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Strategies to control plasmid copy number

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Abstract

Gene therapy holds its promises to silence dysfunctional genes on either transcriptional or translational level or by replacement with functional genes. Not long ago it has revolutionized modern medicine and since then thousand clinical trials have been authorized worldwide.

To put the medical profit of gene therapy and DNA vaccination into practice, methods to produce highly pure plasmid DNA (pDNA) free from bacterial chromosomal DNA, RNA, proteins and endotoxins have to be developed. Since it is known that plasmid copy number (PCN) and plasmid stability are affecting the success of gene therapy to a large extent, the rational design of an optimized vector is an ultimate ambition. To prevent excessive plasmid replication, posing metabolic burden resulting in growth inhibition right up to cell death to the workhorse *Escherichia coli* (*E. coli*), regulation of PCN is to be considered in the rational design of any vector as well.

In this work the main goal was to elaborate a system that regulates plasmid replication of *Col*E1-type plasmids in *Escherichia Coli (E. coli)* by overexpressing $tRNA^{AlaU}$ and *rssB* encoding genes. In the first approach the wt and mutated $tRNA^{AlaU}$ gene, whose expression was controlled by the T7 promoter, was inserted into the *E. coli* chromosome. In shake flask experiments overexpression of the $tRNA^{AlaU}$ gene was induced by addition of IPTG and subsequently its effect on three different *Col*E1-type plasmids has been tested. In the second approach, overexpression of *rssB*, whose expression was also controlled by the T7 promoter, should result in enhanced plasmid concentration after IPTG induction whereas the biological mechanism remains unclear. In shake flask experiments, *rssB* overexpression was also induced by adding IPTG.

All in all, the experimentally gained data have demonstrated clearly that overexpression of *rssB* and $tRNA^{AlaU}$ encoding genes resulted in enhanced plasmid concentration of *Col*E1-type plasmids.

Zusammenfassung

Die Gentherapie erscheint viel versprechend, fehlerhafte Gene auf transkriptioneller oder translationeller Ebene stillzulegen oder diese durch Rekombination mit funktionellen Genen zu korrigieren. Vor nicht allzu langer Zeit revolutionierte die Gentherapie die moderne Medizin und mehr als tausend vorgeschlagene oder laufende klinische Versuche wurden weltweit genehmigt.

Um das Potential der medizinischen Gentherapie und DNA-Impfung in die Praxis umzusetzen, muss die Herstellung hochreiner Plasmid-DNA (pDNA) sichergestellt werden, die frei von jedweder bakteriell chromosomaler DNA, RNA, Proteine und Endotoxine sein muss. Es ist bekannt, dass die Plasmidkopienzahl und Plasmid-Stabilität wesentliche Determinanten sind, die die Gendosis in der Wirtszelle beeinflussen. Daher ist das rationale Design eines pDNA Vektors ein großes Ziel im Rahmen der Gentherapie. Weiters ist bekannt, dass die Regulierung der PCN entscheidend ist, um eine metabolische Belastung der Wirtszelle zu verhindern, da sich eine übermäßige Plasmidreplikation wachstumshemmend auswirkt bis hin zum Zelltod führen kann.

In dieser Arbeit sollte die Konzentration von Plasmiden des *Col*E1-Typs durch Überexpression des Gens *rssB* sowie $tRNA^{AlaU}$ in *Escherichia Col*i (*E. coli*) reguliert werden, die sich in der Literatur als erfolgreich erwiesen haben.

Im ersten Ansatz wurde das für die $tRNA^{AlaU}$ codierende Gen, das unter die Kontrolle des T7 Promoters gebracht wurde, in das *E. coli* Genom inseriert. In Schüttelkolbenexperimenten wurde die Überexpression des $tRNA^{AlaU}$-Gens durch Zugabe von IPTG induziert und anschließend wurde dessen Auswirkung auf drei verschiedene Plasmide des *Col*E1-Typs getestet.

In einem zweiten Ansatz sollte die Überexpression des Gens *rssB*, das ebenso unter der Kontrolle des T7 Promotors stand, zu einer Erhöhung der Plasmidkonzentration nach IPTG-Zugabe führen, wobei der biologische Mechanismus bisher noch nicht vollständig geklärt ist. In Schüttelkolbenexperimenten wurde eine *rssB*-Überexpression durch Zugabe von IPTG induziert.

Insgesamt konnte in dieser Arbeit gezeigt werden, dass eine Überexpression von *rssB* und $tRNA^{AlaU}$ zu einer sichtbaren Erhöhung der Plasmidkonzentration des *Col*E1-Typs geführt hat.

1 Introduction

1.1 Bacterial Plasmids

According to a present definition plasmids are no life form similar to viruses. Plasmids can be accounted for up to 50 % of bacterial DNA and they vary in their size: usually plasmids are small (up to 100 kb) but sometimes a precise distinction between plasmid and bacterial chromosome is hardly possible (e.g. *Vibrio cholerae* and *Rhizobium meliloti*).

Plasmids are extrachromosomal, circular and double-stranded (ds) DNA molecules. These so-called episomes are represented by a specific copy number within a convenient host as well as by its autonomous and self-controlled replication and have been observed in Archaea, Bacteria and Eukarya. Many plasmids contain adequate features making them suitable for a wide host range. Furthermore, plasmid-encoded genes are not essential but grant various advantages for the host such as antibiotic resistance, degradation/conversion of specific substances, pathogenicity and production of virulence factors (12). Plasmids can bring along genes by mechanisms like transposition or recombination contributing to genetic variety in bacterial population and new gene functions. Some plasmids can even break down genetic barriers of different species (26). So, plasmids can be regarded as a reservoir of extrachromosomal DNA which can be interchanged between bacterial populations but despite of various profits, plasmids are still a metabolic burden for the host.

1.1.1 Replication of *Col*E1 plasmids

In molecular biology derivatives of the *Col*E1-type plasmids pBR322, originated from the natural isolate pMB1, are frequently used tools for cloning purposes in *E. coli* (Balbas et *al.*, 2004). According to Cesareni et *al.*, the interaction between Repressor of primer (Rop) and RNAI and/or RNAII is determining the replication of ColE1-type plasmids (17). At least since the turn of the millennium, RNA has highlighted its biologically significance and various regulatory roles in different cellular processes including transcription, translation, RNA processing, and regulation of DNA

replication. Presumptively, plasmid copy number and incompatibility are depending on RNAI/RNAII interactions.

According to Eguchi et *al.*, replication of *Col*E1-type replicons is a complex regulated process which starts 555 bp upstream of the ORI with the expression of plasmid-encoded RNAII species. RNAII persists in *cis* and form a stable duplex with its template DNA only when RNAII fold into a unique tertiary structure. In the next step, a free 3'-OH end of the RNA is generated by RNase H-mediated cleavage of the DNA –RNA hybrid leading to attachment and leading strand synthesis catalyzed by DNA polymerase I (DNAPI) (30).

Negative regulators of plasmid replication are RNAI and Rop/Rom. The plasmid-encoded RNAI is a 108-nt *Col*E1-encoded antisense RNA that is, in contrast to RNAII, active in trans. RNAI is exactly complementary to the 5' part of RNAII and by hybridization to it (known as "kissing structure") it prevents the formation of the RNAII-DNA hybrid (fig.1) (18). Subsequently, after RNAI hybridized with RNAII it will lead to conformational changes in the 5'-end of RNAII and end until the 3'-end of RNAII is reached. As a consequence, RNAII can not act as a primer (25)

The fast and reversible hybridization between these two RNA species has been studied for a long time but if the formation of the kissing complex alone can prevent primer formation or if a complete hybridization between the two RNA species is sufficient is not clear until now.

According to Want et *al.*, RNAI decay or cleavage itself is regulated by four factors: (1) RNaseE impairs endonucleolytic or exonucleolytic activity leading to RNAI decay; (2) polynucleotide phosphorylase act as an exonuclease involved in RNAI decay; (3) RNaseIII participated in RNAI degradation; and (4) poly(A) polymerase I is regulating *Col*E1-like PCN and RNAI degradation (8).

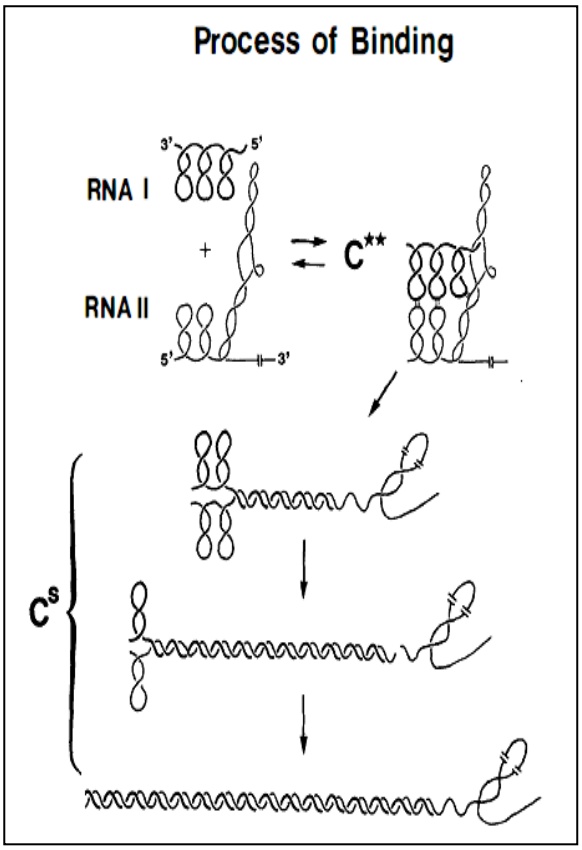


Fig.1: Binding of RNAI to RNAII is a multi-step process involving interaction at their loops. The hybridization process is initiated at the 5'-end of RNAI and comes to a standstill when both, RNAI and RNAII are completely hybridized (30).

Expression of RNAI and RNAII is controlled by a constitutive promoter so that their intracellular concentration correlates always with the plasmid concentration. However, up to now experiments could not make clear the absolute rates of RNAI/RNAII. Lin-Chao et *al.* have measured that RNAI and RNAII molecules are expressed in a ratio of 3 to 1 whereas Polaczek et *al.* suggested that synthesis occur in a ratio of about 5.5 to 1 (41).

The Rop protein is also a negative regulator involved in PCN control. As shown in fig.2, Rop is a small dimeric protein, whose monomers show two tightly packed α-helices connected by a sharp bend (Banner et *al.*, 1987). Helmer-Citterich et *al.* suggested that the Rop protein is facilitating loop-loop interactions between both RNA species and, thus, is directly regulating replication frequency and PCN. They could also show experimentally that Rop binds specifically to RNAI and RNAII stem sequences (and therefore prevents RNAI and RNAII from RNase-mediated

digestion) but they failed to identify the nucleotides that are involved in the interaction. However, they have no proof that the ratio of Rop, RNAI and RNAII is 1:1:1 ratio but several models propose that a number of Rop proteins are to be found in the complex. It is also proposed that the Rop protein is dissociating subsequently after formation of the complex (17).

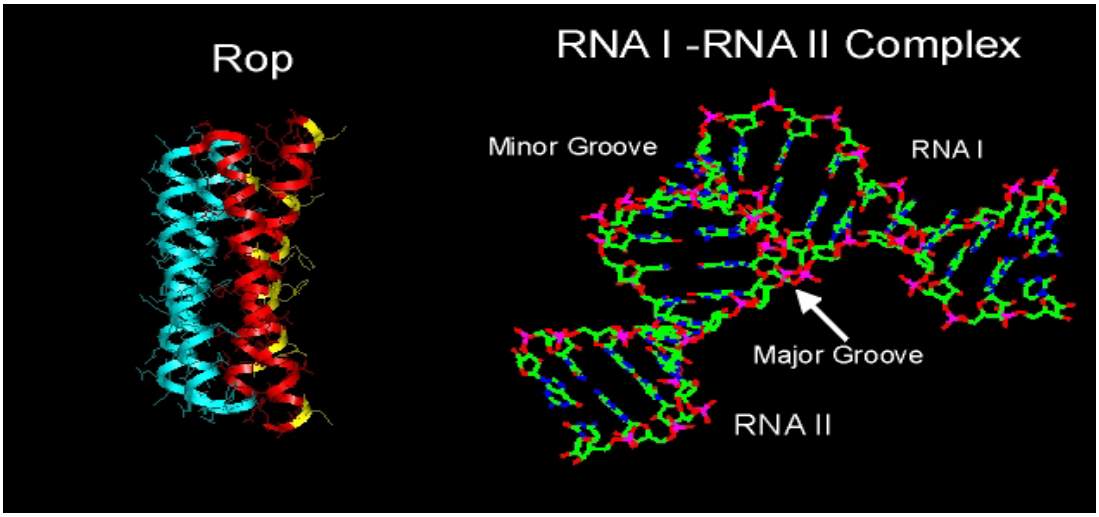


Fig.2: According to structural analysis of the Rop protein, it consists of two helices (helix I in red and helix II in blue). Amino acid residues, involved in RNA binding, are shown in yellow. (B) Loop-loop interaction of RNAI-RNAII leads to formation of minor and major groove (adapted from www.marinolab.umbi.umd.edu).

In vivo and *in vitro* studies have demonstrated that Rop is not acting as a repressor as initially thought rather it enhances RNAI inhibitory activity (Cesareni et *al.*, 1984; Lacatena et *al.*, 1984).

In 1984, Tomizawa and Som could show that Rop increases and accelerate RNAI and RNAII hybridization. Therefore they renamed Rop to Rom ("RNA One Modulator") due to its newly observed function. Though, Rop/Rom is not the only factor involved in regulation of the RNAI-RNAII hybridization. According to current findings the initiator protein of the *Col*E1-unrelated plasmid R6K and the avian retroviral NBP.P12 protein can regulate the hybridization of the two RNA species as well (16). pBR322 and its derivatives applied in the molecular biology field contain the *Col*E1-type origin but lack the Rop encoding gene frequently. *Col*E1-type plasmids and its derivatives like pBR322 are low-copy plasmids (about 15 per cell). Deleting the rop encoding gene, PCN is increased 3-4 times (25).

1.1.2 Inheritance of bacterial plasmids

Resolution of plasmid oligo- or multimers into monomers, regulated plasmid partitioning and killing of plasmid-free descendents are evolved mechanisms essential for stable maintenance of bacterial plasmids (12).

1.1.3 PCN control

Any plasmid has a specific copy number in different bacterial species. The initiation of replication, and thus PCN, is regulated by plasmid-encoded elements. To stably coexist with its host, guarantee stability, maintenance and to reduce the metabolic burden to a minimum extend, plasmids must develop subtle mechanisms to control their replication (Del Solar et *al.*, 2000). To maintain and "sense" the copy number, plasmids implement negative regulatory mechanisms. To be established in the new host, the concentration of the negative regulators is insignificant in this context. This seems to be essential since uncontrolled plasmid replication favours normal copy number immediately. After achieving a specific copy number, it has to be maintained or adjusted to fluctuations by increasing or decreasing the frequency of replication per plasmid copy. Thereby, randomly chosen plasmids are selected for replication. However, plasmids with the same replicons can not be maintained in the same host without selective pressure (Del Solar et *al.*, 1998). Mechanisms such as hemimethylation and supercoiling facilitate identification of newly synthesized plasmids (21, 26).

In response to fluctuations, *E. coli* evolved different mechanisms to control and maintain the PCN: (i) directly repeated sequences (iterons); (ii) binding of antisense RNA to essential RNAs (termed countertranscribed RNAs or ctRNAs); and (iii) antisense RNA in combination with a protein, which can play either an auxiliary or a regulatory role. Especially in Gram-positive bacteria and for few rolling circle-replicating plasmids and theta-replicating plasmids the third type of mechanism prevails.

PCN in R1- and *Col*E1-type plasmids is mainly maintained and regulated by ctRNAs and negatively regulating proteins such as Rom/Rop (21). *Col*E1-type plasmids and its derivatives are popular tools for cloning and transgene expression

since they are small in size and for replication they utilize host-encoded enzymes (19).

1.1.4 Plasmid segregation in *E. coli*

According to Gordon et *al.*, replication of the single *E. coli* chromosome starts at a unique ORI and proceeds bidirectionally until the termination site is reached. In *E. coli* and in *Bacillus subtilis* this process is finished after approximately 40 minutes at 37°C and both, the old and newly synthesized chromosomes are partitioned between the mother and daughter cell. Although the partitioning systems works highly accurate, chromosomeless cells occur with a frequency of less than 10^{-4}. However, it remains unclear if chromosomes are partitioned by either active or passive mechanisms. In bacterial cells there is no proof for the existence of the mitotic apparatus, microtubules or microtubule-like structures as it is well known for eukaryotic cells. To test chromosome and low-copy plasmid segregation in *E. coli*, Gordon et *al.* engineered chromosomes containing tandem lac operator sequences close to the ORI as well as plasmids P1 and F.

Due to the expressed green fluorescent protein (GFP)-LacI fusion protein the partitioning process of chromosomes and plasmids could be followed by fluorescent microscopy. According to their gained results both, chromosomes and plasmids differ in their partitioning behaviour since they are differently localized within the cell. In contrast to the chromosomal oriC region, which stays in touch with the cell pole throughout the entire “cell cycle”, the plasmids P1 and F associate quickly with the site of septum after their replication. After chromosomal duplication, one chromosome continues its association with the old pole and the other is transported to the new cell pole (20).

1.2 Gene therapy

Since genetics proceeded in the 1980s, gene therapy opened a new avenue to treat genetic defects. The identification of genetic abnormalities that effect inherited diseases make the progress of gene therapy possible. Since clinical trials indicated that genetically inherited diseases (such as intestinal cancer, heart disease, diabetes etc.) are passed on to next generation family members gene therapy seems a

promising tool for its treatment. However, not only genetic defects but also environmental factors may affect many diseases and health conditions but it is safe to say that genetic defects make living organisms even more proned to it and that most diseases have a genetic cause.

In contrast to DNA vaccines, whose intentions are to stimulate the immune system of an individual within a short time, gene therapy is usually targeted at persisting expression of the therapeutic gene avoiding stimulation of the immune system. Sometimes, gene therapy is directed at incorporation of the therapeutic gene into the host's chromosome. Unlike DNA vaccines, the therapeutic DNA must be internalized by the target cells and guarantee it’s finely tuned and long-lasting activity when gene therapy is applied. For that reasons gene therapy has not yet achieved success as DNA vaccines did. However, application of gene therapy is focussing on different purposes: (i) the therapeutic gene is encoding proteins to exert new functions in the patient’s cell or to restore the function of mutated proteins; (ii) the therapeutic gene aims to knock down or off the expression of a default gene (38).

For now, both DNA-based (*in vivo*) treatments and cell-based (*ex vivo*) treatments are objects of intense research in the United States. The transfer of the therapeutic gene to the host’s cells can be conducted by applying vectors including viruses or pDNA (“DNA-based gene therapy”). Cell-based gene therapy is characterized by taking patient’s cell from the body to restore dysfunctional genes *in vitro* and to return the restored genes to the patient’s body. Clinicial trials for the treatment of cystic fibrosis (using adenoviral vector), HIV (Human immunodeficiency virus) infection (cell-based), malignant melanoma (cell-based), kidney cancer (cell-based), Gaucher's Disease (retroviral vector), breast cancer (retroviral vector), and lung cancer (retroviral vector) has been conducted until now (14).

At the beginning of gene therapy scientists brought in genes directly into human cells to repair single-gene defects causing cystic fibrosis, haemophilia, muscular dystrophy and sickle cell anemia. This approach has turned out to be more difficult as initially thought since the therapeutic gene prefers recombining randomly in the host genome instead of replacing the dysfunctional gene (36).

1.2.1 Classification of gene therapy

Gene therapy may be classified into two types:

Germ line gene therapy

Reproductive cells (sperm or eggs) are genetically modified to correct inherited genetic defects and the manipulated genome would be transmitted on to the next generations. However, germline therapy would have a lasting effect on the gene pool of the human species. Current laws have put a ban on this kind of application in human beings for ethical and technical reasons.

Somatic gene therapy

Somatic gene therapy targets at modifying the genome of the somatic cell and is restricted only to the person treated with gene therapy. The genetic modification will not be transmitted to the next generations.

1.2.2 Vectors used in gene therapy

1.2.2.1 Viruses

Viral vectors

Viruses attach and transfer their genetic material into their host cells. To ensure replication of viral DNA they hijack the host's cellular machinery. Consequently, the host cell will produce many copies of the virus leading to increasing infection of uninfected cells. To become a permanent component of the host's chromosome some specific viral types evolved mechanisms to insert their genetic material.

Retroviruses

Retroviruses introduce their ribonucleic acid (RNA) including the reverse transcriptase, integrase and other enzymes into the cell. Subsequently, the RNA molecule is reverse transcribed to DNA (known as copy-DNA or cDNA) promoted by the reverse transcriptase. The viral cDNA must enter the nucleus of the host cell by using specific mechanisms to be chromosomally integrated catalyzed by the integrase. Division of the host cell will pass this recombinant genome on to its descendant cells. It can be assumed that insertion of viral genes can occur randomly into the host's genome leading to insertional mutagenesis or even to uncontrolled cell

division. Strategies such as the design of zinc finger nucleases and insertion of specific sequences such as the beta-globin locus control region to facilitate site-specific integration should circumvent this problem. Sometimes gene expression of the retrovirus takes place at a later time.

In the year 1999, 11 boys with X-linked severe combined immunodeficiency (XSID), also known as “bubble-baby syndrome”, were treated with their own bone marrow, which previously was transduced with retroviral vectors to restore the deficient Adenosine Deaminase (ADA) allele (fig.3). XSID is known to be a X-chromosomal linked disease characterized by a rare and disastrous immune disorder. For years, this gene therapy represented a story of success, the immune system was restored but in 2002 2 of the 11 patients came down with a leukemia-like disease. This is probably due to insertional mutagenesis close to the oncogene *LMO*-2 which is known to provoke several human T cell acute lymphoblastic leukemias (T-ALL). Authorities prohibited temporarily this clinical trial together with similar gene transfer trials but the hypothetical risk of random integration into potentially harmful sites has been estimated to be low (Kay et *al.*, 2001). In 2002, after protocol review, clinical trials were continued in the United States, the United Kingdom, France, Italy, and Germany. According to significant hints development of the leukemia-like diseases may have been disease- and protocol-specific.

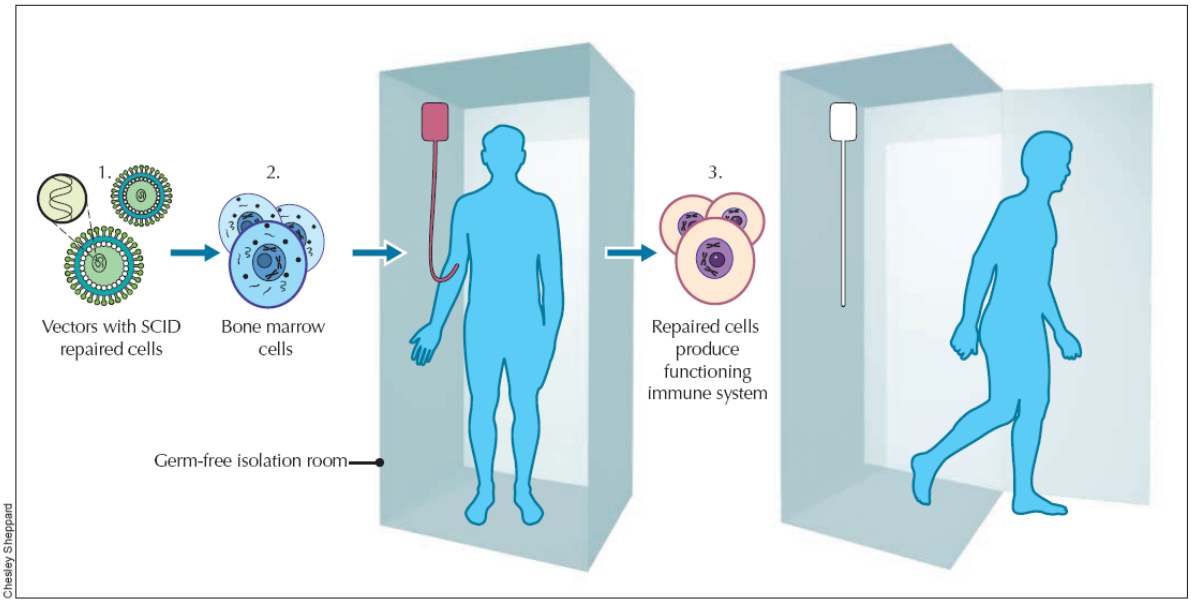


Fig.3: Procedure of cell-based gene therapy. (1) Vectors, mostly crippled viruses, are used as vehicles to transfect patient's cells with the therapeutic gene. (2) Isolated bone marrow cells from the patient are transfected *ex vivo* with the virus. After viral transfection the patient receive transplants of his own bone marrow. (3) Repaired cells restore the patient's immune system (5).

Adenoviruses

Adenoviruses contain a ds DNA. Usually, they cause respiratory (such as common cold), intestinal, and eye infections in humans. The DNA of the adenovirus is neither inserted into the host cell's genome nor is replicated when the replication of the eukaryotic genome is induced, thus leading to transient gene expression. Therefore, successful treatment needs readministration. In China, Gendicine, an adenovirus vector harbouring the tumor suppressor gene p53, is the first gene therapy product to treat cancer and was permitted by the Chinese Food and Drug Administration (FDA) in 2003 to cure head and neck cancer. Application of Gendicine together with chemotherapy and radiotherapy could cure cancer to some extent. By contrast, Advexin, a similar gene therapy administration from Introgen, was denied by the US FDA in 2008. The death of Jesse Gelsinger, a patient participating in a gene therapy trial, caused distrust about the safety of adenovirus vectors leading to preferential treatment with genetically crippled viruses.

Adeno-associated viruses

Adeno-associated viruses (AAV) are small viruses containing single stranded (ss) DNA. The wt type AAV can incorporate DNA site-specifically whereas the recombinant AAV, which is harbouring only the therapeutic gene, does not integrate into the host's chromosome but is producing episomal forms by recombination. These "episomes" guarantee expression of the therapeutic gene in the long run. However, AAV's largest disadvantage as a gene therapy vector could be its low packaging capacity due to the limited size of the AAV virion and technical problems in its manufacturing. AAV is non-pathogenic and, in contrast to wt adenoviruses, it will not be removed by the host's immune system. Nowadays, AAV vectors are used in clinical trials to treat muscle and eye diseases as well as cerebral diseases since dormant cells (such as neurons) are target cells of AAVs as well.

The retroviral encoded *env* gene facilitate recognition of cells which represent a specific receptor on their surface (Adams et *al.*, 1995). So, in contrast to

adenoviruses and adeno-associated viruses, retroviral infection is limited to specific cells.

Replication Vectors

Retroviral vectors are known to be either replication-competent or replication-defective. ONYX-015 (previously called CI-1042) is a replication-competent vector modified to replicate in and induce rapid apoptosis in cells harboring p53 mutations. Replication-defective vectors with multiple deletions of essential genes infect their target cells but after transgene delivery cells will undergo lysis and death.

Cis- and trans-acting elements

Replicative-defective vectors contain always a “transfer construct” including the transgene as well as the packaging sequence, sequences for replication and, sometimes, sequences for reverse transcription. Thus, these sequences are characteristic cis-acting elements, they contain viral sequences on the same chromosome in contrast to the viral trans-acting elements, which can be located not only on the same DNA.

Herpes Simplex Virus

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) are members of the *Herpesviridae* family known to infect humans. The wt HSV-1 can infect neuronal cells, which are usually ignored by the immune system after infection.

Many worries arose since an attenuated viral strain could revert to its pathogenic version (Clark et *al.*, 2008) making gene therapy applications with live viruses even complicated. Some specific live viruses can even circumvent or suppress the immune response.

1.2.2.2 Non-viral methods

Oligonucleotides

Therapeutic oligonucleotides are used in gene therapy to silence the expression of a gene, causing a particular disease, on its transcriptional or translational level. Oligonucleotides include the synthesis of nucleic acids of DNA, RNA or chemical

analogues to knock down or knock off a disease causing messanger RNA (mRNA). However, antisense therapy (such as applications with siRNAs) is a way to treat genetic disorders or infections but is not strictly a kind of gene therapy. Another strategy uses ds oligodeoxynucleotides to titrate transcription factors from the promoter of the default gene leading to decreased expression rates. Furthermore, application of ss DNA oligonucleotides targets at induction of a single-base pair mutation within the disease-causing gene ("targeted gene repair or targeted nucleotide alteration").

Lipoplexes and polyplexes

Lipoplexes (DNA complexed with cationic lipids) and polyplexes (DNA complexed with synthetic polycationic polymers) aim at efficient delivery of DNA into the patient's cells and stabilizing DNA by preventing its degradation. However, DNA-lipid complexes form structures such as micells or liposomes.

In contrast to cationic lipids, the elaborating production of anionic and neutral lipids was neglected. Several features making cationic lipids (because of their positive charge) a proper tool in biopharmaceutical applications such as association with and condensation of the negatively charged DNA, formation and stabilization of lipoplexes and facilitate uptake due to higher affinity to the cell membrane. Still, cationic lipids can cause toxicity in a dose dependent manner making them unsuitable for gene therapy purposes.

Mostly polyplexes can not set their DNA cargo free into the cytoplasma. Therefore, cotransfection with endosome-lytic agents is necessary. Since polymers such as polyethylenimine have intrinsic endosomal activity, they can trigger endosome destruction as well.

Hybrid methods

Some gene transfer techniques are combined such as virosomes (derived from inactivated HIV or influenza virus) complexed with liposomes. It has turned out that virosomes can overcome the epithelial airway barrier easier than application of viral or liposomal methods alone. Viral vectors complexed with cationic lipids or hybridising virus could be used as alternatives in gene therapy applications as well.

Dendrimers

A dendrimer is a spheric synthetic macromolecule which is highly branched. Recently, it has been observed that the use of highly branched macromolecules has significant effects on gene therapy success in contrast to the traditionally used linear polymers. The positive charge of cationic dendrimers enhances its complextion with the negatively charged DNA and the dendrimer-nucleic acid complex may then be internalized by a clathrin-dependent endocytosis (36).

Synthetic vehicles (SV)

For the improvement of therapeutic approaches, synthetic delivery systems are considered promising tools as non-viral vehicles. Chemical bonds of the non-viral vector are only cleaved in response to various stimuli such as environmental or external triggers. Using masked endosomolytic agents (MEAs) is one example for this approach: a pH-labile bond of the vector is only opened when MEAs are entering the acidic endosome. Subsequently, the nucleic acids must be released from the endosome to avoid its lysosomal degradation. Similar to the delivery methods mentioned in 1.3.4, SVs can be used to transport pDNA *in vivo* as well. Recently, MEA-dynamic PolyConjugates (DPCs) have been used as vehicles in clinical trials to provide hepatocytes with siRNAs *in vivo*. (42).

Naked DNA

Unlike viruses, plasmids are "naked" DNA used as gene medicine or DNA vaccine to fight viral, bacterial, parasitic diseases, or cancer (57). Transfection of cells with naked DNA is the easiest method of non-viral transfection since pDNA is chemically simple, easy to produce and characterize, and host cells can be transfected either with naked pDNA, coated with gold or as lipid-DNA complexes (Ledwith et *al.*, 2000).

1.2.2.3 Bacterial gene transfer vectors

Since lately, the bacterial gene transfer vector has been proposed as a third type of vector to be used in gene therapy. Bacterial strains such as *Salmonella*, *Shigella*, *Listeria*, *Yersinia*, and *E. coli* could be qualified for the use as a vehicle for the therapeutical gene. Although the mechanism of how the DNA is passed on from the

bacterial cell to the mammalian cell is yet not clear but *in vitro* and *in vivo* studies demonstrated it definitely (fig.4) (43).

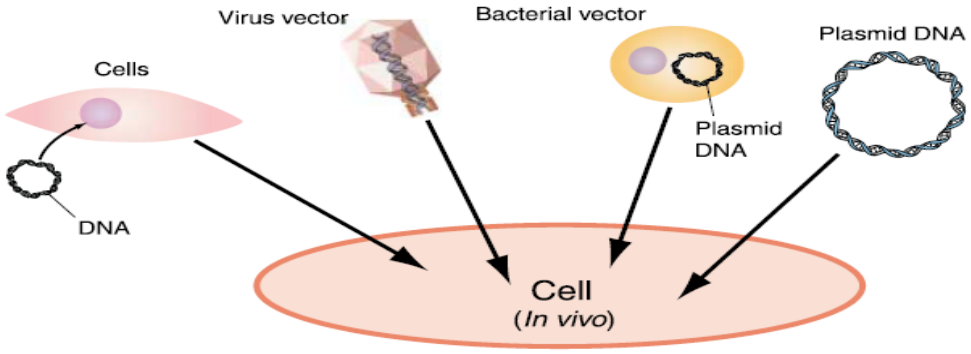


Fig.4: Different methods of application in gene therapy. After removal and *in vitro* transfection of host cells, the "repaired" cells are then returned into the patient's body. Replication deficient and attenuated virus or bacteria deliver the therapeutic gene via transfection of the host cell. Alternatively, cells are transfected with pDNA by using different delivery methods (31).

1.2.2 Optimization of plasmid vectors

According to Carnes et *al.* the production of highly pure pDNA for applications in gene therapy starts with prudent considerations about (i) the rational design of the plasmid vector, (ii) plasmid composition, (iii) yield and quality, and (iv) purity after downstream processing, to be approved by regulatory authorities (13).

It is a fact that PCN is correlating with the choice of the ORI. In general, pDNA for therapeutic purposes contain the *Col*E1 or pMB1 ORI but, according to Carnes the use of pMB1-derived pBR322 plasmids with a G-to-A mutation or the truncated origin in pUC derived plasmids such as p15A multiplicate the PCN. In small scale fermentations temperature-sensitive origins occurring in pMM1, pMM7 and pUC are preferred because PCN can be increased 30- to 40-fold after temperature rise from 30°C to 42°C (10, 44). However, it could be clarified that the G→ A transition in the ORI of pUC vectors leads to minor or even no loop-loop interaction between RNAI and RNAII because the point mutation somehow influence the proper folding of the RNAII (Lin-Chao et *al.*, 1992; Herman et *al.*, 1994). Furthermore, the Rom/Rop protein or a drop in temperature finally leads to normal plasmid concentration. To sum up, the temperature-sensitive G→ A transition can be employed on any pBR322-derived plasmid to multiply pDNA production enormously (33).

1.2.2.1 Rational vector design

The demand for large quantities of highly pure pDNA is arising since gene therapy and DNA vaccines are playing more and more a central role in preclinical studies. The field of therapeutic application of pDNA (DNA vaccines, DNA replicon vaccines, short hairpin RNA (shRNA) vectors, AAV helper plasmids, therapeutic plasmids, gene targeting plasmids) is broad including prevention (viral, bacterial, or parasitic diseases), immunization, therapeutic vaccination (infectious diseases, allergy, autoimmunity), cancer vaccination, or its use as gene therapy vehicles. In contrast to conventional vaccines, new pDNA vaccines can be designed and produced to be applied against new biological pathogens (13) within a short time instead of years.

As a shuttle vector pDNA has to fulfil standardized criteria such as elements essential for its upkeep and reproduction in the bacterial and mammalian host as well as elements crucial for expression of the target gene in the human or animal host (4). Construction of pDNA vectors, which are to be used in gene therapy or DNA vaccine applications, requires observance of certain criteria in the course of its rational design. Thus, pDNA vectors have to obtain: (1) a constitutive, inducible, or tissue-specific promoter with transcriptional activity and a transcription terminator; (2) elements necessary for optimized mRNA processing and translation inclusively Kozak sequence, translational termination, mRNA cleavage, polyadenylation and mRNA-splicing sites; (3) prokaryotic ORI for propagation in *E. coli*; and (4) selection marker for maintaining and positive selection of cells harbouring the pDNA or, alternatively, antibiotic-free selection mechanisms (fig.5). All these pDNA components should be reduced as much as possible to obtain vectors, which vary between 3 and 12 kb without the gene of interest (GOI). Furthermore, in the course of time it became apparent that removing toxic or disturbing sequences from the vector have a beneficial effect on pDNA production. To sum up, the rational vector design is affecting both the production of pDNA and its effective application (2).

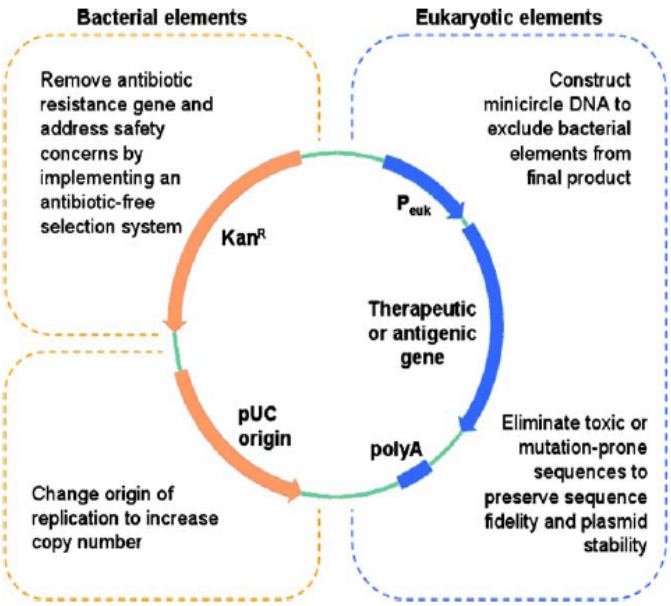


Fig.5: Vector design strategies. For accumulation of pDNA in *E. coli*, selectable markers such as the kanamycine resistance (Kan^R) or alternatively, antibiotic-free selection mechanisms, and the pUC ORI (whereas other ORIs could also be used) are crucial. In the mammalian system, eukaryotic elements such as the promoter/enhancer, the transgene, and polyadenlyation signals are playing a key role in the design of a pDNA vector (7).

Production of plasmid-encoded pharmaceuticals requires specific design of bacterial strains and plasmids. Improvement of plasmid stability, advancement of product safety, accumulation of plasmid amount and simplification of downstream purification processes are getting central objects of strain and vector engineering. At present, for pDNA production *E. coli* strains and plasmid backbones are used that are not specifically suited for production of pDNA. Overall, bacterial strains and pDNA design require alterations to maximize copy number, genetic fidelity, and segregational stability (7).

1.2.2.1.1 Antibiotic-free selection systems

For the propagation, maintenance and selection of recombinant cells, antibiotics and antibiotic resistance genes have been the single choice for a long time. However, their use in gene therapy or other biotechnological fields is more than questionable. For safety reasons, WHO, FDA and the American federal regulation strongly advised against the use of antibiotics and antibiotic resistance genes to prevent undesirable spread of antibiotic resistance in non-resistant bacteria. Constant expression of the

resistance protein is a heavy metabolic burden for the host cell and the antibiotic itself contaminate the final product. Several experiments made clear that removing *bla* (or other antibiotic resistance genes) and other sequences from therapeutic plasmids enhance expression of the transgene clearly and promote pDNA stability and yield in the fermentation process. Furthermore, some patients are hypersensitive to β-lactam antibiotics causing allergic reactions even up to anaphylaxis.

Circumventing the use of conventional antibiotics makes sense for several reasons since resistant bacteria fail to respond, antibiotics are unsuitable for vaccine production and expensive for industrial scale production (Goh et *al.*, 2008). As a result, antibiotic-free selection systems are objectives of research and development and recently many antibiotic-free selection systems are patented. However, non-antibiotic systems demand mutant host strains, specific media or expensive reagents. In the first known antibiotic-free selection system only those auxotrophic bacteria could grow on specific media whose defect gene was complemented by a plasmid-encoded gene. Repression-titration is another method avoiding the use of antibiotics and antibiotic-resistance genes where the lac operator “de-represses” an endogenous essential gene.

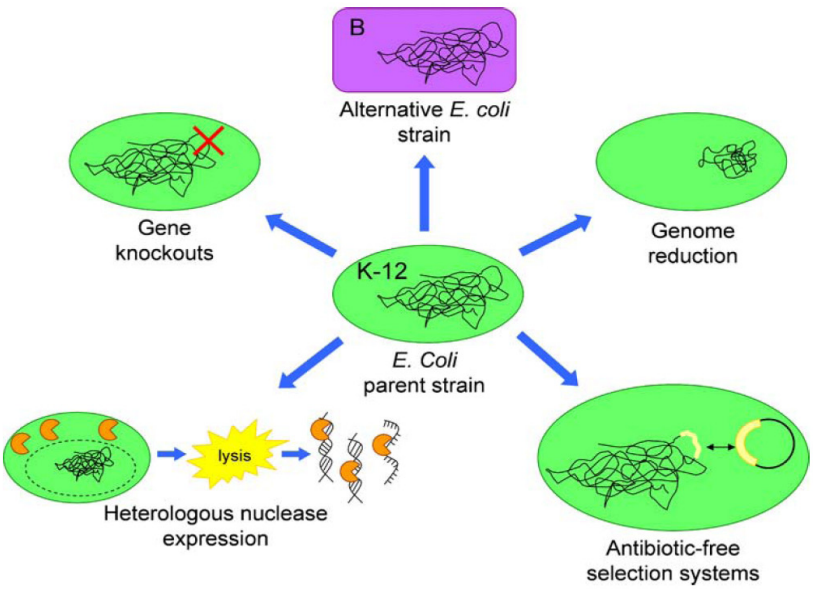


Fig.6: Strain design strategies. The *E. coli* parent strain, presented here in the model, can be any E. coli K-12 strain. Gene knockouts, the use of antibiotic-free selection systems, genomic reduction, removal of mobile elements and, expression of heterologous nucleases to degrade host genomic DNA

(gDNA) and RNA after cell lysis are just a few strategies to engineer strains to be used for the production of pDNA (7).

1.2.2.2 DNA Structure

Besides its canonical Z-form and B-form, DNA can adopt conformations which are known as the noncanonical A-DNA-, C-DNA-, D-DNA- and E-DNA-form. Consequently, for gene therapy only the canonical B-DNA form is relevant since specific DNA structures and sequences can cause instability problems (2). However, the rational vector design requires a clear understanding of both the DNA sequence and DNA structure.

1.2.2.3 Transformation efficiencies

According to Kreiss et *al.* the uptake of pDNA into the nucleus across the nuclear pore complex is influenced clearly by its size. Experimental data demonstrated that transformation with larger plasmids such as the P1 artificial chromosome (PAC) decreases transfection efficiency significantly in contrast to small vectors. Walker et *al.* investigated that the PAC vectors showed lower transformation rates resulting in a consequent lower expression rate of the GOI. However, these effects demand further explanations (35). Furthermore, for pDNA endolysosomal inclusions and degradation, cytosolic sequestration, nuclear exclusion of pDNA, and metabolic degradation are further obstacles to overcome (Lechardeur et *al.*, 2004).

Many barriers provide a biological hindrance to the entry of exogenous DNA including the extracellular matrix, the endosomal/lysosomal environment, the endosomal membrane, and the nuclear membrane making the application of non-viral gene therapy difficult. However, the permeability of the nuclear membrane itself impeding the successful entry of pDNA especially in non-dividing cells where the nuclear envelope is not dissolving due to missing mitosis. However, Dean et *al.* could show experimentally that the 72-bp repeats of the SV40 enhancer is a clear benefit for the delivery of pDNA into the nucleus whereas the activity of the promoter and the ORI are insignificant for the its nuclear localization (44).

1.2.2.4 Plasmid stability

It is safe to assume that specific sequences somehow negatively affect the production of supercoiled plasmids, which are recommended by FDA to prevail in the fermentation process. AT rich regions are proned to the host ss nucleases leading to nicking. Repeats as well as direct or inverted palindrome sequences are affecting pDNA stability in batch fermentation. The same is true for Z DNA-forming sequences such as CpG repeat sequences or formation of multimers. Dimerization of pUC plasmids is promoted by oligopyrimidine or oligopurine sequences leading to unconventional DNA conformations such as triple helices.

However, factors causing segregational instability, such as plasmid size, promote formation of plasmid-free-cells (Mathur et *al.*, 2009). Many high copy plasmids are irregularly disseminated between descendant cells since they lack controlled partitioning systems. Furthermore, high-copy plasmids can overwork the metabolism of their host providing plasmid-free cells with a growth advantage within a fermentation process (10, 13).

According to Yamaguchi et *al.*, OriC-containing plasmids cannot be stably maintained in specific *recA* strains of *E. coli* (due to incompatibility) unlike other *E. coli recA* strains. In many cases it has been noticed that plasmids, having an evolutionary ancestor in common, cannot co-exist in the same host cell and exclude each other (Chakrabarty, 1973). This process is called “incompatibility”. It appears that the quantity of determinant(s), responsible for incompatibility, is directly involved in the loss of plasmids (15).

1.3 DNA Vaccines

1.3.1 Definition of DNA vaccines

According to the definition of FDA DNA vaccines are highly purified plasmids encoding antigens to protect individuals against infectious agents by boosting and/or triggering efficient immune response. In 1990, Wolff et *al.* were among the first suggesting the idea of DNA vaccination. The idea was to synthesize the antigen, delivered by the pDNA molecule, directly in the target organism to stimulate the immune system. To do so, they injected the pDNA-encoded antigen into murine

skeletal muscle. Since then, DNA vaccination is an object of intense research but some DNA vaccine trials have failed due to inappropriate transgene expression (48).

To date, various diseases have not been kept under control by conventional vaccines. Millions of people succumb to fatal diseases every year such as HIV/AIDS (Acquired Immune Deficiency Syndrome), malaria, cancer, and many others. However, viral vectors and DNA vaccines are only used in preclinical trials and currently there are no DNA vaccines as licensed products available (31). Hitherto, FDA has permitted DNA vaccine applications for various infectious diseases such as malaria, Hepatitis B and HIV in phase 1 clinical trials (9).

According to Krishnan, pDNA vaccines provide numerous advantages in contrast to conventional vaccines, such as (i) ease of production, (ii) stability and transport, (iii) no cultivation of pathogens, (v) vaccination against multiple pathogens in a single dose (48).

1.3.2 The immune response to DNA vaccines

DNA vaccination seems to be attractive since both arms of the immune system are involved (Jechlinger, 2006). Complex intracellular and intercellular interactions are necessary for an antigen to promote antibody production and cellular/humoural responses (see fig.7). Recombinant proteins or inactivated virus vaccines fail to stimulate the cytotoxic T lymphocyte (CTL) response, important for an immune response, because in the endolysosome the antigen is degraded into peptides which are incorporated subsequently into major histocompatibility complex (MHC) Class II molecules. T-helper cells (T_H cells) are specifically induced by the peptide-MHC-conglomerate important for both antigen recognition and initiation of immune response.

However, to induce CTL response, association of peptides with MHC Class I molecules are essential. Finally, these MHC Class I-peptide complexes are detected by specific cytolytic T cells that trigger complete destruction of the infected cell. Thus, the idea in DNA vaccine technology is to induce CTL production by expressing antigenic fragments in the MHC Class I receptor leading to apoptosis of the infected cell (31, 45).

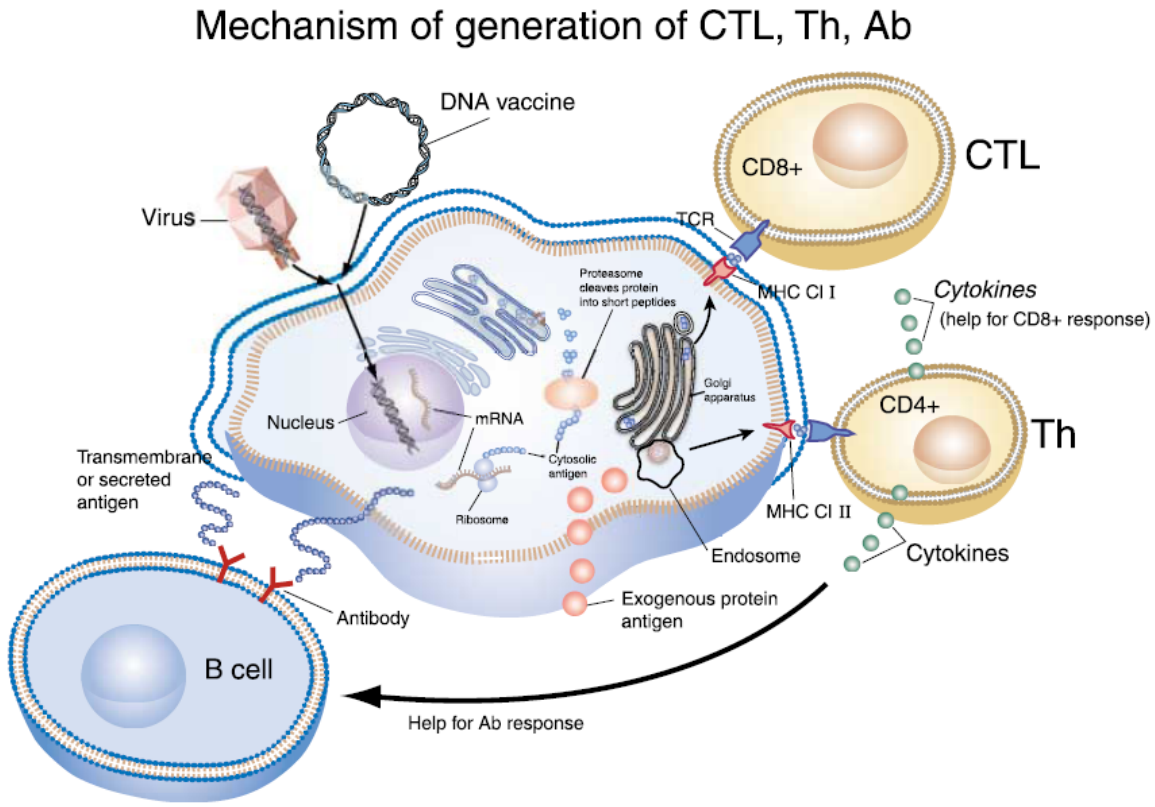


Fig.7: Mechanism of antigen-specific humoural and cellular responses. Professional APCs recognize exogenous antigens and induce its degradation to peptides which are then presented on the surface of MHC Class II molecules. The antigen peptide/MHC Class II conglomerate is detected by specific helper T cells ($CD4^+$ T cells) which will be activated subsequently. Activated $CD4^+$ T cells secrete cytokines to activate B cells and amplification of CTL responses. After recognition of peptide-MHC Class I complexes, presented by APCs, CTL ($CD8^+$ T cells) are activated and induce apoptosis of the infected cell. After detection of extracellular antigens, B cells induce antibody production (31).

1.3.4 Delivery methods

Various delivery methods have been developed to improve and/or ease pDNA applications:

(i) Ballistic delivery or “Gene-Gun delivery” facilitate gene transfer into a broad range of targets and is supposed to stimulate immune response most efficiently. In contrast to injections via syringes, applications with gene-guns are repeatable due to controlled adjustment of gas pressure and do not cause pain.

(ii) Delivery by liposomes: injection, oral or intranasal absorption of cationic liposomes that include pDNA molecules

(iii) Electroporation-assisted delivery: In the first step, nucleic acids are injected in muscle or skin and to speedup pDNA molecules as well as to promote their uptake, an electric current is applied by using electrodes.

(iv) *In vitro* transfection

Among various delivery methods for DNA vaccines, two approaches are currently favoured: injection via a syringe or application of gene gun technique. The mechanism of nucleic acid uptake is still not clear, but it is suggested that DNA uptake occurs via caveolae and/or might involve keratinocytes, fibroblasts and epithelial Langerhans as well, which migrate quickly to regional lymph nodes (Lewis et *al.*, 1999). Furthermore, phagocytosis and/or pinocytosis or specific receptors like a 30 kDa surface receptor or a macrophage scavenger receptor could also play a key role in DNA uptake.

In DNA vaccine applications, intramuscular injections are favoured because muscle cells are considered to take up DNA highly efficient but they fail to generate immune response since they are no professional APCs (31, 32, 46).

1.4 Problems and ethical aspects of gene therapy and DNA vaccination

In gene-based therapy, opinion about the Weissman barrier is divided on its safety issue because, according to this theory, genetic information is only passed on from germ line cells to somatic cells and never the other way round (Sullivan). However, if this soma-to germ line barrier does not exist than genetic information, derived from gene therapy, can be spread to germ cells (“germline transmission”), and would lead to a change in the genetic pool of the human species.

Current problems of gene therapy involve:

- Short-lived nature of gene therapy – the therapeutic DNA must persist in the patient’s cells, expressed at constant levels, and cells harbouring the therapeutic gene must be stable and long-living to be profitable for the patient in the long run.
- Theoretical concerns rise since therapeutic genes can be randomly inserted into the host’s genome leading to activation of oncogenes or inactivation of

tumor suppressor genes. In addition, insertion might lead to chromosomal instabilities by breaking or rearranging DNA.

- Reduction in efficacy of the gene therapy effect – Invaders that already induced immune response are recognized faster by the immune system after a second infection. Thus, using e.g. viral vectors in gene therapy seem impossible when repeated in patients.
- Problems with viral vectors – Viruses used in gene therapy trials can cause problems such as toxicity, immune and inflammatory responses. Since attenuated viral strains could revert to wt their use in gene therapy, to treat specific viral infections such as HIV, is doubted. Furthermore, some viral species evolved strategies to escape or downregulate the host immune response.
- Multigene disorders – Frequently emerging genetic diseases are multifactorial such as heart disease, high blood pressure, Alzheimer's disease, arthritis, and diabetes (36).
- In transient expression assays David Peterson et *al.* identified sequence elements within pBR322 that negatively influence expression of eukaryotic transgenes *in cis*. This region interferes with a negative element, a poison sequence that is known to inhibit DNA replication. Furthermore, Courey et *al.* announced that specific pBR322 sequences can downregulate the activity of the human β-globin promoter. Peterson et *al.* considered these negative elements to act as silencers on eukaryotic genes (47).

1.4.1 Potential safety concerns of DNA vaccines

According to Hodgson et *al.*, careful consideration and questioning is necessary when it comes to pDNA applications in humans depending on approval of WHO, US FDA, or European Agency for the Evaluation of Medicinal Products (EMEA) (57).

Preclinical studies imply that after DNA vaccine application glycoprotein immunoglobulin G (IgG) anti-DNA autoantibodies are secreted due to activated autoreactive B cells but it is unlikely that systemic autoimmunity is generated by DNA vaccination. Concerns are raised that organ-specific autoimmunity could be induced or even declined after DNA vaccine application (9).

Experiences gained by veterinary DNA vaccines might be useful for human DNA applications. Though, reproduction of immune responses, noticed in animal models, in humans is not possible in general but many approaches have been made to improve the human immune response such as enhanced uptake, stabile expression, modulation of the immune response, or in adjuvanting (49).

1.5 Strategies to increase plasmid concentration by overexpression of rssB and tRNA^{AlaU}

1.5.1 *rssB* leading to enhanced *Col*E1 PCN in *pcnB* mutants

By screening multicopy library harbouring randomly integrated chromosomal fragments in *pcnB* mutants, Jain et *al.* found the rssB encoding gene that significantly increased *Col*E1 PCN after its induced overexpression. So far, the mechanism how *rssB* might regulate PCN remains unclear. In *E. coli*, *PcnB* shows its homology to the *cca* gene encoding the tRNA nucleotidyltransferase which binds tRNA. So, one might guess PcnB might be an RNA-binding protein. By binding to RNAI, it blocks the RNAI-RNAII hybrid formation finally leading to enhanced plasmid replication. Alternatively, after sequence analysis Cao and Sarkar identified the *pcnB* locus to be homologous to the *E. coli* poly(A) polymerase I (PAP I) which catalyzes polyadenylation of RNA. So, polyadenylated RNAI is unable to bind RNAII because it is proned to degradation. Deletion of PAPI showed that RNAI intermediates are strongly accumulating within the cell leading to decreased pDNA replication and, thus, decreased PCN of *Col*E1-type plasmids (He et *al.*, 1993; Xu et *al.*, 1993). However, PcnB is known to regulate plasmid replication since strains with deletion in the *PcnB* locus reduce PCN at very low levels and cause plasmid instability.

It is presumed that the *pcnB* defect is suppressed by overexpressing RssB either caused by PAPI-independent polyadenylation (such as the PNPase) of RNAI or by enhanced degradation of nonpolyadenylated RNAI species (Carabetta et *al.*, 2009). Carabetta et *al.* consider the PCN of *Col*E1-derived plasmids is clearly regulated by the RssB-PAPI collaboration (1, 50). It is known that RssB, the ClpXP protease and a cis-acting turnover element (somewhere between methionine 159 (M159) and histidine187 (H187) are somehow involved in the controlled degradation of σ^S (a subunit of RNA polymerase in *E. coli*) (53).

1.5.1.1 RssB

In *E. coli*, the master response regulator RssB (also known as SpreE or MviA) is an element of a two-component signal transduction pathway. The RssB protein is homologous to the N-terminal domain of the response regulator family, but its C-terminal output domain is not homologous to any identified protein. However, depending on phosphorylation of the N-terminal RssB receiver domain RssB recognizes the turnover element (see 1.5.1) within σ^S (53). It is speculated that stress somehow stabilizes σ^S and promote inactivation of RssB by dephosphorylation of its receiver domain.

Hitherto, two interaction partners of σ^S are known: (i) RNAP core enzyme; and (ii) RssB, which mediates σ^S degradation. It is worth noting that the binding site for RssB in σ^S is adjacent to the crucial – 10 promoter element.

Hengge-Aronis et *al.* could clarify experimentally that RssB can be an anti-sigma factor for σ^S *in vivo* as well. When σ^S is accumulating, RssB can act as a repressor of σ^S-dependent genes (28, 29).

Cis-acting element

This cis-acting turnover element is to be detected somewhere between M159 and H187. By site-directed mutagenesis Gisela Becker et *al.* confirmed lysine 173 (K173) and the amino acids around as the turnover element in σ^S. Substitution of K173E (glutamate abbreviated as E) has turned out to prevent ClpPX-mediated proteolysis. However, K173 in σ^S has dual functions: it is essential for σ^S proteolysis but it may play a role in promoter recognition as well (53).

ClpPX

According to Muffler et *al.*, it is not clear if σ^S degradation is catalyzed by either RssB itself or by the ClpPX protease, a complex ATP-dependent protease composed of proteolytic (ClpP) and chaperone (ClpX) subunits. However, there are hints that the protease is only active in the presence of RssB or that RssB might expose σ^S to the ClpPx protease machinery. Experiments in the near future will establish clarity (24).

1.5.1.2 Stability of RpoS/σ^S

Lange *et al.* verified experimentally that *rpoS*/σ^S (σ^S, a subunit of RNAP, is encoded by *RpoS*) expression is enormously complex and is controlled on (i) transcriptional level; (ii) translation level; and (iii) protein stability. RpoS expression is governed by various extrinsically signals such as cAMP, ppGpp, cell density signals, osmotic signals, and starvation signals (fig.8.). Increasing medium osmolarity does not induce necessarily *rpoS* transcription but rather some post-transcriptional mechanisms. Not only σ^S is controlling stationary phase-induced gene expression but various regulating factors as well such as 3', 5'-cyclic AMP-cAMP receptor complex (cAMP-CRP) cAMP-CRP complex, Lrp and integration host factors. Each factor is revealed to be part of a complex regulatory network regulating expression of many genes induced in the stationary phase (27). Both, entry into stationary phase and increased medium osmolarity are enhancing RpoS translation intensely. However, in exponentially growing cells σ^S is a highly unstable protein with a half-life of 1.4 minutes but its clearly more stable in stationary phase (~10.5 min) (27).

All in all, stress signals and transition into stationary phase bring about up-regulation of RpoS and as a transcriptional activator it enhances transcription of 50-100 genes leading to stress resistance and other physiological and/or morphological modifications (53).

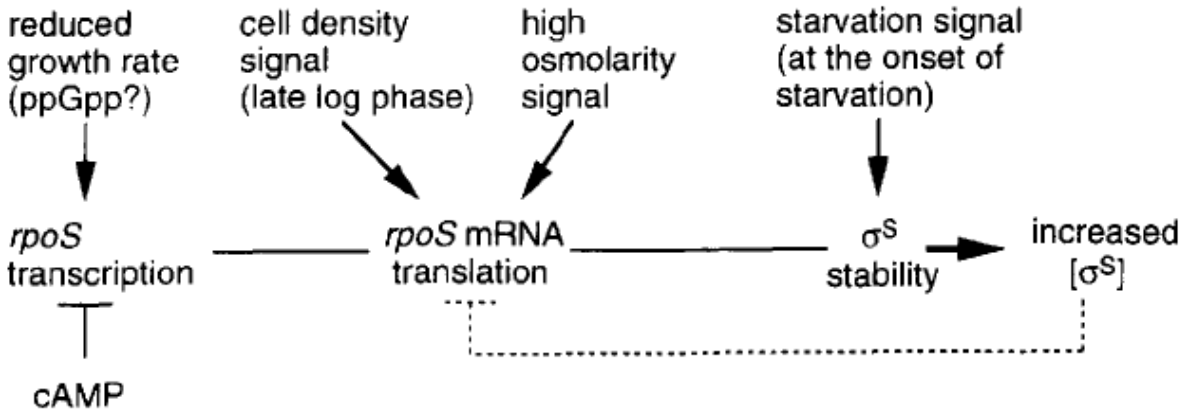


Fig.8: Model of regulation of rpoS/σ^S expression. Various extrinsically signals (indicated in the model) are regulating its expression on transcriptional, translational level as well as its stability (27).

1.5.2 Uncharged $tRNA^{AlaU}$(UGC) in r*elA* mutants

tRNA is not only involved in translation, moreover novel functions has been revealed recently e.g. retroviruses and long terminal repeats (LTR) retrotransposons utilize priming tRNAs for reverse transcription (Marquet et *al.*, 1995), in amino-acid starved *E. coli* tRNAs interact with RNAI and/or RNAII, thus regulating replication of *Col*E1-type plasmids (Wróbel et *al.*, 1997, 1998; Wegrzyn, 1999; Wang *et al.*, 2002). Furthermore, it was shown that the T helper immune response in mice was stronger stimulated after application with $tRNA^{Ala}$ adjuvants (Wang et *al.*, 2006) (8).

It took many years, until a model for tRNA-dependent regulation of *Col*E1-like plasmids replications has been recommended by Yavachev and Ivanov. Before then, Hecker *et al.* were among the first stating that the copy number of the *Col*E1-like plasmid pBR322 was clearly enhanced in amino acid starved *E. coli*. 1994, Herman *et al.* declared that depending on what kind of amino acid was missing in the media, *Col*E1-type plasmids varied in their replication behaviour. The same is true for other *Col*E1-like plasmids (Wróbel and Wegrzyn, 1998). However, still it is not clear why lack of different amino acids result in different replication behaviour of *Col*E1-like plasmids. It has been considered that ppGpp, a global regulator of gene expression in bacteria, can somehow prevent replication of *Col*E1-like plasmids (Herman et *al.*, 1994) (55).

In 1988, Zavachev and Ivanov studied sequence similarity between all 21 tRNAs and RNAI/II, encoded on the *Col*E1 plasmid. According to their homology to RNAI/RNAII, all tRNAs were divided into three classes: (i) tRNAs homologous to RNAI (Arg, His, Leu, Lys, Phe, and Thr); (ii) tRNAs homologous to RNAII (f-Met, Try, and Gly); and (iii) tRNAs homologous to RNAI and RNAII (Met and Val) (Zavachev et *al.*, 1988). The anticodon loop of all tRNAs own seven nucleotides (Hjalt and Wagner, 1992) (18, 56).

According to the model of Wang et *al.*, RNAI-RNAII interaction could be hampered by accumulated uncharged tRNA present in bacterial cells during the relaxed response leading to enhanced plasmid replication. Other experiments argue for primer extension of the 3' CCA sequence of uncharged tRNAs necessary to induce replication (Maizels and Weiner, 1994; Chen and Lambowitz, 1997) (55, 56). In 2006, experiments in amino-acid-starved *RelA* mutants ("relaxed response")

suggested that uncharged $tRNA^{AlaU}$(UGC) catalyze cleavage of RNAI leading to enhanced *Col*E1-like PCN *in vitro* and *in vivo*. The ACCA motif at the 3'-terminus of the $tRNA^{Ala}$ (UGC) is crucial for cleavage of RNAI because those effects have not been seen when the 3'-ACCA sequence was deleted. Based on these data, Wang et *al*. assumed 3'-ACCA of tRNA together with the UGGU motif within the RNAI loops generate a catalytic structure capable of cleaving RNAI molecules only in the presence of Mg^{2+} ions. Want et *al.* stated that both, UGGU sequences and the 3'-ACCA sequences may exert catalytic activity (8).

1.5.2.1 Aminoacylation of tRNA

Aminoacylation is a two-step process where a specific amino acid is attached to its cognate tRNA catalyzed by aminoacyl-tRNA synthetases (aaRS). To generate aminoacyl-tRNA molecules, ATP and the cognate amino acids have to bind to specific aaRS sites. After attachment, inorganic pyrophosphate (PP_i) is released.
To physically link the amino acid to the trinucleotide sequence of its cognate tRNA, the amino acid has to be transmitted from the aa-AMP to the 2'- or 3'-OH group of the 3'-end of the tRNA acceptor stem.

The 20 known aaRS differ in size, differ mostly in their sequences and can occur either as dimeric, trimeric or tetrameric complexes. The aaRS GlnRS, TyrRS, MetRS, GluRS, ArgRS, ValRS, IleRS, LeuRS, TrpRS belong to the class I having the conserved sequence motifs "HIGH" and "KMSKS" in common. According to crystal structures both motifs contain the Rossman fold, a nucleotide binding fold that binds ATP. Aminoacylation of the 2'-OH of an adenosine nucleotide is conducted by the class I synthetases. In contrast to class II synthetases, the class I molecules share two highly conserved sequence motifs. Class II synthetases contain three highly conserved sequence motifs and they catalyze aminoacylation of the 3'-OH of an adenosine nucleotide. Some synthetases conduct proofreading, a process to ensure highly accurate tRNA charging. Non-cognate amino acids are rapidly removed by hydrolyzation of the aminoacyl-tRNA bond (23, 58).

All 21 tRNAs have a trinucleotide sequence in their anticodon loop that can pair with several codons. To conduct correct tRNA aminoacylation, many aaRS do

not confide only in the trinucleotide sequence but utilize identity elements in the acceptor stem of the tRNA as auxiliary device as well.

Recent experiments revealed that the G3•U70 base pair, located in the acceptor stem of $tRNA^{AlaU}$, is an important but not solely identity element to be recognized by a cognate Alanyl-tRNA synthetase (AlaRS). According to McClain et *al.* the recognition mechanism of $tRNA^{AlaU}$ is both, directly and indirectly and not only catalyzed by AlaRS alone, but rather by several synthetases. Concluding from this, minihelices, duplexes, and smaller RNA helices, mimicking the acceptor stem, can be aminoacylated by AlaRS as well (59).

To be recognized by the AlaRS, the first 4 bp encoded on the acceptor stem and the discriminator base A 73 of the $tRNA^{AlaU}$ molecule are critical as it has been shown in *in vivo* and *in vitro* experiments. According to that, base transversion in G2:C71, which is located close by the G3:U70 position, has a tremendous effect on recognition by AlaRS leading to a significant loss of aminoacylation activity. This is true for *in vitro* experiments (6).

1.6 Chromosomal integration of linear DNA fragments via the Red Disruption System

According to Lorenz et *al.* most bacteria refuse the uptake of linear DNA because it is consequently degraded by host encoded exonucleases. However, Datsenko and Wanner had the idea to amplify linear DNA by using PCR primers that are homologous to the target DNA region. To promote homologous recombination with linear DNA, the plasmid-encoded phage λ Red recombinase (fig.9) is necessary, which is controlled by the well-regulated OriR101 promoter. After shifting temperature to 42°C, pKD46 is simply excluded from the cells due to the temperature-sensitive ORI.

Bacteriophages evolved their own homologous recombination system. In contrast to *recD*, *recBC* or *sbcB* mutants the recombination frequency was intensified when the Red-mediated recombination system together with linear DNA fragments was applied. However, the Red system encodes three genes, which are regulated by the *ParaB* promoter: γ, β and exo. After induction with arabinose, the final products

called Gam, Bet and Exo are expressed. The endogenous RecBCD exonuclease V is blocked by Gam facilitating recombination at DNA ends catalyzed by Bet and Exeo (3).

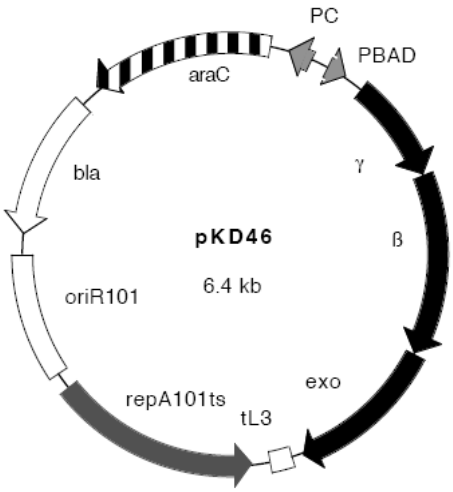


Fig.9: The Red helper plasmid pKD46 enables efficient homologous recombination with linear DNA fragments (adapted from university of Sheffield/Wet Lab).

1.7 Induced gene expression from T7 promoters

In biotechnology, transcription of cloned genes is often initiated by T7 DNA-dependent RNAP. This enzyme is recognizing its promoter highly specifically and relatively few T7 RNAP molecules are sufficient to drive high-level expression. Furthermore, transcription can be reinitiated several times leading to enhanced levels of stable RNAs. According to Studier et *al.* only few hours post induction the protein level will accumulate up to 50% or even more of the entire cell protein concentration Although highly active, the T7 RNAP is overstressing the translational machinery and, thus, the translational efficiency.

T7 RNAPs can direct all basic steps necessary for transcription autonomously independently from host-encoded transcription factors (Ferrari et *al.*, 2004). Structural analysis suggests that transition from initiation to elongation go along with conformational rearrangements particularly in the N-terminal domain of the polymerase (Theis et *al.*, 2004). Compared to the complex RNAPs, the architecture of T7 DNA-dependent RNAPs is rather ordinary with a molecular mass of about 100 kD. According to structural and functional analysis the T7 RNAP, eukaryotic

mitochondrial, chloroplast, other phagelike RNAPs and DNAP of the polymerase I family are not homologous (39, 51).

The chromosomal integrated lambda DE3 prophage is expressing T7 RNAP after induction with IPTG. However, expression of T7 RNAP is controlled by the *lac*UV5 promoter, which is, compared to the wt lac promoter, even active when glucose is added to the media (known s catabolite repression). Because of this, expression of the transgene in DE3 strains might occur. Normally chromosomally integrated λDE3 is inactive in bacterial cells but induction of the SOS cascade may lead to its activation leading to cell lysis. Strains expressing T7 polymerase but lacking integrated DE3 prophages, are rather insensitive after SOS response induction (54). However, it is demonstrated that T7 expressing systems enhance gene expression in untreated cells (despite the high specificity for its own promoter), influence growth of host cells and its effect on induction is rather low compared with gene expression in uninduced cells (Spehr et *al.*, 2000).

2 Objectives

The demand for large quantities of highly pure pDNA is arising since gene therapy and DNA vaccines make their way into preclinical studies (Carnes et *al.*, 2006). Factors such as PCN, structural and segregational stability of plasmids influence the efficiency of recombinant protein production in *E. coli* to a considerable extend (Skulj, 2008). Expression systems such as the T7 system probably cause exhaustion of the protein synthesis machinery but maintenance of the producing population is of particular importance in a fermentation process. Excessive overload of the metabolic burden of the host can be kept within bounds by regulating PCN. However, upkeep of the segregational stability of plasmids is often an intricate problem during the fermentation process. Segregational instability usually leads to the emerging and enhancement of a plasmid-free population resulting in constrained productivity. All in all, retaining low metabolic burden during accumulation of biomass and induction of high PCN in the final part of the fermentation process will lead to enhanced plasmid yield and plasmid quality.

The major object of this work was to evolve a system to regulate the copy number of *Col*E1 plasmids. Two different approaches have been explored: in the first approach, upregulation of plasmid concentration by induced expression of $tRNA^{AlaU}$ wt and mutated $tRNA^{AlaU}$ (mut) should be carried out. For that reason, $tRNA^{AlaU}$ wt and $tRNA^{AlaU}$ mut has been integrated into the *E. coli* chromosome and its expression was controlled by the T7 polymerase. Those strains were transformed with 3 different ColE1-plasmids to test if overexpression leads to an enhanced plasmid concentration. However, in the majority of IPTG-induced and uninduced cells plasmid concentration was marginally increased or even decreased. This was also true for uninduced cells due to the leaky T7 promoter.

In a second attempt *rssB* was cloned in a pBSK plasmid to test whether its overexpression, induced by the T7 polymerase, lead to enhanced plasmid concentration. As in the previous approach, even uninduced cells showed some upregulation of plasmid concentration due to the leaky T7 polymerase.

3 Materials and Methods

3.1 Media

3.1.1 Media and growth conditions

All *E. coli* strains (table 1) used in this work were grown in liquid Luria-Bertani (LB) media (table 2) at 37°C and shaken at 190 rpm. Media was supplemented with antibiotics or other additives as recommended (table 2). Cells were plated on LB-agar-plates containing antibiotics (table 3) in case of need. After electroporation, cells were transferred into SOC medium (table 4) and incubated with agitation at 37°C unless otherwise noted. All media were sterilised by autoclaving for 20 minutes at 121 °C and 2 bar.

Table 1: Bacterial strains

Host	*Source*	*Genotype*	*Resistance*
JM109	Lab stock	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 Δ(*lac-proAB*)	-
MG1655	Lab stock	F^- lambda- *ilvG*- *rfb*-50 *rph*-1	-
BL21 (DE3)	Novagen	F^-, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	-
HMS174 (DE3)	Novagen	F^- *recA*1 *hsdR*(r_{K12}^- m_{K12}^+) (DE3)	Rif^R

Table 2: LB media:

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

LB media components were dissolved in 950 ml sterile deionized water, pH value was set to 7.0 with 5 molar (M) NaOH and volume was adjusted to 1 liter (l) with deionized water. For LB agar, 15 gram (g) agar-agar was added per 1l LB media.

The autoclaved media was cooled down to 50°C in a water bath before adding antibiotics.

Table 3: Antibiotics and other additives:

	Stock solution	*End Concentration*
Ampicillin	100 mg/ml	100 µg/ml
Chloramphenicol	25 mg/ml	25 µg/ml
IPTG	0.1 M	100 µmol
Na-Citrate (pH 5.5)	1 M	10 mM
$CaCl_2$	1 M	5 mM
Glucose	20%	0.2%
$MgSO_4$	1 M	10 mM

Antibiotics and other additives were sterilfiltrated (0,22 µm) or autoclaved and stored at -20°C.

Table 4: SOC medium

A	bacto tryptone	20 g/l
	yeast extract	5 g/l
	NaCl	10 mM
	KCl	3 mM
	$MgCl_2$	10 mM
B	glucose*H_2O	20 mM
C	$MgSO_4$*7H_2O	10 mM

A, B and C were autoclaved separately and mixed subsequently.

3.1.2 Shake flask cultures

The overnight cultures were grown at 37°C with agitation (190 rpm) for 16 hours. After overnight incubation, cultures were diluted 1:1000 in 50 ml LB media containing the corresponding antibiotics. Gene expression was induced by addition of 100 µmol IPTG at an OD_{600} of 0.5. In parallel, uninduced control cells were considered in all experiments.

3.1.3 Bacterial cryostock

For *E. coli* conservation at -80°C cryostocks have been prepared. Cells from a single colony were incubated in 4 ml LB media supplemented with antibiotics at 37°C shaked at 190 rpm. After incubation for 16 hours, 600 µl of cell suspension and 300 µl glycerine (86%) were transferred into a 1.5 ml sterile Eppendorf tube, immediately inverted and frozen at -80 °C.

3.1.4 Bacterial strains and plasmids

Strains

All strains and plasmids used in this work are listed in Table 1 and Table 5.

Table 5: Plasmid list

Plasmids	*Source*	*Resistance*
pBluescript KSII+	Stratagene	Amp^R
pkD46	kindly provided by Joseph Peters	Amp^R, temperature sensitive
pBR322	MBI Fermentas	Amp^R, Tet^R
pUC19	MBI Fermentas	Amp^R
pET11a	Novagen	Amp^R

3.2 Molecular biological Methods

3.2.1 Polymerase Chain Reaction (PCR)

All DNA amplification reactions were carried out in Biometra T3-Thermocycler by using the high-fidelity Phusion polymerase (Finnzymes) or Taq polymerase (Biotools B&M Labs) according to the manufacturer's instruction. Lyophilized primers have been mixed with sterile water to obtain a stock solution with a final concentration of 1 ng/µl. A 1:100 dilution of the primer stock solution gave a final concentration of 10 pmol/µl ready to be used as a primer working solution for PCR reactions. Additionally, 2.5 % dimethyl-sulfoxide (DMSO) was applied in the PCR reaction to avoid formation of secondary structures in the DNA template and DNA primers. The melting temperature (T_m) is the temperature where DNA double helix is separated into its single strands and is necessary to determine the stability of a primer-template hybrid. A simple formula for calculating T_m is:

$$T_m = 4(G + C) + 2(A + T)\ °C$$

3.2.1.1 Colony PCR

Colony PCR techniques have been employed to screen for positive clones by using Taq polymerase. This technique enabled a screening of 8 randomly chosen colonies. Individual colonies were picked with a sterile pipette tip and were inoculated in 10 µl sterilized water. 3 µl of inoculated cells were pipetted into PCR tubes containing the PCR reaction mix.

3.2.1.2 Cloning PCR

To clone genes, the phusion high-fidelity DNAP is a superior choice since it has proofreading function. It has a unique ds-binding domain, fused to a *Pyrococcus*-like proofreading polymerase, which increases the affinity for ds DNA.

3.2.2 Agarose gel electrophoresis and extraction of DNA fragments from acrylamid gels

To separate DNA fragments according to their seize gel electrophoresis (Biorad) was performed using a 1 % agarose gel. To obtain 500 ml of liquid agarose solution, 5 g

agarose and 10 ml of 50x TAE was mixed and filled up to 500 ml with sterile water. For complete dissolution, the solution was heated up in a microwave. After a cool down period, 18 µl ethidium bromide was added. Gels were running using 1x TAE running buffer after 1:50 dilution of a 50x TAE stock solution (table 6). For sizing ds DNA λ DNA *EcoR*I/*Hind*III markers are suitable. 6x DNA loading dye (Fermentas) contains glycerol that causes samples to sink to the bottom of the wells. Analytical gels were run at 130 Volts and preparative gels at 90 Volts. DNA fragments were cut out from preparative gels, purified and used for digestion or cloning. For gel documentation DNA was visualized under UV-light (Bio-Rad).

Table 6: 50x TAE stock solution

Tris base	242 g/l
glacial acid	57.1 ml/l
0,5 M EDTA (pH 8.0)	100 ml/l

3.2.3 Purification of DNA fragments and PCR products

PCR products and DNA fragments were purified with commercially available products (illustra GFX^{TM} PCR DNA and Gel Band Purification Kit from GE Healthcare and Wizard® Plus SV Minipreps DNA Purification System from Promega). The purified nucleic acids were eluted in HPLC-clean water.

3.2.4 Plasmid preparation

pDNA was extracted from 12 ml overnight bacterial culture with the Wizard® Plus SV Minipreps DNA Purification System. Finally, pDNA was eluted with 30 µl nuclease free water. To gain higher pDNA yield, the Promega Wizard® Plus Midipreps DNA Purification System was applied and pDNA was eluted in 600 µl nuclease free water.

3.2.5 Determination of ds-DNA concentration

DNA-concentration was determined in a spectrophotometer by exposing DNA to ultraviolet light at 260 nm. The ratio of A_{260}/A_{280} reflects the purity of a nucleic acid (DNA, RNA) sample and in solution, it averages between 1.8 to 2.

3.2.6 Restriction enzyme digestion of DNA

Restriction digestion with restriction enzymes (NEB) was applied for vector linearization, isolation of insert DNA for cloning purposes and control digestions. Digestions were conducted with restriction enzymes in reaction buffers that came with the restriction enzyme. Some restriction enzymes need Bovine Serum Albumin (BSA) to enhance performance and stabilization of the restriction enzymes. All restrictions were carried out at 37°C (Eppendorf heating block) for two hours. Agarose gel electrophoresis was necessary for analysing or preparation of DNA fragments.

3.2.7 Ligation

To perform successful ligation of insert and vector DNA the T4 DNA Ligase (NEB) was used. The standard ligation reaction for DNA was conducted as follows: a 5:1 molar ratio of insert DNA to vector DNA in a 10 µl set-up is believed to be very efficient. The ligation mix containing T4 DNA ligase, T4 ligase buffer, pDNA, insert and water, was incubated overnight at 16°C and purified and concentrated with 2-butanol precipitation as mentioned in section 3.2.9.

3.2.8 Preparation of electrocompetent cells

A single colony of cells were picked up with a sterile pipette tip and incubated in 20 ml LB medium containing antibiotics overnight at 37°C with agitation (190 rpm). The overnight culture was diluted 1:1000 in a 50 ml fresh LB media and incubated at 37°C with agitation (190 rpm). The OD_{600} was measured every thirty minutes. Once the OD_{600} reached 0.5, the culture was transferred in ice-cold Falcon tubes. The cells have been harvested by centrifugation at 5000 g for 5 minutes at 4°C. The supernatant was decanted and the cell pellet was resuspended in 1 ml ice-cold sterile HPLC-water. The suspension was transferred in an ice-cold Eppendorf tube and centrifuged at 5000 g for 5 minutes at 4°C (the washing step was repeated 5 times). Finally, the pellet was resuspended in 50 µl ice-cold sterile HPLC-water. Cells were prepared freshly for every electroporation.

3.2.9 Concentration and purification of pDNA

Further concentration and purification steps necessary for electroporation of ligated plasmids were carried out with 2-butanol precipitation according to a standard protocol: an equal volume of isopropanol (2-butanol) was added to the DNA as well as 3 M Na-Acetate and 2-butanol. The sample was incubated at room temperature for 10 minutes, and subsequently on ice for further 10 minutes. Centrifugation at full speed for 30 minutes at 4°C was carried out. The supernatant was removed and the pellet was precipitated with 70% ethanol followed by another centrifugation step for 10 minutes at 4°C at top speed. The supernatant was removed and the precipitated DNA was dried at room temperature for 20 minutes. The purified and concentrated DNA was then resuspended in 20 µl sterile water.

3.2.10 Electroporation of purified plasmids

Electroporation was performed by using Biorad gene pulser at 2.5 kV, 1000 Ω and 25 µF. For electroporation, self made and ice-cold cuvettes were used by pasting up cuvettes with aluminium foil. After electroporation, cells were recovered at 37°C in 950 µl SOC-medium for 1 hour with agitation (Eppendorf, Thermomixer) and plated on LB-Agar plates containing antibiotics for selection. The plates were inversely incubated overnight in a 37°C incubator (Heraeus).

3.2.11 Sequencing

DNA sequencing was performed by AGOWA GmbH (Berlin, Germany). Samples had to be diluted with sterile water to the concentrations according to manufacturer's protocol:

Template DNA	**Volume**	**Concentration**
Plasmids	10 µl	100 ng/µl
PCR products:		
200 – 500 bp	10 µl	10 ng/µl

If sequencing with a custom primer was required, 4 µl of a 5 pmol/µl primer solution was added. Universal primers were provided by AGOWA. The sequencing data was available in the download area of the AGOWA online order system.

3.3 Genomic engineering

3.3.1 Construction of the pBR322::TN7<CAT-T7-tRNA^{AlaU} wt> and pBR322::TN7<CAT-T7-tRNA^{AlaU} mut> vector

To construct pBR322::TN7<CAT-T7-tRNA^{AlaU} wt> and pBR322::TN7<CAT-T7-tRNA^{AlaU} mut> pBR322-MCS was used as the backbone vector (fig.10). To create pBR322-MCS, PCR was carried out with the primers KpnI_MCS_for_pBR322 and KpnI_MCS_back_pBR322, the Phusion polymerase and pBR322 as a template. After PCR, fragments were purified (1874 bp) (see 3.2.3) and subsequently restricted with *Dpn*I and *Kpn*I. The *Kpn*I restriction site was located on both primer overhangs essential for religation of pBR322-MCS. In the next step, the linear pBR322-MCS was religated, transformed in electrocompetent JM109, amplified and isolated. After isolation, restriction with *Sac*I and *Kpn*I was done to proof correct ligation because only the correct plasmid can be linearised with these enzymes. After isolating the correct pBR322-MCS, restriction with *Sac*I and *Kpn*I was carried out to clone tRNA^{AlaU} wt and tRNA^{AlaU} mut genes in the plasmid backbone.

To gain the inserts, CAT-T7-tRNA^{AlaU} wt and CAT-T7-tRNA^{AlaU} mut were excised from the vector pBSK::TN7<CAT-T7-tRNA^{AlaU} wt> and pBSK::TN7<CAT-T7-tRNA^{AlaU} mut> by using the restriction enzymes *Sap*I and *Kpn*I. After electrophoresis, the 1800 bp long fragments have been isolated from the preparative gel and purified. Ligation was carried out at 16°C (see 3.2.7) and further concentration and purification steps, necessary for electroporation in electrocompetent cells, were conducted with 2-butanol precipitation (see 3.2.9). Finally, for validation both vectors were cut with the restriction enzymes *Sac*I, *Kpn*I as well as *Sap*I and *Zra*I and have been sent to AGOWA for sequencing (see 3.2.11). The correct vectors pBR322::TN7<CAT-T7-tRNA^{AlaU} wt> and pBR322::TN7<CAT-T7-tRNA^{AlaU} mut> (see fig.10) have been electroporated in BL21(DE3) and HMS174(DE3).

Construction of the vector pBR322::TN7<CAT-T7-tRNA^{AlaU} mut> could not be conducted in this work.

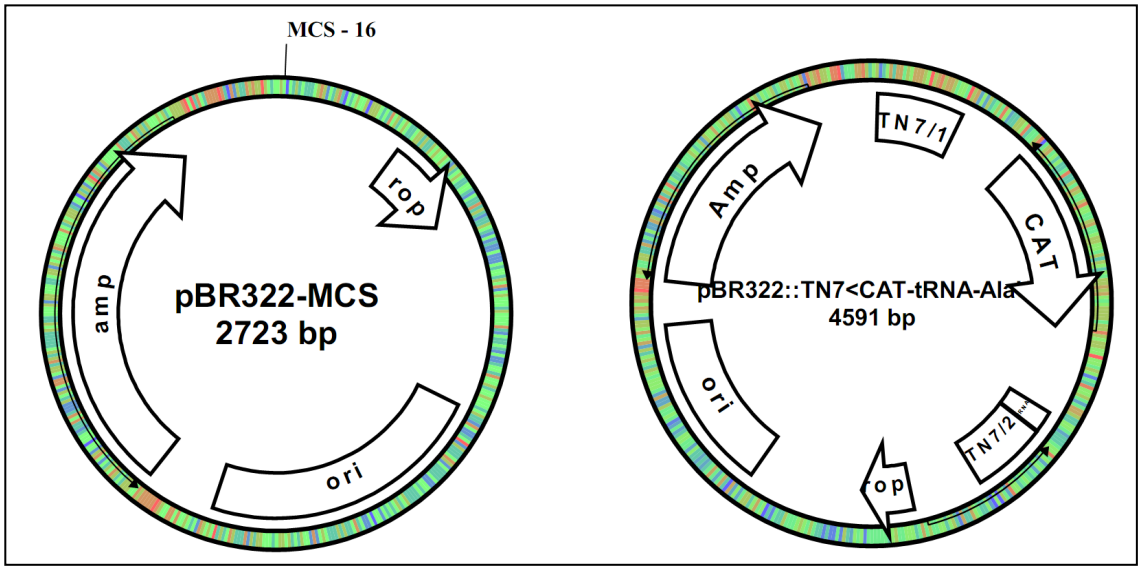


Fig.10: The linear cassette including <CAT-T7-tRNA^{AlaU} wt> was inserted into the backbone vector pBR322-MCS.

3.3.2 Construction of the pBSK::TN7<CAT-T7-*rssB*> vector

To clone *rssB* in the vector backbone pBSK::TN7<CAT-T7-GFP> (fig. 11), GFP (56 bp) was eliminated through restriction with *Bam*H1 and *Nde*I. After restriction, the linearised plasmid has been loaded on a preparative gel to cut out the correct band (4833 bp) after electrophoresis. *rssB* was amplified by conducting colony PCR of JM109 using Phusion DNAP as well as BamH1 rssB_for and NdeI rssB_back as primers. After PCR, *rssB* fragments (1014 bp) have been loaded on a gel and isolated fragments have been purified. Subsequently, *rssB* has been restricted with *Bam*H1 and *Nde*1, cloned in the linear pBSK::TN7<CAT-T7> vector and ligated at 16°C (see 3.2.7). After ligation, pBSK::TN7<CAT-T7-*rssB*> (fig.11) was electroporated in electrocompetent JM109 for amplification. To identify correct plasmids, restriction analysis was performed by using *Bam*H1 and *Xba*I as restriction enzymes. The proper plasmids have been sequenced by AGOWA and afterwords electroporated in HMS174(DE3) and BL21(DE3).

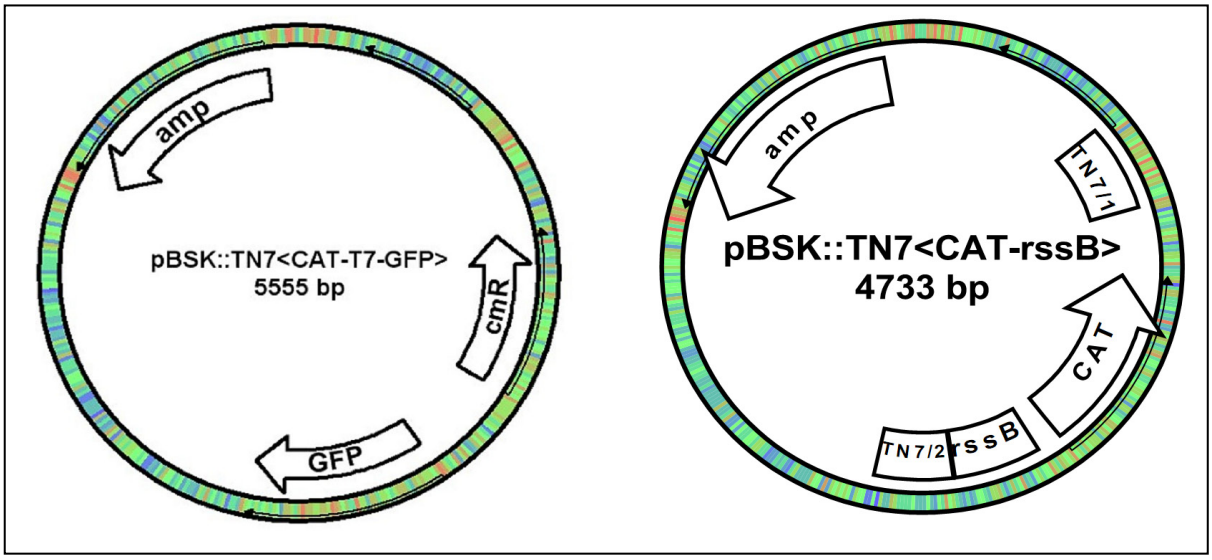


Fig.11: GFP from pBSK::TN7<CAT-T7-GFP> was replaced by the gene *rssB* to create pBSK::TN7<CAT-T7-*rssB*>.

3.3.3 Chromosomal integration via P1 vir phage transduction

To amplify linear DNA fragments encoding $tRNA^{AlaU}$ and $tRNA^{AlaU}$ mut genes (fig.12) pBSK::TN7<CAT-T7-$tRNA^{AlaU}$ wt> and pBSK::TN7<CAT-T7-$tRNA^{AlaU}$ mut> served as a template in the PCR reaction using the primers m1320 and T3 blue script. After PCR, the DNA fragments have been loaded on a preparative gel. After gel electrophoresis bands (1800 bp) were cut out and purified. According to Sauer's P1 transduction protocol, MG1655 cells containing pKD46 were cultivated over night in LB-amp media and the next day, cells were diluted 1:100 in 50 ml LB-amp containing 1% arabinose. Cells were grown at 28°C, shaked until the OD_{600} reached 0.5 and made competent (see 3.2.8). For transformation 400 ng of linear DNA fragments have been used. After electroporation in MG1655, cells have been inoculated in SOC medium for 2 hours at 30°C with agitation and plated on cm-agar plates. Plates have been incubated over night at room temperature. Randomly chosen colonies have been screened with PCR, using m1320 and T3 blue script primers, for the presence of integrated $tRNA^{AlaU}$ wt and $tRNA^{AlaU}$ mut genes and sent to AGOWA for sequencing. Recombinant colonies were cured from pKD46 by incubation at 42°C.

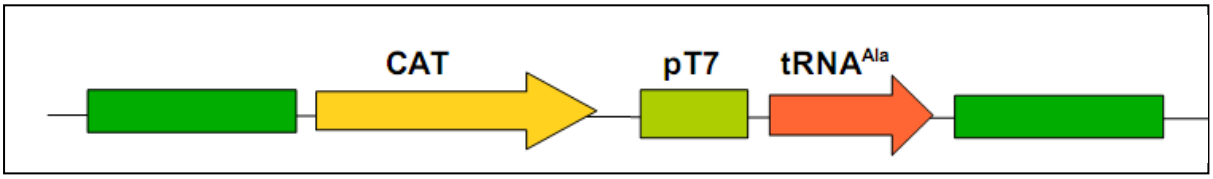


Fig.12: The linear T7-tRNA^{AlaU}-casette, obtained by PCR, contains the cm resistance gene (CAT) to select positive clones. Furthermore, the cassette includes homologous sequences on both ends facilitating integration into the hosts BL21(DE3) and HMS174(DE3).

The donor strains MG1655::TN7 <CAT-T7-tRNA^{AlaU} wt> and MG1655::TN7<CAT-T7-tRNA^{AlaU} mut> have been inoculated in an overnight culture (LB media) containing cm. The next day the MG1655 strains have been diluted 1:100 in fresh LB medium containing 5 mM $CaCl_2$ and 0,2% glucose and incubated with agitation at 37°C for 1 hour. 50 µl of the P1 page lysate was added to the culture and grown at 37°C until the culture has been lysed completely. Several drops of chloroform were added to the lysate and vortexted. The debris was centrifuged away (14,000 rpm for 2 minutes) and the supernatant was transferred to a new tube. A few drops of chloroform was added to the lysate and stored at 4°C.

The recipient strains BL21(DE3) and HMS174(DE3) were grown overnight in 12 ml LB medium. The day after, the cells have been harvested by centrifugation (6,000 rpm for 2 minutes) and resuspended in the original culture volume in fresh 50 ml LB media containing cm, 100 mM $MgSO_4$ and 5 mM $CaCl_2$. 100 µl recipient cells and 100 µl P1 lysate have been mixed and incubated at 37°C for 30 minutes. 200 µl 1 M Na-Citrate (pH 5.5) and 1 ml LB media have been added to the lysate and then incubated at 37°C for 1 hour to enable expression of cm gene. Subsequently, the cells were centrifuged at 6,000 rpm for 3 minutes, resuspended in 100 µl LB media containing 100 mM Na-Citrate (pH 5.5) and plated on cm-containing agar plates. The agar plates have been incubated at 37°C overnight and, the day after, re-streaked on a new plate to isolate cells covering with P1 phage. The re-streaked colonies have been screened with PCR (primers m1320, T3 blue script) for the presence of the tRNA^{AlaU} wt and tRNA^{Ala} mut genes (fig.13) and subsequently sequenced by AGOWA. BL21(DE3) and HMS174(DE3) have been transformed with three different plasmids (pUC19, pBR322 and pET11a).

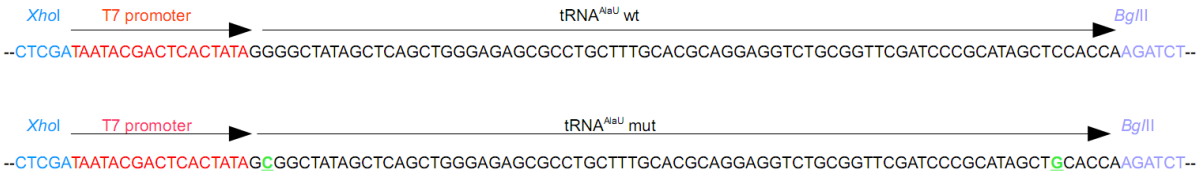


Fig.13: Chromosomal integration of tRNA^{AlaU} wt/mut in BL21(DE3) via P1 transduction. Site-directed point mutations (shown here in green) have been introduced by using mutagenic PCR-primers.

4 Results and Discussion

4.1 Regulation of PCN via IPTG-induced expression of tRNA^{AlaU} wt, tRNA^{AlaU} mut and *rssB*

The object of the following experiments was to increase the PCN in BL21(DE3) and HMS174(DE3) by stable chromosomal integration of tRNA^{AlaU} wt and tRNA^{AlaU} mut controlled by the IPTG-inducible T7 promoter. Homologous recombination at the bacterial transposon *Tn7* donor site was conducted by P1 transduction (see 3.3.3). To investigate the impact of chromosomally integrated tRNA^{AlaU} wt and mut on the replication of *Col*E1-type plasmids, cells have been electroporated with three different plasmids (pET11a, pUC19 and pBR322). A further approach to increase PCN of *Col*E1-derived plasmids was to overexpress *rssB*, cloned in pBSK.

In routine shake flask experiments these transformed strains were grown in LB medium with and without IPTG induction. For selection cm has been used. pDNA concentration was determined at hourly intervals and its concentration was estimated by both gel electrophoresis and spectrophotometry. Shake flask experiments and plasmid preparations were always conducted in triplicates.

4.1.1 Shake flask experiments and determination of plasmid concentration

BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt>, BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} mut>, HMS174(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt>, HMS174(DE3)::TN7<CAT-T7-tRNA^{AlaU} mut> containing pUC19, pBR322 and pET11a, as well as BL21(DE3) pBR322::TN7<CAT-T7-tRNA^{AlaU} wt>, HMS174(DE3) pBR322::TN7<CAT-T7-tRNA^{AlaU} wt>, BL21(DE3) pBSK::TN7<CAT-T7-tRNA^{AlaU} wt>, BL21(DE3) pBSK::TN7<CAT-T7-tRNA^{AlaU} wt>, BL21(DE3) pBSK::TN7<CAT-T7-*rssB*> and HMS174(DE3) pBSK::TN7<CAT-T7-*rssB*> have been inoculated in an overnight culture (12 ml LB-cm media). After 16 hours inoculation, cells have been diluted 1:1000 in fresh LB-cm media. After cells have reached an OD_{600} of 0.5, the expression of the target gene has been induced with 100 µM IPTG. Uninduced control cells have always been considered in this work. In one-hour intervals OD_{600} was measured (see table 7), normalized cells have been isolated and centrifuged at room temperature. After centrifugation plasmids have been isolated and their

concentration has been measured with spectrophotometry. 4 µl of the extracted plasmids have been loaded onto an agarose gel to estimate plasmid concentration.

4.1.1.1 OD_{600} measurement to evaluate bacterial growth

To measure the optical density of bacterial cultures, overnight cultures were diluted 1:1000 in 50 ml media containing antibiotics and 100 µM IPTG was added to induce overexpression of the candidate gene. In parallel, uninduced control cultures have always been included. At one-hour intervals (0h, 1h, 2h, 3h) normalized cells have been isolated and OD_{600} was quantified with a spectrophotometer. Measured OD_{600} growth values are listed in table 7.

Table 7: OD_{600} measurement to evaluate bacterial cell growth

	- IPTG				***+ 100 µM IPTG***			
HOST STRAIN	*0h*	*1h*	*2h*	*3h*	*0h*	*1h*	*2h*	*3h*
BL21(DE3) pBR322-MCS	0.518	1.432	2.864	4.034	0.548	1.344	2.526	3.760
HMS174(DE3) pBR322-MCS	0.550	1.006	1.614	2.292	0.514	0.940	1.390	1.962
BL21(DE3) pBSK	0.542	1.492	3.160	3.876	0.556	1.278	2.936	3.716
HMS174(DE3) pBSK	0.528	0.928	1.458	1.870	0.546	0.835	1.424	1.854
BL21(DE3) pBSK::TN7<CAT-T7-*rssB*>	0.547	1.421	1.983	3.259	0.521	1.298	2.490	3.631
HMS174(DE3) pBSK::TN7<CAT-T7-*rssB*>	0.502	1.510	2.628	3.692	0.502	1.512	2.600	3.500
BL21(DE3) pBR322::TN7<CAT-T7-$tRNA^{AlaU}$ wt>	0.512	1.090	2.496	3.516	0.501	0.990	1.000	1.042
BL21(DE3)::TN7< CAT-T7-RNA^{AlaU} wt> pET11a	0.506	1.394	2.242	3.396	0.589	1.514	2.362	3.378

BL21(DE3)::TN7< CAT-T7-RNA^{AlaU} mut> pET11a	0.584	1.509	2.748	3.760	0.592	1.457	2.718	3.750
BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt> pBR322	0.552	1.406	2.414	3.134	0.558	1.288	2.206	2.860
BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} mut> pBR322	0.594	1.422	2.326	3.080	0.576	1.368	2.184	2.826
BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt> pUC19	0.581	1.522	2.211	2.981	0.513	1.499	2.598	3.265
BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} mut> pUC19	0.532	1.606	2.606	3.872	0.548	1.554	2.512	3.676

In BL21(DE3) pBSK::TN7<CAT-T7-tRNA^{AlaU} wt> and BL21(DE3) pBSK ::TN7<CAT-T7-tRNA^{AlaU} mut> decreasing cell growth rate could be noted (data not shown). However, the data obtained from spectrophotometry and gel electrophoresis, where plasmid concentration was permanently reduced in the course of shake flask experiments, correspond to the decreasing OD_{600} values. One might conclude that overexpression of tRNA^{AlaU}, which may have toxic effects, is the main reason leading to reduced or even decreasing growth.

However, using the same strain BL21(DE3) but transformed with pBR322::T7<CAT-T7-tRNA^{AlaU} wt> showed increasing growth rates in contrast to the strains containing pBSK::TN7<CAT-T7-tRNA^{AlaU} wt> and pBSK::TN7<CAT-T7-tRNA^{AlaU} mut>. In conclusion it is assumed that tRNA^{AlaU} exerted a gene dosage-dependent effect. Furthermore, HMS174(DE3) pBR322::T7<CAT-T7-tRNA^{AlaU} wt and mut> (data not shown), BL21(DE3) pBSK::T7<CAT-T7-*rssB*>, HMS174(DE3) pBSK::T7<CAT-T7-*rssB*> and the chromosomally inserted wt and mut tRNA^{AlaU} genes resulted in enhanced cell growth as well.

4.1.1.2 Determination of plasmid concentration

According to Summers et *al.*, under most growth conditions natural multicopy *ColE*1 plasmids are sustained at steady levels whereas related cloning plasmids are rather instable and are frequently lost (10^{-2}-10^{-5} per cell per generation) because plasmids are irregular distributed between daughter cells. Due to this instability, plasmids are

lost, PCN is constantly decreasing but multimerization is enhanced. However, natural *Col*E1 plasmids encode *cer* promoting resolution of multimers to monomers by XerCD-mediated recombination. Summers et *al.* isolated factors affecting monomerization and stabilisation of *Col*E1 when cloned into vectors (37).

The cloning vectors pBSK and pBR322, used in this work, do not contain the stability region *cer* to maintain plasmids as monomers. In *E. coli*, Chiang and Bremer studied the stability of pBR322-derived plasmids. According to their results, multimerization of those plasmids does not impact instability when cells are growing in antibiotic-free media. However, pBR322 and its derivatives are low copy-number vectors.

4.1.1.2.1 Plasmid concentration of BL21(DE3) pBSK::TN7<CAT-T7-*rssB*> and BL21(DE3) pBSK::TN7<CAT-T7-*rssB*>

BL21(DE3) and HMS174(DE3) have been electroporated with the vector pBSK::TN7<CAT-T7-*rssB*>. After isolation of normalized cells, pDNA was isolated and 4 µl of the extracted plasmids has been loaded onto an agarose gel to estimate plasmid concentration (fig.14).

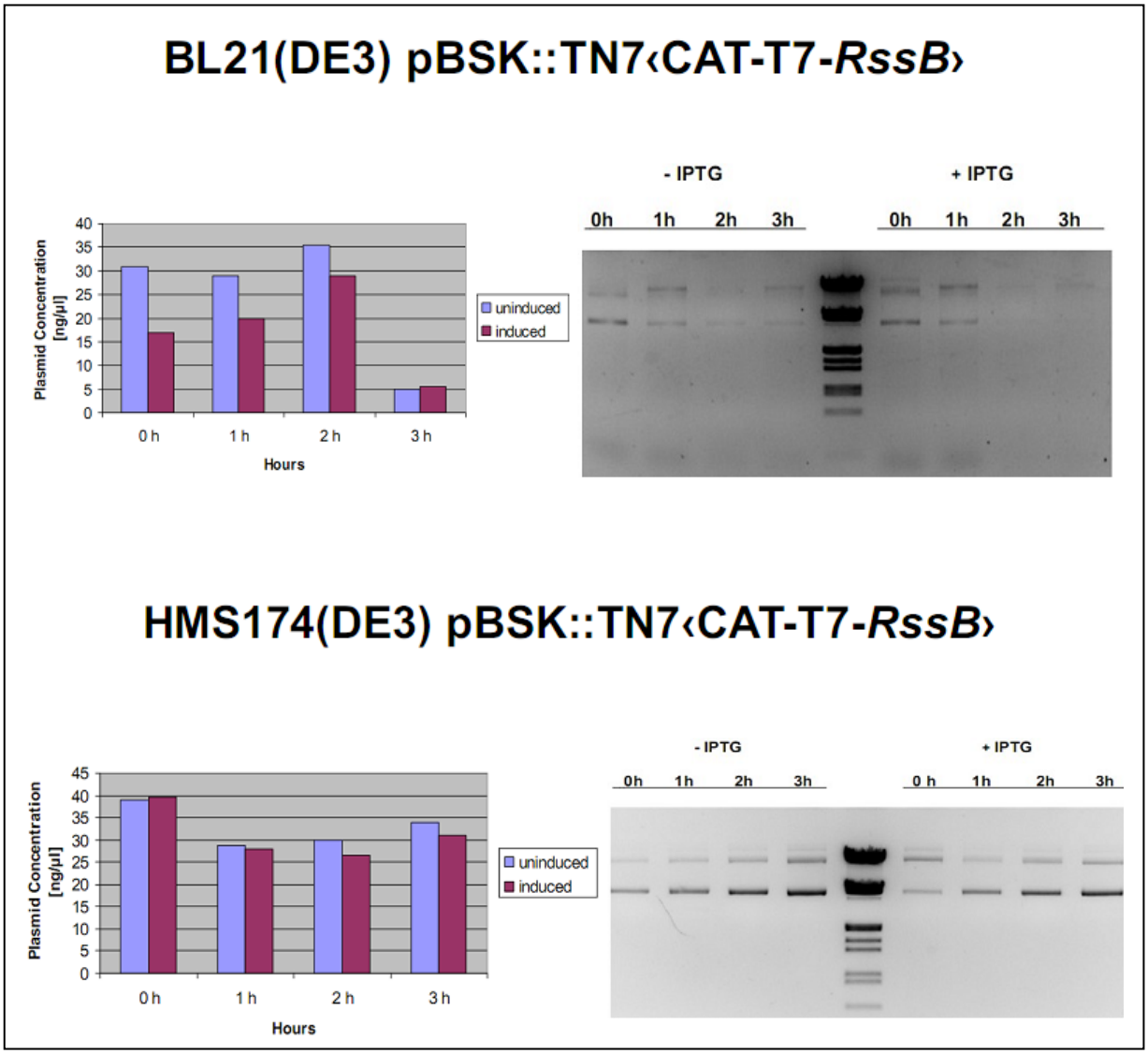


Fig.14: BL21(DE3) and HMS174(DE3) cells have been electroporated with pBSK::TN7‹CAT-T7-*rssB*› whereas *rssB* was under the control of the T7 promoter.

According to the results after electrophoresis, *rssB* was downregulating the plasmid concentration in BL21(DE3) leading to segregational plasmid instability and loss. However, measurement of cell density during shake flask experiments demonstrated stable growth of BL21(DE3) pBSK::TN7‹CAT-T7-*rssB*› (see table 7). Concluding from the control BL21(DE3) pBSK, the plasmid concentration was constantly reduced after IPTG induction. After all, results indicated that *rssB* itself was not causing segregational instability but the plasmid pBSK itself in the host BL21(DE3).

In contrast to BL21(DE3), plasmid concentration in HMS174(DE3) pBSK::TN7‹CAT-T7-*rssB*› was enhanced despite of decreasing plasmid concentration in the control cells HMS174(DE3) harbouring pBSK. That implies that *rssB* expressed in HMS174(DE3) is a factor contributing to enhanced plasmid concentration. Furthermore, in both cases *rssB* was expressed with and without induction due to the T7 promoter.

Results from Sarkar et *al.* results revealed when *rssB* was overexpressed in a K-12 strain, PCN increased four-fold. In contrast to the procedure implemented in this work, they transformed a *pcnB::kan* K-12 strain with the *Col*E1 pET19b-based T7-expression vector containing the *rssB* gene. So, *rssB* was overexpressed in a *pcn*$^-$ background by IPTG-induction but in our experimental set-up both strains are *pcn*$^+$. Concluding from that, *rssB* could not perform its suppressor function in a *pcn*$^+$ background and four-fold upregulation of plasmid concentration was not possible in that extent.

4.1.1.2.2 Plasmid concentration of BL21(DE3) pBR322::TN7<CAT-T7-tRNA^{AlaU} wt

Genetically engineered BL21(DE3) cells have been electroporated with the vector pBR322::TN7<CAT-T7-tRNA^{AlaU} wt>. After isolation of normalized cells, pDNA was isolated. To determine plasmid concentration, spectrophotometry was applied and 4 µl of the extracted plasmids have been loaded onto an agarose gel (fig.15).

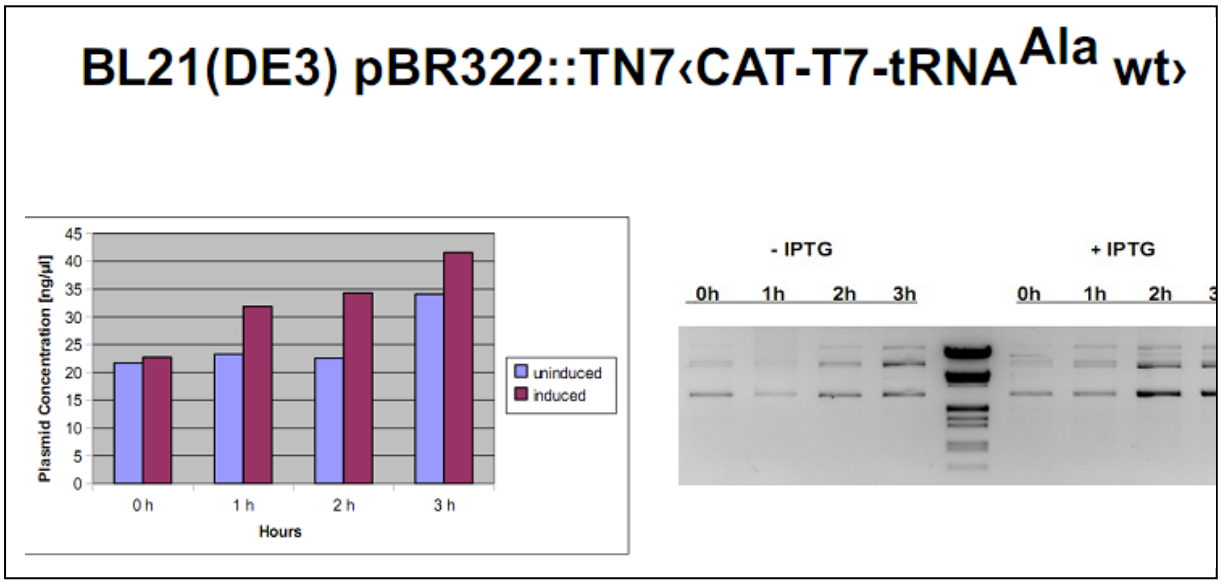


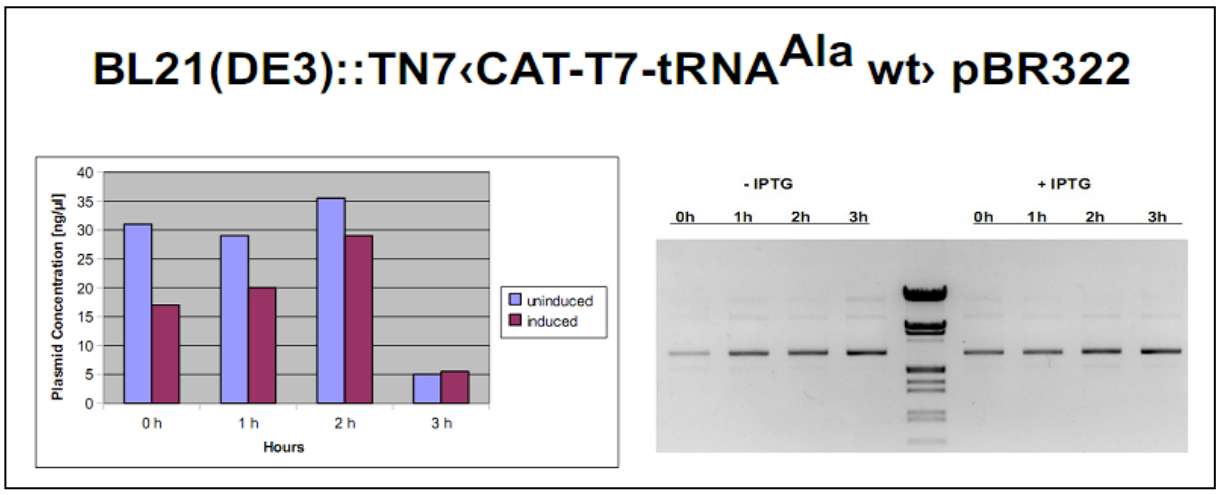
Fig.15: BL21(DE3) cells have been electroporated with pBR322::TN7<CAT-T7-tRNA^{AlaU} wt> whereas tRNA^{AlaU} was under the control of the T7 promoter.

Both data gained from spectrophotometry and gel electrophoresis demonstrated clearly increasing plasmid concentration after IPTG induction. Concluding from that tRNA^{Ala} wt might contribute to increasing plasmid concentration. Results gained from BL21(DE3) pBR322::TN7<CAT-T7-tRNA^{AlaU} mut> could not be demonstrated in this work.

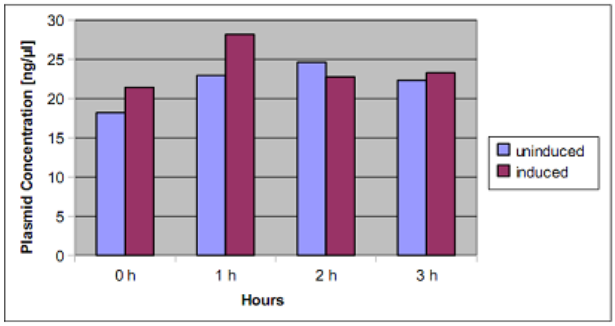
Viacheslavov and Mosevitskiĭ could show that the plasmid pBR322 in *E. coli* B exist in two forms after its isolation as it could be demonstrated in this work (see fig.15): monomers as well as multimers. It is a fact that plasmid multimerization contribute to decreased plasmid concentration still tRNA^{Ala} wt could play its role as an enhancer of plasmid concentration.

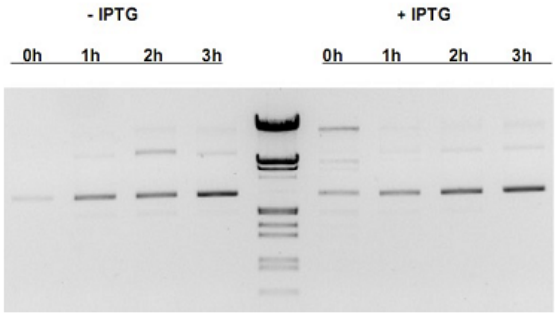
4.1.1.2.3 Plasmid concentration of BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt/mut> pBR322, pUC19 and pET11a

BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt> and BL21::TN7<CAT-T7-tRNA^{AlaU} mut> have been electroporated with the plasmids pBR322, pUC19 and pET11a. After isolation of normalized cells, pDNA was isolated, 4 µl of the extracted plasmids have been loaded onto an agarose gel to estimate plasmid concentration (fig.16).

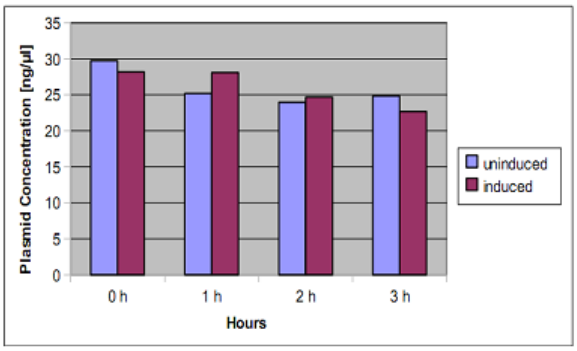


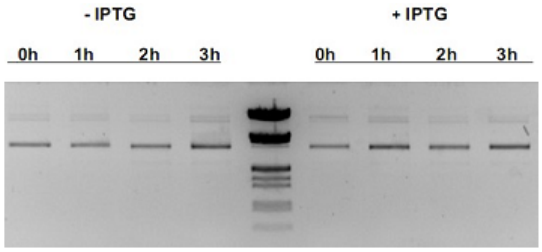
BL21(DE3)::TN7‹CAT-T7-tRNA^{Ala} mut› pBR322



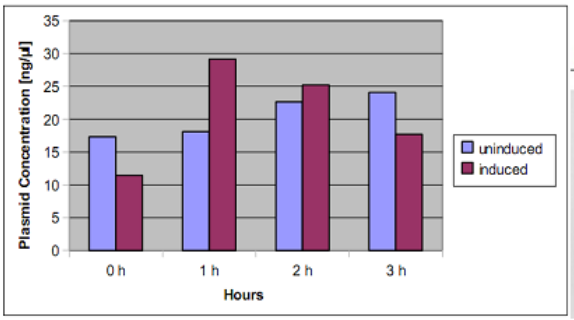


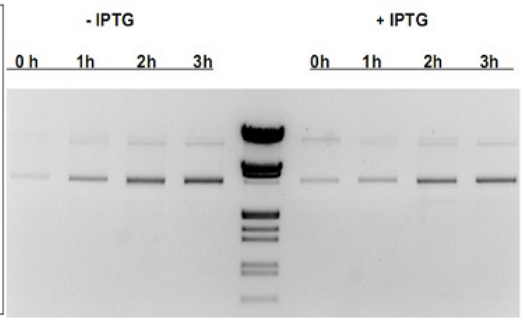
BL21(DE3)::TN7‹CAT-T7-tRNA^{Ala} wt› pET11a





BL21(DE3)::TN7‹CAT-T7-tRNA^{Ala} mut› pET11a





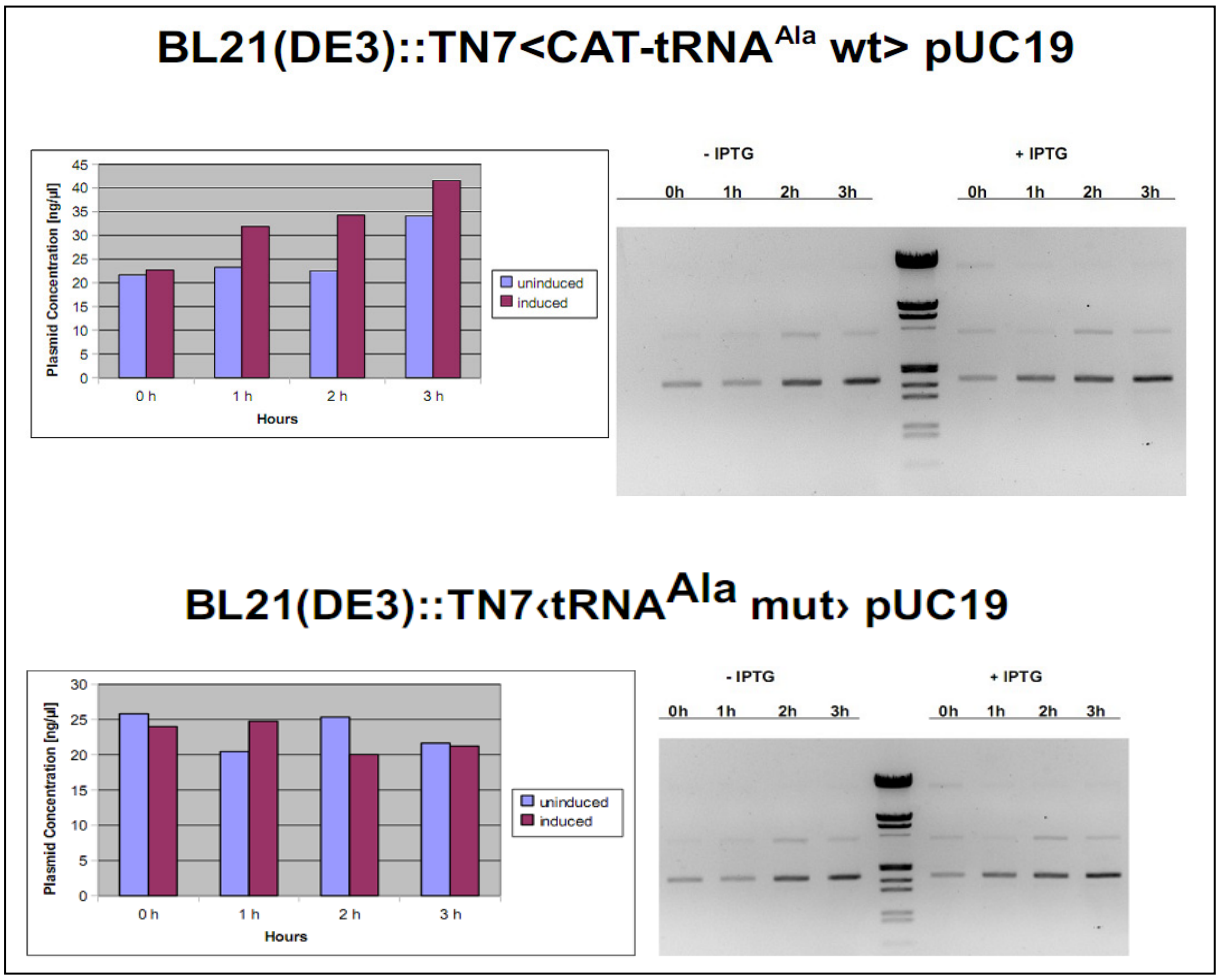


Fig.16: BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt> and BL21::TN7<CAT-T7-tRNA^{AlaU} mut> have been electroporated with the plasmids pBR322, pUC19 and pET11a.

Electrocompetent BL21(DE3)::TN7<CAT-T7-tRNA^{AlaU} wt> and BL21::TN7<CAT-T7-tRNA^{AlaU} mut> cells have been electroporated with three different *Col*E1-type plasmids (pBR322, pET11a and pUC19). The impact of induced overexpression of tRNA^{AlaU} wt and mut on plasmid concentration has been tested in shake flask experiments. Comparing the OD_{600} values of induced, uninduced and control cell lines showed clearly no major difference (see table 7) while overexpression of tRNA^{AlaU} wt and mut had an enhancing effect on plasmid concentration according to the digital photo taken after gel electrophoresis (see fig.16).

Furthermore, experimentally gained data have demonstrated definitely that overexpression of tRNA^{AlaU} wt as well as tRNA^{AlaU} mut influenced plasmid concentration in the same extent in all 6 strains. Contradictory to our assumption tRNA^{AlaU} molecules with mutations in G2 and C71 should result in enhanced plasmid

concentration compared to $tRNA^{AlaU}$ wt. *In vivo* and *in vitro* experiments confirmed that AlaRS is recognizing both the discriminator base A73 of $tRNA^{AlaU}$ and the first 4 bp of the acceptor stem (Beuning et *al.*, 2002) to initiate the aminoacylation process. Recognition of $tRNA^{AlaU}$ helix by AlaRS is clearly reduced after the base transversion G2:C71 and the same is true for its aminoacylation status (6). Concluding from that both point mutations of $tRNA^{AlaU}$ mut should contribute to increasing unloaded $tRNA^{AlaU}$ molecules.

Wang et *al.* have chosen $tRNA^{AlaU}$(UGC) as a model tRNA to enhance PCN by its overexpression in amino-acid starved *RelA* mutants. It is believed that the majority of $tRNA^{AlaU}$(UGC) molecules remain uncharged when media lacking amino-acids have been used. Furthermore, they assumed that the interaction between 3'-ACCA of the uncharged tRNA and the UGGU sequences within the RNAI loops are necessary to produce a catalytic structure that somehow cleave RNAI only in the presence of Mg^{2+} (8). The chosen strains in this work were all $RelA^{+}$ in contrast to the *RelA* mutants applied in this paper. Furthermore, cells used in this work did not grow in amino-acid starved media but to imitate the amino-acid starved situation, the $tRNA^{AlaU}$ encoding gene was mutated by a base transversion at G2:C71 at the second position in the $tRNA^{AlaU}$ helix (6). According to the publication the base transversion turned out to have a tremendous effect on recognition by AlaRS leading to a significant loss of aminoacylation, Thus, mutated $tRNA^{AlaU}$ molecules, used in this work, should be uncharged under these conditions.

Despite the high specificity of the T7 promoter for its own phage-like promoter, expression of the transgene in uninduced cells has been observed as well.

5 Conclusion and Outlook

Most individuals are carriers of defective, usually recessive, genes and some of them have or will come down with genetic diseases or disorders. By now, 2800 monofactorial genetic diseases are known to affect people's medical condition. Nevertheless, diagnostic tests have to be available to allow treatment of genetic diseases.

About $200 million per year will support the Human Genome Program in the U.S. to localize and determine all human genes, thus gaining information which is beneficial for gene therapy progress and prospect. Until now, gene defects causing Duchenne muscular dystrophy, cystic fibrosis, and retinoblastoma have been determined, and further relevant information about various genetic defects will be received in the future. Furthermore, several organizations such as the genetic disease foundation (GDF) have been founded to assist in diagnosis and treatment of genetic disorders. People expect that improvements in the field of molecular medicine will contribute to identification and treatment of various diseases within the next years to improve people's medical condition. Many clinical trials have been successful, whereas other failed.

Nearly all gene therapy trials include treatment of cancer and many of these progress to an advanced stage, involving Phase III. Although serious progress has been made on gene therapy in less than two decades, there is still necessity to evolve enhanced and safer methods to deliver genes into cells. Using naked DNA in gene therapy approaches has turned out as a convenient method but to be applied in individuals, pDNA has to be highly pure and homogenous.

Basically, various factors could raise PCN involved in reducing intracellular concentration of RNAI, increasing RNAII levels or involved in preventing RNAI-RNAII complexes (Sarkar et *al.*, 2002).

In this work the main goal was to elaborate a system that regulates plasmid replication of *Col*E1-type plasmids in *E. coli*. However, both overexpression of the wt and mut $tRNA^{AlaU}$ molecules as well as *rssB* resulted in increased plasmid concentration.

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7 Appendix

Primer sequences:

TN7 extern1	5'-ACCGGCGCAGGGAAGG-3'
TN7 extern2	5'-TGGCGCTAATTGATGCCG-3'
KpnI_MCS_for_pBR322	5'-GGCGGGGTACCCGGGGATCCT CTAGAGATAAGCTGTCAAA-3'
Kpn_MCS_back_for_pBR322	5'-ATGATGGGTACCGAGCTCGAAT TCGCACCATTATGTTCCGGATC-3'
m1320	5‘-TGTAAAACGACGGCCAGTG-3’
T3 blue script	5’-CGCGAGCGATTGTACTGAAG-3’
catbeginfor	5’-TACCTTTTAAGCTTCCTTAGCT-3’
T7prom	5‘-CAACGGTGGTATATCCAGTG-3’
pJensnestedfor	5’-TAATACGACTCACTATAGGG-3’
T7term	5’-GCTAGTTATTGCTCAGCGG-3'

8 Abbreviations

aaRS	aminoacyl-transfer RNA synthetases
AAV	Adeno-associated viruses
ADA	Adenosine Deaminase
AIDS	Acquired Immune Deficiency Syndrome
amp	ampicillin
Amp^R	ampicillin resistance
APC	antigen-processing cell
ATP	Adenosine triphosphate
Bp	base pairs
BSA	Bovine Serum Albumin
Cm	chloramphenicol
Cm^R	chloramphenicol resistance
CTL	cytotoxic T lymphocyte
ctRNAs	countertranscribed RNAs
DMSO	dimethyl-sulfoxide
DNA	desoxyribonucleic acid
DNAP	DNA polymerase
dNTP	deoxy nucleotide triphosphates
DPCs	MEA-dynamic PolyConjugates
E. coli	*Escherichia coli*
EMEA	European Agency for the Evaluation of Medicinal Products
FDA	Food and Drug Administration

GFP	Green Fluorescent Protein
GOI	gene of interest
G	gram
H	hours
HIV	Human immunodeficiency virus
HSV	Herpex simplex virus
IgG	Immunoglobulin G
ITR	inverted terminal repeats
IPTG	isopropyl β-D-thiogalactopyranoside
Kan	kanamycin
Kan^R	kanamycin resistance
L	liter
LB media	Luria-Bertani media
M	molar
MEAs	masked endosomolytic agents
MHC	major histocompatibility complex
mRNA	messenger RNA
mut	mutated
NaCl	sodium chloride
OD_{600}	optical density at a wavelength of 600 nm
ORI	origin of replication
PAC	P1 artificial chromosome
PCN	plasmid copy number
PCR	polymerase chain reaction
pDNA	plasmid DNA

PP_i	inorganic pyrophosphate
ppGpp	guanosine tetraphosphate
RNA	ribonucleic acid
Rom	RNA One Modulator
Rop	Repressor of primer
rRNA	ribosomal RNA
SCID	severe combined immunodeficiency
Ss	single-stranded
SV	Synthetic vehicles
SV40	Simian-Virus 40
Tet	tetracycline
Tet^R	Tetracycline resistance
T_m	melting temperature
tRNA	transfer ribonucleic acid
WHO	World Health Organization
Wt	wild type
XSID	X-linked severe combined immunodeficiency

Lebenslauf

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Volksschule	09/1980 bis 06/1984	Wien
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Handelsschule	09/1988 bis 06/1991	Wien
Handelsakademie	09/1992 bis 02/1994	Wien
Oberstufenrealgymnasium (Zweiter Bildungsweg)	09/1994 bis 09/2001	Wien
Universität Wien, Studium der Ernährungswissenschaft	10/2001 bis dato	
Universität Wien, Studium der Biologie (Studienzweig Genetik/Mikrobiologie)	10/2003 bis 12/2010	