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Formation of an Alternative RNA Polymerase in the Bacterium *Escherichia coli*

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1 Abstract

Antibiotic resistance in medically relevant bacteria causes serious problems for human health. Through conjugative plasmids, such as F-like plasmids, antibiotic resistance genes can be transferred from harmless to pathogenic bacteria. A central activator of F-like conjugative transfer is the transcription factor TraJ.

In this master´s thesis my aim was to investigate the properties of TraJ protein. In previous experiments it was shown *in vitro* that TraJ interacts with subunits of RNA Polymerase of *E. coli* and forms soluble complexes without the α-subunit. In particular I was interested in purifying a possible soluble complex containing TraJ and subunits of RNAP without α-subunit.

For co-expression of protein complexes containing TraJ and subunits of RNAP (β', β and ω) in *E. coli*, different constructs were generated using Gibson cloning or a traditional cloning method. The constructs differ in the tag of TraJ, offering various opportunities for purification.

The proteins were successfully expressed, but most of the overexpressed proteins were insoluble and located in inclusion bodies after co-expression and cell disruption. The insoluble proteins were solubilised with denaturation and further renatured. After renaturation, soluble complexes containing TraJ, β and β' could be analysed over size exclusion and affinity chromatography. Even after gel filtration the co-expressed and renatured complex remained in solution and the formation of a high molecular complex containing β, $\beta'$and TraJ could be shown.

In contrary to our expectation no enhancement of complex formation could be shown in the presence of EF-Tu.

Also, binary interactions between TraJ and the subunits of RNAP were investigated with far western blots. Interactions between TraJ and the subunits of RNAP β', β and σ^{70} could be demonstrated *in vitro*; however, an interaction between TraJ and α-subunit could not be shown.

In conclusion, my results confirm previous experiments, showing that TraJ is involved in the formation of an alternative RNAP. Further experiments have to be conducted to demonstrate transcriptional activity of this alternative RNAP.

Zusammenfassung

Antibiotikaresistenzen in medizinisch relevanten Bakterien können sehr problematisch für die menschliche Gesundheit sein. Durch konjugative Plasmide, wie zum Beispiel F-ähnliche Plasmide, können Antibiotikaresistenzgene von ungefährlichen auf pathogene Bakterien übertragen werden. Ein zentraler Aktivator des konjugativen Transfers von F-ähnlichen Plasmiden ist der Transkriptionsfaktor TraJ.
In dieser Masterarbeit war es mein Ziel, die Eigenschaften von TraJ zu untersuchen. Vorangegangene Versuche zeigten, dass TraJ und Untereinheiten der RNA Polymerase von *E. coli in vitro* miteinander interagieren und lösliche Komplexe auch ohne die α-Untereinheit bilden. Im speziellen lag mein Interesse darin, mögliche lösliche Protein Komplexe - bestehend aus TraJ und Untereinheiten der RNA Polymerase ohne α - aufzureinigen.
Um Proteinkomplexe, bestehend aus TraJ und Untereinheiten der RNAP (β', β und ω), in *E. coli* überexprimieren zu können, wurden unterschiedliche Expressionskonstrukte durch Gibson cloning oder einem herkömmlichen Klonierungsverfahren hergestellt. Die Konstrukte unterscheiden sich durch die verschiedenen Protein-Tags von TraJ, um verschiedene Möglichkeiten für die Aufreinigung der Komplexe zu bieten.
Die Proteine wurden erfolgreich exprimiert, aber der Großteil der überexprimierten Proteine war allerdings nach der Co-expression und dem Zellaufschluss unlöslich in Einschlusskörperchen vorhanden.
Die unlöslichen Proteine wurden durch Denaturierung gelöst und anschließend renaturiert. Nach der Renaturierung konnten lösliche Komplexe, die TraJ β und β' enthalten, über Größenausschlusschromatographie und Affinitätsreinigung analysiert werden. Auch nach der Gelfiltration blieb der co-exprimierte und renaturierte Komplex in Lösung und die Bildung eines hochmolekularen Komplexes, der β', β und TraJ enthält, konnte gezeigt werden.
Wiedererwarten konnte in der Gegenwart von EF-Tu keine Verbesserung der Komplexbildung gezeigt werden.

Auch wurden binäre Interaktionen zwischen TraJ und Untereinheiten der RNAP mittels Far-Western-Blot untersucht. Interaktionen von TraJ mit den RNAP Untereinheiten β, β und σ^{70} konnten nachgewiesen werden, im Gegensatz zu einer Interaktion zwischen TraJ und der α-Untereinheit.

Zusammenfassend bestätigen meine Ergebnisse frühere Experimente, in denen ebenfalls nachgewiesen wurde, dass TraJ bei der Bildung einer alternativen RNAP beteiligt ist. Die Transkriptionsaktivität dieser alternativen RNA Polymerase muss noch in weiteren Experimenten gezeigt werden.

2 Introduction

Antibiotic resistance is a big threat to global health, food security, and development today, according to the WHO.[1] It is complicating the treatment of infections, caused by so called superbugs, for example of *Staphylococcus aureus* and *Mycobacterium tuberculosis*. The increase of resistance is caused on the one hand by extensive and sometimes irresponsible use of antibiotics, which boosts selecting for resistance among pathogenic bacteria.[2]
Already Alexander Fleming warned of the inappropriate use of antibiotics in his Nobel lecture, when winning the Nobel prize for the discovery of penicillin[3]. On the other hand, resistance genes spread due to horizontal gene transfer, which is also a considerable reason for the increase of antibiotic resistance. Bacterial conjugation is a major mechanism of horizontal gene transfer and plays an important role in spreading resistance to *β*-lactams and aminoglycosides to clinically significant organisms.[4] Bacterial conjugation also plays a major role in the microbial evolution, which is another reason why it is important to study bacterial conjugation and its regulation.[5,6]

2.1 Bacterial conjugation

Bacterial conjugation is a horizontal gene transfer (HGT) mechanism, mediated by self-transmissible plasmids or integrated conjugative elements (ICE). It was first described in 1947 by Joshua Lederberg and Edward Tatum in *Escherichia coli* (*E. coli*).[7]
For this cell to cell contact dependent mechanism, ssDNA is unidirectionally transferred through a pilus. This sex-pilus is formed by a cell envelope spanning Type IV secretion system (T4SS), from the donor to the recipient cell.[8,9]
Conjugation can occur only under good environmental conditions, presence of a recipient, initial contact via the pilus or adhesins between donor and recipient, and pilus retraction.[10,11]

2.1.1 F and F-like plasmids

F and F-like plasmids are examples for self-transmissible plasmids, which mediate bacterial conjugation and can spread antibiotic resistance, virulence genes, heavy metal resistances or the capacity to form biofilms.[12]
During F-like plasmid mediated conjugation one strand of the dsDNA is nicked at *oriT* (origin of transfer) and ssDNA is transported through the T4S apparatus into the recipient cell. Simultaneously the plasmid is replicated via rolling circle replication. The transferosome, which connects donor and recipient cells and transports ssDNA, is connected via a coupling protein with the relaxosome, which nicks the DNA at *oriT*.[8,11] After the ssDNA circulates in the recipient cell, the complementary strand is synthesised. Now the recipient cell becomes a donor cell as well.[9, 5]

2.1.2 R1 - a F-like plasmid

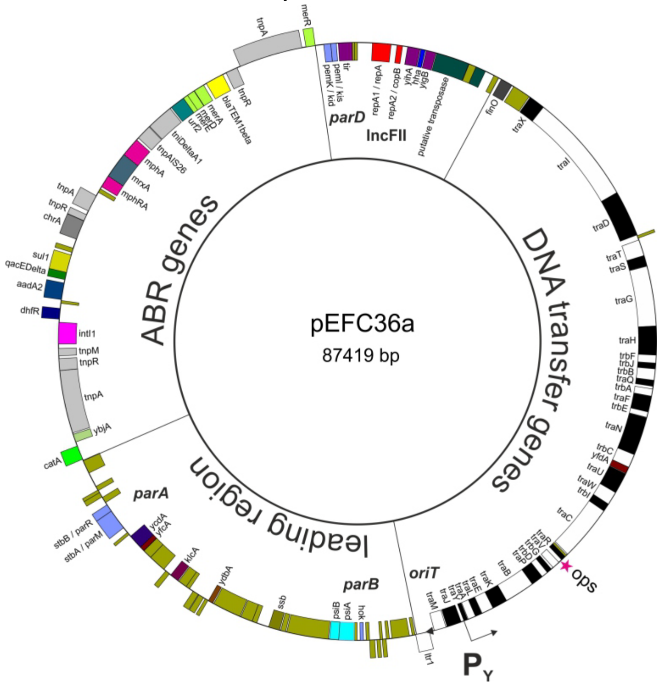

Figure 1: Schematic representation of pEFC36a, *a F-like plasmid with high sequence identity to R1, (Günther Koraimann)*

In this master´s thesis the plasmid R1 was used, which is a F-like plasmid. F-like plasmids are large, low copy number, self transmissible plasmids and are prevalent in commensal and pathogenic enterobacteria such as *Escherichia, Salmonella* and *Klebsiella.*

The R1 plasmid was first isolated in 1965 from a Salmonella infected patient.[13] R1 has a high sequence identity with pEFC36a (figure 1), which was isolated out of a wastewater treatment plant. Plasmid R1 is about 95 kb long[14] and contains genes which confer resistances to kanamycin, ampicillin, sulfonamide, streptomycin and chloramphenicol. It encodes three main regions, the leading region, the resistance region and the transfer (tra) region. The *tra (transfer)* operon encodes most of the type IV secretion machinery components, helicase and relaxase in one 32 kb long transcription unit.[15]

2.1.3 Regulation of the tra operon

Transcription of the *tra* operon is strictly regulated via host- and plasmid encoded factors, as sown in figure 2. The regulation is important for creating a balance between the advantage, given by new genes, and the negative effect that the fitness of the bacterium could experience. Negative effects can include metabolic burden, envelope stress and phage attack.[16,11]

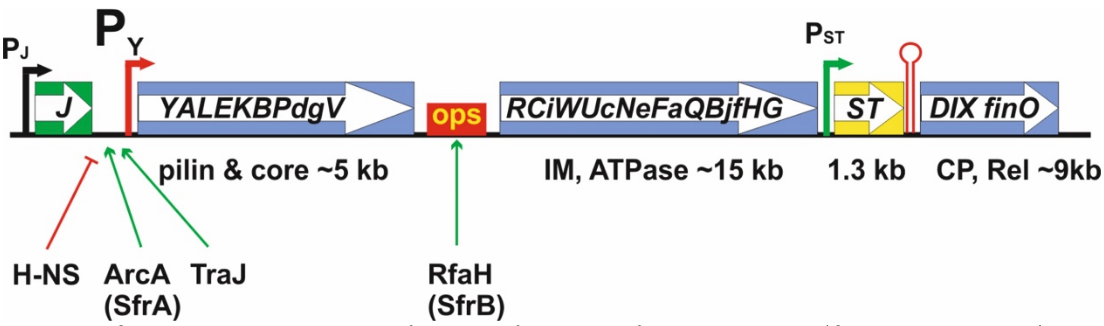

Figure 2: *Schematic representation of the transfer region of F-like plasmids, (Günther Koraimann)*

The main activators of *tra* operon expression are TraJ (plasmid-encoded) and phosphorylated ArcA (host encoded). The transcription factor TraJ and also ArcA bind to the P_Y promoter enabling transcription of DNA transfer genes and together they have to overcome H-NS (histone-like nucleoid structuring stimulation) silencing of the P_Y promotor.[11,5] Only the presence of both activators ensures maximal plasmid transfer.[17,18]

Additionally, TraJ has to escape the post-transcriptional FinOP-control. FinP is a plasmid specific antisense RNA and FinO acts as a co-repressor, and together with Hfq (host factor for Qβ replicase) it regulates the translation of *traJ* mRNA.[19, 20, 21, 22]

CRP (catabolite repressor protein) on the other hand regulates traJ on transcriptional level,[23] as well as Lrp (leucine-responsive protein), which regulates the promotor of *traJ*, P_J.[24, 25]

The chromosomally encoded RfaH (SfrB) is an anti-termination factor, which is required for the transcription of long operons in *E. coli*. In the absence of RfaH the transcription of the whole 32 kb long *tra* operon ceases before the end of the *tra* operon and therefore no conjugation can occur.[26]

Further information and a description of the entire regulating mechanisms, can be found in the review from Frost and Koraimann (2010)[11].

2.1.4 TraJ

The F and F-like plasmids have evolved differently in regard to their regulation, resulting in highly different TraJ proteins. For example, TraJ of R1 cannot activate a P_Y promotor of a F-plasmid, because of their varying recognition sequence. [27,17] Due to the fact that in this research study TraJ of R1 is investigated, the following will confer to TraJ of R1.

The *traJ* gene is located upstream of the *tra* operon, expressed from its own promoter P_J, and is about 25 kDa (228 amino acids; aa).

Figure 3: Schematic representation of TraJ, numbers indicate aa position

It encodes a cytoplasmic protein that binds DNA through its putative C-terminal helix–turn–helix (HTH) DNA-binding motif upstream of P_Y.[23, 28] In addition to its DNA-binding domain, TraJ contains a linker domain (aa 126-151), amino-terminal PAS (Per-ARNT-Sim)- Domain (aa 21- 121) and a C- and N-terminal domain (figure 3). The PAS-domain of the F (PDB: 4KQD) and pSLT (PDB: 4EW7) plasmid encoded

TraJ could be already crystallized (figure 4) in contrast to the rest of the protein. The pSLT encoded TraJ is similar to the R1 plasmid encoded TraJ, because both plasmids belong to the incF plasmids. [27]

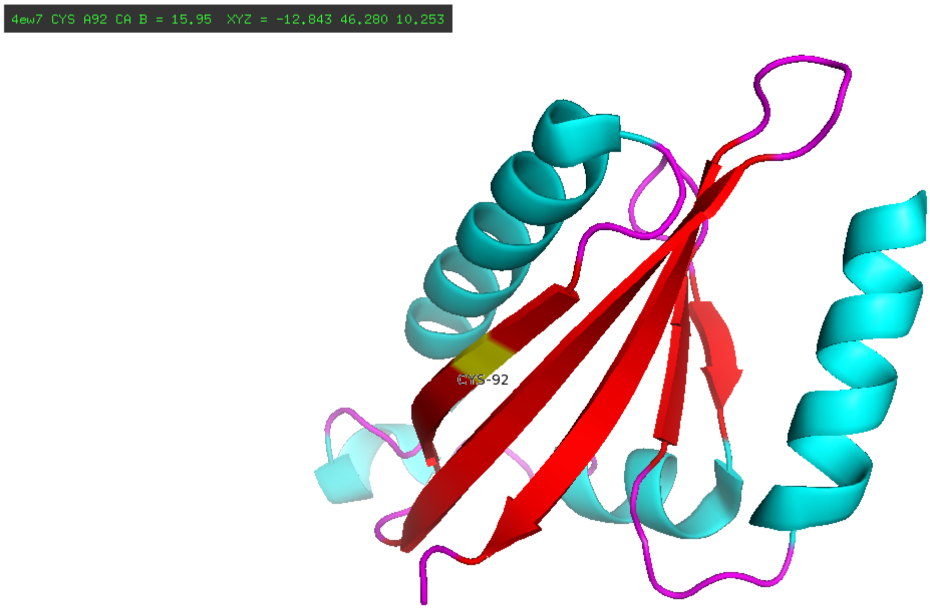

Figure 4: Cristal structure of the PAS domain of TraJ$_{pSLT}$: *characteristic five-stranded antiparallel β-sheet is shown in red and α-helices are shown in cyan (the loops are shown in magenta). The cysteine at position 92 is shown in yellow. The image was created via pymol out of the pdb file 4EW7.*

The PAS domains are flanked by varied numbers of α-helices which most likely facilitate dimerization. These domains are known to be involved in protein interactions and to regulate diverse physiological processes. Therefore, the PAS domain acts as sensors in signaling proteins and is present in all three kingdoms of life. Small-molecule ligands such as metabolites, heme, and flavin nucleotides are bound by some PAS folds to gain their physiological activities.[29, 30, 27, 31, 32] Mutation of multiple cysteine residues (Cys30, Cys41, and Cys67) within the TraJ-F PAS domain significantly inhibits F conjugation, which leads to the hypothesis that the TraJ cysteines form a redox center for sensing oxidative stress. [33]
F-TraJ is found in a soluble form in the cytoplasm, which contains around 2000-4000 monomers per cell.[34] When overexpressed, it is poorly soluble and subject to proteolytic degradation.[35, 36]
Proteolytic degradation of TraJ is mediated by the heat shock chaperon protein GroEL.

2.2 RNA Polymerase in *E. coli*

Transcription describes the synthesis of RNA complementary to the template DNA strand through DNA-dependent RNAP out of Nucleoside triphosphate (NTP´s). The multi-subunit RNA polymerase (RNAP, figure 5) is the vehicle of transcription in all domains of life.

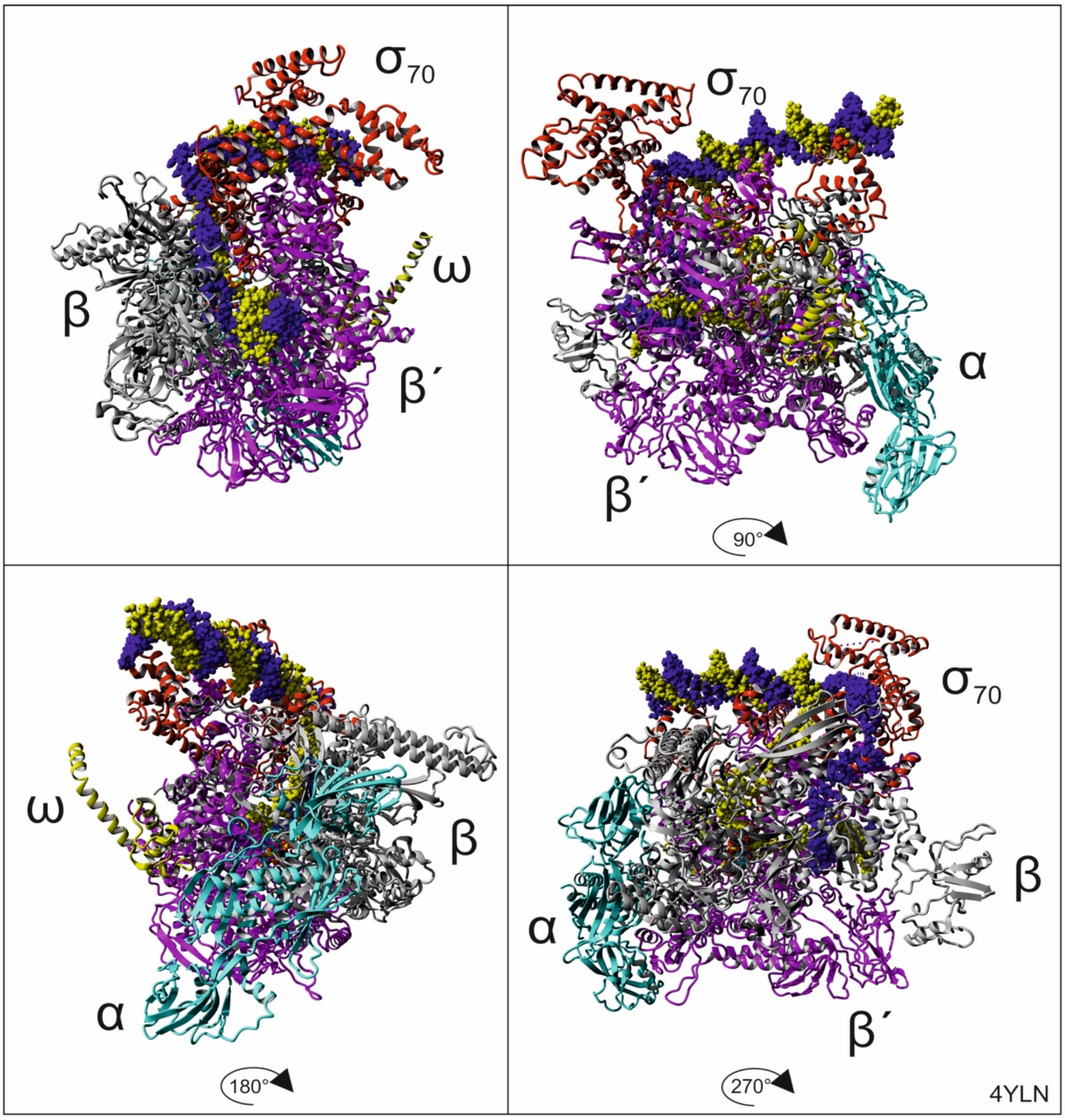

***Figure 5: Open complex formed by the RNAP of* E. coli (PDB: 4YLN)**: *Subunits of the holo-enzyme are colored as follows: β´ magenta, β grey, α cyan, ω yellow and σ^{70} red. DNA is illustrated in blue and yellow, whereby the template strand is yellow and the non template strand is blue. Images were prepared using YASARA software.*

In Eubacteria the core-enzyme consists of five subunits: 2 x α (36 kDa, *rpoA*), β (150 kDa, *rpoB*), β' (155 kDa, *rpoC*), and (10 kDa, *rpoZ*).[37] The α-subunits assemble the RNAP and interacts with regulatory proteins, whereas the β´- and β- subunits form the catalytically active domains. β is involved in catalysis, initiation and elongation and β´ binds DNA (reviewed in Borukhov and Nudler, 2008).[38] The smallest subunit ω has a structural function within the RNAP and can fully restore denaturated RNAP in vitro.[39] It is also known that ω is important during the assembly of RNAP for the incorporation of β´ into the initially formed 2αβ complex.[40,41] The assembly of RNAP is shown schematically in figure 6.

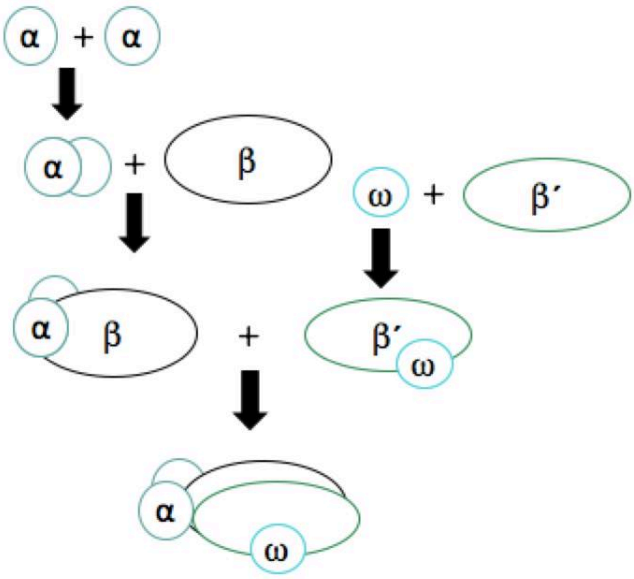

Figure 6: Schematic representation of the assembly process of RNA polymerase from different subunits, drawn according to Gosh et al.[42]

The RNAP has an overall "crab claw" –like structure (150x110x115 Å), in which two "pincers" are formed by β´ and β, while the α subunit lies on the back of the enzyme (figure 5).[37] The 27 Å cleft between β´ and β forms the primary channel for dsDNA. The β-flap domain is part of the exit channel, where newly synthesised RNA passes through.[43] The α-helical motive of the β-flap, called β-tip, is the major interaction site for σ region 4. It also interacts with the N-terminal domain of the α-subunit and with the essential transcription factor NusA.[44] TraJ is also thought to interact with this β-flap domain (Master´s thesis of Ines Aschenbrenner[45]). NusA, which is highly conserved in Eubacteria, is associated to RNAP through the elongation phase of transcription, and regulates pausing, termination and formation of the anti-termination complexes.[46, 44] NusA is also involved in the "immune system" of *E. coli* which supresses toxic activity of foreign genes.[47]

The promoter specific σ70-subunit associates with the core-enzyme, thereby facilitating promoter recognition and forming the holo-enzyme.[43]

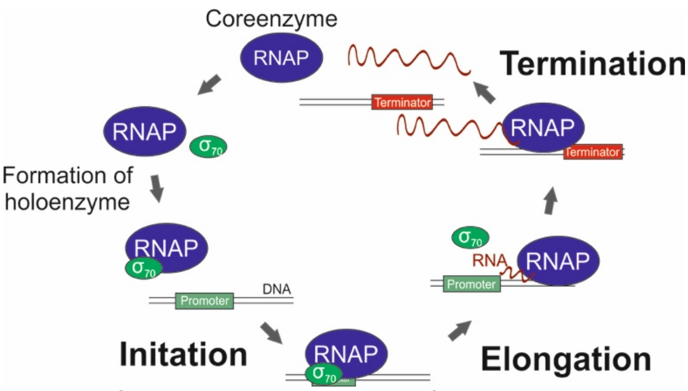

Figure 7: Schematic representation of the transcription cycle

The transcription cycle (figure 7) starts when the holo-enzyme recognizes the promoter.[43] The DNA duplex melts and an open complex is established.[48] After some abortive initiations creating 2-9 nucleotide long transcripts,[49] the RNA transcript with a length of approximately 12 nucleotides gets pushed through the exit channel.

Thereby the σ^{70} factor is released, whose major interaction site for σ region 4 lies at the exit channel. RNAP escapes the promoter and forms a stable transcription elongation complex.[50, 51] Elongation can now begin and proceeds until a termination signal is reached. RNAP releases the RNA molecule. DNA template and RNAP are available for a new transcription cycle.[52], [53]

2.3 Elongation factor thermos unstable (EF-Tu)

A *in vivo* pull down of TraJ from an experiment of Ines Aschenbrenner[45], showed that EF-Tu was also pulled down. For this reason, further investigation concerning involvement of EF-Tu in aRNAP complex formation was performed.

Under rapid growth conditions, the translation elongation factor EF-Tu is the most plentiful protein in most bacterial cells.[54] It belongs to the family of GTPase and therefore hydrolyzes GTP into GDP. The main function of EF-Tu is to deliver amino acyl-tRNA (aa-tRNA) to the A site of the ribosome, to hydrolyze GTP when a correct aa-tRNA is brought and to promote elongation in this way. During this process EF-Tu is subject to massive structural changes, due to hydrolysis and the release of the gamma phosphate, which induces rearrangement of the switch I (effector loop) and the switch II regions. Because of the structural change, GDP-bound EF-Tu has a reduced affinity to aa-tRNA´s. EF-Ts, an exchange factor, promotes the transition to GTP-bound EF-Tu (reviewed in Gregers, 2003 and Hilgenfeld, 1995).[55, 56]
In *Bacillus subtilis,* EF-Tu is also important for the maintenance of cytoskeletal elements by interacting and co-localizing with actin-like MreB protein.[57]

2.4 Aim of this work

Bacterial conjugation can cause great problems for human health, due to the spreading of antibiotic resistance genes. The conjugation of R1, which is an F-like plasmid, is dependent on the activation of the *tra* operon due to TraJ (plasmid encoded) and ArcA-P (host encoded) activation of P_Y promoter. The *tra* operon encodes the majority of the proteins, which are necessary for bacterial conjugation. Our aim is to characterise TraJ, investigate its interaction partners, and reveal its structure, to get a deeper insight into the regulation of bacterial conjugation mediated by F-like plasmids.
Previous experiments in the laboratory of Günther Koraimann, conducted by Ines Aschenbrenner, showed that in the in vivo pull down of TraJ, the subunits α ,β of RNAP, EF-Tu and GroEL were detectable. Further investigation of the interaction between β and TraJ via a two-hybrid-assay showed that TraJ interacts with the β-flap domain.[45]
In this study the interaction between TraJ and different subunits of RNAP should be further explored; also, the other interaction partners, especially EF-Tu, should be considered, to get a better understanding of the interaction between TraJ and RNAP. Far western blot analysis, co-immuno precipitation and soluble complex formation, observed on a size exclusion column, can, due to different elution behaviour, reveal interactions between proteins.
Unpublished data from the laboratory of Günther Koraimann by Karin Bischof suggests, that TraJ forms with subunits of RNAP (αβ´β) a soluble complex over reconstitution. TraJ also formed a soluble complex merely with β´β, leading to the hypothesis that the α subunit is not necessary for a RNAP, which transcribes the *tra* operon together with TraJ. The unique transcription factor TraJ would form an essential part of a non-canonical RNAP (alternative RNAP, aRNAP). This work will entail obtaining a soluble complex of RNAP and TraJ, so as to get a crystal structure and perform activity and other assays.

In order to accomplish this aim, TraJ, β´, β and ω should be co-expressed and purified. The ω-subunit, also part of the core enzyme, is able to restore the denatured RNAP to its full function and is important for the β´ incorporation into the complex. To co-express this complex, expression vectors must be constructed, and to achieve the production of a soluble complex, it may also be necessary to try different expression conditions.

3 Material and Methods

3.1 Bacterial strains

Table 1 shows the *Escherichia coli* (*E. coli)* strains which were worked with in this master´s thesis. In *E. coli* BL-21 the T7-Polymerase is under control of the *lac*UV5-promoter and was used for the expression of proteins. *E. coli* XL-1-Blue was used for plasmid amplification.

Table 1 Bacterial strains used in this work: E. coli *strains with the respective genotypes, sources and descriptions for experimental application used in this work.*

E. coli strains	Genotype	Description/Use	Source/reference	#IMB*
XL1-Blue	*endA1, usdR17* (rK-, mK+), *supE44, thi-1, λ-, recA1, gyrA96, relA1,* [F' p*roAB* *lacI*^{q}ZΔM15 Tn10 (Tet^R)]	used for cloning: transformation; plasmid isolation	Stratagene, La Jolla, CA	393
BL-21 (DE3)	BL21 (*ompT hsdS gal*), λ (DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*])	Used for expression of proteins	G. Sawers, (JIC Norwich, UK)	2233

* number of the strain in the strain collection of the IMB.

3.2 Media and growth

In general, the *E. coli* strains were cultivated in/on LB- or 2x TY-media/agar (table 2) with respective antibiotics (table 3), depending on the used strains and plasmids. The plates were incubated at 37°C in an incubator (Binder, Tuttlingen, Germany) over night. The media was inoculated with one single colony and incubated over night at 37°C and 180 rpm in a Multitron Infors-HT shaker (Infors-HT, Bottmingen, Swizerland), referred to as ONCs (overnight cultures).

Table 2: Media for cultivation of* E. coli *strains
The components were diluted in H_2O_{bidest}. For preparation of media no agar was added.

LB-medium/agar	2xTY-medium/agar
10 g/L tryptone 5 g/L yeast extract 5 g/L NaCl	16 g/L tryptone 10 g/L yeast extract 5 g/L NaCl
15 g/L agar	15 g/L agar

3.3 Antibiotics

According to the antibiotic resistance marker of the used plasmid the appropriate antibiotics were added to agar or media. The pET28a(+) based plasmids carry a Kanamycin resistance maker and the pACYCDuet-1 based plasmids carry a Chloramphenicol resistance maker. The different antibiotics are listed in table 3.

*Table 3: **Antibiotics** used in this work with the respective final concentrations*

Name	Working concentration	Source
Kanamycin	40 µg/mL	Roth, Karlsruhe, Germany
Chloramphenicol	20 µg/mL	Roth, Karlsruhe, Germany

3.4 Plasmids

In order to express proteins, which were used in this master´s thesis, appropriate expression constructs had to be generated.

Most of the plasmid were constructed using Gibson cloning, except pETlysMα, which was constructed with a traditional cloning method. To give an overview over the generated plasmids, they are listed in table 4. The list contains the plasmid description, the source of the plasmid and the number of the strain in the strain collection of the Institute of Molecular Bioscience (IMB), containing the respective plasmid (#IMB).

***Table 4: Plasmids used in this master´s thesis**, their description, source and #IMB (number of the strain in the strain collection of the IMB containing the respective plasmid)*

Plasmid	Description	Source	#IMB*
pACYCDuet-1	Cm^R, P15A ori; *lacI*; IPTG inducible *T7* promoter; used as backbone; 4008 bp	Novagen	
pET28a(+)	Km^R; pBR322 ori; *lacI*; IPTG inducible *T7* promoter; used as backbone; 5369 bp	Boehringer	2367
pJCore1[3]	pACYCDuet-1, 6xHis-*traJ*, *rpoB*CZ, Cm^R 12 977 bp	This work[1]	4153
pJCore2[3]	pACYCDuet-1, Strep-*traJ, rpoB*CZ, Cm^R 12 980 bp	This work[1]	4154
pJCore4S	pACYCDuet-1, Strep-*traJ*, 10xHis-*rpoB, rpo*CZ, Cm^R, 13 040 bp	This work[1]	4245
pJCore5[3]	pACYCDuet-1,Twin-Strep-FLAG-*traJC92S, rpoB*CZ, Cm^R 13 102 bp	This work[1]	4247
pβ´ω	pACYCDuet-1*, rpo*CZ, Cm^R 8102 bp	This work[1]	4244
pETlysM	pET28a(+), *lysM*, Km^R, 5502 bp	This work[1]	4238
pET28lysMα	pETlysM, *rpoA,* Km^R, 6359 bp	This work[2]	4243
pET28tufB[3]	pET28a(+), *tufB*, Km^R, 6413 bp	This work[1]	4242

***** number of the strain in the strain collection of the IMB containing the respective plasmid
[1] Constructed with Gibson cloning
[2] Constructed with a traditional cloning method
[3] DNA-sequences can be found in the supplementary data

In the following part, generated plasmids are described. A more detailed description of the plasmids can be found in the result part.

3.4.1 pJCore

All of the pJCore vectors are constructed on the basis of pACYCDuet-1 expression vector and contain a medium-copy-number p15A origin of replication, which can be propagated in *E. coli* cells containing a second plasmid with the ColE1 origin. This makes it compatible with the pET28a(+) expression vector for co-expression.
It also contains a promoter for bacteriophage T7 RNA polymerase, which is under control of a *lac* operator. At T7 transcription terminator T7 RNA polymerase stops the transcription.
LacI, the *lac* repressor which is also encoded by the plasmid, binds to the *lac* operator to inhibit transcription of the T7 RNAP. This inhibition can be overcome by adding lactose or isopropyl-β-D-thiogalactopyranoside (IPTG).
Furthermore, all pJCore plasmids carry a chloramphenicol acetyltransferase (Cm^R), which confers resistance to chloramphenicol and contains a corresponding promoter.

All pJCore vectors feature cut sites for restriction enzymes *Nco*I and *Hind*III on both sites of *traJ* respectively, so it can be easily exchanged with coding sequences for other TraJ mutants.
The expression vector pJCore1 (figure 8) encodes for JCore1 proteins (TraJ, β, β´, ω) and has the same backbone as the other JCore expression vectors (pJCore2, pJCore4, pJCore5 and pβ´ω) which are listed in table 4 and shown in the results (figures 12, 14, 16, 18 and 20), where a more detailed description of each pJCore plasmid is given.

All pJCore vectors were generated using Gibson cloning, and therefore assembled from overlapping DNA Fragments. All used DNA Fragments are listed in table 5.
pJCore1 contains the DNA fragments PACYC_F1, TEV_TraJ_F1, RpoB_F1, RpoC_F1 and RpoZ_F1.
pJCore2 was assembled from the DNA fragments PACYC_F2, TEV_TraJ_F2, RpoB_F1, RpoC_F1 and RpoZ_F1.
pJCore4S consists of the DNA Fragments FA_F1 (PACYC_F2 and TEV_TraJ_F2), H10RpoB_F3 and FB_F1.
pJCore5S was constructed using the DNA fragments PACYC_F3, TraJ_C92S_F3, RpoB_F2, RpoC_F2 and RpoZ_F1.
pβ´ω was generated with the DNA Fragments pACYC1_F1 and RpoC_F1 and RpoZ_F1.

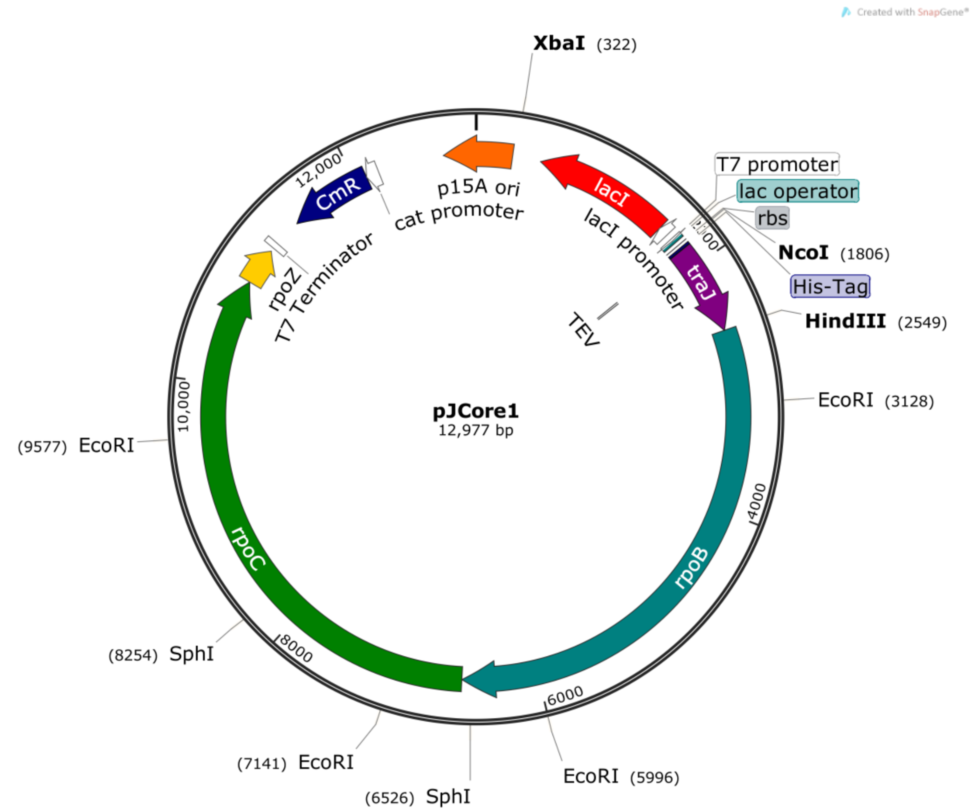

Figure 8: Expression vector pJCore1: *expression vector for JCore1 (His-TEV-TraJ, β, β´, ω); contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator, 6xHis-tag, TEV-cut site,* traJ *(encodes TraJ),* rpoB *(encodes RNAP subunit β),* rpoC *(encodes RNAP subunit β´),* rpoZ *(encodes RNAP subunit ω), T7 terminator,* cat *(chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. This Plasmid was generated using Gibson cloning.*

pJCore1 and pJCore4 contain an N-terminal His-tag (pJCore1 in front of *traJ* and pJCore4 in front of *rpoB*) which allows purification and detection over the His epitope of these proteins.
The vectors pJCore1, pJCore2 and pJCore4S contain a cleavage site (ENLYFQ\S) for TEV (tobacco Etch Virus nuclear-inclusion-a endopeptidase) between the His- or Strep-tag and *traJ*.

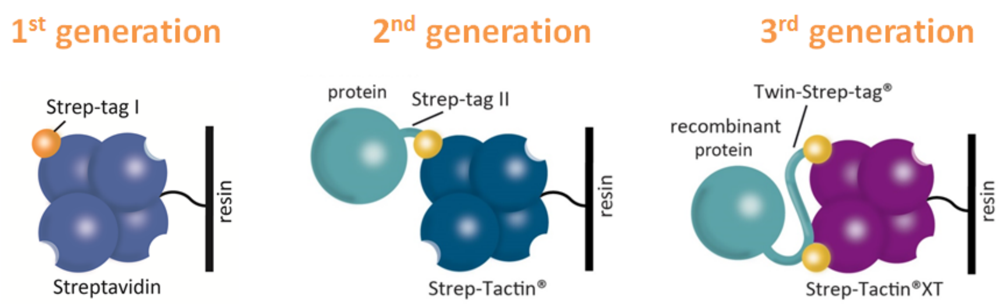

Figure 9:Different types of Strep-tags [58]

Strep-I-tag (figure 9), a peptide that binds Strep-Tactin®, is an engineered form of streptavidin (WSHPQFEK) and is placed in front of *traJ* in pJCore2.
TraJ expressed from pJCore5 vector is tagged with a further developed Strep-tag called Twin-Strep-tag® (figure 9), followed by a FLAG-tag (DYKDDDDK).

3.4.2 pET28

The other expression vectors constructed in this master´s thesis were constructed on basis of the pET28a(+) expression vectors, which are compatible for co-expression with pACYCDuet-1 based expression vectors. In figure 10 pETlysM, which was assembled by Gibson cloning with the DNA fragments PET28a_F1 and LysM_F1 (table 5), is shown as a representative. Other plasmid maps of pET28a(+) based vectors a shown in the result part (figures 22, 24, 26).
All pET28a(+) based vectors carry a aminoglycoside phosphotransferase gene, which confers resistance to kanamycin in bacteria (Kan^R). The high-copy-number ColE1/pMB1/pBR322 origin of replication is also carried on the plasmid. It also contains *bom*, the basis of mobility region from pBR322 and Rop protein coding sequence, which maintains plasmids at low copy number is also part of all pET28a(+) based vectors.

The vector pETlysM (figure 10) was designed to inhibit basal expression of proteins, which are toxic for the cell but should be overexpressed. The coding sequence of any toxic protein can be easily cloned into the multiple cloning site (MCS). The inhibition of the basal expression is useful, if toxic proteins should be expressed and is achieved via *lysM*.
LysM encodes for a lysozyme from bacteriophage T7 (LysM, 151 AA), which is an inhibitor of T7 RNA polymerase. The lysozyme lies behind the T7 terminator sequence in the opposite direction, to ensure only a minimal expression. Nevertheless this minimal expression is enough to inhibit the basal expression. After induction with IPTG the lysozyme is still expressed in a small amount, which does not

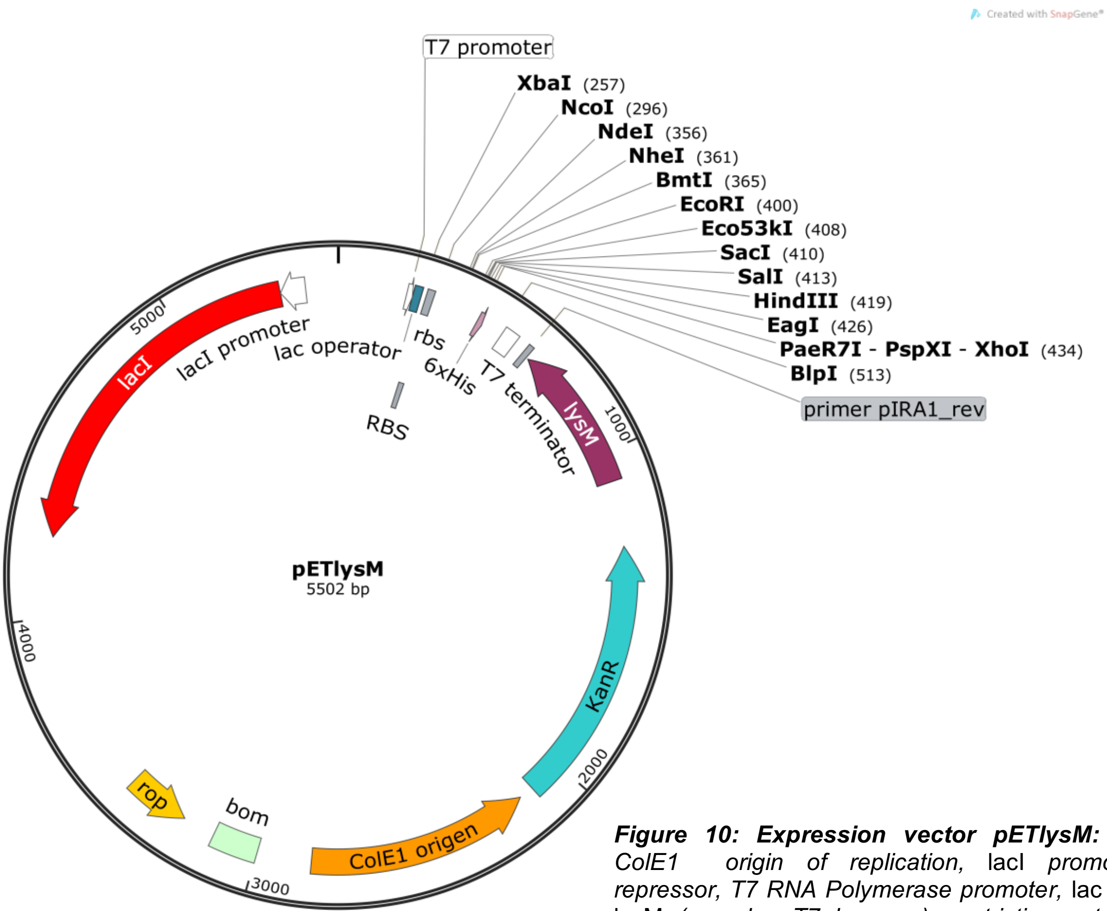

Figure 10: Expression vector pETlysM: *contains ColE1 origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator,* lysM *(encodes T7 lysozym), restriction cut sites for cloning other genes inside the vector , T7 terminator, Kanamycin resistance,* rop *and* bom*. Plasmid was generated using Gibson cloning.*

affect the overexpression.

pETlysMα was made by cloning the DNA fragment RpoA_F1 (encodes *rpoA*) into pETlysM, resulting in expression of the α-subunit without basal expression of the α-subunit.

In order to express EF-Tu, pET28tufB was generated by Gibson cloning, using the fragments EF-Tu, and PET28a_F2 (table 5).

3.4.3 DNA-Fragments

By PCR synthesised DNA-fragments, out of which the plasmids were build, their corresponding primer, used template and size are shown in table 5. The sequences of the oligonucleotides and their #IMB (number of the oligonucleotide in the database of the IMB) are listed in the supplementary data (table 27).
Most of the needed DNA-templates (table 5) were already isolated and purified, ready to use, provided by the laboratory of Günther Koraimann. Only *lysM* had to be amplified out of *E. coli* RosettaD3 [plysS] through cPCR.

*Table 5: **DNA-Fragments** used in this master´s thesis*

Fragment name	Primer #IMB*	Template	Fragment size (bp)
PACYC_F1	2284 & 2293	pACYCDuet-1[3]	3678
TEV_TraJ_F1	2285 & 2286	pSD1002[3]	744
RpoB_F1	*2287 & 2288*	MG1655[1]	4071
RpoC_F1	*2289 & 2290*	MG1655[1]	4264
RpoZ_F1	*2291 & 2292*	MG1655[1]	326
PACYC_F2	2293 & 2294	pACYCDuet-1[3]	3682
TEV_TraJ_F2	2286 & 2298	pSD1002[3]	768
FA_F1	2264 & 2291	pJCore2[3]	4765
FB_F1	2289 & 2292	pJCore2[3]	4590
H10RpoB_F3	*2263* & *2288*	pJCore2[3]	4092
PACYC_F3	2264& 2293	pACYCDuet-1[3]	4465
TSF-TraJ	2280 & 2286	pJR1[3]	844
TraJ_C92S_F3	2280 & 2286	pJR1C92S[3]	844
RpoB_F2	2287 & 2278	MG1655[1]	4078
RpoC_F2	2279 & 2290	MG1655[1]	4277
PET28_F1	2262 & 2281	pET28a+[3]	5091
LysM_F1	2260 & 2261	*E. coli* Rosetta D3 [plysS][2]	602
RpoA_F1	2265 & 1952	pIRA3[3]	1001
TufB_F1	2322 & 2323	MG1655[1]	1236
PET28_F2	2321 & 2324	pET28a+	5231

[*] number of the oligonucleotide in the database of the IMB
[1] chromosomal DNA
[2] DNA prepared as described in (3.4.5)   [3] Plasmid DNA

3.4.4 Polymerase chain reaction (PCR)

The polymerase chain reaction was used in this work to amplify DNA-Fragments and also to add restriction-sites, tag-sequences and overlapping sequences (for Gibson cloning) via primers.

All polymerase chain reactions were conducted as described in the manual[59] of Polymerase Q5 from NEB for a 25 µL reaction volume. If not stated differently 2 µL of DNA [1 ng/µL] were utilised. Two different proofreading DNA Polymerases were used, Phusion or Q5 (NEB, Ipswitch, MA).
In this work two different PCR programs were used in combination with the thermocycler PeqStar Primus 25 Cycler (VWR, Radnor, PA). The standard PCR program (table 6) and Q5 polymerase were used for most of the PCR reactions, but the DNA fragments RpoB_2 and RpoC_2 were amplified using another PCR program (table 7) and Phusion DNA Polymerase.

Table 6: Standard PCR program

Program	Temperature [°C]	Time	
Lid preheating	110		
Initial denaturation	95	30 sec	
Denaturation	95	30 sec	30x
Annealing	60	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	
Cooling	4		

Table 7: PCR conditions used for RpoB_2 and RpoC_2

Program	Temperature [°C]	Time	
Lid preheating	110		
Initial denaturation	98	30 sec	
Denaturation	98	10 sec	30x
Annealing	60	30 sec	
Extension	72	150 sec	
Final extension	72	5 min	
Cooling	4		

3.4.5 Chromosomal DNA preparation from colonies

To amplify DNA fragments out of chromosomal DNA in bacteria, a colony PCR (cPCR) was performed. Therefore, one single colony was resuspended in 50 µL $H_2O_{fres.}$ and heated up for 10 minutes at 98°C (BIOER Biving Block MB-102). After centrifugation for 1 minute at 16,000 rcf (Eppendorf centrifuge, 5415 R), 2 µL of the supernatant were used as template-DNA for colony-PCR, and continued as described in the protocol for a PCR. This method was used for the amplification of the T7 lysozyme *lysM* out of *E. coli* Rosetta DE3 (plysS) (IMB #3524).

3.4.6 DNA agarose gel electrophoresis and DNA purification

DNA agarose gel electrophoresis was conducted according to the standard protocol.[60] Used buffers are listed in table 8.

***Table 8:** Buffers used for DNA agarose gel electrophoresis*

TAE buffer	6x DNA loading dye
40 mM Tris 20 mM acetic acid 1mM EDTA (pH 8)	10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue 0.03% xylene cyanol FF 60% glycerol 60 mM EDTA (pH 8)

For DNA-purification out of the agarose gel the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) was used and the protocol in the manual[61] was followed.

3.4.7 Cloning

3.4.7.1 Gibson cloning

The Gibson assembly is a really fast alternative cloning method, were DNA-fragments with overlapping ends can be assembled to a plasmid. Endonucleases digest DNA starting from the 5`-end resulting in single stranded DNA ends, which then can anneal. Gaps are filled up by a DNA-Polymerase and the DNA ends are linked by a ligase. Its ease-of-use, flexibility and suitability for large DNA constructs are big advantages of this method.
The constructs were designed with NEB builder[62] and for the assembly itself Gibson Assembly Master Mix or the NEBuilder HIFI DNA Assembly Master Mix (NEB, Ipswitch, MA) were used. The for the assembly needed overlapping DNA-fragments (table 5) were amplified with PCR (3.4.4) using oligonucleotides (supplementary data, table 27), which were designed with NEB-builder. The Gibson cloning itself was carried out as described in the manual[63], creating expression-vectors out of the overlapping DNA-fragments.

3.4.7.2 Traditional cloning - pETlysMα

The insert was amplified with oligonucleotides, containing recognition sequences for restriction enzyme sites (NcoI & XhoI). The vector-backbone, which carries the same recognition sequences for restriction enzymes (NcoI & XhoI), was isolated.
Both the vector-backbone, in which the DNA-fragment should be inserted, and the insert itself were cut with 10 U of respective enzymes (*Nco*I & *Xho*I) in 2xTango-buffer for 4 h at 37°C. Then both restriction mixes were analysed on an agarose gel out of which DNA was purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The purified vector-backbone and the insert were then ligated with T4 ligase (ThermoFischer scientific, Waltham, MA) on 16 °C overnight and stored at -20°C before further usage. pETlysMα was constructed with this method. The insert RpoA was cloned into the vector-backbone pETlysM and primer #2265 & #1952 (table 27) were used to amplify the RpoA.

3.4.8 DNA digestion with restriction enzymes

A restriction digestion was done, either to check a plasmid based on the restriction pattern or use the cut DNA for cloning. A restriction mix consisted besides the used restriction enzymes (5U) and the appropriate buffer also of 200-500 ng DNA. According to the used volumes of the DNA, $H_2O_{Fres.}$ was added up to 20 µL or not. The restriction reaction was done at 37°C for the 3-5 hours.

3.4.9 Sequencing

pJCore1, pJCore2, pJCore5 and pET28tufB were sequenced by the company eurofins Genomics (Ebersberg, Germany) using their Mix2Seq kit. Contiguous sequences were assembled using the program CAP3[64] and aligned with the expected sequence with the program Ape[65].

3.4.10 DNA Transformation

3.4.10.1 Electro-Transformation

Electro-competent *E. coli* XL-1 cells were transformed with the correct plasmids following the standard protocol.[60]

Plasmid DNA (directly from Gibson cloning) mixed with 40 µL electro competent *E. coli* XL-1 cells were transferred into sterile (UV radiation for 400 seconds with GS Gene Linker™ UV Chamber, Bio-Rad) cuvettes (GenePulser® Cuvette, 2mm, Bio-Rad, Hercules, CA), which were exposed to an electric shock in an electroporator (Gene Pulser™ Pulse Controller 1652098, from Bio-Rad; 2.5 kV, 25 µF, 200 Ω).

3.4.10.2 Chemical-Transformation

Calcium-chloride-competent ($CaCl_2$) *E. coli* BL21 DE3 cells were transformed with plasmid DNA. For the transformation 30 µL of the $CaCl_2$ competent cells were mixed with 10 ng of the respective plasmid DNA and incubated for 30 minutes on ice. After the incubation a heat shock at 42°C for 45 seconds in a Biving Block MB-102 was conducted, followed by 2 minutes on ice.

700 µL 2x TY medium (37°C) were added to the transformation mixes and then incubated at 37°C for 30 minutes in an incubator (Binder). 150 µL of each mix were plated on selection plates (2xTY agar with the respective antibiotics) and incubated at 37°C overnight in an incubator.

For a co-transformation of two plasmids in one cell 20 ng of each plasmid were used.

3.4.11 Plasmid isolation

After successful transformation the plasmid-DNA was extracted from a 50 mL *E. coli XL-1* ONC in LB-Medium using Nucleobond PC 100 MIDI (Macherey-Nagel, Düren, Germany), following their manua[66]l, and taking into consideration that pACYCDuet-1 is a low copy plasmid and pET28a(+) a high copy plasmid.

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3.5 Proteins

In order to purify soluble protein complexes and to investigate protein-protein interactions, different proteins had to be expressed.
In table 9 a description of proteins used in this master´s thesis is given. The list contains their molecular weight, amino acid length, tag and the construct they were expressed with.

Table 9: Proteins, *which were used in this master´s thesis are listed, containing their molecular weight (calculated with Ape[65]), their amino acid (AA) length, their tag and the construct they were expressed with.*

Protein	Organism	Molecular weight (kDa)	AAs length	tag	Construct
EF-Tu-His	*E. coli* MG1655	44.137	400	C-terminal 6xHis	pET28tufB
α (full length)	*E. coli* MG1655	37.355	329 335 (+ tag)	C-terminal 6xHis	pET28lysM pDuRNAP329[1]
α (without C-terminal domain)	*E. coli* MG1655	29.307	256 262 (+ tag)	C-terminal 6xHis	pDuRNAP256[1]
σ^{70}	*E. coli* MG1655	71.216	620	N-terminal 6xHis	pLNH12His[67]
His-β	*E. coli* MG1655	153.122	1363	N-terminal 10xHis	pJCore4
β	*E. coli* MG1655	150.279	1342	-	pJCore1 pJCore2 pCore5+C92S
β´	*E. coli* MG1655	152.016	1379	-	pJCore1 pJCore2 pJCore4 pCore5+C92S pβ´ω
His-TraJ	Plasmid R1	28.426	245	N-terminal 6xHis	pJCore1
FLAG-TraJ	Plasmid R1	27.482	236	N-terminal FLAG	pTKNF4[1]
Strep-TraJ	Plasmid R1	28.557	246	N-terminal Strep	pJCore2 pJCore4
Twin-Strep-FLAG-TraJ	Plasmid *R1*	30.900	270	N-terminal Twin-Strep-FLAG	pJCore5

[1] made by Karin Bischof

3.5.1 *Expression*

In order to overexpress proteins, ONCs (2-5 mL) of *E. coli* BL21 DE3, containing the respectively plasmid, were grown and a main culture of 50 mL or 350 mL was inoculated with the ONC to a OD_{600} of 0,1.
The main culture was grown in a shaker (Infors-HT, Bottmingen, Swizerland) for 1,5 hours at 37°C and 180 rpm before the overexpression was induced with 0,5 mM IPTG (Isopropyl-β-D-thiogalactopyranosid) (Sigma Aldrich, St. Louis, MO). After induction, protein production was going on for 2,5 hours or overnight at the indicated temperature (16°C/30°C/37°C). The cell pellet was then collected with centrifugation at 4000 g for 10 minutes, and the washed twice with 10 mL of PBS (table 10). If not stated differently, cells were incubated for 2,5 hours at 37°C.

Table 10: *PBS buffer to wash harvested cells*

PBS
150 mM NaCl 20 mM sodium phosphate, pH 8

3.5.2 *Cell disruption*

Three different cell disruption methods were used:

3.5.2.1 *Mixer*

The same protocol, used for RNA Polymerase purification, after Zhi[68] was used to lyse cells expressing JCore. This protocol contains cell disruption with a blender, Polyethylenimin (PEI) precipitation, a sodium chloride wash and elution step and ammonium sulfate precipitation. The used blender was smoothie 2 Go, from Kenwood.
For the cell disruption, only 4 g of frozen cell pellet were used and only a quarter of the stated buffer volume was added. Furthermore, the 20 minutes stirring was reduced to four times two minutes with five minutes break in between, due to massive foam formation. The DNA was only sheared once for 30 seconds by high speed mixing.

3.5.2.2 *French Press Protocol*

To disrupt cells with a French press, first the 50 mL or 350 mL cell pellet was resuspended in 3.5 mL or 8 mL lysis buffer (table 11), respectively. Then it was disrupted 3 times at 1100 bar with the French pressure baby cell or the French pressure cell (Heineman, Schwäbisch Gmünd, Germany) respectively. The followed pre-purification contained again a PEI precipitation, NaCl wash and elution step and ammonium sulfate precipitation as described by Zhi. [68]

Table 11*: Buffer used for cell disruption using French press*

Lysis buffer for French press
50 mM Tris-HCl, pH 8 5% (v/v) glycerol 1 mM EDTA 5 mM DTT 1 mM PMSF

3.5.2.3 Sonication

For sonication a cell pellet from a 350 mL culture was resuspended in 8 mL buffer and the protocol form Borukhov[69] for the first sonication step for the β-subunit was followed.
Despite the original protocol from Borukhov no lysozyme was added to the buffer (table 12), due to precipitation, and the subunits of aRNAP were expressed together and not expressed separately. Sonication was carried out with 5x15 seconds with a 30% amplitude (Branson sonifier 250, Microtip) and 15 seconds break on ice water.
For native purification of EF-Tu, buffer A (table 12) was used during sonication.

***Table 12**: buffer used for sonication*

Buffer A	Buffer for sonication
50 mM Tris/HCl, pH 7.6 60 mM NH_4Cl 7 mM $MgCl_2$ 7 mM 2-mercaptoethanol 1 mM phenylmethylsulfonyl fluoride 15% glycerol	40 mM Tris-HCl, pH 8 300 mM KCl 10 mM EDTA 1 mM PMSF 0.2% Sodium deoxycholate

3.5.3 Inclusion body - purification

Since most of the overexpressed proteins were in inclusion bodies (IB), the proteins were purified out of them. To purify proteins out of IB, the Borukhov protocol[70] was followed, starting with the 2nd sonication step.
This protocol consists of three sonication steps, with centrifugation at 15 000 rpm (Avanti(R) J-26XP (rotor JA10), Beckman coulter, Indianapolis, IN) in between. Every time after centrifugation the supernatant was discarded and the pellet was resuspended in 8 mL of the next buffer (lysis buffer A-C, table 13). Through this procedure inclusion bodies and proteins contained in these were purified.
In the next step the proteins were denaturised by resuspending the pellet, which contains the purified inclusion bodies, in the denaturation buffer (table 13). Through this step the proteins are solubilized and are called “crude proteins”. They can be stored at -20°C without any damage.

Table 13: Lysis buffer A-C, used for IB-purification and denaturation buffer, used for solubilising the proteis

Denaturation buffer	Lysis buffer A	Lysis buffer B	Lysis buffer C
50 mM Tris HCl, pH 8 6 M guanidine-HCl 10 mM $MgCl_2$ 10 µM $ZnCl_2$ 1 mM EDTA 10 % Glycerol 10 mM DTT	40 mM Tris-HCl, pH 8 300 mM KCl 10 mM EDTA 1 mM PMSF 0.2% Sodium deoxycholate	40 mM Tris-HCl, pH 8 300 mM KCl 10 mM EDTA 1 mM PMSF 0.2% n-octyl β-D-glucopyranoside	40 mM Tris-HCl, pH 8 300 mM KCl 10 mM EDTA 1 mM PMSF 0.2% n-octyl β-D-glucopyranoside 1 mM DTT

3.5.4 *Protein renaturation*

To renature and refold the proteins again the protocol by Tang[71] was followed. Guanidine hydrochloride is dialysed out of the buffer so the proteins can refold. Subunits of aRNAP were co-expressed and directly purified out of inclusion bodies and 500 (if reconstituted with EF-Tu) - 800 µL of the crude protein was used for dialysis. Glycerol (to an final concentration of 30%), PMSF (to a final concentration of 1 nM) and H_2O_{fres} (if needed) were added up to 1 mL of the dialysis mixes. The mixes were dialysed at 4°C in a Float-A-lyzer® G2 dialysis devices (MWOC: 8-10 kDa; Spectrum Laboratories, Inc., Rancho Dominguez, CA) against 300 mL of renaturation buffer (table 15), and the buffer was renewed after 4h and then dialysed overnight.

The same method was also used to reconstitute the aRNAP, parts of it or with possible interaction partners. Therefore, separate denatured proteins were renatured together in so called reconstitution mix, which allows the proteins to refold slowly and form a soluble complex.
The used amount of each aRNAP subunit and EF-Tu during reconstitution in 1 mL is shown in table 14.

Table 14: Amount of the subunits for renaturation in 1 mL

Subunit	***µg***	***source***
β'+ω	300	Karin Bischof 30.6.16 (p$\beta'\omega$)
β	150	Karin Bischof 18.3.16
FLAG-TraJ	40	Karin Bischof 18.3.16
EF-Tu	40	This work
σ^{70}	40	Karin Bischof 12.8.15 (A+B)

To optimize the renaturation, Burgess[70] optimization protocol was followed and the optimal glycerol concentration was determined for refolding of aRNAP.
Glycerol was added to the dialysed sample to an end concentration of 30% so it could be frozen at -20°C for storing without any damage.

Table 15: Renaturation buffer used for renaturation of denaturised proteins

Renaturation buffer
50 mM Tris HCl, pH 8
10 mM $MgCl_2$
10 µM $ZnCl_2$
1 mM EDTA
200 mM KCl
30 % Glycerol
2 mM DTT

3.5.5 *Fast protein liquid chromatography (FPLC)*

FPLC is a protein purification method were a liquid and a solid phase is used. The purpose of the solid phase is to separate the protein(s) of interest from other proteins in a certain manner. In this work size exclusion and affinity purification chromatography is used. In the liquid phase the proteins are solved and through its change (length of elution or a specific elution buffer) the proteins which were hold back from the solid phase are eluted in the liquid phase. In this master´s thesis an ÄKTAFPLC system (GE Healthcare, Chicago, IL) was used in combination with the Unicorn software.

To avoid damages of the equipment all used buffers were sterile-filtered with a sterile unit (Merck Millipore, Darmstadt, Germany) and degassed with SONOREX ultrasound (BANDELIN, Berlin, Germany). The FPLC system was located in a 4°C room. Before protein purification the whole system of the FPLC (pumps, loops, fraction collector, collumn) was washed with water and next equilibrated with the first buffer, which was used during the protein purification.
After the protein purification the whole system was washed again with water and the columns were stored in 20% ethanol at 4°C.

In table 16 all used columns used in this master´s thesis are listed.

***Table 16: Columns** used in this master´s thesis*

His GraviTrap TALON (GE Healthcare, Chicago, IL)
His spinTrap (GE Healthcare, Chicago, IL)
HisTrap (1mL) (GE Healthcare, Chicago, IL)
PD-10 Desalting Column (GE Healthcare, Chicago, IL)
StrepTrap HP (1mL) Column (GE Healthcare, Chicago, IL)
Superdex 200 Increase 10/300 GL (GE Healthcare, Chicago, IL)
Superose 6 Increase 10/300 GL (GE Healthcare, Chicago, IL)

3.5.5.1 *Affinity purification of proteins and protein complexes*

His-tagged proteins and their interaction partners were purified out of (pre-purified) cell lysate (3.5.2), denatured (3.5.3.) or renatured proteins (3.5.4) by immobilized metal ion affinity chromatography. His-columns (His GraviTrap TALON, His spinTrap and HisTrap (GE Healthcare, Chicago, IL)) were used for this propose.
To purify Strep-tagged proteins and their possible partners out of (pre-purified) cell lysate (3.5.2) or renatured proteins (3.5.4) StrepTrap HP Column was used.

3.5.5.1.1 StrepTrap HP Column

This column was used in combination with an ÄKTAFPLC (GE Healthcare, Chicago, IL) system, and was hold at all times at 4°C. The maximal applied pressure was 0,5 MPa, and the used flowrate was 0,5 mL/min. First the column was washed using 5 mL SC-binding buffer or TEGED buffer (table 17). The sample ((pre-purified) cell lysate (3.5.2) or renatured proteins (3.5.4) containing Strep-tagged proteins) was centrifuged at 4°C and 16 000 g for 15 minutes in order to load 500 µL of the supernatant on the column. After washing the column with 10 mL SC-binding buffer, two times 5 mL SC-elution buffer (table 17) was used.
The flow through, washing and elution steps eluates were collected and analysed over a SDS-Page and WB. For the analysation 100 µL were acetone-precipitated and

then resuspended in 40 µL FSB. 10 µL were analysed on a SDS-PAGE and western blot (3.5.7).

Table 17: buffers used for protein purification over StrepTrap HP Column

SC-binding buffer	TEGED buffer	SC-elution buffer
50 mM Tris HCl, pH 8 10 mM $MgCl_2$ 10 µM Zinc chloride 1 mM EDTA 200 mM KCl 2 mM DTT	10 mM Tris HCl pH 8 5% (v/v) glycerol 0.5 mM NaCl 0.1 mM EDTA 0.1 mM DTT	2.5 mM desthiobiotin in SC-binding buffer

3.5.5.1.2 His-Trap

His spin trap:
For purification of His-tagged TraJ 600 µL of pre-purified cell lysate out of a 50 mL *E. coli* BL-21[pJCore1] culture were applied to the His spin column (GE Healthcare, Chicago, IL). The purification was proceeded as described in the manual[72]. A wash buffer 1 was applied and then the wash step was repeated with wash buffer 2 and eluted in Elution buffer. For the analysation 100 µL of each wash step and eluate was acetone-precipitated and then resuspended in 40 µL FSB. 10 µL were analysed on a SDS-PAGE and western blot (3.5.7.).

Table 18: Buffers used for protein purification over His-spin trap

binding buffer 1	Wash buffer 1	Wash buffer 2	Elution buffer
20 mM Tris-HCl pH 8 5% (v/v) glycerol 0.5 mM b-mercaptoethanol 1M NaCl	binding buffer 5 mM imidazole	binding buffer 40 mM imidazole	binding buffer 250 mM imidazole

Denaturing conditions
The 1 mL His-Trap column (GE Healthcare, Chicago, IL) was used under denaturing conditions to purify His-tagged proteins out of crude proteins (3.5.3) and was handled with a 5 mL syringe (Braun). First it was washed with 4 mL of $H_2O_{fres.}$ and then equilibrated with 5 mL of binding buffer 2 with a flowrate not exceeding 1 mL/minute.
After equilibration, 8 mL of the supernatant from the centrifuged (15 minutes, 16 000rcf) sample (denaturised protein (3.5.3)), was applied with a 10 mL syringe. 15 mL of wash buffer was then applied to the column. Bound proteins were eluted with 3 mL of elution buffer. The input of the column and the eluate were collected. For the analysation 100 µL were ethanol-precipitated and then resuspended in 40 µL FSB and analysed on a SDS-PAGE and western blot (3.5.7).

Table 19: Buffers used for protein purification over His-Trap column under denaturation conditions

Binding buffer 2	Wash buffer	Elution buffer
50 mM Tris-HCl, pH 8 0.5 M NaCl 5 mM imidazole 6 M guanidine hydrochloride complete	50 mM Tris-HCl, pH 8 0.5 M NaCl 5 mM imidazole 6 M guanidine hydrochloride	50 mM Tris-HCl, pH 8 0,5 M NaCl 500 mM imidazole 6 M guanidine hydrochloride

3.5.5.1.3 His-GraviTrap-TALON column

The His-GraviTrap-TALON column was used to purify His-tagged protein and its interaction partners out of (pre-purified) cell lysate (3.5.2).
When pre-purified cell lysate was obtained by cell disruption with a mixer (3.5.2.1) and was further used with the His-GraviTrap-TALON column, a puffer change from TEGED-buffer to binding buffer 1 had to be done. This is necessary because the TEGED-buffer is not compatible with this column. This was done using PD-10 column as described in the manual[73]. The sample, now in binding buffer 1, was loaded on a His-GraviTrap-TALON column, after it was equilibrated with 10 mL of binding buffer 1.
When the sample was going very slow into the column volume, 20 µL DNase (ThermoFisher Scientific, Waltham, MA, 1 U/µL) and 2 mL wash buffer 1 was added to the sample.
To wash the column, 10 mL of wash buffer 1 was applied to the column and then the same amount of wash buffer 2 was applied. The proteins were eluted with 3 mL of each elution buffer, which contained binding buffer 1 and the indicated concentration of imidazole.
100 µL of the flow through, the wash steps and the elution were acetone precipitated and analysed on a SDS-PAGE and western blot (3.5.7). Used buffers are listed in table 17 and 18.

Before applying cell lysis supernatant containing EF-Tu (3.5.2.3) to the column, it was centrifuged for 15 minutes at 10 000 cfu. 3 mL of the supernatant were mixed with 5 mL of binding buffer 3 and applied to the column, after the column was equilibrated with 20 mL of binding buffer 3. The column was washed with 10 mL of wash buffer. Protein elution followed with 3 mL elution buffer. 100 µL of the flow through, the wash steps and the elution were acetone precipitated and analysed on a SDS-PAGE and western blot (3.5.7). Used buffers are listed in table 20.

Table 20: buffers used for His-GraviTrap-TAON column

Binding buffer 3	Wash buffer	Elution buffer
250mM Sodium phosphate 300 mM NaCl	binding buffer 3 5 mM imidazole	binding buffer 3 150-1000 mM imidazole

3.5.5.2 Size exclusion chromatography

The size exclusion columns (Superdex 200 Increase 10/300 GL and Superose 6 Increase 10/300 GL) were used to separate protein complexes from free proteins out of renaturation mixes (3.5.4). This was achieved by separation based on the size of

the proteins. By trapping smaller molecules in the pores of the stationary phase, lager molecules, which are to large to enter the pores, flow quicker through the column. The smaller the molecule, the longer the retention time, the larger the molecule the shorter the retention time.
The columns were used in combination with an ÄKTAFPLC (GE Healthcare, Chicago, IL) system and were hold at 4°C at all time. The maximal applied pressure was 2.2 MPa, and the used flowrate was 0,5 mL/min. The sample (refolded protein complexes) to be analysed was centrifuged at 4°C and 16 000 g for 15 minutes in order to load 500 µL of the supernatant on the column.
First the column was washed with water and then with SC-binding buffer for 36 mL. Each run consisted of 5 mL equilibration with SC-binding buffer (table 17) followed by the sample injection and 36 mL of elution with SC-binding buffer. Each mL was collected separately, starting at 1.5 mL after injection. 100 µL of the indicated millilitres were then acetone precipitated analysed on a SDS-PAGE and western blot (3.5.7).

3.5.6 Protein interaction studies

3.5.6.1 Far western blot

The far western blot is a fast and easy method to investigate protein interactions of two proteins.
Interaction partners are discovered by overlaying the membrane of a western blot with a protein. The overlaying protein probes possible interaction partners on a western blot, which were in the previous separated on a SDS-PAGE and then subject to a western blot. Then the protein, with which the membrane was overlaid, is detected with AB, as usual for a western blot. When a band with the height of the protein, which was separated on the SDS-PAGE is detected, an interaction between the overlaying and Protein on the SDS-PAGE is discovered.
The far western blot was done as described for a normal western blot (3.5.7). However, instead of a primary AB, 0.2 µM purified Protein (FLAG-TraJ, His-α or His-EF-Tu) was used. Then the respective primary and secondary AB against the before used Protein (FLAG-TraJ, His-α or His-EF-Tu) were used to detect where the applied Protein had interacted with the Proteins on the membrane.

3.5.6.2 Co-Imunoprecipitation with FLAG-agarose beads

Protein interactions can also be shown with Co-Immunoprecipitation. In this master´s thesis FLAG-agarose beads are used for this method, were antibodies against FLAG are immobilized on agarose beads, which can bind FLAG-tagged proteins, in this case FLAG-TraJ. Through a washing step all proteins are washed away, except of FLAG-TraJ which binds to the antibodies on the agarose beads and proteins which interact with TraJ. This allows to indirectly capture proteins that are bound to TraJ, which can be shown when the proteins are eluted from the beads and are analysed on a SDS-PAGE.

First 40 µL of Anti-FLAG M2-Agarose (Sigma Aldrich, St. Louis, MO) were blocked with 3% Blotto for 30 minutes at room temperature to block unspecific binding sites. Then it was centrifuged for 90 seconds at 16800 rcf (Eppendorf centrifuge, 5415 R) and the supernatant was discarded. The beads were then washed four times with

500 µL TBS, which contained resuspension (by inverting the Eppendorf tube) of the beads in TBS, centrifuge for 90 seconds at 16800 rcf and then wasting the supernatant.

After the wash-steps, the beads were equilibrated with 500 µL dilution buffer, which consisted of resuspension (by inverting the Eppendorf tube) of the beads in dilution buffer, centrifuge for 90 seconds at 16800 rcf and then wasting the supernatant. From this step onwards all steps were on 4°C or on ice. Then the beads were incubated with 90 µL of the indicated protein mix and 710 µL dilution buffer overnight under constant rotation with a tube rotator (VWR Tube Rotator). Next the sample was centrifuged for 90 seconds at 8000 rcf. The supernatant was separated form the pellet and both were analysed.
The pellet, containing the beads and the bound proteins, was washed three times with 500 µL of TBS and then resuspended in 40 µL 2 x FSB. Before applying the sample to a SDS-PAGE, in was denatured at 65°C for 10 minutes and then centrifuged. In this way the protein, which before was bound and pulled down from the beads, was now detached from the beads and located in the supernatant.

200 µL of the supernatant and 600 µL acetone was precipitated at -70°C for 30 minutes. After the precipitation it was centrifuged for 15 minutes at 8000 rcf and the supernatant was wasted. The pellet was dried on room temperature and then resolved in 40 µL 1xFSB.
15 µL of the pellet and supernatant were loaded on a SDS-PAGE followed by a western blot. (3.5.7.4 & 3.5.7.5) Used buffers are listed in table 21.

Table 21: *Buffers used for Imuno-co-precipitation*

3% Blotto	TBS	Dilution buffer
50 mM Tris-HCl, pH 7.5 150 mM NaCl 0.1% Tween 20 (v/v) 3% milk powder	20 mM Tris HCl, pH 7.5 150 mM NaCl	20 mM Tris HCl, pH 7.5 150 mM NaCl 2 mM EDTA 1% Triton X-100

3.5.7 *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot*

3.5.7.1 *Whole cell analysation preparation*

The cell pellet was resuspended in 1xFSB (table 22) and heated at 95°C for 5 minutes. Then the sample was centrifuged and the supernatant was further used.

3.5.7.2 *Acetone precipitation*

To precipitate soluble proteins in order to analyse them on a SDS-PAGE, three volumes of pre-cooled acetone (-20°C) were mixed with the sample and then the mix was stored at -20°C overnight or 20 minutes at -70°C. Afterwards it was centrifuged for 10 minutes at 16,000 g and 4°C. All of the supernatant was carefully removed. The pellets were dried for 15 minutes at 37°C with an open lid in an incubator (Binder, Tuttlingen, Germany). Then the pellets were resuspended in 40 µL 1x FSB (table 22), heated up to 95°C for 5 minutes and then loaded on SDS gels for SDS-PAGE or western blot analysis (3.5.7.4-5)

3.5.7.3 *Ethanol precipitation*

When proteins were denaturised with guanidine hydrochloride because of insolubility, they could not be precipitated with previous described acetone precipitation for following analysation on a SDS-PAGE. Ethanol precipitation on the other hand did precipitate the denaturised proteins so they could be analysed on a SDS-PAGE.
100 µL of denatured protein were mixed with 900 µL cold absolute ethanol (-20°C) and precipitated on -20°C overnight or at least one hour.
Then the the precipitated proteins were centrifuged for 15 minutes at 20 817 g and 4°C. The supernatant was thrown away and the tubes were dried with bottom up on paper towels.
900 µL 70% ethanol was added without mixing and the sample was centrifuged for 10 minutes at 20 817 g at 4°C. All of the supernatant was put away, using a pipette and the protein pellet was dried for 10-15 minutes or more at 37°C till it was dry. The dry pellet was resolved in 40 µL 1xFSB and heated up at 95°C for 5 minutes.

3.5.7.4 *SDS-PAGE*

With SDS-PAGE, protein separation in a polyacrylamide gel through an electrical field is realised, and was conducted according to standard protocols.[60] The samples were prepared by adding 5xFSB (table 22) to a final concentration of 1x and then heated for 5 minutes at 95°C. Then 10 µL of the samples were loaded on a 12.5% or a 15% polyacrylamide running gel with a stacking gel on top (table 23). 4 µL of the PageRuler^{TM} pre-stained protein ladder (ThermoFisher Scientific, Waltham, MA) were also always loaded on the gel. After the separation, the gel was shortly washed with dH_2O and either stained with Kang staining solution (table 22) under swivelling or it was used further for western blotting (3.5.7.5)

Table 22: buffers used for SDS-PAGE

5x conc. FSB	Electrophoresis buffer	Kang staining soultion
0.3 M Tris-HCl, pH 6.8 0.6 M DTT 10% SDS 50% glycerol 0.02% bromophenol blue	250 mM Tris 1.92 M glycine 1% SDS	0.02%, Coomassie Blue CBB G-250 5%, Aluminium-(14-18)- hydrate-sulfate 10% Ethanol (96%) 2% Ortho-Phosphoric acid (100%)

Table 23: Gels used for SDS-PAGE

12,5% running gel	15% running gel	Stacking Gel
375 mM Tris-HCl, pH 8.8 12.5 % AA:BA = 37.5:1 0.1 % SDS 0.87% TEMED 0.027% APS	375 mM Tris-HCl, pH 8.8 15 % AA:BA = 37.5:1 0.1 % SDS 0.87% TEMED 0.027% APS	125 mM Tris-HCl, pH 6.8 4.5% AA:BA = 37.5:1 0.1 % SDS 0.135% TEMED 0.04% APS 0.001% bromophenol blue

3.5.7.5 Western blot

To detect different proteins on SDS-PAGE with different antibodies, the proteins have to be transferred on a membrane. This method is called western blotting and was conducted according to standard protocols.[60] The membrane (Immobilon-P transfer PVDF membrane, Merck Millipore, Darmstadt, Germany) was activated with methanol for 1 minute.

After the transfer, the membrane was put in 1xTST containing 1 or 3% milk powder and the unspecific binding sites were blocked over night at 4°C or 30 minutes at room temperature. The membrane was washed three times with 1xTST for 5 minutes (wash step) and then incubated with the respective antibodies (table 25), which again was followed by a wash step. Western blotting Detection Reagents (Clarity ™ or Select ™ Western ECL substrate, GE Healthcare, Chicago, IL) were used for detection.

The detection of the chemiluminescence was conducted with a molecular Imager® Gel Doc™ XR System (Bio-Rad, Hercules, CA) for 1-15 minutes, depending on the signal intensity. After detection the membrane was stripped with stripping buffer for 25 minutes at 50°C in a shaking water bath 1083 (GFL, Burgwedel, Germany), washed 5 times for 5 minutes with 1x TST under the outlet and stored between to filter papers in the dark until it was used again. Before a new antibody was used the membrane had to be blocked with 1x TST and 3% milk powder for 45 minutes. Buffers used for western blotting are listed in table 24.

Table 24: Buffers used for western blotting

TST	Caps buffer	Stripping buffer
50 mM Tris-HCl, pH 7.5 150 mM NaCl 0.1% Tween 20 (v/v)	10 mM CAPS 10% Methanol pH 11	62.5 mM Tris-HCl,pH 6.8 2% SDS 100 mM β-mercaptoethanol

3.5.7.6 Antibodies

Antibodies, which were used for the detection of proteins during western blots, are listed in table 25.

***Table 25: Antibodies** used for western blot analyses, with the respective dilutions and sources*

Name	Dilution[1]	Source/Company
anti-His peroxidase conjugate[3]	1:10,000	Sigma-Aldrich, St. Louis, MO
anti-Strep MAB–Classic, HRP[2] conjugate[3]	1:32,000	IBA Lifescience, Goettingen, Germany
anti-β[3]	1:2,000	Neoclone, Madison, WI
anti-σ^{70} [3]	1:2,000	Neoclone, Madison, WI
anti-EF-Tu[3]	1:1,000	Hycult Biotech, Uden, Netherlands
anti-α-NTD[3]	1:2,000	Neoclone, Madison, WI
anti-FLAG[3]	1:8,000	Sigma-Aldrich, St. Louis, MO
anti-mouse IgG, HRP[2] conjugate[4]	1:15,000	GE Healthcare, Chicago, IL

[1] the dilution was made in 1% dry milk powder in 1xTST. [3]produced in mouse
[2] horseradish peroxidase conjugate [4]produced in rabbit

3.6 Water quality

According to the different experiments deionised-, double distilled- or nuclease free Fresenius water (Fresenius Kabi, Bad Homburg, Germany) was used.

3.7 Laboratory equipment

Table 26 lists all laboratory equipment used in this master´s thesis.

Table 26: Laboratory equipment used in this master´s thesis

ÄKTAFPLC system (GE Healthcare, Chicago, IL)
Avanti(R) J-26XP centrifuge (rotor JA10) (Beckman coulter, Indianapolis, IN)
French press baby cell (Heineman, Schwäbisch Gmünd, Germany)
Eppendorf centrifuge, 5415 R (Eppendorf, Hamburg, Germany)
Gene Pulser™ Pulse Controller 1652098 (Bio-Rad, Hercules, CA)
GS Gene Linker™ UV Chamber (Bio-Rad, Hercules, CA)
Heating block Biving Block MB-102 (Bioer Technology, Hangzhou, China)
Incubator (Binder, Tuttlingen, Germany)
light microscope (Leitz Biomed/Leica, wetzlar, Germany)
Mighty Small Transphor electrophoresis Unit (GE Healthcare, Chicago, IL)
Molecular Imager® Gel Doc™ XR System (Bio-Rad, Hercules, CA)
Multitron standard shaker (Infors-HT, Bottmingen, Swizerland)
nanoDrop 1000 (ThermoFischer scientific, Waltham, MA)
PeqStar Primus 25 Cycler (VWR, Radnor, PA)
PowerPac Basic (Bio-Rad, Hercules, CA)
French press pressure cell (Heineman, Schwäbisch Gmünd, Germany)
SE250 Mighty Small II gel electrophoresis units (Hoefer, Holliston, MA)
shaking water bath 1083 (GFL, Burgwedel, Germany)
smoothie 2 Go (Kenwood, Havant, UK)
Sonifier 250 with microtip (Branson, Dietzenbach, Germany)
SONOREX super Ultrasound bath (BANDELIN, Berlin, Germany)
SPECTROstar Nano (BMG Labtech, Ortenberg, Germany)
Tube Rotator (VWR, Radnor, PA)
UV transilluminator CN-3000-WL (Biovision, Milpitas, CA)
Vision Capt software
Wide Mini-Sub® Cell GT (Bio-Rad, Hercules, CA)

4 Results

4.1 Construction and description of expression-vectors used in this master´s thesis

To express protein complexes expression constructs had to be designed.
Gibson assembly (pJCore1, pJCore2, pJCore4S, pJCore5, pβ´ω, pETlysM and pET28tufB) or traditional cloning (pETlysMα) was used to assemble the plasmids, which are listed in table 4 and described in the following part. More detailed information on the backbones pET28(a)+ and pACYCDuet-1 are given in 3.4

4.1.1 pJCore expression vectors

Figure 11 gives an overview over the different constructs, which encode aRNAP and describes the different components of each pJCore plasmid.

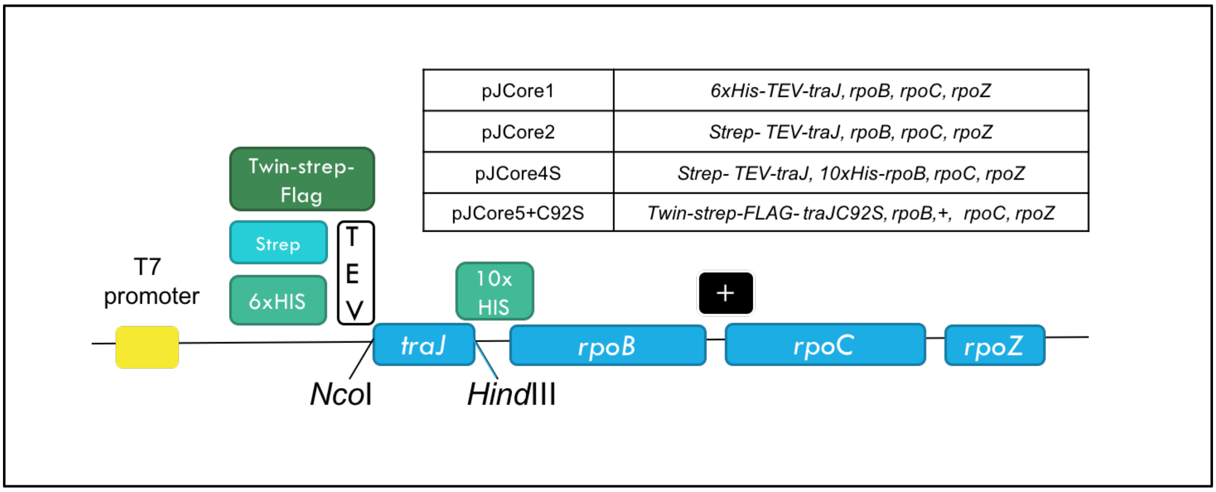

***Figure 11: Schematic overview of the different constructs, which encode the aRNAP**. The different N-terminal tags (green) and the TEV-cut site are shown, the plus represents the intergenic region between* rpoB *(β) and* rpoC *(β´). All exact constructs are shown in the the following part (figure 12, 14, 16, 18) and listed in table 4.*

4.1.1.1 pJCore1

The expression-vector pJCore1 (figure 12, consists of the fragments PACYC_F1, TEV_TraJ_F1, RpoB_F1, RpoC_F1 and RpoZ_F1 (table 5) and is based on the backbone pACYCDuet-1. It contains sequences encoding 6x His-tagged TraJ, β´-, β- and ω- subunit of RNAP of *E. coli.* His-tag can be cleaved off via a TEV-protease, because a TEV-protease cleavage site is placed between TraJ and the His-tag. This can be useful either during purification or after purification to obtain purified TraJ without any tag, which might interfere with its confirmation or interaction partners.
Furthermore, the coding sequence of TraJ can be exchanged with different mutants of TraJ or other proteins, because it contains restrictions endonuclease cutting sites for *Nco*I at the N-terminus and *Hind*III on the C-termnius of *traJ*.

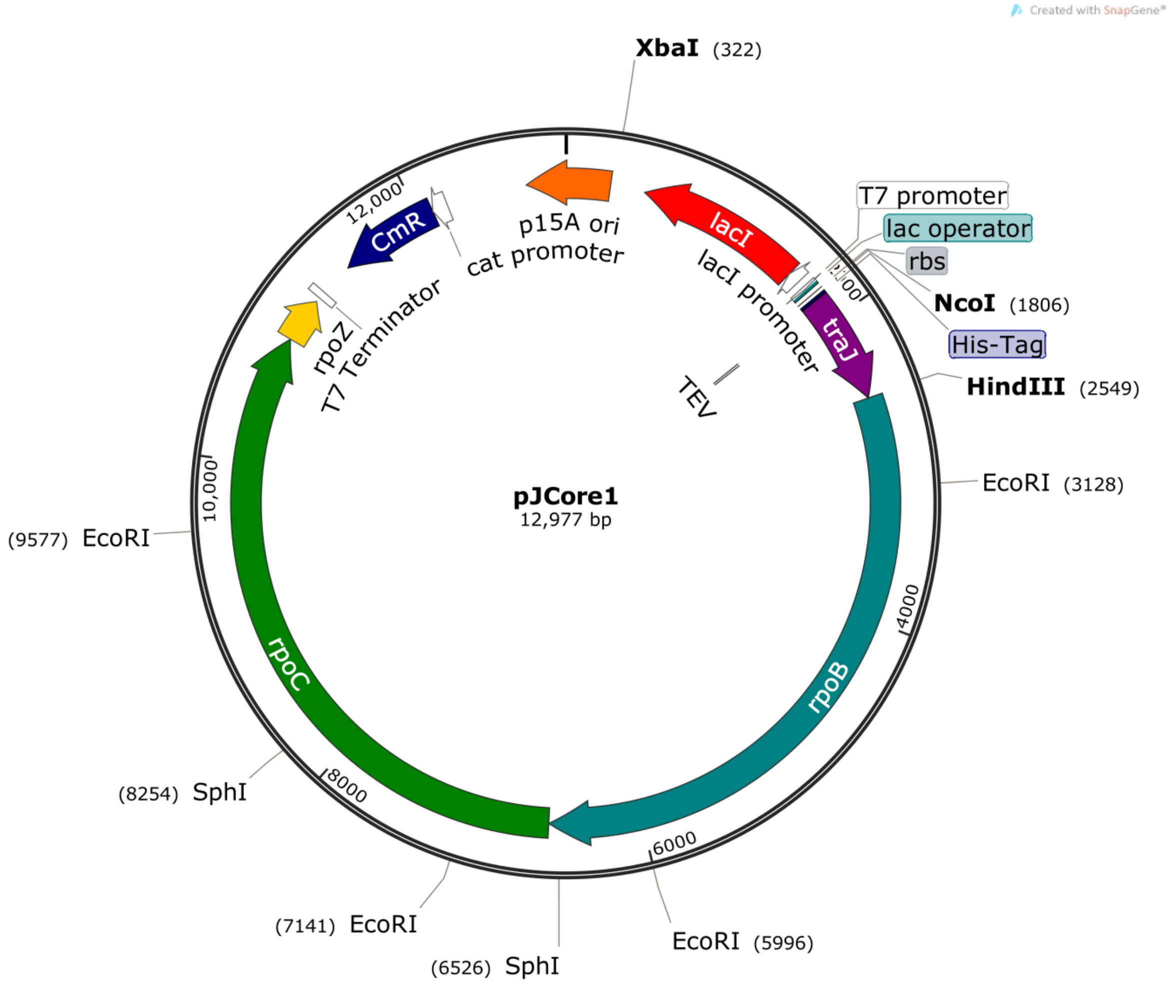

Figure 12: Expression vector pJCore1: *expression vector for JCore1 (encodes His-TEV-TraJ, β, β´, ω); contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator, 6x His-tag, TEV-cut site,* traJ *(encodes TraJ),* rpoB *(encodes RNAP subunit β),* rpoC *(encodes RNAP subunit β´),* rpoZ *(encodes RNAP subunit ω), T7 terminator,* cat *(chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. The plasmid was generated using Gibson assembly out of the fragments PACYC_F1, TEV_TraJ_F1, RpoB_F1, RpoC_F1 and RpoZ_F1.*

This construct allows to overexpress TraJ together with parts of RNAP (β´, β and ω). Furthermore, TraJ and its interaction partners can be purified over its His-tag.

To construct this plasmid, the fragments were synthesised by PCR with respective primers. Figure 27 shows an agarose gel where DNA fragments, synthesised by PCR, were analysed for their right size. The fragments and their respective size, the used primers and templates are listed in table 5. To analyse and also get pure DNA-fragments the obtained fragments were separated on an agarose gel and purified out of the gel.
The purified fragments were then assembled with Gibson assembly to a plasmid. *E. coli* XL-1 cells were transformed with the assembled pJCore1 to amplify the plasmid. After culturing the cells, pJCore1 was isolated out of the cells again. To screen the constructed plasmid, pJCore1 was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 13). The pattern can be seen on a agerose gel where the different sized DNA fragments are separated. For this purpose, pJCore1 was cut with EcoRI resulting in fragments with the size 6528, 2868, 2436 and 1145 bp. Furthermore, the plasmid was cut with XbaI and SphI resulting in fragments with

the size 6200, 5049 and 1728 bp. Both analysations of the restriction patterns, obtained with EcoRI and XbaI and SphI showed the expected pattern.

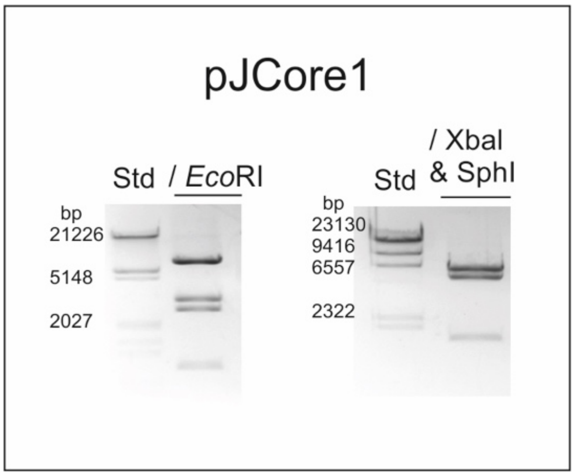

Figure 13: Verification of pJCore1*, via restriction-pattern-analysation. After the isolation out of* E. coli *XL-1. pJCore1 was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments are 6528, 2868, 2436 and 1145 bp for the digestion with EcoRI and for the digestion with XbaI and SphI 6200, 5049 and 1728 bp. For size comparison λ/EcoRI & HindIII and λ/HindIII standards were used. The shown pictures are out-takes of pictures, taken with a digital camera.*

To verify the the sequence pJCore1 was also sequenced and the obtained sequence is shown in the supplementary data. The obtained contiguous sequences were assembled with the software CAP3[74]. The sequences were aligned with the expected sequence of the plasmid. The sequencing results and sequencing primers can be found in the supplementary data. No mutation was found.

4.1.1.2 pJCore2

The expression-vector pJCore2 (figure 14) is assembled out of the fragments PACYC_F2, TEV_TraJ_F2, RpoB_F1, RpoC_F1 and RpoZ_F1 (table 5). This vector is similar to pJCore1, differing only in the tag of TraJ, which is a N-terminal Strep-tag in the case of pJCore2.

In order to synthesise the plasmid, the fragments were generated by PCR with respective primers. Figure 27 shows an agarose gel where DNA fragments were analysed for their right size. The fragments and their respective size, the used primers and templates are listed in table 5. The obtained fragments were analysed on an agarose gel and purified out of the gel.

The purified fragments were then assembled with Gibson assembly to a plasmid and *E. coli* XL-1 cells were transformed with the assembled pJCore2 to amplify the plasmid. After culturing the cells, pJCore2 was isolated out of the cells again. To check if the plasmid was constructed in the right way, the plasmid was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 15). For this purpose, pJCore2 was cut with EcoRI resulting in fragments with the size 6531, 2868, 2436 and 1145 bp. Furthermore, the plasmid was cut with XbaI and SphI resulting in fragments with the size 6203, 5049 and 1728 bp. Both restriction pattern analysations of the plasmid, obtained with EcoRI and XbaI and SphI showed the expected pattern.

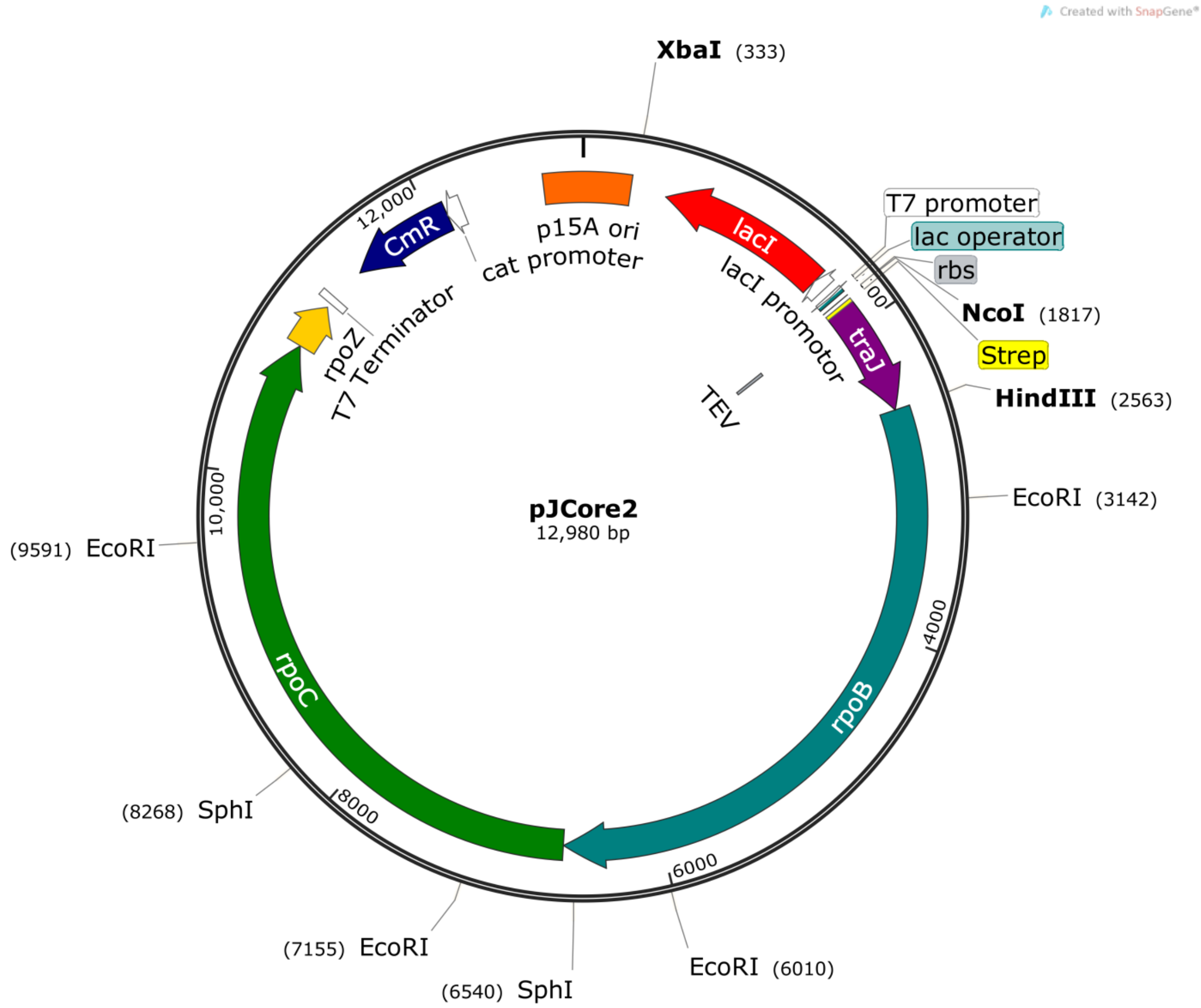

Figure 14: Expression vector pJCore2: *expression vector for JCore2 (Strep-TEV-TraJ, β, β´, ω); contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator, Strep-tag, TEV-cut site,* traJ *(encodes TraJ),* rpoB *(encodes RNAP subunit β),* rpoC *(encodes RNAP subunit β´),* rpoZ *(encodes RNAP subunit ω), T7 terminator,* cat *(chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. The plasmid was generated using Gibson assembly with the fragments PACYC_F2, TEV_TraJ_F2, RpoB_F1, RpoC_F1 and RpoZ_F1.*

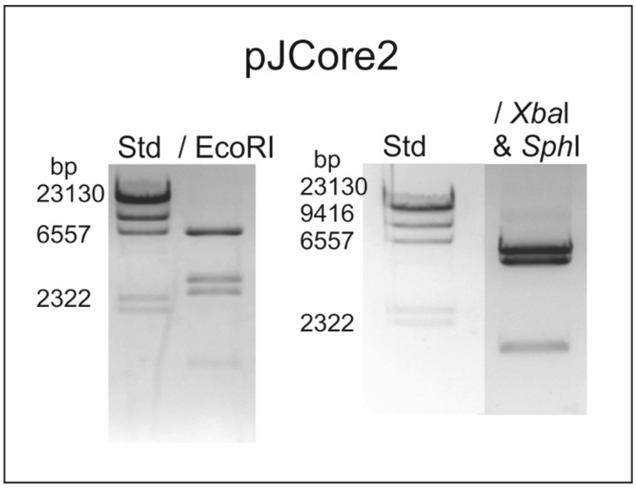

Figure 15: Verification of pJCore2*, via restriction-pattern-analysation. After the isolation out of* E. coli *XL-1. pJCore2 was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments are 6531, 2868, 2436 and 1145 bp for the digestion with EcoRI and for the digestion with XbaI and SphI 6203, 5049 and 1728 bp. For size comparison λ/EcoRI & HindIII and λ/HindIII standards were used. The shown pictures are out-takes of pictures, taken with a digital camera.*

To verify the the sequence pJCore2 was also sequenced, and the obtained sequence is shown in the supplementary data. The obtained contiguous sequences were assembled with the software CAP3[74]. The sequence was aligned with the expected sequence of the plasmid. The sequencing results and sequencing primers can be found in the supplementary data. No mutation was found.

4.1.1.3 pJCore4S

The fragments FA_F1 (PACYC_F2 and TEV_TraJ_F2), H10RpoB_*F3* and FB_F1 (containing *rpo*C and *rpo*Z) (table 5) were assembled to the expression vector pJCore4S (figure 16). A similar vector pJCore4H was also constructed, but FA_F1 was exchanged with pACYC_F1 and TEV_TraJ_F1 (encoding His-tagged TraJ). Because it was not further used it is not described in this master´s thesis. This construct differs to the other construct because not only TraJ is N-terminally Strep-tagged, but also the β-subunit is tagged N-terminally with a 10xHis-tag, which allows two approaches to to purify the alternative RNAP.

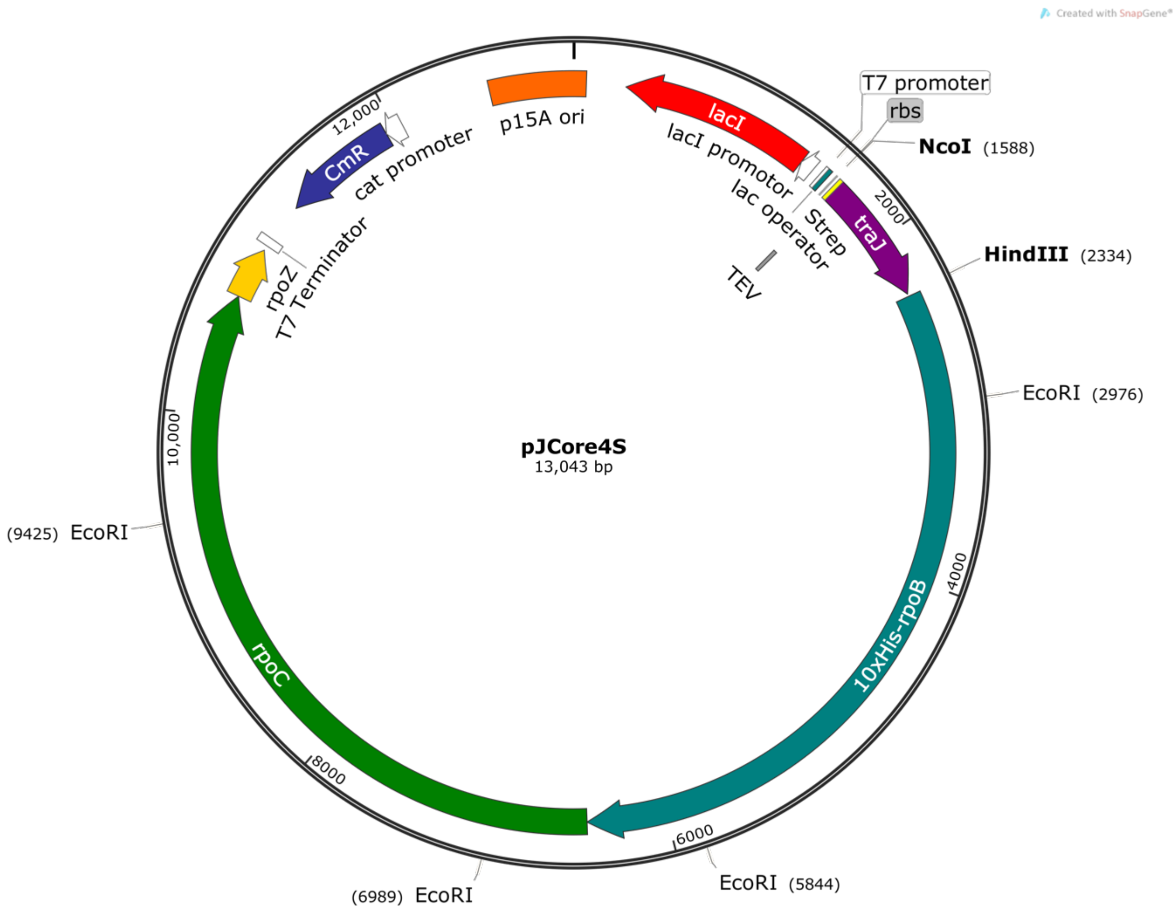

Figure 16: Expression vector pJCore4S: *expression vector for JCore4 (Strep-TEV-TraJ, His-β, β´, ω); contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator, Strep-tag, TEV-cut site,* traJ *(encodes TraJ), 10xHis-tag,* rpoB *(encodes RNAP subunit β),* rpoC *(encodes RNAP subunit β´),* rpoZ *(encodes RNAP subunit ω), T7 terminator,* cat *(chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. The plasmid was generated with Gibson assembly, using the fragments FA_F1, H10RpoB_*F3 *and FB_F1.*

In order to synthesise the plasmid, the fragments were synthesised by PCR (figure 27) with respective primers. The fragments and their respective size, the used primers and templates are listed in table 5. To analyse the obtained fragments, they were separated on an agarose gel and purified out of the gel.

The purified fragments were then assembled with Gibson assembly to a plasmid and *E. coli* XL-1 cells were transformed with the assembled pJCore4S to amplify the plasmid. After culturing the cells, pJCore4S was isolated out of the cells again. To check if the plasmid was constructed in the right way, the plasmid was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 17). For this purpose, pJCore4S was cut with EcoRI, which gave a distinct pattern on an agarose gel with fragments of 6573, 2868, 2436 and 1145 bp.

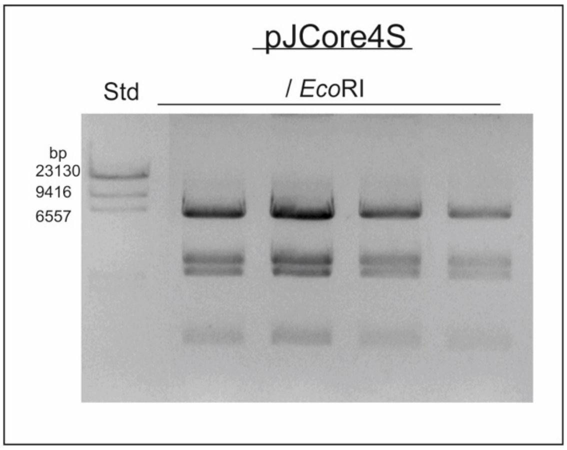

***Figure 17: Verification of pJCore4S**, via restriction-pattern-analysation. After the isolation out of* E. coli *XL-1. pJCore4S was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments after digestion with EcoRI are 6573, 2868, 2436 and 1145 bp. For size comparison λ/HindIII standard was used. The shown pictures are out-takes of pictures, taken with a digital camera.*

4.1.1.4 pJCore5

The expression vector pJCore5 (figure 18) is assembled out of the fragments PACYC_F3, TraJ_C92S_F3, RpoB_F2, RpoC_F2 and RpoZ_F1 (table 5). This vector allows overexpression of Twin-Strep-FLAG-tagged TraJ mutant C92S, which results in better function (unpublished data of the laboratory of Günther Koraimann), together with subunits of RNAP (β´, β and ω). Twin-Strep-tag is an improved version of the Strep-tag, which should assure better binding to the StrepTrap HP column and better detection of Strep-tag. The purpose of the FLAG-tag is to make sure the Twin-Strep-tag is accessible. Furthermore, the expression of β´ was also tried to improve by adding the intergenic region between *rpoB* and *rpoC* which is contained in the fragments RpoB_F2 and RpoC_F2.

To synthesise the plasmid, the fragments were synthesised by PCR (figure 27) with respective primers. The fragments and their respective size, the used primers and templates are listed in table 5. The obtained fragments were separated on an agarose gel and purified out of the gel.

The purified fragments were then assembled with Gibson assembly to a plasmid and *E. coli* XL-1 cells were transformed with the assembled pJCore5 to amplify the plasmid. pJCore5 was isolated out of the cells again, after culturing the cells. To check if the plasmid was constructed in the right way, the plasmid was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 19). For this purpose, pJCore4S was cut with EcoRI, resulting in fragments with 6623, 2868, 2436 and 1145 bp.

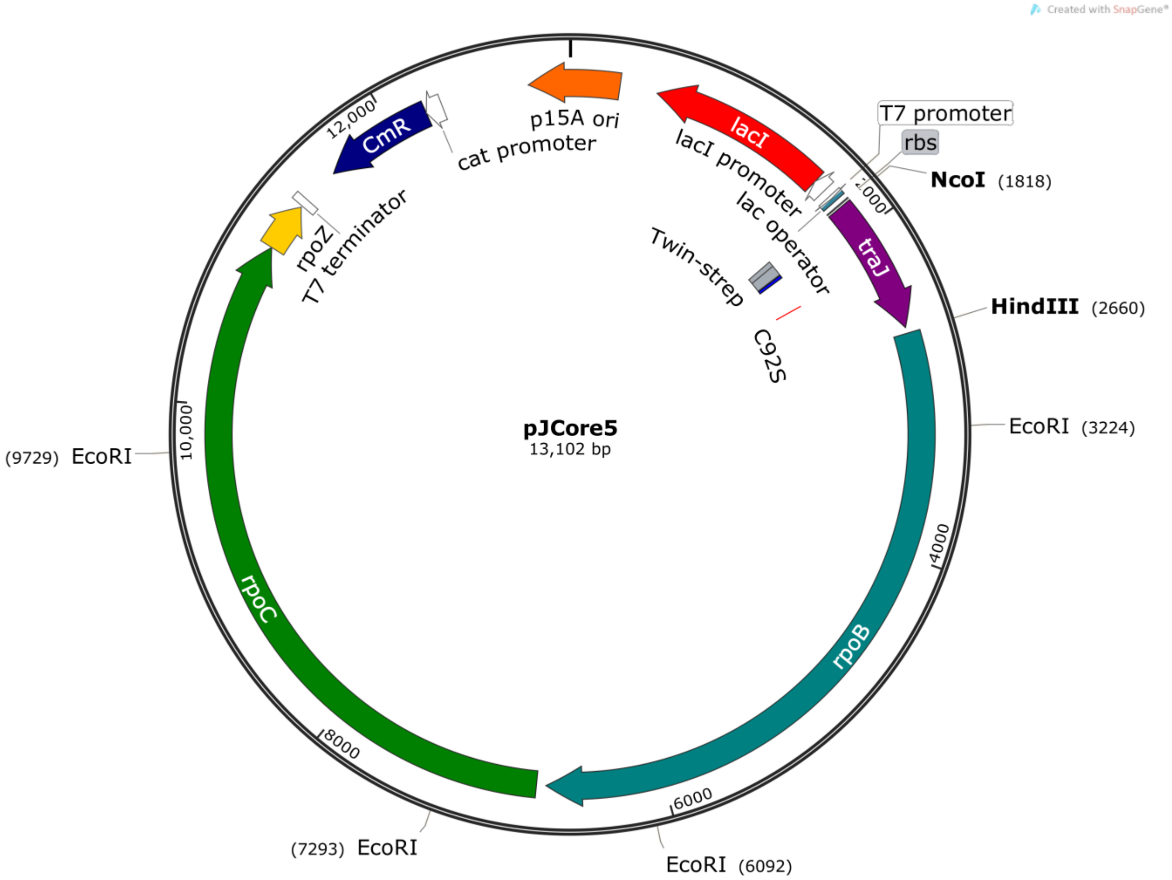

Figure 18: Expression vector pJCore5: *expression vector for JCore5 (Twin-Strep-FLAG-TraJ C92S, β,intergenic region, β´, ω); contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator, Twin-Strep-tag, FLAG-tag, traJ (encodes TraJ), rpoB (encodes RNAP subunit β), intergenic region, rpoC (encodes RNAP subunit β´), rpoZ (encodes RNAP subunit ω), T7 terminator, cat (chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. The plasmid was generated using Gibson assembly, with the DNA-fragments PACYC_F3, TraJ_C92S_F3, RpoB_F2, RpoC_F2 and RpoZ_F1.*

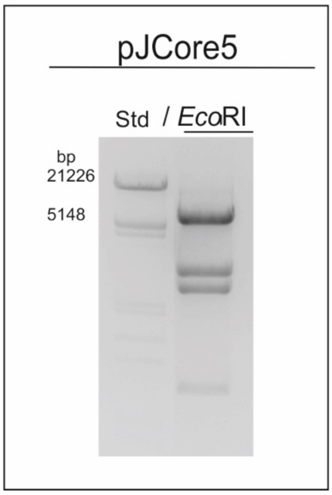

Figure 19: Verification of pJCore5*, via restriction-pattern-analysation, after the isolation out of* E. coli *XL-1. pJCore5 was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments after digestion with EcoRI are 6623, 2868, 2436 and 1145 bp. For size comparison λ/EcoRI+HindIII standard was used. The shown pictures are out-takes of pictures, taken with a digital camera.*

The sequence of pJCore5 was also verified by sequencing, and the obtained sequence is shown in the supplementary data. The obtained contiguous sequences were assembled with the software CAP3[74] and thenaligned with the expected sequence of thed plasmid. The sequencing results and sequencing primers can be found in the supplementary data. No mutation was found.

4.1.1.5 pβ´ω

To only overexpress β´and ω subunit together the expression-vector pβ´ω (figure 20) was designed, which consists of the fragments pACYC1_F1, RpoC_F1 and RpoZ_F1 (table 5). The overexpressed β´- and ω-subunits were purified out of inclusion bodies and were used for reconstitution mixes.

To construct this plasmid, the fragments were synthesised by PCR (figure 27) with respective primers. The fragments and their respective size, the used primers and templates are listed in table 5. To analyse and also get the right DNA-fragments the obtained fragments were separated on an agarose gel and purified out of the gel. The purified fragments were then assembled with Gibson assembly to a plasmid.

E. coli XL-1 cells were transformed with the assembled pβ´ω to amplify the plasmid and after culturing the cells, pβ´ω was isolated out of the cells again. To verify the constructed plasmid, pβ´ω was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 21). For this purpose, pβ´ω was cut with EcoRI and BamHI, resulting in fragments with the size 4375, 2436 and 1390 bp.

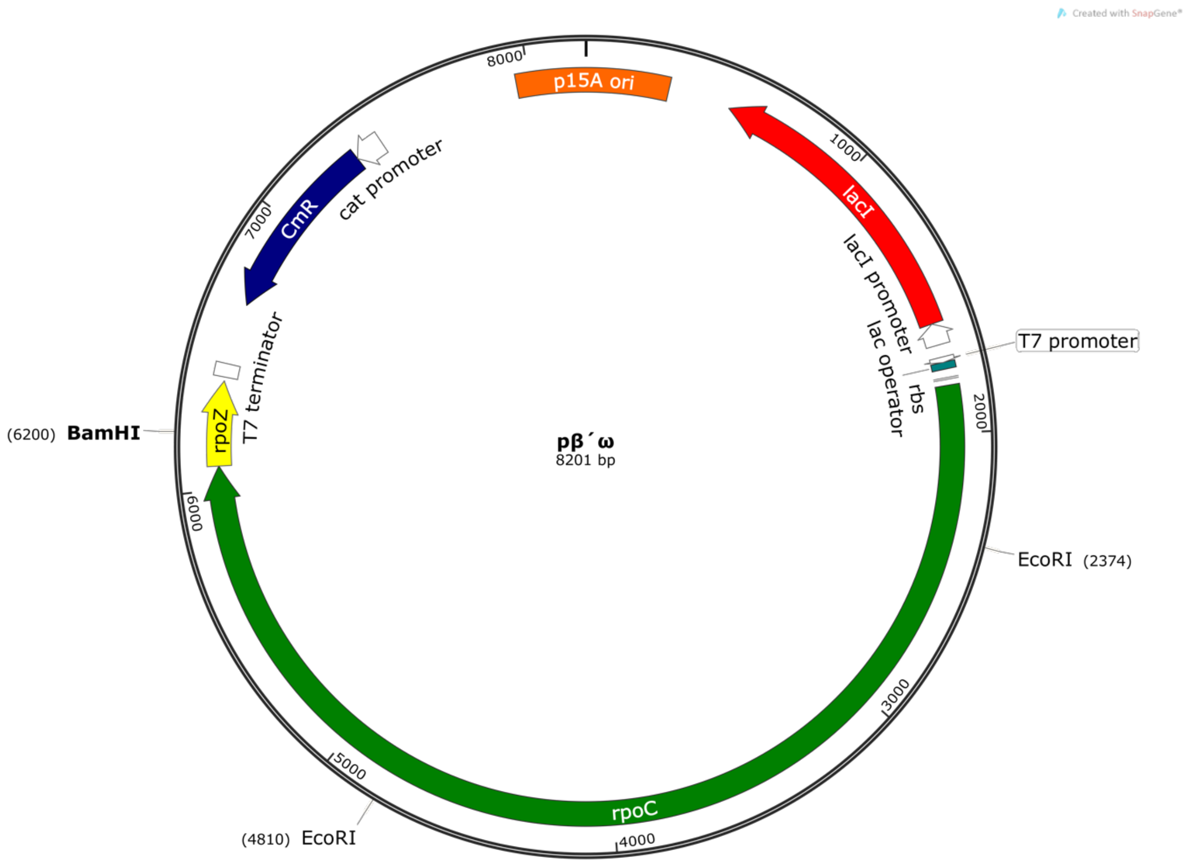

Figure 20: Expression vector pβ´ω: *contains p15A origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator,* rpoC *(encodes RNAP subunit β´),* rpoZ *(encodes RNAP subunit ω), T7 terminator,* cat *(chloramphenicol acetyltransferase) promoter and Chloramphenicol resistance. The expressed β´ and ω were purified out of inclusion bodies and used as part of the reconstitution mixes. The plasmid was generated using Gibson assembly, with the fragments pACYC1_F1 and RpoC_F1 and rpoZ_F1.*

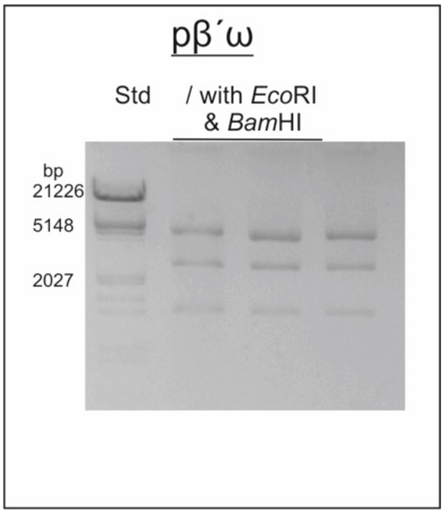

***Figure 21: Verification of pβω**, via restriction-pattern-analysation. After the isolation out of E. coli XL-1. The digested plasmids were analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments after double digestion with EcoRI and BamHI are 4375, 2436 and 1390 bp. For size comparison λ/EcoRI+HindIII standard was used. The shown pictures are out-takes of pictures, taken with a digital camera.*

4.1.2 pET28a expression vectors

pETlysM The expression vector pETlysM (figure 22) was designed to inhibit basal expression of overexpressed protein via LysM, an inhibitor of T7 polymerase, which is useful for overexpression toxic proteins. This vector contains the fragments PET28a_F1 and LysM_F1 (table 5).

In order to generate the plasmid, the fragments were synthesised by PCR (figure 27) with respective primers. The fragments and their respective size, the used primers and templates are listed in table 5. The obtained fragments were analysed on an agarose gel and purified out of the gel.

The purified fragments were then assembled with Gibson assembly to a plasmid pETlysM and *E. coli* XL-1 cells were transformed with the assembled plasmid to amplify it. After culturing the cells, pEtlysM was isolated out of the cells again. To check if the plasmid was constructed in the right way, the plasmid was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 23). For this purpose, pETlysM was double digested with BamHI and HincII, resulting in fragments with the size of 3335, 1429, 719 and 19 bp. The smallest fragment of 19 bp can not be seen on the gel and the fragment at 719 bp can be seen only slightly.

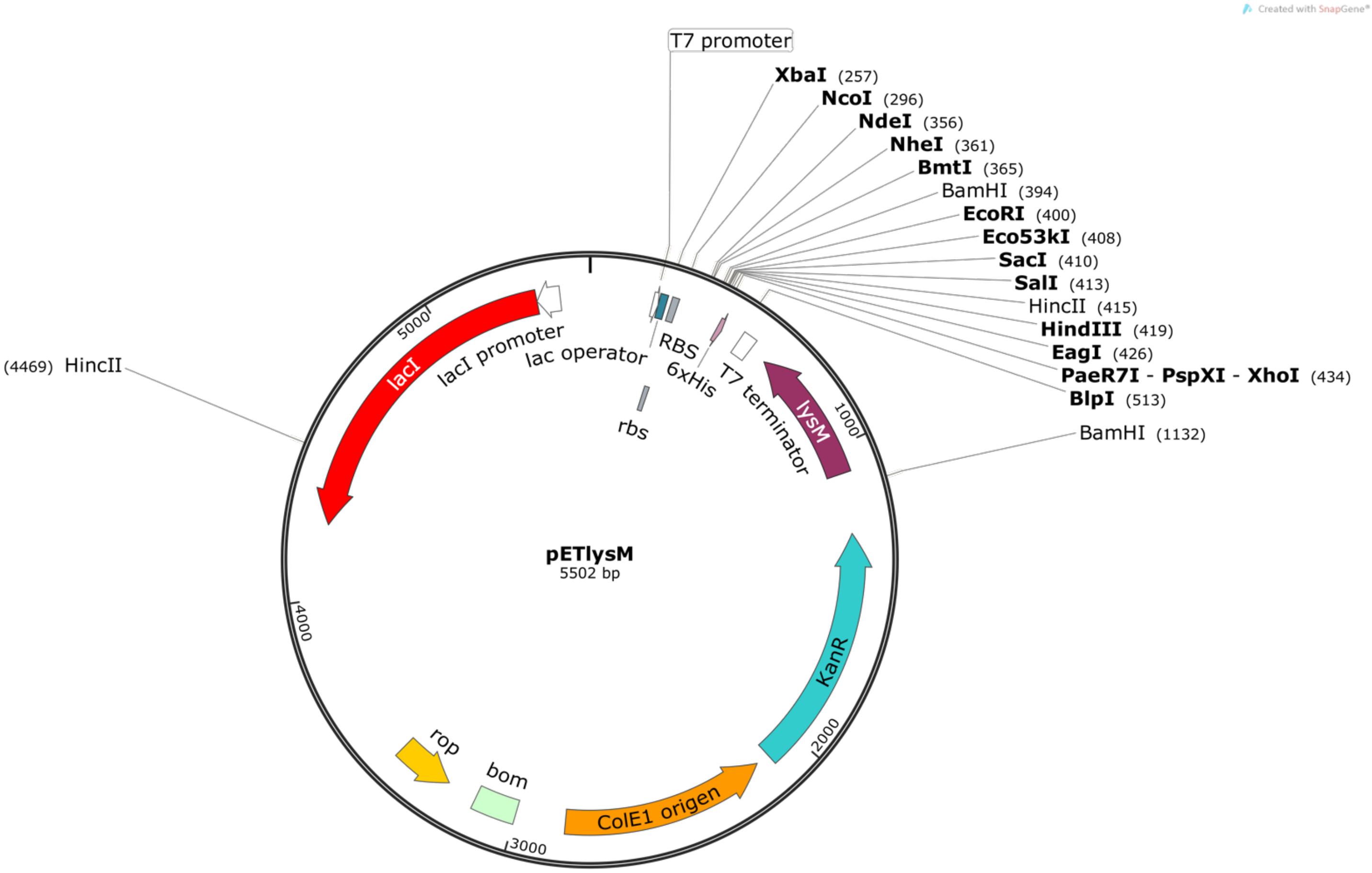

Figure 22: Expression vector pETlysM: *contains ColE1 origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator,* lysM *(encodes T7 lysozym), restriction cut sites for cloning other genes inside the vector, T7 terminator, Kanamycin resistance,* rop *and* bom*. The plasmid was generated with Gibson assembly, using the fragments PET28a_F1 and LysM_F1.*

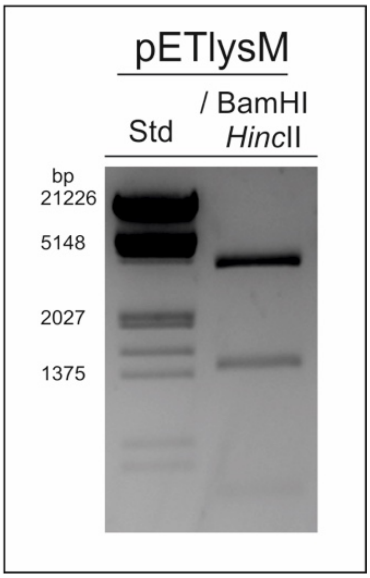

Figure 23: Verification of pETlysM *via restriction-pattern-analysation. After the isolation out of* E. coli *XL-1. petlysM was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments after double digestion with HincII and BamHI are 3335, 1429, 719 and 19 bp. For size comparison λ/EcoRI+HindIII standard was used. The shown pictures are out-takes of pictures, taken with a digital camera*

4.1.2.1 pETlysMα

In this master´s thesis the gene encoding the full length α-subunit (*rpoA*) of RNAP of *E. coli* was cloned into pETlysM using a traditional cloning method. This was necessary because without the inhibition of the basal expression of the α-subunit the cells died when the α-subunit was expressed together with the alternative RNAP (encoded by pJCore4S). The vector pETlysMα (figure 24), consisting of pETlysM and the DNA-fragment RpoA_F1 (table 5), was suitable for co-expression of aRNAP and α without resulting in cell death. The α-subunit is C-terminally 6x-His-tagged in this construct.

In order to generate the plasmid, the DNA fragment RpoA_F1 (table 5) was synthesised by PCR (figure 27) with the primers #2265 and #1952 (table 27). RpoA_F1 and pETlysM were cut with the restriction enzymes NcoI and XhoI. The obtained cut DNA was analysed on an agarose gel and purified out of the gel.

RpoA_F1 was then cloned into pETlysM with traditional cloning and *E. coli* XL-1 cells were transformed with the resulting plasmid pETlysMα. After culturing the cells, pEtlysMα was isolated out of the cells again. To check if the plasmid was constructed in the right way, the plasmid was cut with restriction enzymes, which cut the plasmid in specific patterns (figure 25). For this purpose, pETlysMα was double digested with SphI and ClaI, resulting in fragments with the size 3522, 1591, 449, 421, 275 and 101 bp. As can be seen in figure 25, the double digestion was not complete, and the uncut vector can also be seen at the size of 3259 bp. The smaller fragment sizes can not be seen on the agarose gel. Nevertheless, the other fragments have the expected size.

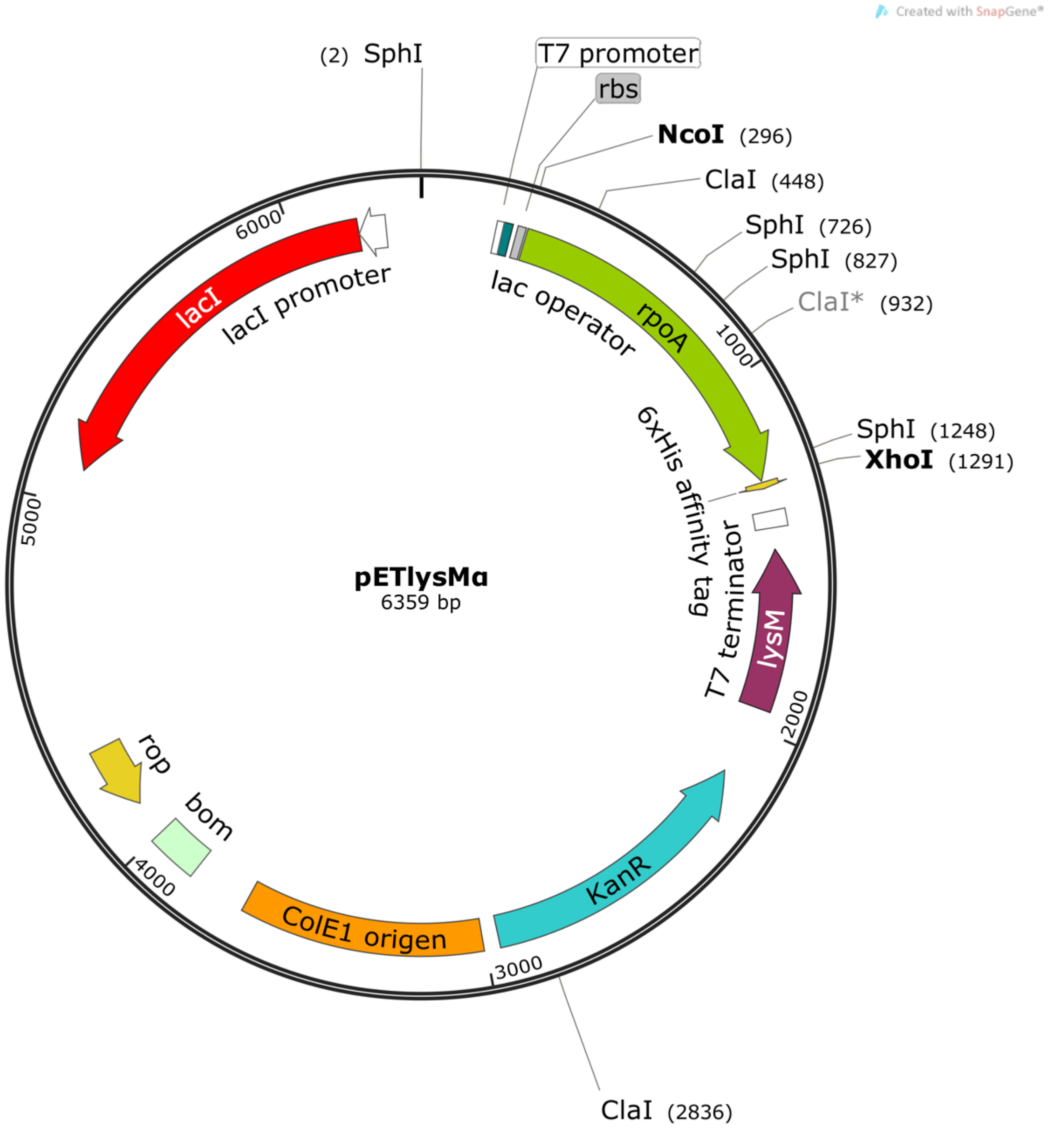

Figure 24: Expression vector pETlysMα: *contains ColE1 origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator,* rpoA *(encodes for α-subunit), T7 terminator,* lysM *(encodes T7 lysozym), Kanamycin resistance,* rop *and* bom*. The plasmid was generated using traditional cloning method, were the RpoA_F1 was cloned into pETlysM.*

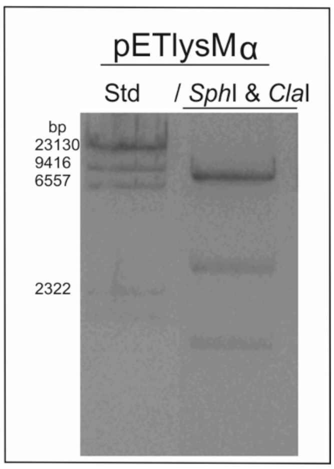

Figure 25: Verification of pETlysMα *via restriction-pattern-analysation. After the isolation out of* E. coli *XL-1. pETlysMα was analysed with electrophoretic separation on a 1.2% agarose gel, and DNA was made visible with Ethidium Bromide. The sizes of the expected fragments after double digestion with SphI and ClaI are 3522, 1591, 449, 421, 275 and 101 bp. For size comparison λ/HindIII standard was used. The shown pictures are out-takes of pictures, taken with a digital camera*

4.1.2.2 pET28tufB

To investigate interactions between the alternative RNAP and EF-Tu, an expression-vector pET28tufB (figure 26) was designed to overexpress EF-Tu. The expression-vector pET28tufB was assembled from the fragments TufB_F1, containing the gene *tufB* which encodes EF-Tu, and PET28a_F2 (table 5). Following *tufB* a 6xHis-tag-sequence was placed in frame, resulting in the opportunity to express a C-terminally His-tagged EF-Tu.

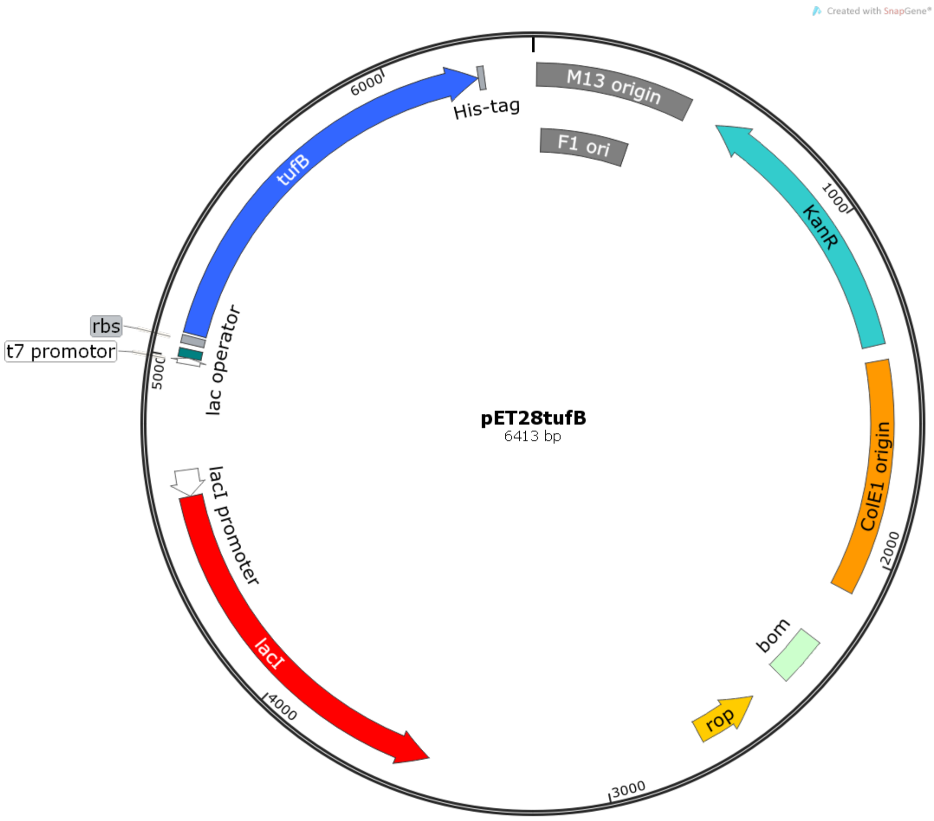

Figure 26: Expression vector pET28tufB: *contains ColE1 origin of replication,* lacI *promoter and repressor, T7 RNA Polymerase promoter,* lac *operator,* tufB *(encodes EF-Tu), 6xHis, restriction cut sites for cloning other genes inside the vector , T7 terminator, Kanamycin resistance,* rop *and* bom. *The plasmid was generated by Gibson assembly, using the DNA fragments TufB_F1 and PET28a_F2.*

To generate the plasmid, the fragments were synthesised by PCR (figure 27) with respective primers. The fragments and their respective size, the used primers and templates are listed in table 5. The obtained fragments were separated on an agarose gel and purified out of the gel.

The purified fragments were then assembled with Gibson assembly to a plasmid and *E. coli* XL-1 cells were transformed with the assembled pET28tufB to amplify the plasmid. pET28tufB was isolated out of the cells again, after culturing the cells.

To validate the sequence of *tufB,* it was sequenced. The obtained contiguous sequences were assembled with the software CAP3[74]. The sequences were aligned with the expected sequence of the plasmid. The sequencing results and sequencing primers can be found in the supplementary data. The *tufB* sequence showed mutations, which are not changing the amino acid sequence, and one mutation E202D, which should not interfere with function and structure, because no domains are affected.

4.1.2.3 Fragments used for plasmid assembly

The required DNA-fragments to assemble the plasmids, were obtained with a standard PCR with adequate Primers and templates, which are listed in table 5. The PCR products were analysed on an agarose gel (figure 27) for the right size and purity.

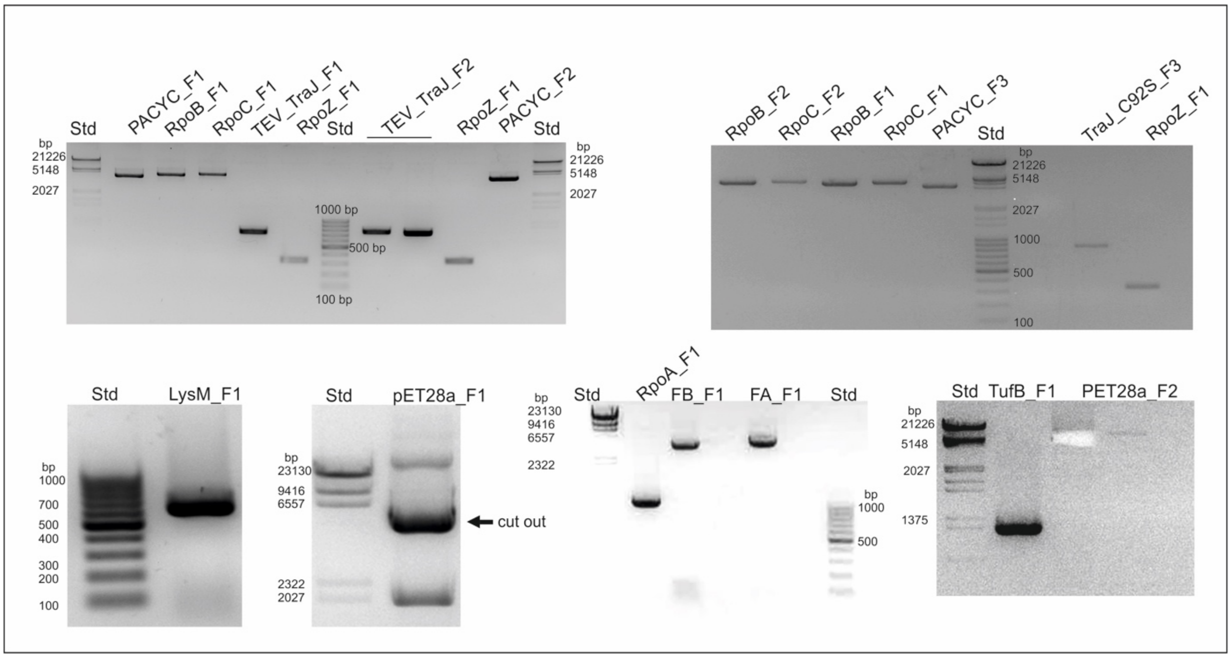

Figure 27: DNA-Fragments for the Gibson Assembly: *The size of DNA-fragments (table 5) for Gibson-Assembly were verified by separating them electrophoretic a 1.2% agarose gel. DNA was made visible with Ethidium bromide. For size comparison Gene Ruler 100 bp DNA Ladder, λ/ EcoRI & HindIII, λ/HindIII and GeneRuler™ 100 bp DNA Ladder Standards were used. The shown pictures are out-takes of pictures, taken with a digital camera.*

4.2 Expression and purification of the alternative RNAP

4.2.1 Almost no soluble aRNAP complex is formed

Chemical-competent *E. coli* BL21 cells were transformed with the respective plasmid. Protein expression was induced by adding IPTG to the culture and the cells were incubated for additional two hours. The cells were harvested, washed, and after the whole cells were disrupted, they were analysed on a SDS-Page and followed western blot (figure 28) and compared to a not induced control.

As expected the induced samples show an expression of the respective proteins, and the not induced controls show less or no expression.

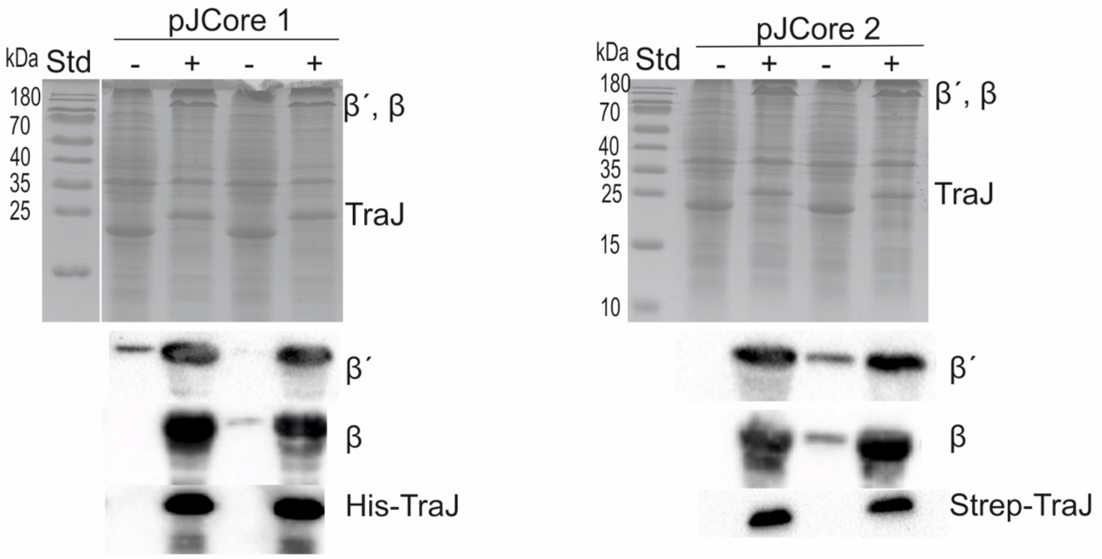

Figure 28: Analysation of expression level in induced (+) and not-induced (-)* E. coli *BL-21 (DE3) cells with pJCore1 and pJCore2 *After transformation of* E. coli *BL21 (DE3) with respective plasmid, protein expression was induced with 0,5 mM IPTG and then incubated for two hours at 37°C. 0.1 OD disrupted whole, induced and not-induced cells were analysed on a 12,5% SDS-PAGE and western blot. 4 µL of the PageRulerTM pre-stained protein ladder were used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

E. coli BL 21 (DE3) cells, transformed with the plasmid pJCore1 or pJCore2 were lysed with a French press, followed by a buffer change from lysis buffer to binding buffer 1. Plasmid pJCore1 or pJCore2 express the protein complexes referred to as JCore1 (His-TraJ, β´,β,ω) and JCore2 (Strep-TraJ, β´,β,ω).

Only a small amount of the overall overexpressed proteins was found to be in the supernatant and most proteins were located insoluble in the pellet (figure 29). The Strep-tag of TraJ could not be detected, and also not much β and β´ can be found in supernatant or pellet fraction.

Also, different temperatures for heating up the sample before applying it to the gel were compared. The heating has the effect of destroying SDS-resistant complexes, which could not migrate into the gel. Most of all it is important for the β´-subunit as can be seen in figure 29, and the best temperature to separate and detect β and β´ is heating the sample up to 95°C for 5 minutes.

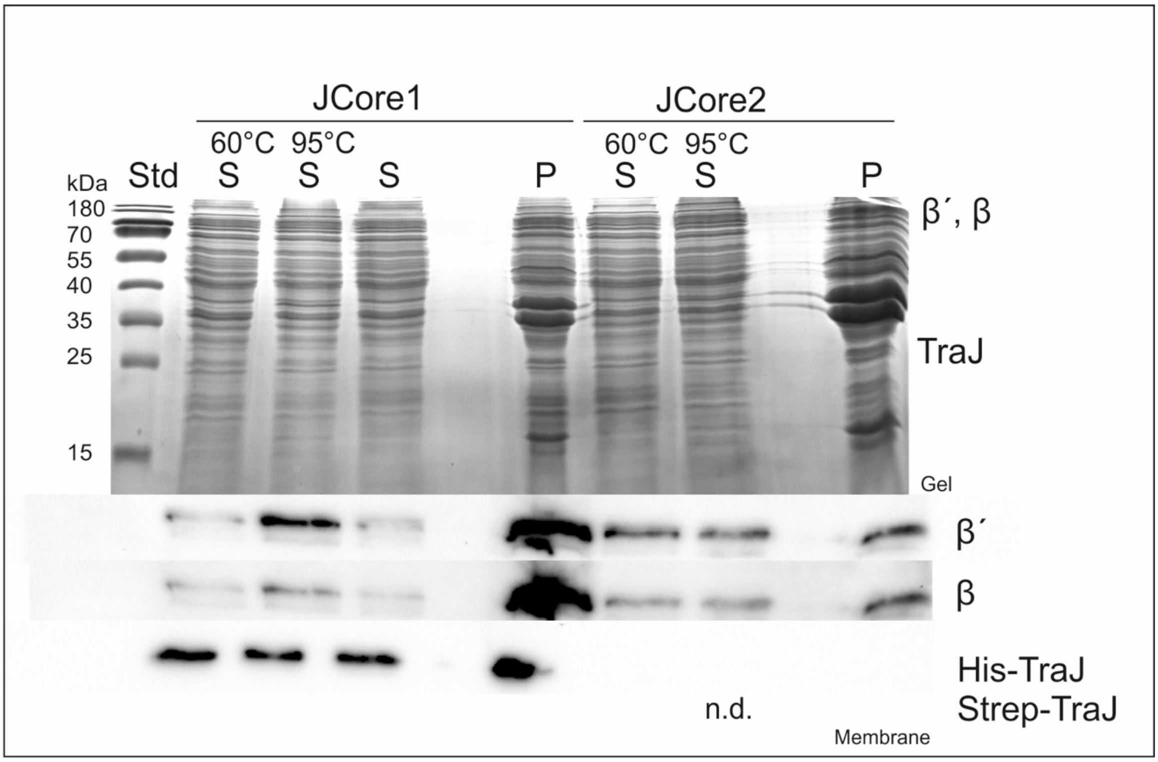

Figure 29: Preparation optimisation for SDS-PAGE samples. *After induction of protein expression, E. coli BL21 (DE3) cells, containing pJCore1 or 2 respectively, were disrupted with a French press. The pellet (P) and the supernatant (S) were analysed on a 15% SDS-PAGE followed by a western blot. The supernatant was treated with three different Temperatures (RT/ 60°/ 95°C) before applying it to the Gel. 4 µL of the PageRuler^{TM} pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). The Strep-tag of TraJ could not be detected. Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

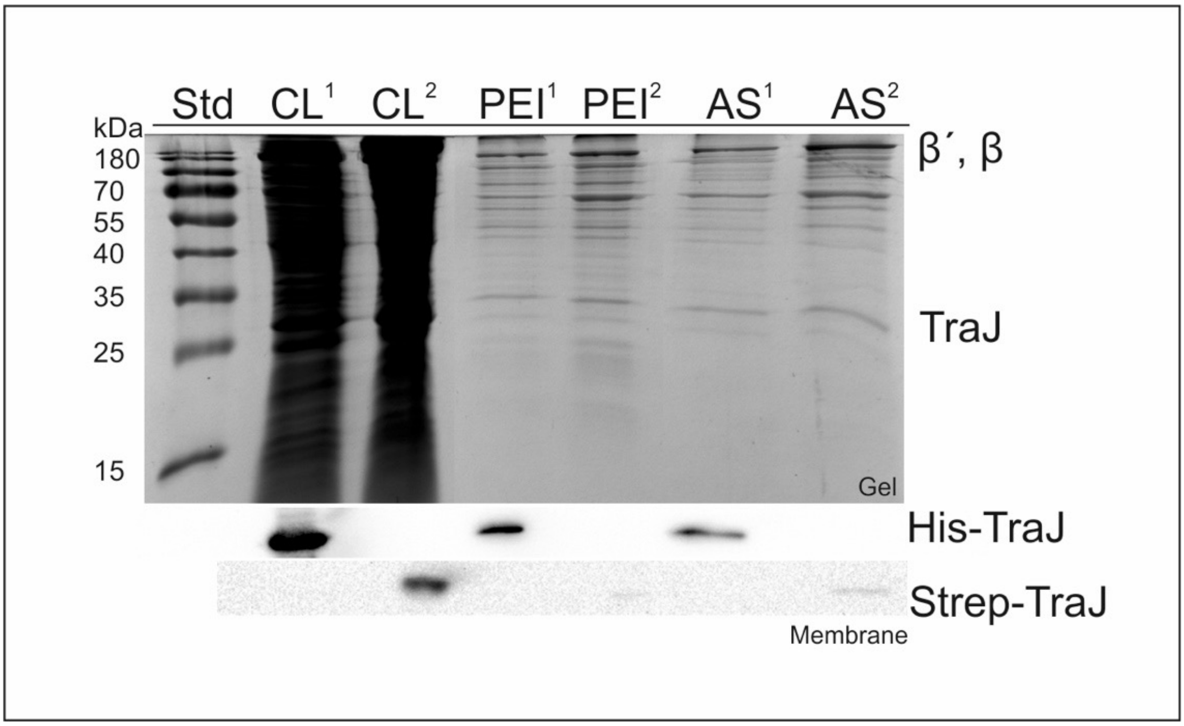

Figure 30: Aliquots of different steps of the pre-purification of aRNAP (JCore1 ([1]) and pJCore2 ([2])) *cell lysate (CL), PEI precipitation (PEI) and Amonium sulfate-precipitation (AS) were analysed on a 12,5% SDS-PAGE and western blot. 4 µL of the PageRuler^{TM} pre-stained protein ladder was used as a standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

Nevertheless, the soluble aRNAP was pre-purified and during the different purification steps (clear cell lysate, PEI precipitation and AS-precipitation) aliquots were collected. The protein contained in the aliquots was precipitated with acetone and analysed on a SDS-Page followed by a western blot (figure 30). A lot of TraJ was lost during this prepurification, mostly during the PEI precipitation.

The pre-purified proteins were further subject to a purification step on a His-GraviTrap-TALON column (JCore1) (figure 31A) or on a StrepTrap HP column (JCore2) (Figure 31B). The His- or Strep-tagged TraJ elution was carried out with imidazole or desthiobiotin, respectively. TEGED buffer was used as binding buffer for the StrepTrap HP column. Again aliquots were analysed on a SDS-Page (figure 31).

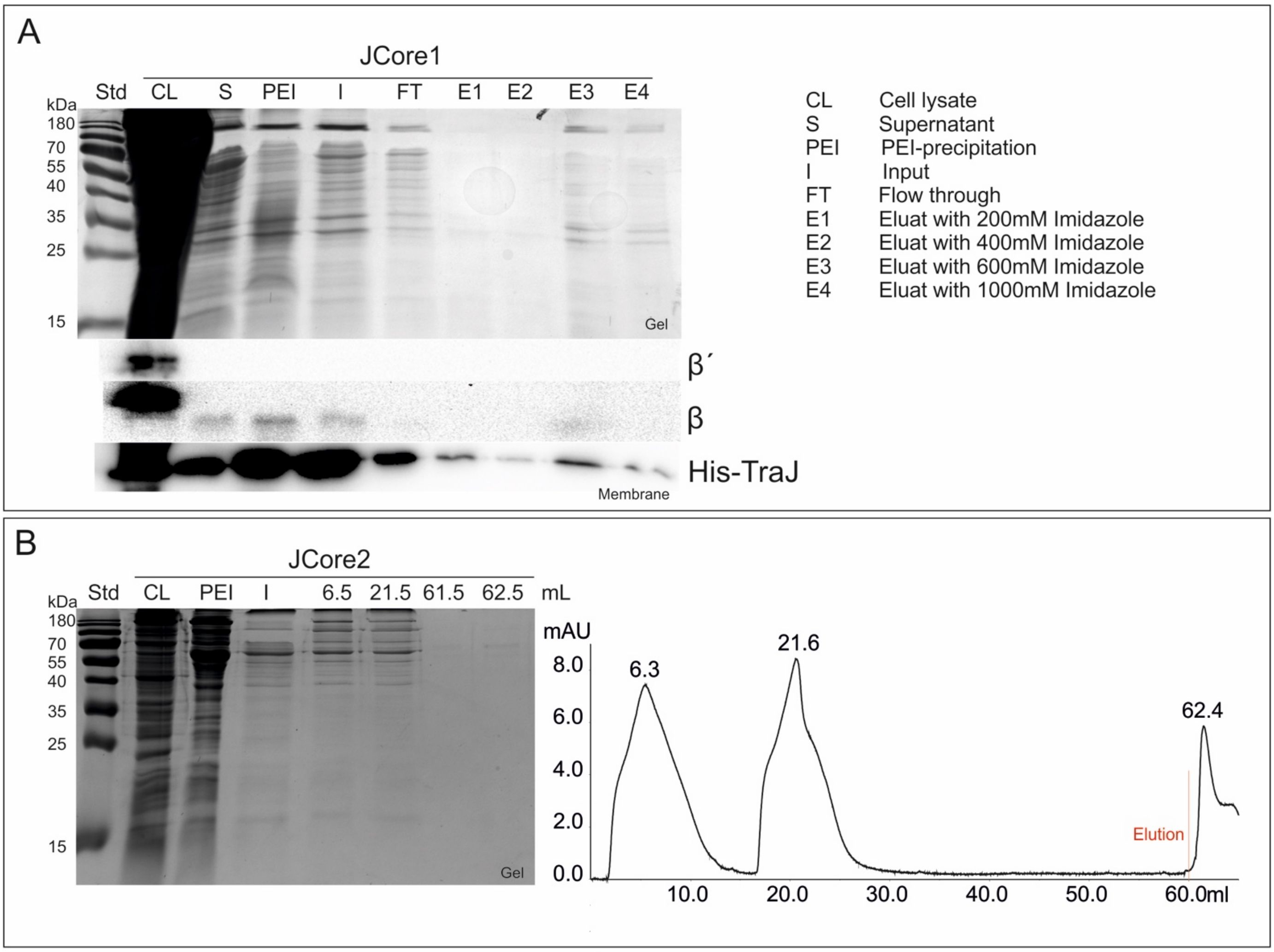

Figure 31: Native aRNAP-purification over His-GraviTrap-TALON/ StrepTrap HP column: *(**A**) alternative RNAP (JCore1) was tried to purify on a His-GraviTrap-TALON column under native conditions, after native RNAP pre-purification. Proteins were eluted with 200, 400, 600 and 1000 mM Imidazole. (**B**)The alternative aRNAP (pJCore2) was attempted to purify over a StrepTrap HP column in combination with an ÄKTAFPLC system, after native aRNAP pre-purification. (**A, B**) Aliquots of both native aRNAP pre-purifications and affinity column purifications were analysed a 12,5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as a standard. The SDS-PAGE was dyed with Kang-dye and on the western blots, proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System and the corresponding chromatogram.*

As can be seen in figure 31 each method had their problems. Both columns had a lot of TraJ in the flow through, which can be seen on the SDS-PAGE and chromatogram. This indicates poor binding of the tagged protein to the column. Another problem with the His-GraviTrap-TALON column was the unspecific protein binding, which then eluted with higher Imidazole concentrations. Also β-subunit had a strong, not specifically interaction with the column material and it did not elute even with 1 M of Imidazole. Almost no β´ can be found in the any step of the purification, maybe due to insufficient expression of the β´-subunit. This lack of β´ may interfere with the formation of a soluble aRNAP complex.
The purification over the StrepTrap HP column did not result in any purification of TraJ, due to very poor binding.
Both approaches were therefore not useful, as no aRNAP was enriched nor was it purified.

When the same experiment was carried in a small scale a 50 mL cell culture and 1 mL His-spin-trap was used, and a small amount of TraJ eluted together with β and β´. This could be shown on a SDS-PAGE (figure 32) and through mass spectrometry done Rechberger Gerald, Mag. Dr. rer. nat. (Data not shown).

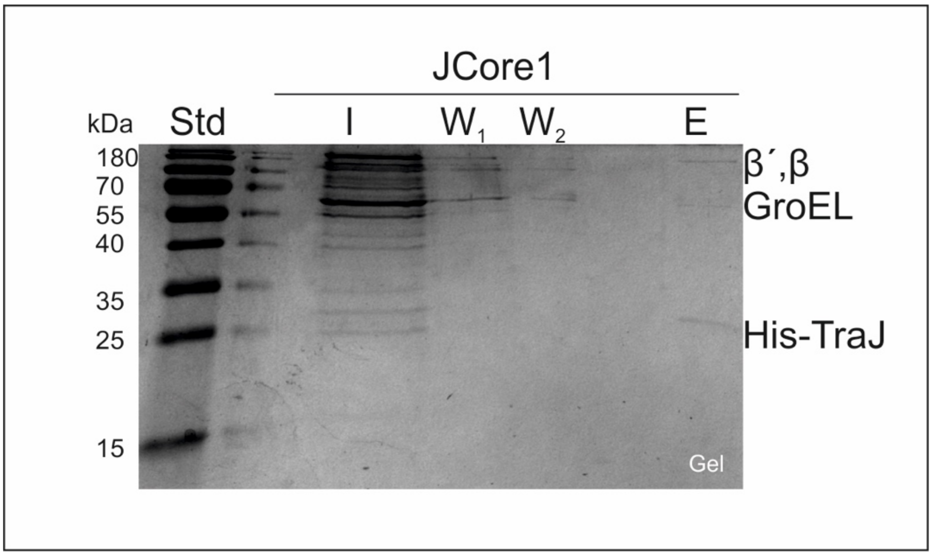

Figure 32: Native purification of aRNAP over a His-spin-trap. *After pre-purification of aRNAP the small-scale sample was loaded on a His-spin-Trap. The input (I), which was applied to the column, two wash steps with 5 mM (W1) and 40 mM (W2) Imidazole and the eluate (250 mM Imidazol) were analysed on a SDS-PAGE, which was Kang-dyed after electrophoretic separation (A). 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The visual bands of the eluate were cut out and analysed with MS from Rechberger Gerald, Mag. Dr. rer. nat. Shown is a take out of a picture, which was taken with ChemiDoc™ MP System.*

Only a small amount could be purified under native conditions and GroEL, a heat shock chaperon, which mediates the proteolytic degradation of TraJ[35], was co-purified. The co-purification of GroEL indicates not fully folded complex. The eluted protein probably also contains free TraJ. β and β´ do co-purify as well, which indicates an interaction with TraJ.

Even though it could be shown that β and β´ were eluted with His-tagged TraJ from the His-GraviTrap-TALON column, this could only be shown in a small amount. Through this approach no pure alternative RNAP could be purified, and only in a small amount.

To increase the amount of soluble protein, lower expression temperatures (16°C and 30°C) were tested (data not shown). The co-purification of GroEL (figure 32) hinted a not right folded complex. Lower temperature can increase solubility due to the fact that cells grow slower and more time for folding of the overexpressed proteins is given. But also the more provided time for the complex to fold properly, did not increase the solubility of overexpressed aRNAP.

In the original protocol after Zhi[68] for RNAP-purification a mixer was used for cell disruption. This protocol was followed but did not lead to any increase in the amount of soluble aRNAP (data not shown).

Considering all made experiments, the expression vectors pJCore1 and pJCore2 are not suitable to produce large amounts of soluble protein complexes containing TraJ, β, β\` and ω.

4.2.2 Co-expression with α-Subunit does not enhance formation of soluble aRNAP complexes

It was speculated, that α is necessary for the formation of aRNAP.
The idea was that the α subunit may be part of an intermediate complex which is necessary to form the final aRNAP without the α-subunit. Hints leading to this theory were given by an *in vivo* pull down, executed by Sahra Trunk, where the α-subunit was co-purified with FLAG-TraJ[60]. One explanation of this phenomena could be intermediate complex formation, which then leads to the formation of an aRNAP.
The α-subunit and aRNAP, encoded by pJCore4S, were overexpressed together. The pJCore4 was used because of its advantage to purify aRNAP over two tags, due to the fact that it contains a N-terminally 10xHis-tagged β-subunit and a N-terminally Strep-tagged TraJ. This gives the opportunity to purify aRNAP over the a His and Strep column.

Due to basal expression, cells expressing aRNAP and α (encoded by pIRA3) did not grow and died (observation). This could be due to toxic intermediates, formed with α and the subunits of aRNAP, which have a toxic effect on the cells only in high concentrations. This intermediates could play a role in the formation of aRNAP and may not be toxic in lower concentration, which is usually present in the cell.
This phenomenon could be eliminated with the help of expression vector pETlysM, due to the fact that it carries a T7 lysozym, which inhibits T7 polymerase and prevents basal expression. *RpoA* was cloned into pETlysM, and α could be expressed together with aRNAP without killing the cells, after co-transformation of *E. coli* BL21 (DE3) with pETlysMα and pJCore4S. The expression of the ω-subunit, which is also encoded on the pJCore expression vectors, could not be detected the entire thesis due to the lack of antibodies against ω-subunit.
As can be seen on the SDS-PAGE in figure 33, almost only the α-subunit was overexpressed and only little β, β´ and TraJ were expressed.

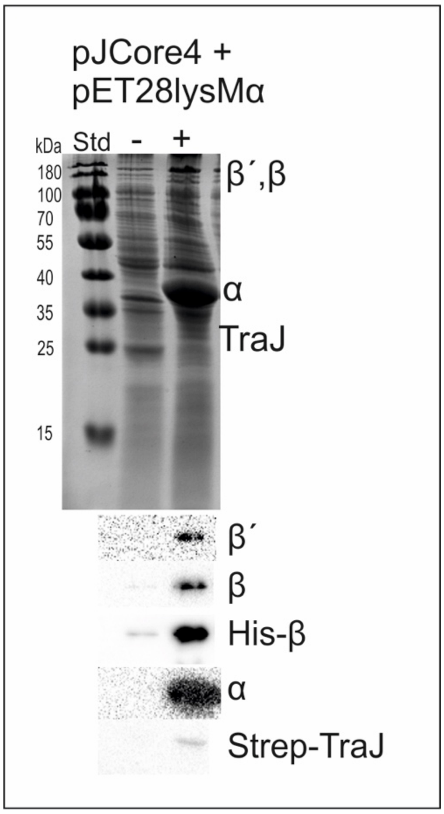

Figure 33**: Analysation of expression level in induced (+) and not-induced (-)* **E. coli** ***BL-21 (DE3) cells containing pJCore4 and pETlysMα *After transformation of* E. coli *BL21 (DE3) with pJCore4 and pETlysMα, protein expression was induced with 0,5 mM IPTG and then incubated for two hours at 37°C. 0.1 OD disrupted whole, induced (+) and not-induced (-) cells were analysed on a 12,5% SDS-PAGE and/or western blot. 4 µL of the PageRuler™ pre-stained protein ladder were used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots, proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System*

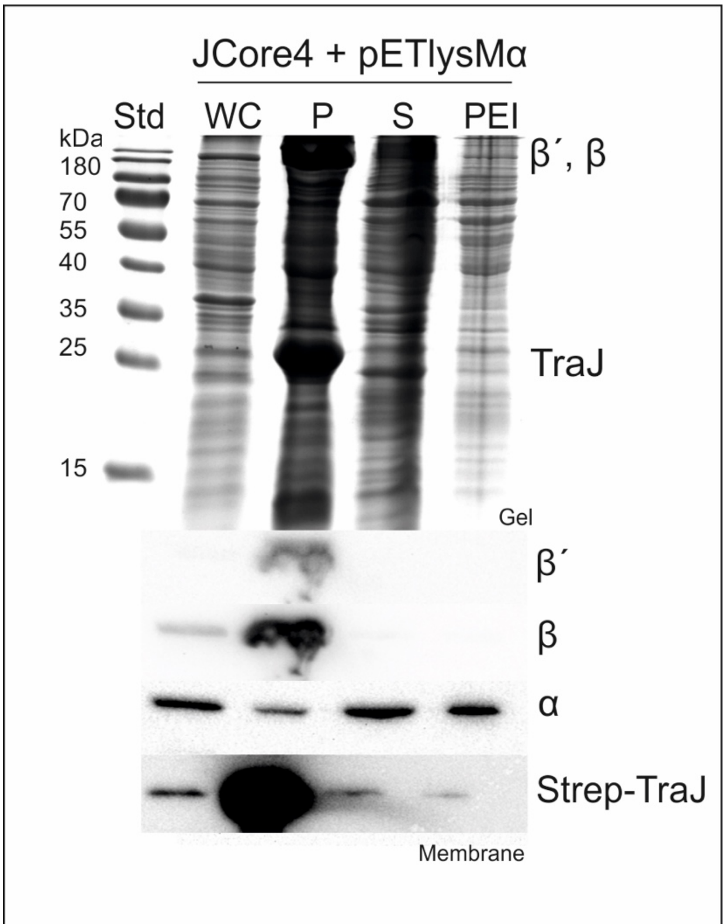

***Figure 34: aRNAP (JCore4) and α (pETlysMα) were co-expressed** and pre-purified. Aliquots of whole cells (WC), pellet (P), supernatant (S) and PEI-precipitation (PEI) were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

The cells containing TraJ, β, β´, ω and α were lysed with a mixer and pre-purified with PEI-precipitation. Aliquots of indicated steps were once again analysed on a SDS-PAGE, followed by a western blot (figure 34).
No improvement of aRNAP solubility was reached by co-expressing the α-subunit. Due to the fact that almost only the α-subunit is found in the soluble fraction, no further purification was attempted.
But it could also be shown, that β and β´ were not found in the soluble part together with α-subunit, and only α was soluble. But to interpret this result without any doubt, RNAP without TraJ should be purified using the same protocol.

4.3 Soluble aRNAP is obtained by refolding of aRNAP from solubilized inclusion bodies

All attempts to increase the solubility of the aRNAP complex faild and most of the overexpressed protein was always found in the pellet. This might be due to still missing factors or co-factors of aRNAP, which can be accessed in the cell in low concentration under normal conditions. But when aRNAP is overexpressed, not a sufficient amount of co-factors is present in the call, which would be necessary for the overexpressed complexes. This could be a reason for the large amount of insoluble protein.
The cells were looked at in greater detail and under the light microscope (Leitz Biomed/Leica, wetzlar, Germany) massive formation of inclusion bodies, were overexpressed protein is stored, could be observed in the cells (figure 35).

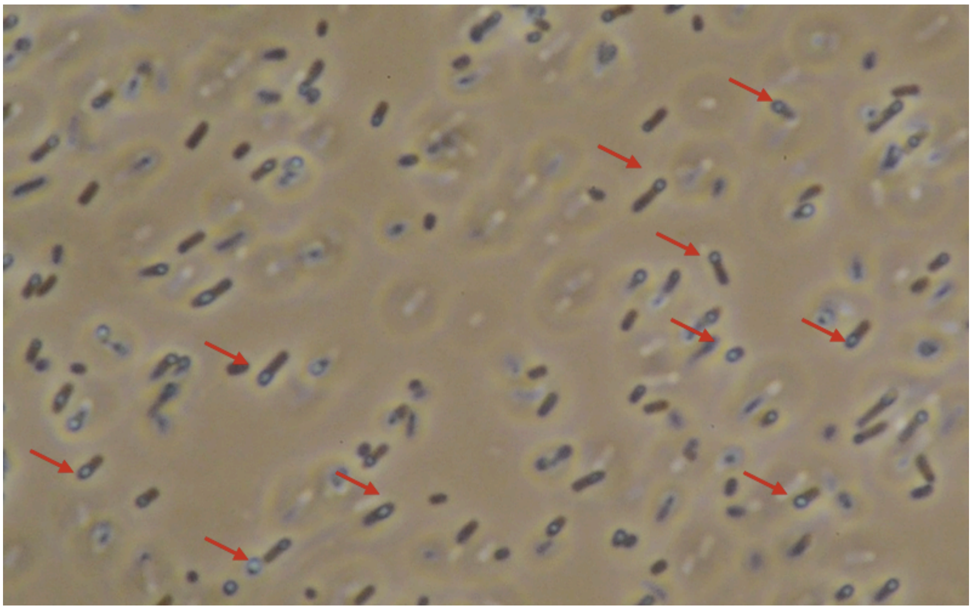

***Figure 35: Inclusion bodies under a light microscope**: E. coli BL21 (DE3) [pJCore2], induced with IPTG for 1.5h, with a lot of inclusion bodies (marked with red arrows), pictured with a canon camera on a light microscope 80x objective.*

4.3.1 Optimisation of refolding conditions

There are existing protocols to purify RNAP out of inclusion bodies, because it was also an issue for years when attempting to purify RNAP. One of these protocols is described by Borukhovs [69], which was followed.

After the purification of the proteins out of the inclusion bodies (“crude protein”), they were renatured, by dialysing the guanidine hydrochloride out of the buffer.

The aRNAP alone (encoded by pJCore2), as well as aRNAP co-expressed with α-subunit, were purified out of inclusion bodies and renatured via dialysis.
But after renaturation most of aRNAP was still in the pellet fraction.
In figure 36 the pellets of renatured proteins encoded by pJCore1/2 with and without α are analysed on a SDS-PAGE and western blot. The soluble (IBR) and the precipitated protein pellet (IBRP), which is still insoluble after renaturation with 20% glycerol in the renaturation buffer, was compared. It becomes apparent that almost all of α is soluble in the supernatant and TraJ is only in the insoluble pellet, with and without α. β is with and without α almost equally distributed in pellet and soluble fraction and it seem that β´ is more soluble with α (figure 36).

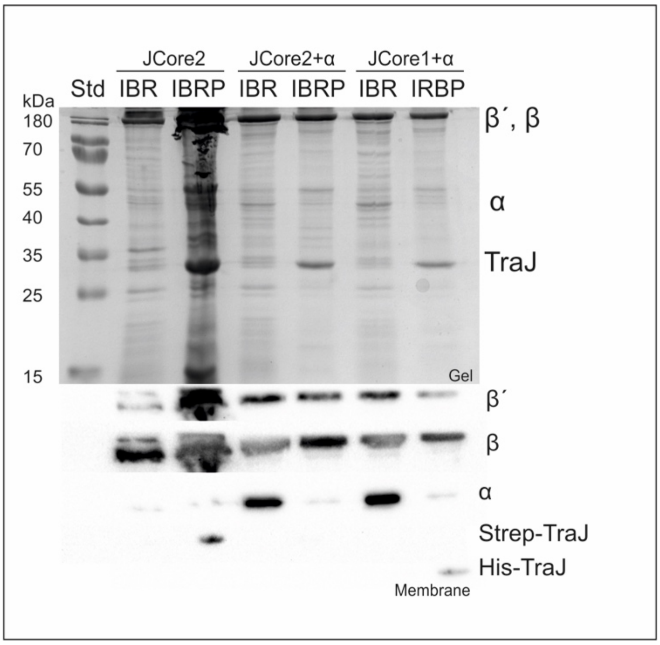

Figure 36: renatured aRNAP with and without α: *aRNAP (JCore2/1) was purified out of inclusion bodies and purified α was added optional (+α), before it was renatured by dialyses. The dialysed proteins with 20% glycerol in the renaturation buffer were separated by centrifugation in pellet (IBRP) and supernatant (IBR), which were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

Because most parts of aRNAP were still insoluble after renaturation, the conditions for the renaturation were optimized by a small pre-experiment, where the best glycerol concentration was determined. The smaller the value for OD_{320} is, the more protein could be folded back under this condition and did not precipitate. From the results, which are shown in figure 37, it can be concluded that the best refolding of the protein is completed at 4°C and with 30% glycerol concentration in the buffer.

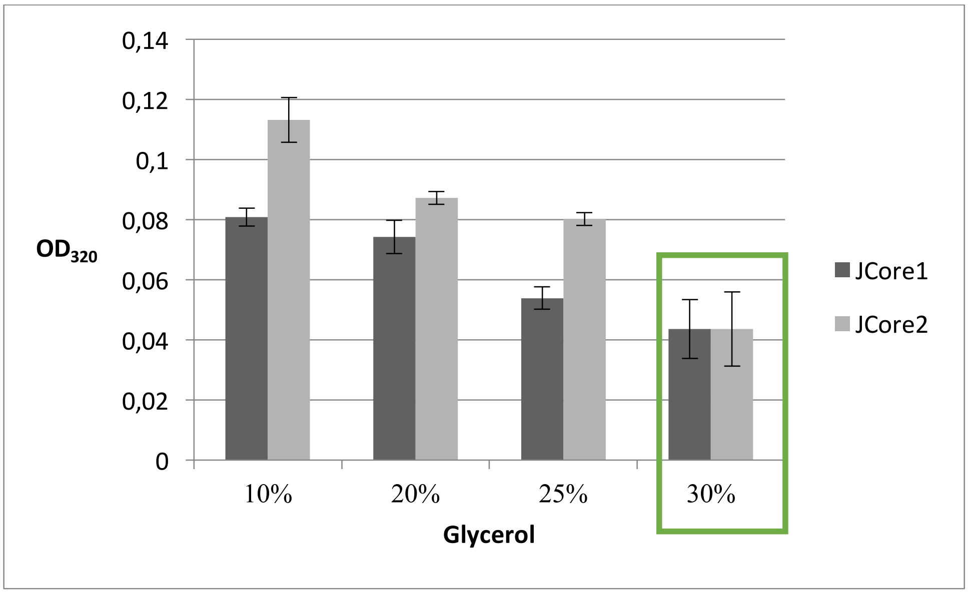

***Figure 37: aRNAP (JCore1/2) refolding optimization**: renaturation buffers with different glycerol concentrations (10%, 20%, 25% and 30%) were used to dialyse aRNAP at 4°C. How well the proteins renatured was indicated with OD_{320}, the lower OD_{320} the better the renaturation was completed.*

The denatured and then with the optimized conditions renatured aRNAP was analysed on a SDS-Page followed by a western blot. As can be seen in figure 38 more TraJ, β´ and β did refold under optimized renaturation conditions. But a lot of other proteins did refold as well, and the aRNAP has to be purified further.

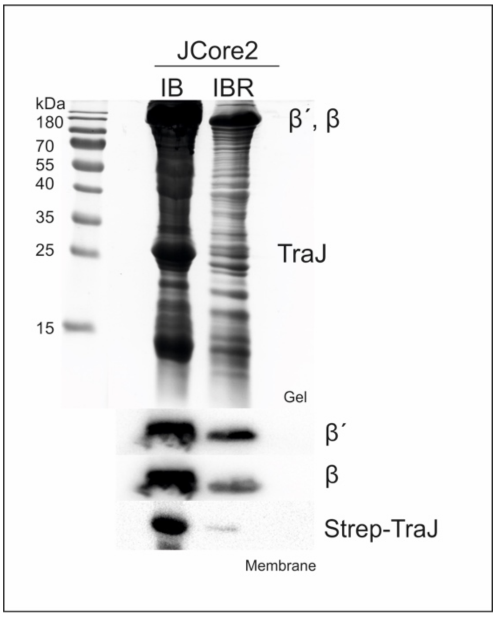

***Figure 38: denatured and renatured aRNAP** (JCore2) was purified out of inclusion bodies, then it was renatured by dialyses. Denatured and renatured aRNAP were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

4.3.2 Strep-tagged TraJ does not bind to StrepTrapHP column

For further purification of renatured aRNAP there were different choices of affinity column to choose from.
Because of the already experienced trouble with the His-GraviTrap-TALON column and the β-subunit, this column was not further used to purify aRNAP, due to the fact that the β- subunit binds unspecific to this column.
Instead a StrepTrap HP column (1 mL) for Strep-tagged TraJ was used, because we thought that the now folded complex can bind better to the StrepTrap HP column than in previous attempts. The renatured proteins were first separated based on their size over a Superdex 200 increase 10/300 GL, to ensure that mostly the formed complex aRNAP is applied to the column and therefore can bind better. SC-binding buffer served as running buffer for the StrepTrap HP column purification.

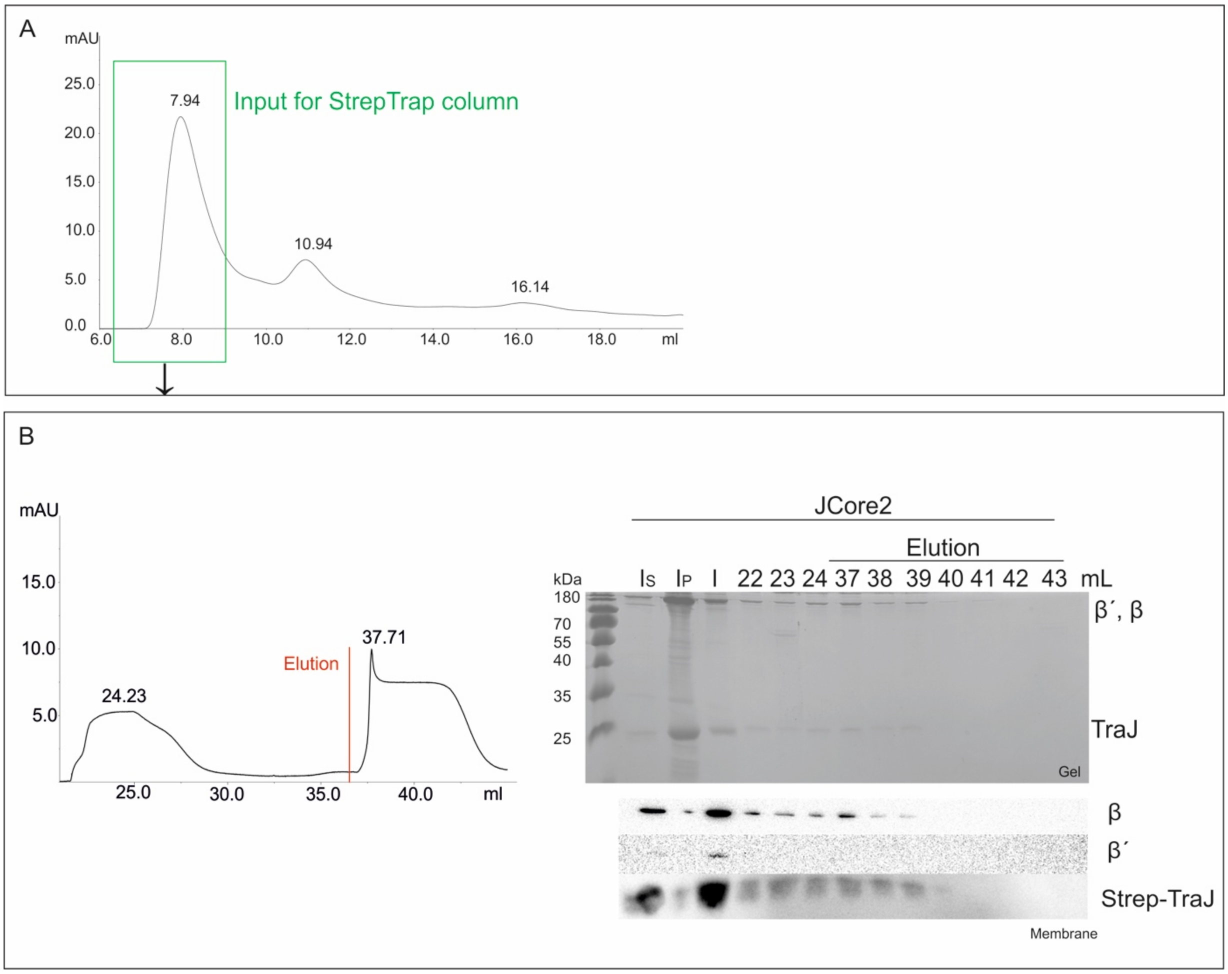

Figure 39: aRNAP separated on a Superdex 200 increase 10/300 GL column (A) followed by StrepTrap HP column purification (B)*. Renatured aRNAP (Jcore2) was separated on a Superdex 200 increase 10/300 GL column (A). Then mL 6.5-8.5 (I) were applied together on a StrepTrap HP column, where aRNAP was eluted with desthiobiotin (B). The renatured aRNAP was separated in pellet (Ip) and supernatant (Is), and only the supernatant was applied on the Superdex 200 increase 10/300 GL column. Aliquots of the inputs (Is, Ip and I) of both columns, the wash (22-24 mL) and eluate fractions (37-43 mL) of the StrepTrap HP column purification were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler^{TM} pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System and the corresponding chromatograms.*

In figure 39 it gets apparent, that Strep-tagged TraJ still does not bind specifically strong to the StrepTrap HP column and gets eluted mostly during wash steps together with β. But in the first eluate fraction (37 mL) an increase of β elution is evident, compared to the flow through. A slight improvement in binding was achieved compared to the previous native purification (figure 31B).
Not for the first time β' is again only detectable in a small amount, and therefore the ratio between the subunits of aRNAP is not optimal, which can prevent the formation of the aRNAP complex. This could be caused by a bad expression of the β'-subunit. A reason for this bad expression may be due to the previous used expression vectors (pJCore1, pJCore2, pJCore4S) did not contain the intergenic region between *rpoB* and *rpoC*. This intergenic region is present in the chromosomal DNA of *E. coli* and might be important for the proper expression of the β'- subunit. This issue should be addressed to ensure proper aRNAP complex formation.

4.4 Improvement of aRNAP expression and purification from IBs

Due to the existing problem of poor binding of Strep-tagged TraJ to the StrepTrap HP column and the poor expression of β', pJCore5 was designed.

This construct contains a Twin-Strep-tagged TraJ (figure 9), which should improve binding of the Strep-tag to the StrepTrap HP column, and also improve the Strep-tag detection, which was not reliable in the past (figure 29 and unpublished data).
In between the Twin-Strep-tag and the TraJ a FLAG-tag was placed, to ensure accessibility of the tag, due to previous good experiences with FLAG-tag TraJ (Laboratory of Günther Koraimann).

Furthermore, TraJ contains a mutation, where a cysteine is substituted by a serine on position 92, which enhances the activity in TraJ (unpublished data). In this construct the expression of β' was also tried to improve, due to previous bad expression (31, 34, 39 and observations), by adding the intergenic region between *rpoB* and *rpoC*.

The proteins could be expressed successfully (figure 40). Better detection of Strep-tag and better expression of β' was accomplished with the expression of aRNAP, encoded by the new plasmid pJCore5.

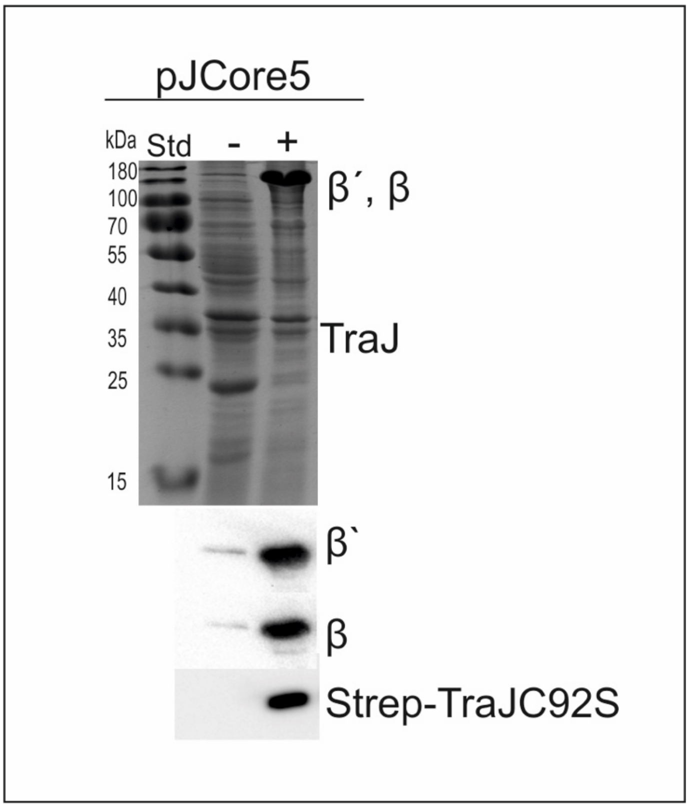

Figure 40: : Analysation of expression level in induced (+) and not-induced (-)* E. coli *BL-21 (DE3) cells containing pJCore4 and pJCore5. *After transformation of* E. coli *BL21 (DE3) with pJCore5, protein expression was induced with 0,5 mM IPTG and then incubated for two hours at 37°C. 0.1 OD disrupted whole, induced and not-induced cells were analysed on a 12,5% SDS-PAGE and/or western Blot. 4 µL of the PageRulerTM pre-stained protein ladder were used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System*

Only a slight improvement of binding to the column of only TraJ could be seen and most of the proteins did not stay in solution during the run (figure 41A). But proteins, which were eluted with desthiobiotin did stay in solution. Even though β´ and β could not be detected very well on the western blot, there is a band at the respective size of β and β´ on the gel. The result suggests that a few stable and soluble complexes could be formed by β, β´ and TraJ, which mostly eluted at 12.5 mL.

Due to precipitation of proteins during the run, glycerol was added up to 30% (final concentration) to the running buffer (SC-binding buffer), which resulted in β´ and β elution together with TraJ and no protein was precipitating during the run (figure 41B). Glycerol in the running buffer however made the run difficult, due to high pressure in the system
Most of the tagged protein was still not binding specifically to the column. (figure 41B).

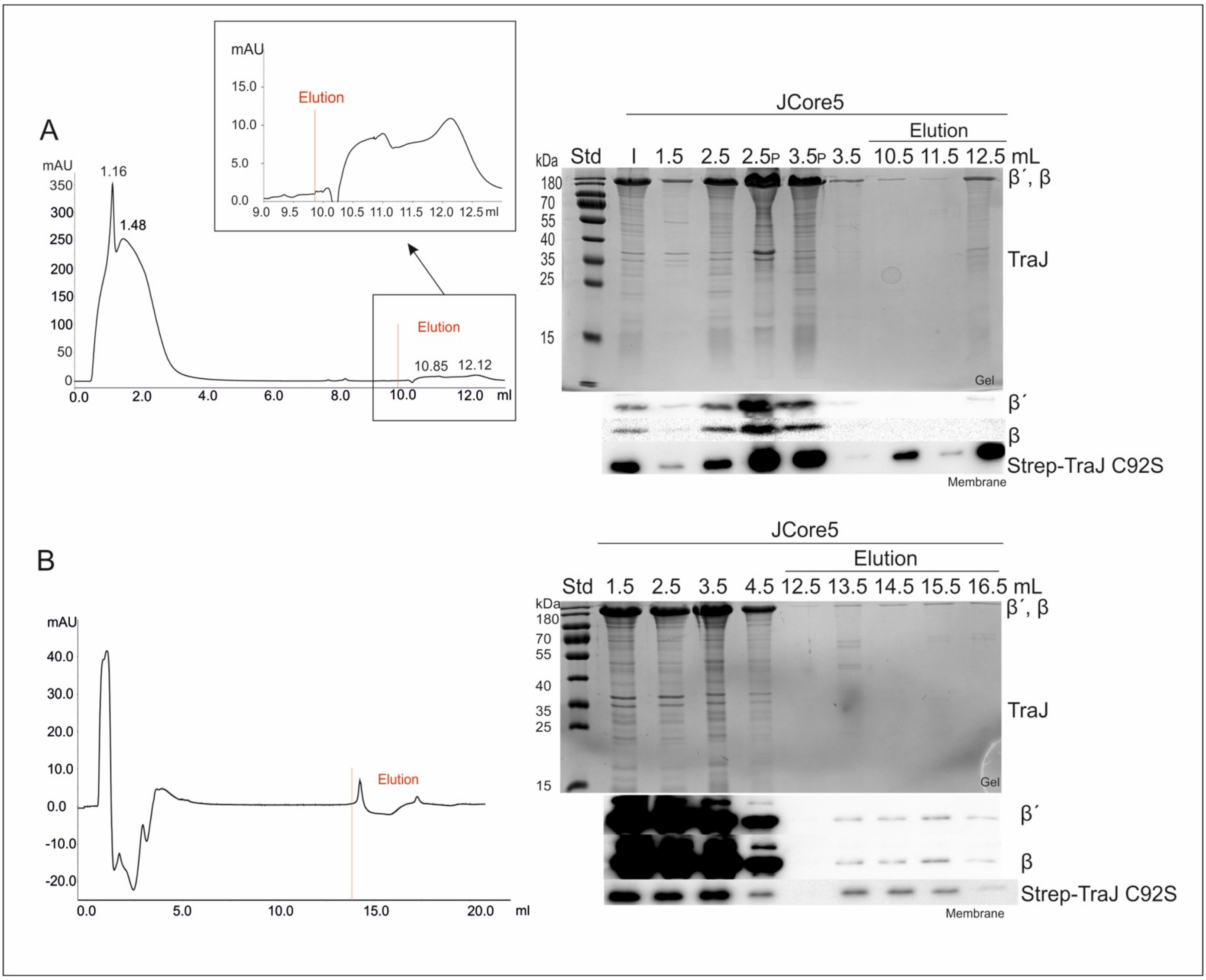

***Figure 41: aRNAP purified over a StrepTrap HP column**. Renatured aRNAP (JCore5) was purified over a StrepTrap HP column, where aRNAP was eluted with desthiobiotin. Aliquots of the input (I), flow through (1.5 mL), wash (2.5-4.5) and eluate fractions (10.5-12.5 mL) of the StrepTrap HP column purification were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System and the corresponding chromatograms. **B** shows the same run as A, but glycerol was added to the running buffer to a final concentration of 30%, to avoid protein precipitation as happened in run A (2.5_P and 3.5_P).*

4.5 Compex formation of renaturated aRNAP can be observed on a Size exclusion column

Another approach to gain more insight in the aRNAP complex formation, and to separate aRNAP from free subunits and aggregates of the IB-purification, the size exclusion column Superdex 200 increase 10/300 GL was used. JCore1 was purified out of inclusion bodies, renatured and then separated over the Superdex 200 increase 10/300 GL.

As shown in figure 42, most of TraJ, which was renatured with β and β´, elutes together with β and β´ at 7.5 and 8.5 mL. Some TraJ elutes at 14.5 mL, which corresponds to the size of a dimer of TraJ, hinting that TraJ is not a limiting factor in complex formation. Due to this result a high molecular weight complex containing 2xTraJ (27 kDa), β (150 kDa) and β´ (155 kDa) is suspected to be formed, with an calculated size of 360 kDa (with ω). The comparison with the gel filtration standard (Bio-Rad, Hercules, CA, figure 43) however, does not necessarily reflect the true size of the complex, due to the fact that the standard is measured with globular proteins. The crystal structure of β and β´ are elliptically and therefore seem bigger in comparison to the standard (at 670 kDa). RNAP core can form dimers[75], so the JCore may also form dimers and elutes even earlier (740 kDa).

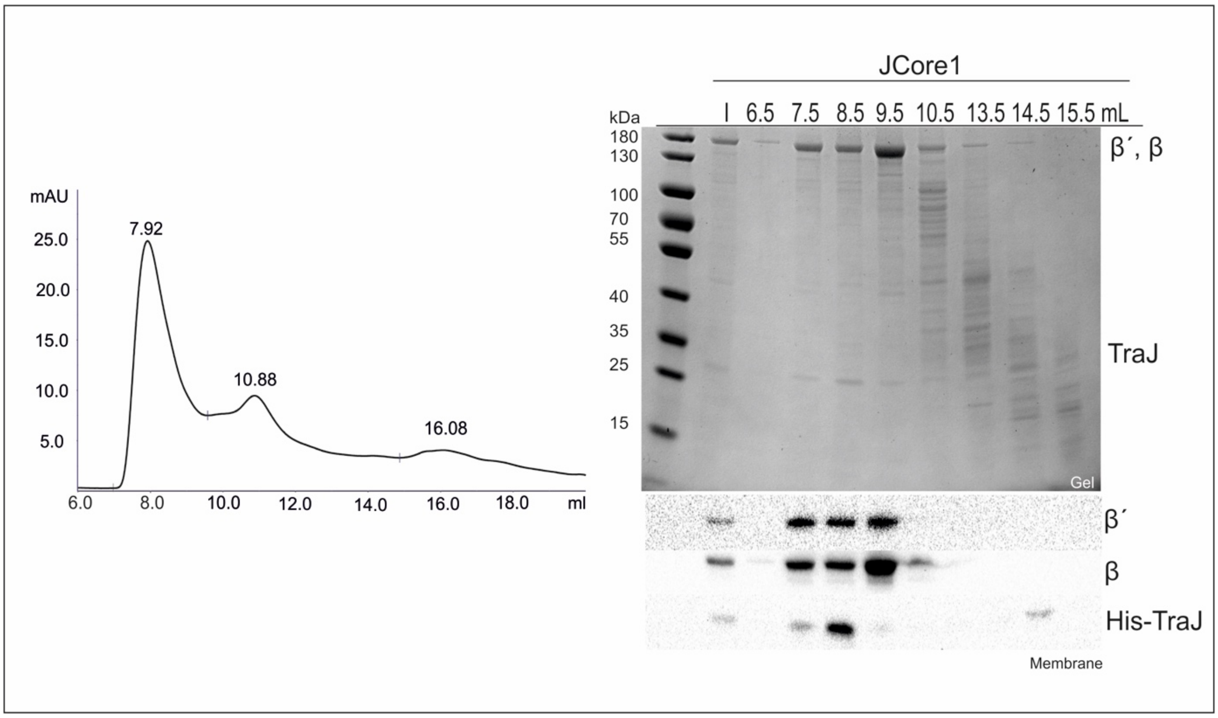

Figure 42: Formation of a high molecular weight complex containing β´, β and His-TraJ (JCore1) *analysis by size exclusion chromatography (Superdex Increase 10/300 GL). Complexes were allowed to form during renaturation of proteins purified form over-expressed JCore1 proteins. The obtained fractions were subject to SDS-Page and western blotting. 4 µL of the PageRuler™ pre-stained protein ladder were used as standard. Through comparison with the runs of the single proteins (supplementary data) and with the standard run it can be concluded that β´, β and TraJ form a high molecular weight complex (Jcore). The indicated mL were then subject to an western blots. Proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System and the corresponding chromatogram.*

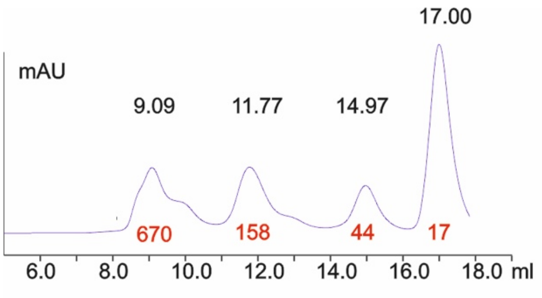

Figure 43: Gel Filtration Standard *(Bio-Rad, Hercules, CA) was separated over a Superdex 200 increase 10/300 GL (GE Healthcare, Chicago, IL). The sizes of the proteins included in the standard are indicated beneath the representing peek (670, 158, 44 and 17 kDa) in the chromatogram.*

The separation of β, β´, His-tagged TraJ (and ω) over a Size exclusion column could show the formation of a high molecular complex (aRNAP). This could be also shown in previous experiments in the laboratory of Günther Koraimann with reconstituted aRNAP containing FLAG-tagged TraJ, done by Karin Bischof. Apparently the His- and the FLAG-tag are suitable to tag TraJ, without interfering with the formation of aRNAP complex.

4.6 EF-Tu interacts with JCore but not with JHolo

A *in vivo* pull down of TraJ from a previous experiment of Ines Aschenbrenner[45], showed that EF-Tu was also pulled down. We wanted to investigate, whether EF-Tu also is part of aRNAP, if it helps with the assembly of aRNAP or enhances the stability of aRNAP and therefore increases the solubility or whether it inhibits aRNAP. QR, a RNA dependent RNAP (Replicase) from phage Qβ contains EF-Tu in its structure as active core subunit[76], which encouraged us to proceed with the investigation regarding if EF-Tu is involved in aRNAP complex formation.

4.6.1 Native EF-Tu purification

To conduct further experiments EF-Tu had to be expressed and made available.
EF-Tu expression via pET28tufB caused a spherical growth shape, which can be associated with MreB-EF-Tu interaction. During this interaction, EF-Tu is bound to GDP (Guanosine diphosphate)[57], which hints that EF-Tu is in this specific conformation.

EF-Tu was overexpressed from the pET28tufB plasmid in *E. coli* BL21 cells were transformed with the respective plasmid. Whole cells with induced and not induced EF-Tu overexpression were compared on a SDS-Page and western blot (figure 44).

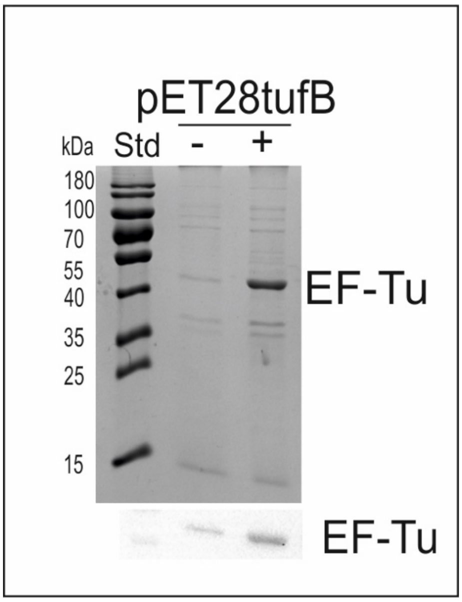

Figure 44: Analysation of expression level in induced (+) and not-induced (-)* E. coli *BL-21 (DE3) cells containing pJCore4 and pET28tufB *After transformation of* E. coli *BL21 (DE3) with pET28tufB, protein expression was induced with 0,5 mM IPTG and then incubated for two hours at 37°C. 0.1 OD disrupted whole, induced and not-induced cells were analysed on a 12,5% SDS-PAGE and/or western blot. 4 µL of the PageRuler™ pre-stained protein ladder were used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System*

Then it was purified under native conditions over a His-GraviTrap-TALON column. Aliquots from some steps of the purification were analysed over a SDS-PAGE and a western blot (figure 45), which shows that that most of the overexpressed protein is in the supernatant and that the protein purification was successful. The native purified EF-Tu was later used for far western blot with EF-Tu (figure 51).

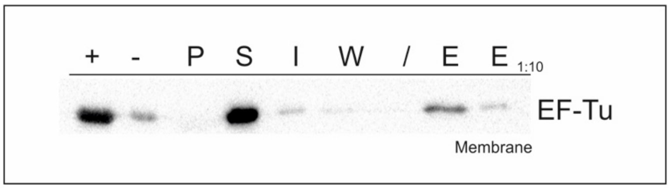

Figure 45: EF-Tu purified under native condition over a His-GraviTrap-TALON column: *After overexpression from the pET28tufB plasmid, cells were disrupted and His-tagged EF-Tu was purified under native conditions over a His-GraviTrap-TALON column. Aliquots of not induced (-), induced (+), pellet fraction (P), supernatant (S), sample loaded onto the column (I), wash step (W), eluate with 150 mM imidazole (E) and a 1:10 dilution of the eluate were subject to western blotting. On the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

4.6.2 EF-Tu purification under denaturing conditions

It was speculated that EF-Tu would help the aRNAP to assemble, for which purpose purified denatured EF-Tu was needed, so it could be renatured with aRNAP together. In order to obtain a relative pure EF-Tu, it was purified over a His-trap-column under denaturing conditions. Aliquots from the purification-steps were analysed on a SDS-PAGE and a western blot (figure 46) and from which can be concluded, that the purification was successful. Elution 2 and 3 was further used, because of the highest yield of EF-Tu.

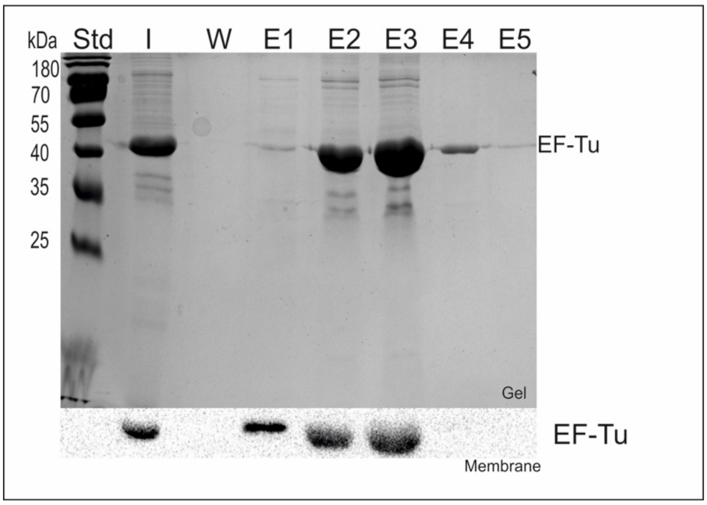

Figure 46: Purification of EF-Tu under denaturing conditions via His-tag*: denatured His-tagged EF-Tu, purified out of IB, was purified over a His-Trap column (1 mL) and eluted with 5 mL of 500 mM imidazole. The elution was collected in fractions of one mL (E1-5). Aliquots of the input (I), wash (W) and eluate fractions (E1-5) of the His-trap column purification were then analysed on a 12.5% SDS-PAGE and western blot. 4 µL of the PageRuler™ pre-stained protein ladder was used as standard. The SDS-PAGE was dyed with Kang-dye and on the western blots proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

4.6.3 The alternative RNAP interacts with EF-Tu

Denatured EF-Tu and aRNAP could be renatured together to soluble proteins. To find out whether EF-Tu can bind to aRNAP, if it can be reconstituted to one soluble complex and also to separate it from free parts of the subunits and aggregates, EF-Tu and aRNAP were analysed over size exclusion columns (Superdex 200 and Superose 6 increase 10/300 GL). The size exclusion column Superdex 200 increase 10/300 GL was used, due to good results in the group of Günther Koraimann in the past. Before the proteins were applied to the column, denaturised EF-Tu was renatured with either the whole aRNAP or parts of it (reconstitution mixes). Single subunits of aRNAP were expressed and purified out of inclusion bodies by Karin Bischof using the protocol for purification out of inclusion bodies used in this work.

When the reconstitution mix, which contains TraJ and EF-Tu was separated over a Superdex 200 increase 10/300 GL column, TraJ eluted with EF-Tu already at 13.5 mL (figure 47A) and not at 14.5 mL, as the TraJ-Dimer normally does. Therefore, an interaction between EF-Tu and TraJ is likely. The comparison with the gel filtration standard (Bio-Rad, Hercules, CA, figure 47E) however, does not necessarily reflect the true size of the complex, due to the fact that the standard is measured with globular proteins.

When β (150 kDa), TraJ (27 kDa) and EF-Tu (43 kDa) are reconstituted and separated over the Superdex 200 increase 10/300 GL column, they elute together at 8.5 mL (around 670 kDa compared to the standard), what hints interactions between them (figure 47B).
In figure 47C JCore5 (complex, which consists of proteins expressed from pJCore5) is separated after reconstitution together with EF-Tu and separated over a size exclusion column. It shows a similar elution behaviour as the complex formed of β, TraJ and EFTU. Also reconstituted TraJ-holo-RNAP (with σ^{70}) and EF-Tu was analysed over the Superdex 200 increase 10/300 GL column, and the result (figure 47D) suggests that the JHolo-enzyme elutes with EFTU at 6.5 mL.
But a good resolution at 7.5 mL is not given because of the dead-volume of the column, therefore another size exclusion column was used.

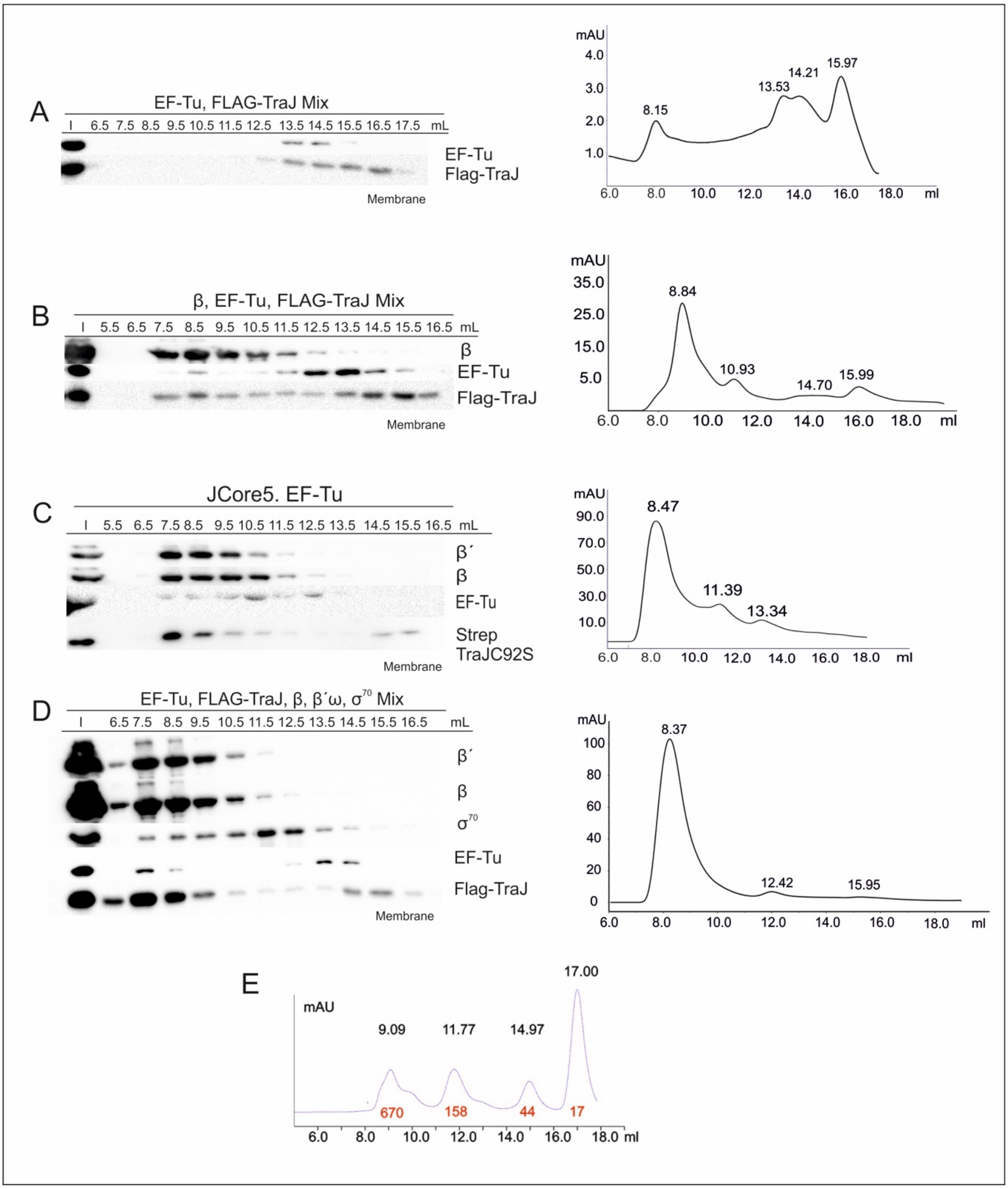

Figure 47: ***Parts of aRNAP, whole aRNAP and JHolo with EF-Tu separated on a Superdex increase 10/300 GL:*** *the indicated proteins were renatured together and then separated over Superdex increase 10/ 300 GL column. The indicated mL were then subject to a western blots. Proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System (Bio-Rad, Hercules, CA) and the corresponding chromatograms. Separated reconstitution mixes:* ***A*** *EF-Tu and FLAG-TraJ and EF-Tu;* ***B*** *β, FLAG-TraJ and EF-Tu;* ***C*** *JCore5 and EF-Tu;* ***D*** *JHolo and EF-Tu + 0.1 mg/mL Heparin.* ***E*** *Gel Filtration Standard (Bio-Rad, Hercules, CA)*

Because a better resolution of the separation of big protein complexes was expected, the Superose 6 increase 10/300 GL was used. To estimate the sizes of the eluted protein complexes the same gel filtration standard as for the Superdex 200 increase 10/300 GL column was applied on the superpose 6 increase 10/300 GL column and is shown in figure 48E. Considering the standard and the expected size of the reconstituted complex, containing JCore and EF-Tu (around 400 kDa), the complexes would elute at around 14 mL and considering the shape of the complex it would probably elute around 12-13 mL.

When JCore5 and EF-Tu were reconstituted and separated on a Superpose 6 increase 10/300 size exclusion column EFTU, β, β´ and TraJ eluted at 12.5 and 13.5 mL. Most of EF-Tu, separated alone over the superpose column, elutes at 15.5 mL (figure 48A), which again indicates an interaction between aRNAP and EF-Tu. β, β´ and TraJ probably form aggregates, which elute earlier (8.5-11.5 mL). Proteins eluted later are probably intermediate complexes, which contain only parts of the complex.
A reconstitution mix, consistent of EF-Tu and aRNAP with FLAG-tagged TraJ, separated over the Superose 6 increase 10/300 GL column (figure 48D) shows a similar elution behaviour as the aRNAP with the Twin-Strep-FLAG-tagged TraJ (figure 48B).

When EF-Tu together was reconstituted with the JHolo and separated over the superpose 6 size exclusion it becomes apparent that at 12.5-13.5 mL, where the elution of the protein complex is expected, almost no EF-Tu is eluted together with the holo-enzyme. It seems as if EF-Tu is prevented to bind to aRNAP when σ^{70} is present. This suggests that EF-Tu is not a part of the initiative complex of aRNAP. But it could be necessary for the assembly, the elongation or interacts with the the JCore enzyme with the purpose of inhibition.

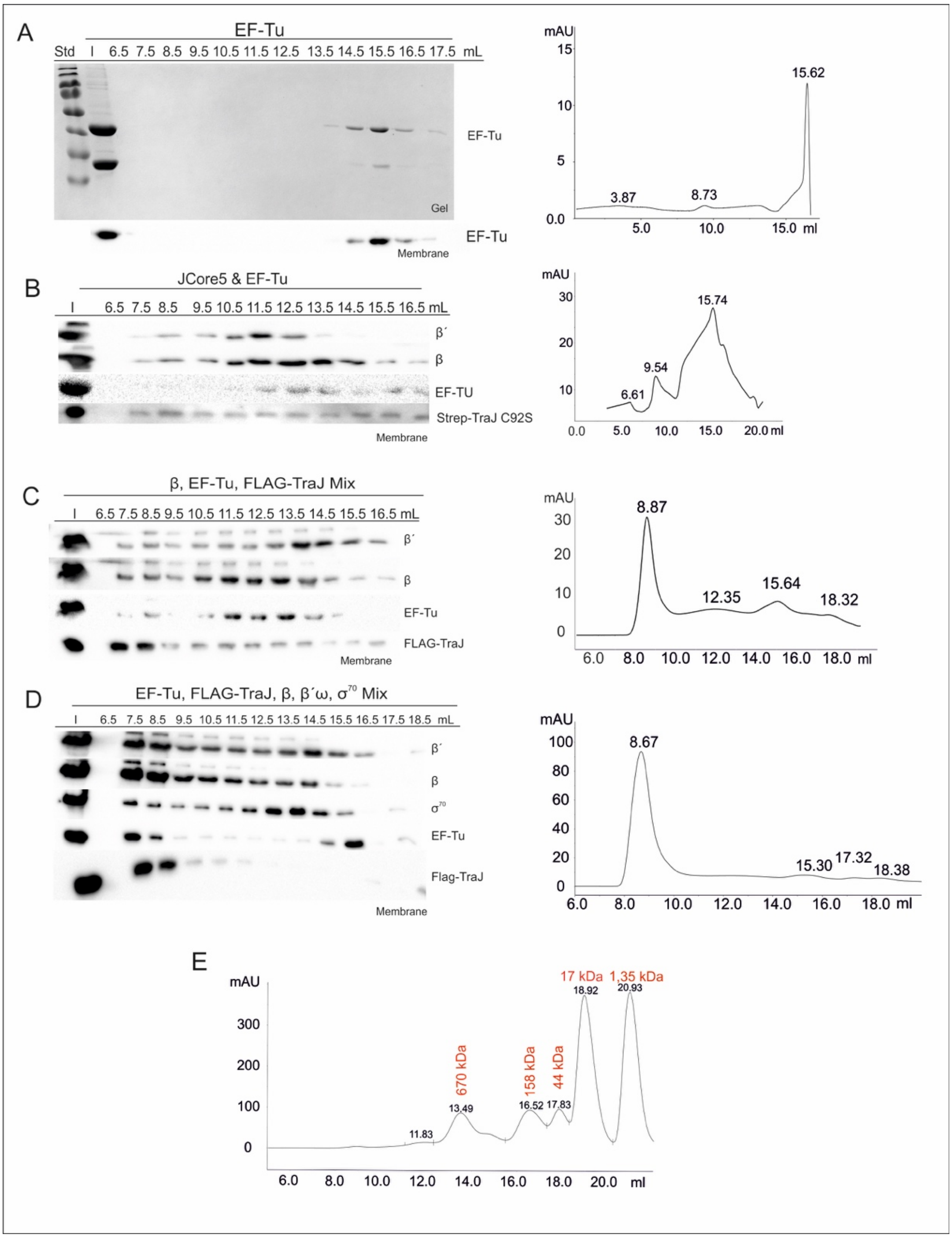

Figure 48: Parts of aRNAP and whole aRNAP with EF-Tu separated on a Superose 6 increase 10/300 GL: *the indicated proteins were renatured together and then separated over Superose 6 increase 10/300 GL column. The indicated mL were acetone precipitated and then subject to western blots. Proteins were detected with indicated Antibody (listed in table 25). Shown are take outs of pictures, which were taken with ChemiDoc™ MP System (Bio-Rad, Hercules, CA) and the corresponding chromatograms.* ***Reconstitution mixed: A*** *EF-Tu alone for reference;* ***B*** *EF-Tu and JCore (JCore5);* ***C*** *JCore and EF-Tu;* ***D*** *JHolo and EF-Tu;* ***E*** *Gel Filtration Standard (Bio-Rad, Hercules, CA)*

Considering all separations, it becomes apparent, that at the separation of the TraJ-holo-enzyme and EF-Tu, EF-Tu elutes earlier (8.5/9.5 for Superose 6 and 7.5 for Superdex 200) than without σ^{70}. The reason behind that phenomena might be the bigger size of the protein complex, but in comparison with the standards of the columns respectively, the eluted proteins can only be aggregates. Considering the standard and the expected size, the complex would be eluted around 14 mL, and with consideration of the shape of the complex, it would elute around 12-13 mL.

4.7 Binary interactions between subunits of aRNAP

4.7.1 TraJ interacts with β, β' and σ^{70} individually

Binary interactions of TraJ, β, β´ and σ^{70} became already apprehend in previous purification experiments (affinity purification and size exclusion).
To confirm the binary interactions between TraJ and the other subunits of aRNAP far western blots (FWB) were performed. Renatured FLAG-tagged TraJ, which was purified out of inclusion bodies by Karin Bischof, was used to discover interaction partners by overlaying the membrane of a western blot, were in the previous step different RNAP subunits were separated on a SDS-PAGE. Purified FLAG-TraJ was put on the western blot membrane for the first step of western blot detection. When TraJ interacts with one of the subunits, the proteins will be detected and a band can be seen on the membrane at the height of the interacting protein. TraJ itself was also detected on the western blot, serving as a positive control because it can interact with itself.

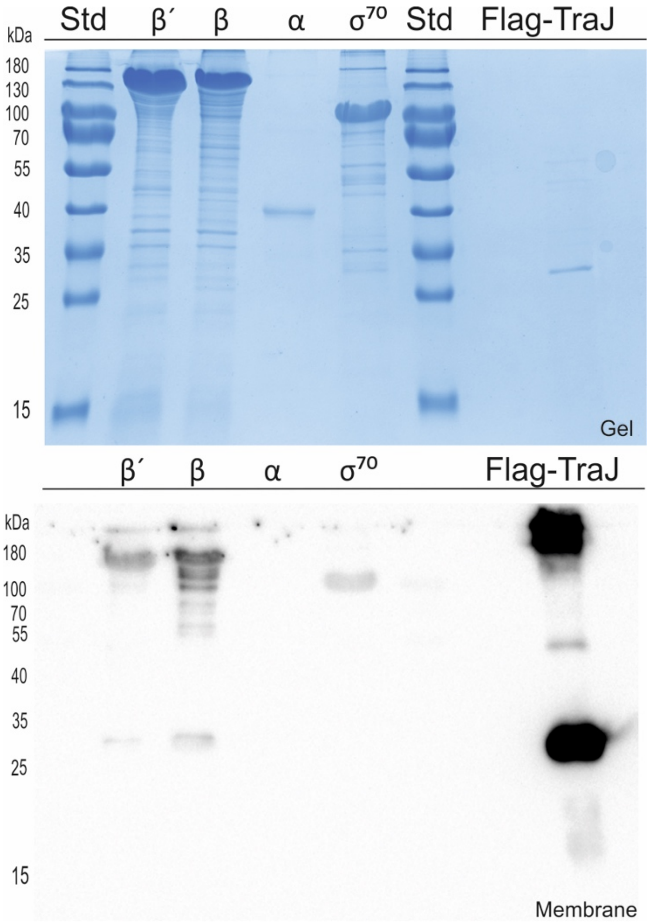

***Figure 49: Interaction of TraJ with subunits of RNAP**. Indicated, purified proteins were subject to SDS-PAGE and subsequent WB. Purified FLAG tagged TraJ (FLAG-TraJ, purified by Karin Bischof) was used to overlay the membrane in the first incubation step. Anti-FLAG (mouse) antibody (AB) and anti-mouse-POX conjugated AB were used as primary and secondary AB, respectively. As shown here, TraJ interacts with σ^{70}, β and β`, but not with the α-subunit (full length, 329 AA). 4 µL of the PageRuler^{TM} pre-stained protein ladder was used as standard. Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

As shown in figure 49 the interaction between TraJ and β, β' and σ^{70} respectively, could be confirmed via a far western blot.

The same western blot was repeated, but this time it was detected with purified α (256 AA), which lacks the C-terminus.
As expected, α interacts with β, β' and surprisingly with TraJ (figure 50). On this FWB it also becomes apparent that β' also contains some α.

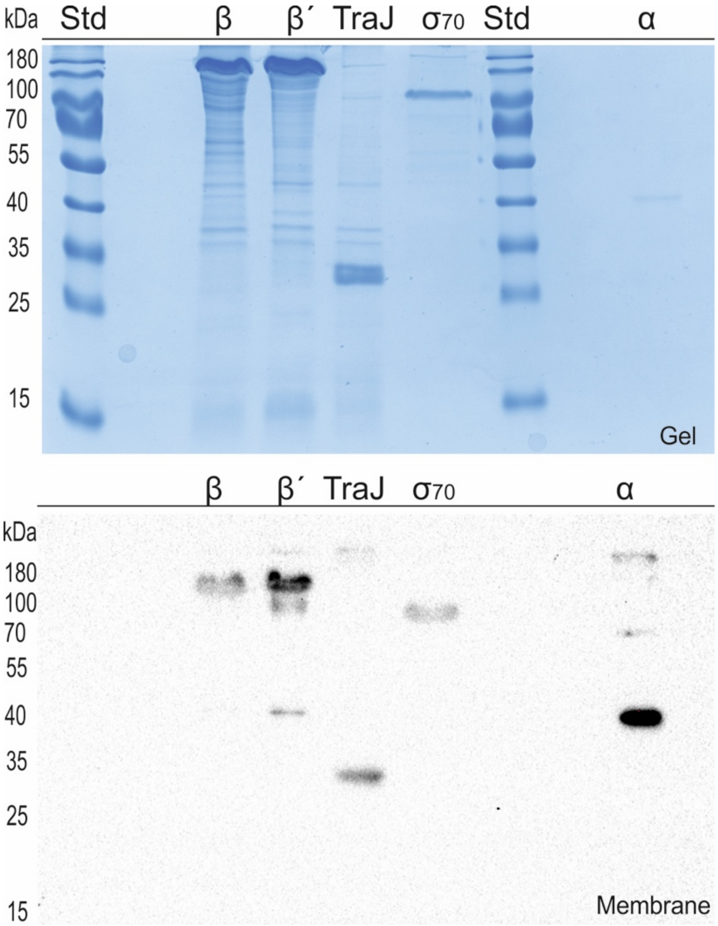

***Figure 50: Interaction of α (256 AA) with subunits of aRNAP**. Indicated proteins were subject to SDS-PAGE and subsequent WB. Purified His-tagged α (Karin Bischof) was used to overlay the membrane in the first incubation step. Anti-α (mouse) antibody (AB) and anti-mouse-POX conjugated AB were used as primary and secondary AB, respectively. As shown here, α interacts with σ70, β and β`, and with the TraJ. 4 µL of the PageRulerTM pre-stained protein ladder was used as standard. Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

From this FWB can be concluded that TraJ interacts only with the short version of α (256AA), which misses the C-terminus.

4.7.2 Confirmation of EF-Tu interaction with aRNAP

An interaction between EF-Tu and aRNAP was also suggested, due to the results of the size exclusion experiments and the in vivo pull down. Through a far western Blot no interaction could be confirmed between EF-Tu and the subunits of aRNAP (figure 51). However, the FWB showed an interaction between σ^{70} and EF-Tu.
Even though no interaction except with σ^{70} was shown, the far western blot shows, that EF-Tu is contained in TraJ, β and β' samples, which were purified out of inclusion bodies alone. Probably it could be also found in the inclusion bodies

because it is the most plentifully contained protein in most of *E. coli* cells. This impurity should be eliminated by a more exact purification of the single parts of the aRNAP.
This is a major problem if EF-Tu has an inhibiting character, so the focus has to be purifying the components of aRNAP without EF-Tu. The far western blot was conducted with native purified EF-Tu over a His-Gravi-TALON column (figure 45).

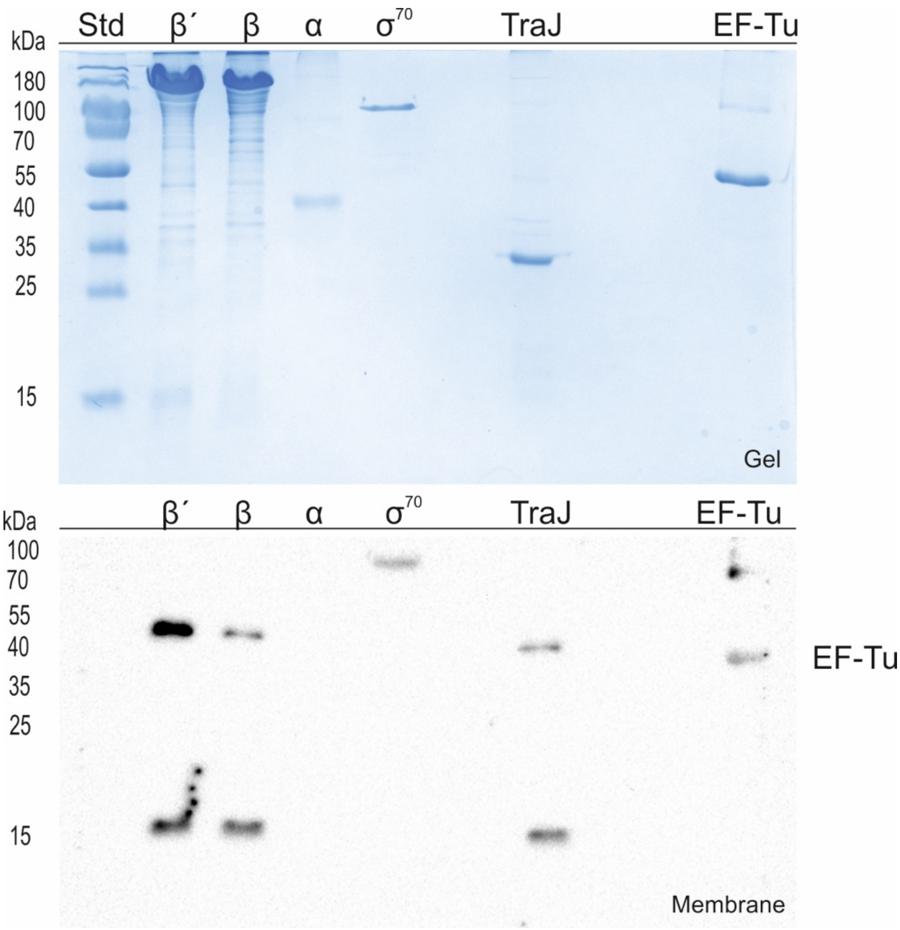

Figure 51: Interaction of EF-Tu with subunits of RNAP. *Indicated proteins were subject to SDS-PAGE and subsequent WB. Purified His-tagged EFTU was used to overlay the membrane in the first incubation step. Anti-EFTU (mouse) antibody (AB) and anti-mouse-POX conjugated AB were used as primary and secondary AB, respectively. No interaction except with σ^{70} could be shown, but with EF-Tu itself, which is present in Beta beta´ and TraJ 4 µL of the PageRulerTM pre-stained protein ladder was used as standard. Shown are take outs of pictures, which were taken with ChemiDoc™ MP System.*

Another method to show protein interaction is called Co-Immunoprecipitation and was used to investigate the interaction between EF-Tu and parts of aRNAP. For that purpose, Agarose-FLAG-beads were used to pull down FLAG-tagged TraJ (purified by Karin Bishop) and any protein bound to it out of reconstitution mixes with EF-Tu and/or β. Reconstituted β and EF-Tu without TraJ served as negative control.
The pulled down proteins were analysed on a SDS-PAGE, followed by a western blot (figure 52). Because the detection of EF-Tu with antibodies on the membrane caused some problems, the band, which was thought of as EF-Tu, was cut out and is to be analysed with MS in the near future.

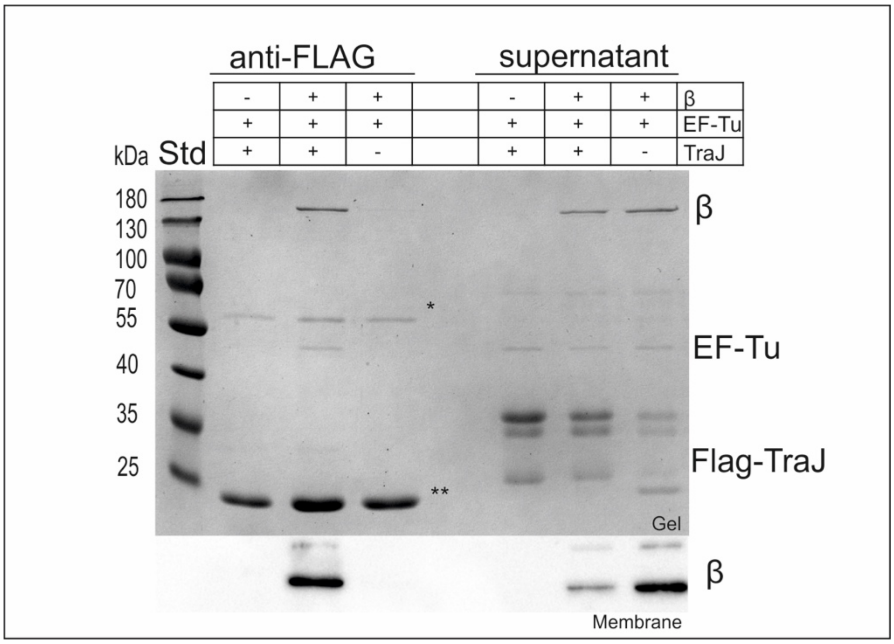

Figure 52: FLAG-TraJ pulls down all subunits of RNAP*. The protein mixtures 1 (reconstitution mix TraJ, EF-Tu), 2 (reconstitutions mix TraJ, β, EF-Tu), 3 (β, EF-Tu, negative control) were subject to an anti-FLAG-IP and were analysed by SDS-PAGE and western blotting. The bands of the heavy * (55 kDa) and light ** (25kDa) chains of the antibodies served as loading control. In samples 2 FLAG-TraJ pulls down β and EF-Tu.*

The result hints, that EF-Tu interacts with Beta and TraJ, but not or only week with TraJ alone. Because when only EF-Tu was renatured with FLAG-TraJ and FLAG-TraJ was pulled down, almost no EF-Tu can be detected in the pull down. The contrary can be observed when β-subunit is renatured together with EF-Tu and FLAG-TraJ, were a lot of β and EF-Tu can be found in the pulldown of FLAG-TraJ, suggesting a interaction between these three proteins. As expected no proteins, only the heavy and light chains of the antibodies are visible in the pull down of the negative control, consisting of EF-Tu and β without FLAG-TraJ.

Therefore an interaction between EF-Tu, TraJ and β´could be confirmed with co-immunoprecipitation.

5 Discussion

Self-transmissible F-like plasmids are responsible for the spread and persistence of antibiotic resistance and virulence genes in medically relevant bacteria. Conjugative DNA transfer is mediated by a type IV secretion system, whose components are mostly encoded in the *tra* operon. The main activator of *tra* operon expression of F-like plasmids is TraJ. The transcription factor TraJ binds to the P_Y promoter enabling transcription of DNA transfer genes.
It could be shown that TraJ interacts with subunits of RNAP β´, β and σ^{70} individually and together they form a soluble core- and holo-complex. Kathrin Froschauer was recently able to show that aRNAP can bind to DNA, and more specifically, to the promoter P_Y (unpublished data). In this modified RNAP, TraJ is a unique transcription factor forming an essential part of a non-canonical RNAP. We propose that this RNAP contains TraJ instead of α and its function is to ensure transcription of the complete *tra* operon.

One hypothesis is, that TraJ is interfering at an early step with the RNAP assembly and functionally replaces α.
Our model of the assembly of the alternative RNAP (figure 53) starts with the interaction of RNAP subunit β and a dimer of TraJ. The following steps are the same ones as in the assembly of the known RNAP of *E. coli* (figure 6). TraJ would therefore take the place of the α subunit.

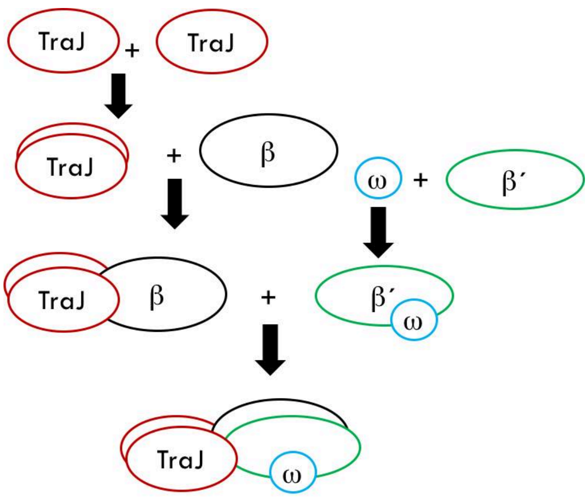

Figure 53: schematic model of the assembly process of the alternative RNAP from different subunits

In favour of this model it could be shown that TraJ and β can form a soluble complex when reconstituted together (unpublished Data). Additionally Ines Aschenbrenner showed in her master´s thesis, that TraJ interacts with the β-flap domain[45]. In her study also no interaction of full length α and TraJ could be shown via a bacterial two hybrid system[45] and the far western blot with TraJ, conducted in this work, did not show any interaction with the full length α neither. Sarah Trunk observed in her master´s thesis[60] a co-purification of the α-subunit with TraJ, which could be explained, if a RNAP would form a mixed dimer with an aRNAP. Another explanation would be that GroEL, which is also co-purified with TraJ, binds to α. An interaction between TraJ and α (256 AA) was suggested from the far western blot with α (256 AA), which lacks the C-terminal domain.

The theory that α assists in the assembly of the alternative RNAP was addressed by co-expressing aRNAP and the α subunit. This co-expression resulted in cell death, however even with controlled basal expression no increase in solubility and therefor complex formation was observed. Even most of β-subunit stayed in the pellet fraction with TraJ after cell disruption, and was therefore almost not detectable in the soluble fraction with α, suggesting a stronger interaction between TraJ and β than β and α .

In general, very little soluble complex of JCore could be purified out of the supernatant of cells, overexpressing aRNAP. Most of the overexpressed proteins were located inside inclusion bodies. The same problem was faced during first attempts to purify RNAP, resulting in the availability of well-established protocols for purifying RNAP out of inclusion bodies and for renaturation of RNAP, which were used in this thesis.

One of the reasons why TraJ and JCore are not soluble in a high concentration may be their low concentration in the cell[34]. It might be just another regulation of the bacterial conjugation, which is preventing the cell from too much cellular stress. TraJ probably aggregates at higher concentration, forming SDS-resistant complexes with itself, as well as with other proteins. This phenomenon could also be observed on SDS-PAGE and size exclusion chromatography.
aRNAP also aggregated when observed under an electron microscope, unless a concentration of 3 nM or less was applied to the grip (figure 54).
The electron microscopy pictures also show, that the formed complexes of JCore and JHolo are similar to the formed complexes of RNAP Core- and holo-enzymes concerning their size and shape.

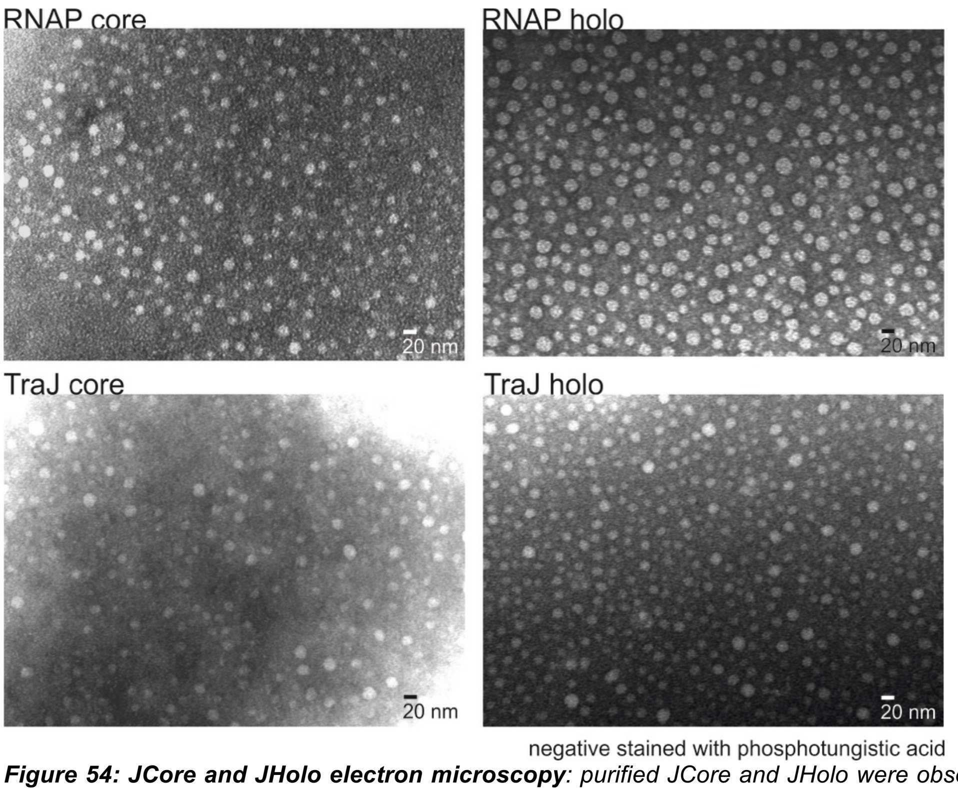

***Figure 54: JCore and JHolo electron microscopy**: purified JCore and JHolo were observed under an electron microscopy with a 28 500x magnification after it was negative stained with phosphotungistic acid. For comparison RNAP core (NEB) and holo was also observed. (Kathrin Froschauer and Günther Zellnig)*

An also likely explanation might be, missing co-factors or additional proteins stabilising the alternative RNAP. This seems logical, considering GroEL was co-purified with JCore in a native purification, which indicates a not right folded complex. A reason for a not right folded complex could be a missing interaction partner. One hypothesis was, that EF-Tu could be an additional component of the aRNAP, as previous observation (co-purification and in-vivo-pull down) in our lab suggested an interaction between TraJ and EF-Tu. The results of this work hints that EF-Tu binds TraJ and β simultaneously, which enhances the binding affinity, because interactions between EF-Tu -TraJ and EF-Tu - β individually seem to be a weaker interaction than the interaction EF-Tu-TraJ-β. Corresponding to the energy level of the cell, the GDP-bound EF-Tu form regulates the translation and the cytoskeleton formation over MreB. Through binding the core enzyme of aRNAP it could maybe also regulate the transcription of the *tra* operon.
An interaction between EF-Tu and the JCore could be shown, but not with the TraJ-holo-enzyme, where it seems that EF-Tu is replaced by σ^{70}. Unpublished results of Kathrin Froschauer suggest as well that EF-Tu is inhibiting the P_Y promotor activation.
Furthermore, the complex formation of aRNAP under physiological conditions might be enhanced or dependend on co-factors, which are available in the cell in low concentration but not enough to provide for overexpressed aRNAP complexes, which prevents the complex formation.

The special feature of this new non-canonical RNAP is that it can transcribe the whole 32 kb long *tra* operon. The substitution of the α subunit with TraJ might prevent NusA from binding, because both are binding to the β-flap domain. This might inhibit the regulation of NusA concerning the termination of the transcription.
Already 1989, Koraimann was able to show that the polycistronic mRNA of the *tra* operon contains a secondary structure between *traA* and *traL.* The hairpin structure prevents the transcription of *traL* when expressed with the T7 expression system[77]. Also RfaH, an anti-termination factor, can not overcome this termination (unpublished data). Maybe the aRNAP is necessary to overcome this secondary structure and to transcribe the whole operon. This possibility should be investigated further in coming research.
A cryo-electron microscopy or crystal structure of aRNAP could give more insight into the structure and an *in vitro* assay could show transcriptional activity of the TraJ-holo-RNAP. For all these methods pure aRNAP is needed. This might only be achieved *in vitro* and with a Flag-tagged aRNAP construct, because all other purifications and *in vivo* experiments caused a lot of trouble.
If the transcriptionally activity of aRNAP can be proved with an *in vitro* transcription assay, TraJ-holo-RNAP would represent the first known bacterial multisubunit transcription machinery without α. We propose that TraJ-holo-RNAP is necessary for transcription initiation from the P_Y promoter, open complex formation as well as complete transcription of the *tra* operon of F-like plasmids.

6 Abbreviations

aa amino acids
aa-tRNA aminoacyl transfer Ribonucleic acid
aRNAP alternative RNA Polymerase
Bp Base pairs
cPCR colony Polymerase chain Reaction
DNA Deoxyribonucleic acid
DTT dithiothreitol
E. coli *Escherichia coli*
EDTA ethylenediaminetetraacetic acid
EF-Tu Elongation factor thermos unsteable
FWB Far western blot
Fwd Forward Primer
IPTG Isopropyl-β-D-thiogalactopyranosid
Kb kilo bases
kDa kilo Dalton
mRNA messenger RNA
ONC over night culture
PBS Phosphate-buffered saline
PCR Polymerase chain Reaction
PEI Polyethylenimin
PMSF Phenylmethanesulfonyl fluoride
Rcf relative centrifugal force
Rev Reverser Primer
RNA Ribonucleic acid
RNAP DNA dependend RNA Polymerase
Rpm Revolutions per minute
SDS-Page sodium dodecyl sulfate polyacrylamide gel electrophoresis
tra transfer
TAE Tris-acetate-ethylenediaminetetraacetic acid

7 Literature

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8 Suplementary Data

8.1 Oligonucleotides

The used oligonucleotides (table 27) were synthesised by eurofins Genomics (Hvidovre, Denmark).

Table 27: Oligonucleotides *which were used in this work and their sequence; #IMB (number of the oligonucleotide in the database of the IMB)*

	Sequence	#IMB*
Alpha_fwd		1952
T7lysozym_fwd	cgccctgtagcggcgCAATTAATTATCCACGGTCAGAAGTGACCAGTTCG	2260
T7lysozym_rev	cgaaaagtgccacctgGGCGCCAGCAACCGCACC	2261
pIRA1_fwd	CAGGTGGCACTTTTCGGGGAAATGTG	2262
H10rpoB_fw	CATCATCATAGCAGCGGCCTGGAAGTGCTGTTTCAGGGCCCGGTTTACTCCTATACCGAGAAAAAC	2263
pJCore1_rev	CAGGCCGCTGCTATGATGATGATGATGATGATGATGATGATGcatagggttcctcagctc	2264
alphaC329stop	tataCTCGAGttactcgtcagcgatgcttgc	2265
rpoC_fwd_001	atgggcagcagcagcACTCCGACGGGAGCAAAT	2269
Twin_Strep_Flag_2	gacgCCATGGactctgcctggtctcacccgcaattcgagaaaggcggcggtagcggtggcggcagcggtggtagcgcg	2276
Twin_Strep_Flag_1	ggcagcggtggtagcgcgtggagccatccgcagtttgaaaaactcgagGACTACAAAGACGACGATGACAAGT	2277
rpoB+_rev	atccgctgccgggttttaacccgacagcagtg	2278
rpoC+fwd	tgtcgggttaaaacccggcagcggattgtgc	2279
TSFJ_fwd	gtttaactttaataaggagatatacgacgccatggacagcgcc	2280
pIRA1_rev	CGCCGCTACAGGGCGCGT	2281
T7lysozym_fwd	cgccctgtagcggcgCAATTAATTATCCACGGTCAGAAGTGACCAGTTCG	2282
T7lysozym_rev	cgaaaagtgccacctgGGCGCCAGCAACCGCACC	2283
pACYCDuet-1_rev	GCTGTGGTGATGATGGTGATGG	2284
TEV_TraJ_fwd_01	atcaccatcatcaccacagcGAGAACCTGTACTTCCAG	2285
TEV_TraJ_rev	tcctcagctcaagcttTTATTACTTAACACCATAAAATTCACG	2286
rpoB_fwd	ttaagtaataaaagcttGAGCTGAGGAACCCTATG	2287
rpoB_rev	ccgtcggagtTTACTCGTCTTCCAGTTC	2288
rpoC_fwd	agacgagtaaACTCCGACGGGAGCAAATc	2289
rpoC_rev	ctccacaggtTTACTCGTTATCAGAACCGC	2290
rpoZ_fwd	taacgagtaaACCTGTGGAGCTTTTTAAG	2291
rpoZ_rev	agcggtggcagcagcctaggTTAACGACGACCTTCAGC	2292
pACYCDuet-1_fwd	CCTAGGCTGCTGCCACCG	2293
pACYCDuet-2_rev	tttttcgaactgcgggtgagaccaGCTGCTGCCCATGGTATATCTC	2294

TEV_TraJ_fwd_02	gatataccatgggcagcagctggtctcacccgcagttcgaaaaaGAGAACCTGTACTTCCAG	2298
pEt28lysM_rev	ATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTC	2321
tufB_fwd	aagaaggagatataccatATGTCTAAAGAAAAGTTTGAACGtacaaaaccg	2322
tufB_rev	tagcagccggatctcttagtgatgatggtggtgatgGCTCAGAACTTTTGCTACaacgccc	2323
pEt28lysM_fwd	catcaccaccatcatcactaaGAGATCCGGCTGCTAACAAAG	2324

* number of the oligonucleotide in the database of the IMB

Primers used for sequencing are listed in table 28.

Table 28: Sequencing primers

pJCore1	
2301	GAGTGGGTTTTACCTTTGTCGGAGTC
2302	CATACGACCAACCGCAGACAAGTC
2303	CGTAGTTGCCTTCTTCGATAGCAG
2304	GATGTCAGCGGTGATCTCTTCC
2305	CACCCTTGTTACCGTGACGACC
2306	CGATTTCAGGAACCAGATGTGCG
2307	GGTATGGACCTACGGTGATTACAG
2308	GCGGTAGCAGGTGTTCAGC
2309	CGTCTTCAGCAGTTACACGACCC
2310	ATCAGAACGTCGTTACCCTGAGCA
2311	GAGCAGATCCTCGACCTGTTCTT
2312	CCAAGCCGATTTCCGCAGCAGT
2313	GTGTCGTTCAGTACGTGGATGCTTCC
2314	CGAAGAAATGCAGCTCAAACAGG
2315	CAGATCCGCCTGTACGATGGTCGC
2316	CGCTGGTTCCGCTGGATGG
2317	CTCAGGACGTTGTACTGGGTCTGTA
2318	GTATGCGTGGTCTGATGGCGAAG
2319	CGGTCGTACTAAAGAAAGCTACAAAGT
2320	GTGGTGTTCATGCTGTTACTCG
2284	GCTGTGGTGATGATGGTGATGG
2285	atcaccatcatcaccacagcGAGAACCTGTACTTCCAG
2286	tcctcagctcaagcttTTATTACTTAACACCATAAAATTCACG
2287	ttaagtaataaaagcttGAGCTGAGGAACCCTATG
2288	ccgtcggagtTTACTCGTCTTCCAGTTC
2289	agacgagtaaACTCCGACGGGAGCAAATc
2290	ctccacaggtTTACTCGTTATCAGAACCGC
2291	taacgagtaaACCTGTGGAGCTTTTTAAG
2292	agcggtggcagcagcctaggTTAACGACGACCTTCAGC
2293	CCTAGGCTGCTGCCACCG
pJCore2	
2294	tttttcgaactgcgggtgagaccaGCTGCTGCCCATGGTATATCTC

2298	gatataccatgggcagcagctggtctcacccgcagttcgaaaaaGAGAACCTGTACTTCCAG
2286	tcctcagctcaagcttTTATTACTTAACACCATAAAATTCACG
2287	ttaagtaataaaagcttGAGCTGAGGAACCCTATG
2288	ccgtcggagtTTACTCGTCTTCCAGTTC
2289	agacgagtaaACTCCGACGGGAGCAAATc
2290	ctccacaggtTTACTCGTTATCAGAACCGC
2291	taacgagtaaACCTGTGGAGCTTTTTAAG
2292	agcggtggcagcagcctaggTTAACGACGACCTTCAGC
2293	CCTAGGCTGCTGCCACCG
2301	GAGTGGGTTTTACCTTTGTCGGAGTC
2302	CATACGACCAACCGCAGACAAGTC
2303	CGTAGTTGCCTTCTTCGATAGCAG
2304	GATGTCAGCGGTGATCTCTTCC
2305	CACCCTTGTTACCGTGACGACC
2306	CGATTTCAGGAACCAGATGTGCG
2307	GGTATGGACCTACGGTGATTACAG
2308	GCGGTAGCAGGTGTTCAGC
2309	CGTCTTCAGCAGTTACACGACCC
2310	ATCAGAACGTCGTTACCCTGAGCA
2311	GAGCAGATCCTCGACCTGTTCTT
2312	CCAAGCCGATTTCCGCAGCAGT
2313	GTGTCGTTCAGTACGTGGATGCTTCC
2314	CGAAGAAATGCAGCTCAAACAGG
2315	CAGATCCGCCTGTACGATGGTCGC
2316	CGCTGGTTCCGCTGGATGG
2317	CTCAGGACGTTGTACTGGGTCTGTA
2318	GTATGCGTGGTCTGATGGCGAAG
2319	CGGTCGTACTAAAGAAAGCTACAAAGT
2320	GTGGTGTTCATGCTGTTACTCG

pJCore5	
2301	GAGTGGGTTTTACCTTTGTCGGAGTC
2302	CATACGACCAACCGCAGACAAGTC
2303	CGTAGTTGCCTTCTTCGATAGCAG
2304	GATGTCAGCGGTGATCTCTTCC
2305	CACCCTTGTTACCGTGACGACC
2306	CGATTTCAGGAACCAGATGTGCG
2307	GGTATGGACCTACGGTGATTACAG
2308	GCGGTAGCAGGTGTTCAGC
2309	CGTCTTCAGCAGTTACACGACCC
2310	ATCAGAACGTCGTTACCCTGAGCA
2311	GAGCAGATCCTCGACCTGTTCTT
2312	CCAAGCCGATTTCCGCAGCAGT
2313	GTGTCGTTCAGTACGTGGATGCTTCC
2314	CGAAGAAATGCAGCTCAAACAGG
2315	CAGATCCGCCTGTACGATGGTCGC
2316	CGCTGGTTCCGCTGGATGG

2317	CTCAGGACGTTGTACTGGGTCTGTA
2318	GTATGCGTGGTCTGATGGCGAAG
2319	CGGTCGTACTAAAGAAAGCTACAAAGT
2320	GTGGTGTTCATGCTGTTACTCG
2278	atccgctgccgggttttaacccgacagcagtg
2279	tgtcgggttaaaacccggcagcggattgtgc
2280	gtttaactttaataaggagatatacgacgccatggacagcgcc
tufB (in pJR1TufB) done by Kathrin Froschauer	
1693	gaagGCGGCCGCaGGTATTACAGACAGAGCATCTG
1095	GCTACGGCGTTTCACTTCTG

8.2 Sequencing results

The sequences and maps of all plasmids can be found under AGKoraiman(X:):

Sequence of pJCore1:
The plasmid was sequenced from 1566-11891 bp.

```
   1 gtccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggaaagacatgc 60
  61 aaaagcaccactggcagcagccactggtaattgatttagaggagttagtcttgaagtcat 120
 121 gcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagt 180
 181 tacctcggttcaaagagttggtagctcagagaaccttcgaaaaaccgccctgcaaggcgg 240
 241 ttttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatcatc 300
 301 ttattaatcagataaaatatttctagatttcagtgcaatttatctcttcaaatgtagcac 360
 361 ctgaagtcagccccatacgatataagttgtaattctcatgttagtcatgccccgcgccca 420
 421 ccggaaggagctgactgggttgaaggctctcaagggcatcggtcgagatcccggtgccta 480
 481 atgagtgagctaacttacattaattgcgttgcgctcactgcccgctttccagtcgggaaa 540
 541 cctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtat 600
 601 tgggcgccagggtggtttttcttttcaccagtgagacgggcaacagctgattgcccttca 660
 661 ccgcctggccctgagagagttgcagcaagcggtccacgctggtttgccccagcaggcgaa 720
 721 aatcctgtttgatggtggttaacggcgggatataacatgagctgtcttcggtatcgtcgt 780
 781 atcccactaccgagatgtccgcaccaacgcgcagcccggactcggtaatggcgcgcattg 840
 841 cgcccagcgccatctgatcgttggcaaccagcatcgcagtgggaacgatgccctcattca 900
 901 gcatttgcatggtttgttgaaaaccggacatggcactccagtcgccttcccgttccgcta 960
 961 tcggctgaatttgattgcgagtgagatatttatgccagccagccagacgcagacgcgccg 1020
1021 agacagaacttaatgggcccgctaacagcgcgatttgctggtgacccaatgcgaccagat 1080
1081 gctccacgcccagtcgcgtaccgtcttcatgggagaaaataatactgttgatgggtgtct 1140
1141 ggtcagagacatcaagaaataacgccggaacattagtgcaggcagcttccacagcaatgg 1200
1201 catcctggtcatccagcggatagttaatgatcagcccactgacgcgttgcgcgagaagat 1260
1261 tgtgcaccgccgctttacaggcttcgacgccgcttcgttctaccatcgacaccaccacgc 1320
1321 tggcacccagttgatcggcgcgagatttaatcgccgcgacaatttgcgacggcgcgtgca 1380
1381 gggccagactggaggtggcaacgccaatcagcaacgactgtttgcccgccagttgttgtg 1440
1441 ccacgcggttgggaatgtaattcagctccgccatcgccgcttccactttttcccgcgttt 1500
1501 tcgcagaaacgtggctggcctggttcaccacgcgggaaacggtctgataagagacaccgg 1560
1561 catactctgcgacatcgtataacgttactggtttcacattcaccaccctgaattgactct 1620
1621 cttccgggcgctatcatgccataccgcgaaaggttttgcgccattcgatggtgtccggga 1680
1681 tctcgacgctctcccttatgcgactcctgcattaggaaattaatacgactcactataggg 1740
1741 gaattgtgagcggataacaattcccctgtagaaataattttgtttaactttaataaggag 1800
1801 atataccatgggcagcagccatcaccatcatcaccacagcGAGAACCTGTACTTCCAGGG 1860
1861 TTGTGCGCTGGACCGTAGAGAAAGGCCACTTAACAGTCAATCTGTAAATAAATACATCCT 1920
1921 TAACGTTCAGAATATCTACAGAAATTCTCCCGTTCCGGTTTGTGTCCGTAACAAAAACCG 1980
1981 GAAAATCCTTTATGCCAATGGGGCTTTTATTGAACTCTTTTCCAGAGAAGATAAACCCTT 2040
2041 ATCCGGAGAGAGTTATATACGTCTGCAGGTTGAAATTTTTCTTTCATCACTTGAACTGGA 2100
2101 ATGCCAGGCTCTTGGACATGGCTCTGCATTTTGTCGTCGTTTTAATTTTCATGGCGAAAT 2160
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2161 CTATCAGATAAGGATGGAGAATGTTTCTTTTTATAATGACGAATCTGTTGTTTTATGGCA 2220
2221 AATTAATCCGTTTCCTGATTATCCATTTTTTGCGTTAAATCAGAGTGGAAGTAATACAAA 2280
2281 TACTTCTGATAAATTAACGATATGGAATGATCTTTCTCCAGGGACATTGGTTGTTTTCTC 2340
2341 TTTTTATATGCTGGGTGTTGGTCACGCAACAATTGCCAGAGAGTTGGGTATTACAGACAG 2400
2401 AGCATCTGAGGATCGAATTAAACCAGTTAAACGGAAAATAAAAGAATTTTTTGAACACTT 2460
2461 TGATTTATTCAGAGTGTCATGTATCTATAAAGGAGAAATAGATTCGCTATTAAGTATAAT 2520
2521 TCGTGAATTTTATGGTGTTAAGTAATAAAAGCTTGAGCTGAGGAACCCTATGGTTTACTC 2580
2581 CTATACCGAGAAAAAACGTATTCGTAAGGATTTTGGTAAACGTCCACAAGTTCTGGATGT 2640
2641 ACCTTATCTCCTTTCTATCCAGCTTGACTCGTTTCAGAAATTTATCGAGCAAGATCCTGA 2700
2701 AGGGCAGTATGGTCTGGAAGCTGCTTTCCGTTCCGTATTCCCGATTCAGAGCTACAGCGG 2760
2761 TAATTCCGAGCTGCAATACGTCAGCTACCGCCTTGGCGAACCGGTGTTTGACGTCCAGGA 2820
2821 ATGTCAAATCCGTGGCGTGACCTATTCCGCACCGCTGCGCGTTAAACTGCGTCTGGTGAT 2880
2881 CTATGAGCGCGAAGCGCCGGAAGGCACCGTAAAAGACATTAAAGAACAAGAAGTCTACAT 2940
2941 GGGCGAAATTCCGCTCATGACAGACAACGGTACCTTTGTTATCAACGGTACTGAGCGTGT 3000
3001 TATCGTTTCCCAGCTGCACCGTAGTCCGGGCGTCTTCTTTGACTCCGACAAAGGTAAAAC 3060
3061 CCACTCTTCGGGTAAAGTGCTGTATAACGCGCGTATCATCCCTTACCGTGGTTCCTGGCT 3120
3121 GGACTTCGAATTCGATCCGAAGGACAACCTGTTCGTACGTATCGACCGTCGCCGTAAACT 3180
3181 GCCTGCGACCATCATTCTGCGCGCCCTGAACTACACCACAGAGCAGATCCTCGACCTGTT 3240
3241 CTTTGAAAAAGTTATCTTTGAAATCCGTGATAACAAGCTGCAGATGGAACTGGTGCCGGA 3300
3301 ACGCCTGCGTGGTGAAACCGCATCTTTTGACATCGAAGCTAACGGTAAAGTGTACGTAGA 3360
3361 AAAAGGCCGCCGTATCACTGCGCGCCACATTCGCCAGCTGGAAAAAGACGACGTCAAACT 3420
3421 GATCGAAGTCCCGGTTGAGTACATCGCAGGTAAAGTGGTTGCTAAAGACTATATTGATGA 3480
3481 GTCTACCGGCGAGCTGATCTGCGCAGCGAACATGGAGCTGAGCCTGGATCTGCTGGCTAA 3540
3541 GCTGAGCCAGTCTGGTCACAAGCGTATCGAAACGCTGTTCACCAACGATCTGGATCACGG 3600
3601 CCCATATATCTCTGAAACCTTACGTGTCGACCCAACTAACGACCGTCTGAGCGCACTGGT 3660
3661 AGAAATCTACCGCATGATGCGCCCTGGCGAGCCGCCGACTCGTGAAGCAGCTGAAAGCCT 3720
3721 GTTCGAGAACCTGTTCTTCTCCGAAGACCGTTATGACTTGTCTGCGGTTGGTCGTATGAA 3780
3781 GTTCAACCGTTCTCTGCTGCGCGAAGAAATCGAAGGTTCCGGTATCCTGAGCAAAGACGA 3840
3841 CATCATTGATGTTATGAAAAAGCTCATCGATATCCGTAACGGTAAAGGCGAAGTCGATGA 3900
3901 TATCGACCACCTCGGCAACCGTCGTATCCGTTCCGTTGGCGAAATGGCGGAAAACCAGTT 3960
3961 CCGCGTTGGCCTGGTACGTGTAGAGCGTGCGGTGAAAGAGCGTCTGTCTCTGGGCGATCT 4020
4021 GGATACCCTGATGCCACAGGATATGATCAACGCCAAGCCGATTTCCGCAGCAGTGAAAGA 4080
4081 GTTCTTCGGTTCCAGCCAGCTGTCTCAGTTTATGGACCAGAACAACCCGCTGTCTGAGAT 4140
4141 TACGCACAAACGTCGTATCTCCGCACTCGGCCCAGGCGGTCTGACCCGTGAACGTGCAGG 4200
4201 CTTCGAAGTTCGAGACGTACACCCGACTCACTACGGTCGCGTATGTCCAATCGAAACCCC 4260
4261 TGAAGGTCCGAACATCGGTCTGATCAACTCTCTGTCCGTGTACGCACAGACTAACGAATA 4320
4321 CGGCTTCCTTGAGACTCCGTATCGTAAAGTGACCGACGGTGTTGTAACTGACGAAATTCA 4380
4381 CTACCTGTCTGCTATCGAAGAAGGCAACTACGTTATCGCCCAGGCGAACTCCAACTTGGA 4440
4441 TGAAGAAGGCCACTTCGTAGAAGACCTGGTAACTTGCCGTAGCAAAGGCGAATCCAGCTT 4500
4501 GTTCAGCCGCGACCAGGTTGACTACATGGACGTATCCACCCAGCAGGTGGTATCCGTCGG 4560
4561 TGCGTCCCTGATCCCGTTCCTGGAACACGATGACGCCAACCGTGCATTGATGGGTGCGAA 4620
4621 CATGCAACGTCAGGCCGTTCCGACTCTGCGCGCTGATAAGCCGCTGGTTGGTACTGGTAT 4680
4681 GGAACGTGCTGTTGCCGTTGACTCCGGTGTAACTGCGGTAGCTAAACGTGGTGGTGTCGT 4740
4741 TCAGTACGTGGATGCTTCCCGTATCGTTATCAAAGTTAACGAAGACGAGATGTATCCGGG 4800
4801 TGAAGCAGGTATCGACATCTACAACCTGACCAAATACACCCGTTCTAACCAGAACACCTG 4860
4861 TATCAACCAGATGCCGTGTGTGTCTCTGGGTGAACCGGTTGAACGTGGCGACGTGCTGGC 4920
4921 AGACGGTCCGTCCACCGACCTCGGTGAACTGGCGCTTGGTCAGAACATGCGCGTAGCGTT 4980
4981 CATGCCGTGGAATGGTTACAACTTCGAAGACTCCATCCTCGTATCCGAGCGTGTTGTTCA 5040
5041 GGAAGACCGTTTCACCACCATCCACATTCAGGAACTGGCGTGTGTGTCCCGTGACACCAA 5100
5101 GCTGGGTCCGGAAGAGATCACCGCTGACATCCCGAACGTGGGTGAAGCTGCGCTCTCCAA 5160
5161 ACTGGATGAATCCGGTATCGTTTACATTGGTGCGGAAGTGACCGGTGGCGACATTCTGGT 5220
5221 TGGTAAGGTAACGCCGAAAGGTGAAACTCAGCTGACCCCAGAAGAAAAACTGCTGCGTGC 5280
5281 GATCTTCGGTGAGAAAGCCTCTGACGTTAAAGACTCTTCTCTGCGCGTACCAAACGGTGT 5340
5341 ATCCGGTACGGTTATCGACGTTCAGGTCTTTACTCGCGATGGCGTAGAAAAAGACAAACG 5400
5401 TGCGCTGGAAATCGAAGAAATGCAGCTCAAACAGGCGAAGAAAGACCTGTCTGAAGAACT 5460
5461 GCAGATCCTCGAAGCGGGTCTGTTCAGCCGTATCCGTGCTGTGCTGGTAGCCGGTGGCGT 5520
5521 TGAAGCTGAGAAGCTCGACAAACTGCCGCGCGATCGCTGGCTGGAGCTGGGCCTGACAGA 5580
5581 CGAAGAGAAACAAAATCAGCTGGAACAGCTGGCTGAGCAGTATGACGAACTGAAACACGA 5640
5641 GTTCGAGAAGAAACTCGAAGCGAAACGCCGCAAAATCACCCAGGGCGACGATCTGGCACC 5700
5701 GGGCGTGCTGAAGATTGTTAAGGTATATCTGGCGGTTAAACGCCGTATCCAGCCTGGTGA 5760
5761 CAAGATGGCAGGTCGTCACGGTAACAAGGGTGTAATTTCTAAGATCAACCCGATCGAAGA 5820
5821 TATGCCTTACGATGAAAACGGTACGCCGGTAGACATCGTACTGAACCCGCTGGGCGTACC 5880

5881 GTCTCGTATGAACATCGGTCAGATCCTCGAAACCCACCTGGGTATGGCTGCGAAAGGTAT 5940
5941 CGGCGACAAGATCAACGCCATGCTGAAACAGCAGCAAGAAGTCGCGAAACTGCGCGAATT 6000
6001 CATCCAGCGTGCGTACGATCTGGGCGCTGACGTTCGTCAGAAAGTTGACCTGAGTACCTT 6060
6061 CAGCGATGAAGAAGTTATGCGTCTGGCTGAAAACCTGCGCAAAGGTATGCCAATCGCAAC 6120
6121 GCCGGTGTTCGACGGTGCGAAAGAAGCAGAAATTAAAGAGCTGCTGAAACTTGGCGACCT 6180
6181 GCCGACTTCCGGTCAGATCCGCCTGTACGATGGTCGCACTGGTGAACAGTTCGAGCGTCC 6240
6241 GGTAACCGTTGGTTACATGTACATGCTGAAACTGAACCACCTGGTCGACGACAAGATGCA 6300
6301 CGCGCGTTCCACCGGTTCTTACAGCCTGGTTACTCAGCAGCCGCTGGGTGGTAAGGCACA 6360
6361 GTTCGGTGGTCAGCGTTTCGGGGAGATGGAAGTGTGGGCGCTGGAAGCATACGGCGCAGC 6420
6421 ATACACCCTGCAGGAAATGCTCACCGTTAAGTCTGATGACGTGAACGGTCGTACCAAGAT 6480
6481 GTATAAAAACATCGTGGACGGCAACCATCAGATGGAGCCGGGCATGCCAGAATCCTTCAA 6540
6541 CGTATTGTTGAAAGAGATTCGTTCGCTGGGTATCAACATCGAACTGGAAGACGAGTAAAC 6600
6601 TCCGACGGGAGCAAATCCGTGAAAGATTTATTAAAGTTTCTGAAAGCGCAGACTAAAACC 6660
6661 GAAGAGTTTGATGCGATCAAAATTGCTCTGGCTTCGCCAGACATGATCCGTTCATGGTCT 6720
6721 TTCGGTGAAGTTAAAAAGCCGGAAACCATCAACTACCGTACGTTCAAACCAGAACGTGAC 6780
6781 GGCCTTTTCTGCGCCCGTATCTTTGGGCCGGTAAAAGATTACGAGTGCCTGTGCGGTAAG 6840
6841 TACAAGCGCCTGAAACACCGTGGCGTCATCTGTGAGAAGTGCGGCGTTGAAGTGACCCAG 6900
6901 ACTAAAGTACGCCGTGAGCGTATGGGCCACATCGAACTGGCTTCCCCGACTGCGCACATC 6960
6961 TGGTTCCTGAAATCGCTGCCGTCCCGTATCGGTCTGCTGCTCGATATGCCGCTGCGCGAT 7020
7021 ATCGAACGCGTACTGTACTTTGAATCCTATGTGGTTATCGAAGGCGGTATGACCAACCTG 7080
7081 GAACGTCAGCAGATCCTGACTGAAGAGCAGTATCTGGACGCGCTGGAAGAGTTCGGTGAC 7140
7141 GAATTCGACGCGAAGATGGGGGCGGAAGCAATCCAGGCTCTGCTGAAGAGCATGGATCTG 7200
7201 GAGCAAGAGTGCGAACAGCTGCGTGAAGAGCTGAACGAAACCAACTCCGAAACCAAGCGT 7260
7261 AAAAAGCTGACCAAGCGTATCAAACTGCTGGAAGCGTTCGTTCAGTCTGGTAACAAACCA 7320
7321 GAGTGGATGATCCTGACCGTTCTGCCGGTACTGCCGCCAGATCTGCGTCCGCTGGTTCCG 7380
7381 CTGGATGGTGGTCGTTTCGCGACTTCTGACCTGAACGATCTGTATCGTCGCGTCATTAAC 7440
7441 CGTAACAACCGTCTGAAACGTCTGCTGGATCTGGCTGCGCCGGACATCATCGTACGTAAC 7500
7501 GAAAAACGTATGCTGCAGGAAGCGGTAGACGCCCTGCTGGATAACGGTCGTCGCGGTCGT 7560
7561 GCGATCACCGGTTCTAACAAGCGTCCTCTGAAATCTTTGGCCGACATGATCAAAGGTAAA 7620
7621 CAGGGTCGTTTCCGTCAGAACCTGCTCGGTAAGCGTGTTGACTACTCCGGTCGTTCTGTA 7680
7681 ATCACCGTAGGTCCATACCTGCGTCTGCATCAGTGCGGTCTGCCGAAGAAAATGGCACTG 7740
7741 GAGCTGTTCAAACCGTTCATCTACGGCAAGCTGGAACTGCGTGGTCTTGCTACCACCATT 7800
7801 AAAGCTGCGAAGAAAATGGTTGAGCGCGAAGAAGCTGTCGTTTGGGATATCCTGGACGAA 7860
7861 GTTATCCGCGAACACCCGGTACTGCTGAACCGTGCACCGACTCTGCACCGTCTGGGTATC 7920
7921 CAGGCATTTGAACCGGTACTGATCGAAGGTAAAGCTATCCAGCTGCACCCGCTGGTTTGT 7980
7981 GCGGCATATAACGCCGACTTCGATGGTGACCAGATGGCTGTTCACGTACCGCTGACGCTG 8040
8041 GAAGCCCAGCTGGAAGCGCGTGCGCTGATGATGTCTACCAACAACATCCTGTCCCCGGCG 8100
8101 AACGGCGAACCAATCATCGTTCCGTCTCAGGACGTTGTACTGGGTCTGTACTACATGACC 8160
8161 CGTGACTGTGTTAACGCCAAAGGCGAAGGCATGGTGCTGACTGGCCCGAAAGAAGCAGAA 8220
8221 CGTCTGTATCGCTCTGGTCTGGCTTCTCTGCATGCGCGCGTTAAAGTGCGTATCACCGAG 8280
8281 TATGAAAAAGATGCTAACGGTGAATTAGTAGCGAAAACCAGCCTGAAAGACACGACTGTT 8340
8341 GGCCGTGCCATTCTGTGGATGATTGTACCGAAAGGTCTGCCTTACTCCATCGTCAACCAG 8400
8401 GCGCTGGGTAAAAAAGCAATCTCCAAAATGCTGAACACCTGCTACCGCATTCTCGGTCTG 8460
8461 AAACCGACCGTTATTTTTGCGGACCAGATCATGTACACCGGCTTCGCCTATGCAGCGCGT 8520
8521 TCTGGTGCATCTGTTGGTATCGATGACATGGTCATCCCGGAGAAGAAACACGAAATCATC 8580
8581 TCCGAGGCAGAAGCAGAAGTTGCTGAAATTCAGGAGCAGTTCCAGTCTGGTCTGGTAACT 8640
8641 GCGGGCGAACGCTACAACAAAGTTATCGATATCTGGGCTGCGGCGAACGATCGTGTATCC 8700
8701 AAAGCGATGATGGATAACCTGCAAACTGAAACCGTGATTAACCGTGACGGTCAGGAAGAG 8760
8761 AAGCAGGTTTCCTTCAACAGCATCTACATGATGGCCGACTCCGGTGCGCGTGGTTCTGCG 8820
8821 GCACAGATTCGTCAGCTTGCTGGTATGCGTGGTCTGATGGCGAAGCCGGATGGCTCCATC 8880
8881 ATCGAAACGCCAATCACCGCGAACTTCCGTGAAGGTCTGAACGTACTCCAGTACTTCATC 8940
8941 TCCACCCACGGTGCTCGTAAAGGTCTGGCGGATACCGCACTGAAAACTGCGAACTCCGGT 9000
9001 TACCTGACTCGTCGTCTGGTTGACGTGGCGCAGGACCTGGTGGTTACCGAAGACGATTGT 9060
9061 GGTACCCATGAAGGTATCATGATGACTCCGGTTATCGAGGGTGGTGACGTTAAAGAGCCG 9120
9121 CTGCGCGATCGCGTACTGGGTCGTGTAACTGCTGAAGACGTTCTGAAGCCGGGTACTGCT 9180
9181 GATATCCTCGTTCCGCGCAACACGCTGCTGCACGAACAGTGGTGTGACCTGCTGGAAGAG 9240
9241 AACTCTGTCGACGCGGTTAAAGTACGTTCTGTTGTATCTTGTGACACCGACTTTGGTGTA 9300
9301 TGTGCGCACTGCTACGGTCGTGACCTGGCGCGTGGCCACATCATCAACAAGGGTGAAGCA 9360
9361 ATCGGTGTTATCGCGGCACAGTCCATCGGTGAACCGGGTACACAGCTGACCATGCGTACG 9420
9421 TTCCACATCGGTGGTGCGGCATCTCGTGCGGCTGCTGAATCCAGCATCCAAGTGAAAAAC 9480
9481 AAAGGTAGCATCAAGCTCAGCAACGTGAAGTCGGTTGTGAACTCCAGCGGTAAACTGGTT 9540
9541 ATCACTTCCCGTAATACTGAACTGAAACTGATCGACGAATTCGGTCGTACTAAAGAAAGC 9600

9601 TACAAAGTACCTTACGGTGCGGTACTGGCGAAAGGCGATGGCGAACAGGTTGCTGGCGGC 9660
9661 GAAACCGTTGCAAACTGGGACCCGCACACCATGCCGGTTATCACCGAAGTAAGCGGTTTT 9720
9721 GTACGCTTTACTGACATGATCGACGGCCAGACCATTACGCGTCAGACCGACGAACTGACC 9780
9781 GGTCTGTCTTCGCTGGTGGTTCTGGATTCCGCAGAACGTACCGCAGGTGGTAAAGATCTG 9840
9841 CGTCCGGCACTGAAAATCGTTGATGCTCAGGGTAACGACGTTCTGATCCCAGGTACCGAT 9900
9901 ATGCCAGCGCAGTACTTCCTGCCGGGTAAAGCGATTGTTCAGCTGGAAGATGGCGTACAG 9960
9961 ATCAGCTCTGGTGACACCCTGGCGCGTATTCCGCAGGAATCCGGCGGTACCAAGGACATC 10020
10021 ACCGGTGGTCTGCCGCGCGTTGCGGACCTGTTCGAAGCACGTCGTCCGAAAGAGCCGGCA 10080
10081 ATCCTGGCTGAAATCAGCGGTATCGTTTCCTTCGGTAAAGAAACCAAAGGTAAACGTCGT 10140
10141 CTGGTTATCACCCCGGTAGACGGTAGCGATCCGTACGAAGAGATGATTCCGAAATGGCGT 10200
10201 CAGCTCAACGTGTTCGAAGGTGAACGTGTAGAACGTGGTGACGTAATTTCCGACGGTCCG 10260
10261 GAAGCGCCGCACGACATTCTGCGTCTGCGTGGTGTTCATGCTGTTACTCGTTACATCGTT 10320
10321 AACGAAGTACAGGACGTATACCGTCTGCAGGGCGTTAAGATTAACGATAAACACATCGAA 10380
10381 GTTATCGTTCGTCAGATGCTGCGTAAAGCTACCATCGTTAACGCGGGTAGCTCCGACTTC 10440
10441 CTGGAAGGCGAACAGGTTGAATACTCTCGCGTCAAGATCGCAAACCGCGAACTGGAAGCG 10500
10501 AACGGCAAAGTGGGTGCAACTTACTCCCGCGATCTGCTGGGTATCACCAAAGCGTCTCTG 10560
10561 GCAACCGAGTCCTTCATCTCCGCGGCATCGTTCCAGGAGACCACTCGCGTGCTGACCGAA 10620
10621 GCAGCCGTTGCGGGCAAACGCGACGAACTGCGCGGCCTGAAAGAGAACGTTATCGTGGGT 10680
10681 CGTCTGATCCCGGCAGGTACCGGTTACGCGTACCACCAGGATCGTATGCGTCGCCGTGCT 10740
10741 GCGGGTGAAGCTCCGGCTGCACCGCAGGTGACTGCAGAAGACGCATCTGCCAGCCTGGCA 10800
10801 GAACTGCTGAACGCAGGTCTGGGCGGTTCTGATAACGAGTAAACCTGTGGAGCTTTTTAA 10860
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10921 TACTGGTCGCCGCGCGTCGCGCTCGTCAGATGCAGGTAGGCGGAAAGGATCCGCTGGTAC 10980
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Sequence of pJCore2:

The plasmid was sequenced from 1575-11904 bp.

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Sequence of pJCore5

The plasmid was sequenced from 1623-12004 bp.

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12661 gagaaaaaaggctgcaccggtgcgtcagcagaatatgtgatacaggatatattccgcttc 12720
12721 ctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatggcttacga 12780
12781 acggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggcc 12840
12841 gcggcaaagccgtttttccataggctccgcccccctgacaagcatcacgaaatctgacgc 12900
12901 tcaaatcagtggtggcgaaacccgacaggactataaagataccaggcgtttccccctggcg 12960
12961 gctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttat 13020
13021 ggccgcgtttgtctcattccacgcctgacactcagttccgggtaggcagttcgctccaag 13080
13081 ctggactgtatgcacgaacccc 13102

Sequence of pET28tufB:
The plasmid was sequenced from 5071-6255 bp

1 TGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCG 60
61 CAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTC 120
121 CTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGG 180
181 GTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTC 240
241 ACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTT 300
301 CTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTC 360
361 TTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTA 420
421 ACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTCAGGTGGCACTTT 480
481 TCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA 540
541 TCCGCTCATGAATTAATTCTTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTAT 600
601 TCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAA 660
661 ACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTC 720
721 GTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGA 780
781 AATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCC 840
841 AGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAAC 900
901 CGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGAC 960
961 AATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATAT 1020
1021 TTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAG 1080
1081 TGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCA 1140
1141 TAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTAC 1200
1201 CTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTG 1260
1261 TCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCA 1320
1321 TGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACAC 1380
1381 CCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGACCAAAATCCCTTAA 1440
1441 CGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGA 1500
1501 GATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG 1560
1561 GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGC 1620
1621 AGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAG 1680
1681 AACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC 1740
1741 AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCG 1800
1801 CAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC 1860
1861 ACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGA 1920
1921 AAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTT 1980
1981 CCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAG 2040
2041 CGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCG 2100
2101 GCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA 2160
2161 TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGC 2220
2221 AGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGATGCGG 2280
2281 TATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATATGGTGCACTCTCAGTA 2340
2341 CAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTG 2400
2401 GGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCT 2460
2461 GCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAG 2520
2521 GTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTC 2580
2581 GTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAG 2640
2641 AAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTT 2700
2701 GGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAA 2760

2761 ACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACG 2820
2821 TTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGG 2880
2881 TCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCC 2940
2941 TGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTA 3000
3001 CGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCA 3060
3061 GCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACC 3120
3121 CCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGGGCCGC 3180
3181 CATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAA 3240
3241 GGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGC 3300
3301 GCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTAC 3360
3361 GAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCA 3420
3421 CCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTA 3480
3481 ATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAA 3540
3541 CCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTAT 3600
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3661 CCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAA 3720
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3781 ATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCATTG 3840
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4081 GCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTGTCT 4140
4141 GGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCTTCCACAGCAATGG 4200
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4381 GGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTG 4440
4441 CCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACTTTTTCCCGCGTTT 4500
4501 TCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGAAACGGTCTGATAAGAGACACCGG 4560
4561 CATACTCTGCGACATCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCT 4620
4621 CTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGA 4680
4681 TCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAGCAGCCCAGTAGTAGGTTGAGG 4740
4741 CCGTTGAGCACCGCCGCCGCAAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCC 4800
4801 CCGGCCACGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGG 4860
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4981 AATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAA 5040
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5101 AACCGCACGTTAACGTCGGTACTATCGGCCACGTTGACCATGGTAAAACAACGCTGACCG 5160
5161 CTGCAATCACTACCGTACTGGCTAAAACCTACGGCGGTGCTGCTCGCGCATTCGACCAGA 5220
5221 TCGATAACGCGCCGGAAGAAAAAGCTCGTGGTATCACCATCAACACTTCTCACGTTGAAT 5280
5281 ACGACACCCCGACCCGTCACTACGCACACGTAGACTGCCCGGGGCACGCCGACTATGTTA 5340
5341 AAAACATGATCACCGGTGCTGCGCAGATGGACGGCGCGATCCTGGTAGTTGCTGCGACTG 5400
5401 ACGGCCCGATGCCGCAGACTCGTGAGCACATCCTGCTGGGTCGTCAGGTAGGCGTTCCGT 5460
5461 ACATCATCGTGTTCCTGAACAAATGCGACATGGTTGATGACGAAGAGCTGCTGGAACTGG 5520
5521 TTGAAATGGAAGTTCGTGAACTTCTGTCTCAGTACGACTTCCCGGGCGACGACACTCCGA 5580
5581 TCGTTCGTGGTTCTGCTCTGAAAGCGCTGGAAGGCGACGCAGAGTGGGAAGCGAAAATCC 5640
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5881 GTGCTGGTGAGAACGTAGGTGTTCTGCTGCGTGGTATCAAACGTGAAGAAATCGAACGTG 5940
5941 GTCAGGTACTGGCTAAGCCGGGCACCATCAAGCCGCACACCAAGTTCGAATCTGAAGTGT 6000
6001 ACATTCTGTCCAAAGATGAAGGCGGCCGTCATACTCCGTTCTTCAAAGGCTACCGTCCGC 6060
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6181 ACGACGGTCTGCGTTTCGCAATCCGTGAAGGCGGCCGTACCGTTGGCGCGGGCGTTGTAG 6240
6241 CAAAAGTTCTGAGCcatcaccaccatcatcaCTAAGAGATCCGGCTGCTAACAAAGCCCG 6300
6301 AAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGC 6360
6361 CTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT 6413