DEPARTMENT OF CROP SCIENCES
DIVISON OF PLANT PROTECTION

Protein A fusion constructs

A concept for secratory expression of antimicrobial peptides in *E. coli*

Masterthesis

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Abstract

Antimicrobial peptides (AMPs) are part of the defence system of plants. AMPs have been isolated from different plant structures and have activities towards phytopathogens and even human pathogens. Therefore, plant AMPs are considered as promising antibiotic compounds in different fields.

Plant AMPs are usually positively charged and their three-dimensional structure is stabilised by several disulfide bonds. Moreover, these bonds cannot be formed properly in the cytoplasm of *E. coli* bacteria. Hence, it is an advantage to secrete them into the extracellular space, where the correct disulfide bond formation can be provided.

This masterthesis describes a concept for secratory expression of AMPs in *E.coli*. Therefore, in a recent paper, a protocol which deals with the improved extracellular expression of *Staphylococcus aureus* recombinant protein A, in *E.coli* strain BL21(DE3), was tested. Interestingly, no verification for the increased protein A yield could be given. Nevertheless, amplifications of the 5 homologous domains (A-E), of the N-terminal part of protein A, were made and fragment-D was cloned into a pET-vector derived from pETtrx_1a.

In further experiments, these fragments could be cloned each into the modified pET-vectors. It would be possible to use these vector constructs as fusion carriers for AMPs and test them for improved extracellular expression with the expression protocol referred to above.

Zusammenfassung

Antimikobielle Peptide (AMP) sind Teil des Abwehrsystems von Pflanzen. AMP wurden von verschieden Pflanzenstrukturen bereits isoliert und wirken gegen Phytopathogene und sogar gegen Humanpathogene. In Folge dessen, gelten pflanzliche AMP als vielversprechende Stoffe für Antibiotika und andere Bereiche. Pflanzliche AMP sind normalerweise positiv geladen und ihre dreidimensionale Struktur wird durch mehrere Disulfidbrücken stabilisiert. Diese Brücken können sich jedoch nicht richtig im Zytoplasma von *E. coli* Bakterien bilden. Aus diesem Grund, ist es vorteilhaft AMP in den extrazellulären Raum zu exprimieren, indem die korrekte Disulfidbrückenbildung gewährleistet ist.

Diese Masterarbeit beschreibt ein Konzept für die selektive Expression von AMP in *E.coli*. Dafür wurde ein Artikel herangezogen, indem ein Protokoll für die erhöhte Expression von rekombinanten *Staphylococcus aureus* Protein A, im *E.coli* Stamm BL21 (DE3), beschrieben wurde. Dieses Protokoll wurde getestet, aber interessanterweise wurde keine erhöhte Expression des Proteins in induzierten Zellen festgestellt.

Dennoch wurden die 5 homogenen Domänen (A-E), des N-terminalen Endes von Protein A, amplifiziert und Fragment-D wurde in einen pET-Vektor, der von pETtrx_1a abstammt, kloniert.

In weiterführenden Experimenten könnten die anderen Fragmente in die pET-Vektoren kloniert werden. Es wäre möglich diese Konstrukte als Fusionsträger für AMPs zu nutzen und diese auf erhöhte extrazelluläre Expression, mit dem oben genannten Expressionsprotokoll, zu testen.

Composition

1. Introduction

1.1. Plant defense

The defense system of plants against herbivores and pathogens, includes a wide variety of constitutive and inducible mechanisms. They have developed different strategies such as structural, chemical, and protein-based defenses, to protect themselves from invading organisms and stop them before they are able to cause significant damage (Beattie, 2008).

The constitutive defense consists of chemical and physiological barriers, and is continuously present in the plant. The cell wall, waxy epidermal cuticles, hairs, trichomes, thorns, spines, and thicker leaves or the production of toxic chemicals such as terpenoids, alkaloids, anthocyanins, phenols, and quinones, are just a few examples of this defense system (War et al,. 2012).

In addition, plants have an inducible defense, which is only active when needed. Plants can produce toxic chemicals, pathogen-degrading enzymes or even practice cell suicide (Beattie, 2008; Gilchrist, 1998).

Moreover, plant cells are able to recognize with pattern recognition receptors (PRRs), microbe/pathogen-associated molecular patterns (MAMPs/PAMPS) including specific proteins, cell wall components and other elicitors which are commonly found in microbes and other organisms. Flagellin (Flg), elongation factor Tu (EF-Tu), peptidoglycan (PGN), lipoploysachharides (LPS), fungal chitin, and β-glucans from oomycetes are recognized by plant surface localized PRRs (Newman et al. 2013).

Furthermore, recognition with PRRs triggers the induction of additional compounds such as phytoalexins (Jeandet et al,. 2013), different antimicrobial peptides (AMPs) (Nawrot et al, 2014), for instance lipid transfer proteins (Edstam 2013; Kader 1996), thionins (Abbas et al. 2013; Bohlmann et al. 1988; Epple, 1997), hevein-like peptides (Nawrot et al. 2014), knottin-like peptides (Nawrot et al, 2014), glycine-rich peptides (Sachetto-Martins et al, 2000) and snakins (Nawrot et al, 2014). In addition, also two phytohormones, salicylic acid and jasmonic acid, are part of the defence mechanism (Tamaoki et al. 2013; Hammond-Kosack et al, 1996).

1.2 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are part of the defence system of plants as mentioned before. AMPs have been isolated from different parts of the plant such as roots, seeds, flowers, stems, and leaves of a wide variety of species and face activities towards phytopathogens and even human pathogens (Barbosa Pelegrini et al, 2011). Therefore, plant AMPs are considered as promising antibiotic compounds in different fields as described in (Gordon et al, 2005, Diamond et al, 2009).

1.2.1 AMP families

AMPs are found in different species and are grouped into several families, but have some characteristics in common. Most of the natural occurring antimicrobial peptides have a length of 10-50 amino acids and range in size from 2-9kDa. 17% of the amino acids of AMPs in plants are positively or negatively charged (Barbosa Pelegrini et al. 2011), contain a high position of hydrophobic amino acid, are stabilised by several disulfide bonds, occur in a three-dimensional structure and have the feature to target outer membrane structures (Nawrot et al, 2014).

AMPs are grouped on the basis of their amino acid sequence, number of cysteine residues and their spacing. Furthermore, they can be restricted in anionic AMPs (AAMPs) and cationic AMPs (CAMPs) (Nawrot et al, 2014).

In summary, the main families of AMPs display thionins, lipid transfer proteins, defensins, cyclotides, snakins, and hevein-like proteins, which are briefly discussed in the following pages.

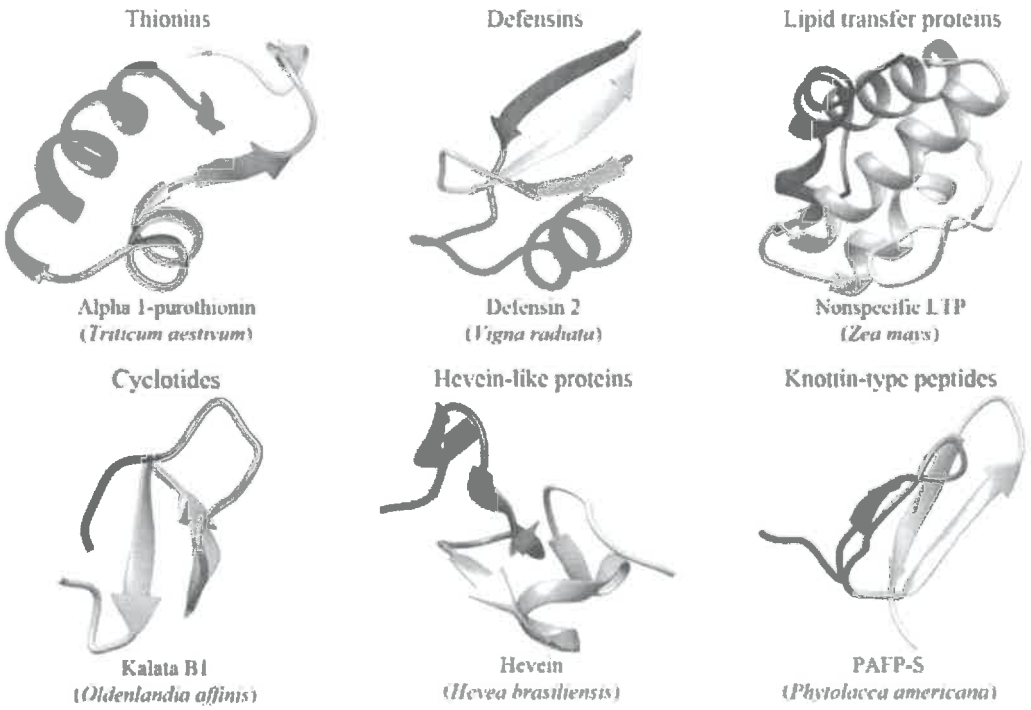

Figure 1: Selection of three-dimensional structures of the main families of AMPs by (Nawrot et al, 2014).

In the following table, the most common AMP families are listed and their characteristics are displayed. All AMPs need correct folded disulfide bridges for their stability and activity, which has to be considered within expression experiments, as proven in (Haag et al, 2012).

Table 1: Summary of the most common AMPs with structural characteristics are shown (Nawrot et al, 2014).

AMP family	Characteristics
Thionins	Low molecular weight ~5kDa; rich in arginine, lysine and cysteine residues. Two antiparallel α-helices and an antiparallel double-stranded β-sheet, linked with 4 conserved disulfide bridges
Defensins	Initially classified as a γ-thionin; ~5kDa molecular weight; basic, cysteine rich peptide, 45-54 amino acids; positively charged; Triple-stranded β-sheet with an α-helix in parallel and stabilized by 4 disulfide bridges
Lipid transfer proteins	Capable of exchanging lipids between membranes in vitro and all show a common structure of a hydrophobic cavity, enclosed by 4 α-helices, connected with 4 disulfide bonds

Puroindolines	Have a unique tryptophan-rich domain; weight of 13kDa, contain 5 disulfide bridges
Snakins	Comprise the cellwall-associated peptide snakin-1 (StSN1) and snakin-2 (StSN2), 63 amino acid residues; ~6.9kDa molecular weight. Only 38% sequence similarity but have identical antimicrobial activity. All own 12 conserved cysteine residues and 6 disulfide bonds
Cyclotides	Circular proteins with high sequence similarities and a structural identity; They consist of 28–37 amino acids, contain a head-to-tail cyclised backbone; 3 intermolecular disulfide bonds, which are arranged in a cysteine backbone knot topology (cyclic cysteine knot, CCK)
Hevein-like proteins	4.7kDa molecular weight; bind chitin; 20–40 amino acids with several cysteine and glycine residues at conserved positions--> chitin-binding domain, which is responsible for binding carbohydrates; they differ in the number of disulfide bonds; most of them possess 8 cysteine residues, which form 4 disulfide bonds

Table 2: Additional occurring plant AMPs (Nawrot et al, 2014).

Other plant AMPs	
Ib-AMPs	Four smallest (20-mer) antifungal and antibacterial peptides. Contain a well-defined loop structure, stabilized by 2 disulfide bonds
Knottin-type peptides	Consists of 6 cysteine residues, which form 3 disulfide bonds, an amide backbone of approximately 30 amino acids; resulting in a notably characteristic 'pseudo-knotted' structure. Can be further divided in subclasses (Reinwarth et al, 2012)
2S albumin proteins	Are synthesized as a single large precursor polypeptide of 18–21kDa. Two subunits of 8–14 and of 3–10kDa. Have 4 helices and 4 disulfide bonds. Found in the α-amylase/trypsin inhibitors and nonspecific lipid transfer proteins

1.2.2 Plant AMPs potential for pharmacy and biotechnology

One of the advantages of AMPs is, that they are encoded in small genes with conserved regions, thus the possibility of targeted amplification is given. Highly promising are also transgenic experiments, which can lead to an increased production of AMPS with specific activity and selected peptides. Therefore, AMPs have a high potential for therapeutic use in healthcare and agriculture (Datta et al, 2015). They can be used as natural antibiotics as an alternative for their chemical counterparts in the plant and animal kingdom. Major problems faced by the expression of AMPs, are their intrinsic toxicity, low stability of some of their compounds and the difficulty to ensure correct disulfide bond formations in the compartment where they are expressed.

Future prospects consist of developing a plan, regarding to fulfil the following aspects. First, it would be a point to ensure AMPs to be less toxic for their compartment, where they are expressed (Montesinos et al, 2007), but still preserve their antimicrobial activity. Moreover, they should maintain their correct disulfide formation and thus, their stability and activity. Additionally, a decrease in production costs is necessary, to ensure as well lower costs in the end product (Nawrot et al, 2014).

In conclusion, this thesis should be a concept of how AMPs could be expressed in a higher yield by following the above mentioned approaches.

1.3 Exogenous expression in *E.coli* bacteria

Endogenous overexpression of proteins, in the actual host, is rather expansive and protein yields are often quite low after purification. Beside this method, an exogenous expression is possible in bacteria. It is always necessary to bear in mind, in which extend the expression system can fulfil the scientific question and considers the properties of the desired protein or peptide.

E.coli bacteria are simple, cost effective hosts for the production of high amounts of specific proteins or peptides. Nevertheless, recombinant protein expression in *E. coli* is limited by their physiological features and leads often to difficulties in obtaining the native form of the desired protein. Some specific structural classes, such as polypeptides, that undergo extensive post-translational modifications, can be aggregated and intermediates are folded, instead of yielding the native proteins (de Marco 2009).

Another problem is faced with the production of proteins, which depend on disulfide bridges for their stability and activity, thus AMPs are counted to them.

Procaryotes have an reducing cytoplasm which doesn't allow proper disulfide bond formation and hence cannot be active under these conditions. Therefore, bacteria have export systems and enzymatic activities, which ensure the formation and the quality control of disulfide bonds in the oxidizing periplasm (Seras-Franzoso et al, 2012). In the article of (de Marco 2009), different strategies are explained how to exploit the physiological mechanisms of bacteria, to produce properly folded disulfide-bonded proteins. Other articles certify the importance of considering oxidizing mutant strains, plasmids for the overexpression of chaperones and foldases and several other expression alternatives (Samuelson, J.C., et al, 2012; Lobstein et al, 2012; Saaranen et al, 2012).

Furthermore, they indicate, that an optimized DNA sequence, improved expression conditions, the choice between cytoplasmic and periplasmic folding and accumulation of the target protein, are important factors. Moreover, the right selection of leader sequences (Low et al, 2013), suitable bacteria strains, and the optimized purification strategy, are necessary for a successful purification of the target protein.

In this study, plant AMPs were considered as expression product, which are usually positively charged and their three-dimensional structure is stabilized by several disulfide bonds. Disulfide bond formation in proteins is critical in the biogenesis of proteins and has been discussed by (Saaranen et al, 2012). The reducing cytoplasm of *E. coli* is therefore not suitable for AMP expression. This environment would lead to incorrect folded AMPs, which is caused by glutaredoxin (gor) and thioredoxin-reductase pathways. In the study of (Lobstein et al, 2012), they modified these pathways in a new *E. coli* strain called SHuffle C3030. This strain holds a mutation in the peroxidase ahpC, which leads to a suppression of the trxB and the gor reductase and therefore, an oxidizing environment in the cytoplasm is given (Bessette et al. 1999; Faulkner et al, 2008).

Protein translocation:

In this study the Sec- and two-arginine Tat-pathways are the most important ones. In the figure in the end of the text, the Sec-pathways are labelled with A/B and the Tat-pathway with C. They give a visual overview of these systems. The Sec-pathway is the most common pathway for protein translocation. Over 90% of the proteins produced in *E. coli* are translocated via the Sec-pathway.

The Sec machinery is a complex system, wherein SecB first binds to the pre-protein to make it transportable. SecA then functions as a translocation ATPase through binding with the pre-protein and adenosine triphosphate (ATP). Furthermore, SecA inserts into the membrane and forms a translocation complex with the Sec translocase, SecY/SecE/SecG. A protein-conductin channel is formed with SecE and SecY, while SecG stimulates the translocation. Proteins are translocated in an unfolded state using energy from ATP hydrolysis and a proton gradient. As a final step in translocation, the signal peptidase cleaves the signal peptide and SecD catalyzes the release of the mature protein from the cytoplasmic membrane. Furthermore, the protein is directed to the SecA/SecYEG complex, with the presence of SecB or the signal recognition particle (SRP). Moreover, SecB targets less hydrophobic signal peptides post-translational. SRP targets highly hydrophobic signal peptides and membrane proteins co-translationally. The SRP pathway includes the *E. coli* SRP (ffh) and FtsY. The SRP binds to the new signal peptide, which targets the ribosome to the translocase via the FtsY receptor.

The Sec independent pathway is called the twin-arginine transport (Tat) system. Tat machinery consists of three different membrane proteins, TatA, TatB and TatC. The TatBC complex recognizes the target proteins and activates the recruitment of the TatA complex. This forms a translocon within the cell membrane. One of the characteristics of the Tat-pathway is, that it can recognize folded proteins for export to the periplasm. Proteins that own cofactors for folding or have the ability to fold independently in the cytoplasm can be transported within this pathway (Low et al, 2013).

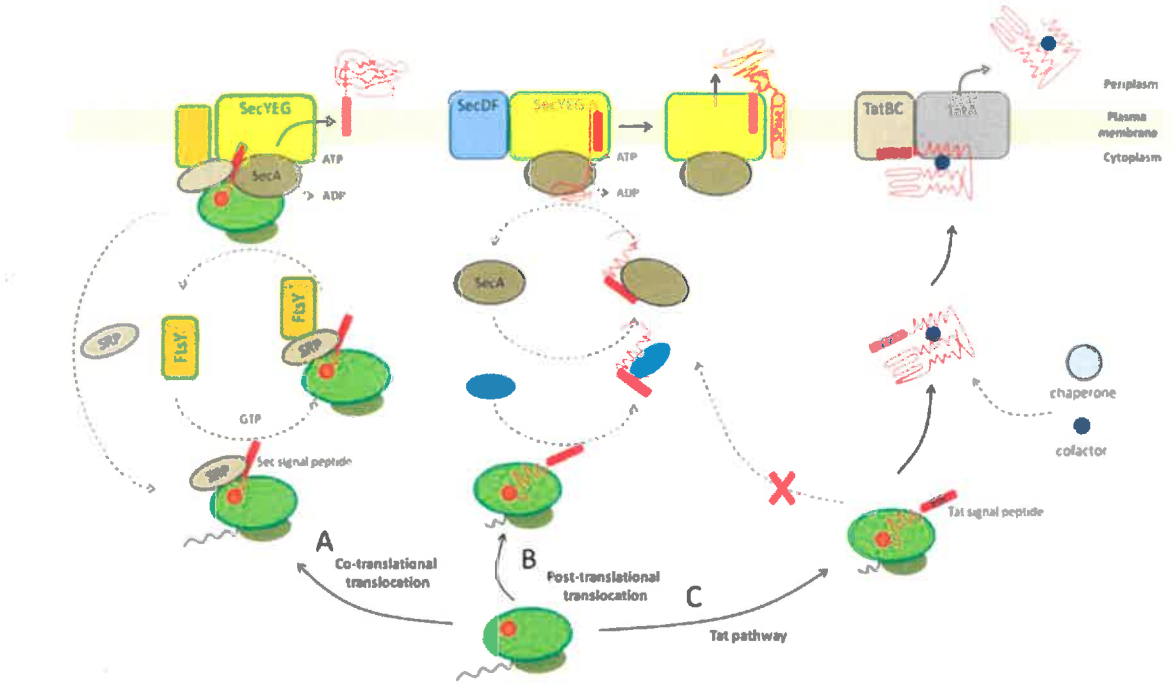

Figure 2: In this figure the Sec-pathways and the Tat-pathway are shown. Pathway A shows the co translational-translocation. Pathway B shows the post-translational translocation and pathway C shows the Tat route (Low et al, 2013).

Types of protein secretion:

In the following figure, the different bacterial secretion systems of proteins are displayed.

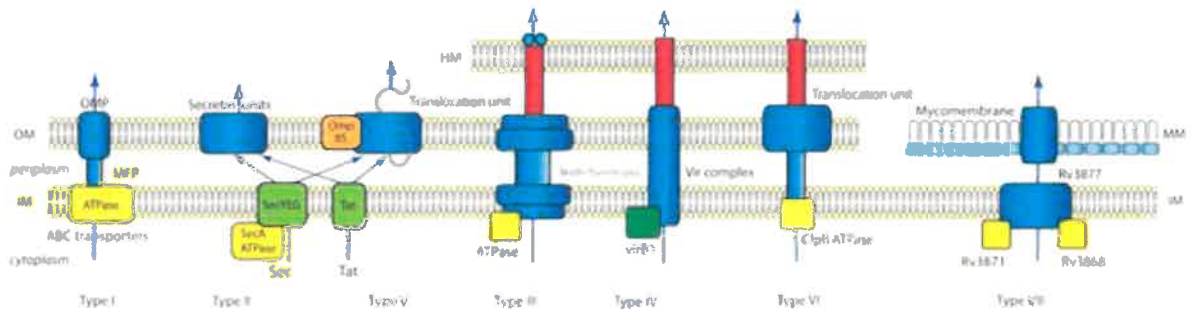

Figure 3: Bacterial secretion systems
HM: host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow (Tseng et al, 2009).

Figure 3 summarizes the main secretion systems known in gram-negative bacteria. Some proteins are secreted and exported across the inner and outer membranes in a single step via the type I, type III, type IV or type VI pathways. However, some proteins are first exported into the periplasmic space via the universal Sec or two-arginine (Tat) pathways and then translocated across the outer membrane via the type II or type V machinery. In a few cases also the type I or type IV machineries are used (Tseng et al, 2009).

Disulfide bond formation in the periplasm:

As stated in (de Marco et al, 2009) disulfide bond formation is a slow progress in the periplasm and has to be enzymatically catalyzed. The five members of the Dsb protein system (DsbA, B, C, D, G), regulate protein oxidation in the periplasm. All proteins belong to the thioredoxin protein family, except DsbB, and are involved in both disulfide-bond formation and rearrangement. The soluble monomer DsbA gives its disulfide bond to the new synthesized polypeptides, as seen in the figure below. DsbA then binds specific to partially unfolded proteins by hydrophobic interactions with the use of a chaperone-like recognition mechanism and is re-charged by the inner membrane DsbB (de Marco et al, 2009).

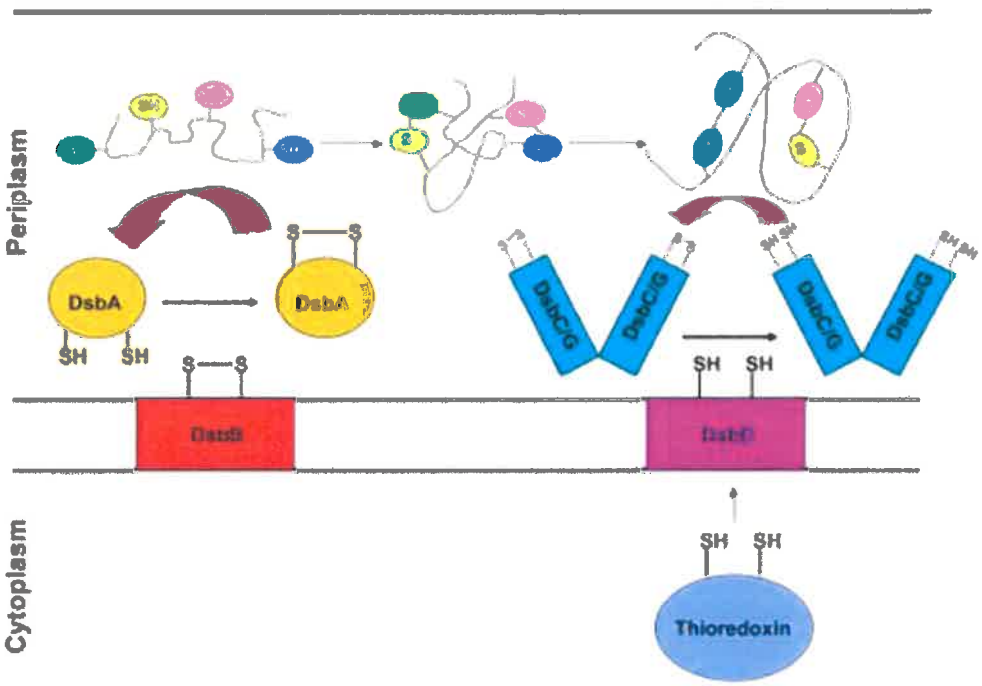

Figure 4: Protein oxidation and isomerization in the procaryotic periplasm.

DsbA couples cysteines providing the disulfide bond and is re-charged by the inner membrane DsbB. Not correct disulfide arrangements of oxidized proteins are scrambled by DsbC and DsbG. The involved isomerases are kept reduced by the inner membrane DsbD. DsbD itself is kept reduced by cytoplasmic thioredoxin, which ultimately obtains electrons from NADPH through thioredoxin reductase (de Marco et al, 2009).

As already mentioned, in this study the *E. coli* strain BL21 (DE3) have been used for periplasmic protein expression. The T7 RNA polymerase (T7 RNAP) -based protein production strain BL21(DE3) in combination with T7-promoter based expression vectors is widely used (Zhang et al, 2015). In this thesis an expression protocol for protein A was implemented with this T7 system and is discussed in the following chapter.

1.3.1 T7 expression system

Many expression systems need a regulated expression of T7 RNA polymerase, which allows them to produce large amounts of protein. T7-RNA polymerase is an enzyme, which is originally encoded in the DNA of bacteriophage T7. This polymerase transcribes the DNA, which begins with a specific 23-bp promoter sequence called the T7-promoter. Copies of the T7-promoter are located at several sites on the T7 genome, but are not present in the chromosome of *E. coli*. Therefore, T7-infected cells express mainly viral genes. This property can be used for expressing foreign DNA in *E.coli* chromosomes. In this study *E.coli* BL21 (DE3) cells were used. The recombinant *E. coli* cells carry the gene encoding the T7 RNA polymerase next to the *lac*-promoter. These cells are then transformed with plasmid vectors that carry a copy of the T7- promoter and, adjacent to it, the cDNA encoding the desired protein. In this case they carried the sequence of protein A. The protein expression is induced with IPTG, by adding it to the culture medium containing the transformed BL21 (DE3) cells. The T7 RNA polymerase is expressed by transcription from the *lac*-promoter. The polymerase then binds to the T7-promoter on the pET20bHis vector and catalyzes the transcription of protein A. In each *E. coli* cell many copies of the expression vector are present, which lead to numerous mRNAs, that are translated to protein (Lodish et al, 2000).

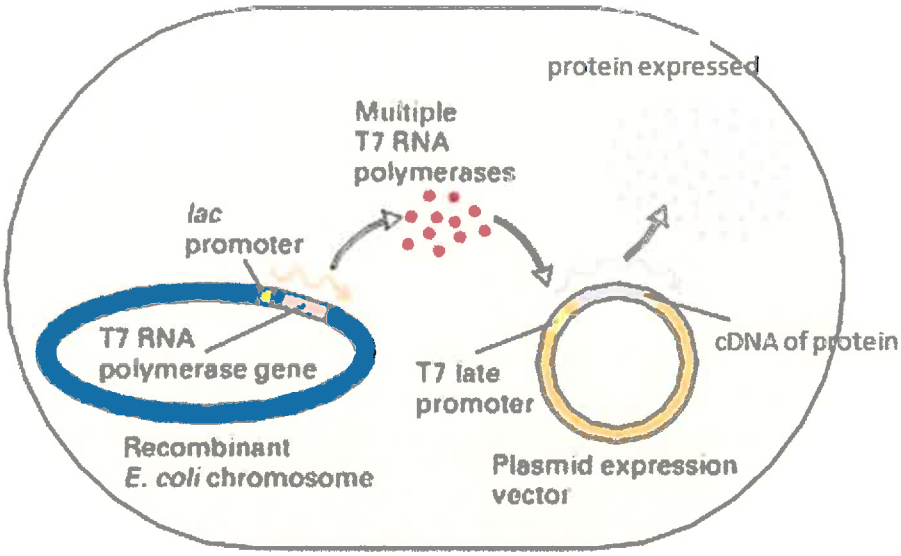

Figure 5: Protein expression in an *E. coli* host, displaying the T7 expression system (Biotechnology Information Space, Azhar et al, 2012).

1.3.2 Alternative prokaryotic expression systems

Escherichia coli is probably the most common expression host. Nevertheless, other bacterial expression systems have been taken in consideration.

The *Lactoccocus lactis* system has proven to be a viable choice for membrane proteins. Moreover, several *Pseudomonas* systems were developed and achieved product yields comparable to *E. coli* systems. Furthermore, bacterial systems such as *Streptomyces*, coryneform bacteria, and halophilic bacteria have different advantageous properties, which can promote niche research. With these alternative expression systems, different aspects can be taken in consideration with challenging proteins (Chen, 2012).

1.4 Protein A

In pathogenesis of *Staphylococcus aureus*, Staphylococcal protein A (SpA) plays an important role. It's advantageous properties lead to its widely use in biotechnology (Rigi et al, 2014).

SpA has a high affinity to several classes of Immunoglobulin G (IgG) and has been used for antibody purification. SpA is ~42kDA heavy, type 1 membrane protein, which consists of three different functional regions. The N-terminal region harbours the 5 homologous domains (A-E), which are responsible for the interaction with

different classes of IgG (Moks et al, 1986). SpA holds as well a secretion signal sequence and a cell wall linkage region. The whole protein A has been purified from *Staphylococcus aureus*, even though only in low yields, by (Hammond et al. 1990). The 5 different domains have been attempted to be isolated as well, successfully, fragments B and E were already cloned in expression experiments (Moks et al, 1986). Further studies even revealed, that properties of fragment D were sufficient for the secretion of various target proteins into the periplasmic space of *E. coli* (Heel et al, 2010). Moreover, even studies demonstrated SpA as a potential fusion protein and had a closer look on its structure (Graille et al, 2000).

In the following figure the composition of protein A is shown. Additionally, the crystal structure of SpA is displayed. The specific amino acid sequence of protein A is shown in chapter 3.2.1.

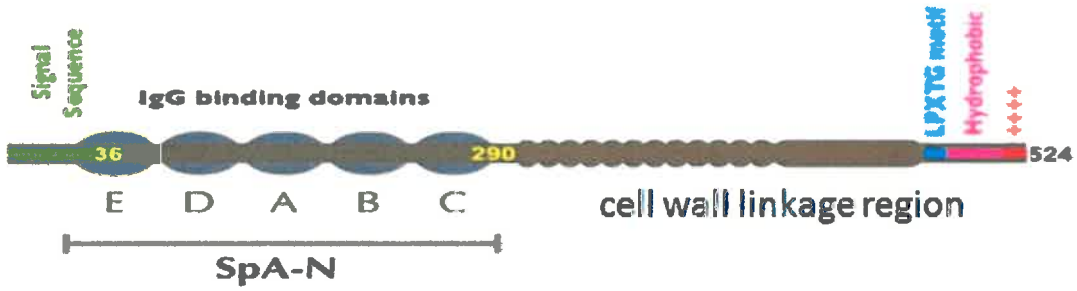

Figure 6: Protein A.
The N-terminal part with the 5 IgG binding domains, the signal sequence and the cell wall linkage region are shown (Oas Lab, Duke University, Staphylococcal Protein A).

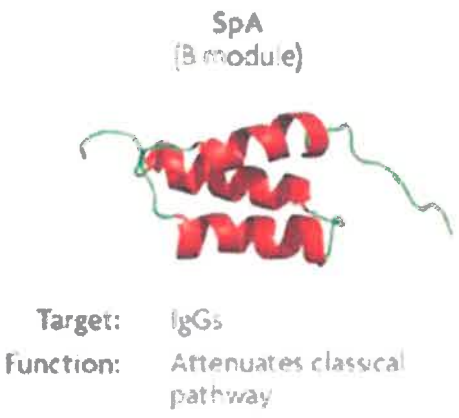

Figure 7: Crystal structure of *Staphylococcal aureus* protein A (Lambris et al, 2008).

Protein A, has been studied since the early 1980s and is a well promising partner for recombinant protein expression in *E.coli*. In the following chapter, a protocol is described which managed to gain a high yield of SpA through secreting it into the medium. In this study, the following paper acted as an incentive for the idea of using the SpA as fusion protein for periplasmic expression of AMPs.

1.5 Protein expression protocol by (Freiherr von Roman et al, 2014)

In this protocol they exceeded to gain an extensive yield of protein A.
They took the N-terminus part of the protein A gene sequence coding for the five IgG binding domains. Additionally, they fused it to a pelB signal peptide, a sequence of amino acids, which when attached to a protein, directs it to the bacterial periplasm. There the sequence is removed by a signal peptidase, which is responsible for periplasmic localization. The protein is then translocated into the periplasmic space of *E.coli*.
They used a pET20bHis vector carrying a T7-promotor and the SpA sequence. The transformation was done with BL21 (DE3) cells, which carry the T7-polymerase gene. Furthermore, they induced the protein expression with IPTG and added Tris-HCl pH8.5 up to a final concentration of 180mM. With this optimized release protocol, more than 380mg/L of protein A were obtained. Therefore, a protocol was developed for the extracellular production of SpA in a stirred tank bioreactor yielding 5.5g/L of the secreted protein A.
This protocol is an attractive scientific approach to use the properties of protein A and to produce therefore as well high yields of different proteins of interest.

1.6 Aims of this study

The aim of this Masterthesis is to develop a concept for a fusion carrier for extracellular expression of antimicrobial peptides (AMPs) in *E.coli* bacteria.
As discussed prior, plant AMPs are usually positively charged and their three-dimensional structure is stabilised by several disulfide bonds. Moreover, these bonds cannot be formed properly in the cytoplasm of *E. coli* bacteria. Hence, it is an advantage to secrete them into the extracellular space, where the correct disulfide bonds can be provided.

The protocol discussed in the chapter before, which deals with the improved extracellular expression of protein A in *E. coli* strain BL21 (DE3), has been taken as basis of this masterthesis.

The following points resemble the main steps of the achieved concept.

- Verification of the improved protein A expression protocol.
- Amplification of protein A sequence, serving as a template for the amplification of the five homologous domains (A-E) of the N-terminal part of the protein A sequence.
- Construction of a fusion carrier, a pET-vector derived from pETtrx_1a.
    - Elimination of the HindIII restriction side of pETtrx_1a, by cutting with HindIII and filling up with Klenow.
    - Insertions of HisLinker, by cutting with XbaI and BamHI.
    - Insertions of an amplified protein A fragment by cutting with XbaI and HindIII.

2. Material and methods

2.1 Cultivation methods

2.1.1 Liquid and solid media

The composition of the media used in this study are shown in the following tables.

Table 3: LB-medium

Ingredients	Amount per 1L
Tryptone	10g
Yeast extract	5g
NaCl	10g
(Agar)	15g

For 1L of LB-medium, the ingredients of the table above were dissolved in 1L ddH_2O (for solid media, 15g agar were added) and autoclaved. For selective media antibiotics were added after autoclavation.

Table 4: Composition of M9-minimal media.
The ingredients in green were dissolved in 1L ddH_2O and autoclaved. Ingredients in blue and red were added afterwards sterile filtered. The pH was set at 7.4.

Ingredients	Amount per 1L
Na_2HPO_4	12.8g
KH_2PO_4	3.0g
NaCl	0.5g
NH_4Cl	2.0g
glucose	20.0g
$CaCl_2$	0.1mM
$MgSO_4$	1.0mM
$FeCl_3$	10.0µM
pH 7.4	

For 1L M9-minimal media, 12.8g Na_2HPO_4, 3.0g KH_2PO_4, 0.5g NaCl, 2.0g NH_4Cl were dissolved in 900ml ddH_2O and autoclaved. After autoclavation 20g of glucose

were dissolved in 100ml ddH_2O, sterile filtered with a syringe filter with a pore size of 0.22µm, and added. Stock solutions with a concentration of 1M of $CaCl_2$, $MgSO_4$ and $FeCl_3$ were produced. The solutions were again sterile filtered and 100µl $CaCl_2$, 1000µl $MgSO_4$ and 10µl $FeCl_3$ were taken from the stock solutions and pipetted to the medium to obtain the final concentrations listed in the table above. The final pH-value was set to 7.4.

2.1.2 Competent cells for transformation

The cells of the different *E. coli* strains used in this study were made chemical competent as described below. The detailed composition of the buffers used, is described in the following tables.

Bacteria were grown in 250ml LB-medium without antibiotic to an OD_{590} (optical density at 590nm) of 0.6 for BL21 (DE3) and to 0.8 for DH10B at 37°C on a rotary shaker in the incubator. After checking the specific OD, the flasks with the bacterial culture were cooled on ice. The cold bacteria were centrifuged for 15min with 4500rpm at 4°C. The supernatant was discarded and the pellet was dissolved in 100ml ice cold TFB1 buffer. After incubation of the resuspended cells on ice, they were centrifuged again for 10min with 3000rpm at 4°C. The supernatant has been discarded again and the pellet was resuspended in 20ml of ice cold TFB2 buffer. The cells were incubated for 30min on ice. In the meanwhile a rack with 1.5ml Eppendorf-caps was put in an ice box with some liquid nitrogen. After the incubation time, aliquots of 100µl of the cells, were pipetted in the precooled Eppendorf-caps and stored at -80°C.

Buffer solutions:

Table 5: Components of buffer TFB 1.

Ingredients	Amount per 1L
KAc	30mM
RbCl	100mM
$CaCl_2 . 2H_2O$	10mM
$MnCl_2 . 4\ 2H_2O$	50mM
glycerol	15%
pH 5.8 with HA (acetic acid)	

Table 6: Components of TFB 2.

Ingredients	Amount per 1L
MOPS (3- Morpholinopropanesulfonic acid)	10mM
$CaCl_2 . 2H_2O$	75 mM
RbCl	10mM
Glycerol	15%
pH 6.8 with KOH	

All Buffers were prepared by sterile filtration with a syringe filter with a pore size of 0.22µm.

2.1.3 Cultivation of Bacteria

Cultivation of DH10B:

Competent cells of the *E.coli* strain DH10B were prepared and used for cloning experiments with the pET-vector constructs, which had a kanamycin resistance. The transformed cells were grown in LB-medium or on LB-agarplates with 50µg/ml kanamycin.

Cultivation of BL21 (DE3) and gene expression:

Competent cells of the *E.coli* strain BL21 (DE3) were prepared and used for protein A expression experiments.

BL21 (DE3) were transformed with the vector pET20bHis containing the sequence for protein A expression. Transformation steps were performed as described in 2.2.1. 5ml of LB-medium were supplemented with 100µg/mL ampicilin and inoculated with a single colony of the transformed BL21 (DE3). The 5ml tubes were incubated for 8 hours at 37°C shaking with 180rpm to obtain a seed culture. For the protein expression, doublets of 100ml M9 minimal medium in a 250ml flask were inoculated with 2ml seed culture and cells were grown for 16h at 37°C up to an OD_{600} of 3.5 on a shaker with 180rpm. One flask was induced and the other served as control and was only treated with Tris-HCL solution. Finally, the temperature was lowered to 30°C and induction was proceeded with 1mM IPTG (Isopropyl-β-D-thiogalactopyranosid) and Tris-HCL was added up to a final concentration of 180mM. Furthermore, a stock of a 2molar Tris-HCL solution with a pH of 8.5 was provided.

Ten percent (v/v), which equals 10ml of the stock culture, were added with sterile filtration to the induced cells and to the control flask. Moreover, a 1molar stock of IPTG was prepared. For an end concentration of 1mM IPTG, in an end volume of 112ml, 112µl of the stock solution were used for induction. The expression of protein A was examined after 24 hours.

2.2 Molecular and genetic methods

2.2.1 Transformation of *E.coli*

The vectors used in this study were transformed in different *E. coli* strains.

The pET20bHis vector was transformed in BL21 (DE3) cells. The pETtrx_1a constructs were transformed in DH10B cells. Therefore, 100µl of competent *E. coli* were taken out of the -80°C freezer and thawed on ice. 5µl of the vector were pipetted to the competent cells and vortexed. After 15 minutes incubation on ice, they were heat shocked at 42°C for 1 minute and put again on ice for additional 5 minutes. Then 200µl of LB-medium were added and incubated at 37°C for 45 minutes shaking at 800rpm. Afterwards, 200µl of the cells were plated on a LB-agarplates. The DH10B cells were grown on plates with 50µg/ml kanamycin and BL21 (DE3) on plates with 100µg/ml ampicilin. Both were incubated over night at 37°C.

2.2.2 Plasmid preparation

The plasmids used for transformation and PCR templates, were prepared and extracted due to the manual of the GeneJET Plasmid Miniprep Kit (Thermo Scientific). In figure 9 pET20bHis containing the protein A template and pETtrx_1a used for constructing a new expression vector are shown.

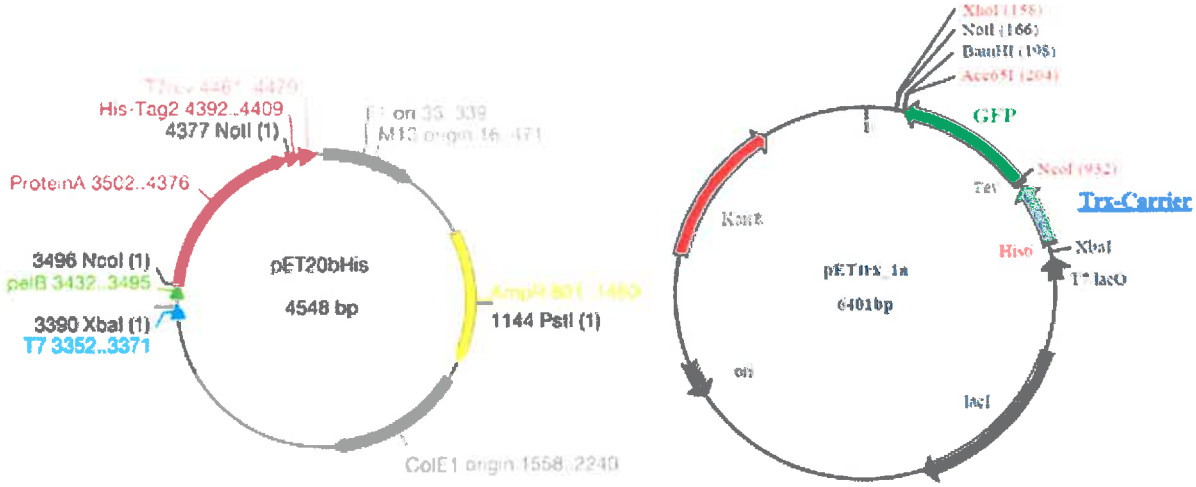

Figure 8: Plasmids used in this study.
pET20bHis with protein A template and pETtrx_1a used for constructing a modified expression vector (LaVallie, et al, 1993).

2.2.3 PCR- techniques

PCR (Polymerase chain reaction) was proceeded in a Mastercycler Gradient (Eppendorf) with Phusion High Fidelity Polymerase (Thermo Scientific). Polymerase HF Buffer (Thermo Scientific), 10mM dNTPs Mix (Thermo Scientific), 10mM specific primers (Sigma-Aldrich) and DNase free water were also used. The denaturation temperature was set with 98°C for 30 seconds (1 cycle).

The periodical denaturation at 98°C was proceeded for 10 seconds. The annealing temperatures from 47°C to 67°C for 20 seconds were depending on whether the whole protein A sequence was amplified or the 5 different domains of protein A (35 cycles). The final elongation temperature was set at 72°C for 10 minutes and was then held at 4°C.

The gradient PCR was done in the same cycler. The mean value was set with 57°C and a 10 degree gradient, ranging from 47°C to 67°C to obtain the optimal annealing temperatures for the single fragments.

To check if the right fragment has been transferred, a colony PCR was performed. Therefore, Taq-Polymerase and Polymerase Buffer (Peqlab) were used and the denaturation temperature was set at 94°C. The same primers to amplify the whole protein A sequence, pETforII and pETrev, were used to detect the inserts of the new constructed pETtrx_1a.

Table 7: Primer sequences used for insert construction

Primer	Length [bp]	Neucleid Acid Sequence 5'-3'
pETforII	20	TCCCGCGAAATTAATACGAC
Protein A-ArevHind	24	TATAAGCTTTGGTGCCTGAGACTC
Protein A-BrevHind	24	TATAAGCTTCGGTGCTTGGGCATC
Protein A-CrevHind	24	TATAAGCTTCGGAGCCTGCGCGTC
Protein A-DrevHind	24	TATAAGCTTAGGAGCCTGTGATTC
Protein A-ErevHind	24	TATAAGCTTCGGTGCCTGGCTATC
pETrev	20	ACTAGCATAACCCCTTGGGG

2.2.4 DNA purification

The DNA has been purified between the working steps from remaining enzymes and primers with a GeneJET PCR Purification Kit (Thermo Scientific) as in the manufacturers manual.

2.2.5 Agarose gel electrophoresis

The amplified PCR-products and vector constructs were checked by agarose gel electrophoresis. The gel was prepared with TAE-buffer (40mM Tris, 0.11% (v/v) glacial acetitic acid, 1mEDTA) and 1%-1.5% agarose, depending on the different sizes of the fragments. The agarose was dissolved by heating in the microwave and afterwards ethidiumbromide was added to a final concentration of 0.8µg/ml. The gel chamber (Bio-Rad) was filled with the same TAE-buffer. The samples were prepared with 6x DNA loading buffer (Thermo Scientific) and loaded to the wells. As DNA ladders 100bp or 1kb Gene Ruler (Thermo Scientific) were used. The current was set 85V for 35 minutes provided by a PowerPac Basic power supply system (Bio-Rad). To visualize the DNA with ethidiumbromide an UV lamp with 245nm Gel Doc XR+ system supported by a LabTM Software version 4.0.1 (Bio-Rad) was used.

2.2.6 HisLinker preparation

The fragments HisLinkerA and HisLinkerB (in a ratio 1:1) were constructed to a double strand by heating them up to 60°C for 5 minutes and cooling them down to room temperature. They were inserted in the target vector by double digestion with the enzymes XbaI (Fermentas) and BamHI (Fermentas), as described in the following chapter. The presence of this linker in the vector, created a new HindIII

restriction site and allows the protein A fragments to be inserted in the vector. Moreover, the HisLinker posses 6 histidine residues. The sequence of the HisLinker is shown in figure 10.

Figure 9: The HisLinker sequence.
The colours green, yellow and red mark the restriction sites. The histidine residues are highlighted in blue (Source: author).

2.2.7 Digestion of plasmids and other constructs

The restriction enzymes and buffers were stored at -21°C. For the double digestions with XbaI and BamHI and XbaI and HindIII the 10x buffer Tango (Fermentas) was used. For a double digest of a total volume of 20µl, 1µl of each enzyme, 2µl buffer, 10µl vector and 6µl ddH_2O were used. The digestions were left for 4 hours at 37°C and stored at 4°C over night, if not used the same day. A single digest was proceeded the same way as a double digest with the exception, that for a single digestion with HindIII 10x buffer R (Fermentas) was used.

Table 8: Restriction enzymes with the sequence they cut.

Restriction enzyme	Sequence cut
XbaI	TCTAGA
BamHI	GGATCC
HindIII	AAGCTT

2.2.8 Klenow - DNA blunting

The pETtrx_1a plasmid was cut with HindIII (Fermentas) and Klenow (Thermo Scientific) was then used to create DNA blunting by fill in 5'-overhangs. For a 50µl reaction, 37µl pETtrx_1a cut with HindIII, 5µl Klenow Buffer (Thermo Scientific), 1.5µl Klenow (Thermo Scientific) and 1.25µl of 10mM dNTPs Mix (Thermo Scientific) were used. This mixture was left 1 hour at 37°C and then inactivated for 10 minutes at 75°C. This removed the HindIII restriction site and created a new NheI site.

2.2.9 Dephosphorylation

To prevent the plasmid from ligation with itself, phosphatase has to be added. This enzyme removes the phosphate backbone and linearizes the DNA. For a 60µl reaction, 1µl of phosphatase (Fast AP, Thermo Scientific), 50µl of the vector, 6µl of the buffer (Fast AP, Thermo Scientific) and 3µl of ddH_2O were incubated for 30 minutes at 37°C. The plasmid has then been again purified.

2.2.10 Ligation

For a 10µl ligation reaction 1µl T4 DNA ligase (Fermentas), 5µl of the vector, 3µl of the insert and 1µl of ligase buffer (Thermo Scientific) were used and incubated at room temperature for 4 hours. To check if the vector contains the correct insert, 4 transformations with DH10B where performed.

Table 9: Ligation reactions, values in µl

Ligation	1	2	3	4
Vector	2µl	2µl	2µl	2µl
Insert	1µl	5µl	x	x
Buffer	1µl	1µl	1µl	1µl
Ligase	1µl	1µl	1µl	x
ddH_2O	5µl	1µl	6µl	7µl
Total volume	10µl	10µl	10µl	10µl

The highest CFU (colony forming units) should be seen in 1 and 2, where the insert is present. Without insert and without ligase no colonies should be formed.

2.2.11 Colony selection and colony PCR

Seven colonies were selected and added to 100µl LB-medium and then incubated for 4 hours at 37°C, shaking with 450rpm. 1µl of the culture served as a template for a colony PCR as described in section 2.2.3.

2.3 Biochemical methods

2.3.1 BCA Protein Assay

To check the amount of total Protein produced with BL21 (DE3) the Thermo Scientific Pierce™ BCA Protein Assay Kit was used, following the manufacturers manual. This test is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein.

2.3.2 Tricine-SDS-Page

The protein A fragment has a size of about 40kDA and was resolved on tricine SDS polyacrylamide gels referring to (Schägger, 2006). The samples were all mixed with 2x sample laemmli buffer (12% SDS (w/v), 30% glycerol (w/v), 0.1% coomassie brilliant blue G-250 (w/v), Tris-HCl (pH 7.0) and 20mM dithiothreitol (DTT)) referring to the amount of protein 1:1 or 1:4. Then they were heated up to 95°C for 10 minutes to denature the proteins and loaded, after cooling to room temperature, on the gels. The composition of the buffers and solutions are referring to (Schägger, 2006). The gels were fixed in a Mini-PROTEAN Tetra cell system (Bio-Rad) and the cathode and anode buffers were filled into the device. First, the voltage was set to 30V for about 10 minutes until 75% of the samples wandered into the stacking gel. Then the voltage was increased to 150V until the coloured loading buffer reached the bottom of the gel. As a further step, the gels were fixed in a fixing solution (50% methanol, 10% acetic acid) for 40 min and then stained in coomassie solution (0.025% coomassie brilliant blue G-250 (w/v) in 10% acetic acid) for 30 minutes. Destaining was performed in 10% acetic acid, changing the solution every 2 hours, while letting them shake for 6 hours.

Table 10: Components of the SDS-Page gel.
Ammonium persulfate (APS) is used for polymerization and tetramethxlethylendiamine(TEMED) has the function of a catalyst.

Components	4% stacking gel	16% separating gel
AB3	333µl	3300µl
3x Gel buffer	1000µl	3304µl
Glycerol	x	1g
ddH_2O	2664µl	2500µl
APS 10%	30µl	33µl
TEMED	3µl	3.3µl

3. Results

3.1 Protein A expression following the protocol of (Freiherr von Roman et al, 2014)

3.1.1 Protein A expression in E. coli BL21 (DE3)

The vector pET20bHis has been transformed in *E. coli* BL21 (DE3), which were grown in M9 minimal medium. The induction was done with IPTG as described in chapter 2.1.3. BL21 (DE3) cells have also been cultivated with the same conditions without receiving the pET20bHis vector to prove strain specific protein bands on the gels. The samples of the induced cells and the not induced cells have been loaded on a SDS-Page gel and proteins were separated according to their size. The following figure shows two gels with samples of BL21 (DE3) cells. The left one displays the BL21 (DE3) which received the vector. Samples of cell pellets and supernatants, induced and not induced, were loaded on the gel. There are several bands visible in the cell pellets but only 2 bands can be seen in every sample. The bands at 35kDa might represent protein A. There are also bands in every sample with about 37kDA. To prove, that they are strain specific, BL21 (DE3) have been cultivated under the same conditions with the exception of not receiving the specific vector carrying protein A. The samples have been loaded as well on a SDS-Page gel and bands at about 37kDA have been detected. Moreover, there has been no difference of band intensity of induced and not induced cells in all samples.

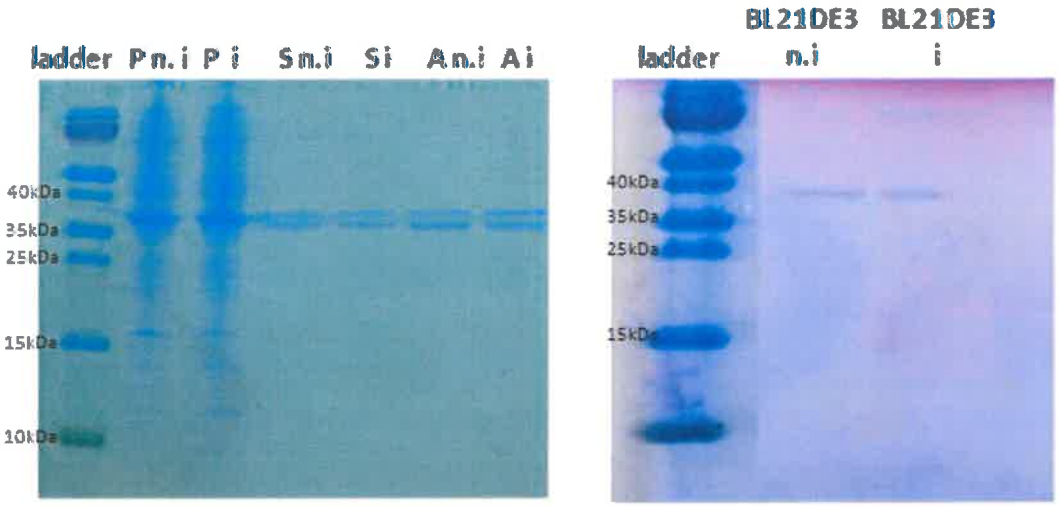

Figure 10: SDS-Page protein A.
On the left: Lane 1: marker, lane 2: cell pellet not induced, lane 3: cell pellet induced, lane 4: supernatant not induced, lane 5: supernatant induced, lane 6: acetone precipitation not induced, lane 7: acetone precipitation induced. On the right: Lane 1: marker, lane 2: BL21 (DE3) without a specific vector not induced, lane 3: BL21 (DE3) without specific vector induced.

3.1.2 Protein A expression - BCA test

The amount of protein in the samples has been measured with the BCA-test, as described in chapter 2.3.1. The not induced and induced samples had protein amounts higher than 1.5mg/ml. In the table below the values of the standards and of the supernatant of the BL21 (DE3) cells are given. As already seen in the gels, protein is present but no difference can be seen in induced and not induced samples.

Table 11: BCA-Test.
The protein concentrations of the standards and the induced and not induced cells are shown.

Content	Linear regression fit based on raw data in µg/ml (OD562)
Standard 1	1984,23
Standard 2	1478,20
Standard 3	1029,42
Standard 4	714,57
Standard 5	631,83
Standard 6	222,25
Standard 7	156,32
Not induced	1642,33
induced	1982,26

3.2 Construction of a fusion carrier with protein A fragments

3.2.1 Amplification of the protein A sequence

The whole protein A sequence on pET20bHis had to be amplified. Therefore, specific pET-primers, pETtrxfor2 and pETrev, were used to gain the N-terminal protein A sequence with some 3'- and 5'-overhangs. In the next steps this PCR product functioned as a template for the amplification of the 5 domains of protein A to receive the different fragments for cloning.

The following figure shows the N-terminal protein A sequence. The sequences of the forward primer pETforII (red) and the reverse primer pETrev (blue) are shown in the top and the bottom of the figure. The areas where the primers bind on the sequence, are indicated in the same colours. The yellow area of the sequence indicates the 5 domains of protein A.

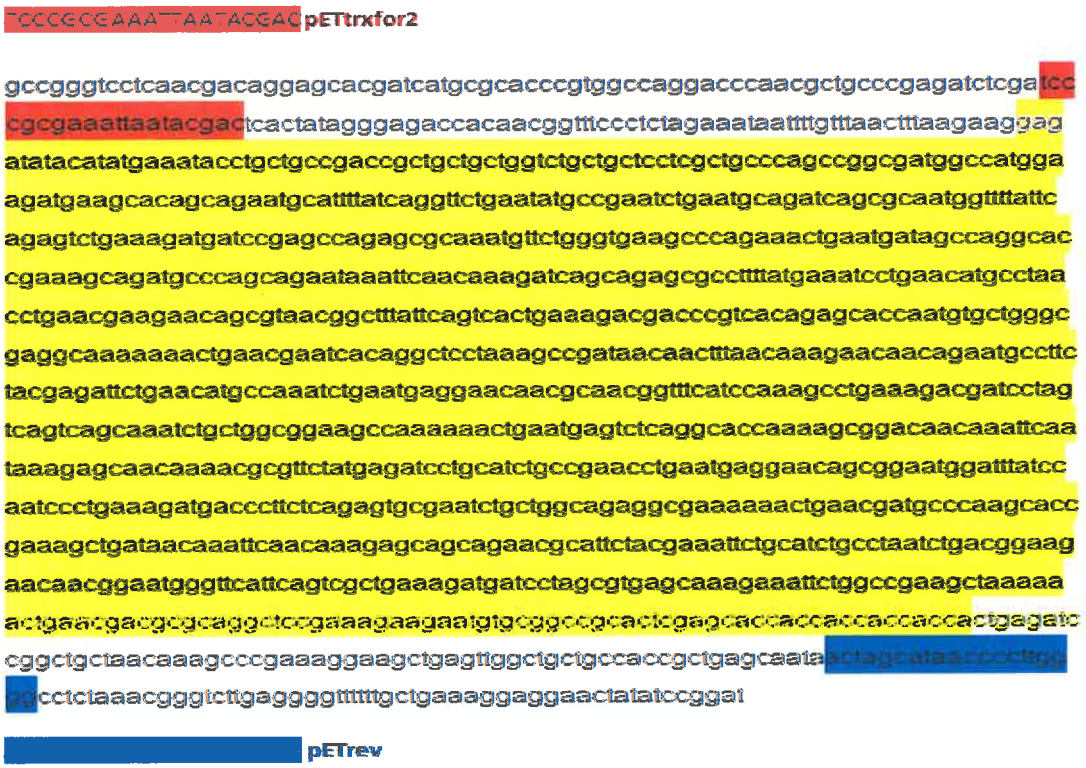

Figure 11: N-terminal sequence of protein A.
The yellow marked part represents the sequence which acts as a template for the 5 domains. Red indicates the binding site of the forward primer and blue the reverse primer. (Source: author)

The PCR was proceeded as described in chapter 2.2.3. The protein A sequence was successfully amplified, as seen in the figure below. The band of the amplified sequence is between 1000bp and 1500bp and has the right size of the protein A sequence with 1226bp.

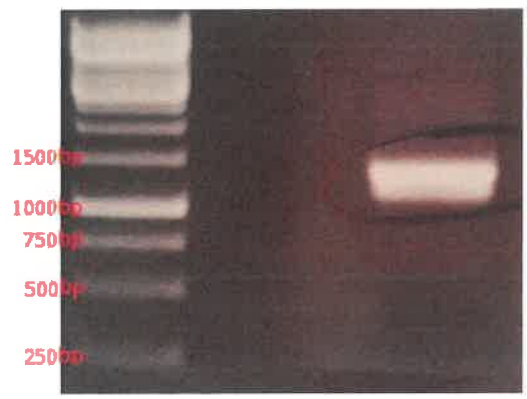

Figure 12: SpA isolated. 1.2% Agarose Gel. Lane 1: 1kB ladder, lane 2: SpA

This PCR product has been purified and served as a template for the following amplification steps. The following figure shows the N-terminal protein A sequence (amino acid sequence). The different colours show the specific primer binding sites and the length of the expected fragments after PCR amplification.

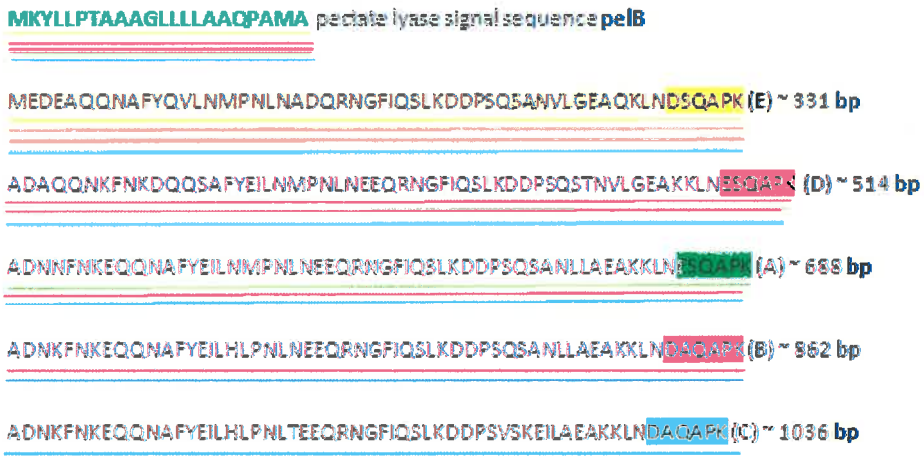

Figure 13: N-terminal sequence of protein A.
Colours indicate the specific primer binding sites for the 5 domains A-E. Yellow fragment-E; red fragment-D; green fragment-A; magenta fragment-B and cyan fragment-C. The numbers on the right, represent the approximate fragment sizes. The pectate lyase signal peptide, which is not part of the Protein A itself, is shown in green (Source: author).

3.2.2 Gradient PCR of protein A fragments

A gradient PCR was performed for all 5 domains of the protein A sequence as described in chapter 2.2.3. The optimal annealing temperatures for the single fragments were obtained: fragment-A 47°C; fragment-B 67°C; fragment-C 52°C; fragment-D 47°C and fragment-E 49°C.

In the following figure, the gradient PCR products of fragment-B with a size of 826bp are shown on a 1.5% gel.

Figure 14: Gradient PCR of fragment-B on a 1.5% gel.
Lane 1: 100bp ladder, lanes 2-8 fragment B at different temperatures.

Even though bands with the correct size could be produced, as well bands in other sizes could be seen on the gels. This can be explained because of the similarity of the 5 homologous regions. Therefore, the specific primers can bind to multiple sites of the protein A sequence.
In the following figure it can be seen, that all the fragments have been successfully amplified with their specific annealing temperatures.

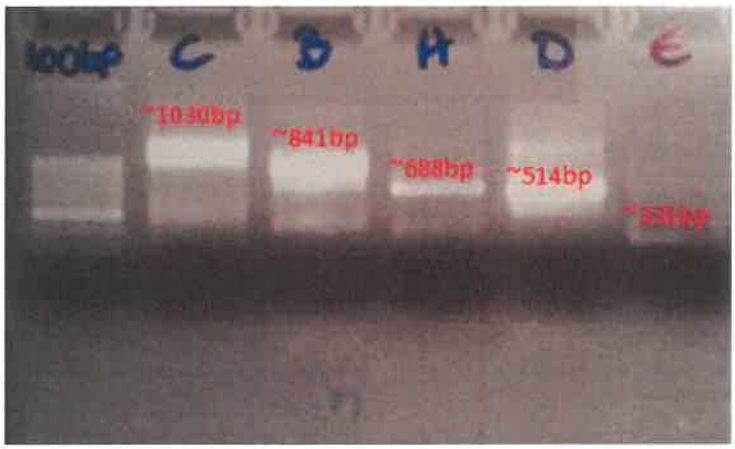

Figure 15: Fragments A-E. 1.5% agarose gel.
Lane 1: 100bp ladder, lane 2: fragment-C, lane 3: fragment-B, lane 4: fragment-A, lane 5: fragment-D, lane 6: fragment-E. Sizes of the fragments are marked in red.

To prevent losses of the amount of PCR products through DNA purifications, PCR has been done several times for each fragment to ensure a high quantity of PCR products for further cloning steps. The following figure shows the multiple amplification of fragment-D.

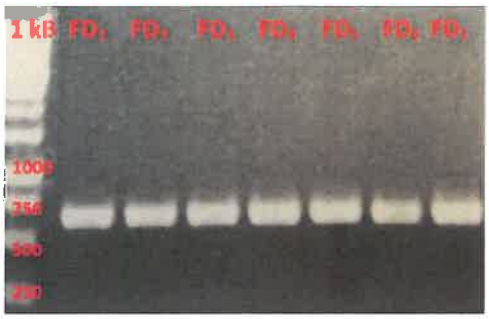

Figure 16: Fragment-D on a 1.5% agarose gel.
Lane 1: 1kB ladder; lane 2-8: fragment-D.

3.3 Construct pETtrx_1a

A modified pET vector derived from pETtrx_1a was used in this study to construct a new expression vector. The original vector has a Trx-GFP fragment, which had to be replaced by a HisLinker. This linker should allow the cloning of the protein A fragments through restrictions with XbaI and HindIII. In the following figure the pETtrx_1a vector is shown with the TRX-GFP fragment and the specific restriction sites.

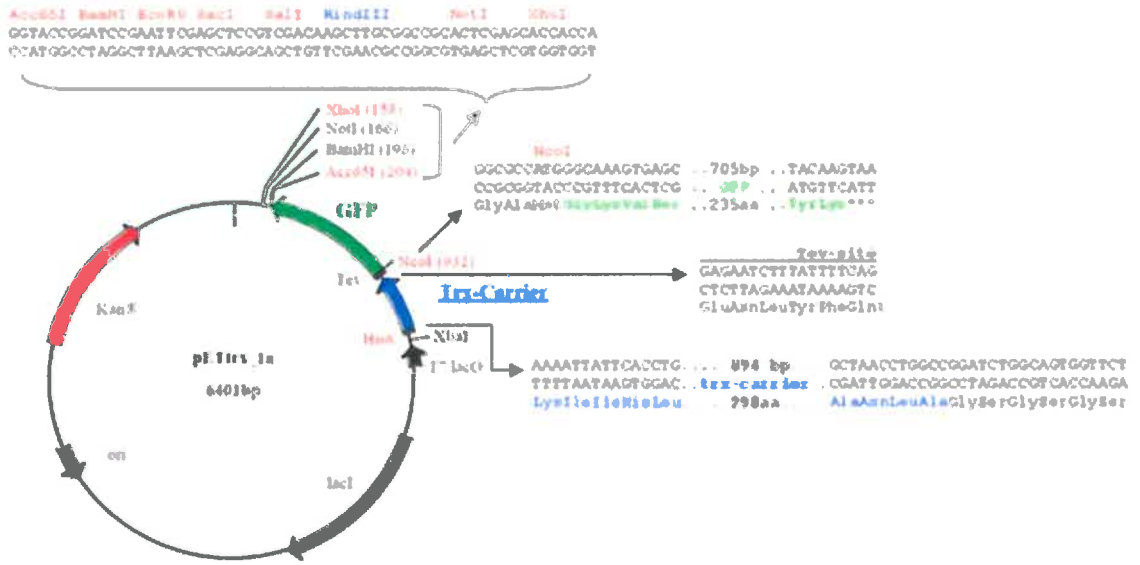

Figure 17: pETtrx1a construct.
The TRX-GFP fragment (blue and green) is shown, which can be cut out with XbaI and BamHI for linker insertion. Behind the GFP Sequence the restriction sites are shown in brackets. A single digest with HindIII is made to remove the restriction site (blue) and create a new NheI site (LaVallie et al, 1993)

3.3.2 Elimination of HindIII restriction site and creation of new NheI site

The pETtrx_1a plasmid was cut with HindIII and Klenow was then used to create DNA blunting by fill in 5'-overhangs. It was tested to cut with HindIII to prove, that the restriction site was removed.

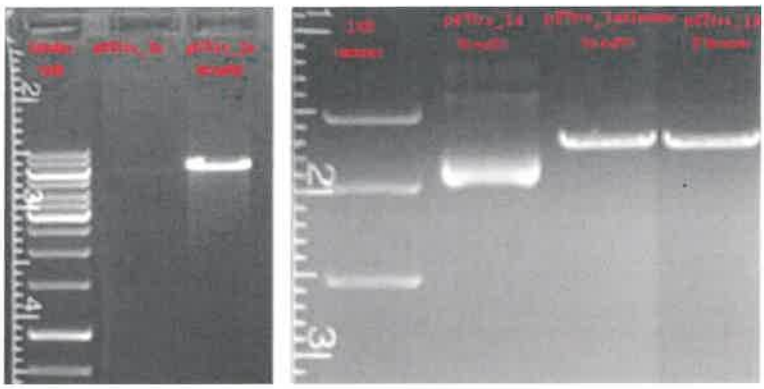

Figure 18: Two 1% agarose gels with pETtrx_1a constructs.
Left gel: lane 1: 1kB marker; lane 2: pETtrx_1a uncut; lane 3: pETtrx_1a cut with HindIII. Right gel: lane 1: 1kb marker; lane 2: pETtrx_1a cut with HindIII; lane 3: pETtrx_1a with Klenow cut with HindIII; lane 4: pETtrx_1a with Klenow uncut.

On the left gel picture can be seen, that the uncut vector and the cut vector have the same size. To prove, that pETtrx1a with Klenow cannot be cut with HindIII anymore a digestion with this enzyme has been done. As seen on the right gel picture, the pETtrx_1a with Klenow after digestion with HindIII, has the same size as pETtrx_1a with Klenow uncut. Therefore, it can be assumed, that the HindIII restriction site has been removed.

3.3.3 HisLinker and modified pETtrx_1a

The double stranded HisLinker (HisLinker construction in 2.2.6) and the Klenow-modified plasmid were cut with XbaI and BamHI. In the following figure the cut pETtrx_1aKlenow vector was loaded on a 1.5% agarose gel. The TRX-GFP fragment, which should be cut out with XbaI and BamHI, has a size of ~500bp and is indicated by the red arrow.

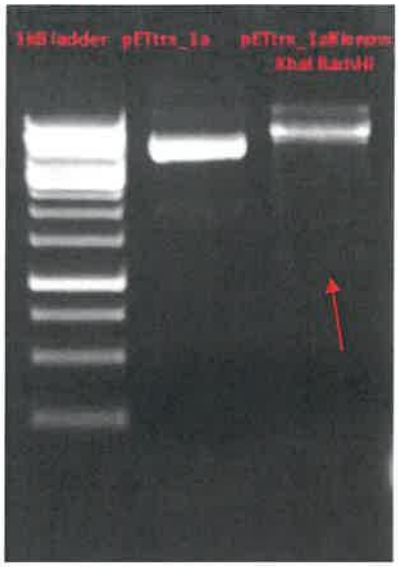

Figure 19: 1.5% Agarose gel with pETtrx_1a constructs XbaI/BamHI.
Lane 1: 1kB ladder, lane 2: pETtrx1a uncut, lane 3: pETtrx_1aKlenow cut with XbaI and BamHI, The red arrow indicates the fragment, that has been cut out of the vector.

3.4 Insertion of the Protein A fragments in the modified expression vector

The 5 fragments, obtained by amplifying them as described in 2.2.3, and the vector pETtrx_1aHisLinker were cut in the same way as described in section 2.2.7. Afterwards they were ligated as described in 2.2.10.

After ligation of the new constructed vector and the HisLinker, the fragment D has been selected to be cloned. The vector and the fragment have been cut with BamHI and HindIII. The HindIII restriction site is provided by the inserted HisLinker. The cut vector and the cut fragment were ligated as described in 2.2.11. The vector was checked for the specific insert with a colony PCR as described in 2.2.11. In the following figure the pETtrx_1aHisLinker construct was checked for the correct insert, fragment D. The red arrow indicates the sample that was sent for sequencing.

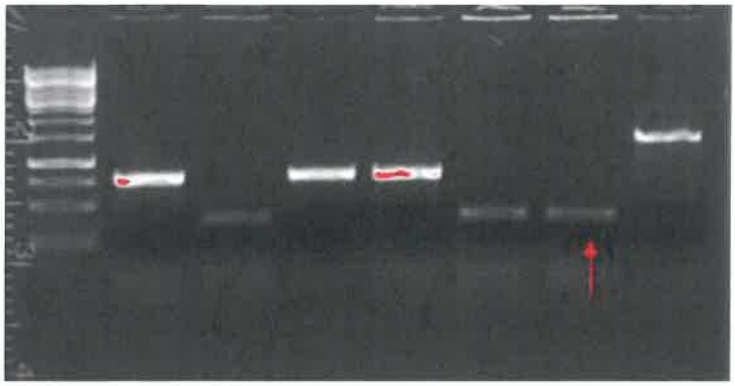

Figure 20: 1.5% Agarose Gel with colony PCR products.
Lane 1: 100bp ladder, lane 2-8 colony PCR products of pETtrx_1aHisLinker with fragment-D.

3.5 Sequencing

After sequencing the chosen section, this part contained the HisLinker and histidine rich parts of the old construct of pETtrx_1a. The inserted fragment-D was a nonsense sequence and the BamHI restriction site could not be found. Hence, it would be plausible, that the BamHI restriction site got lost through cloning procedures and therefore no correct sequence could be transformed into the vector.

4. Discussion

4.1 Protein A expression

The expression of foreign protein in *E. coli* BL21 (DE3) was successful. The highest amounts achieved for total protein were 1.9mg/ml. With SDS-Page, bands in the specific size of the protein A were found. To prove the presence of protein A, it would have been interesting to do a His-Tag purification, which was not done, due to lack of time. Moreover, the high affinity of the antibody binding domains of protein A could be used for specific protein detection. Western Blot and IgG affinity chromatography could be taken in consideration.

Interestingly the protein expression was different in this study compared to the protocol of the paper, even though every step has been implemented as in the protocol. Furthermore, bands of protein A were visible in the same intensity in induced and not induced samples (see figure 10).

Protein expression can be influenced by different factors (Gräslund et. al, 2008). In the paper of (Duong-Ly et al, 2014), they mentioned the possibility of temperature change at the induction. Moreover, they stated, that a low temperature can prevent the protein from secretion, between 15–28°C the protein secretion should be sufficient. Furthermore, the amount of IPTG can be optimized, lower concentrations of IPTG favour slow folding of proteins.

In this study protein expression was induced at 30°C after gaining an OD_{600} of 3.5. A not sufficient oxygen supply for the cells could result from the high OD. Another approach would be the very high concentration of 20g/L glucose in the M9 medium. Probably they used this high amount of glucose to ensure the growth of the cells until the specific OD. The problem, that could arise of this might be the following. The lac-promotor for the protein A expression can be induced with IPTG. If IPTG is not present there is allways a basal level of expression from this promotor. If glucose is present in high amounts, it can bind and inhibit IPTG induction. There is the possibility, that at the time of induction not all the glucose was used up and therefore, no induction could be done at this point (De Bellis et al, 1990). Moreover, this could also explain the band intensity of induced and not induced cells in the SDS-Page.

In conclusion, first it would be necessary to have a validation of the expressed protein being the specific protein A. Additionally, temperature and IPTG concentration adaptations could be made. Moreover, some adaptations of the amount of glucose in

the M9-minimal media or a later point of induction, to make sure the glucose has been used up, could be taken into consideration.

4.2 Amplification of the protein A sequences

The amplification of the whole protein A and thus the 5 domains was successful. The bottleneck of this part was the amplification of the fragments directly from the pET20bHis vector, which led to many unspecific bands.

Unfortunately the primers were binding also at other parts of the vector. Therefore, it was indispensable to isolate the protein A sequence first and to do the amplification steps with this PCR product as template. Thereby, the fragments obtained had the right sizes. Additionally, to obtain even a better output, the suggestions of the PCR-Phusion Protocol (Chester et al, 1993), could be taken in consideration, such as taking more template, optimizing the annealing temperature, increasing the extension time and the number of cycles, which was unfortunately not done, because there was no urgently need for it.

4.3 Construction of the pETtrx_1a vector construct

The vector has been constructed satisfyingly until the step of fragment insertion. The BamHI restriction site has been lost, probably during cloning events. Without this restriction site, no fragments could be inserted. The HindIII restriction site has been removed, the Klenow filling worked as well and the HisLinker insertion was also successful. Unfortunately the absence of the BamHI restriction site prevented further experiments. Nevertheless, NEB (New England Biotechnologies) website, offers a large troubleshooting protocol, dealing with not working enzymes and not sufficient cloning events (Restriction Enzyme Troubleshooting Guide, NEB). The optimal incubation time of the reaction mix should be considered to ensure, that the enzymes have enough time to evolve their activity Due to lack of time, no new expression vector was constructed and the cloning events have not been revised.

4.4 Future prospects

The protein A expression protocol should be retried and it would be interesting to adapt the temperature and IPTG concentrations. What would be also intriguing is the fact, that the cultivation in the M9-minimal media has been in half the volume as written in the paper. They worked with 200ml cell culture in 1L flasks, and in this study 100ml in 250ml flasks were used. Perhaps this leads to different qualities of cell

growth e.g. oxygen supply. It should not make any difference, but could be a fact to bear in mind. Moreover, the glucose amount in the M9 medium could be lowered to assure induction with IPTG or to proceed the induction at a later time.

The construction of the vector should be revised. It would be reasonable to check the correct restriction sites through the cloning process. It has been shown, that a sufficient incubation time of the reaction mix is highly recommended to ensure, that the enzymes have enough time to evolve their activity. The correct fragment insertions would lead to a next interesting step; the transformation of the vector with the different fragments in the BL21 (DE3) cells and implement the protein A expression protocol. If expression of single fragments is possible, the fragment with the best expression rate should be selected for fusion experiments with antimicrobial peptides. Different aspects have been picked up to express AMPs in other hosts such as *E.coli* (Guerreiro et al, 2008). Among this, the construction of a fusion carrier with protein A fragments and AMPs, following the protocol of protein A expression (Freiherr von Roman et al, 2014), may give the chance of a high yield of AMPs. With further work on this field of science, antibiotics and other pharmaceutical products could benefit of the antimicrobial features of AMPs.

5. Appendix

5.1 Sequence of the pre-protein of protein A (DNA)

MKYLLPTAAAGLLLLAAQPAMAMEDEAQQNAFYQVLNMPNLNADQRNGFIQSLKD
DPSQSANVLGEAQKLNDSQAPKADAQQNKFNKDQQSAFYEILNMPNLNEEQRNGF
IQSLKDDPSQSTNVLGEKKLNESQAPKADNNFNKEQQNAFYEILNMPNLNEEQRNG
FIQSLKDDPSQSANLLAEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQRN
GFIQSLKDDPSQSANLLAEAKKLNDAQAPKANKFNKEQQNAFYEILHLPNLTEEQRN
GFIQSLKDDPSVSKEILAEAKKLNDAQAPKEECAAALEHHHHHH*

pelB-signal sequence

5.2 Figures:

5.3 Tables:

6. Acknowledgements

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