest.

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List of Abbreviations

µl	Microliter
µM	Micromolar
6xHis	Hexahistidine
ABP	Antibacterial Peptide
bp	Base pair
BSA	Bovine Serum Albumin
℃	Degree Celsius
DNA	Deoxyribonucleic Acid
CFU	Colony Forming Unit
CV	Column volume
E. coli	Escherichia coli
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography
g	Gram
GDP	Gross Domestic Production
GFP	Green Fluorescence protein
GST	Glutathione S-transferase

h	Hour
IF	Insoluble Fraction
IMAC	Immobilized Metal Affinity Chromatography
kDa	Kilo-Dalton
m	Minute
MBP	Maltose Binding Protein
MDR	Multi-Drug Resistant
MIC	Minimum Inhibition Concentration
mM	Milimolar
ml	Millimeter
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin Resistant *staphylococcus aureus*
Msch	Mechanosensitive Channel Protein
NCCLS	National Committee for Clinical Laboratory Standard
ng	Nanogram
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Hydrogen Potential
pI	Isoelectric Point

rpm	Rotation per minute
RT	Room Temperature
s	Second
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SF	Soluble Fraction
S. aureus	Staphylococcus aureus
SUMO	Small Ubiquitin-like Modifiers
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WHO	World Health Organization

Chapter 1
Introduction

1.1 Background

Recombinant expression systems have been widely practiced producing commercially valuable proteins and peptides. *Escherichia coli* have earned the status of most popular choice as a host organism for recombinant protein expression due to various reasons. One of the most promising features of *E. coli* is its ability to produce fusion proteins (Hayat et al. 2018). The goal of a recombinant expression is to get the purified protein in higher concentrations with minimum purification steps. Recombinant expression systems have been inextricably linked in mitigating antibiotic resistance (AMR), which is a persuasive global health challenge. By providing profound procedures to produce protein-based antimicrobials and biologics, these systems have provided invaluable options for studying the molecular basis of this phenomenon (Rosano and Ceccarelli 2014). Researchers are using recombinant expression to produce bacterial enzymes that confer resistance to various antibiotics, enabling detailed investigations into their structure, function, and interactions with antibiotics. This knowledge can be leveraged to develop novel antibiotics that circumvent resistance mechanisms or to design strategies to inhibit the activity of resistance-conferring proteins (Papaneophytou and Kontopidis 2014). Furthermore, recombinant expression is being employed to produce engineered proteins that can degrade or neutralize antibiotics, helping to understand the mechanisms of antibiotic inactivation and inform the development of countermeasures (Hwang et al. 2016). According to

Precedence Research, the global recombinant proteins market has shown strong growth, and the market size has increased from $3.13 billion in 2023 to $3.52 billion in 2024. It is poised to reach about $10.26 billion by 2033, growing at a CAGR of 12.6% during the forecast period from 2024 to 2033 ("Recombinant Proteins Market Size to Hit USD 10.26 Billion By 2033," n.d.). The market size has witnessed such a staggering increase is due to many elements falling in place. This is in response to an increased prevalence of chronic diseases, such as cancer, diabetes, and autoimmune disorders. Moreover, biologics, especially recombinant proteins, are increasingly favored over traditional small-molecule drugs due to often better efficacy and safety profiles (Miller 2023). Technical development in recombinant protein technology involves improved expression systems coupled with more efficient purification techniques, enabling more complicated and stable proteins, therefore driving the market further. Increased investments in biotechnology and pharmaceutical research also increase demand for recombinant proteins toward therapeutic and research applications (Jayakrishnan et al. 2024). The advent of antibiotics has marked a significant milestone in 20th century when the antibiotic infections emerged as a major threat to humanity in the post-World War II era. The discovery and general application of antibiotics dramatically declined the mortality rates resulting from infectious diseases (that included pneumonia, meningitis, and tuberculosis) (Miller 2023). However, the alarming rate at which antibiotics have been overused and misused has contributed to the emergence and spread of antibiotic-resistant bacteria reporting the first case of penicillin resistance in 1947. Overuse and improper use of antibiotics played a pivotal role in driving this resistance. The prescribing of antibiotics for inappropriate conditions, such as viral infections, and failure to take a full course of treatment undoubtedly promote the

emergence of resistant bacteria. Besides, wide antibiotic usage in agriculture and animal husbandry spreads resistant genes into the environment (Ventola 2015). Antibacterial resistance occurs when bacteria develop a sort of mechanism that enables them to tolerate the action of antibiotics. Microorganisms evolve mechanism of self-defense and present resistance through the elaboration of enzymes that render antibiotics inactive, through modification of the target sites within the bacteria, and creation of protective barriers. When infection by resistant bacteria occurs, these are much more difficult to treat, thus creating longer illnesses, greater health care costs, and even death (F. Zhang and Cheng 2022). Antibiotic resistance has rendered most of antibiotics ineffective against infections, making it hard to cure common infections and make them likely to cause serious complications and death. A recent study published in Nature, discovered that, between 1990 and 2021, drug-resistant infections claimed the lives of over a million people year; by 2050, this number may rise to about two million. According to the analysis, greater access to proper antibiotics and improved infection treatment could save an estimated 92 million lives between 2025 and 2050 (Naddaf 2024). Considering the severity of the issue, the paradigm of research is now focusing on alternatives to conventional antibiotics which include the study of **A**nti**M**icrobial **P**eptides (AMPs). These naturally occurring molecules, produced by various organisms, have demonstrated potent antimicrobial activity against a wide range of pathogens, including antibiotic-resistant bacteria (Mba and Nweze 2022). By studying the mechanism of action and optimizing the properties of antimicrobial peptides, researchers hope to develop new therapeutic agents to fight antibiotic-resistant infections and secure the future of effective antimicrobial therapy. AMPs are small molecules, usually a ranging from 10 to 50 amino acid residues where majority of AMPs

weigh less 10 kDa and the trend of new additions to the AMP database is also seen more faster towards the low molecular weight AMPs (Luong, Thanh, and Tran 2020). Although the smaller size brings several advantages, like increased diffusion rates through biological membranes thereby, a lower degree of immunogenicity as well. However, their relatively small size makes AMPs rather challenging for recombinant production and purification. The choice of the fusion tag becomes critical onus is again on their smaller size. Smaller fusion tags are generally favored for AMPs to minimize its impact on structure and function. Large fusion tags may interfere in the antimicrobial activity of AMPs or in its interaction with target molecules (Luong, Thanh, and Tran 2020). Besides, smaller tags reduce the overall molecular weight of the fusion protein, thus improving expression yields and purification efficiency. The recovery of peptide from the attached fusion tag is also challenging, thus a strategy that allow functional studies surpassing the tag removal would be readily accepted by scientists. Earlier to address the problem of antimicrobial resistance, multiple AMPs have been produces using SmbP as fusion tag as small as 10 kDa (Perez-Perez et al. 2020).

1.2 Problem Statement

However, despite their numerous advantages, existing fusion tags do suffer from some disadvantages that raise the demand for the development of new tags. The most relevant one is that many of these conventional fusion tags are quite larger in size. Large tags often disrupt the native structure or activity of the host protein and are less convenient to work with in their true form (Köppl et al. 2022a). For instance, a tag like GST is about 26 kDa and may interfere with the folding or activity of smaller proteins, thereby reducing their functionality. In addition, some tags function less well in each expression system or in

different organisms. An example might be that a tag working efficiently in bacterial systems fails to work correctly in eukaryotic cells, which limits the versatility of these tools (Francis and Page 2010). A general disadvantage with classical affinity tags involves troublesome removal after purification. Most of the fusion tags require harsh chemical or enzymatic treatment to detach the tag from target protein, which may involuntarily cause damage to the target protein. Another reported issue with the removal of tag is 'the incomplete removal', thus leaving residual fragments of the tag (Costa et al. 2014). The latter might interfere with further experimental applications. For example, incomplete cleavage of the tag can affect the biological properties of the protein or its interaction with other molecules. Since most of the commonly protein tags used for recombinant expression are quite large, there presence along with the protein under study may hinder the biological activity, hence, the removal become inevitable. Harsh conditions often drive up the overall cost and complexity of a purification process and can limit its overall scalability or feasibility for a particular application (Mahmoudi Gomari et al. 2020). These limitations have, in turn, driven interest in alternative tag designs and removal strategies. These are designed to provide the smoothest and easiest purification process with minimal damage by proteolytic activity, maintaining target molecule integrity for downstream analysis or applications. This drives the continuous search for novel fusion tags that address these issues. Efforts are directed at designing smaller and more versatile tags that function across multiple expression systems without disrupting protein function(Schwinn et al. 2020). As a matter of fact, such advanced fusion tags have great importance in the development of more efficient protein-based research and in designing better therapeutic agents. It is concluded that, in current molecular biology, fusion proteins and tags represent a widely

used tool. At the same time, technologies today have significant limitations, and there is an ever-growing demand for more effective fusion tag discovery. These will further enhance production, stability, and usability in many recombinant proteins for basic research and medical applications (Saraswat et al. 2013). They also aid in the protein purification step by immobilizing to the column during affinity chromatography. The aforementioned aspects of fusion proteins made them a preferred choice of scientists for recombinant expression analyses (Pina, Lowe, and Roque 2014; Kimple, Brill, and Pasker 2013).

1.3 Justification

The small metal-binding protein, also known as SmbP, is a naturally produced metal scavenging polypeptide by *Nitrosomonas europaea* to expel out toxic metal ions. Its molecular weight is 9.9 kDa and it has a natural ability to bind multiple divalent and trivalent metal ions (Perez-Perez et al. 2021a). Both properties make SmbP ideal candidate to be used as fusion protein. Immobilize metal ion chromatography using Ni(II) ions is the most used purification method for recombinant proteins and as the SmbP naturally binds Ni(II), it is ideal to be used as fusion protein (Vargas-Cortez et al. 2016a). Although, there are other fusion protein tags including GST and MBP, but they both don't guarantee high quality protein purification for all type of proteins. It is a well-established fact that fusion protein tag efficacy is a variable value which depends a lot on protein of interest. Lower yield and formation of inclusion bodies have been reported with both GST and MBP, so, it is highly recommending having more options to be used as fusion proteins(Köppl et al. 2022b). This will increase the range of target proteins that can be produced through recombinant expression. The prominent feature of SmbP is its smaller molecular size, around 10 kDa which is very small as compared to previously used fusion proteins GST

(26 kDa) and MBP (42 kDa) which aids in higher yields after tag removal. Another attribute of SmbP is its signal sequence at N-terminus that results in the periplasmic expression of target protein. In the absence of this signal sequence, the truncated version of SmbP results in cytoplasmic expression of protein (Vargas-Cortez et al. 2016b). In this project, we will be testing a new smaller variant of SmbP which will be referred as "SmallTalk." The 3-Dimensional crystallographic structure of SmbP (PDB ID 3U8V) clearly shows that it consists of two chains, A and B (https://www.rcsb.org/structure/3U8V). Our hypothesis is that the Ni(II) binding capacity of SmbP is only specific to one of its two chains. Hence, we will be testing constructs with SmallTalk as fusion protein to test our hypothesis. Previous experiment with SmbP has shown the dissociation of this double chain peptide into two halves indicated by a separate band in SDS-PAGE. The positive results testing our hypothesis could give us an even shorter fusion protein. It will be considered revolutionary in field of protein expression and purification due to its smaller size and simpler purification steps. Proper folding into mature protein is a crucial step in recombinant expression of proteins. Not all fusion proteins promise proper folding of all type of target proteins. In such scenario, identifying and establishing new fusion proteins will increase the number of valuable proteins that can be produced through recombinant expression (MacConmara, Nwadei, and Kirk 2015; Köppl et al. 2022b). Furthermore, the purification of recombinant proteins using SmbP also needs to be established and optimized with varying sizes of proteins of interest and check its ability to return properly folded protein. For this purpose, also, we have targeted a protein of interest with sequentially smaller in size as compared to previously expressed proteins in our laboratory with SmbP. The antimicrobial peptides selected to be expresses as target proteins are Bin1b

a beta defensin (X. Li et al. 2023), MP1106 a plectasin (X. Li et al. 2023) and Enterocin DD14 a bacteriocin (Montfort-Gardeazabal, Balderas-Renteria, et al. 2021a). The Bin1b and MP1106 are peptides that contain sulfur bridges and hence beta sheets are present in their quaternary structure. The presence of these structure has made the recombinant expression of these peptides challenging in the earlier reports with other fusion tags (S. Zhang and Corin 2017). So, the attempt to express and purify them with a newly designed tag will provide valuable insight into the functional credibility of the newly developed fusion tag. The bacteriocin selected also hold therapeutical significance as this class of antimicrobial peptides are considered as antibiotic alternatives (Simons, Alhanout, and Duval 2020).

1.4 Hypothesis

A smaller version of SmbP referred to as 'SmallTalk' shows recombinant protein and peptide purification potential using immobilized metal-affinity chromatography. Because of its 5 kDa size, it will simplify protein and peptide production since it will not be necessary to remove the SmallTalk tag for bioactivity assays.

1.5 Objectives and Goals:

i. Specific objectives

- To design and produce a smaller workable variant of SmbP, referred to as SmallTalk, and compare its protein expression efficiency with SmbP.
- To produce a novel bacteriocin via recombinant expression using SmalllTalk and assay its antimicrobial.
- To produce antimicrobial peptides with sulfur bridges and analyze their expression capacities while tagged with SmallTalk.
- To calculate the Minimal Inhibitory Concentrations (MIC) of heterologously expressed antimicrobial peptides against Gram-positive and Gram-negative bacteria.

ii. Goals

- Obtain Doctoral degree by publishing the results in good quality journal.
- Present the research work in national and international conferences and develop progressive communication with scientific community through research.
- To publish the project work in peer reviewed journal to serve the scientific community by sharing the knowledge discovered.

Chapter 2
Literature Review

2.1 Heterologous Expression System

Bacterial expression systems have been at the center of pharmaceutical industry due to their efficiencies, ease of use and cost-effectiveness in recombinant protein production. E. coli is the most widely used organism as expression host for the heterologous expression of protein for research, therapeutic and industrial enzymes. *E. coli,* from it first landmark use in production of recombinant insulin, has never fade its celebrity status and still serves as a preferred choice due its various benefits that include rapid growth rate, high yield and scale-up abilities (Fakruddin et al. 2013). Growth hormones and interferons are few other exemplary products that *E. coli* based recombinant expression systems has delivered to this world (Rezaei and Zarkesh-Esfahani 2012) (Morowvat et al. 2014). A detailed schematic flowchart is presented in **Figure 1.1** that describes the complete working mechanisms and all the key players in the heterologous expression systems. Despite being well studied and highly optimized working conditions, problems with protein solubility, misfolding, and purification frequently occur in process of large-scale expression. Usually, these concerns arise when attempts to produce eukaryotic proteins are done in a prokaryotic system. So, the three issues just reported are all inter-dependent, as an insoluble and misfolded protein often undergoes inclusion body formation and renders the purification procedures. A misfolded protein cannot be taken for functional analysis as well (Bhatwa et al. 2021). This is where the fusion protein tags come in play. These tags are genetically engineered along

with the target protein and are aimed to improve solubility, proper folding, and stabilize the proteins, thereby increasing their functionality and simplifying purification process. The following section provides a detailed insight into the importance of fusion tags(Kimple, Brill, and Pasker 2013).

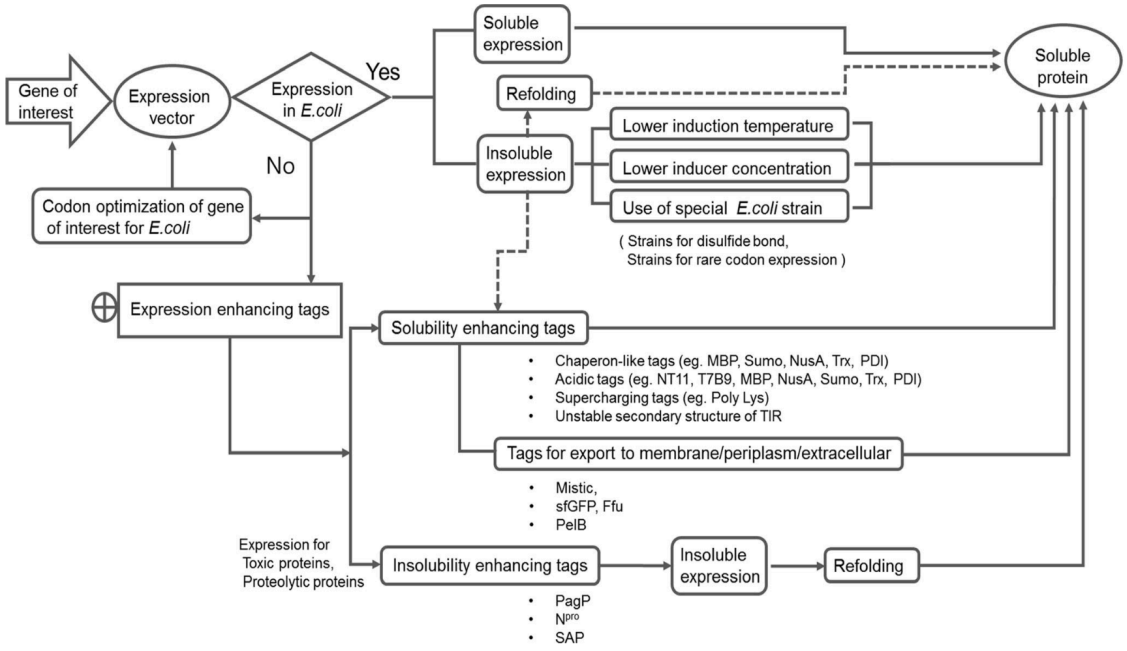


Figure 1.1 Schematic Flowchart summarizing the strategies in heterologous protein production using expression-enhancing tags through an *E. coli* expression system. Generally, the gene of interest is expressed by an expression vector containing a promoter and an antibiotic resistance gene used for selection, and His-tag used for purification when heterologous gene is expressed in *E. coli*. If the protein is not produced as a soluble form, then several conditions should be taken into consideration to facilitate the soluble production of target protein. The soluble expression can be increased either by lowering the incubation temperature or decreasing the amount of inducer such as IPTG or by both. In the case of expressed protein or gene product of *E. coli,* if they form disulfide bonds,

accordingly, a special strain can be used. Alternatively, at this stage, soluble-enhancing tags can be used to induce soluble expression of the gene of interest. Codon optimization for *E. coli* or fusing the target gene with an expression-enhancing tag, will be considered if it's not expressed. Insolubility-enhancing tags are often used on purpose with the aim of getting an insoluble protein. Under denaturing or mild solubilizing conditions, an insoluble protein can be refolded to get back its soluble form. Commonly, heterologous proteins are expressed in the cytoplasm of *E. coli,* but certain tags transport a passenger protein to compartments other than the cytoplasm, such as membranes, periplasm, or extracellular.

2.2-Fusion proteins

Fusion proteins are artificially designed proteins created by combining two or more different gene sequences into one protein that contains several functional elements (Mahmood et al. 2023). A fusion tag can either be a short amino acid sequence or even a large domain, which is genetically fused with a target protein for facilitating its expression, purification, stability, or detection (Riggs 2001). Among the main reasons why fusion proteins are so important is the simplification and enhancement they can allow for in the expression, purification, and detection of proteins within various systems. They also play their part in investigating protein localization, trafficking, and interactions and thus providing massive insights into cellular processes (Terpe 2003). Due to its wide range of applications, it has become one of the foundations in therapeutic development. They are also applied in medicine whereby drugs are being made with higher potency, such as antibody-drug conjugates, in which an antibody specific for the cancer cell is fused to a toxin or drug (Lu et al. 2020). The delivery will be directly targeted to the tumor, essentially while minimizing harm to healthy cells. Other examples include therapeutic enzymes,

where fusion proteins can be engineered to enhance drug stability, increase circulation time in the blood, or improve their targeting ability at specific tissues (J. Li et al. 2023).

Below are the various roles they play summarized:

1. Improvement in Solubility

Fusion tags improve the solubility of otherwise aggregating or misfolded recombinant proteins. The common tags applied to enhance such solubility include: MBP: Maltose Binding Protein, GST: Glutathione S-Transferase, Thioredoxin (Trx). These fusion tags prevent the formation of inclusion bodies and enhance the yield of functional protein (Riggs 2001; Köppl et al. 2022b) (Harper and Speicher 2011).

2. Easy Purification

Most of the fusion tags contain sequences which facilitate easy purification through affinity chromatography. Examples include: His-tag (6xHis) binds to nickel or cobalt ions in Ni-NTA or IMAC columns. GST-tag allows purification using a glutathione affinity column. Strep-tag binds to Strep-Tactin resins for purification. This hence makes the purification of the recombinant protein faster, more efficient, and selective (Harper and Speicher 2011).

3. Improvement in Protein Stability: Fusion tags can improve the stability of the recombinant protein during expression or storage. This is particularly desirable with proteins that are prone to degradation and/or unstable in their native state. Tags like MBP and SUMO can increase folding, stability, and resistance to proteolytic degradation of the protein (Butt et al. 2005) (Reuten et al. 2016)

4. Detection: Several fusion tags are being used for the detection or visualization of protein by applying specific antibodies or fluorescence. His-tag is usually detected with anti-His antibodies, FLAG-tag, Myc-tag are small peptide tags that are conveniently monitored with tag-specific antibodies. GFP is a fluorescent tag that allows the real-time observation of the protein in cells (Yadav et al. 2016).

5. Facilitating Proper Folding and Localization: Some fusion tags facilitate proper folding of the target protein, particularly if expressed in heterologous systems such as *E. coli*. The tags may also target the protein to appropriate cellular compartments, if required. For example, a signal peptide can direct the protein into the secretory pathway (de Marco 2009).

6. Permitting Protease Cleavage: The fusion tag must be cleavable since mostly the functional studies require the tag to be removed, if the tag interferes with the function of the protein. Common examples of proteases are as follows: TEV protease, Factor Xa, Thrombin (Jenny, Mann, and Lundblad 2003).

2.3-Types of Fusion tags

Narrowing down to most fundamental challenge in the production of heterologous proteins in *Escherichia coli*, are low protein expression due to misfolding into insoluble aggregates; the inclusion bodies. Mostly these expression problems are caused by weak promoters, presence of rare codons or poor translation initiation. Some of the underutilized and inherently difficult approaches to alleviate these problems are by using rare tRNAs supplemented E.coli strains and the introduction of corrective sequences in the gene while designing overexpression plasmids (Bhatwa et al. 2021). The most commonly opted approach to circumvemt these problems is by attaching a highly-translated native gene as a fusion tag on the N-terminal of the heterologous protein. This pormises improved yields and added benefits of increasing the solubility of the target protein; both of which are corelated. This form the basis of solubility tags which is one of the two most important classes of fusion tags. The second most important class is affinity tags which are crucial during protein purification (Ki and Pack 2020). These tags deploy a variety of strategies to bind the heterologous target protein onto a specialized affinity matrix. Different classes

of fusion tags used for specific purposes are catagorized in **Table 1.1**, however, the most commonly used tags are solubility tags and affinity tags. In most cases, one tag from each of the two classes has to be engineered with the target protein, one for enhancing solubilty and one for the specific affintiy to resin being used for purification. Interestingly, some of the protein tags function in both affinity and solubility roles—for example, the glutathione-S-transferase (GST) tag improves solubility of some proteins while providing purificartion basis owing to its inherent affinity to glutathione and maltose-binding protein (MBP) can be used for purification by deploying its maltose binding affinity for purification of attached target protein (Harper and Speicher 2011; Reuten et al. 2016). This class of dual function tags mentioned in table as well, contains another example of metal binding proteins. This is relatively a newly discovered members of this class but gaining immense attention due to reasons. Metal binding proteins has an inherent quality of affinity fo diavalent or trivalent metal ions, a property that is used in Immobilized Metal Ion Chromatography, one of the most common and fundamental purification strategy details of which are provided in next section.

Table 1: A detailed description of Types of Fusion tags with Examples

Tag Name	Description	Examples
Affinity Tags	Aid in affinity purification of the target protein using specific ligands.	-His-tag (6xHis) -GST (Glutathione S-Transferase) -Strep-tag -FLAG-tag
Solubility Tags	Improve solubility and prevent aggregation of difficult-to-express proteins.	-MBP (Maltose Binding Protein) -NusA -Thioredoxin (Trx) -SUMO (Small Ubiquitin-like Modifier)
Detection Tags	Facilitate identification, tracking, or detection of proteins using antibodies or other methods.	-Myc-tag -HA-tag (Hemagglutinin tag) -FLAG-tag - V5-tag
Cleavable Tags	Enable the removal of tags after expression using specific proteases.	-His-tag with TEV protease site -GST-tag with Factor Xa cleavage site -SUMO-tag with SUMO protease
Fluorescent Tags	Allow visualization of protein localization and interaction through fluorescence.	-GFP (Green Fluorescent Protein) -mCherry/RFP (Red Fluorescent Protein) -YFP (Yellow Fluorescent Protein)

Enzymatic Tags	Catalyze reactions to detect or track proteins via enzymatic activity.	-β-Galactosidase (LacZ) -Alkaline Phosphatase (AP) - Luciferase
Dual Function Tags	Combine multiple functionalities like solubility and affinity purification.	-His-MBP -GST-His - Metal-binding proteins (e.g., SmbP, Ferritin)
Targeting/Localization Tags	Direct proteins to specific cellular compartments for functional studies.	- Signal Peptide Tags (for secretion) - Nuclear Localization Signal (NLS) - Mitochondrial Targeting Sequences

2.4-Immobilized Metal-Affinity Chromatography

IMAC is a reliable protein purification technique that is extensively employed in research and development (R&D) laboratories to purify synthetic and recombinant proteins associated with protein tags. This technique relies on the strong affinity of fusion proteins commonly referred to as protein tags such as small metal binding proteins SmbP, CusF, CusF3H+, Glutathione S-transferases (GST), Maltose binding protein (MBP), FLAG49 peptide, and the Hexa-histidine peptide (6X His tag) genetically engineered at the N or C-terminal with the amino acid residues of the target protein thereby allowing target recombinant proteins to be expressed as recombinant fusion proteins with respective tags (Adamíková *et al*., 2019; Riguero *et al*., 2020). Although, chromatographic technique comes with some purification challenges which might compromise the purity and yield of proteins considering the type of column resin and the metal chelators employed, the method

is highly reliable coupled with efficient protein purity and yield as reported by several studies (Adamíková *et al*., 2019; Afzal *et al*., 2021; Riguero *et al*., 2020). Recently new IMAC techniques are being developed and used to address chromatographic purification impacts on recombinant proteins of therapeutic applications. This includes the use of column resins coated with prominent metal chelators like iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) permitting the conjugation of resins with affinity metal ions such as Cu(II), Zn(II), and Ni(II). The principle of working of IMAC is shown in **Figure 1.2**. The affinity of protein tags to the metal ions on the column resins allows for the chromatographic separation of expressed recombinant proteins associated with above mentioned fusion proteins (Santos *et al*., 2019).

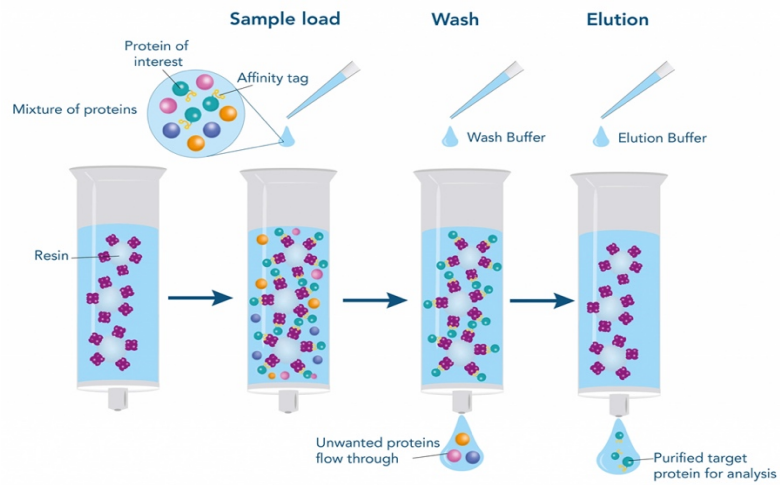


Figure 1.2 Schematic representation of Principle of Immobilized Metal Ion Affinity Chromatography (IMAC) (Adapted from https://www.neuromics.com/protein-affinity-chromatography)

At the center of IMAC is the strategic use of metal binding proteins, which are genetically fused to the target protein. These proteins have high-affinity binding with certain metal ions-such as nickel or cobalt ions-along the lines of the so-called universal histidine tag, or

His-tag (Riguero et al. 2020). Immobilized on a column of chromatography, these metal ions will serve like a molecular magnet, entangling the target protein through its fused metal-binding protein. Due to the specificity of the interaction between the metal ion and the metal binding protein, there is no match for it, which allows attachment only to the desired protein onto the column while impurities get washed away. This selectivity is one of the integral ingredients in the procurement of pure protein samples that might be required for a wide range of applications such as structural and functional studies, drug development, and diagnostics. In addition to specificity, there are other advantages offered by IMAC (Charlton and Zachariou 2008). Relatively, it is a mild way of purification that has minimal chances of denaturation or degradation for the proteins. Furthermore, using IMAC allows the purification of a wide variety of proteins independent of their size, charge, or hydrophobicity. Its versatility has made IMAC a tool that modern biochemistry and molecular biology cannot ignore. The basis of success in IMAC rests with the metal binding proteins (Block et al. 2011). They provide a capture means for the target protein but also contribute to stability and solubility. Quite often, fusing a metal binding protein to the target protein will result in an improvement in the general properties of the target protein, making it easier to purify and handle (Bhatwa et al. 2021).

2.5-Case Study: Small Metal Binding Protein (SmbP)

SmbP is an excellent example of a small and efficient fusion tag. SmbP represents a protein with very strong and specific affinity for the divalent metal ions Ni(II) and Co(II). SmbP originates from organisms that have a whole range of such proteins, relying on the presence

of metal-binding proteins for a variety of critical functions like metal ion homeostasis and enzyme cofactor regulation (Barney, LoBrutto, and Francisco 2004). This inherent ability for tight binding of metal ions has been exploited in biotechnology, where SmbP serves as a fusion tag for the purification of recombinant proteins. Applied to immobilized metal affinity chromatography, SmbP provides a highly selective method for recovering the target protein from complex mixtures such as bacterial lysates. The fusion protein will bind to immobilized metal ions on the column, while impurities are washed away. Because of its strong interaction with the metal ions, SmbP enables efficient capture of the tagged protein even under conditions where this normally would be difficult (Vargas-Cortez et al. 2016b). Elution readily occurs by adding imidazole, which competitively inhibits binding of SmbP to the metal ions, or by changing the pH below the level at which the metal-protein interaction occurs. Besides the high binding affinity, this small size is a significant advantage of SmbP over larger tags like GST or MBP by minimizing possible steric interference with target protein structure or biological activity. Thus, SmbP has applications in situations where native function is crucial, such as functional assays or structural biology studies (Montfort-Gardeazabal, Claudio, et al. 2021). Overall, SmbP represents a versatile, efficient, and non-disruptive fusion tag to produce highly pure, recombinant proteins that have immense value in both research and industrial biotechnology.

Previously, the utilization of SmbP as new fusion proteins has been established in Protein Expression and Purification Laboratory, UANL, Mexico. It is naturally produced by *Nitrosomonas europaea*, to carry toxic metal ions out of the cell. This protein was first considered as fusion protein due to its metal ion binding ability which later manipulated

for purification steps of recombinant protein expression. Furthermore, due to comparatively low molecular weight, the final yield of purified proteins is enhanced in comparison to those obtained with larger fusion proteins. The recombinant VpDef peptide has been produced using SmbP. VpDef is a defensin that possesses antimicrobial activity against Gram positive and Gram-negative bacteria. Structurally, it contains eight cysteine residues that form four critical disulfide bonds considered necessary for correct folding of peptide and its antimicrobial activity. Thus, the experiment laid to the effective use of small metal-binding proteins to produce mature peptide. It simplifies the process by eliminating the step of *in-vitro* refolding (Montfort-Gardeazabal et al., 2020).

Montfort-Gardeazabal et al., 2021 has also shown that SmbP has successfully been used to express mature and functional Bin1b which was earlier turned into inclusion bodies using previous methodologies. Bin1b is also a beta-defensin which is known for its antimicrobial activity. The produced Bin1b in the experiment was tested for its antimicrobial activity and showed inhibitory effects against *Staphylococcus aureus*. Similarly antimicrobial peptide LL-37 and human growth hormone hGH has also been successfully produced using SmbP (Perez-Perez et al. 2021b). The cathelicidin peptide LL-37 is an important bioactive molecule with many biological activities that also include antimicrobial activity. The highlight of the experiment was that SmbP_LL-37 conjugated molecule also possessed the antimicrobial activity before the separation of free LL-37 with enterokinase. Hence the use of SmbP as fusion protein considerably cut short the purification time before the detection of antimicrobial activity.

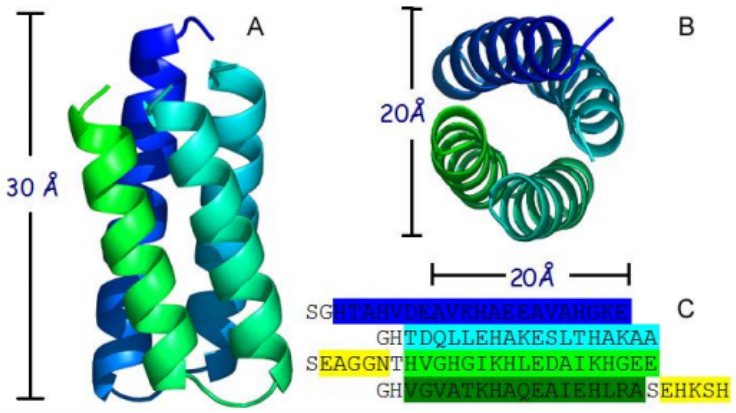


Figure 1.3 Crystallographic structure of SmbP (**A**): Longitudinal view (**B**) Circular View (**C**) Amino acid sequence of SmbP

2.6-Antimicrobial Resistance and alternate options: AMPs

With the ever-increasing threat of antibiotic resistance in pathogenic bacteria, the urge to discover new antimicrobial bioactive compounds has become inevitable. The decline in new antibiotic discovery has led scientists to look for alternate options. Bacteriocins are of great importance in this regard due to their potential antimicrobial activity (Simons, Alhanout, and Duval 2020). In addition, their smaller molecular size made them a fascinating option for large scale production. In this project, we aim to produce novel bacteriocin shown to possess antimicrobial activity in previous studies. This bacteriocin is selected based on its smaller size and novelty in terms of recombinant expression. Enterocin DD14 is a bacteriocin isolated from *Enterococcus faecalis* 14 which consists of two small peptides with molecular weight of 5206.41 Da and 5200.74 Da (Ladjouzi et al. 2023). The two peptides, DD14A and DD14B share highly similar amino acid sequence and possess no signal peptide. This makes Enterocin DD14 a member of class IIb that consists of leaderless two-peptide bacteriocin. Enterocin DD14 has inhibitory effect

against *Clostridium perfringens* which is a causative agent of Necrotic enteritis (NE) in broiler chicken (Caly et al. 2017) (la Mora et al. 2020). It is a severe disease that leads to high mortality in broiler chickens and thus poses serious economical threats to poultry industry. *Clostridium perfringens* is a Gram-positive, anaerobic pathogen frequently found in the gastrointestinal tract of humans and animals. The said Enterocin also showed antimicrobial activity against Methicillin-Resistant *S. aureus* (MRSA) in combination with methicillin. MRSA defy antibiotic treatment due to its ability of biofilm formation. The ability of *S. aureus* to grow in multicellular communities with the formation of biofilms through self-produced extracellular matrix, allow it to spread and persist several infections. Enterocin DD14 has the potential to be used as a viable treatment option against *S. aureus*. Hence, its recombinant expression will be valuable as it proposes an effective solution to the problems of *S. aureus* mediated infections (Belguesmia, Spano, and Drider 2021) (Tuon et al. 2023). Bacteriocins are antimicrobial peptides or proteins produced by bacteria responsible for preventing the growth of related or even unrelated bacterial species. They contribute to competitive interactions within microbial communities and are a component of a bacterial defense mechanism. Bacteriocins being ribosomally synthesized and have a great potential to be produced as biopreservatives using recombinant protein expression (Benítez-Chao et al. 2021). The discovery of new bacteriocins is rapidly increasing, with databases continually expanding as more of these antimicrobial peptides are identified. Despite this growing catalog, there remains a significant gap in research focused on the recombinant expression of bacteriocins. Recombinant expression, which involves producing bacteriocins using engineered host organisms like *E. coli* or yeast, could greatly enhance their large-scale production and application. However, challenges such as low

yield, host toxicity, and improper protein folding have hindered progress in this area. Overcoming these obstacles is crucial, as efficient recombinant expression would enable cost-effective, scalable production and allow for genetic modifications to improve bacteriocin stability and efficacy. Addressing this gap is essential to fully harness the potential of bacteriocins in antimicrobial therapies, food preservation, and biotechnology. Current situation of increased pathogenic attacks demands novel therapeutic agents in market. The rise in antibiotic resistance, coupled with the limited discovery of new antibiotics, has positioned bacteriocins as a promising alternative for combating microbial infections. However, for bacteriocins to be commercially viable as antimicrobial agents, significant improvements are needed in their recombinant expression systems. Optimizing expression designs will allow for higher yield, stability, and bioactivity while minimizing toxicity to the host cells. This will enable large-scale production and pave the way for their application in medicine, agriculture, and food preservation. Advances in genetic engineering and bioprocess optimization are essential to fully unlock the commercial potential of bacteriocins as effective alternatives to traditional antibiotics. This project is therefore, focusing on the production of such novel bacteriocin that have been reported to possess antimicrobial activity against putative disease-causing pathogens. As the world is facing the shortage of new antibiotics with increased number of pathogenic attacks, bacteriocin produced through recombinant expression offers a potential alternative to antibiotics. This project can also be carried forward for further research to establish the analytics required for the industrial scale production of expressed bacteriocin.

Chapter 3
Materials and Methods

3.1-Study Area & Waste Disposal:

This research study was conducted at the Protein Expression and Purification Laboratory of the Graduate School of Chemical Sciences, Autonomous University of Nuevo Leon (UANL), Mexico. All experimental methods right from gene design, propagation, molecular cloning, plasmid construct design and transformation, protein expression, purification, and finally bioactivity assays were carried out in the Protein Expression and Purification Laboratory, except for synthesis of synthetic DNA fragments encoding the target recombinant hybrid peptide. All wet laboratory experiments were conducted under aseptic conditions with standard laboratory protocols and practice supervised by the principal investigator. Laboratory waste generated were disposed according to the guidelines described by the Graduate Faculty of Chemical Sciences (FCQ, UANL, MX). All the waste collected during work for this project will be subjected to disposal considering the biosafety rules. 3 routes criteria will be followed; non-hazardous material will be collected in container (A), solutions disposals will be collected in separate container (B) and will be neutralized according to pH if required. Solid organic waste in the form of agarose and acrylamide gels will be collected in special containers (C) double lined by trash bags. All three types of collected wastes will be disposed via university's chemical waste program.

3.2 Experimental Part: Bioinformatics

3.2.1 Sequence identification corresponding to the putative new molecule- The SmallTalk

The amino sequence of SmbP and its 3-D structure in PDB format was retrieved from RCSB PDB. The accession ID of SmbP from *Nitrosomonas europaea* in PDB is 3U8V. BLAST tool available on NCBI website was used for the confirmation of SmbP amino acid sequence. The accession number of SmbP in NCBI is WP_011112924.1 and PDB determined 3-D structure of SmbP is also linked with the said accession numbers. The Nickle affinity of SmbP is attributed to PubChem compound ID of Nickel in the obvious specification of this protein. The structure of SmbP consists of four alpha helices. To perform the sequence to structure alignment, UCSF Chimera was used. The aim is to select the amino acid sequence referring to 3rd and 4th alpha helices of the SmbP rightly not to disrupt the conformation.

3.2.2 In-silico structure characterization and validation of SmallTalk

Structure modelling of SmallTalk from its putative nucleotide sequence was done using two different methods of structure prediction: through homology modeling using Swiss Modeler and by Threading using i-Tasser. The resulting structure was analyzed through QMEAN value and Ramachandran plot. Ramachandran plot were generated using PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/). The 3D structure of SmallTalk-Bin1b was predicted using i-Tasser which is homology independent tool and generate the model using iterative threading approach.

3.2.3 Primer designing for the newly hypothesized fusion peptide tag

Once the nucleotide sequence of SmallTalk is selected, primers were designed to amplify the designated region of SmallTalk through PCR amplification. The restriction site of *NdeI* was added to flanking side of forward primer and *KpnI* restriction site was added to flank the reverse primer. These restriction sites have been chosen to clone SmallTalk DNA fragment in a pET-30a (+) (EMD Millipore, Burlington, MA) parent plasmid already containing GFP tagged with SmbP flanked by NdeI and KpnI. Additional 9 to 10 nucleotides clamp was added upstream to both reverse and forward primers for the efficient binding of DNA polymerase. Melting temperature of both primers were in standard range of 50-to-60-degree Celsius and the melting temperature difference of both primers were kept in between the range of 5 degree Celsius.

3.3 Molecular Cloning

3.3.1 Amplification of SmallTalk through Polymerase Chain Reaction

The amplification of SmallTalk was performed using standard Polymerase Chain Reaction using ThermoFisher Taq DNA Polymerase. PCR cycle steps deployed for thermal cycler are as follows: Initial denaturation for 1 min at 95 °C, denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 45 seconds, elongation at 72 °C for 50 seconds, and final elongations at 72 °C for 5 min, 32 reaction cycles of amplification. The PCR composition as per the reaction volume is given in **Table 2.1** and Polymerase Chain Reaction cycle parameters are given in **Table 2.2**. The amplified product was visualized under UV using 1.5% agarose gel stained with ethidium bromide.

Table 2.1: PCR mixture Composition

Components	Reaction volume (20µl)	Concentration
10 X reaction Buffer	05 µl	1X
10 mM DNTPs	02 µl	5 µM
Forward Primer	01 µl	0.5 µM
Reverse Primer	01 µl	0.5 µM
Template DNA	01 µl	≈10 ng
DNA Polymerase Enzyme	01 µl	5 units
Nuclease free water	09 µl	09 µl

Table 2.2: PCR Cycle Parameters for the amplification of SmallTalk

Step	Temperature	Time
Cycle 1: Initial Denaturation	95 C	2 min
Cycle 2: Denaturation	95 C	30 sec
Annealing	61 C	45 sec
Elongation	72 C	30 sec
Cycle 3: Final Elongation	72 C	5 min

3.3.2-Molecular Cloning of Synthetic Plasmid DNA Molecules With Protein Of Interest Tagged With New Fusion Peptide Tag

I. Construction of plasmid for the expression of SmallTalk tagged GFP

The amplified band at the exact expected size was cut immediately and preserved in a closed eppendorf in nuclease free conditions. The amplification product was purified with the MEGAquick-spin™ Plus Total Fragment DNA Purification kit. pET-30a (+) cloned with SmbP-GFP already prepared in previous study of Protein Expression and Purification Laboratory was restricted using *KpnI* and *NdeI* restriction enzymes. In a 1.5 ml eppendroff tube was added 1 µg of plasmid DNA, 5 µl of 10X NEBuffer to a final concentration of 1X, 1 µl of each enzyme and nuclease free water up to 50 µl. After mixing the components by gently tapping the eppendroff, microfuged the tube briefly so that to collect all components at the bottom. The reaction mixture was incubated at 37 °C for 2 hours. Gently mixed the components and microfuge briefly. Same reaction was performed with the purified DNA fragment of amplified Smalltalk. As the SmbP and SmallTalk fragments are very small in size, the reaction mixture was loaded on 2% agarose gel stained with ethidium bromide instead of usual 1%. Restricted pET-30a (+) backbone DNA containing GFP and SmallTalk DNA were purified using MEGAquick-spin™ gel extraction kit. Ligation reaction mixture was prepared by mixing 2 µl of ligase Buffer, 50 ng of restricted pET-30 a (+) plasmid DNA and 18 ng of SmallTalk following 1:10 vector to insert ratio, 1 µl of T4 DNA Ligase and nuclease free water up to 20 µl. The reaction mix was incubated at 4 °C over-night. After incubation, ligase enzyme was heat inactivated at 65 °C for 10 minutes. 2 to 5 µl of ligation mixture was transformed for propagation into chemically competent DH5α cells by heat shock method (42 °C for 45 seconds). Transformants cells

were screened for positive clones and after plating cells were incubated at 37 °C for over-night on Lauria-Bertani agar-kanamycin with a final concentration of 30 µg/ml. Multiple perfectly formed and segregated single colonies were picked and grown in 2 ml of Lauria-Bertani broth containing marker antibiotic kanamycin for 16 hours. Cells were harvested and plasmid DNA isolation was performed using iNtRON DNA-Spin^{TM} Plasmid DNA Purification kit. The presence of gene in designated area of plasmid with correct orientation was confirmed by PCR amplification of T7 region using 5′-T7 promoter forward primer (5′-TAATACGACTCACTATAGGG-3′) and the 3′-T7 terminator reserve primer (3′-GCTAGTTATTGCTCACGG-5′) pair. Amplicons were analyzed on agarose gel and visualized under UV illuminator. Confirmed plasmid constructions were sequenced by STARSEQ (Instituto de Biotecnologia, UNAM, Mexico) and results were analyzed using BioEdit (version 7.7) software. Pairwise sequence alignment of company returned sequence and theoretically curated nucleotide sequence of SmallTalk-GFP constructions was conducted using Emboss Needle (https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle).

II. Construction of pET-30a(+) SmallTalk-Bin1b

The earlier elucidated amino acid sequence of SmallTalk was used while the amino acid sequence of Bin1b was retrieved from Antimicrobial Peptide Database (https://aps.unmc.edu/home) under the accession number ‘AP01592’. A 118 nucleotides sequence encoding SmallTalk-Bin1b optimized according to the codon usage preferences in *E. coli* was synthesized commercially cloned in pUC57. The protein coding DNA region is flanked by *NdeI* and *XhoI* restriction sites. Both pET-30a (+) and pUC57 cloned with SmallTalk-Bin1b were digested with *NdeI* and *XhoI* restriction enzymes. Restricted

fragments were loaded on 1% agarose gel stained with ethidium bromide. The target DNA fragments on approximate size were cut and purified using MEGAquick-spin DNA purification kit and were ligated together with T4 DNA ligase. The ligation mixture was propagated by transformation in chemically competent DH5α cells by heat shock method. Positive transformants were selected on LB-Kanamycin agar plates (30 µg/ml) and multiple colonies were further confirmed for the presence of designed plasmid DNA molecule through PCR amplification of T7 region followed by sanger sequencing.

III. Construction of pET-30a(+) SmallTalk-MP1016:

For the construction of SmallTalk-MP1106 gene cloned in the expression vector, previously engineered construction harboring CusF3H+_MP1106 was used as backbone. SmbP sequence is flanked by *NdeI* and *KpnI* restriction sites so the nucleotide sequence corresponding to SmbP was removed by treating with respective restriction enzymes. The amplified SmallTalk sequence also adjoined by same restriction sites through plasmid incorporation was treated with enzyme digestion to leave the flanks, cut and open, thus sticky. The digested plasmid backbone with MP1106 gene and all the elements of expression plasmid, except the SmbP (as it is removed through restriction digestion) was ligated with the digested SmallTalk. As a simplified overview, SmbP was cut at restriction sites using restriction enzymes and SmallTalk having the same sticky ends as treated with same restriction enzymes was pasted in place of SmbP, in the expression plasmid pET-30a(+) and joined by the formation of phosphodiester linkages via T4 DNA ligase. The reconstructed plasmid molecules were transformed into DH5α for in-vivo propagation.

IV. Construction of pET30a (+) SmallTalk-Enterocin DD14:

Enterocin DD14 being a member of class II-b bacteriocin, comprises of two peptides unit, both of which are required to exhibit the antimicrobial activity. Through literature review, the different strategies were found to be successfully implemented, either to clone both genes within same plasmid separated by linker or to clone the genes in two different plasmid and produce the two peptide units separately. For this study, second approach was considered. Enterocin DD14A and DD14B genes were independently cloned in pET30 a expression vector in software based simulation. After codon optimization based on *E. coli* preferences using GenScript online codon optimization tool, the sequence was sent to GenScript BioTech for de Novo synthesis.

3.3.3-Propagation and Storage of Plasmid in DH5α: Ensuring Stability and Amplification

In vivo propagation of the designed and synthesized plasmid constructs was performed in transformation-ready *E. coli* DH5α by heat shock method. Propagation in DH5α is necessary as it amplifies the plasmid DNA, not only generating enough needed for PCR but also ensures the stability of the plasmid. It does so by preserving its genetic integrity and preventing any potential degradation or mutations before confirmation of plasmid's structure and sequence through PCR. All the plasmid constructions (ST-GFP, ST-Bin1b, ST-MP1106, ST-Enterocin DD14A & Enterocin DD14B) were transformed in *E. coli* DH5α. ST-GFP, ST-Bin1b and ST-P1106 were ligated using T4 DNA ligase and the ligation mixture was transformed into DH5**α** competent cells. ST-Enterocin DD14 A & B were directly synthesized in expression vector pET30a, hence the 4 ug of lyophilized DNA in the company vial was diluted 10 folds Heat shock method was employed for

transforming ST-GFP and ST-Bin1b into chemically competent DH5α while electroporation was conducted for transforming ST-MP1106 and ST-Enterocin DD14 constructs into electrically competent DH5α. Transformed cells containing under-study constructions were precisely labelled and stored in -80 for long-term storage and back-up.

Tables 3.1, 3.2 and 3.3 are populated with the protocols followed for reconstitution and dilution of lyophilized plasmids, transformation followed for heat shock methods and transformation protocol deployed for electroporation respectively.

Table 3.1 Reconstitution and Dilution Protocol for Lyophilized Plasmid DNA from GenScript:

- Keep the vial sealed until ready to use. Centrifuge at 6,000 x g for 1 minute at 4°C.
- Open the vial and add 20 µl of sterilized water to dissolve the DNA.
- Close the lid and vortex the vial for 1 minute.
- If necessary, heat the solution at 50°C for 15 minutes to dissolve the DNA.
- https://www.genscript.com/gsfiles/techfiles/Gene%20User%20Instructions.pdf?678702735

Table 3.2 Transformation protocol by Heat Shock Method

- Take competent cells out of -80°C and thaw on ice (approximately 20-30 mins).
- Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.
- Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
- Incubate the competent cell/DNA mixture on ice for 20-30 mins.
- Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
- Put the tubes back on ice for 2 min.
- Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.
- Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
- Incubate plates at 37°C overnight.
- https://www.addgene.org/protocols/bacterial-transformation/

Table 3.3 Transformation protocol by Electroporation

- Remove a 50 µL aliquot of cells from -80ºC storage and place them directly onto ice to thaw, and, once thawed, do not handle the cells vigorously, as this will reduce the transformation efficiency or use freshly prepared ice-cold electro-competent cells.
- 2. (Optional) Aliquot 50 µL of cells into ice-cold electroporation cuvette (0.1-cm gap) Test an aliquot of cells to check that the sample does not arc, using the electroporation conditions in step 6. If arching does occur, wash cells in 1 mL of ice-cold sterile distilled water and resuspend in 50 µL ice-cold sterile 10% glycerol, until arcing does not occur.
- 3. To the 50-µL cell aliquot, add prechilled plasmid DNA (between 5 pg and 100 ng) in a low volume (< 5 µL). Mix by gentle tapping and incubate on ice for 10 to 30 minutes.
- 4. Place the 0.1 cm electroporation cuvette in Ice to chill.
- 5. Place all the DNA/cell mix into the prechilled electroporation cuvette, making sure it has no bubbles. Then into the apparatus (IMPORTANT: wipe the sides of the cuvette making sure its completely dry).
- 6. Set the electroporation conditions on a Bio-Rad Gene Pulser to 1.8 kV, 25 µF, and 200 Ω. This conditions may change depending on the Bacterial strain and the pulse apparatus, check it beforehand. To deliver an electric pulse, press the pulse button until a beep sounds and a time constant appears in the apparatus window. If there is a popping sound, the sample has arced, probably because the plasmid DNA has too high a salt concentration.
- 7. In E. Coli, time constants Should be between 4,5 and 5, lower values probably mean a very low transformation or cell death.
- 8. Immediately after cells have been pulsed, add 400 µL of room temperature S.O.C. medium and gently resuspend the cells. Transfer the cells to an eppendorf and incubate at 37ºC with vigorous shaking for 1 hour.
- 9. Spread aliquots of the cells onto LB agar plates containing an antibiotic appropriate for the selection of transformants. Several different dilutions of the cell suspension should be spread onto the plate to obtain single colonies.
- 10. If a high number of transformants is expected, the original aliquot of cells can be diluted, 1 in 10 serial dilutions of 100 µL should be spread onto plates. If a low number of transformants is expected, it is better to spread aliquots of 200 µL onto five plates, rather than the whole 1 mL on one agar plate, to avoid growth inhibition because of dead cells.
- 11. Invert the plates and incubate at 37ºC overnight.

3.4-Confirmation of Designed Recombinant Plasmids

3.4.1-Confirmation by PCR amplification of plasmid's T7 region

Since the plasmid under investigation contains the kanamycin resistance gene, the growth of certain colonies on LB-agar plates supplemented with kanamycin suggests successful uptake of the plasmid by these colonies. The presence of this gene allows the colonies to grow despite the presence of kanamycin, confirming the plasmid's functional expression of antibiotic resistance. However, to eliminate the chances of false positives (colonies containing intact plasmid but lacking the gene of interest), more accurate and specific analysis, conventional colony-based PCR analysis was carried out for the identification and confirmation of designed constructs. Multiple single well-rounded colonies were picked form the agar plates grown over night with the transformed cells potentially harboring the constructed plasmids. ST-GFP cloned in pET-30a(+) reconstructed from PCR amplified SmallTalk and GFP containing pET-30a(+) backbone via sticky end ligation was assimilated by competent cells were grown in LB-kanamycin-agar plate. The same way, ST-MP1106 plasmid clone was reconstructed from amplified and restriction enzyme digested SmallTalk and MP1106 containing plasmid backbone. ST-Bin1b was reconstructed by joining ST-Bin1b gene insert isolated from commercially synthesized pUC57 and ligated into MCS of pET-30a(+) digested with same restriction as used to remove ST-Bin1b gene. In the order of occurrence, ST-Enterocin DD14 A & B constructs were designed in the last, upon reviewing the results from prior constructs. So, they were directly synthesized in the expression vector. The company return plasmid DNA in this case was ready to express plasmid DNA molecule, so was generally first propagated in DH5a for storage and propagation. For the identification and characterization of T7 region

of plasmid backbone encompassing gene of interest, T7 promoter and terminator primers were employed. The nucleotide sequence of 5'-T7 promoter used as forward primer is **5'-TAATACGACTCACTATAGGG-3'** and that of 3'-T7 terminator reversed primer is **3'-GCTAGTTATTGCTCACGG-5'**. Following components DNase-free water, 5 mM dNTPs, 10 ng plasmid DNA template, 0.5 µM primer, 5 U Taq polymerase (Table 3.6 and 3.7). The reaction conditions were carried out as follows, initial denaturation at 95°C for 1 min, denaturation at 95°C for 30 s, primer annealing at 55°C for 45 s, elongation at 72°C for 50 s and final elongations at 72°C for 5 min, 32 reaction cycles were carried out. Samples were stored at 4°C until required for further use. From the prepared Master Mix, 20 µl was dispensed in each (sterile) PCR microtube. For the preparation of template plasmid DNA, a sterile micropipette tip was used to touch the colony and dispense in 10 µl of Molecular Grade sterile water. For the PCR reaction, 2 µl of this colony mix was added to the PCR master mix and remaining colony DNA was stored at 4 degrees Celsius to proceed with plasmid DNA isolation procedures for the confirmed colonies. Master mix was prepared as per the recipe mentioned in **Table 3.4**.

Table 3.4: PCR Cycle Parameters for the amplification of T7 region of pET-30(+) a

Step	**Temperature**	**Time**
Cycle 1:	95 C	2 min
Initial Denaturation		
Cycle 2:	95 C	30 sec
Denaturation		
Annealing	55 C	45 sec

Elongation	72 C	30 sec
Cycle 3:	72 C	5 min
Final Elongation		

3.4.2-Visualization of PCR amplicons

The PCR amplicons were viewed on a 1% agarose gel stained with Ethidium bromide (EtBr). Agarose gel was prepared as follows; 0.3 g agarose powder (molecular grade) was dissolved in 30 ml of 1X Tris-acetate−EDTA (TAE) buffer under microwave (RIVALTM Microwave) heat for 90 s. the dissolved gel was mixed with 5 µl Ethidium bromide (1µg/ml), appropriately mixed by gentle shaking and subsequently casted into gel casting tray with comb fixed. The gel was allowed to solidify at room temperature for 30 min. Casted agarose gel was then used for electrophoresis analysis of the PCR amplicons under 70 V for 5 min followed by 110 V for 25 min. The stained gel was visualized under UV light (UVP White/UV Transilluminator). A 10 kb DNA molecule (New England Biolabs Inc.) was used as DNA marker for fragment size estimation.

3.4.3-Sequencing of designed plasmid constructs

In our attempt to further confirm the DNA nucleotide accuracy and specificity of the synthetic DNA inserts CusF3H+_LL-37_Renalexin and SmbP_LL-37_Renalexin in the designed recombinant plasmid constructs named above. The Sanger DNA sequencing method was employed and carried out at the DNA synthesis and sequencing laboratory of the Institute of Biotechnology, National Autonomous University of Mexico (UNAM). A 16 µl sample volume consisting of 15 µl of each plasmid construct at a final concentration

of 600 ng, and a 1 µl T7 promoter forward primer at 10 µM concentration was prepared in a 1.5 ml Eppendorf tube and transported on ice to the DNA sequencing laboratory at UNAM for Sanger sequencing. Since the three construct ST-GFP, ST-Bin1b and ST-MP1106 were cloned into pET-30a(+), hence they were carried forward for the confirmation of correct placement of gene in the vector backbone. However, the final construction ST-Enterocin was directly synthesized into pET-30a(+) by GenScript which rendered it sequencing unnecessary.

3.5-Protein Expression and Purification:

3.5.1 Small Scale Expression of Recombinant Fusion Proteins

The confirmed pET30a (+) plasmid DNA molecules were cloned into BL21 DE3 cells (New England Biolabs, Ipswich, MA), the expression strain of *E. Coli*. ST-GFP and ST-Bin1b were transformed via heat shock method into chemically competent cells, while ST-MP1106 and ST-Enterocin clones were transformed into electrically competent cells through electroporation. Transformants were plated on kanamycin LB agar plates (to select the clones possessing kanamycin resistance gene within the plasmid) and grown at 37 °C for 16 hours. Single well rounded and evenly formed colony was picked and used to inoculate 2 ml of LB broth with 2 µl of 1000X kanamycin to obtain a final concentration of 30 ug/ml. After 16 hours of incubation at 37 degrees with shaking at 220 rpm, culture was cool down to room temperature. From the over-night grown culture, 100 µl was used to inoculate 10 ml (As per recommended ratio i.e. 1:100) of LB in small flasks with already added 10 µl of 1000X kanamycin (30 µg/ml). Bacterial culture was grown at 37 °C temperature and 220 rpm shaking speed until an optical cell density; OD_{600nm} of 0.4-0.6 was obtained. At this point, flasks were allowed to cool down at room temperature from

37 degree Celsius after taking out of incubator and then the bacterial culture were induced with Isopropyl-β-D-thiogalactopyranoside, 10 µl of 0.1 Molar IPTG for the expression of recombinant protein at a final concentration of 0.1 mM. Expression was evaluated at varying values of time (4 hrs and 16 hrs) and temperature (37 °C and 25 °C) to optimize the best conditions. Cells were harvested by centrifugation at 16,250 x g for 5 minutes in 2 ml eppendroff tube and the supernatant was discarded. Cell pellet can be stored at -20 °C at this stage of experiment or proceed directly to sample preparation (Section 5.1 below).

3.5.2-Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of small-scale expression: A detailed guide to SDS PAGE analysis

- *Sample preparation*

For the characterization and visualization of the expressed fusion proteins and track their presence in soluble or insoluble fractions, samples were prepared from collected cell pellet. Cell pellet is washed once with DI water and centrifuged and decanted again. To this washed and dried (by inversion on paper towel) cell pellet, 120 µl of lysis buffer 1 (50 mM Tris–HCl, pH: 8.0) was added along with 150 µl of glass beads followed by vortexing for 5 minutes. After removing the glass beads, mixture was centrifuged at 16,250 X g for 5 min, the supernatant was taken into a new tube. This comprises the soluble fraction. To obtain the insoluble fraction, the remaining pellet was washed from the previous step by adding 120 µl of water, vortex, centrifuge, remove water, and repeat. Next was added 120 µl of 8 M urea solution and pellet was resuspended using a vortex followed by boiling for 10 min, and then centrifuge at 16,250x g for 10 min. Supernatant was removed and cell pellet was discarded. This collected supernatant is the insoluble fraction. Sample buffer was added to the supernatant at a final concentration of 1X (5 volume of protein sample: 1 volume of sample buffer) to both soluble and insoluble fractions and mix was boiled for

10 min. Samples were analyzed using 12% SDS PAGE. Sample Buffer used in the sample preparation is given in **Table 3.5** with ingredients and their function.

Table 3.5 Sample buffer recipe:
2.4 ml of 1 M Tris-HCl buffer at pH 6.8 (this provides the buffering system that preserve the overall structure of peptides under analysis),
0.8 g of SDS (Regardless of the initial charge, SDS aids to linearize the proteins by denaturing them and giving them a net negative charge. By doing this, the difference in charge and shape that may normally distort the mobility of the proteins in the gel is reduced), **0.5 ml of β-mercaptoethanol** (This chemical is used to disrupt disulfide bonds. In addition to SDS, beta-mercaptoethanol guarantees that the bands are caused by individual polypeptides rather than molecular complexes),
4 ml of 100% glycerol (provides density to the peptide sample so that it can settle in the gel well smoothly),
4 mg of Bromophenol blue (It's the tracking dye to visually indicate the position of bands on gel).
https://sharebiology.com/laemmli-buffer-preparation/

- *Gel preparation*

A 15% resolving gel and a 4% stacking gel were prepared with the compositions mentioned in **Tables 3.6 and 3.7**. TEMED and APS are added in the last after mixing all the other components, right before pouring the gel into gel caster assembly. A Online tool is available at following address: "https://www.cytographica.com/lab/acryl2.html" to calculate varying amounts of components to prepare required percentage of gel as it has to be selected based on the size of target peptide.

Table 3.6 Resolving Gel Recipe (15 %)

Component	***Amount***	***Amount***
	40% Acrylamide/bisacrylamide solution (29:1)	30% Acrylamide/bisacrylamide solution (19:1)
Distilled water	1.825 ml	1.15 ml
Resolving gel buffer (1.5 M Tris, pH 8.8)	1.25 ml	1.25 ml
Acrylamide/bisacrylamide solution	1.875 ml	2.5 ml
SDS solution (10%)	50 µl	50 µl
TEMED	5 µl	5 µl
Ammonium persulfate (10%)	40 µl	40 µl

Table 3.7 Stacking Gel (15%)

Component	**Amount**	**Amount**
	40% Acrylamide/bisacrylamide solution (29:1)	30% Acrylamide/bisacrylamide solution (19:1)
Distilled water	1.6 ml	0.8 ml
Stacking gel buffer (0.5 M Tris, pH 8.8)	1.25 ml	1.25 ml
Aacrylamide/bisacrylamide solution (40%)	1.875 ml	0.35 ml
SDS solution (10%)	50 µl	50 µl
TEMED	5 µl	10 µl
Ammonium persulfate (10%)	40 µl	30 µl

The resolving gel was prepared and casted first into a two vertical glass slides and covered with 200 µl Iso-butanol to facilitate the solidification and to prevent air bubbles formation in the gel. After 20 min solidification of the resolving gel, the Iso- butanol was removed, and the gel layer was then rinsed three times with distilled water before the 4% stacking gel was cast with a 15-well comb fixed. A total of 5 µl of prepared protein samples were loaded onto the gel, and a 3 µl of a 120 kDa standard protein (PAGE−MASTER protein standard plus) was employed as the molecular marker for protein size characterization.

- *Voltage and time settings for SDS-PAGE apparatus (Running the gel):*

  The gel electrophoresis analysis was run under the following conditions, 80 volts for 10 min followed by 120 volts for 1.3 h. After the gel electrophoresis, the gel was then washed in distilled water 3 to 4 times.

- *Staining and Destaining of Gel:*

  The gel was stained in 80 ml SDS staining solution for overnight. After overnight staining, the gel was washed twice in distilled water for 5 min and destained for 45 min in 80 ml destaining solution. The composition of staining and destaining solution is listed in **Tables 3.8 and 3.9**. The stained protein bands in the gel were visualized under white light (UVP White/UV Transilluminator).

Table 3.8 Recipe of Staining Solution

Component	**Final Concentration**
Methanol	50%
Glacial acetic acid	10%
Coomassie brilliant blue R250	0.1%

Table 3.9 Recipe of Destaining Solution

Component	Final Concentration
Methanol	50%
Glacial acetic acid	10%

3.6-Large Scale Expression

A generalized protocol applied for the large-scale expression (1 Liter) of all four constructs is explained here. Large scale expression was carried out in a sequential order for the four fusion peptides, one construct at a time to avoid human errors, since this is a tedious experiment. For large scale expression intended to purify the protein of interest, one-liter Lauria-Bertani media was prepared, distributed 125 ml into eight baffled flasks, and sterilized. Before inoculating with over-night grown starter culture, 125 µl of kanamycin (1000X) was introduced into each flask and inoculated with 125 µl of overnight inoculum. Cells were allowed to grow at 37 °C and 220 rpm shaking until they reach OD_{600} of 0.4-0.6. Recombinant expression was induced with 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) flasks were cooled to room temperature expression was induced by adding 125 µl of 0.1 M IPTG (at a final concentration of 0.1-mM). Cells were allowed to express at under previously optimized conditions (Temperature 25 °C, revolution 220 rpm and time 16 h). Cells pellets were harvested in 50 ml Falcon tubes by centrifugation at 17000 x g, for 15 minutes in refrigerated centrifuge.

3.6.1-Sample preparation from pellet collected from 1 L of bacterial culture

The pellet of bacterial cell cultures from the four types of expressed clones were subjected to two different methods of cell lysis in order to obtain protein content of the culture. Bacterial clones expressing ST-GFP and ST-Bin1b were disrupted using mechanical lysis procedures while ST-MP1106 and ST-Enterocin was resuspended using sonicator for the cell envelope disruption. The detailed protocol is mentioned in sections

- Mechanical Lysis using glass beads: Cell pellets harboring the recombinant protein expression were resuspended in 30 ml of chilled lysis buffer (500 mM NaCl, 50 mM Tris-HCl; pH 8.0) with 0.1 mm glass beads. After resuspension of cell pellet and before vortexing, 30 µl of Antifoam 204 and 30 µl of protease inhibitor cocktail was added. Cells were lysed by vortexing for 10 minutes while maintaining the refrigerated conditions. Once the lysis was complete, Falcon tubes were centrifuged at 8500 × g, 4 °C for 15 min resulting in pelleted cell debris and clear soluble cell lysate. This step can be repeated twice to get a very clear sample, devoid of cell debris.
- Lysis by Sonication: Place the falcon tube in a beaker full of ice and insert the tip to the bottom of falcon. Once reached, elevate the tip slightly until it does not touch the bottom or any wall but still stays inside the liquid. Repeat 3 Cycles with constant amplitude of 0.2 J/s of 30 seconds *ON* + 90 Seconds *OFF* (to let cool down the sample). After each cycle, readjust the falcon position with the help of the ice. Final sample should look more transparent and less viscous as compared to the starting conditions. Centrifuge at 14000 rpm for 15 minutes at 4 ºC. Transfer supernatant to a new falcon tube and proceed with sample preparation for SDS-PAGE analysis. It

can be stored at -20 °C at this stage (https://static.igem.org/mediawiki/2020/a/ad/T--UPF_Barcelona-CellLysisbySonification.pdf).

3.6.2 Purification of recombinant proteins; ST-GFP, ST-Bin1b by Immobilized Metal Ion Affinity Chromatography (IMAC)

Recombinant proteins (ST-GFP & ST-Bin1b) tagged with SmallTalk were purified individually deploying the principle of Immobilized Metal Ion Affinity Chromatography (IMAC) using ÄKTA Prime Plus System (GE HealthCare) for FPLC. 1-ml HisTrap FF agarose resin charged with Ni (II) was used for the purification process. Following steps were performed as per the manual of ÄKTA Prime Plus System (GE HealthCare). The composition of all the buffers used in the purification procedures are listed with final concentrations in **Table 3.10**.

1. 'System Wash' was performed by navigating through options from the software of machine "Template => Application Template => System wash (Select a, b . . . . **ok**)". This process was carried out twice, first with 20% ethanol and then with DI water.
2. Charging the valves and column before sample loading: Valve 'a' was placed in equilibrium buffer and valve 'b' was placed in Elution buffer. Valve 'b' was charged with 12 column volume of elution buffer (Manual Run => b=100% , flow rate = 1, pressure= 0.5 select injecting valve= Waste) and Valve a is charged with 5 column volume of equilibrium buffer (Manual Run => a=100% , flow rate = 1, pressure= 0.5 select injecting valve = Load), this will equilibrate the column with equilibrium buffer. It is crucial to charge valve 'b' first with elution buffer in order

to avoid ambiguity & during charging the valves, flow rate could increase up to 1 but not while the sample is passing.

3. Sample loading: The clarified whole-cell lysate was then charged onto the column under persistent conditions of 0.5 ml/min flow rate and 0.5 MPa pressure to avoid over pressure error. System settings will remain the same as column equilibration (Manual Run => a=100%, flow rate = 1, pressure= 0.5 select injecting valve = Load), system will just be 'paused' and valve 'a' will be placed into sample now (while taking out from equilibrium buffer) and continue 'Run'. Sample must be placed on ice during this whole time of charging. During a single run, upto 50 ml of sample can be charged. When the curve becomes steady horizontally, fraction of flowthrough is collected to be analyzed on electrophoresis.
4. Afterwards, the column was washed with 3 Column Volumes of Wash Buffer or until a steady baseline has achieved. The reason of the column wash is to eliminate contaminants and unbound or non-specifically bound proteins so that only the target protein is left on the resin. The standard is to run at least ten column volumes (CV) of wash buffer through the column, however, it can be lower down to 5 CV. One fraction is collected at this stage as representative of column wash in SDS-PAGE.
5. The elution of recombinant protein was carried out loading elution buffer. Collected elution fractions, column flow through and cell lysate were analyzed to visualize the presence of target recombinant protein using 15 % SDS-PAGE (Santos et al. 2019a). The purity of bands was quantified using ImageJ software provided by National Institute of Health (NIH) (Adamíková, Antošová, and Polakovič 2019).

Table 3.10 Buffers used for Purification

Equilibrium Buffer (also called Lysis Buffer**)**:- 50 mM Tris–HCl, 500 mM NaCl, pH: 8.0. Weigh 6.06 g Tris, 29.22 g NaCl, and dissolve them in water to a volume of 900 mL; adjust pH with HCl, make up to 1 L with water. Store at 4 C.
Washing buffer: 50 mM Tris–HCl, 500 mM NaCl, 5 mM imidazole, pH of 8.0: Weigh 6.06 g Tris base, 29.22 g NaCl, 0.34 g imidazole, and dissolve them in water up to 900 mL, adjust pH with HCl, and make up to 1 L with water.
Elution buffer: 50 mM Tris–HCl, 500 mM NaCl buffer, 200 mM imidazole, pH of 8.0: Weigh 6.06 g Tris, 29.22 g NaCl, 13.61 g imidazole and dissolve them in water to a volume of 900 mL. Adjust pH with HCl. Make up to 1 L with water. Store at 4 C.

Critical Points to consider while using AKTA

- Using a 2–5 mM imidazole concentration range in Equilibrium Buffer avoids the binding of unwanted proteins to the column. Just make sure to add the imidazole before adjusting the final pH.
- The imidazole concentration in the washing buffer can be increased up to 10 mM.
- All the components (Buffers and dI water) must pass through 0.22 0.22 µm filter and sterilized.

3.6.3 Purification of recombinant proteins; ST-MP1106 & ST-Enterocin DD14 by Gravity Column Chromatography

Large-scale expression of these two peptides was carried out in flasks. NEBExpress® Ni Resin (1 ml) was used to carry out the purification procedures. Gravity column purification is the manual version while AKTA prime plus is the automated version, however, the principle in both cases is the same which is affinity chromatography. Affinity chromatography is a powerful technique for purifying specific proteins, which in our study are GFP and antimicrobial peptides, by exploiting their interactions with a ligand having affinity for Nickle (which is SmallTalk in this study) and Ni resin for binding. Gravity columns provide a simple and cost-effective method for small-scale purification, though slower compared to automated systems. Since most of the laboratories still work with gravity column chromatography, Smalltalk was also tested with this method of column

chromatography as well as with the FPLC version. The ÄKTA which is a representation of FPLC, a sophisticated liquid chromatography platform that enables precise, high-throughput protein purification with real-time monitoring and greater reproducibility.

Resin Preparation

- Gently shake the Ni Resin bottle to fully resuspend the slurry, then transfer the desired amount to a closed column. Let the resin settle.
- Open the column and allow the storage buffer to drain.
- Equilibrate the resin by adding 5 column volumes (CV) of Lysis/Binding buffer, and let it flow through.

Binding

- Load the protein sample lysate onto the column and let it interact with the resin for at least 5 minutes.
- Collect the flow-through in a clean tube and reserve it for SDS-PAGE analysis.
- *Note:* Binding efficiency for some His-tagged proteins can improve with longer incubation times. To maintain the activity of the target protein, place the column at 4°C if extending the binding time. However, prolonged incubation may increase non-specific binding.

Column Wash

- Add at least 10 CV of Wash buffer to the column and let it flow through. Monitor the flow-through by absorbance at 280 nm to ensure complete removal of unbound lysate.
- Collect the flow-through in fractions and reserve for SDS-PAGE analysis.

Protein Elution

- Elute the fusion protein using Elution buffer.

  Note: It is recommended to use a minimum of 3 CV of elution buffer to elute the target protein. Optimal volumes may vary and should be determined empirically for different substrates.

- Collect the flow-through in fractions and monitor protein elution by measuring absorbance at 280 nm or performing SDS-PAGE.
- Analyze the protein sample lysate (load), flow-through, washes, and eluates using SDS-PAGE.
- *Note:* To remove imidazole for downstream applications, use gel filtration (e.g., Zeba™ Spin Desalting Columns) or dialysis (e.g., Slide-A-Lyzer™ Dialysis Cassettes).

Ni Resin Cleaning Guidelines

When using a gravity column, determine the flow rate before cleaning to ensure accurate contact time with the cleaning agent. To remove lipoproteins, lipids, and proteins bound by **hydrophobic interactions**, choose one of the following methods:

- Add 1 M NaOH to the column, allow a contact time of 1 to 2 hours, then wash with 10 CV of Lysis/Binding Buffer.
- Add 30% isopropanol to the column, allow a contact time of 15-20 minutes, then wash with 10 CV of water.
- Add 0.1 – 0.5% non-ionic detergent in 0.1 M acetic acid to the column, allow a contact time of 1 – 2 hours, then wash with 10 CV of water.

To remove proteins bound by **ionic interactions**:

- Add 1.5 M NaCl to the column, allow a contact time of 10 – 15 minutes, then wash with 10 CV of water.

3.6.4 Dialysis

The purified SmallTalk-GFP in multiple elution fractions were pooled together in semi-permeable dialysis membrane and was dialyzed against 1X PBS Buffer to exchange the elution hence desalting the purified fusion protein. The dialysis membrane pouch containing purified fusion protein was dipped by clamping horizontally in dialysate (1X PBS Buffer). The volume of dialysate must range 200 to 500 times the volume of protein in the pouch to allow efficient desalting. The dialysis setup was incubated on a magnetic stirrer with gentle shaking for 1 hour at room temperature. The dialysate was renewed with fresh 1X PBS Buffer and after another hour of incubation at room temperature with shaking, assembly was shifted to 4 °C for overnight.

3.6.5 Quantification of Purified fusion protein

i. **Bradford Assay**: ST-GFP and ST-Bin1b was quantified using Bradford assay. Dialyzed fusion protein was quantified using BSA (Bovine Serum Albumin) as reference protein (Kielkopf, Bauer, and Urbatsch 2020). The concentration of the dialyzed recombinant fusion peptide was measured and quantified by Bradford analysis as described by (Bradford, 1976; Colyer and Walker, 1996; Wingfield, 2007). Briefly, the Bovine Serum Albumin (BSA) as protein standard calibration curve was performed in milligram per milliliter (mg/ml) with concentrations ranging from 1.3 – 0.1 mg/ml. A 10 µl of BSA and the purified recombinant fusion peptide were separately mixed in a 96-well microtiter plate with 200 µl Bradford

reagent (50 mg Coomassie brilliant blue G-250, Methanol and 85% Phosphoric acid) and incubated in the dark for 5 – 10 min, absorbance readings were taken at 595 nm. The calibration curve for BSA was established in Microsoft excel and the concentration of purified recombinant fusion peptide was estimated using the linear equation of the standard BSA calibration curve. The complete procedures carried

Table 3.11 Protocol Followed for Bradford Assay

1. Prepare the Bradford reagent according to the manufacturer's instructions. Briefly, the Bradford reagent can be prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 mL of 95% ethanol. Then, add 100 mL of 85% phosphoric acid and 850 mL of distilled water. Mix well and store the solution in a brown glass bottle.
2. Prepare a series of BSA standard solutions of known concentrations (e.g. 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) by diluting a stock solution of BSA in distilled water.
3. Dilute the protein sample to an appropriate concentration using distilled water. The concentration of the protein sample should be within the linear range of the standard curve.
4. Add 20 µL of the protein sample or BSA standard to a cuvette containing 980 µL of distilled water. Then, add 1 mL of Bradford reagent to each cuvette and mix well by inverting the cuvette several times.
5. Incubate the cuvettes at room temperature for 5 minutes. During this time, the Bradford reagent binds to the protein, resulting in a shift in the dye's absorption spectrum and a change in color from brown to blue.
6. Measure the absorbance of the samples and standard at 595 nm using a spectrophotometer. Set the spectrophotometer to zero using a blank cuvette filled with distilled water. Then, measure the absorbance of the standard solutions and the protein sample.
7. Calculate the protein concentration of the samples by comparing the absorbance values to the standard curve obtained from the BSA standard solutions. Plot the absorbance values of the standard solutions against their corresponding protein concentrations to create a standard curve. Use the standard curve to determine the protein concentration of the sample based on its absorbance.
8. Repeat steps 4 to 7 for each sample and standard to obtain multiple measurements.
9. Calculate the mean protein concentration of the samples by averaging the results of the multiple measurements.

Tips for a Successful Bradford Protein Assay:

- Ensure that the Bradford reagent is freshly prepared and stored in a brown glass bottle to prevent photodegradation.
- Use distilled water to prepare all solutions and dilute the protein sample.
- Use BSA as a standard protein because it has a well-defined structure and is stable in solution.
- Measure the absorbance of the samples and standard within 5 to 10 minutes after adding the Bradford reagent, as prolonged incubation can lead to a decrease in the accuracy of the assay.
- Avoid high concentrations of detergents or reducing agents in the protein sample, as these can interfere with the binding of the dye to protein and affect the accuracy of the assay.

ii. **BCA Assay**: ST-MP1106 and ST-Enterocin DD14 was quantified by bicinchoninic acid assay (BCA assay). The bovine serum albumin standards were used as reference protein which are provided with the kit. Complete assay was performed as per the manufacturer's protocol provided with the kit tagged in the following link (Pierce™ BCA Protein Assay Kits - Thermo Fisher Scientific). The calibration curve was drawn in Microsoft Excel and the quantities were estimated using the standard curve of BSA.

3.7-Antimicrobial Activity of SmallTalk tagged Antimicrobial Peptides

Minimum inhibitory concentration (MIC) of SmallTalk-Bin1b, ST-MP1106 and ST-Enterocin was determined via Dose-response assay ("Broth Microdilution Antibacterial Assay of Peptides Laszlo Otvos and Mare Cudic," n.d.). The antimicrobial activity was tested against gram-positive bacteria *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) and gram-negative strains *Escherichia coli* and *Pseudomonas aeruginosa.* All the test pathogens were provided from the University Hospital. The minimum inhibition concentration assays were carried out in accordance with the National Committee for Clinical Standards (NCCLS) set for broth micro-dilution method ("Global Laboratory Standards for a Healthier World," n.d.). Single colonies of sensitive bacterial strains were picked to inoculate 5 ml TSB (Tryptone: 17.0 g, Soytone; 3.0 g, Glucose: 2.5 g, Sodium Chloride 5.0 g and Dipotassium phosphate: 2.5 g; pH 7.3) and incubated at 37 °C and 220 rpm for overnight. This medium is particularly nourishing

for a range of species and is preferably selected for the growth of pathogenic bacteria, since it contains digests of soybean meal and casein, which give amino acids and other nitrogenous compounds. As a pancreatic digest of casein, tryptone is made up of larger peptides and a variety of amino acids, including certain essential amino acids. The next morning, 5 µl of overnight culture was used to inoculate 5 ml TSB and was incubated with shaking at 37 °C until optical cell density OD_{600} reaches 0.8-1.0. Cells in this mid-log growth phase were serially diluted in 1X PBS buffer to 1 x 10^5 CFU/ml. (PBS Buffer: Sodium Chloride 8 g, Potassium Chloride 0.2 g, Sodium Phosphate Dibasic 1.44 g, Potassium Phosphate Monobasic 0.245 g; pH 7.4). A 96-well microtiter plate was used to perform 2-fold broth microdilution assay with a total of 200 µl assay volume in each well. The composition in each well consisted of 100 µl of diluted peptide (concentrations ranging from 30 to 0.5 µM) 80 µl of bacterial suspension and 20 µl TSB to support the growth of bacteria. A gentle mix with the pipette tip was given very carefully avoiding cross mixing after adding all three components in each well while considering. Two control samples were also added to the experimental design, one positive control comprising kanamycin and bacterial suspension while the second negative control with constituting bacterial suspension with 1X PBS buffer. Plate was incubated for 16 hours at 37 °C with shaking. After the incubation, the absorbance of bacterial culture mixed with antimicrobial peptide was measured using spectrophotometer to quantify MIC value (Montfort-Gardeazabal, Balderas-Renteria, et al. 2021b). Minimum Inhibitory Concentration is defined as the concentration of antimicrobial agent that hinders the visible turbidity of bacterial growth as compared to negative control (Kowalska-Krochmal and Dudek-Wicher 2021).

Table 3.12 Tryptic Soy Broth (TSB) composition

Compound name	Amount
Pancreatic digest of casein (Tryptone)	17.0 g
Papaic digest of soya bean (Soytone)	3.0 g
Glucose/Dextrose	2.5 g
Sodium Chloride	5.0 g
Dipotassium phosphate (K_2HPO_4: MW=174.2 g/mol)	2.5 g
	pH 7.3

Table 3.13 PBS Buffer Composition

Compound	Molecular Formula	Weight (in grams)
Sodium Chloride (NaCl)	(NaCl)	8.0 g
Potassium Chloride	(KCl)	0.2 g
Sodium Phosphate Dibasic	(Na_2HPO_4)	1.44 g
Potassium Phosphate Monobasic	(KH_2PO_4)	0.245 g
		pH 7.4

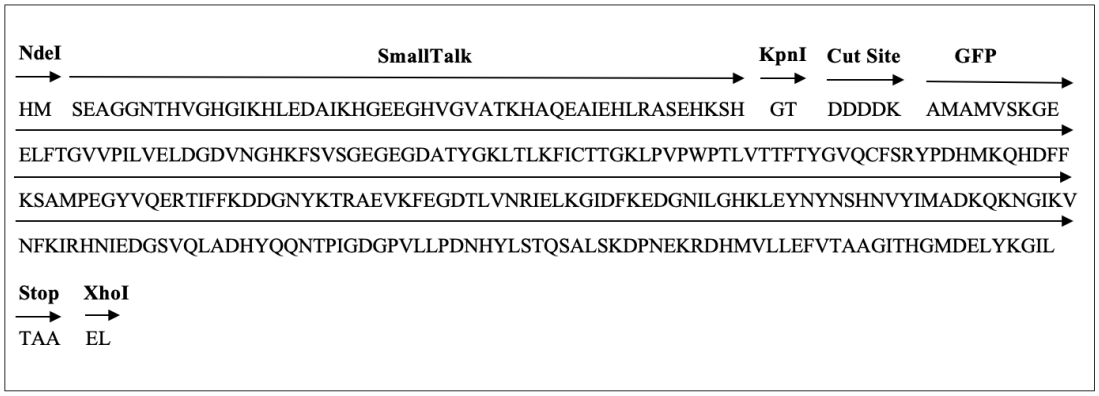
Chapter 4
Results

Section I- Molecular Design and Sequence Analysis

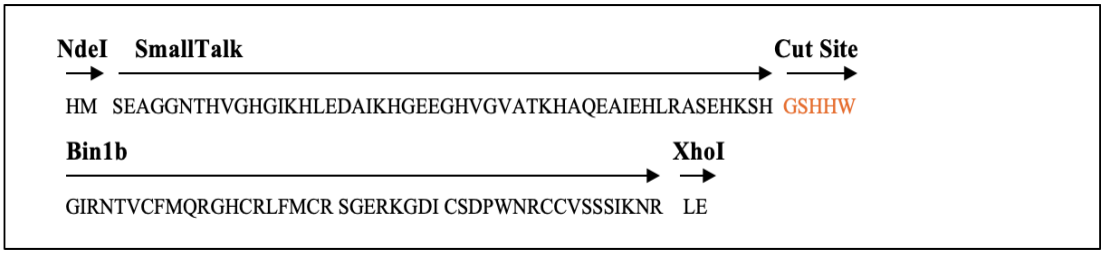
4.1.1-Amino acid Sequence and Expression Cassettes of constructs

The amino acid sequences of gene constructs designed to encode recombinant proteins ST-GFP, ST-Bin1b, ST-MP1106 and ST-Enterocin DD14 are depicted in **Figure 4.1 (A, B, C & D)** respectively. In sequence of time, ST-GFP and ST-MP1106 constructs were designed first from the SmbP-GFP-pET-30a(+) and CusF3H+-MP1106-pET-30a(+) parent clones available in the PEP laboratory. The constructions for the expression of ST-Bin1b was next in experimental design and hence it was commercially synthesized by GenScript cloned in pUC57, flanked by NdeI and XhoI restriction site. The final construction in the project, ST-Enterocin was also commercially synthesized directly in pET-30a(+) as the functionality of SmallTalk has been tested in the previously made constructions. The amino acid sequence of 'DDDDK' serve as enzymatic cleavage site in three constructs i.e. ST-GFP, ST-MP1106 and ST-Enterocin, while 'GSHHW' in ST-Bin1b. Bin1b has the Antimicrobial Peptide Database ID of 'AP01592' and the amino acid sequences used in the construction was taken from Antimicrobial Peptide Database. The length of ST-Bin1b fusion protein in terms of number of amino acids is 100 which will be the expression product from protein expression bacterial strain. The amino acid sequence of MP1106 is adapted from plectasin that is a fungal defensin with accession number (UQI49964.1). The theoretical amino acid Enterocin DD14 sequence was is reported in the first report of discovery of this peptide (DOI 10.1016/j.ijantimicag.2016.11.016). The number of amino acid residues for ST-DD14A are 99 which will comprise the final structure of working fusion protein. All the sequences are flanked by NdeI at the start and XhoI at the end, where stop codon lies just beside the

XhoI site. Since ST-GFP and ST-MP1106 are reconstructed from previously constructed clones (SmbP-GFP and cUSF3H+-MP1106) by replacing SmbP with SmallTalk, there amino sequence also contains KpnI just at the ending of SmallTalk.

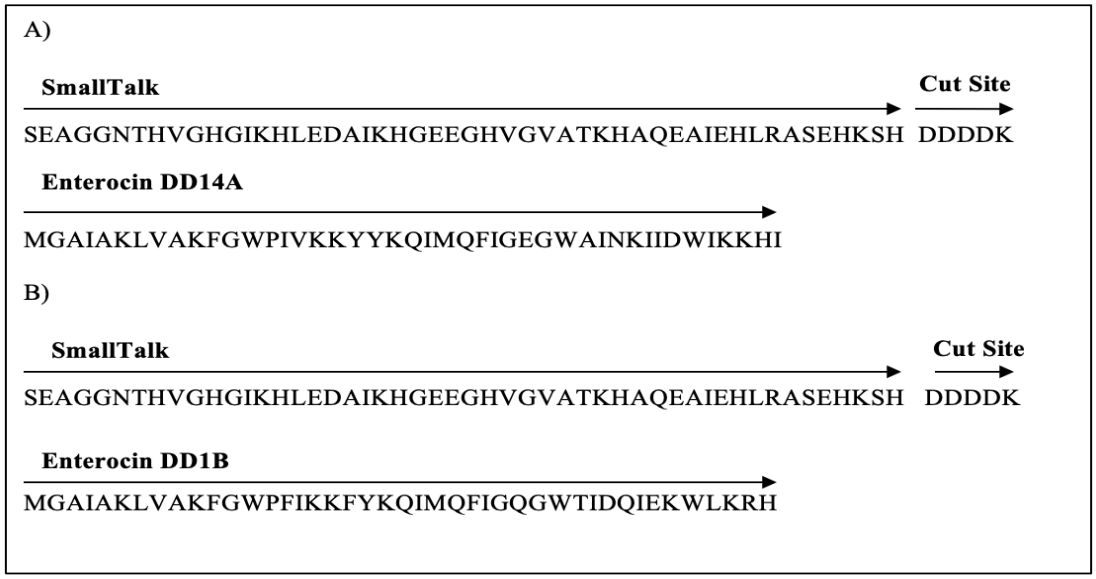


A)



B)

C)



D)

Figure 4. 1: Amino acid sequence flowchart representation of the expression cassette encoding for the chimeric protein. (A) Expression cassette encoding for ST-GFP. (B) Expression cassette encoding for ST-Bin1b (C) Expression cassette encoding for ST-MP1106 (D) Expression cassette encoding for ST-Enterocin DD14 (A & B)

4.1.2-DNA nucleotide sequence encoding for the hybrid peptide

The synthetic DNA nucleotide sequence of the target gene molecularly cloned into the plasmid expression vector pET30a+ as DNA insert for ST-GFP, ST_Bin1b, ST-MP1106 and ST-Enterocin are provided in Figure 4.3 (A, B, C & D). The DNA (gene) nucleotide sequence was chemically synthesized by GenScript Biotechnology Company, USA. The ST-Bin1b has 312 nucleotide base pairs (bp), MP1106 (294 bp) the Enterocin DD14A (312 bp) and DD14B (309). The restriction site NdeI (CATATG) has been added to the start of sequence to be expressed while XhoI (CTCGAG) is placed at the ending of the sequence.

A)

ST-GFP Nucleotide Sequence

CATATGTCTGAAGCGGGTGGAAACACCCACGTTGGTCACGGCATTAAGCATCTCG
AGGATGCAATCAAGCACGGCGAAGAGGGTCATGTTGGCGTTGCTACCAAGCACGC
GCAGGAGGCCATTGAACATTTGCGCGCATCCGAGCACAAAAGCCATGGTACCGAC
GACGACGACAAGGCCATGGCTATGGTATCTAAAGGTGAAGAACTGTTCACGGGTG
TAGTGCCGATCCTGGTAGAACTGGACGGTGACGTTAACGGCCATAAATTCTCCGTT
TCCGGCGAAGGTGAAGGTGATGCGACCTACGGCAAACTGACCCTGAAATTCATCT
GTACCACCGGTAAACTGCCGGTTCCGTGGCCTACCCTGGTCACCACCTTCACGTAT
GGTGTTCAGTGCTTCTCTCGCTACCCGGATCACATGAAACAGCACGATTTCTTTAA
ATCTGCCATGCCGGAAGGCTATGTACAGGAACGTACCATCTTCTTTAAAGATGACG
GTAACTATAAAACCCGTGCGGAAGTAAAATTTGAAGGTGACACTCTGGTTAACCG
CATCGAACTGAAAGGCATCGACTTCAAGGAAGACGGCAACATCCTGGGTCATAAA
CTGGAATACAATTACAACTCTCACAACGTATACATTATGGCAGACAAGCAGAAAA
ACGGCATTAAAGTGAATTTCAAAATTCGCCACAATATCGAAGACGGTTCTGTACA
GCTGGCCGACCACTATCAGCAGAACACTCCGATCGGCGATGGTCCGGTGCTGCTG
CCTGATAACCATTATCTGTCTACTCAGTCTGCTCTGTCCAAAGATCCTAACGAAAA
ACGCGACCACATGGTGCTGCTGGAATTCGTCACTGCTGCCGGCATCACCCACGGTA
TGGACGAGCTGTATAAAGGCATCCTGTAA CTCGAG

B)

ST-Bin1b Nucleotide Sequence:

TCAGAAGCTGGAGGTAATACACACGTAGGGCACGGCATCAAGCACTTGGAGGACG
CGATTAAGCACGGCGAGGAAGGTCACGTGGGTGTTGCAACGAAACACGCCCAAGA
GGCGATCGAGCACCTGCGTGCTAGCGAACATAAAAGCCACGGTTCCCATCATTGGG
GTATTCGCAACACCGTTTGTTTTATGCAGCGTGGTCATTGTCGTCTGTTCATGTGCCG
TAGCGGTGAACGTAAAGGCGACATCTGCAGCGATCCGTGGAATCGCTGCTGCGTGT
CCTCTTCGATTAAGAACCGC CTCGAG

Nucleotide Sequence of ST-MP1106

TCTGAAGCGGGTGGAAACACCCACGTTGGTCACGGCATTAAGCATCTCGAGGATGCAAT
CAAGCACGGCGAAGAGGGTCATGTTGGCGTTGCTACCAAGCACGCGCAGGAGGCCATT
GAACATTTGCGCGCATCCGAGCACAAAAGCCATGGTACCGACGACGACGACAAGGCCA
TGGCTGGTTTCGGCTGCAACGGTCCGTGGAGCGAAAAGGATATGCACTGCCACAACCAC
TGCAAGAGCATCAAAGGTTACAAGGGTGGCTATTGCGCGAAAGGTGGCTTTATTTGCAA
GTGCTACTAA CTCGAG

C)

ST-Enterocin DD14A
CATATGTCAGAAGCTGGAGGTAATACACACGTAGGGCACGGTATTAAGCACTTGGA
AGATGCTATCAAGCACGGCGAGGAAGGTCATGTTGGTGTGGCTACCAAACATGCAC
AAGAGGCGATCGAGCACCTGCGTGCGAGCGAACATAAATCCCATGATGACGACGA
CAAAATGGGTGCGATCGCCAAGCTGGTTGCGAAGTTCGGCTGGCCGATTGTGAAAA
AGTATTACAAACAGATTATGCAGTTTATCGGCGAGGGCTGGGCAATCAACAAAATC
ATCGATTGGATTAAGAAGCACATT TAA CTCGAG
ST-Enterocin DD14B
CATATG
TCAGAAGCTGGAGGTAATACACACGTAGGGCACGGTATTAAGCACCTGGAAGATGC
GATCAAGCACGGCGAGGAAGGTCATGTTGGTGTCGCTACGAAACACGCGCAAGAG
GCTATCGAGCACTTGCGCGCAAGCGAACATAAGTCCCATGATGACGACGACAAAAT
GGGTGCGATCGCCAAGCTGGTGGCGAAGTTCGGCTGGCCGTTTATTAAGAAATTCT
ACAAACAAATCATGCAGTTTATCGGCCAGGGCTGGACCATTGATCAGATTGAGAAA
TGGCTGAAACGTCAT TAA CTCGAG

D)

Fig 4.2- Synthetic DNA nucleotide sequences of the target genes (DNA insert) (A) Synthetic DNA nucleotide sequence encoding for SmallTalk-GFP fusion protein (B) Synthetic DNA nucleotide sequence encoding for SmallTalk-Bin1b Fusion protein (C) Synthetic DNA nucleotide sequence encoding for SmallTalk-MP1106 Fusion protein (D) Synthetic DNA nucleotide sequence encoding for SmallTalk-Enterocin Fusion protein.

Section II: Computational Modelling and Analysis

4.2-Bioinformatics Data Interpretation and its derived molecules

4.2.1. Analysis of 3D structure of SmallTalk:

Since the nucleotide sequence of SmallTalk is extracted from SmbP, initially homology modelling method of structure prediction was opted. Homology modeling is the most accurate method of structure prediction when a template with significant sequence similarity already exists (Hameduh et al. 2020). Swiss Model is homology modelling based web server that search for the most suitable template based on BLAST and HHblits search algorithms (Kumar and Sharma 2023). From the list of templates returned by Swiss Model, crystallographic structure of SmbP (PDB Id 3u8v.1) was selected to build model. A predicted model is qualified by its QMEANDisCo and GMQE values. The QMEANDisCo global score is an advanced version of QMEAN score with value ranging from 0 to 1, where higher values represent accuracy. Predicted model's QMEANDisCo is 0.7 while Global Model Quality Estimate (GMQE) 0.71 which represents that model is reliable. The second validation of newly designed molecule was carried out using I-TASSER. **I**-TASSER, or Iterative Threading ASSEmbly Refinement, is a bioinformatics technique that assists in the construction of a three-dimensional structure model of a protein molecule on the basis of its amino acid sequence. It principle of working is fold recognition. Proteins adopt intricate 3-Dimensional Structures, which are formed by the interactions between the amino acids in the protein chain. These types of folds mostly produce secondary structures such as alpha helices and beta sheets, which ultimately combine to form the tertiary structure of a protein. Most proteins have a specific sequence and therefore end up with similar structures known as folds. A fold is extremely important in understanding the biological functioning of the protein and thus can be inferred from the amino acid sequence. The predicted models from both softwares are virtually identical and reside in complete confidence with each other. A ribbon diagram of SmallTalk predicted model is shown in **Fig. 4.3 (A)**.

Ramachandran plot was generated using PDBSum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) is presented in **Fig. 4.3 (B).** the plot describes that 95.35% of residues are in most favored region where the acceptable range starts from 85%. Further the values of Ramachandran outliers 0.00 %, MolProbity Score 1.20, Clash score 0.00, bad bonds 0/343 and bad angles 10/460 showed the model to be a true fit (Hollingsworth and Karplus 2010a; Chen et al. 2010). As Ramachandran plot indicates the prediction of structural stereochemical properties of new molecules, the plot of SmallTalk is depicting that 95.35% of residues are falling in the favorable regions. It shows that the conformation is energetically favorable and consistent with allowed stuctures of proteins (Hollingsworth and Karplus 2010b).

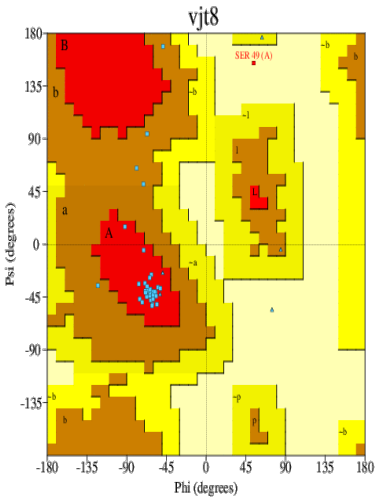
A)  B) 

Figure 4.3 (A): 3-Dimensional predicted model of SmallTalk using SmbP (3U8V) as lead structure **(B)** Ramachandran plot of predicted model of SmallTalk with 95% of residues in favorable region depicting the conformational confidence of newly developed molecule.

The MolProbity score is a single score that is adjusted to be on the same scale as X-ray resolution by combining the clashscore, rotamer, and Ramachandran evaluations. Since SmallTalk is a newly designed prottein molecule, its structure validation was carried out that include genertaion of Molprobity scores as well. The Molprobity score of 1.2 for SmallTalk indicates that generated model is of very good quality and bond angels and bonds lengths are very closer to ideal values with minimum steric clashes. C-beta deviation

value is a measure that shows displacement of C-beta atom from its ideal geomtery. Our predicted structure returned zero value of C-beta which means C-beta atoms are positioned exactly a expected provided C-beta is a key structural component of an amino acid. Physicochemical properties of SmallTalk were calculated using ProtParam (http://www.expasy.org/tools/proparameter). Solubility of SmallTalk was calculated using Protein-Sol (https://protein-sol.manchester.ac.uk/) which results that the solubility index of SmallTalk is 0.89 where the average of experimental dataset representing average soluble protein from *E. coli* is 0.45 and values above this depicts higher solubility. This value makes SmallTalk a solubility enhancing molecule since it is completely alpha helical structure. Alpha helices are solubility enhancing structure in three-dimensional structure of proteins. The use of SmallTalk as a fusion protein tag indicates that it would be attached to a protein of interest under study, hence it would be adding additional alpha helices to the final structure of chimeric protein thereby imparting a positive impact on solubility of target protein. The *pI* of SmallTalk also gives insight towards the hydrophilic nature of the tag and thus coincides with estimated solubility index values (Table 4.1 & 4.2).

Table 4.1: MolProbity Results *using MolProbity version 4.4*

MolProbity Score	1.20
Clash Score	0.00
Ramachandran Favoured	95.35%
Ramachandran Outliers	0.00%
Rotamer Outliers (A8 HIS)	3.12%
C-Beta Deviations	0
Bad Bonds	0 / 343
Bad Angels	10 / 460

Table 4.2: Physicochemical parameters calculated using Protaparam

Amino acid sequence	SEAGGNTHVGHGIKHLEDAIKHGEEGHVGHAT KHAQEAIEHLRASEHKSH
Number of amino acids	50
Molecular weight	5372.78 da
Theoretical pI	6.33
Aliphatic index:	62.60
Grand average of hydropathicity (GRAVY)	-1.068
The instability index (II)	31.01 (This classifies the protein as stable)

4.2.2-In-silico Modelling of Chimeric proteins tagged with new SmallTalk:

I. SmallTalk-Bin1b:

The 3D structure of ST-Bin1b was also generated using I-TASSER software that works using ab-Initio modelling methods. The generated structure of SmallTalk-Bin1b is shown in **Figure 4.4 (A)**. Differential color coding has been implied to ditinctly describing the component sof chimeric protein. The alpha helices of SmallTalk are shown in purple color and the cyan colored structure with two embedded beeta sheets shown in red color are corresponding structure to Bin1b. As the structure of Bin1b represents it comprises of sulfur bridges which have an impact on the expression and purification strategies in the wet lab experiments. These insights from the In-silico analysis make it essential to be carried out before

running the experiment so that the expression and purification strategies must be selected accordingly. Ramachandran plot of the predicted model of ST-Bin1b is shown in **Figure 4.4 (B)** which shows the clustering of blue dots representing amino acid residues of molecule in the favorable region (brown and red colored area) of graph. Ramachandran plot of predicted model of SmallTalk-Bin1b generated using PDBSum is shown in **Figure 4.2 (B)**. The plot results showed that 88% residues fall in favorable regions (60% most favorable, 24% additional and 10% generously allowed regions) while only 10% fall in disallowed regions. The high percentage of disallowed residues and residues in additional and generously favorable regions a compared to most favorable regions can be explained by the presence of 12 glycine residues in a 100 amino acid structure. As the presence of high number of glycine increase the conformational freedom and overall flexibility of protein structure.

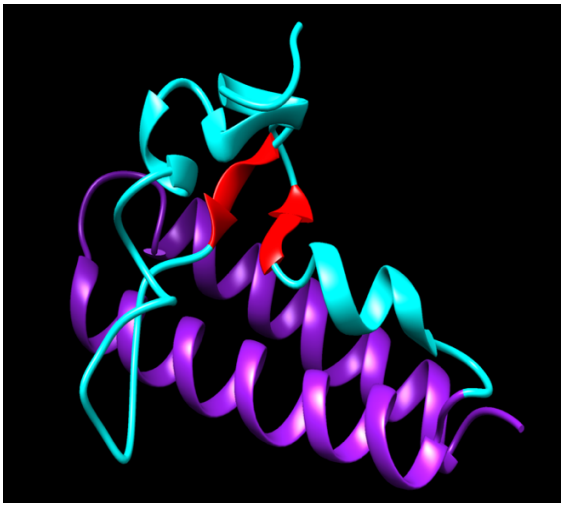
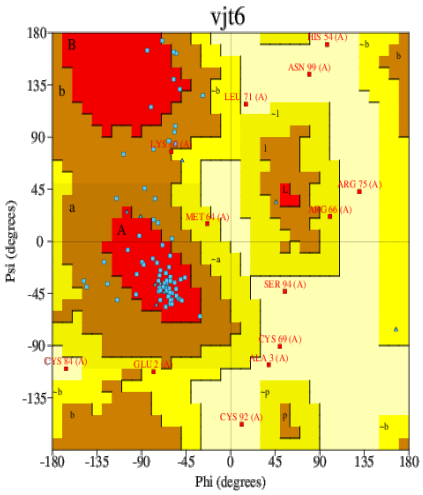
A)  B) 

Figure 4.4 (A): 3-Dimensional predicted model of SmallTalk-Bin1b using I-TASSER. Alpha helices of Smalltalk are depicted in purple color, Cyan colored structure with embedded red colored beta sheets is representing Bin1b **(B)** Ramachandran plot of predicted model of SmallTalk-Bin1b generated using homology independent and iterative threading-based modelling.

II. SmallTalk-MP1106:

The C-score is a confidence score that is used by I-TASSER to estimate the quality of predicted models. It is computed using the convergence parameters of the structure assembly simulations and the importance of threading template alignments. Typically, the C-score falls between [-5,2], where a larger value denotes a model with a higher level of confidence and vice versa. The beta sheets are represented by blue colored arrows hanging in between magenta colored alpha-helix. Hence, as per the ribbon diagram representation in **Figure 4.5 (A)**, the SmallTalk alpha helices depicted in purple-colored ribbons are adding more helical content to the overall molecular structure that is designed to be produced in the experimental laboratory. The Ramachandran plot shown in **Figure 4.5 (B)** is representing that the amino acid residues depicted by blue dots are accumulated in the favored regions (Red and Brown area) of the chart.

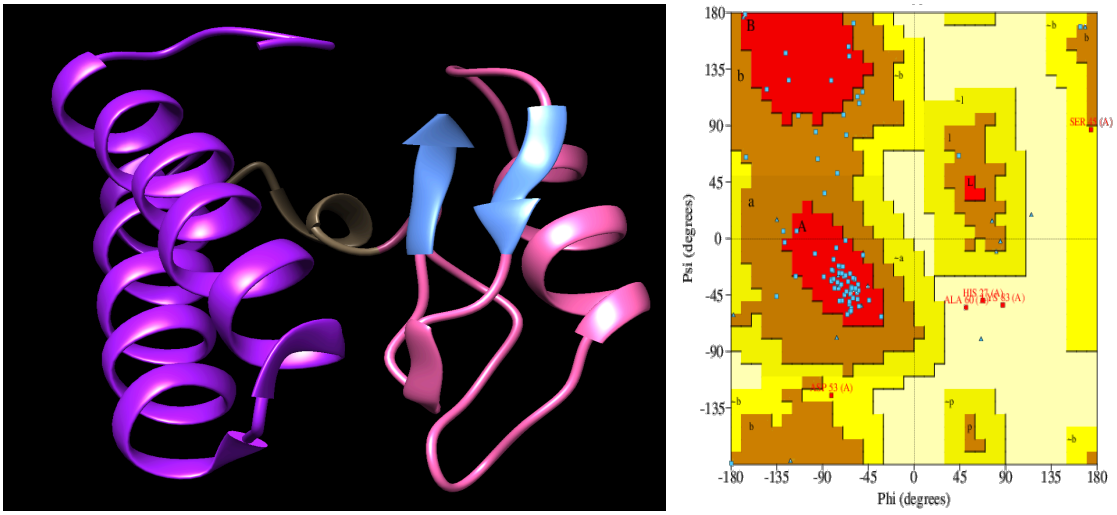


Figure 4.5 (A): 3-Dimensional predicted model of SmallTalk-MP1106 using I-TASSER. Alpha helices of Smalltalk are depicted in purple color, Magenta colored structure with embedded, blue-colored beta sheets is representing MP1106 **(B)** Ramachandran plot of predicted model of SmallTalk-MP1106 generated using homology independent and iterative threading-based modelling.

III. SmallTalk-Enterocin DD14 A&B

The Enterocin DD14 belongs to a class of bacteriocins known as two peptide bacteriocins that is made up of two different peptide chains, or subunits. In the case of Enterocin DD14, these chains may work synergistically to exert their antimicrobial properties, and they have distinct amino acid sequences. The predicted 3-Dimensional structure of both chains was designed using ab initio modeling, th same used for earlier constructs. The ribbon representation illustrates, both the chains majorly comprise of alpha helices. This indicates towards a smoother and easier expression BL21 strain. It is especially favored for use with T7 promoter-based systems regulation and high yields as compared to SHuffle® T7. In the natural host, this two-peptide is expressed by the genes present on same operon and the products act synergistically against the invading bacteria, as host's natural defense mechanism. In our study, we cloned the two genes separately on isolated pET-30a (+) and the products were than later used in fixed proportions for the MIC determination. The ribbon structures are shown in **Figure 4.6 (A**) and **Figure 4.7 (A)**, where purple-colored ribbons are depiction of SmallTalk and the orange ribbons in **Figure 4.6 (A)** are representation of DD14 A. The enterokinase cleavage site (DDDDK) in this case has intrinsically assimilated in the helical structure of DD14. Hence, it presents a solid case for the testing the functionality chimeric protein, despite the cleavage. Ramachandran plots for the predicted models of DD14 A & B are shown in **Figures 4.6 (B)** and **4.7 (B).** The occurrence of most amino acid residues falls in the favorable regions (red and brown area) labelled by blue dots. The plot shows that the models are indicating a confident molecule workable *in-vivo.*

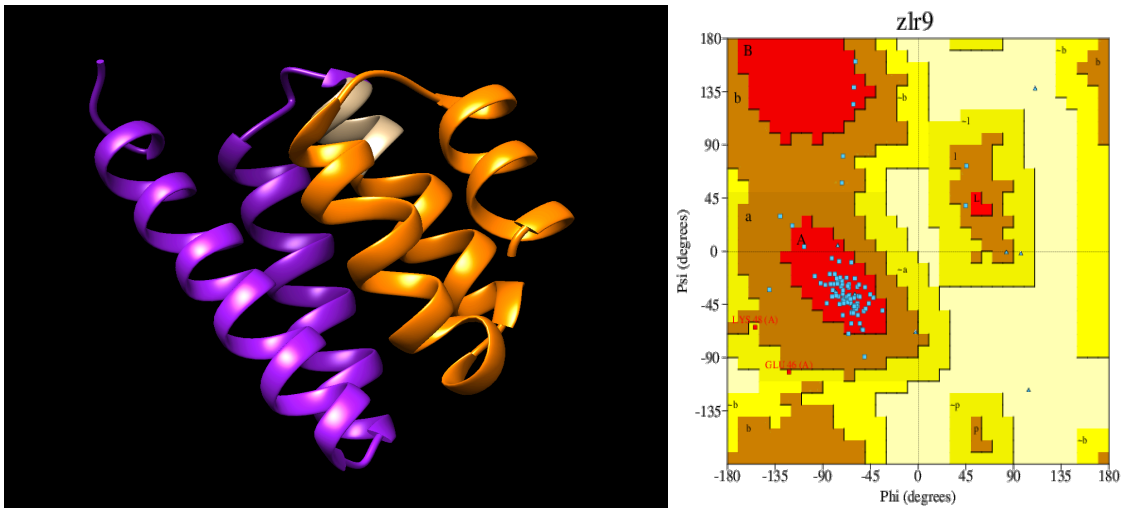


Figure 4.6 (A): 3-Dimensional predicted model of SmallTalk-Enterocin DD14A using i-Tasser. Alpha helices of Smalltalk are depicted in purple color, Orange colored helices are representation of Enterocin DD14A **(B)** Ramachandran plot of predicted model of SmallTalk-Enterocin DD14A generated using homology independent and iterative threading-based modelling

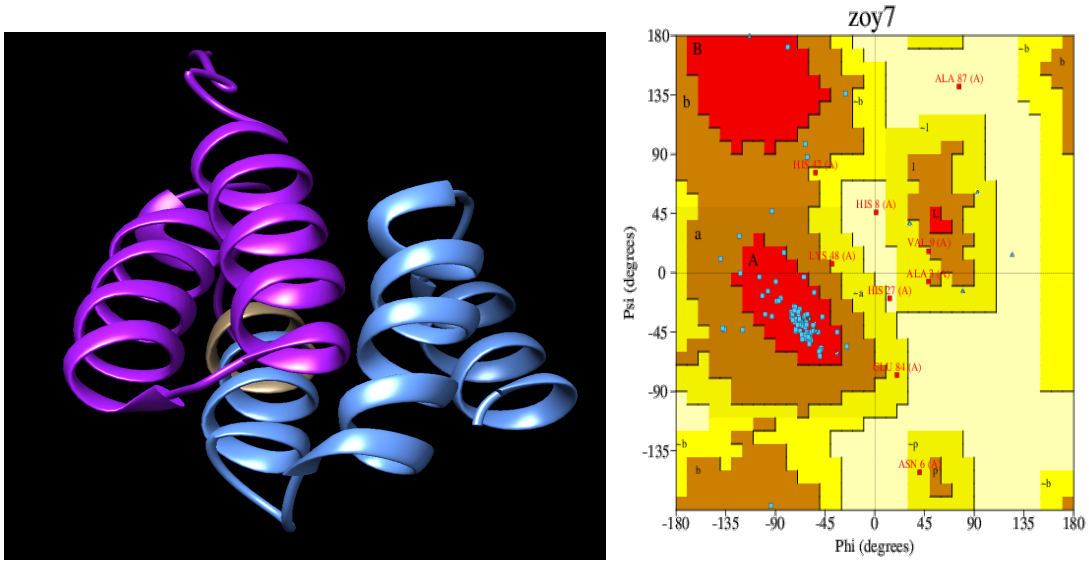


Figure 4.7 (A): 3-Dimensional predicted model of SmallTalk-Enterocin DD14B. Alpha helices of Smalltalk are depicted in purple color; Blue-colored helices are representation of Enterocin DD14B **(B)** Ramachandran plot of predicted model of SmallTalk-Enterocin DD14B

Section III- Empirical Research Outcomes; The Transcription Realm

4.3-Design and Construction of Recombinant plasmids

4.3.1-PCR Amplification of SmallTalk from SmbP and visualization amplified DNA molecule in Agarose gel under UV

Forward and reverse primers were designed targeting the 3rd and 4th alpha helices of SmbP. The specific nucleotide sequence was selected in UCSF Chimera (https://www.cgl.ucsf.edu/chimera/) using the Match-Align tool provided in the analysis section. The selection was cross referenced using the Sequence-to-Structure validation option. Once the nucleotide sequence was selected, primers were designed from the starting and ending length of selected nucleotide sequence. The melting temperature for both primers was set at closest possible, and the GC content was assured in ideal range (i.e. 50-60%). Primer length was also ascertained to be in the ideal window (18 to 24 base pairs). The designed primers for the amplification of SmallTalk encoding DNA molecule are given in **Table 4.3**.

Table 4.3 Synthesized Primers sequence with key parameters

Name	Primer Sequence	Length	T_m	GC%
Forward Primer	T*GAGTCACT***CATATG**TCTGAAGCGGG TGGAAACACC	21 bps	61 ℃	57%
Reverse Primer	*ATGATGATGC***GGTACC**ATGGCTTTTGT GCTCG	22 bps	61 ℃	55%

***Bold letters are restriction site, italicized are leader sequence**

The design of first construction with the expression cassette shown in Figure 4.3 (A), was achieved by cloning amplified SmallTalk into a parent plasmid pET30a (+) containing SmbP-GFP expression cassette. While amplifying the DNA molecule of SmallTalk, *NdeI* and *KpnI* restriction sites were introduced at both flanks through PCR primers. Same restriction sites were present around SmbP in SmbP-GFP-pET30a (+) backbone which was replaced by SmallTalk using regular restriction and ligation reaction. The enterokinase cleavage site (DDDDK) is present at the N-terminal of GFP to allow molecular cleavage of SmallTalk to release tag free GFP. The final construction as depicted in Figure 4.9 A was obtained. The amplified SmallTalk containing 158 base pairs is shown in Figure 4.8 A. This band is restricted using the inserted restriction sites hence the purified DNA fragment will have sticky ends. The backbone of SmbP-GFP-pET30a (+) was also treated with same restriction site and the larger band (the DNA molecule -GFP-pET30a (+)) was purified and contains same sticky ends, band is shown in Figure 4.8 B.

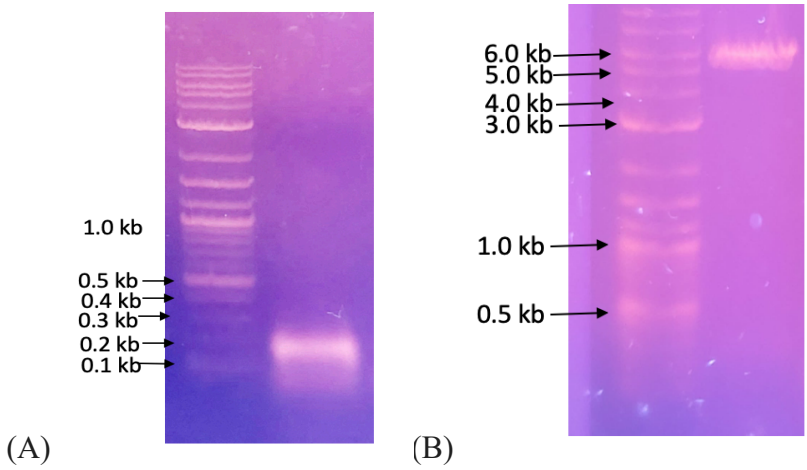


Figure 4.8 Agarose gel analysis of restriction enzyme digestion of plasmid DNA. (A) 1.5% Agarose gel analysis of amplified SmallTalk: Lane1; DNA ladder, Lane 2: Amplified SmallTalk (158 bps). (B) 1% Agarose gel analysis of restriction digestion: Lane1; DNA ladder, Lane2; pET30a_SmbP_GFP cut

4.3.2-In-silico modeling of molecular cloning and the design of recombinant plasmids.

I. pET30 a (+)-SmallTalk-GFP

An *In-silico* modeling of the molecular cloning reaction to be performed under wet laboratory conditions were ascertained using SnapGene (www.SnapGene.com), an advance online molecular biology bioinformatics tool to evaluate the efficiency and reliability of the wet laboratory molecular ligation reaction. The schematic flowchart (Figure 4.9) below shows the *In-silico* modeling of two recombinant plasmid expression vectors.

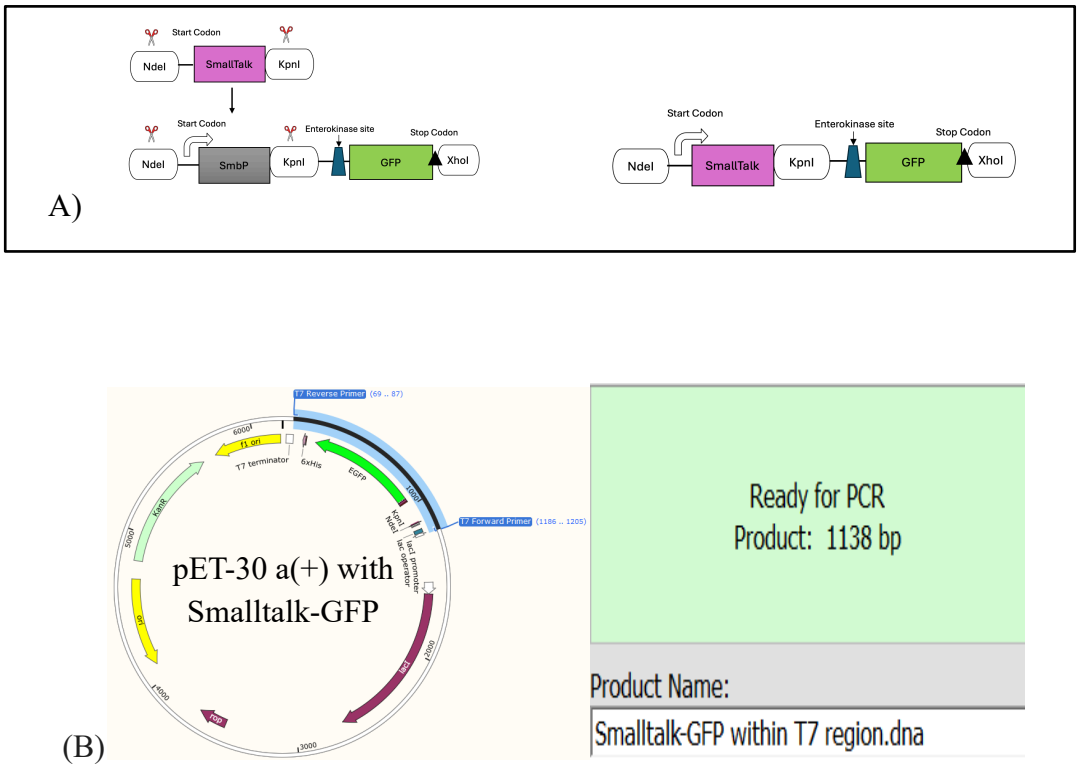


Figure 4.9 Schematic representation of *in silico* modulation of molecular cloning in SnapGene. (A) Graphical representation of designing of new molecule SmallTalk-GFP from existing SmbP-GFP. (B) *In-silico* modeling of recombinant plasmid construct pET30a_CusF3H+_LL-37_Renalexin. (B) *In-silico* PCR simulation of T7 region of recombinant plasmid construct pET30a_SmallTalk_GFP

II. pET30 a (+)-SmallTalk-Bin1b

For the SmallTalk-Bin1b construct, as per the design shown in **Figure 4.10 (A),** the coding DNA sequence was chemically synthesized by GenScript. The amino acid sequence was downloaded from Antimicrobial Peptide Database (https://aps.unmc.edu/) under the accession number AP01592 and was optimized for codon usage preference in *E. coli*. The chemically synthesized coding DNA sequence was flanked by *NdeI* and *XhoI* and was cloned into pET30a (+) after treating both synthetic gene fragment of SmallTalk-Bin1b and pET30a (+) plasmid DNA molecule. Restricted digests were ligated using T4 DNA ligase producing the desired construction shown in **Figure 4.10 B**. The ligation mixture was propagated in DH5α and were confirmed through amplification of T7 region using T7 promoter and T7 terminator primers in PCR reaction. The size of band visualized in agarose gel (**Figure 4.10 C**) corresponds to the size of 0.48 kb as theoretically ascertained by *In-silico* PCR using SnapGene. Second screening of positive clones was done by sanger sequencing.

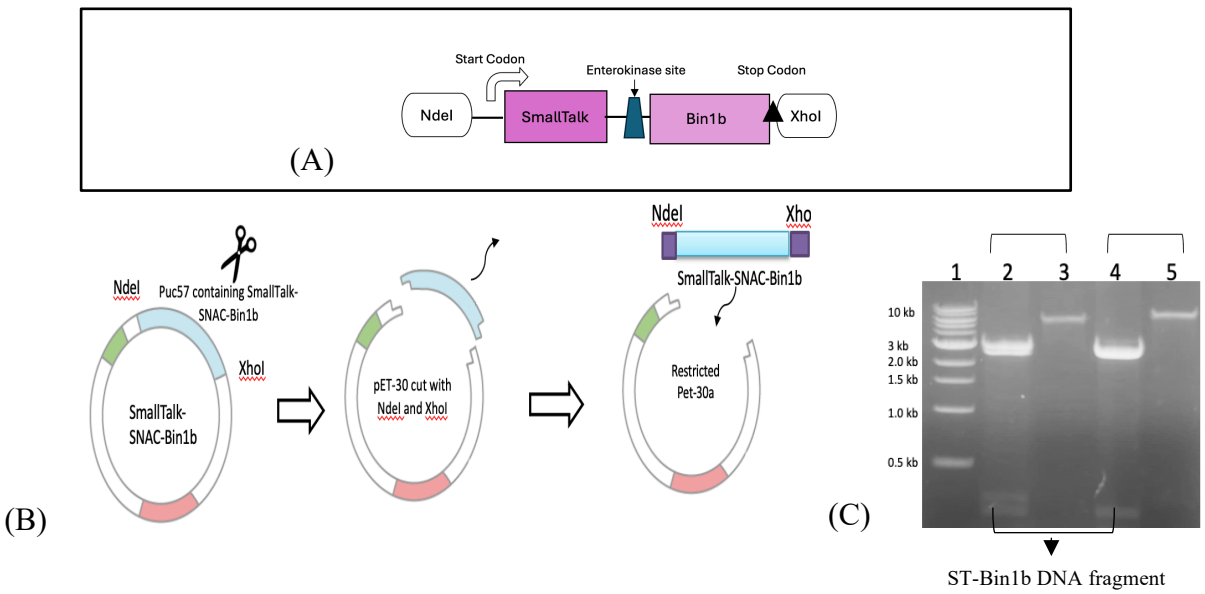
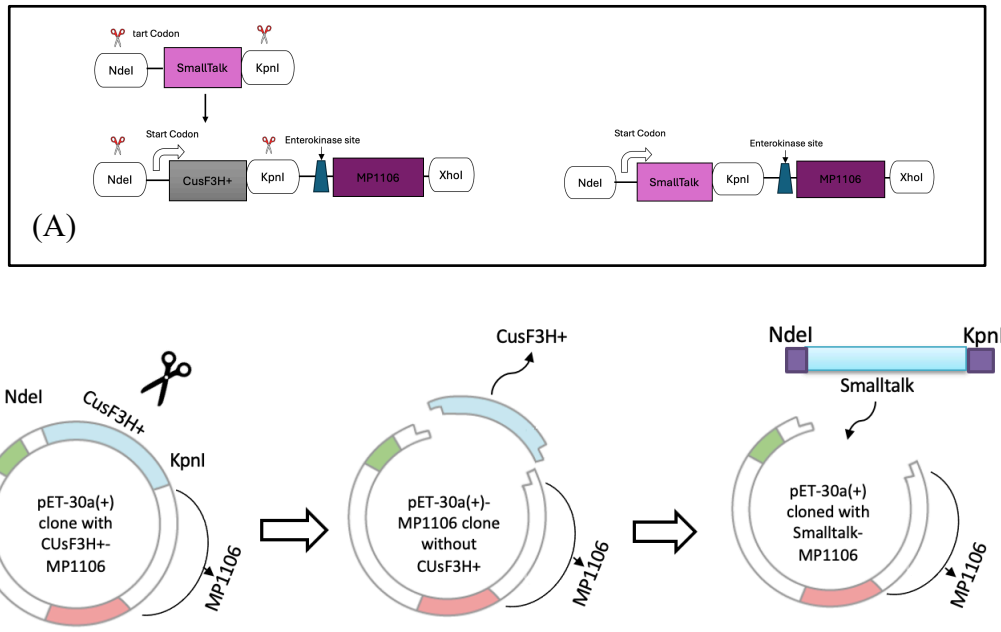


Figure 4.10 (A) Gene map of expression cassette for SmallTalk-Bin1b **(B)** Schematic representation of cloning of SmallTalk-Bin1b from pUC57 into pET-30 a (+) through restriction digestion **(C)** Restricted pUC57 and Bin1b fragment; Lane 1 DNA Marker, Lane

2 & 4 freed pUC57 and SmallTalk_Bin1b fragment (318 bps); Lane 3 pET30 a (+) restricted plasmid (5.4 kb),

III. pET30 a (+)-SmallTalk-MP1106

MP1106 was previously studies with conjugation of cUSf3H+ in PEP laboratory, so this construction was used as a parent molecule. This engineering was done in a manner like the synthesis of first molecule of SmallTalk-GFP. So, the cUSF3H+ fusion tag in the parent molecule is flanked by restriction sites, NdeI at the start and KpnI at the end of cUSF3H+. The same restriction sites have been incorporated in the primers of SmallTalk amplification so that the amplified SmallTalk would be possessing these very restriction sites on both ends. After treating pET-30 a (+)-cUSF3H+-MP1106 and amplified SmallTalk with *KpnI* and *NdeI*, the pET30a (+)-MP1106 backbone was ligated with amplified SmallTalk. The diagrammatic representation of phenomenon is shown in Figure 4.11. The occurrence of chemical reaction at nucleotide sequence level is represented through initial and final expression cassettes of intended molecules **Figure 4.11 (A & B).**



(B)

Figure 4.11 (A) Gene map of expression cassette for SmallTalk-Bin1b **(B)** Schematic representation of cloning of SmallTalk with MP1106 containing pTE30 a (+) backbone

IV. pET30 a (+)-SmallTalk-Enterocin DD14

Sequentially, Enterocin DD14 constructs were designed towards the end of the project when the previous constructions have shown positive results. So, the constructions were directly designed in pET-30 A (+); the expression vector. This direct design and synthesis lead to a fast experimental design aiming directly towards the expression, purification and functional studies surpassing the molecular cloning experiments. The expression cassette is represented in the **Figure 4.12,** the amino acid sequence of which was directly sent to GenScript to be cloned in pET-30 a (+)**.**

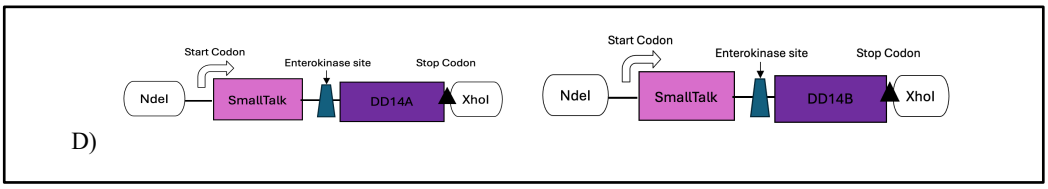


Figure 4.12 Gene map of expression cassette for SmallTalk-Enterocin DD14 A & B.

4.4.3-Clone confirmation

a. Using T7 region amplification by PCR

Since the three clones, pET30 a (+)-SmallTalk-GFP, pET30 a (+)-SmallTalk-Bin1b and pET30 a (+)-SmallTalk-MP1106 were synthesized by cloning in PEP Laboratory, the next step is to confirm the synthesized clones by T7 region amplification by PCR. The procedure for confirming a clone by the amplification of the T7 region involves the use of PCR to generate the sequence between the T7 promoter and terminator, thereby confirming that the target gene cassette is successfully inserted into the intended vector, pET-30 a (+) in this case. The primers are available on AdGene website (https://www.addgene.org/mol-bio-reference/sequencing-primers/) and given in the methodology section were used to anneal the bordering regions. The resultant PCR amplicon is subjected to agarose gel analysis and visualization under UV. The size of the product provides verification of the insertion and orientation of the target gene into the required

location. In evaluating the presence of the target gene that encodes for recombinant fusion proteins SmallTalk-GFP, SmallTlak-Bin1b and SmallTalk-MP1106 independently in the plasmid constructs designed via molecular ligation, a 20 µl conventional polymerase chain reaction (PCR) was employed using T7 promoter and T7 terminator-specific primers pairs (provided in Methods section). These primer pairs anneal strongly to their target sites on pET-30 a (+), within which the synthetic DNA insert was cloned at the multiple cloning sites; and under the influence T7 promoter. Figure 4.13 depicts the PCR conditions that favor the amplification reaction. PCR amplicons were analyzed on a 1% agarose gel stained with Ethidium bromide (1 µg/ml) and visualized on a UV trans-illuminator (Figure 4.13). (This confirmation and the following (Sanger Sequencing) was not carried out for Enterocin DD14 since they were directly cloned in pET-30 a (+) by GenScript, so they were directly taken for expression.)

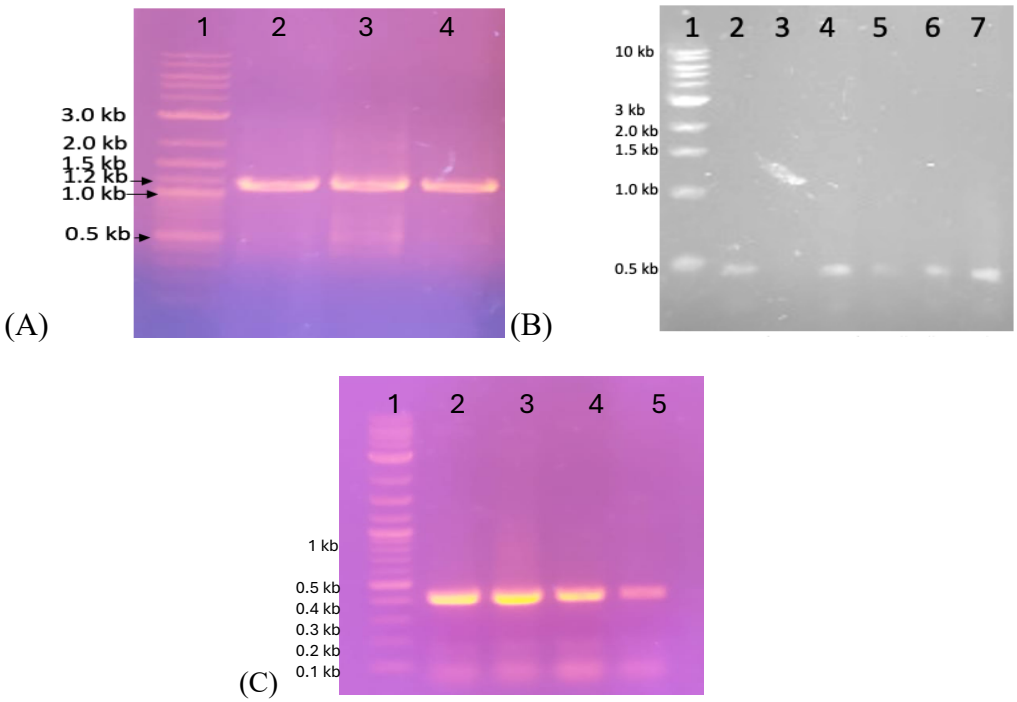


Figure 4.13 PCR analysis of DNA insert in the designed recombinant plasmid constructs. (A) 1% Agarose gel analysis of SmallTalk GFP DNA amplicons (estimated size calculated form In-silico PCR is 1138 bps): Lane 1; DNA ladder, Lane 2, 3 & 4; PCR amplicons. (B) 1% Agarose gel analysis of SmallTalk-Bin1b DNA amplicons: Lane 1; DNA ladder, Lane:

2,4,5,6 & 7; PCR amplicons (estimated DNA size 410 bps). (C) 1% Agarose gel analysis of SmallTalk-MP1106 DNA amplicons: Lane 1; DNA ladder, Lane: 2,3,4 & 5; PCR amplicons (estimated DNA size 413 bps).

b. Verification of Clone Integrity Through Sanger Sequencing and Theoretical Sequence Alignment using EMBOSS Needle

The plasmid DNA molecules after confirmation through T7 region amplification, clones were sent for Sanger Sequencing with added T7 forward primer in the sequencing reaction tube. The resulting sequence chromatogram and DNA nucleotides generated from the Sanger sequence analyzer were viewed, analyzed, and evaluated using the European Molecular Biology Laboratory online bioinformatics tool (www.ebi.ac.uk/tools/sssEMBOSS.com) for sequence alignment between the synthetic DNA nucleotide sequences (amplified in lab) and the designed plasmid PCR amplicon sequence (Designed *In-silico*). The Finch TV software (www.finchTV.com) tool was used to display sequence chromatogram which shows distinctively clear chromatograms with nicely sharped peaks suggesting a good plasmid constructs purity. Pairwise DNA sequence alignment analysis (Figure 4.14) was performed using the Needleman−Wunsch algorithm in EMBOSS to assess and evaluate the degree of similarity and identity between the designed plasmid DNA insert and the synthetic DNA construct (Oladipo *et al*., 2023).

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#=======================================

EMBOSS_001       54 TCTGAAGCGGGTGGAAACACCCACGTTGGTCACGGCATTAAGCATCTCGA    103
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        1 TCTGAAGCGGGTGGAAACACCCACGTTGGTCACGGCATTAAGCATCTCGA     50

EMBOSS_001      104 GGATGCAATCAAGCACGGCGAAGAGGGTCATGTTGGCGTTGCTACCAAGC    153
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       51 GGATGCAATCAAGCACGGCGAAGAGGGTCATGTTGGCGTTGCTACCAAGC    100

EMBOSS_001      154 ACGCGCAGGAGGCCATTGAACATTTGCGCGCATCCGAGCACAAAAGCCAT    203
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      101 ACGCGCAGGAGGCCATTGAACATTTGCGCGCATCCGAGCACAAAAGCCAT    150

EMBOSS_001      204 GGTACC    209
                    ||||||
EMBOSS_001      151 GGTACC    156


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# Length: 156
# Identity:     156/156 (100.0%)
# Similarity:   156/156 (100.0%)
# Gaps:           0/156 ( 0.0%)
# Score: 780.0
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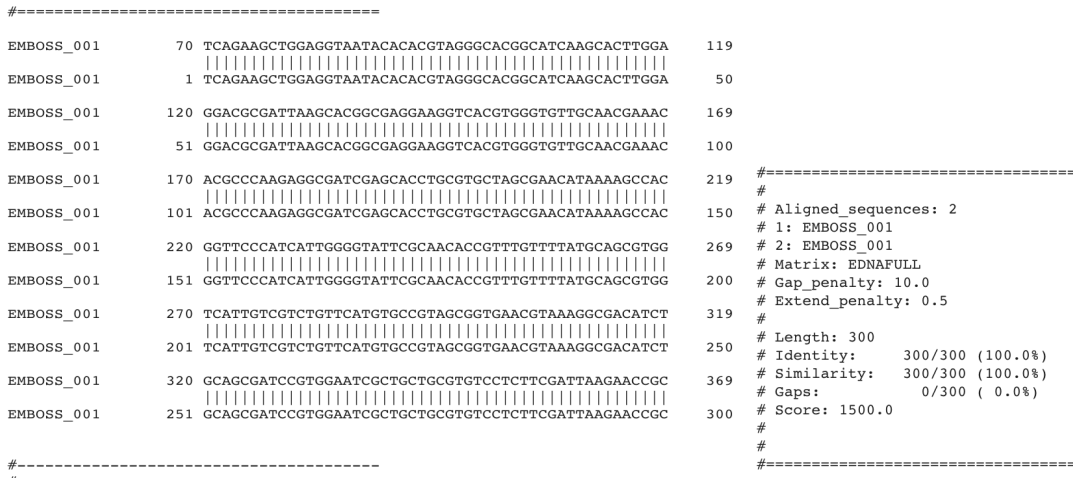
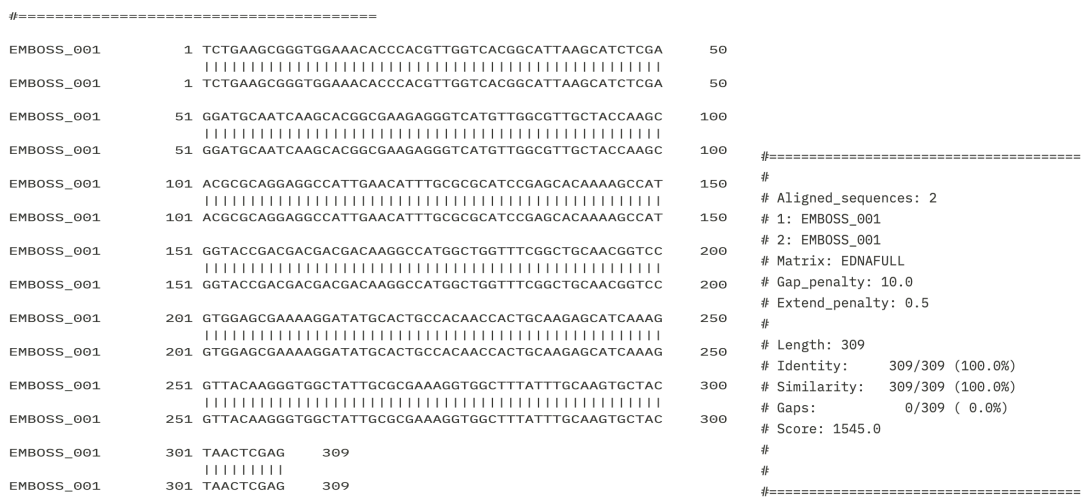
(A)



(B)



(C)

Figure 4.14 DNA nucleotide sequence alignment using the Needleman−Wunsch algorithm for pairwise analysis between the synthetic DNA sequence resultant from Sanger Sequencing and designed plasmid DNA insert (A) Nucleotide Sequence Alignment for ST-GFP DNA amplicon with theoretically designed DNA molecule. (B) Nucleotide Sequence Alignment for ST-Bin1b DNA amplicon with theoretically designed DNA molecule (C) Nucleotide Sequence Alignment for ST-MP1106 DNA amplicon with theoretically designed DNA molecule.

Section IV-Empirical Research Outcomes; The Translation Realm

4.4 Small Scale Expression of Recombinant Proteins Tagged with Newly Designed SmallTalk

4.4.1- A comparison of Heterologous Expression of SmbP-GFP and SmallTalk-GFP

The *E. coli* strain Bl21 (DE3) was selected for the recombinant production of SmallTalk-GFP due to its engineered capacity of being protease deficient and producing its own T7 polymerase under the control of lac promoter, a combination that guarantees higher level of expression. Earlier the use of SmbP as carrier protein has shown significant advantages in recombinant expression and purification of fusion protein in soluble form including GFP (Vargas-Cortez et al. 2017; 2016c; Santos et al. 2019b) and Bin1b (Montfort-Gardeazabal, Balderas-Renteria, et al. 2021c). SmallTalk being a variant of SmbP is expected to give the same results in terms of soluble expression and efficient purification using IMAC. To conduct a comparative analysis of expression levels of SmbP-GFP (previously constructed clone in PEP laboratory) and SmallTalk-GFP (constructed during this study), both constructs were expressed at different predefined sets of growth parameters. Cell pellets collected from 2 ml of IPTG-induced overnight grown cell culture was lysed to obtain clear cell lysate with soluble recombinant protein.

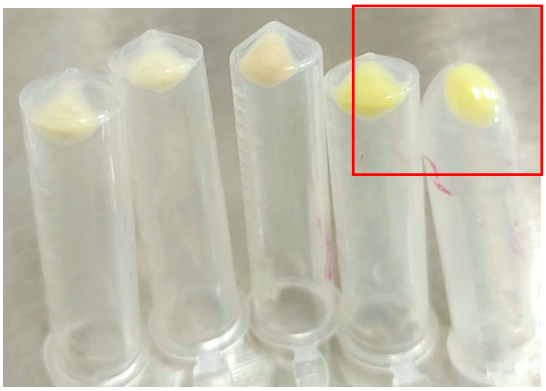


Figure 4.15: Green color of GFP is visible in small scale expression pellet collected from 2 ml of expressed culture media

A 12% SDS-PAGE (Figure 4.16) was run to visualize the expression of Green Fluorescent Protein tagged with SmallTalk where 5 µl aliquots of cell lysate were charged in the successive wells along with the negative control (carrying plasmid without gene of interest). The gel image in figure 5 shows profound evidence of expression of SmallTalk-GFP at comparable levels to the expression of SmbP-GFP. Lanes 2,4 and 6 are representing the expression of SmbP-GFP while lane 3, 5 and 7 are showcasing the aliquot of SmallTalk-GFP and lane 8 being the negative control. Results suggests that the conditions of 25 °C and 16 hours are the best optimization for the recombinant expression tagged with SmallTalk. Same conditions were applied for the pilot scale expression of SmallTalk-GFP.

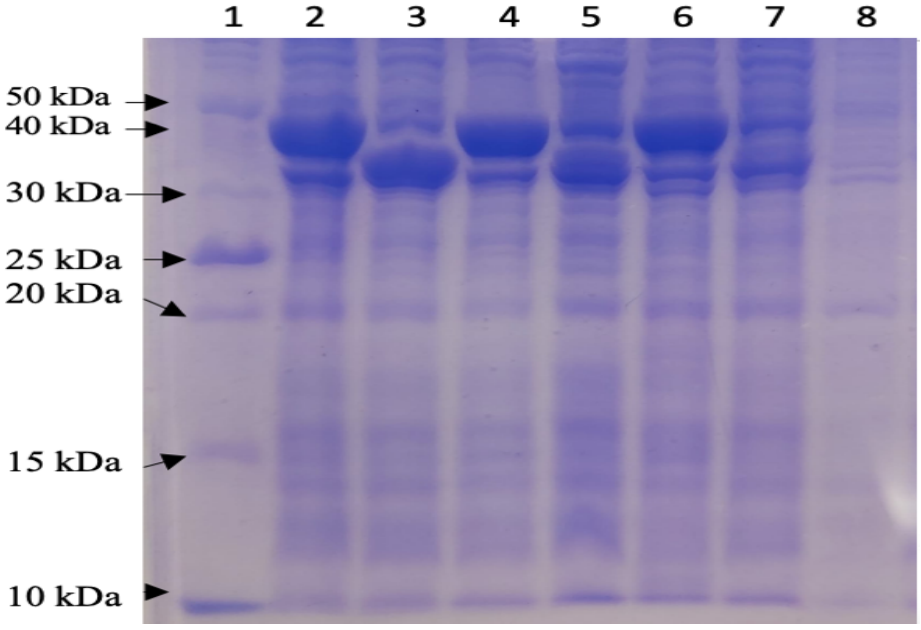


Figure 4.16: Small scale expression of fusion proteins SmallTalk-GFP and SmbP-GFP expressed in *E. coli* Bl21 (DE3) run on 12 % SDS-PAGE. Lane 1: Protein Marker, Lane 2: SmbP-GFP (25 °C 16 h), Lane 3: ST-GFP (25°C, 16 h), lane 4: SmbP-GFP (37 °C, 16 h), lane 5: ST-GFP (37 °C, 16 h) lane 6: SmbP-GFP (37 °C, 4 h) lane 7: ST-GFP (37 °C, 4 h), Lane 8: Negative control (Bl21 untransformed cells)

4.4.2- Small Scale expression of ST-Bin1b

For the expression of Bin1b, SHuffle® T7 strain of *E. coli* was selected since the *In-silico* results has sown that bin1b possess sulfur bridges and Bl21 strain show difficult expression with sulfur bridges containing proteins. The small-scale expression of SmallTalk-Bin1b is shown in **Figure 4.17** represents an analysis of 5 µl of clear cell lysate prepared form 2 ml of expressed cell culture under varying sets growth parameters (time and temperature) and at constant rotation of 220 rpm. Successful evidence of recombinant protein expression in soluble form at all the growth parameters can be observed, except only at 37 °C for 4 hrs. which can be attributed to inclusion bodies formation due to higher temperature and lesser time (**Figure 4.17**). Remarkably, efficient expression was attained at 25 °C for 4 hrs., significantly reducing the time for consequent experiments as well strongly suggesting a faster set of growth parameters for antimicrobial peptide expression tagged with SmallTalk.

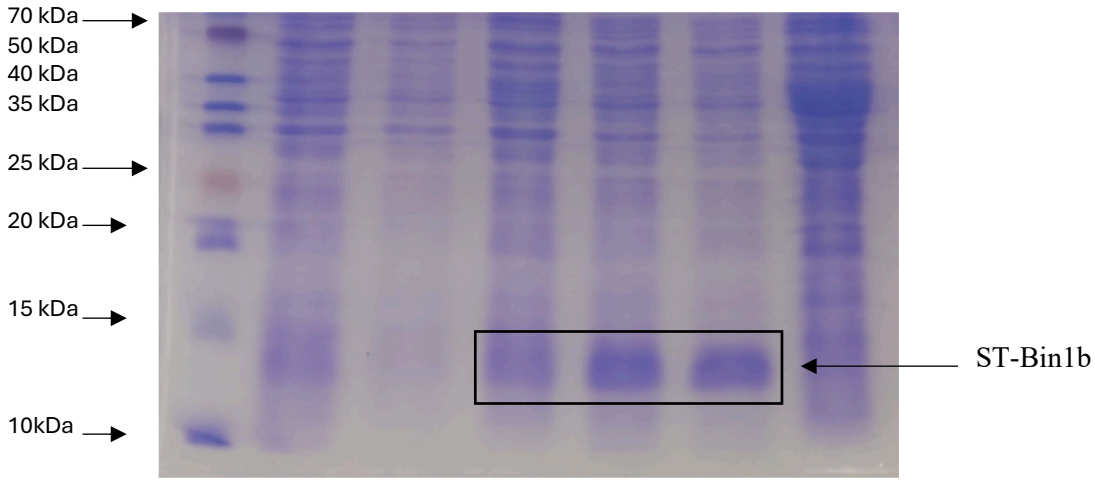


Figure 4.17: Expression of SmallTalk-Bin1b in different conditions using *E. coli* Shuffle, Lane 1 Size Marker, Lane 2: uninduced Shuffle T7 No induction 37 °C 16 h, lane 3: SmallTalk-Bin1b 37 °C, 4 h, lane 4: 37 °C 16h, lane 5: 25 °C 4h, lane 6: 25 °C 16h, lane 7: Shuffle T7 blank (without plasmid)

4.4.3- Small Scale expression of ST-MP1106 & ST-Enterocin DD14 A and B

In evaluating the expression of the chimeric peptides ST-MP1016 in *E. coli lemo21* (DE3) (**Figure 4.18**) and ST-Enterocin DD14 A and B (17 kDa) in *E. coli* BL21(DE3) (**Figure 4.19 A & B**), soluble cell lysates were prepared by lysing cell pellets collected from 2 ml overnight cultures in a 150 µl lysing buffer (50 mM Tris-HCl pH 8.0). 5µl of each soluble lysate was treated with 6X sample buffer (final concentration of 1X) and analyzed on a 15% Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE). To access the cytoplasmic expression of the target fusion proteins in *E. coli lemo* (DE3) and *E. coli* BL21(DE3) transformed cells, both soluble and insoluble cell lysate fractions were made and analyzed. Two *E. coli* BL21(DE3) and SHuffle T7(DE3) control samples were made, transformed cells without IPTG induction and untransformed cells.

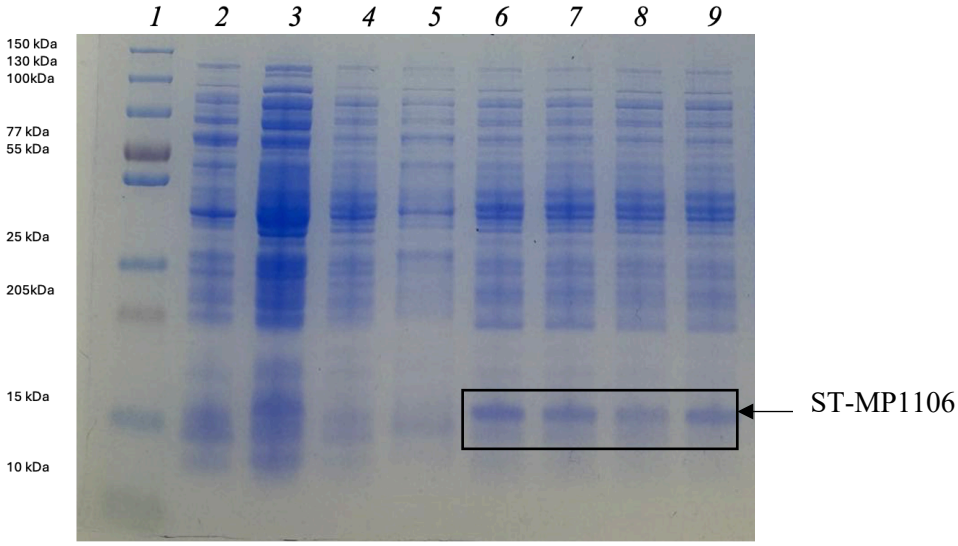


Figure 4.18 Small scale expression of fusion proteins SmallTalk-MP1106 expressed in *E. coli* lemo (DE3), Analysis of soluble fractions run on 12 % SDS-PAGE. Lane 1: Protein Marker, Lane 2, Positive Control i.e. ST-Bin1b Lane 3, 4: Negative controls (Untransformed Bl21 Cells expressed at 25 °C for 16 h) Lane 5 : Soluble fraction at conditions 25 °C for 4 h, Lane 6: ST-MP1106 Soluble fraction at conditions 25 °C for 16

h, lane 7: ST-MP1106 Soluble fraction at conditions 37 °C for 4 h, lane 9 : ST-MP1106 Soluble and Insoluble fractions at 37 °C for 4 h.

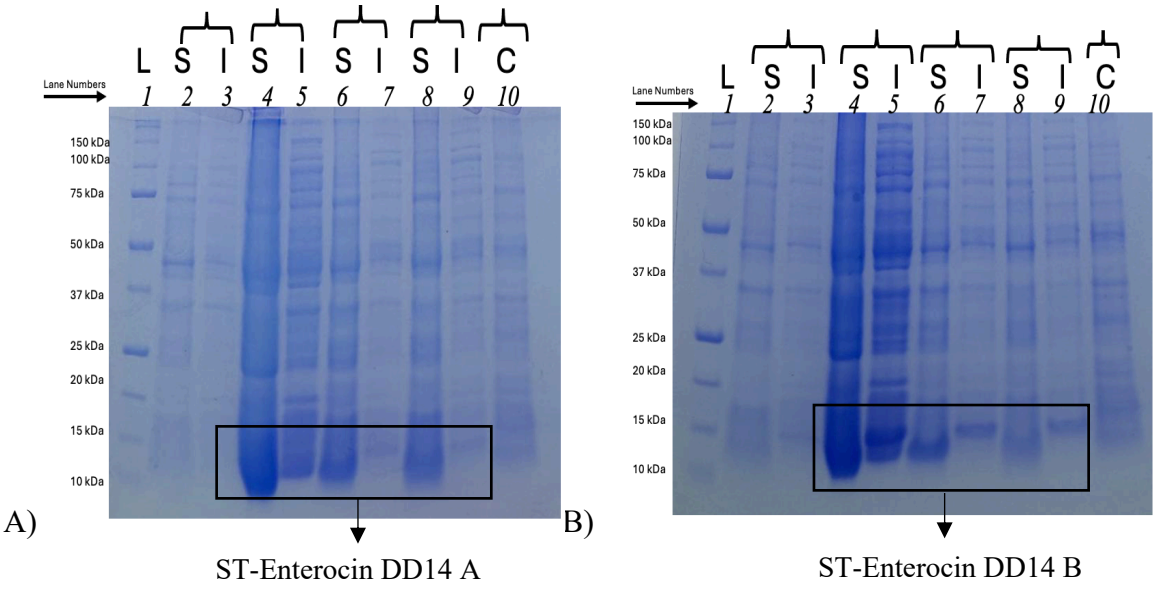


Fig 4.19 (A) Small scale expression of fusion proteins SmallTalk-Enterocin DD14A expressed in *E. coli* Bl21 (DE3), Analysis of soluble and insoluble fractions run on 12 % SDS-PAGE. Lane 1: Protein Marker, Lane 2 & 3: ST-DD14A Soluble fraction and Insoluble fraction at conditions 25 °C for 16 h, Lane 4 & 5: ST-DD14A Soluble and Insoluble Fraction at conditions 25°C for 16 h, lane 6 & 7: ST-DD14A Soluble and Insoluble fractions at conditions 37 °C for 16 h, lane 8 & 9 : ST-DD14A Soluble and Insoluble fractions at 37 °C for 4 h lane 10: Untransformed Bl21 Cells expressed at 25 °C for 16 h

Fig 4.19 (B) Small scale expression of fusion proteins SmallTalk-Enterocin DD14B expressed in *E. coli* Bl21 (DE3), Analysis of soluble and insoluble fractions run on 12 % SDS-PAGE. Lane 1: Protein Marker, Lane 2 & 3: ST-DD14B Soluble fraction and Insoluble fraction at conditions 25 °C for 16 h, Lane 4 & 5: ST-DD14B Soluble and Insoluble Fraction at conditions 25°C for 16 h, lane 6 & 7: ST-DD14B Soluble and Insoluble fractions at conditions 37 °C for 16 h, lane 8 & 9 : ST-DD14B Soluble and Insoluble fractions at 37 °C for 4 h lane 10: Untransformed Bl21 Cells expressed at 25 °C for 16 h.

Section V: Functional Analysis of Chimeric Protein Molecules

4.5.1-Large Scale Expression and purification of ST-GFP

BL21 DE3 strain were grown in a volume of 1 Liter under above mentioned conditions and cells were harvested in refrigerated centrifuge. Cells were lysed in cold room by mechanical vortexing method using 0.1 mm glass beads. Cell lysate was collected by centrifugation at 4 °C, the green color of GFP was evidently visible in the lysate.

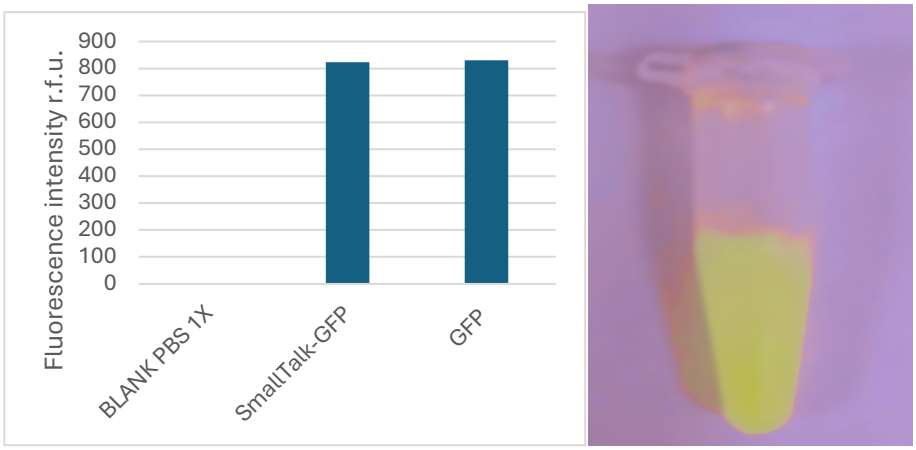


Figure 4.20 (A) Fluorescence spectrum analysis of SmallTalk-GFP, GFP and PBS Buffer as blank. **(B)** Purified GFP fraction collected from IMAC analyses

Cleared cell lysate by repeated centrifugation was charged onto the IMAC column as protein source. ÄKTA Prime Plus by GE health care systems was used for purification of recombinant proteins by fast protein liquid chromatography deploying the principle of immobilized metal ion chromatography. SmallTalk-GFP were loaded onto a 1-ml HisTrap FF column charged with Ni(II) ions in separate runs. The hypothesis that, SmallTalk could exhibit affinity to Nickle ions bound to the resin of HisTrap FF column thereby allowing the separation of SmallTalk bound proteins from the rest of cellular proteins present in the cell lysate, was tested at this point. Resulting fractions were analyzed in 15% SDS PAGE.

The presence of SmallTalk-GFP in elution fractions provides insights into the effectiveness of SmallTalk as a fusion tag. SmallTalk successfully allowed separation of tagged GFP, the absence of SmallTalk-GFP in flow through fractions of SDS-PAGE analysis shows evidence of successful binding of SmallTalk with Ni (II) charged agarose resin (**Figure 4.21 B**). These results can be further verified form the chromatogram (**Figure 4.21 A**) where the broadened curve shows the adsorption of fusion protein onto the resin and a sharp peak representing successful elution using 200 mM imidazole.

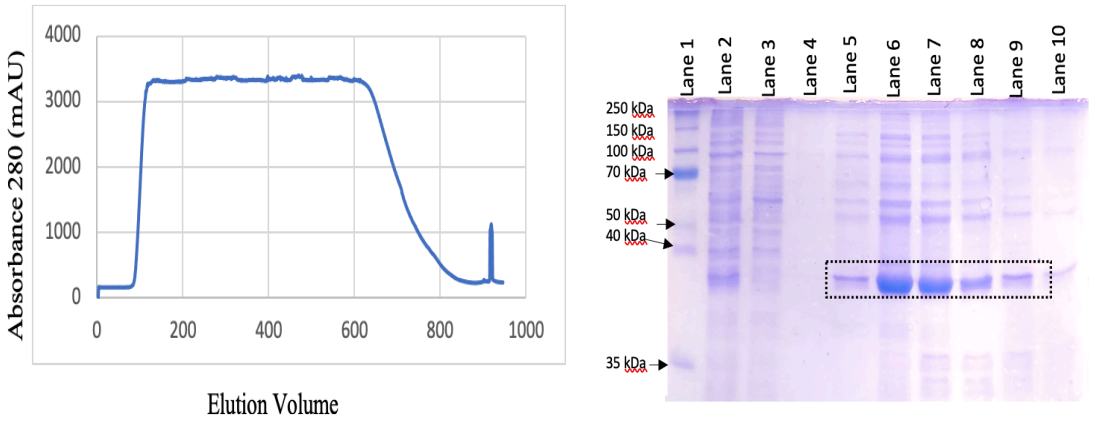


Figure 4.21 (A) Chromatogram from the purification of SmallTalk-GFP using the AKTA Prime Plus FPLC system using a 5-mL HisTrap FF column. the broad first peak is the column flow through. The second narrow peak is the column eluent of SmallTalk-GFP with 200 mM imidazole**. (B):** 12% SDS-PAGE analysis of Purified fractions of SmallTalk-GFP using IMAC chromatography, Lane 1 Protein Marker, Lane 2 Lysate, Lane 3 Flow through, Lane 4 to 10 Elution Fractions (B) Purified GFP expressed using SmallTalk as fusion partner.

The green color of GFP was visible with naked eye in the eppendroff representing that expressed protein is functionally active (Figure 4.20 A & B). The yield of SmallTalk-GFP from one liter of culture media is 7.2 mg which is higher as compared to SmbP-GFP (5.6 mg/L) (**Table 4.4**). This marks the successful use of SmallTalk resulting high yield that was hypothesized initially at the start of project. However, the purity of SmallTalk-GFP came out to be lower than SmbP-GFP.

Table 4.4: Comparison of yield of GFP expressed using SmbP and SmallTalk as fusion protein

Fusion protein	Recombinant peptide yield	Reference
SmbP-GFP	5.6 mg/L	(Vargas-Cortez et al. 2016c)
SmallTalk-GFP	7.2 mg/L	This study

ST-GFP was the first construct that was studied using SmallTalk as a fusion protein tag, aiming towards the visual evidence of SmallTalk on SDS-PAGE gel, the enterokinase cleavage analysis was performed. The cleavage reaction mixture was run on 15% Tricine gel. The analysis showed a very clear distinct three bands representing SmallTalk-GFP chimeric protein, GFP alone and SmallTalk alone (Figure 4.22).

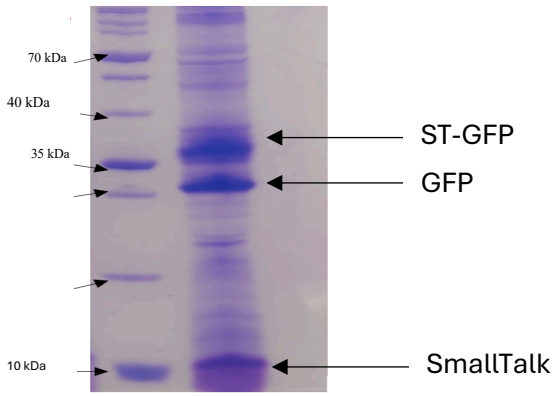


Figure 4.22: 15% Tricine SDS-PAGE analysis of Enterokinase cleavage of SmallTalk-GFP; Lane 1 Protein marker, lane 2 SmallTalk-GFP uncut (32 kDa), GFP (27 kDa) and SmallTalk (5 kDa)

4.5.2-Purification of ST-Bin1b

For large scale expression of SmallTalk-Bin1b aimed to purify the antimicrobial peptide via IMAC, same growth conditions were applied to one liter of expression volume. Overnight grown *E coli SHuffle® T7* cells with SmallTalk-Bin1b cloned pET30a (+), induced with 0.1 mM IPTG were harvested in a refrigerated centrifuge. After cell lysis through mechanical vortexing using 0.1 mm glass beads, collected cell lysate was used as protein source for immobilized metal ion chromatography.

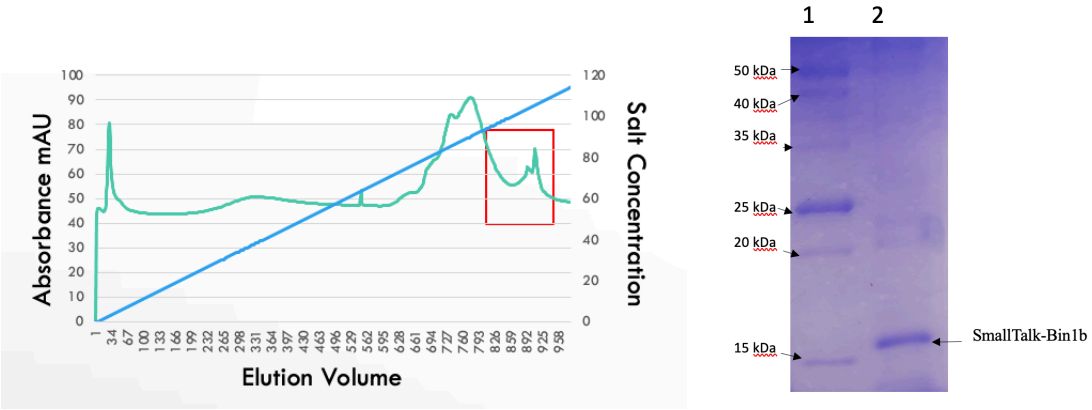


Figure 4.23: Anion Exchange Purification of SmallTalk-Bin1b (A) Chromatogram of SmallTalk-Bin1b from the purification using AKTA prime plus system with a 1 mL His-Trap FF Column (B) SDS-PAGE Analysis of purified fragment after Immobilized Metal Ion and Anion Exchange Chromatography: Lane 1 Protein Marker, Lane 2 Purified SmallTalk-Bin1b

Once the purification of Bin1b was confirmed with the newly designed tag by visualizing the protein content of lysate in SDS-PAGE, SmallTalk-Bin1b was further purified to ascertain its antimicrobial activity by anion exchange chromatography. Sodium chloride (NaCl) gradient was used to elute the protein and fractions highlighted by chromatogram peaks to possess the protein was pooled together and analyzed by electrophoresis. Purified SmallTalk-Bin1b was run on 15% SDS-PAGE and results are shown in Figure 8. The final yield of SmallTalk-Bin1b resulting from 1 liter of culture media is **6.4 mg**. A comparison of yield of Bin1b tagged with different tags have been made in **Table 4**. Tagged protein

was taken for antimicrobial testing as shown in some previous examples including GB1 and Ig-Fc tagged Bin1b was tested for bioactivity (Guo et al. 2009; Ahmed et al. 2021).

Table 4.5: Comparison of Yield of Bin1b Expressed using Multiple Fusion Protein Tags

Fusion protein	Recombinant peptide Yield	Reference
GST	2.4 mg/L	(Vargas-Cortez et al. 2016c)
GB1-His	5-6 mg/L	(Guo et al. 2009)
SmbP	11.4 mg/L	(Vargas-Cortez et al. 2016c)
SmallTalk	6.5 mg/L	This study

4.5.3-Purification of ST-MP1106 and Enterocin DD14

Since the *In-silico* results has already shown that MP1106 possess beta sheets so the expression was carried out Bl21 and *lemo21* (DE3). The SDS-PAGE analysis of purified fragments revealed higher expression from lemo21 (DE3) as compared to BL21 (DE3). The elution with varying concentrations of imidazole ranging from 10 mM to 200 mM imidazole concentrations. Our results show that imidazole as low as 50 mM worked to elute the chimeric protein off the resin. This was in alliance with another hypothesis that since SmallTalk is smaller in size than SmbP, it would require lower concentrations of imidazole. With such low concentrations i.e. 50 mM, further downstream analysis can be carried out even without removing imidazole, depending on specific functional studies of the fusion protein. A comparison of purification fractions of ST-MP1106 expressed in Bl21 (DE3) and *lemo21* (DE3) is shown in **Figure 4.24.** The analysis depicts comparatively lower overall expression of ST-MP1106 in Bl21(DE3) than *lem21*(DE3), however, in both cases the elution occurrence is at 50 mM imidazole concentrations.

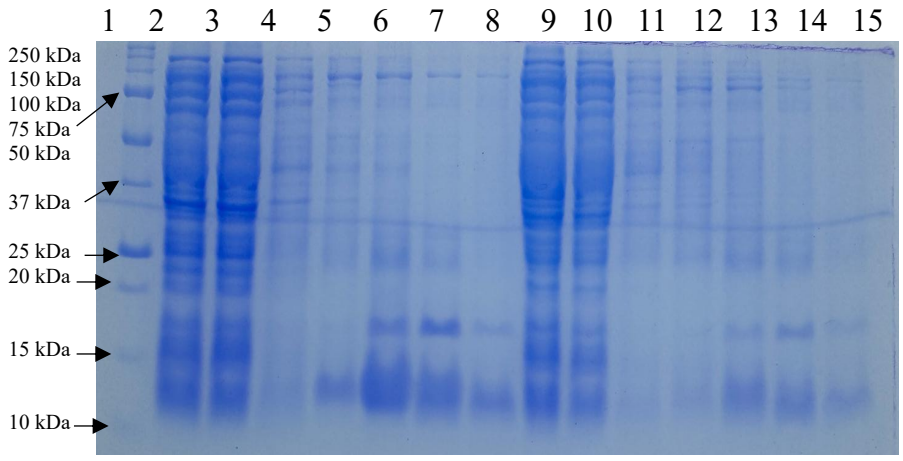


Figure 4.24 Purification of MP1106 in Lemo21 DE3 (Lane 2 to 8) and BL21 DE3 (lane 9 to 15) using different concentrations of Imidazole. Lane 1: Protein Size Marker, Lane 2:Cell lysate, Lane 3: Flowthrough, Lane 4 Column Wash, Lane 5 Elution with 10 mM Imidazole, Lane 6 Elution with 50 mM Imidazole, Lane 7 Elution with 100 mM Imidazole, Lane 8 Elution with 200 mM Imidazole, Lane 9 Lane 9: Cell lysate, Lane 10: Flowthrough, Lane 11 Column Wash, Lane 12 Elution with 10 mM Imidazole, Lane 13 Elution with 50 mM Imidazole, Lane 14 Elution with 100 mM Imidazole, Lane 15 Elution with 200 mM Imidazole

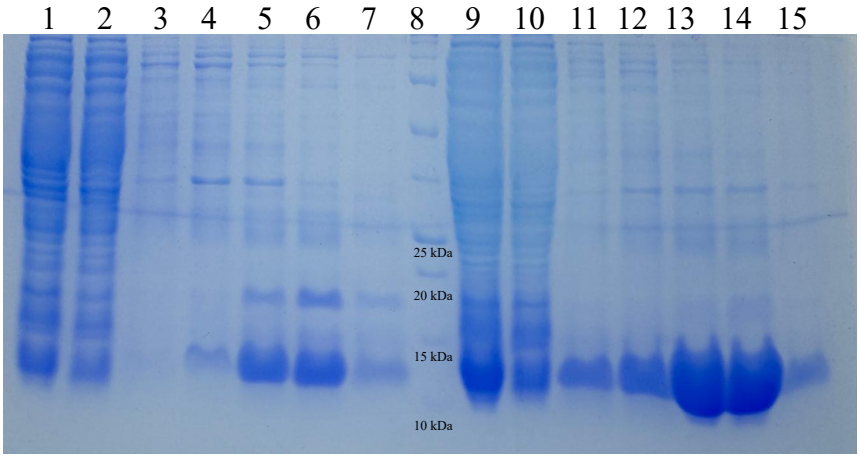


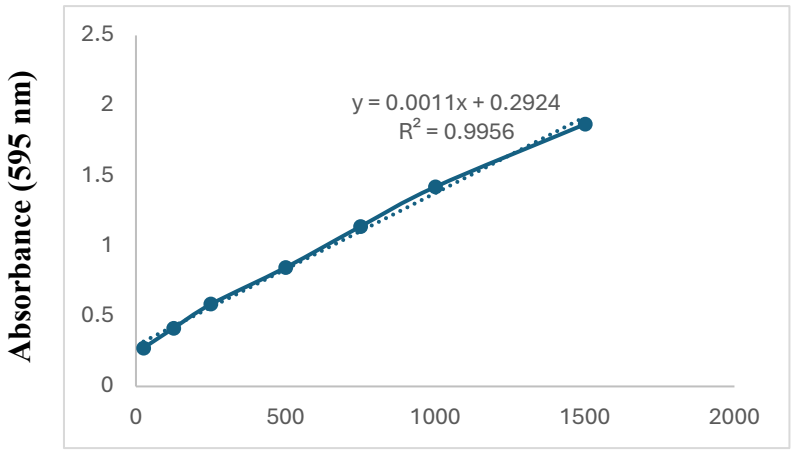
Figure 4.25 Purification of DD14-A (Lane 1 to 7) & DD14-B (Lane 9 to 15) using different concentrations of Imidazole. Lane 1: Cell lysate, Lane 2: Flowthrough, Lane 3: Column Wash, Lane 4: Elution with 10 mM Imidazole, Lane 5: Elution with 50 mM Imidazole, Lane 6: Elution with 100 mM Imidazole, Lane 7: Elution with 200 mM Imidazole, Lane 8: Protein Size Marker, Lane 9: Cell lysate, Lane 10: Flowthrough, Lane 11 Column Wash,

Lane 12 Elution with 10 mM Imidazole, Lane 13 Elution with 50 mM Imidazole, Lane 14 Elution with 100 mM Imidazole, Lane 15 Elution with 200 mM Imidazole.

MP1106 was expressed in both *BL21(DE3)* and *lemo21(DE3)* since the former strains has shown troubled expression in earlier reports due to the presence of sulfur bridges in quaternary structure. Our results showed the expression in both strains, however, *lemo21 (DE3*) showed clearly more expression than *BL21(DE3)* as this strain is designed for proteins containing cysteine residues. The yields of MP1106 from 1 L culture medium of *lemo21 (DE3)* clones containing pET-30a(+)-SmallTalk-MP1106 plasmid is 6.5 mg and form *BL21 DE3* is slightly lesser at 4.7 mg from one liter of expression culture.

Enterocin DD14 was expressed in its constituents; DD14 A and DD14 B in separate badges of recombinant expression. The yield of DD14 A from 1 L of expression medium is 7.6 mg whereas the yield of DD14 B from 1 L of expression medium is 9.5 mg. Our study is the first report of recombinant production of Enterocin DD14. Earlier it has been expressed in its natural host with final yield as low as 0.35 mg from 1 L of native host Enterococcus faecalis (Belguesmia et al. 2024).

(B)



(A)

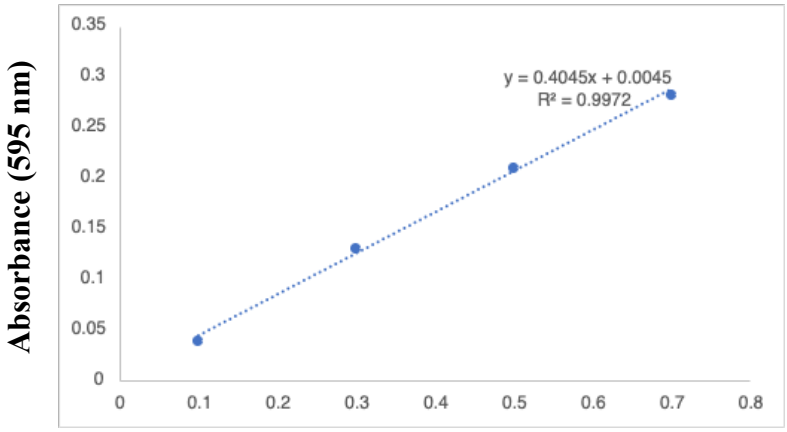


Figure 4.26 Bovine Serum Albumin (BSA) standard calibration curve and protein quantification. (A) Calibration standard curve for recombinant fusion protein SmallTalk-MP1106 (with disulfide bond) and SmallTalk-Enterocin DD14 A and Enterocin DD14 B by Bicinchoninic Acid Protein Assay. (B) Calibration standard curve for recombinant fusion protein SmallTalk-GFP and SmallTalk-Bin1b (with disulfide bond) by Stanford Protein Assay.

4.6-Antimicrobial activity of SmallTalk-Bin1b, SmallTalk-Enterocin DD14 and SmallTalk-MP1106

MIC is defined as the concentration of antimicrobial agent which is the peptide SmallTalk-Bin1b in our case, that prevent visible growth of sensitive pathogen observed in terms of turbidity. The antimicrobial capacity of SmallTalk-Bin1b was tested against Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) clinical isolates. The lowest concentration of Bin1b at which no visible growth was observed and quantified in spectrophotometer was considered as proposed MIC of tag attached Bin1b. The MIC values against each of the test pathogen has been reported in **Table 4.6**. The MIC values of SmallTalk-Bin1b calculated in our study are lower than previously reported MIC values of Bin1b fusion protein. This shows a positive impact on the activity of bound target protein. Bin1b has known to present

solubility issues. For any functional protein, solubility is very crucial. The enhanced functional activity can be attributed to increased percentage of alpha helical content. Because of the way their structure exposes hydrophilic side chains to the solvent, alpha helices are frequently more soluble than beta sheets, which improves the water-solvent interaction. On the other hand, hydrophobic regions can become less soluble when beta sheets expose them. The probability of exposing hydrophobic surfaces is further decreased by the compact form of alpha helices. The discrepancy in solubility can be explained by this change in surface exposure.

Table 4.6: MIC Values of Bin1b Expressed in *SHuffle® T7 using SmallTalk as Fusion Protein, a*gainst different Pathogens

Test pathogen	**MIC (μM) of SmallTalk-Bin1b**	
Escherichia coli	20	
Klebsiella pneumoniae	20	
Pseudomonas aeruginosa	7.5	This study
Staphylococcus aureus	22.5	

The antimicrobial potency of the purified bacteriocin, Enterocin DD14, against clinical isolates of *Listeria monocytogenes* (ATCC 19115) and *Salmonella enterica* (ATCC 14028) was evaluated, and the minimum inhibitory concentrations (MICs) determined via the broth microdilution assay in 96-well plate using plate reader for over night growth determination. Since the bacteriocin possess two components, the synergistic effect was also analyzed, so the tests were conducted with Enterocin DD14 A only, Enterocin DD14 B only and combination of Enterocin DD14 A & Enterocin DD14 B. our results has shown that both components are required for optimal bioactivity but not necessarily synergistic. The range for differential concentrations of antibacterial agent was made from 10 μg to 200 μg, for all the drugs: Enterocin DD14 A only, Enterocin DD14 B only and combination

of Enterocin DD14 A & Enterocin DD14 B. Enterocin DD14 is a newly discovered bacteriocin and our study is the first report of producing bacteriocin through heterologous recombination (**Table 4.8**).

The antimicrobial capacity of SmallTalk-MP1106 was tested against Gram positive (*Listeria monocytogenes*) and Gram negative (*Salmonella enterica*) clinical isolates. The lowest concentration of MP1106 at which no visible growth was observed and quantified in spectrophotometer was considered as proposed MIC of tag attached MP1106. The MIC values against each of the test pathogen has been reported in **Table 4.7**. The MIC values of SmallTalk-Bin1b calculated in our study are lower than previously reported MIC values of Bin1b fusion protein.

Table 4.7: MIC Values of MP1106 Expressed in *Lemo21(DE3) using SmallTalk as Fusion Protein,* a*gainst Gram-positive Listeria monocytogenes and Gram-negative Salmonella enterica*

Test Pathogen	**MIC Enterocin DD14**	
Listeria monocytogenes	40 μM	
Salmonella enterica	35 μM	This study

Table 4.8: MIC Values of Enterocin DD14 (Combined A and B) Expressed in *Bl21 (DE3) using SmallTalk as Fusion Protein,* a*gainst Gram-positive Listeria monocytogenes and Gram-negative Salmonella enterica*

Test Pathogen	**MIC Enterocin DD14**	
Listeria monocytogenes	45 uM	
Salmonella enterica	60 uM	This Study

All the MIC calculation experiments were conducted while the tag being attached to the antimicrobial to test the hypothesis whether the presence of tag will allow the successful expression of biological activity of the target protein. In our experiments, comparable MICs were exhibited by Enterocin DD14 and MP1106 whereas Bin1b has expressed even enhanced values of MIC which implies that our newly design tag has positive impact on the overall structural formation of Bin1b thus improving the functional activity of target protein.

Chapter 6
Discussion

E. coli has been the most well-established heterologous system for the recombinant expression of proteins that hold tremendous significance due to their role in cellular functioning, therapeutic medicine, biotech industry and biological studies. Being a prokaryotic organism, this bacterial host often present difficulties in successful expression of eukaryotic proteins which requires post translational modifications, improper folding, low solubility, and inclusion body formation. Fusion proteins have been widely used to circumvent these problems; however, the selection of carrier molecule has been a trial-and-error experience. Solubility enhancing tags and affinity tags are generally two different classes of fusion proteins, and usually both must be added to a target protein molecule for effective expression and purification purposes. There is also a class of solubility enhancing tags that serves affinity purposes as well including GST, MBP and Sumo tags. SmallTalk has also been successfully shown to give soluble expression of model protein GFP and a eukaryotic defensin in its functional formation. This property of SmallTalk could be attributed to various factors that are generally considered while selecting a fusion protein. The pI of SmallTalk is 6.3 which makes it an acidic fusion protein, thus suggested to enhance solubility by inhibiting protein aggregation via electrostatic repulsion. SmallTalk serves as N-Terminal tag which suggests that it might also take advantage of its natively presented translation initiation sites. Further, the structural conformation of SmallTalk comprises of alpha helices that tend to present hydrophilic residues on the surface, hence contributing to solubility of carrier molecule. The negative GRAVY value of SmallTalk also indicates its hydrophilicity advocating its solubilizing capabilities (Table 2). Additionally, and most importantly, the principle of purification that SmallTalk works on is its inherent affinity for Ni(II) that has been successfully implied in IMAC system. Affinity chromatography has been the most widely used purification techniques in protein production realm providing the most efficient, cost effective and straight-forward protein isolation and purification strategy. Expression, solubility, and purification must be

considered equally important altogether as important factors in efficient recombinant protein production. SmallTalk can be tested as a suitable candidate for testing as a fusion tag offering comparable potential in heterologous expression. Functional assays are often deployed to ascertain the bioactivity of target protein purified after heterologous expression. To analyze the solubility of expressed protein, these functional analyses are carried out with and without the tag. Once the efficiency of SmallTalk as a carrier protein has been tested with model protein GFP, second attempt was done with a eukaryotic cationic antimicrobial peptide that has been expressed with variety of fusion tags as it represents an important class of *β*-defensin. Being a cationic antimicrobial peptide first discovered from rat epididymis, Bin1b has shown to act as innate immune effector against sexually transmitted bacteria. Further, its structural consistence with other members of defensin family make it a suitable candidate to carry out structural and functional analyses that could be implied to remaining class members. However, the presence of three sulfur bridges resulting from six conserved cysteine residues which reduces the rate of successful expression and purification of this beta-defensin. Earlier, it has been expressed using fusion proteins like GB1, GST and Fc-IgG and in this study SmallTalk has been employed for its expression and purification. Bin1b expressed with GST has shown it only exhibited bioactivity while the tag being attached to it while the tag removed Bin1b could not show significant bioactivity. The final yield of GST-Bin1b reported was 2.4 mg/L of culture media (Sun et al. 2004). This low yield could also be attributed to the size imbalance between carrier protein and target peptide since the same resources of cell machinery are deployed for the translation of carrier molecule as well, hence resulting in lesser final yield. SmallTalk-Bin1b expression resulted in yield of 6 mg/L of culture which is only lower than SmbP (lead molecule of SmallTalk) and higher than GST, GB1 and IgG Fc tagged Bin1b (Table 4). The heterologous expression of Bin1b tagged with GB1 was additionally aided with His-tag where the former was intended for the correct folding while later serving the purification purposes. Similarly, the IgG Fc tag was also employed for the recombinant expression in a rather non-conventional lentiviral system for purification. After SmbP, SmallTalk has been used successfully to express functional form of Bin1b in the most used *E. coli* expression system and purification was also achieved using the most employed IMAC purification process. Our newly proposed fusion protein tag has successfully shown

to serve both solubilization and purification purposes using most conventionally used IMAC system. The purity of SmallTalk-GFP as calculated using ImageJ software is 91% and that of SmallTalk-Bin1b is 86%. The purity percentage of SmbP-GFP was same as achieved by SmallTalk however, SmallTalk-Bin1b was slightly less pure as compared to SmbP-Bin1b. As an independently new biological molecule, SmallTalk has potential to perform varyingly with different target proteins attached to it. The isoelectric point of SmbP is 6.1 whereas that of SmallTalk is 6.33 as resulted by ProtParam. As pI is a crucial factor that effects solubility, purification and stability of fusion protein, SmallTalk offers a new option in tag library with slightly different *pI* than SmbP. Overall, like all other fusion tags, SmallTalk offers its unique characteristics and its ability to solubilize and purification using nickle affinity for IMAC procedures make it a very suitable candidate to be selected for heterologous expression of relatively smaller peptides.

Conclusion

A fusion tag that holds the ability to allow antimicrobial testing without removing the tag is preferred in recombinant expression of antimicrobial peptides. Most of the existing fusion tags had to be removed prior to bioactivity testing because they are large enough in size to interfere with protein function or stability. In contrast to other tags, the newly designed SmallTalk fusion tag in this study facilitated AMP solubilization and expression without interfering with its attached protein's bioactivity. Therefore, this study has eliminated the requirement to purify and cleave off the tag prior to testing some losses decreased the efficiency of processes. A tag like SmallTalk that does not affect the functional integrity of the protein-represented the most important progress in producing and testing AMPs, making this process even more effective for researchers working with antimicrobials. The development and application of alternative antimicrobial agents, such as antimicrobial peptides (AMPs), have emerged as promising strategies to combat these resistant bacteria. In this study, recombinant antimicrobial peptides, ST-Bin1b, ST-MP1106, and ST-Enterocin DD14, were designed, produced, and purified through the innovative use of the newly developed fusion protein tag, the SmallTalk. This fusion tag facilitated enhanced bacterial expression using various *E. coli* strains, including BL21(DE3), Lemo21(DE3), and SHuffle T7(DE3). In case of Bin1b and MP1106, both of which are not only eukaryotic peptides but also contain beta sheets in their quaternary structure, were shown to express significantly better than previous reports of recombinant expression using other tags. Inclusion body formation of eukaryotic protein while being expressed in a prokaryotic system is a major drawback faced by

scientists, yet the highly optimized expression conditions of *E. coli* urge them to pick bacteria compared to other eukaryotic hosts. SmallTalk has shown the potential to aid in the expression of eukaryotic peptides by solubilizing the peptide and adding extra helical content to the spatial structure. It also allowed for the efficient purification of the recombinant peptides through immobilized metal affinity chromatography (IMAC). The nickel affinity of SmbP was shown to possessed by its derived molecule, SmallTalk as well. IMAC allows one step purification pathways which make it the most used purification system for recombinant expressions. SmallTalk has established itself as a fusion protein quite decently with significant ability to purify the target proteins, which is tested by four examples in this study (GFP, Bin1b, MP1106 and Enterocin DD14). Testing the antimicrobial peptides against both Gram-positive and Gram-negative bacteria demonstrated their antimicrobial potency, even with the SmallTalk tag attached. The results revealed that the fusion tag significantly improved the solubility of beta-sheet-containing peptides like Bin1b and MP1106, outperforming previously used tags. Furthermore, Bin1b, MP1106 and Enterocin DD14 retained their antimicrobial activity with the tag, further highlighting the ability of SmallTalk to preserve the bioactivity of the fused proteins. SmallTalk has also shown to elute the target protein from the charged matrix with significantly lower concentrations of imidazole, as low as 50 mM tested in the gravity column chromatography. Low imidazole concentrations prevent denaturation or loss of activity of target protein by allowing a more gradual and selective elution of target proteins. These findings underscore the potential of SmallTalk as an effective fusion tag that not only enhances the solubility and expression of AMPs but also maintains their bioactivity. The SmallTalk fusion tag did not interfere with the bioactivity of the AMPs, as the fusion proteins were found to be as active as the unfused AMPs against a variety of bacterial pathogens. This study represents an important advancement in the use of fusion tags to produce

AMPs and provides a foundation for further research into their therapeutic applications as alternatives to traditional antibiotics.

References

1. Adamíková, Jana, Monika Antošová, and Milan Polakovič. 2019. "Chromatographic Purification of Recombinant Human Erythropoietin." *Biotechnology Letters* 41 (4–5): 483–93. https://doi.org/10.1007/s10529-019-02656-8.
2. Ahmed, Shiraz, Jiang Tao, Miaochen Wang, Yue Zhai, Wenhai Liu, Muthukumaran Jayachandran, Chen Chu, et al. 2021. "An Improved Lentiviral System for Efficient Expression and Purification of β-Defensins in Mammalian Cells." *Biotechnology Journal* 16 (9). https://doi.org/10.1002/biot.202100023.
3. Barney, Brett M., Russell LoBrutto, and Wilson A. Francisco. 2004. "Characterization of a Small Metal Binding Protein from Nitrosomonas Europaea." *Biochemistry* 43 (35): 11206–13. https://doi.org/10.1021/BI049318K/SUPPL_FILE/BI049318KSI20040706_020600.PDF.
4. Belguesmia, Yanath, Louis David Guay, Radja Teiar, Md Ramim Tanver Rahman, Elodie Dussert, Eric Biron, and Djamel Drider. 2024. "Synthesis, Antimicrobial Activity, and Mechanistic Studies of Enterocin DD14, a Leaderless Two-Peptide Bacteriocin." *International Journal of Biological Macromolecules* 280 (November):135716. https://doi.org/10.1016/J.IJBIOMAC.2024.135716.
5. Belguesmia, Yanath, Giuseppe Spano, and Djamel Drider. 2021. "Potentiating Effects of Leaderless Enterocin DD14 in Combination with Methicillin on Clinical Methicillin-Resistant Staphylococcus Aureus S1 Strain." *Microbiological Research* 252 (November):126864. https://doi.org/10.1016/J.MICRES.2021.126864.
6. Benítez-Chao, Diego Francisco, Angel León-Buitimea, Jordy Alexis Lerma-Escalera, and José Rubén Morones-Ramírez. 2021. "Bacteriocins: An Overview of Antimicrobial, Toxicity, and Biosafety Assessment by in Vivo Models." *Frontiers in Microbiology* 12 (April). https://doi.org/10.3389/FMICB.2021.630695.
7. Bhatwa, Arshpreet, Weijun Wang, Yousef I. Hassan, Nadine Abraham, Xiu Zhen Li, and Ting Zhou. 2021. "Challenges Associated With the Formation of Recombinant Protein Inclusion Bodies in Escherichia Coli and Strategies to Address Them for Industrial Applications." *Frontiers in Bioengineering and Biotechnology* 9 (February). https://doi.org/10.3389/FBIOE.2021.630551.
8. Block, Helena, Barbara Maertens, Anne Spriestersbach, Nicole Brinker, Jan Kubicek, Roland Fabis, Jörg Labahn, and Frank Schäfer. 2011. "WITHDRAWN: Reprint of: Immobilized-Metal Affinity Chromatography (IMAC): A Review." *Protein Expression and Purification*, September. https://doi.org/10.1016/J.PEP.2011.08.021.
9. "Broth Microdilution Antibacterial Assay of Peptides Laszlo Otvos and Mare Cudic." n.d.

10. Butt, Tauseef R., Suzanne C. Edavettal, John P. Hall, and Michael R. Mattern. 2005. “SUMO Fusion Technology for Difficult-to-Express Proteins.” *Protein Expression and Purification* 43 (1): 1. https://doi.org/10.1016/J.PEP.2005.03.016.
11. Caly, Delphine L., Mickaël Chevalier, Christophe Flahaut, Benoit Cudennec, Ahmed Khassaf Al Atya, Gabrielle Chataigné, Romain D’Inca, Eric Auclair, and Djamel Drider. 2017. “The Safe Enterocin DD14 Is a Leaderless Two-Peptide Bacteriocin with Anti-Clostridium Perfringens Activity.” *International Journal of Antimicrobial Agents* 49 (3): 282–89. https://doi.org/10.1016/J.IJANTIMICAG.2016.11.016.
12. Charlton, Adam, and Michael Zachariou. 2008. “Immobilized Metal Ion Affinity Chromatography of Native Proteins.” *Methods in Molecular Biology (Clifton, N.J.)* 421 (December):25–35. https://doi.org/10.1007/978-1-59745-582-4_2.
13. Chen, Vincent B., W. Bryan Arendall, Jeffrey J. Headd, Daniel A. Keedy, Robert M. Immormino, Gary J. Kapral, Laura W. Murray, Jane S. Richardson, and David C. Richardson. 2010. “*MolProbity* : All-Atom Structure Validation for Macromolecular Crystallography.” *Acta Crystallographica Section D Biological Crystallography* 66 (1): 12–21. https://doi.org/10.1107/S0907444909042073.
14. Costa, Sofia, André Almeida, António Castro, and Lucília Domingues. 2014. “Fusion Tags for Protein Solubility, Purification, and Immunogenicity in Escherichia Coli: The Novel Fh8 System.” *Frontiers in Microbiology* 5 (FEB). https://doi.org/10.3389/FMICB.2014.00063.
15. Fakruddin, Md., Reaz Mohammad Mazumdar, Khanjada Shahnewaj Bin Mannan, Abhijit Chowdhury, and Md. Nur Hossain. 2013. “Critical Factors Affecting the Success of Cloning, Expression, and Mass Production of Enzymes by Recombinant E. Coli.” *ISRN Biotechnology* 2013 (September):1–7. https://doi.org/10.5402/2013/590587.
16. Francis, Dana M., and Rebecca Page. 2010. “Strategies to Optimize Protein Expression in E. Coli.” *Current Protocols in Protein Science* 61 (1): 5241. https://doi.org/10.1002/0471140864.PS0524S61.
17. “Global Laboratory Standards for a Healthier World.” n.d. www.clsi.org.
18. Guo, Chenyun, Hua Diao, Yandong Lian, Heguo Yu, Hongchang Gao, Yonglian Zhang, and Donghai Lin. 2009. “Recombinant Expression and Characterization of an Epididymis-Specific Antimicrobial Peptide BIN1b/SPAG11E.” *Journal of Biotechnology* 139 (1): 33–37. https://doi.org/10.1016/j.jbiotec.2008.10.003.
19. Hameduh, Tareq, Yazan Haddad, Vojtech Adam, and Zbynek Heger. 2020. “Homology Modeling in the Time of Collective and Artificial Intelligence.” *Computational and Structural Biotechnology Journal* 18:3494–3506. https://doi.org/10.1016/j.csbj.2020.11.007.
20. Harper, Sandra, and David W. Speicher. 2011. “Purification of Proteins Fused to Glutathione S-Tranferase.” *Methods in Molecular Biology (Clifton, N.J.)* 681:259. https://doi.org/10.1007/978-1-60761-913-0_14.
21. Hayat, Seyed Mohammad Gheibi, Najmeh Farahani, Behrouz Golichenari, and Amirhossein Sahebkar. 2018. “Recombinant Protein Expression in Escherichia Coli (E.Coli): What We Need to Know.” *Current Pharmaceutical Design* 24 (6): 718–25. https://doi.org/10.2174/1381612824666180131121940.
22. Hollingsworth, Scott A., and P. Andrew Karplus. 2010a. “A Fresh Look at the Ramachandran Plot and the Occurrence of Standard Structures in Proteins.” *BioMolecular Concepts* 1 (3–4): 271–83. https://doi.org/10.1515/bmc.2010.022.

23. ———. 2010b. “A Fresh Look at the Ramachandran Plot and the Occurrence of Standard Structures in Proteins.” *BioMolecular Concepts* 1 (3–4): 271–83. https://doi.org/10.1515/bmc.2010.022.
24. Hwang, In Young, Elvin Koh, Hye Rim Kim, Wen Shan Yew, and Matthew Wook Chang. 2016. “Reprogrammable Microbial Cell-Based Therapeutics against Antibiotic-Resistant Bacteria.” *Drug Resistance Updates* 27 (July):59–71. https://doi.org/10.1016/J.DRUP.2016.06.002.
25. Jayakrishnan, Achuth, Wan Rosalina Wan Rosli, Ahmad Rashidi Mohd Tahir, Fashli Syafiq Abd Razak, Phei Er Kee, Hui Suan Ng, Yik Ling Chew, et al. 2024. “Evolving Paradigms of Recombinant Protein Production in Pharmaceutical Industry: A Rigorous Review.” *Sci 2024, Vol. 6, Page 9* 6 (1): 9. https://doi.org/10.3390/SCI6010009.
26. Jenny, Richard J., Kenneth G. Mann, and Roger L. Lundblad. 2003. “A Critical Review of the Methods for Cleavage of Fusion Proteins with Thrombin and Factor Xa.” *Protein Expression and Purification* 31 (1): 1–11. https://doi.org/10.1016/S1046-5928(03)00168-2.
27. Ki, Mi Ran, and Seung Pil Pack. 2020. “Fusion Tags to Enhance Heterologous Protein Expression.” *Applied Microbiology and Biotechnology* 104 (6): 2411–25. https://doi.org/10.1007/S00253-020-10402-8.
28. Kielkopf, Clara L., William Bauer, and Ina L. Urbatsch. 2020. “Bradford Assay for Determining Protein Concentration.” *Cold Spring Harbor Protocols* 2020 (4): pdb.prot102269. https://doi.org/10.1101/pdb.prot102269.
29. Kimple, Michelle E., Allison L. Brill, and Renee L. Pasker. 2013. “Overview of Affinity Tags for Protein Purification.” *Current Protocols in Protein Science / Editorial Board, John E. Coligan ... [et Al.]* 73 (SUPPL.73): Unit. https://doi.org/10.1002/0471140864.PS0909S73.
30. Köppl, Christoph, Nico Lingg, Andreas Fischer, Christina Krößß, Julian Loibl, Wolfgang Buchinger, Rainer Schneider, Alois Jungbauer, Gerald Striedner, and Monika Cserjan-Puschmann. 2022a. “Fusion Tag Design Influences Soluble Recombinant Protein Production in Escherichia Coli.” *International Journal of Molecular Sciences* 23 (14). https://doi.org/10.3390/IJMS23147678/S1.
31. ———. 2022b. “Fusion Tag Design Influences Soluble Recombinant Protein Production in Escherichia Coli.” *International Journal of Molecular Sciences* 23 (14). https://doi.org/10.3390/IJMS23147678/S1.
32. Kowalska-Krochmal, Beata, and Ruth Dudek-Wicher. 2021. “The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance.” *Pathogens* 10 (2): 165. https://doi.org/10.3390/pathogens10020165.
33. Kumar, Raj, and Ajay Sharma. 2023. “Computational Strategies and Tools for Protein Tertiary Structure Prediction.” In *Basic Biotechniques for Bioprocess and Bioentrepreneurship*, 225–42. Elsevier. https://doi.org/10.1016/B978-0-12-816109-8.00015-5.
34. Ladjouzi, Rabia, Elodie Dussert, Radja Teiar, Yanath Belguesmia, and Djamel Drider. 2023. “A Review on Enterocin DD14, the Leaderless Two-Peptide Bacteriocin with Multiple Biological Functions and Unusual Transport Pathway.” *Antibiotics* 12 (7). https://doi.org/10.3390/ANTIBIOTICS12071188.
35. Li, Jianmin, Qingluo Wang, Guoyu Xia, Nigela Adilijiang, Ying Li, Zhenqing Hou, Zhongxiong Fan, and Jinyao Li. 2023. “Recent Advances in Targeted Drug Delivery Strategy for Enhancing Oncotherapy.” *Pharmaceutics* 15 (9). https://doi.org/10.3390/PHARMACEUTICS15092233.

36. Li, Xuan, Ya Hao, Na Yang, Ruoyu Mao, Da Teng, and Jianhua Wang. 2023. “Plectasin: From Evolution to Truncation, Expression, and Better Druggability.” *Frontiers in Microbiology* 14 (December):1304825. https://doi.org/10.3389/FMICB.2023.1304825/BIBTEX.
37. Lu, Ruei Min, Yu Chyi Hwang, I. Ju Liu, Chi Chiu Lee, Han Zen Tsai, Hsin Jung Li, and Han Chung Wu. 2020. “Development of Therapeutic Antibodies for the Treatment of Diseases.” *Journal of Biomedical Science 2020 27:1* 27 (1): 1–30. https://doi.org/10.1186/S12929-019-0592-Z.
38. Luong, Huy Xuan, Tung Truong Thanh, and Tuan Hiep Tran. 2020. “Antimicrobial Peptides – Advances in Development of Therapeutic Applications.” *Life Sciences* 260 (November):118407. https://doi.org/10.1016/J.LFS.2020.118407.
39. MacConmara, Malcolm, Ifeoma Nwadei, and Allan D. Kirk. 2015. “Immunosuppressive Biologic Agents.” *Transplantation of the Liver: Third Edition*, January, 1343–53. https://doi.org/10.1016/B978-1-4557-0268-8.00096-8.
40. Mahmood, Tehreem, Areej Shahbaz, Nazim Hussain, Rahat Ali, Hamid Bashir, and Komal Rizwan. 2023. “Recent Advancements in Fusion Protein Technologies in Oncotherapy: A Review.” *International Journal of Biological Macromolecules* 230 (March). https://doi.org/10.1016/j.ijbiomac.2023.123161.
41. Mahmoudi Gomari, Mohammad, Neda Saraygord-Afshari, Marziye Farsimadan, Neda Rostami, Shahin Aghamiri, and Mohammad M. Farajollahi. 2020. “Opportunities and Challenges of the Tag-Assisted Protein Purification Techniques: Applications in the Pharmaceutical Industry.” *Biotechnology Advances* 45 (December):107653. https://doi.org/10.1016/J.BIOTECHADV.2020.107653.
42. Marco, Ario de. 2009. “Strategies for Successful Recombinant Expression of Disulfide Bond-Dependent Proteins in Escherichia Coli.” *Microbial Cell Factories* 8 (1): 1–18. https://doi.org/10.1186/1475-2859-8-26/FIGURES/3.
43. Mba, Ifeanyi Elibe, and Emeka Innocent Nweze. 2022. “Focus: Antimicrobial Resistance: Antimicrobial Peptides Therapy: An Emerging Alternative for Treating-Resistant Bacteria.” *The Yale Journal of Biology and Medicine* 95 (4): 445. /pmc/articles/PMC9765339/.
44. Miller, Frederick W. 2023. “The Increasing Prevalence of Autoimmunity and Autoimmune Diseases: An Urgent Call to Action for Improved Understanding, Diagnosis, Treatment and Prevention.” *Current Opinion in Immunology* 80 (February):102266. https://doi.org/10.1016/J.COI.2022.102266.
45. Montfort-Gardeazabal, Jorge M., Isaias Balderas-Renteria, Nestor G. Casillas-Vega, and Xristo Zarate. 2021a. “Expression and Purification of the Antimicrobial Peptide Bin1b in Escherichia Coli Tagged with the Fusion Proteins CusF3H+ and SmbP.” *Protein Expression and Purification* 178 (February). https://doi.org/10.1016/J.PEP.2020.105784.
46. ———. 2021b. “Expression and Purification of the Antimicrobial Peptide Bin1b in Escherichia Coli Tagged with the Fusion Proteins CusF3H+ and SmbP.” *Protein Expression and Purification* 178 (February):105784. https://doi.org/10.1016/J.PEP.2020.105784.
47. ———. 2021c. “Expression and Purification of the Antimicrobial Peptide Bin1b in Escherichia Coli Tagged with the Fusion Proteins CusF3H+ and SmbP.” *Protein Expression and Purification* 178 (February). https://doi.org/10.1016/j.pep.2020.105784.
48. Montfort-Gardeazabal, Jorge M., Pilar C. Morales-San Claudio, Nestor G. Casillas-Vega, and Xristo Zarate. 2021. “Expression and Purification of the VpDef Defensin in Escherichia Coli

Using the Small Metal-Binding Proteins CusF3H+ and SmbP." *Protein and Peptide Letters* 28 (1): 108–14. https://doi.org/10.2174/0929866527666200610133407.

49. Mora, Zuamí Villagrán De la, María Esther Macías-Rodríguez, Jenny Arratia-Quijada, Yesica Sughey Gonzalez-Torres, Karla Nuño, and Angélica Villarruel-López. 2020. "Clostridium Perfringens as Foodborne Pathogen in Broiler Production: Pathophysiology and Potential Strategies for Controlling Necrotic Enteritis." *Animals : An Open Access Journal from MDPI* 10 (9): 1–28. https://doi.org/10.3390/ANI10091718.
50. Morowvat, Mohammad Hossein, Valiollah Babaeipour, Hamid Rajabi-Memari, Hossein Vahidi, and Nader Maghsoudi. 2014. "Overexpression of Recombinant Human Beta Interferon (RhINF-β) in Periplasmic Space of Escherichia Coli." *Iranian Journal of Pharmaceutical Research : IJPR* 13 (Suppl): 151. /pmc/articles/PMC3977065/.
51. Naddaf, Miryam. 2024. "40 Million Deaths by 2050: Toll of Drug-Resistant Infections to Rise by 70." *Nature* 633 (8031): 747–48. https://doi.org/10.1038/D41586-024-03033-W.
52. Papaneophytou, Christos P., and George Kontopidis. 2014. "Statistical Approaches to Maximize Recombinant Protein Expression in Escherichia Coli: A General Review." *Protein Expression and Purification* 94 (February):22–32. https://doi.org/10.1016/J.PEP.2013.10.016.
53. Perez-Perez, David A., Elizeth Pioquinto-Avila, Eder Arredondo-Espinoza, Jose Ruben Morones-Ramirez, Isaias Balderas-Renteria, and Xristo Zarate. 2020. "Engineered Small Metal-binding Protein Tag Improves the Production of Recombinant Human Growth Hormone in the Periplasm of Escherichia Coli." *FEBS Open Bio* 10 (4): 546. https://doi.org/10.1002/2211-5463.12808.
54. Perez-Perez, David A., Teresa de J. Villanueva-Ramirez, Adriana E. Hernandez-Pedraza, Nestor G. Casillas-Vega, Patricia Gonzalez-Barranco, and Xristo Zarate. 2021a. "The Small Metal-Binding Protein SmbP Simplifies the Recombinant Expression and Purification of the Antimicrobial Peptide LL-37." *Antibiotics* 10 (10). https://doi.org/10.3390/ANTIBIOTICS10101271.
55. ———. "The Small Metal-Binding Protein SmbP Simplifies the Recombinant Expression and Purification of the Antimicrobial Peptide LL-37." *Antibiotics* 10 (10). https://doi.org/10.3390/ANTIBIOTICS10101271.
56. Pina, Ana Sofia, Christopher R. Lowe, and Ana Cecília A. Roque. 2014. "Challenges and Opportunities in the Purification of Recombinant Tagged Proteins." *Biotechnology Advances* 32 (2): 366. https://doi.org/10.1016/J.BIOTECHADV.2013.12.001.
57. "Recombinant Proteins Market Size To Hit USD 10.26 Billion By 2033." n.d. Accessed October 2, 2024. https://www.precedenceresearch.com/recombinant-proteins-market.
58. Reuten, Raphael, Denise Nikodemus, Maria B. Oliveira, Trushar R. Patel, Bent Brachvogel, Isabelle Breloy, Jörg Stetefeld, and Manuel Koch. 2016. "Maltose-Binding Protein (MBP), a Secretion-Enhancing Tag for Mammalian Protein Expression Systems." *PloS One* 11 (3). https://doi.org/10.1371/JOURNAL.PONE.0152386.
59. Rezaei, Marzieh, and Sayyed H. Zarkesh-Esfahani. 2012. "Optimization of Production of Recombinant Human Growth Hormone in Escherichia Coli." *Journal of Research in Medical Sciences : The Official Journal of Isfahan University of Medical Sciences* 17 (7): 681. /pmc/articles/PMC3685787/.
60. Riggs, P. 2001. "Fusion Proteins." *Encyclopedia of Genetics*, 739–40. https://doi.org/10.1006/RWGN.2001.0487.

61. Riguero, Valeria, Robert Clifford, Michael Dawley, Matthew Dickson, Benjamin Gastfriend, Christopher Thompson, Sheau Chiann Wang, and Ellen O'Connor. 2020. "Immobilized Metal Affinity Chromatography Optimization for Poly-Histidine Tagged Proteins." *Journal of Chromatography. A* 1629 (October). https://doi.org/10.1016/J.CHROMA.2020.461505.
62. Rosano, GermÃ¡n L., and Eduardo A. Ceccarelli. 2014. "Recombinant Protein Expression in Escherichia Coli: Advances and Challenges." *Frontiers in Microbiology* 5 (April). https://doi.org/10.3389/fmicb.2014.00172.
63. Santos, Bryan D., Jose Ruben Morones-Ramirez, Isaias Balderas-Renteria, Nestor G. Casillas-Vega, David W. Galbraith, and Xristo Zarate. 2019a. "Optimizing Periplasmic Expression in Escherichia Coli for the Production of Recombinant Proteins Tagged with the Small Metal-Binding Protein SmbP." *Molecular Biotechnology* 61 (6): 451–60. https://doi.org/10.1007/s12033-019-00176-4.
64. ———. 2019b. "Optimizing Periplasmic Expression in Escherichia Coli for the Production of Recombinant Proteins Tagged with the Small Metal-Binding Protein SmbP." *Molecular Biotechnology* 61 (6): 451–60. https://doi.org/10.1007/s12033-019-00176-4.
65. Saraswat, Mayank, Luca Musante, Alessandra Ravidá, Brian Shortt, Barry Byrne, and Harry Holthofer. 2013. "Preparative Purification of Recombinant Proteins: Current Status and Future Trends." *BioMed Research International* 2013. https://doi.org/10.1155/2013/312709.
66. Schwinn, Marie K., Leta S. Steffen, Kris Zimmerman, Keith V. Wood, and Thomas Machleidt. 2020. "A Simple and Scalable Strategy for Analysis of Endogenous Protein Dynamics." *Scientific Reports 2020 10:1* 10 (1): 1–14. https://doi.org/10.1038/s41598-020-65832-1.
67. Simons, Alexis, Kamel Alhanout, and Raphaël E. Duval. 2020. "Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria." *Microorganisms* 8 (5). https://doi.org/10.3390/MICROORGANISMS8050639.
68. Sun, Xiang-Jun, Dong-Ning Wang, Wei-Jie Zhang, and Xiang-Fu Wu. 2004. "Expression of an Antimicrobial Peptide Identified in Rats 185 MOLECULAR BIOTECHNOLOGY Expression of an Antimicrobial Peptide Identified in the Male Reproductive System of Rats." *Molecular Biotechnology*.
69. Terpe, K. 2003. "Overview of Tag Protein Fusions: From Molecular and Biochemical Fundamentals to Commercial Systems." *Applied Microbiology and Biotechnology* 60 (5): 523–33. https://doi.org/10.1007/S00253-002-1158-6.
70. Tuon, Felipe Francisco, Paula Hansen Suss, Joao Paulo Telles, Leticia Ramos Dantas, Nícolas Henrique Borges, and Victoria Stadler Tasca Ribeiro. 2023. "Antimicrobial Treatment of Staphylococcus Aureus Biofilms." *Antibiotics* 12 (1). https://doi.org/10.3390/ANTIBIOTICS12010087.
71. Vargas-Cortez, Teresa, Jose Ruben Morones-Ramirez, Isaias Balderas-Renteria, and Xristo Zarate. 2016a. "Expression and Purification of Recombinant Proteins in Escherichia Coli Tagged with a Small Metal-Binding Protein from Nitrosomonas Europaea." *Protein Expression and Purification* 118 (February):49–54. https://doi.org/10.1016/J.PEP.2015.10.009.
72. ———. 2016b. "Expression and Purification of Recombinant Proteins in Escherichia Coli Tagged with a Small Metal-Binding Protein from Nitrosomonas Europaea." *Protein Expression and Purification* 118 (February):49–54. https://doi.org/10.1016/J.PEP.2015.10.009.

73. ———. 2016c. “Expression and Purification of Recombinant Proteins in Escherichia Coli Tagged with a Small Metal-Binding Protein from Nitrosomonas Europaea.” *Protein Expression and Purification* 118 (February):49–54. https://doi.org/10.1016/j.pep.2015.10.009.
74. ———. 2017. “Production of Recombinant Proteins in Escherichia Coli Tagged with the Fusion Protein CusF3H+.” *Protein Expression and Purification* 132 (April):44–49. https://doi.org/10.1016/j.pep.2017.01.006.
75. Ventola, C. Lee. 2015. “The Antibiotic Resistance Crisis: Part 1: Causes and Threats.” *Pharmacy and Therapeutics* 40 (4): 277. https://doi.org/Article.
76. Yadav, Dinesh K., Neelam Yadav, Sarika Yadav, Shafiul Haque, and Narendra Tuteja. 2016. “An Insight into Fusion Technology Aiding Efficient Recombinant Protein Production for Functional Proteomics.” *Archives of Biochemistry and Biophysics* 612 (December):57–77. https://doi.org/10.1016/j.abb.2016.10.012.
77. Zhang, Fusheng, and Wei Cheng. 2022. “The Mechanism of Bacterial Resistance and Potential Bacteriostatic Strategies.” *Antibiotics* 11 (9). https://doi.org/10.3390/ANTIBIOTICS11091215.
78. Zhang, Shuguang, and Karolina Corin. 2017. “Peptide Surfactants in Membrane Protein Purification and Stabilization.” *Peptide Applications in Biomedicine, Biotechnology and Bioengineering*, January, 485–512. https://doi.org/10.1016/B978-0-08-100736-5.00020-X.