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Abstract

The application of naked DNA instead of therapeutic proteins has become a main subject of medical investigation. The development of new therapeutic methods like gene therapy and DNA vaccination has caused a growing demand for high quality plasmid DNA (pDNA).
For an optimal yield of pDNA, it is important to be able to regulate plasmid copy number (PCN) as extensive plasmid replication exerts metabolic burden on the host cell, and as a consequence leads to growth cessation.

In this work, control of PCN of ColE1-type plasmids should be achieved by RNA-RNA interaction as well as by RNA-tRNA interaction.
In a first approach the RNAI-gene was inserted into the *E.coli* chromosome and put under the control of the T7 promoter. By the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG), RNAI-expression was induced in shake flask experiments and the influence on different ColE1-type plasmids was followed. It could be shown that extensive RNAI expression resulted in a dramatic decrease of PCN. As this effect was also observed in the case of no IPTG addition the leaky T7-system was exchanged by the pL_{lacO-1} promoter. By the use of this promoter, RNAI expression was restricted to IPTG addition.

In a second approach regulation of PCN should be achieved by tRNA over expression. It was reported recently, that uncharged tRNAs can interact with RNAI, thereby mediating cleavage of RNAI. Reduction of functional RNAI should further lead to an increase in the number of uninhibited RNAII molecules and thus, in enhanced initiation of plasmid replication. Therefore, a chromosomal copy of the $tRNA^{Ala}$-gene was brought under the control of the T7 promoter. Because of two point mutations charging of tRNA molecules was inhibited. Expression was again induced by the addition of IPTG in shake flask experiments and PCN of ColE1-type plasmids was determined.

With this work it could be demonstrated, that PCN of ColE1-type plasmids can be regulated and reduced, respectively by the expression of a chromosomal copy of the RNAI-gene under the control of the pL_{lacO-1} promoter. The over expression of

uncharged tRNAs however, showed an unexpected effect. Contrary to publications, predicting an increase in plasmid replication, expression of tRNA led to a clear reduction of PCN.

Zusammenfassung

Die therapeutische Verwendung von DNA anstelle von Proteinen ist zu einem bedeutenden Gegenstand medizinischer Forschung geworden. Die Entwicklung neuer Therapieformen wie der Gentherapie und DNA-Impfstoffen hat den Bedarf an hochwertiger Plasmid-DNA stark gesteigert. Die Regulation der Plasmidkopienzahl (PCN) ist dabei für eine optimale DNA-Ausbeute ausschlaggebend. Eine zu starke Plasmidreplikation würde nämlich zu einer Überlastung und schließlich zum Zusammenbruch des bakteriellen Stoffwechsels führen.

Gegenstand dieser Arbeit war es die Replikation von Plasmiden mit ColE1-ori mittels RNA-RNA-Interaktion sowie tRNA-RNA-Wechselwirkung regulierbar zu machen. Dazu wurde in einem ersten Schritt das RNAI-Gen unter der Kontrolle des T7-Promotors ins bakterielle *Escherichia coli (E.coli)* Genom integriert. In Schüttelkolbenversuchen wurde anschließend die RNAI-Expression durch die Zugabe von Isopropyl-β-D-thiogalactopyranosid (IPTG) induziert und ihre Auswirkung auf die Replikation verschiedener ColE1-Plasmide untersucht. Es konnte gezeigt werden, dass die Überexpression von RNAI zu einer starken Reduktion der PCN führt. Da es sich beim T7-System allerdings um ein sehr undichtes handelt, wurde dieser Effekt auch im uninduzierten Zustand sichtbar. Deshalb wurde der T7-Promotor in einem weiteren Schritt durch den pL_{lacO-1} Promotor ersetzt wodurch eine regulierbare RNAI-Expression möglich wurde. Nur im Fall der Induktion durch IPTG wurde die PCN gesenkt.

In einem weiteren Ansatz wurde versucht die Plasmidreplikation durch die Überexpression von unbeladenen tRNAs zu steigern. Dazu wurde das $tRNA^{Ala}$-Gen unter die Kontrolle des T7-Promotors gebracht und wiederum ins *E.coli* Genom integriert. Zwei Punktmutationen in diesem Gen verhinderten dabei die Beladung der tRNA-Moleküle mit Alanin. Auch in diesem Fall wurde die Genexpression in Schüttelkolbenversuchen durch die Zugabe von IPTG induzierte, und deren Auswirkung auf die Replikation von ColE1-Plasmiden untersucht. Laut bereits existierenden Publikationen, sollte es zur Wechselwirkung zwischen unbeladener tRNA und RNAI kommen. Der daraus resultierende Anstieg an freier RNAII sollte in weiterer Folge zu einer Erhöhung der PCN führen.

Mit dieser Arbeit wurde gezeigt, dass die PCN von ColE1-Plasmiden über die Expression einer chromosomalen Kopie des RNAI-Gens unter dem pL_{lacO-1} Promotor reguliert bzw. reduziert werden kann. Die Expression des chromosomalen $tRNA^{Ala}$-Gens bewirkte, entgegen den Erwartungen, eine Senkung der PCN.

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1 Introduction

1.1 Bacterial Plasmids

The majority of bacteria have only one circular chromosome suspended in the cytoplasm without a surrounding nuclear membrane. The chromosome size in a typical bacterial cell can range from 0.6-6 megabase paires (Mbp) (1) and is 4.64 Mbp in *Escherichia coli* (*E. coli*) (2). In addition, tiny separate minichromosomes or so called plasmids exist in the cytoplasm of bacteria. Plasmid size can vary from 1 to over 200 kilobase pairs (kbp) and the number of identical plasmids within a single cell can range anywhere from one to even thousands. Plasmids are not required for normal growth but might become essential under special environmental conditions. They carry a handful of genes that usually have a protective role in the cell. These genes provide selective/competitive advantage to the plasmid-containing host as they often carry genes involved in antibiotic resistance, degradation of substrates foreign to the species or tolerance to heavy metals. Plasmids can also be transferred from one bacterial cell to another by 3 different mechanisms namely conjugation, transformation and transduction. This enables cells to exchange their genetic material and new genes can be transferred between members in the “common gene pool” (3). Generally bacterial plasmids are inherited in a very stable manner, independently from the cell chromosome. In the case of high-copy-number plasmids, the copy number and cell division ensure a very low frequency of plasmid loss. Low copy number plasmids posses certain mechanisms which enable their maintenance during cell growth in non selective conditions (4). So called plasmid addiction systems reduce the number of plasmid free cells. Two genes are necessary for a functional killer system: one gene encodes a stable toxin which is always a protein and the second gene encodes for an antidote with short lifetime which can either be an antisense RNA or a protein. In the case of plasmid loss, the toxin would kill its host cell, as there is no antidote that can neutralize the stable toxin. Different postsegregational killer systems exist in bacteria and are reviewed in (5).

One example for such a mechanism is the toxin colicin E1 which is produced by the ColE1 plasmid and kills plasmid free cells. Further, there are also some plasmids that

are able to produce conjugation inhibitors that prevent other plasmids to enter the same host. Each plasmid has an origin of replication (ori) to provide for duplication of the plasmid within the host cell (6). Basically, plasmids can replicate in an autonomous and self-controlled way but they also extensively use the replication machinery of the host (7).

1.1.1 ColE1 replication mechanisms

There are several different replicons existing in *E.coli* including F, R and ColE1. The most widely used plasmids in biotechnology are derivatives of the plasmid pBR322 which carry a ColE1 origin of replication. The characteristics of these plasmids are a theta replication mechanism, the production of high plasmid copy numbers, a relatively low molecular weight and the requirement of only host-encoded proteins. ColE1 plasmids have been extensively studied and used as vectors for gene therapy and DNA vaccines (8, 9).

Two different RNA molecules are responsible for replication of ColE1 plasmids. They are encoded in a region of about 600 base pairs. RNAII is a 555 nucleotide molecule which acts as a primer for DNA Polymerase I and thus, mediates plasmid replication. When RNAII binds to the plasmid, it is cleaved by the host-encoded enzyme RNase H. Due to this cleavage the 3'OH of RNAII is released and can be then elongated by DNA Polymerase I. The second important RNA molecule that regulates plasmid replication is the 110 nucleotide RNAI. This molecule is able to bind to RNAII thereby inhibiting the primer function of RNAII and thus negatively controls plasmid replication. Interaction of RNAI and RNAII requires a folded structure of both molecules. In a first step RNAI and RNAII form a transient so called "kissing complex" which is followed by RNA duplex formation. The initial interaction of RNAI and RNAII is stabilized by a small, 63 amino acids long, dimeric protein that is called Rom or Rop. Rom/Rop processes the complementary loops for the formation of the "kissing complex" (Fig.1.1.) (8, 9, 10).

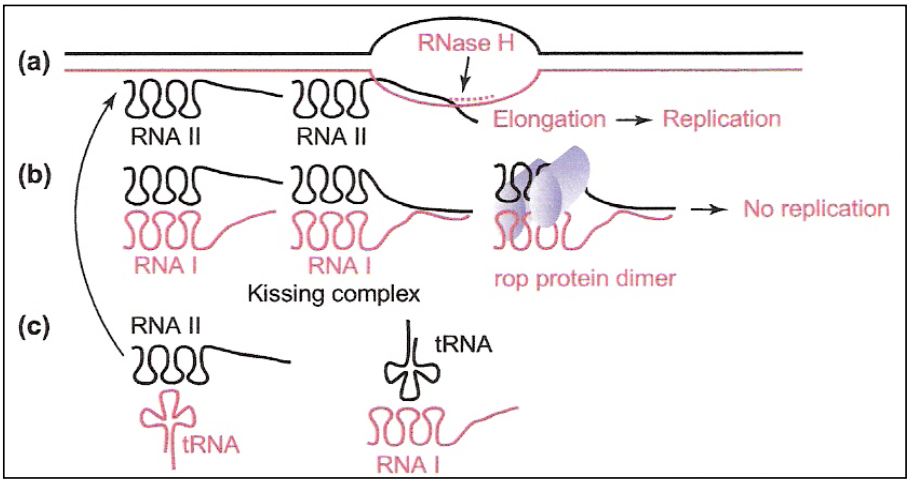

Fig.1. 1: Regulation of plasmids with ColE1 replication origin. (a) RNAII acts as a primer for DNA Polymerase I after being processed by RNase H. (b) By the binding of RNAI and the stabilization of the "kissing complex by Rop/Rom, plasmid replication is inhibited. (c) Uncharged tRNAs can interact with RNAI resulting in free RNAII which in turn enhances plasmid replication (picture taken from Grabherr et al., 2002).

Different mutations concerning the different members of ColE1-replication machinery have been used to convert pBR322-derived plasmids into high copy number plasmids. The pUC19 plasmids for example carry a point mutation in the RNAII sequence providing high copy numbers (11, 12).

Interestingly also uncharged tRNAs are able to influence the ColE1-replication mechanism. In *E.coli* wild type cells, amino acid starvation results in the appearance of large amounts of the alarmones guanosine pentaphosphate (pppGpp) and guanosine tetraphosphate (ppGpp). Production of these molecules is promoted by the ribosome associated protein RelA. RelA itself is activated by the binding of uncharged tRNAs to the ribosomal A site. Production of the alarmones results in the cessation of transcription of stable RNAs (rRNAs and transfer RNAs). This effect is termed the stringent response. As expression of ribosomal protein genes is controlled by rRNA levels, the stringent response includes a large-scale downregulation of the translation apparatus (13). In relaxed mutant cells (*relA*), production of ppGpp is inhibited. As a result, uncharged tRNAs are accumulating inside the cytoplasm. It was shown that in the presence of large amounts of tRNA, three products of specific cleavage of RNAI could be observed. It is not yet sure, if cleavage of RNAI is

mediated by a direct or an indirect effect of tRNA. However, this tRNA-induced RNAI decay further leads to free RNAII which can prime replication efficiently thereby leading to an increase in plasmid copy number (14).

Recent studies showed that the ColE1-replication mechanism can also be used for an antibiotic free plasmid selection system. Although the injection of pDNA containing an antibiotic resistance gene is currently tolerated by regulatory authorities because of no alternatives, there are some risks that cannot be excluded involving for example gene transfer to environmental organisms. In this antibiotic free plasmid selection system, RNAI is able to bind to the mRNA of a certain modified repressor (TetR). The repressor itself inhibits the expression of an essential gene (*murA*) that is located on the host's chromosome and involved in bacterial cell wall synthesis. If the ColE1-like plasmid is present in the host, RNAI is expressed and can bind to the repressor mRNA. As the repressor is inhibited by RNAI, the essential gene can be expressed and cells owning the plasmid are thus able to grow (10).

1.2 DNA as a pharmaceutical product

Plasmids are currently evaluated for DNA vaccination or gene therapeutic applications, both for human and veterinary indications. Thus, there is a rapidly growing need for high quality plasmid DNA for drug applications (8).

1.2.1 Therapeutic plasmids

Therapeutic plasmids used for medical purpose must carry several important genetic components: a prokaryotic replication origin, a eukaryotic promoter, a certain target gene a selection marker and a polyadenylation signal sequence (poly-A) (Fig.1.2.). The prokaryotic replication origin is necessary for the amplification and propagation of the plasmid within the host. The eukaryotic promoter is responsible for the expression of the target gene which encodes a therapeutic protein or RNA molecule. Usually, the marker gene constitutes an antibiotic resistance gene which allows plasmid propagation and selection for plasmid carrying clones. The poly-A sequence

is necessary for successful termination of transcription and further provides stability as well as effective translation (8).

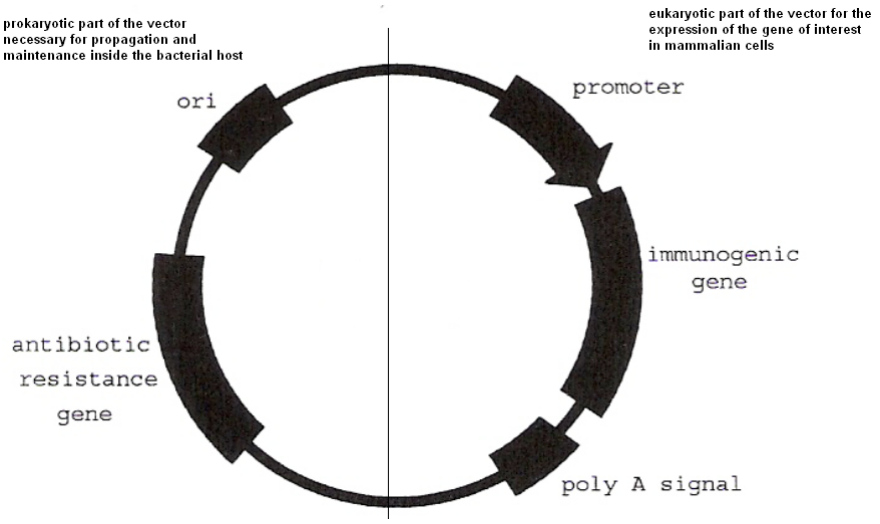

Fig.1. 2: Design of therapeutic plasmid consisting of genetic components necessary for medical purpose (picture taken from Dhama et al., 2008).

1.2.2 pDNA for Gene Therapy and DNA vaccines

Plasmid DNA has become especially important in the field of gene therapy and DNA vaccination, and, more recently, plasmid-based gene silencing is gaining importance. Whereas gene therapy involves the introduction of a gene into cells to produce a therapeutic protein to treat genetic diseases, DNA vaccination involves the introduction of a gene into cells to produce antigen to prevent infectious diseases. The expression of dsRNA from plasmids is another technology of rising medical interest as it is much cheaper than the chemical synthesis of double strand RNA and further allows large scale application (8).

When dsRNA of a certain gene is expressed in the cells, it gets degraded by different enzymes. These enzymes are then screening other mRNAs for the same sequence. Once found, the mRNA gets degraded and no protein can be produced. This mode of action leads to silencing of the targeted gene.

The advantage of plasmid-based drugs compared with protein-based gene therapy and vaccination is that they allow for better processing, quality control, and large-

scale production (11). Today the delivery of DNA instead of whole proteins is gaining more and more in importance (8).

1.2.2.1 DNA vaccines

Today, plasmid DNA is also used for the development of so called DNA vaccines. These vaccines constitute an effective way for the in vivo expression of desired proteins to initiate immune response. DNA vaccines should work as follows: The plasmid DNA carries a gene encoding a viral, parasitic or bacterial antigene that can be expressed in mammalian cells. When the plasmid is injected into the host, the gene is transcribed and translated into the antigenic protein. This protein then causes a specific immune response and antibodies are produced against the expressed antigen. Further, the class I-restricted MHC antigen presentation pathway for cytotoxic T-cell induction can be activated. The advantages of DNA vaccines include the simplicity of manufacture, biological stability, cost effectiveness and safety, ease of transport in lyophilized form and the ability to act in presence of maternal immunity. Furthermore, it is possible to combine different genes simultaneously, giving rise to multivalent vaccines (15).

Another advantage of using DNA isolated from bacteria is the presence of unmethylated CG sequences in prokaryotic cells. These sequences act as natural adjuvants, stimulating the innate immunity through toll-like receptor (TLR) 9. Expression of the encoded protein will be automatically at a site where resident antigen presenting cells (APCs) such as dendritic cells are activated through stimulated TLR9. Once activated APCs cause local inflammation, enhanced antigen processing and presentation, venue of immune cells from the blood as well as migration of APCs to the lymphoid organs. All these effects are achieved by the injection of naked pDNA without any additional adjuvants. The major disadvantage of pDNA vaccines is their capacity to persist in vivo and their putative ability to modify the genetic information of some host cells by integration into the chromosome (16).

Plasmid DNA is also used for anti-cancer vaccination. In order to be distinguishable from normal tissue cells by the immune system, cancer cells should present target structures on their cell surface. The ideal target antigen would be exclusively expressed in tumor cells with no expression in normal adult tissues. More and more

tumor associated antigens are getting identified today. They are classified into four different groups: differentiation antigens, cancer/testis (CT) antigens, mutated antigens and ubiquitous antigens, which are overexpressed in cancer cells. The first anti-tumor vaccine which was recently authorized is a pDNA coding for the human melanoma antigen tyrosinase and is used for the vaccination against melanoma in dogs (16).

In the field of veterinary medicine, the last few years have seen numerous trials of DNA vaccines against various animal diseases like foot and mouth disease (FMD) and herpes virus infection in cattle, Aujeszky's disease and classical swine fever in swine, rabies and canine distemper in canines, and avian influenza, infectious bronchitis, infectious bursa disease and coccidiosis in bird (15).

Concerning DNA vaccine development for humans, there is a promising progress in the field of the treatment of multiple sclerosis. Three companies, namely Orchestra Therapeutics, Opexa Therapeutic and Bayhill Therapeutics have designed DNA vaccines to treat multiple sclerosis, which are already in phase II development and could reach the market by 2012 (17, 18, 19).

Large Scale Production

For the production of large amounts of plasmid DNA, recombinant *E.coli* cells are used routinely. Alternatively, in vitro methods for DNA production are very expensive and would afford specialized equipment. Purification of DNA is one of the most challenging parts of production procedure as it has to be separated from the very similar host DNA. In the pDNA production process there are three main base unit operations: fermentation, cell lysis and downstream processing (20).

Plasmid Vector optimization

Vectors have to be optimized before used in fermentation processes as they must ensure acceptable fermentation production yield and quality as well as an acceptable purity after downstream processing. Quality and yield of DNA are strongly affected by the organization of elements, as well as the specific elements included. AT rich regions for example have a negative influence on the supercoiling of DNA. These regions are susceptible to endogenous single stranded nucleases as their binding to each other is not that strong. Enrichment of supercoiled DNA is desirable because it

is generally regarded as the physiologically active conformation and thus optimal for the transfection of eukaryotic cells. (20, 21, 22).

Further it is important to inhibit the formation of replication intermediates. These linear fragments are observed when the origin is close to and parallel with the CMV promoter, as in this case, replication tends to terminate. The function of plasmids in eukaryotic cells can also be disturbed because of certain sequence orientations (46, 23, 24, 25). It was shown for example, that the orientation of the kanamycin resistance gene dramatically affects expression from the adjacent CMV promoter, as well as overall plasmid yield (26). All the points mentioned above have to be kept in mind when designing an effective plasmid vector.

In order to obtain high yield of plasmid DNA, high copy number plasmids are used for pDNA production. However, a too extensive plasmid replication can also lead to a metabolic overload of the host. Therefore it is important to be able to regulate PCN. Therapeutic plasmids typically contain a ColE1 or pBR322 derived replication origin carrying certain mutations to increase replication frequency. Such mutations include for example a *rop* deletion or a G to A point mutation (28). Further there are temperature sensitive origins (e.g. pUC, pMM1, pMM7) which allow a 30- to 40-fold increase in PCN when temperature is shifted from 30°C to 42°C (27, 28)

1.3 Genomic Engineering of pDNA production hosts

Genomic engineering comprises different techniques. However, they all involve DNA manipulation. Today it possible to isolate single genes from an organism, determine the gene sequence, to manipulate or alter the base sequence and thereby the function of the protein and to transfer the isolated gene from one organism to another. One field of genomic engineering is the integration of linear DNA fragments into the chromosome of a certain host via homologous recombination (6). The mechanism of homologous recombination in *E.coli* is described in the next chapter.

1.3.1 Homologous recombination in *E.coli*

The mechanism of homologous recombination is more fully established in *E.coli* than in eukaryotic cells. However, it is likely, that both cell types have much in common in this respect. The event of genetic recombination naturally occurs in vivo between two separated chromosomes that share homologous sequences. This genetic mixing is very important as it constitutes the basic for genetic diversity.

The first step of homologous recombination is the so called single strand invasion which is mainly catalyzed by the enzyme RecA. During single strand invasion a single strand of DNA invades the DNA duplex molecule at a site of complementary base sequences. Multiple copies of RecA bind to the single stranded DNA and the 3'end of the single strand inserts into the DNA duplex. It hybridizes via complementary sequences and displaces the original strand creating a structure called D-loop. The affinity of RecA to the DNA single strand is ATP dependent. When ATP is bound to RecA, it mediates strand invasion by binding to the DNA. Hydrolysis of ATP leads to loss of affinity and RecA dissociates again from the DNA.

In order to create the free 3'ends of the invading strand, one strand of each DNA duplex must be nicked by an endonuclease. Both strands then invade complementary DNA duplexes thereby resulting in the formation of the so called Holliday junctions. The cross-over junction randomly moves along the chromosomes just as long as there is homology. This movement is called branch migration.

In a last step, the DNA duplex bound together must be separated. This is done by nicking of the non invading strands. But before this can be carried out, the Holliday junction is rearranged in three dimensions to uncross the strands, a process called isomerization. The subsequent nicking of the noninvading strands results in the exchange of chromosome arms and in genetic recombination (6, 29).

As the mechanism of homologous recombination is based on the presence of complementary sequences, it constitutes an appropriate tool for the insertion of DNA fragments flanked by homologous regions. In this way, external genes can be inserted into the host’s chromosome or functional genes can be disrupted.

1.3.2 Chromosomal integration of linear DNA fragments

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1.3.2.1 Insertion of linear DNA fragments by RecET recombinase

The problem of transforming bacteria with linear DNA fragments is that there are exonucleases present inside the cells that degrade linear DNA. It has been shown, however, that mutants lacking exonucleases V of the RecBCD recombiation complex are transformable with linear DNA (22). The single mutation of *recD* has led to mutant strains that were still able of recombination but lacked exonuclease activity. But, as already known for a long time, many bacteriophages own recombination systems, much more efficient than those of the *recD* mutant strains. One of these efficient recombination systems is the λ Red system of phage lambda. This system includes three genes: *exo, ß* and *γ* whose products are called, Exo Bet and Gam. Gam inhibits the RecBCD exonuclease V. In this way Bet and Exo can gain access to DNA ends to promote recombination. Plasmids have been produced encoding the λ Red system like for example the pKD20 or pKD46 vector where the recombination system is under the control of an arabinose-inducible promoter (30). For integration into the host genome, linear DNA fragments with 36- to 50-nt homology extensions are produced by PCR. These fragments can than be used to transform *E.coli* cells carrying a Red helper plasmid (pKD20, pKD46, pRED/ET). Expression of the genes of the λ Red system promotes integration of the linear DNA fragment in a site specific way according to the complementary flanking sequences (30).

1.3.2.2 Insertion of linear DNA fragments by P1 Transduction

Another, older, method of chromosomal integration constitutes the use of the phage P1. As the γ-red gene, which is necessary for homologous recombination, is toxic under some conditions, chromosomal integration is not applicable in *recA*- strains of *E.coli*. (31). Since all production hosts for recombinant plasmid or protein production are *recA*-, as this mutation secures genomic stability, chromosomal engineering is performed in the wild-type environment of MG1655 and then transferred into the strain of choice by P1 viral transduction. Phage transduction is used to move selectable genetic markers from one “donor” strain to another “recipient” strain. Phage P1 is commonly used as a transducing agent because it is a generalized transducer giving rise to “transducing particles”. During the replication and lysis of the phage in a culture of bacteria, a small percentage of the phage particles will contain a

genome segment that contains the gene of interest. P1 packages approximately 90 kb of DNA, so it is possible to transduce genes that are linked to a selectable marker. Once a phage population has been generated from a donor strain, the phage is used to infect a recipient host. Most of the bacteria are lysed by phage that packaged P1 genomes, but a fraction of the phage inject a genome segment derived from the donor host. Homologous recombination then allows the incoming genomic segment to replace the existing homologous segment. The infected recipient bacteria are plated on a medium that selects for the genome segment of the donor bacteria.

All of this would not work if the infectivity of the phage could not be controlled. Otherwise, phage released from neighbouring cells would infect and lyse the bacteria that had been infected with transducing particles. Phage P1 requires calcium for infectivity. Therefore, P1 infectivity can be controlled by growing in the presence and absence of calcium.

1.4 Inducible expression of genes

1.4.1 The T7-System

The T7-system is one of the most widely used systems for recombinant protein production. It permits controlled induction of gene expression via IPTG and as the T7-promoter is a very strong one, leads to high transcription levels and protein yield. This strategy was developed in 1986 by W.F. Studier and B.A. Moffatt, who created an expression system which was highly selective for bacteriophage T7 RNA polymerase. The initial system involved two different methods of maintaining T7 RNA polymerase in the host cell – in one method, a lambda bacteriophage was used to insert the gene which codes for T7 RNA polymerase, and in the other, the gene for the polymerase was inserted into the host chromosome. This expression system has become known as the pET system. The pET vector contains several important elements –a T7 promotor which is specific to only T7 RNA polymerase, a *lac* operator which can block transcription, a multiple cloning site, an f1 origin of replication, a resistance gene, a ColE1 origin of replication and occasionally, some plasmids also carry a *lacI* gene which codes for the *lac* repressor protein, (Fig.1.3.).

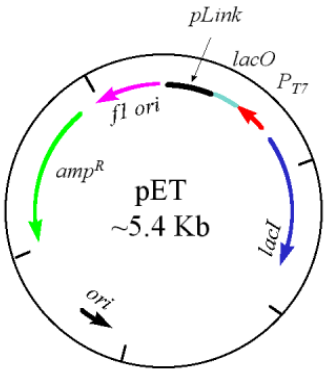

Fig.1.3: The pET vector (picture from Alberghina et al., 2000)

The first step in this expression system is to clone the gene of interest (GOI) into the vector at the multiple cloning site. Transcription of the GOI is restricted to the presence of the T7 RNA polymerase. The host cell is genetically engineered to incorporate the gene for T7 RNA polymerase, the *lac* promoter and the *lac* operator in its genome. The inducible promoter of the polymerase gene can then be activated by IPTG. The T7 RNA polymerase is expressed and quickly begins to efficiently transcribe the GOI (32).

1.4.2 Tight regulation of gene expression via highly controllable promoters

Promoters like those of phage T7 or phage lambda, as well as the promoter of the lac operon have so far been widely used for the control of gene expression. However, all of these promoters also have serious limitations. Whereas the T7 system is a very strong and leaky one, which induces gene expression even in the absence of the inducer, the promoter of phage lambda P_L is induced by the inactivation of its repressor cI 857 via a temperature shift. This principle does not permit quantitative control on the one hand, and causes pleiotropic effects on the other hand. In the case of P_{lac}, the activation of this promoter by CRP/cAMP also affects many other operons and thus profoundly changes the metabolic state of the cell. For the construction of a tightly controllable promoter, the promoter P_L of phage lambda was altered. This promoter is a strong promoter in vivo and can be efficiently repressed by cI, the lambda repressor. In order to create a promoter that can be activated by IPTG, the cI binding site was replaced by the lacO1 sequence. This operator 1 encoding a sequence that comprises a 18bp of the lacO1 core sequence in the spacer region and a 22bp sequence upstream of the promoter. In the absence of IPTG the $P_{LlacO-1}$ promoter is tightly repressed by the lac repressor (33).

The $P_{LtetO-1}$ promoter constitutes another tightly controllable promoter system and is obtained by the integration of the tetO2 sequence in the same way as described above. This promoter is tightly repressed by the Tet repressor and can be activated by the supply with anhydrotetraclycline (33, 12).

For the construction of the $P_{lac/ara-1}$ promoter, the P_{lac} promoter was altered in different steps. In vivo this promoter is depressed by IPTG and activated by CRP/cAMP and shows intermediate activity (62, 30). The $P_{lac/ara-1}$ promoter contains the following alterations and mutations: a point mutation at position -8 (T to A), a lac operator sequence in the spacer region, a wild-type operator sequence upstream of the promoter and a deleted CRP/cAMP binding site substituted by a recognition site of AraC. Due to the listed modifications, this promoter is well controllable and can be activated by either IPTG or L(+) arabinose (33, 34).

All of the described inducible promoter systems require, of course, host strains, that are expressing appropriate repressors. The *E.coli* strain DH5αZ1 for example carries the genes encoding the TetR, the LacR as well as the spectinomycin resistance

marker in its genome. The repressors are thereby under the control of a constitutive promoter (33).

The development of tightly regulatable promoters allows the expression of single genes or RNA in well defined amounts. Furthermore, it is important, that these promoters are at the same time highly depressable by the constitutive expression of its corresponding repressor protein. The quantitative and temporal regulation of gene expression allows the study of the impact of single genes on cellular physiology (33).

2 Objectives

High production rates cause substantial metabolic stress for the host cell. In particular, strong expression systems or heavy plasmid replication tend to exceed the metabolic capacity of the host. Therefore, in order to maintain viability of a productive population during the entire bioprocess, it is important to be able to control plasmid replication rates. Via the control of PCN, the metabolic load can be tuned, therefore overstraining of the cell factory is avoided and thus, the yield of protein and plasmid DNA is maximized (9).

The aim of the current work was to develop strategies for regulating the copy number of plasmids with a ColE1 replication origin in *E.coli*. Two different approaches were investigated:

- Inducible expression of RNAI, the negative regulator of PCN

and

- Inducible expression of uncharged $tRNA^{Ala}$, positively regulating the PCN

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In order to achieve the aims of the current studies, the different working steps, listed below, were followed:

1) Design and cloning of expression cassettes for the inducible expression of RNA or $tRNA^{Ala}$, respectively.

2) Chromosomal insertion of the cloned constructs.

3) Investigation of plasmid copy number behaviour upon induction of the expression constructs using various assays.

3 Material and Methods

3.1 Media, growth conditions and storage of bacterial strains

3.1.1 Media

Bacterial cells were routinely grown in LB-media at 37°C shaking (table 3.1.). If cells did carry a resistance gene LB-media containing the appropriate antibiotic was used (table 3.2.). Cells were plated on LB-agar-plates supplied with antibiotics, if necessary. All antibiotics and other additives are listed in table 3.2. After electroporation, cells were put into SOC-medium for maximizing the transformation efficiency of competent cells (table 3.3.). All media were sterilised by autoclaving for 20 minutes at 121°C and 2 bar.

Table 3. 1: LB-medium (Luria-Bertani medium)

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

The components for the LB-medium were dissolved in 950 ml sterile deionized water and pH was set to 7.0 with 5 N NaOH. Volume was adjusted to 1 liter. For LB-agar, 15 g agar-agar was added per 1 liter LB-Medium. The autoclaved media was cooled to approximately 50°C before addition of an antibiotic stock solution.

Table 3. 2: Additives

	STOCK SOLUTION	FINAL CONCENTRATION
ANTIBIOTICS		
ampicillin	100 mg/ml	100 µg/ml
kanamycin	50 mg/ml	50 µg/ml
chloramphenicol	25 mg/ml	25 µg/ml
tetracycline	15 mg/ml	15 µg/ml
OTHER ADDITIVES		
IPTG	0.1 M	100 µmol
NaCitrate	1 M	10 mM
$CaCl_2$	1 M	5 mM
glucose	20%	0.2%
l-arabinose	20%	0.2%

Table 3. 3: 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 afterwards.

3.1.2 Shake flask cultures

Over night cultures were grown at 37°C shaking. The next day, cultures were diluted 1:100 in LB-medium supplied with the corresponding antibiotic. Induction of gene expression was achieved by adding 100 µM IPTG at an OD_{600} of 0,3 or by adding IPTG directly to freshly inoculated cultures. Cells were grown on 37°C at 180 rpm after induction. Uninduced control flasks were always included in the experiments.

3.1.3 Bacterial kryostocks

For conservation of bacterial strains, kryostocks were prepared. Therefore, an *E.coli* over night culture was grown in 3 ml of appropriate medium, at 37°C shaking. The next day, 600 µl of cell suspension and 300 µl of glycerine (80 %) were mixed in a 1,5 ml Eppendorf tube, and frozen at -80°C.

3.2 Bacterial strains and vectors

3.2.1 Bacterial strains

Bacterial strains listed in table 3.4. were used for the different experiments.

Table 3. 4: Description of bacterial strains

HOST	SOURCE	GENOTYPE	RESISTANCE
HMS174 (DE3)	Novagen	F– recA1 hsdR(rK12– mK12+) (DE3)	Rif^R
DH5α	Invitrogen	F- 80dlacZM15 (lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 - thi-1 gyrA96 relA1	-
JM109	Lab stock	recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi ∆(lac-proAB)	-
MG1655	Lab stock	F- lambda- ilvG- rfb-50 rph-1	-
NEB10ß	New England Biolabs (NEB)	araD139 Δ(ara-leu)7697 fhuA lacX74 galK (Φ80 Δ(lacZ)M15) mcrA galU recA1 endA1 nupG rpsL Δ(mrr-hsdRMS-mcrBC)	

3.2.2 Plasmids

For the different experiments, plasmids listed in table 3.5. were used.

Table 3. 5: Description of vectors used

VECTOR	PROVIDED BY	RESISTANCE
pBluescript KSII+	Stratagene	Amp^R
pkD46	Datsenko, 2000; kindly provided by Joseph E. Peters	Amp^R, temperature sensitive
pTSA29recA	Philips, 1998; kindly provided by Joseph E. Peters;	Amp^R, temperature sensitive
pBR322	MBI Fermentas	Amp^R, Tet^R
pBad	Invitrogen	Amp^R
pET11a	Novagen	Amp^R
pRED/ET	Gene Bridges	Amp^R, Tet^R
pUC19	MBI Fermentas	Amp^R
pUC19Δ*RNAI*	Lab stock	Amp^R

3.3 Molecularbiological methods

3.3.1 Polymerase chain reaction (PCR)

All PCRs were carried out in a Biometra T3-Thermocycler. Lyophilized primers, obtained from Sigma-Aldrich (St.Louis, MO, USA) were dissolved with sterile water, resulting in a stock solution with a concentration of 1 ng/µl. The primer working solution was obtained by a 1:100 dilution of the primer stock giving a final concentration of 10 pmol/µl. In the case of templates with a complex secondary structure, 1-5 % dimethyl-sulfoxide (DMSO) was added to the reaction mix. The melting temperature is dependent on the amount of Adenine (A), Thymine (T), Guanine (G) and Cytosine (C), and was calculated according to the following formula:

T_m[°C] = 4 x (amount of G and C) + 2 x (amount of A and T)

3.3.1.1 Colony PCR

For the screening of different colonies in consideration of the expected genotype, a colony PCR using a Taq polymerase, purchased from Biotools B&M Labs (Madrid, Spain), was used. The reaction mix was prepared as follows:

3 µl 10x buffer
1,2 µl $MgCl_2$ (2mM)
0,3 µl dNTPs (10mM)
0,3 µl sense primer
0,3 µl antisense primer
0,15 µl polymerase
24,75 µl H_2O
Σ 30 µl

Colonies were picked with a sterile pipette tip, and were directly inoculated into a PCR tube containing the reaction mix. Tubes were placed into the Thermocycler and the following program was performed, as described below:

initial denaturation	95°C	2min
	94°C	30sec
30 cycles	lower primer Tm-3°C	30sec
	72°C	1min/kb
final extension	72°C	5min

3.3.1.2 Cloning PCR

As the Taq polymerase has no proofreading activity, it is not suitable for cloning purposes. In such cases, the Phusion DNA polymerase, purchased from Finnzymes (Espoo, Finnland) or the KOD DNA polymerase, purchased from Merck (Darmstadt, Germany), were used, respectively.

The Phusion High-Fidelity DNA Polymerase combines a *Pyrococcus*-like enzyme with a processivity-enhancing domain. By fusing a double-strand DNA binding domain to the polymerase, its processivity can be increased. As a result of this combination, shorter extension times, more robust and high yield amplification, and

the ability to copy long templates in a fraction of time can be achieved. The reaction mix for a PCR using the Phusion polymerase was prepared as follows:

10 µl Phusion HF Buffer
1 µl dNTPs (10mM)
2,5 µl sense primer
2,5 µl antisense primer
x µl template
0,5 µl polymerase
up to 50 µl with nuclease free water
Σ 50 µl

The following program was use:

initial denaturation	98°C	30sec
	98°C	5-10 sec
25-35 cycles	45-72°C (lower primer Tm+3°C)	10-30 sec
	72°C	15-30 sec
final extension	72°C	5-10 min

For complex and long templates the KOD polymerase was used. This DNA polymerase is a recombinant form of *Thermococcus kodakaraensis* KOD1 DNA polymerase. It is a high fidelity thermostable polymerase, amplifying target DNA up to 6 kbp. The KOD Polymerase produces blunt-ended DNA products. The standard reaction set up is described below:

5 µl 10x buffer
3 µl $MgCl_2$ (25mM)
5 µl dNTPs (2mM)
2 µl sense primer
2 µl antisense primer
0,4 µl KOD DNA Polymerase
x µl template DNA (0,006-6ng)
up to 50 µl with nuclease free water
Σ 50 µl

For DNA amplification using KOD polymerase, the following program was used:

Cycling parameters	**0,5kbp target DNA**	**1-2kbp target DNA**	**3-4kbp target DNA**	**5-6 kbp target DNA**
1. Denature	15s 98°C	15s 98°C	15s 98°C	30s 98°C
2. Anneal	1-30 s 68°C	2s lowest primer Tm°C	5s lowest primer Tm°C	30s lowest primer Tm°C
3. Extend	none	20s 72°C	40s 72°C	60s 72°C
4. Repeat steps 1-3	20-30 cycles	20-30 cycles	20-30 cycles	20-30 cycles

If a polymerase with an exonuclease activity was necessary, the Deep Vent polymerase, purchased from New England Biolabs (NEB) (Ipswich, MA, USA) was used. This enzyme posses a 3`- 5` proofreading exonuclease and strand displacement ability. The term strand displacement describes the ability to displace downstream DNA, encountered during synthesis. Deep Vent polymerase is therefore able to digest primers that bind more than once to a certain template (used during cloning of the RNAI expressing plasmids).

Deep Vent polymerase is purified from a strain of *E. coli* that carries the Deep Vent DNA polymerase gene from a *Pyrococcus* species. The native organism was isolated from a submarine thermal vent at 2010 meters and is able to grow at temperatures as high as 104°C. This polymerase yields very long primer extensions with the longest product length to date of 14 kb thereby producing blunt-ended DNA fragments.
In a standard PCR using the Deep Vent polymerase, the following 50 µl reaction was prepared in a 0,5 ml PCR tube on ice:

5 µl TermoPol Reaction Buffer (10x)
1 µl Deoxynucleotide Solution Mix (10mM)
4 µl $MgSO_4$ (100mM)
2 µl sense primer
2 µl antisense primer
0,5 µl Deep Vent polymerase
1 – 10 ng template DNA
x µl nuclease free water
∑ 50 µl

For amplification of DNA using the Deep Vent polymerase, the following program was used:

Initial Denaturation	95°C	2-5 minutes
	95°C	15-30 seconds
20 – 30 cycles	55-65°C	15-30 seconds
	72°C	1 minute / 1000 bp
Final Extension	72°C	5 minutes
Storage	4°C	∞

All primers and their sequences are listed in point 7. “Appendix”.

3.3.2 Assembly of oligonucleotides and fill-in, using DNA Polymerase I (Klenow)

Two oligonucleotides with short overlapping sequences were mixed in a PCR tube (500 pmol of each oligo) and put into the Biometra T3-Thermocycler. The oligo mix was heated to 99°C and slowly cooled down to 36°C (0,5°C / minute). By this controlled drop of temperature the overlapping ends of the oligos could properly bind to each other. In order to fill up the remaining single stranded ends of the oligonucleotides, the following Klenow reaction mix was prepared:

x µl oligo mix
1 µl dNTPs (25mM)
1 µl NEB 3 buffer
1 µl Polymerase I Klenow

The reaction mix was incubated for 15 minutes at room temperature and was inactivated for 20 minutes at 75°C, afterwards.

3.3.3 Agarose gel electrophoresis

In order to separate DNA according to its size, gel electrophoresis was performed using a 1 % agarose gel. To obtain 360 ml of liquid agarose solution, 3,6 g agarose and 7,2 ml of 50x TAE were mixed and filled up to 360 ml with sterile water. To melt the agarose, the solution was heated in the microwave until the agarose was completely dissolved. After the solution had cooled down, 10 µl of ethidium bromide

were added. After mixing carefully, gels were poured. Before samples were loaded onto the agarose gel, 1x BX buffer was added. Analytical gels were run at 130 V and preparative gels at 90 V. As running buffer 1x TAE buffer was used. After the samples were separated on the gel, DNA was visualized under UV-light. In the case of a preparative gel, DNA-bands were cut out using a small scalpel.

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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

6x BX-loading buffer

bromophenol blue	0.25 % (w/v)
xylene cyanol FF	0.25 % (w/v)
glycerol in H_2O	30 % (v/v)

3.3.4 Purification of DNA

DNA was purified either by using a preparative agarose gel or by direct purification of a PCR reaction or other enzymatic reactions using the Qiaquick PCR and Gel Purification kit (Qiagen, Hilden, Germany). The purification steps were carried out according to the protocols. For the purification of small DNA fragments, the QiaexII Gel extraction kit (Qiagen, Hilden, Germany) was used or ethanol precipitation was carried out. In the case of ethanol precipitation, 2-3 volumes of 100 % ethanol as well as 1/10 volume of 3 M sodium acetate (pH 5.2) were added to the sample. After mixing, the solution was frozen over night at -20°C. Alternatively, the precipitate can also be frozen at -80°C for 20 minutes to 1 hour. The solution was then centrifuged at top speed in a microcentrifuge at 4°C for 30 minutes. The supernatant was discarded later on and the pellet was dried at room temperature. After drying, the DNA was dissolved in a defined amount of sterile water. After ligation, DNA was extracted by isopropanol precipitation. The same amount of isopropanol was added to the samples. Everything was mixed, and incubated for 15 minutes at room temperature.

The precipitate was centrifuged for 15 minutes at top speed and the DNA pellet was washed by adding 70 % ethanol (1,5fold of the amount of the original sample). After centrifugation, the supernatant was discarded and the pellet was dried at room temperature. The DNA was resuspended in a defined amount of sterile water.

3.3.5 Restriction enzyme digestion

Different restriction enzymes from NEB were used for vector linearization and control digestions. Every enzyme is provided with a certain buffer for an optimal working efficiency (NEB 10x buffers 1-4). In few cases it is also necessary to add BSA (100x stock solution) to the reaction mix. The amount of enzyme should never exceed 1/10 of the total reaction solution. According to their working temperatures, the restrictions were carried out at either 25°C or 37°C. The reaction mixes were incubated in an Eppendorf heating block for about 2 hours. Some enzymes had to be heat inactivated by placing them at 65°C for 20 minutes.

3.3.6 Ligation

All ligations were carried out using the T4-DNA Ligase, purchased form NEB. Reaction mixes were either incubated at room temperature for 2 hours or at 16°C over night. Standard ligation was performed with a 5:1 molar ratio of insert DNA to vector DNA in a 10 µl set-up. The insert-vector-ratio was calculated as follows:

Example for the calculation of vector-insert ratio when 3µl of vector DNA are used:

vector (3000 bp): 24 ng/µl
insert (200 bp): 15 ng/µl

$$\frac{(3\ \mu l \times 24\ ng/\mu l)\ 72}{3000\ bp} = 0{,}024$$

0,024 x 5 = 0,12

0,12 x 200 bp = **24 ng/µl** of insert DNA

24 ng/µl / 15 ng/µl = **1,6 µl** of insert should be used for an optimal ligation efficiency

If the insert was obtained by PCR, it had to be phosphorylated in order to ligate it to the vector backbone. For this phosphorylation step, the T4 Polynucleotide Kinase from NEB was used. A 30 µl standard phosphorylation mix contained the following ingredients: 1 µl PNK, 3 µl T7-ligase buffer and 26 µl insert solution. The reaction mix was incubated at 37°C for 30 minutes.
In order to inhibit relegation after lineariziation, vectors were dephosphorylated before ligation. Therefore 1 µl of Antarctic Phosphatase (NEB), 2 µl of Antarctic Phosphatase buffer and 27 µl of vector solution were mixed in a 30 µl preparation, incubated at 37°C for half an hour and finally inactivated at 65°C for 5 minutes.

3.3.7 Plalsmid preparation

Plasmids were isolated from cells by using the Promega Miniprep kit. Plasmid preparation was carried according to the appropriate protocols. For all Minipreps, cells from 3ml of an over night culture were harvested by centrifugation and lysed as described in the protocols. Plasmid DNA was isolated via columns and eluted with either the provided elution buffer or nuclease free water. Plasmid DNA was eluted with 50-80µl buffer/water depending on the required plasmid concentration.
For higher concentrations of plasmid DNA, the Promega Pure Yield^{TM} Midiprep kit was used following the protocol. In the case of a Midiprep, cells were harvested from a 50ml over night culture and finally eluted in 600µl of elution buffer or nuclease free water.

3.3.8 Sequencing

DNA sequencing was carried out by either AGOWA GmbH (Berlin, Germany) or Eurofins MWG GmbH (Ebersberg, Germany). Samples that were sequenced by AGOWA had to be diluted with sterile water to the concentrations listed above.

Plasmids	10 µl	80 ng/µl
PCR products		
150-500 bp	10 µl	10 ng/µl
500-1000 bp	10 µl	20 ng/µl
1000-2000 bp	10 µl	40 ng/µl
custom primers	4 µl	5 pmol/µl

Samples sequenced by Eurofins MWG were diluted to the following concentrations:

Plasmids	15 µl	50-100 ng/µl
PCR products		
< 300 bp	15 µl	2 ng/µl
300-1000 bp	15 µl	5 ng/µl
> 1000bp	15 µl	10 ng/µl
custom primers	15 µl	2 pmol/µl

3.3.9 Electro transformation

For the transformation of plasmids, electrocompetent cells of the strains NEB10ß, DH5α or JM109 were used. Cells were grown over night in 20 ml LB-medium at 37°C, shaking. The next day the over night culture was diluted 1:100 in 400 ml LB-medium and incubated again at 37°C shaking until an OD_{600} of 0,6-0,8 was reached. The following steps were all carried out on ice and using a cooled centrifuge (4°C), respectively. All solutions were precooled to 4°C. Cells were harvested by centrifugation (Beckman Avanti J-20 XP centrifuge, JLA10.500 rotor, 4000 rpm, 8 minutes, 4°C) and the supernatant was discarded. Cells were washed three times with 1 mM HEPES (500 ml, 250 ml, 100 ml). After the last washing step, the pellet was resuspended in 30 ml 10 % glycerine. The suspension was transferred into JA20 cups and centrifuged again. The pellet was finally resuspended in 3 ml 10 % glycerine. Aliquots of 50 µl were pipetted into iced 1,5 ml Eppendorf tubes and shock frozen in liquid N_2. Electrocompetent cells were stored at -80°C.

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Electroporation was done by using Biorad gene pulser at 2.5 kV, 1000 Ω and 25 µF. For electroporation, self made cuvettes were used: Brand Ultravette cuvettes (12,5 x 12,5 x 45 mm) were pasted up with conductive aluminium foil. After transformation cells were recovered at 37°C in 500 µl SOC-medium for 30 minutes before plating them on LB-Agar plates, containing the appropriate antibiotic.

For the transformation of linear DNA-fragments, MG1655 cells containing the pkD46 plasmid were used. Cells were prepared freshly for every electroporation. Therefore, an over night culture was diluted 1:100 in 50 ml LB-amp-medium containing 500 µl 20 % arabinose. Cells were grown at room temperature until an OD_{600} of 0,4-0,6 was reached. The pellet was washed four times with 1ml of ice cold water (Eppendorf microcentrifuge, 5000 rpm, 5 minutes, 4°C) and was finally resuspended in 150 µl of ice cold water. After electroporation, cells were incubated in 900 µl SOC-medium at 37°C for 3-4 hours.

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3.4 Genomic engineering

3.4.1 Chromosomal integration of linear DNA by homologous recombination

Linear DNA fragments were obtained by PCR or by cutting them out of the corresponding vector. Fragments were loaded onto a preperative agarose gel, cut out and extracted according to section 3.3.4. *E. coli* MG1655 carrying the Red helper plasmid pKD46 (Datsenko, 2000) were grown in LB-amp medium over night. The next day, cells were diluted 1:100 in 50ml LB-amp containing 1% arabinose (20%). Cells were grown at room temperature, shaking until OD_{600} of 0,4-0,6 and made competent (see section 3.3.9.). Electroporation was done by using Biorad gene pulser at 2.5 kV, 1000 Ω and 25 µF. 100-300 ng of the gel purified linear cassette were used for transformation. After a recovery time of several hours in 900µl SOC medium, cells were plated onto appropriate agar plates and incubated over night at 37°C.

3.4.1.1 P1 Transduction

P1 phage lysat preparation

MG1655 cells, with the linear DNA fragment integrated into their genome, were grown over night in LB-cm medium supplied with 5mM CaCl2. The over night culture was diluted 1:100 in 5 ml LB-cm medium containing again 5 mM CaCl2. 20 µl of a high titer P1 phage lysate were added to the donor cells. At the same time, controls only consisting of MG1655 cells without the phage lysat, were also put at 37°C shaking. The cultures were checked for lysis hourly (1h-6h). After cells were lysed, several drops of chloroform were added and the lysate was vortexed (chloroform kills phage-resistant bacteria and removes cell wall debris). The debris was removed by centrifugation (3500 g, 15 minutes, Jouan C312) and the supernatant was transferred to a new tube. In order to get rid of any remaining cells, the lysate was further pressed through a filter (22µm) and transferred to a light-safer container. The lysate was stored at 4°C.

Transduction

E.coli HMS174(DE3) carrying the pTSA29 (carries all genes necessary for homologous recombination) plasmid were grown over night in LB-amp medium supplied with 5mM CaCl at 30°C shaking. The next day, 300 µl of HMS174(DE3) cells were mixed with 100 µl of P1-lysate in 15ml tubes. The following working steps also include cells only and phage only controls. The cell-lysate, as well as the controls were incubated static at 37°C for 25 minutes. Then 5 ml of LB-medium supplied with 10 mM NaCitrat were added. The solutions were mixed and cells were pelleted (3500 g, 15 minutes, Jouan C312). The supernatant was discarded and the cell pellets were resuspended in 1 ml LB-NaCitrat solution. Cells were transferred into 1,5 ml Eppendorf tubes and incubated for at least 1 hour at 37°C shaking. Finally, cells were spread on selective agar plates (LB-cm) and incubated at 37°C. Colonies were cured from pTSA29 plasmid.

3.4.1.2 Chromosomal integration of linear DNA fragments using the pRed/ET system

Alternatively, chromosomal integration of linear DNA fragments was achieved by using the Quick & Easy *E. coli* gene deletion kit, purchased from Genebriges GmbH (Heidelberg, Germany).

With this kit, direct integration of a linear DNA fragment is possible and no P1 transduction has to be carried out. Plasmid pRed/ET (figure 3.1.), which basically can be introduced in any *E.coli* strain, represents the core of the Red/ET technology. In contrast to the pKD46 plasmid, pRed/ET can be introduced also in *recA*$^-$ negative strains because the plasmid additionally carries the *recA* gene. pRed/ET encodes for the phage λ recombination machinery. Redγ stabilizes the linear recombination partner so that exonuclease Redα and the DNA-binding protein Redß can mediate recombination between target molecules. Expression of the corresponding genes is under control of an inducible arabinose promoter. The addition of the inducer, followed by a temperature rise (30°C to 37°C) results in a transient recombineering activity, since pRed/ET is no longer replicated due to its thermosensitivie ori pSC101 and finally gets lost.

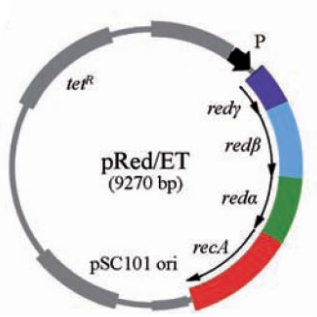

Fig.3 1: The pRed/ET plasmid for direct integration of linear DNA fragments (picture from Genebridges GmbH).

Following the protocol, the pRed/Et plasmid was transformed into competent E.coli JM109 cells. An over night culture was inoculated in LB-amp medium. Half of the standard ampicillin concentration was used (50 µg/ml), due to the low copy nature of the pSC101 ori. The next day the over night culture was diluted 1:50 in 10 ml LB-amp medium and grown to an OD_{600} of 0,3 at 30°C shaking. The dilution was prepared two times, one of them representing the uninduced control. When cells had grown to the proper OD_{600}, one of the cultures was induced by adding 0,3 % - 0,4 % arabinose (10 %). Both cultures were further incubated for one hour at 37°C shaking to allow expression of the pRed/ET genes. 2,5 ml of culture were harvested afterwards (Eppendorf microcentrifuge, 4 minutes, 5000 rcf) and washed 4 times with 1 ml of ice cold sterile water. After the last washing step, the supernatant was decanted, and the cell pellet was resuspended in the remaining water. 300-400 ng of linear fragment was mixed with ~40 µl of competent cells. Electroporation was done by using Biorad gene pulser at 2.5 kV, 1000 Ω and 25 µF. After electroporation, cells were transferred

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to 500 µl SOC-medium and placed at 37°C shaking for 3 hours. Finally cells were spread on a selective agar plate and grown over night at 37°C.

3.5 Analytical methods

3.5.1 Determination of Plasmid Copy Number

3.5.1.1 ***Calculation of PCN using Agilent 2100 Bioanalyzer (Agilent Technologies)***

For the quantitative analysis of DNA, a chip has to be loaded which can then be inserted into the bioanalyzer for quantification of DNA. The chip accommodates sample wells, gel wells and a well for an external standard (DNA ladder). Micro-channels create interconnected networks among these wells. During chip preparation, the micro-channels are filled with a sieving polymer and fluorescence dye. Then, twelve samples and standard are loaded in each well.

Once the wells and channels are filled, the chip becomes an integrated electrical circuit. The 16-pin electrodes of the cartridge are arranged so that they fit into the wells of the chip. Each electrode is connected to an independent power supply that provides individual control and flexibility.
Charged biomolecules like DNA or RNA are electrophoretically driven by a voltage gradient, similar to slab gel electrophoresis. The molecules are separated by size. Dye molecules intercalate into DNA/RNA strands and these complexes are detected by laser-induced fluorescence. Data is translated into electropherograms (peaks).

During the chip run, the dye intercalates directly with the DNA/RNA and all bands pass the detector at different time points. An extra “lower” marker fragment is run with each of the samples to compensate for drift effects that may occur during the course of a chip run.
The software automatically compares the unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify the ribosomal DNA/RNA peaks.

Preparation of DNA samples

All solutions used for plasmid DNA preperation were taken from Promega Miniprep Kit. Centrifugation steps were carried out by using an Eppendorf microcentrifuge.

An over night culture of the cells under investigation was inoculated in LB-media and grown at 37°C shaking. The next day OD_{600} was measured, and a normalized number of cells (3 / OD_{600} = xml) was harvested. Cell pellets were resuspended thoroughly in 250µl of resuspension solution. 250µl of lysis buffer were added and tubes were inverted 4 times. At this point, 1µg of the internal standard (in our case pMCP-1 (4892 bp)) was added to the samples to compensate for losses during purification. In a next step, 10µl of alkaline protease solution was added, the samples were inverted again 4 times, and incubated at room temperature for 5 minutes. Afterwards 350µl of neutralization solution was added. After inverting the samples for another 4 times, they were centrifuged at top speed for 10 minutes. The supernatants were transferred to miniprep columns and centrifuged for 1 minute at top speed. The DNA was washed 2 times with wash buffer (750 µl, 250 µl) and columns were dried by centrifugation for 2 minutes at top speed. Finally plasmid DNA was eluted with 50µl nuclease free water by centrifugation for 1 minute at top speed.

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Plasmid linearization

For later quantitative analysis of DNA, both plasmids, from the internal standard as well as from the samples, had to be linearized. Therefore, an enzyme was chosen, with only one restriction site in both vectors. A master mix containing the enzyme, the buffer and if necessary sterile water was prepared. 25 µl of sample solution were mixed with 5 µl of the master mix and the restriction was incubated at the appropriate temperature for at least 1,5 hours. Finally, the restriction enzyme was inactivated by placing the reaction solution at 65°C for at least 30 minutes. Samples were stored at -20°C.

Loading the Biochip

For proper handling of the bioanalyzer and all corresponding chemicals, the different points, described in the break “Essential Measurement Practice” of the Agilent protocol, were followed.

Preparing the gel-dye mix

Before starting, the DNA dye concentrate, as well as the DNA gel matrix, were equilibrated to room temperature for 30 minutes. The dye concentrate was vortexed for 10 seconds and spinned down. 25 µl of the dye concentrate were pipetted into a DNA gel matrix vial. The dye concentrate was stored again at 4°C in the dark. The tube containing gel and dye was vortexed for another 10 seconds and the mix was transferred to the top receptacle of a spin filter. The spin filter was placed in an Eppendorf microcentrifuge and spinned for 10 minutes at room temperature at 4000rpm. Afterwards, the filter was discarded.

Loading the gel-dye mi

Before the mix was loaded to the biochip, it was equilibrated, if necessary, to room temperature for 30 minutes. During this time, the mix had to be protected from light. A new DNA chip was taken out of its sealed bag and placed on the chip priming station. 9 µl of the gel-dye mix were pipetted at the bottom of the well marked G and the mix was dispensed. The plunger of the syringe of the chip priming station was pressed down until it was held by the clip. After 30 seconds the plunger was released. When the plunger had moved back to the 0,3 ml mark, it was slowly pulled back to the 1ml position after 5 seconds and the chip priming station was opened. Further, 9 µl were pipettet in two other wells marked.

Loading the marker

5 µl of the DNA marker were pipetted into the well marked with the ladder symbol and into each of the 12 sample wells.

Loading the ladder and the samples

1 µl of the DNA ladder were pipetted in the well marked with the ladder symbol. In each of the 12 sample wells, 1 µl of sample or 1 µl of deionized water was pipetted. The loaded chip was placed horizontally in the adapter of the IKA vortex mixer and

vortexed for 60 seconds at 2400 rpm. The chip was put into the Agilenta 2100 bioanalyzer and the run was started within 5 minutes.

3.5.1.2 Determination of dried biomass

An appropriate amount of beakers was marked, and placed at 100°C over night. The next day, the beakers were transferred to the essicator and dried for 2-3 hours. Within the first half hour, the valve of the essicator remained open to allow the hot air to escape. The dried breakers were weighted using an analytical balance, and the measurements were noted..

An over night culture of *E.coli* cells under investigation was inoculated in 20ml LB-media and grown at 37°C shaking. The next day, exactly 10 ml of each cell suspension were transferred to a 15ml plastic tube and centrifuged for 15 minutes at 5000 rcf at 4°C (Eppendorf centrifuge 5804R). The cell pellets were washed 3 times with 1ml of ice cold water (5 minutes, 5000 rcf, 4°C, Eppendorf centrifuge 5804R). The pellets were resuspended in water and poured quantitatively into the according dried beakers. The beakers were again placed at 100°C over night and dried in the essicator the next day. The beakers containing the dried biomass were again weighed. The biomass was calculated and values were used for the determination of PCN per cell.

3.5.1.3 Determination of total amount of DNA by fluorimetric measurement

If not otherwise stated, all chemicals were purchased from Merck.

10x TNE-buffer	Tris 1M
	EDTA 10mM
	NaCl 1M
	pH 7,4 with HCl

Dilution buffer: 1:10 dilution of 10x TNE buffer (prepared freshly)

Hoechst reagent: 100 ml dilution buffer + 10 µl Hoechst dye 33258 (10 mg/ml)

This reagent has to be prepared freshly when needed. It must be cooled and has to be protected from light.

Solution I	glucose 50 mM
	EDTA 10 mM
	Tris/HCl (pH 8,0) 25 mM

SDS solution (Biorad): 0,5 %

DNA-standard solution (Sigma): 1 mg of calve thymus DNA is dissolved in 1 ml of sterile water

Lysozyme solution (Sigma): 10 mg/ml of lysozyme is dissolved in sterile water

Determination of the total amount of DNA is achieved by fluorimetric measurement, using bisbenzimidazol (Hoechst-dye 33258). Bisbenzimidazol binds to DNA and therefore its fluorescence can be measured at 458 nm, with an excitation wavelength of 365 nm.
Samples were prepared as follows:
The OD_{600} of an over night culture was measured and a normalized number of cells was harvested (3 / OD600 = xml). 150 µl of solution 1 were added and the cell pellet was resuspended thoroughly. 50 µl of lysozyme solution were added and the sample was mixed again. After an incubation time of 10 minutes at 37°C 200 µl of SDS solution (0,5 %) were added, mixed and incubated for another 10 minutes at 37°C. 8,6 ml dilution buffer were exhibited in 15 ml tubes and the viscous DNA solution was added. With 1 ml of dilution buffer the remaining DNA solution was washed out and transferred into the tube. The tubes were vortexed thoroughly and kept on ice until measurement.
For calibration, different dilutions of calve thymus DNA in a concentration range of 0-6 µg/ml were used.
15 µl of sample and standard solution were pipetted into a 96 well plate and 280 µl of the Hoechst reagent were added using a multichannel pipette. Measurements were carried out using a Molecular Devices fluourimeter from Spectramax Gemini.

Calculation of DNA concentration was performed automatically based on the calibration curve obtained by the calve thymus DNA standard solution.

3.5.1.4 Final calculation of PCN per cell

The calculation of the PCN did require the results of the bioanalyzer assay, the amount of dried biomass as well as the results from the fluorimetric measurements of the total DNA. All data was combined and the PCN per cell was calculated using the following formula:

$$\frac{\text{size of chromosome [bp]} \times \text{pDNA}}{\text{average size of plasmid [bp]}} \times \text{chromosomal DNA [µg]}$$

3.5.2 Pour-plate method

In order to check, whether plasmids were segregationally stable, the pour-plate method according to Koch was applied. Therefore, *E.coli* cells were grown over night in LB-medium without any antibiotics at 37°C shaking. The next day, cell cultures were diluted to a normalized OD_{600} value again in LB-medium. After 3 hours of growth at 37°C, gene expression was induced by adding 100 µM IPTG. After another 3 hours, cells were diluted in SOC-media to an appropriate amount of cells per millilitre. Three dilutions were plated on LB-plates and LB-selective-plates, respectively. The plates were incubated over night at 37°C. The next day, growing colonies were counted and a mean value from the three dilutions was calculated.

4 Results and Discussion

4.1 Down regulation of plasmid copy number via inducible expression of RNAI

The replication of ColE1-type origin is regulated by the two RNA molecules RNAI and RNAII. Both molecules are encoded on the plasmid itself. The 555-nucleotide RNAII can bind at the origin of replication thereby acting as a primer for DNA polymerase I. This is only possible when RNAII is not bound by RNAI. The RNAI molecule is a short 110-nucleotide antisense molecule and acts as an inhibitor of RNAII (35).

In the following experiments, regulation of plasmid copy number was tried to be achieved by a chromosomal copy of RNAI under the control of an inducible promoter. Therefore two different linear fragments were designed. The first consisted of the T7 promoter, followed by the RNAI sequence. The second fragment was composed of the RNAI sequence under the control of the pL_{lacO-1} promoter. In both cases chloramphenicol resistance was used as selection marker.
The RNAI-fragments were integrated into the chromosome of HMS174(DE3) via homologous recombination. In order to investigate the impact of RNAI overexpression, HMS174(DE3) cells were transformed with different plasmids. Replication of the pUC19 plasmid, a RNAI-deleted version of the pUC19, designated pUC19Δ*RNAI* and, in some cases, the low copy number plasmid pBR322 were investigated. In the case of the pUC19Δ*RNAI* plasmid, the -10 and the -35 box of the RNAI promoter were deleted and thus, no RNAI was expressed from this plasmid. Shake flask experiments, wherein cells were grown in medium with and without IPTG, were carried out, and the plasmid copy numbers were determined

4.1.1 Regulation of plasmid copy number via inducible expression of RNAI under the control of the T7 promoter

4.1.1.1 Construction of the T7-RNAI plasmid

For the construction of a plasmid carrying the RNAI sequence under the control of the T7 promoter, the pBSK::TN7<CAT-T7-GFP>shown in figure 4.1., was used as a template vector. The pBSK::TN7<CAT-T7-GFP> vector did carry the reporter gene GFP which was already under the control of the T7 promoter and terminated by the T7 terminator. Primers with overlapping ends binding to the T7 promoter on the one hand and to the T7 terminator of the GFP gene on the other hand were designed. The overlapping ends were thereby representing the sequence of RNAI. Two PCRs were carried out using the pBSK::TN7<CAT-T7-GFP> as template and the primer pair T7prom-RNAI overlap and m13-20back as well as the primer pair T7term-RNAI and ColEIfor. For these PCRs, the Deep Vent polymerase was used because of its strand displacment activity. As primers could also bind to the ori, this strand displacement property enables the polymerase to digest the binding primers and to proceed with the PCR. The two fragments with a length of 1350 bp and 1554 bp respectively were loaded onto a preparative agarose gel. The fragments were cut out and DNA was extracted by using the Qiagen Qiaquick PCR Purification and Gel Purification kit. One of the two fragments was phosphorylated with T4 Polynucleotide Kinase as described in section 3.3.6. The fragments were ligated at room temperature and precipitated with isopropanol. The emerging plasmid is shown in fig. 4.1.

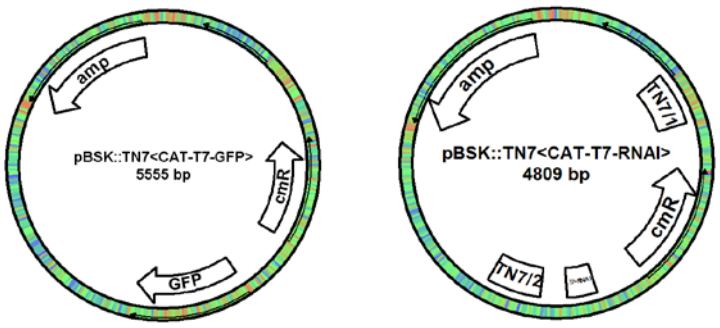

Fig.4 1: Original pBSK vector a) and vector carrying the RNAI gene under the control of the T7 promoter b). This vector was created by the use of primers with overlapping ends constituting the RNAI.

The pBSK::TN7<CAT-T7-RNAI> was transformed into competent NEB10ß cells. Growing colonies were checked by PCR screening, using the primers m13-20 back and T3. Plasmids of positive colonies were isolated by a Miniprep kit and cut with the restriction enzyme *Hind*III. As the control restriction showed the expected bands on an agarose gel, plasmids were sequenced.

4.1.1.2 Amplification of linear T7-RNAI fragment

In order to obtain a linear T7-RNAI fragment the pBSK::TN7<CAT-T7-RNAI> vector was used as template. For the amplification of the fragment, a PCR was carried out, using KOD or Phusion DNA polymerase, respectively together with primers m13-20 back and T3. A linear T7-RNAI fragment as shown in fig. 4.2. was obtained. The PCR product was loaded onto an agarose gel, cut and purified according to the manufactures instructions. To get rid of any remaining vector template, the extracted DNA was digested using the enzyme *Dpn*I that only cuts methylated DNA. After digestion, the T7-RNAI fragment was purified again using a PCR-purification Kit.

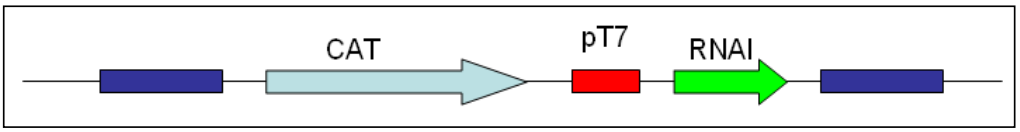

Fig.4 2.: Linear T7-RNAI fragment obtained by PCR. The fragment carries a chloramphenicol resistance gene as selection marker and is flanked by homologous sequences (dark blue bars) for the later intergration into the HMS174(DE3) genome.

4.1.1.3 Integration of T7-RNAI fragment into HMS174 (DE3) chromosome

For chromosomal integration, MG1655 cells were transformed with the linear T7-RNAI fragment by electorporation. Growing colonies were screened using a primer pair, that bound on the one hand to the cm-resistance gene of the linear fragment (cat begin for) and on the other hand in the MG1655 genome (TN7/2-HO-for), resulting in a 781 bp band. To make sure, that the growing colonies did not gain their antibiotic resistance from a remaining undigested pBlueScript vector, another PCR screening was carried out, using plasmid ori- primers (RNAII 115 back, RNAII 625 for) resulting in a 497 bp fragment.

The T7-RNAI fragment was transferred to the recipient strain HMS174(DE3) by P1 transduction.

A successful homologous recombination of the T7-RNAI fragment was checked by PCR using the primers cat begin for and TN7/2 extern. A fragment of 831 bp was obtained indicating that the recombination event had taken place at the right genome locus. As this screening PCR was carried out with the Phusion polymerase, the amplified fragments could be directly sequenced.

Competent HMS174(DE3) cells were transformed with the pUC19 plasmid as well as with the pUC19Δ*RNAI* plasmid. As controls, HMS174(DE3), that did not carry the RNAI genome modification, were transformed with these plasmids.

4.1.1.4 Bacterial cell growth under induced and uninduced conditions

In order to check whether IPTG influences cell growth, over night cultures of the strains HMS174(DE3)::TN7<CAT-T7-RNAI>, HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19 and HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19*ΔRNAI* were inoculated in 10 ml LB-medium, supplied with the appropriate antibiotics (cm, cm/amp). The next day, cell cultures were diluted to the same OD_{600} in another 10 ml medium and different concentrations of IPTG were added. Cell growth was observed by measuring the OD_{600} after one, two and three hours. Measured growth values are listed in table 4.1.

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Table 4. 1: Bacterial cell growth of different HMS strains with and without IPTG

	HMS174(DE3)::TN7<CAT-T7-RNAI>			**HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19**			**HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19Δ*RNAI***		
	1h	2h	3h	1h	2h	3h	1h	2h	3h
no IPTG	0,087	0,432	1,333	0,098	0,292	0,843	0,083	0,345	0,969
100 µM IPTG	0,078	0,319	0,603	0,078	0,199	0,348	0,077	0,259	0,364
25 µM IPTG	0,073	0,395	1,199	0,1	0,327	0,815	0,098	0,346	0,881
2,5 µM IPTG	0,074	0,355	1,193	0,86	0,273	0,824	0,093	0,369	0,932

Low concentrations of IPTG (2,5 µM, 25 µM) did not influence cell growth of any of the three strains, significantly. The addition of 100 µM of IPTG however led to a lower

growth rate, as the OD_{600} measured herein, reached only half the value of IPTG-free cell cultures. At this point it can be stated that IPTG, when added in concentrations of 100 µM, had a toxic effect on the cells as it reduced cell growth in the case of all three *E.coli* strains. Andersson et al. already showed, that induction with 0,1 µM IPTG results in a decrease of cell growth as well as of cell mass (36). Previous publications could demonstrate, that the addition of IPTG to wild-type *E.coli* leads to elevated expression of several stress response proteins and therefore to a reduced cellular growth rate (37). Andersson et al. further showed that the presence of plasmids also effects cell growth and causes a decline in the colony forming ability of cells (36). As those cells that were carrying plasmids are showing a reduced OD_{600} value even in the absence of IPTG, this might also be the case in this experiment. Addition of IPTG should induce RNAI overexpression, and thus lead to an inhibition of plasmid replication. If plasmid replication is inhibited plasmid loss and therefore growth stagnation could be expected under selection pressure as this was the case for the induced cell cultures.

4.1.1.5 Pour plate assays

In the next experiment, over night cultures of the strains listed in table 4.2. were grown in 10 ml LB-medium. The next day, cell suspensions were diluted to a normalized OD_{600} value in fresh LB-medium (10 ml). After 2 hours of growth, IPTG (100 µM) was added to the cells and OD_{600} was measured hourly (table 4.2.). After 3 hours, two different dilutions from each cell culture were spread on LB- as well as on LB-amp agar plates and incubated over night at 37°C. The next day, colony forming units (cfu) were determined (table 4.3.).

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Table 4. 2: Bacterial cell growth in LB-medium after addition of IPTG

	OD_{600} when IPTG was added (100 µM)	OD_{600} after 1 hour	OD_{600} after 2 hours	OD_{600} after 3 hours
HMS174(DE3)+pUC19	0,107	0,151	0,524	1,020
HMS174(DE3)::TN7<CAT-T7-RNAI>	0,387	0,998	1,912	2,460
HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19	0,578	1,364	2,416	3,025
HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19∆*RNAI*	0,519	1,109	1,874	2,515
HMS174(DE3)+*ΔRNAI*	0,508	1,030	2,116	3,250

Table 4. 3: Cfu's on LB- and LB-amp plates after 3 hours of growth in liquid LB-medium supplied with 100µM IPTG

	D1		**D2**	
	LB	**Amp**	**LB**	**Amp**
HMS174(DE3)+pUC19	600	468	57	39
HMS174(DE3)::TN7<CAT-T7-RNAI>	463		2	
HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19	522	0	61	0
HMS174(DE3)+pUC19*ΔRNAI*	350	9	59	1
HMS174(DE3)::TN7<CAT-T7-RNAI>pUC19∆RNAI	230	0	44	0

The results of the pour plate assay showed, that the pUC19 plasmid could be stably propagated. As the number of growing colonies on LB-amp agar plates was not significantly lower than the number of colonies that grew on LB agar plates, it was shown, that the majority of cells did still carry the pUC19 plasmid. In the case of the HMS174(DES)::TN7<CAT-T7-RNAI>pUC19 strain, cells that were spread on LB-amp plates could not grow any more. This indicated, that the pUC19 plasmid was lost, because of an overexpression of RNAI and the resulting inhibition of plasmid replication. Concerning the pUC19*ΔRNAI* plasmid it seemed that this plasmid was not propagated stably. Just a few colonies were still able to grow on the LB-amp plates indicating, that nearly all of the cells had lost their pUC19*ΔRNAI* plasmid. The induction of RNAI expression in the HMS174(DE3)::TN7<CAT-T7-

RNAI>pUC19Δ*RNAI* strain did result in the complete loss of the plasmid as no colonies were able to grow anymore on the ampicillin agar plates.

4.1.1.6 Determination of pDNA concentration

The different *E.coli* strains were grown over night in 5 ml LB-medium with and without 100 µM IPTG. The next day, OD_{600} was measured and normalized cell numbers were pelleted. Plasmid DNA was extracted from the cell pellets and the DNA was eluted in 50 µl elution buffer. By using the Nanophotometer, the concentrations of the extracted plasmid DNA were determined (table 4.4.). In order to confirm and to visualize the measured concentration values, 2 µl of extracted plasmids were loaded onto an agarose gel. A picture of the gel is shown in figure 4.3.

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Table 4. 4: Concentration of extracted plasmid DNA after induced and uninduced cell growth in LB-medium.

Host/Vector pair	**pDNA yield**
HMS174(DE3)+pUC19	138 ng/µl
HMS174(DE3)+pUC19+IPTG	**136 ng/µl**
HMS174(DE3)::TN7<T7-RNAI>pUC19	33 ng/µl
HMS174(DE3)::TN7<T7-RNAI>pUC19+IPTG	**27 ng/µl**
HMS174(DE3)+pUC19*ΔRNAI*	43 ng/µl
HMS174(DE3)+pUC19*ΔRNAI*+IPTG	**41 ng/µl**
HMS174(DE3)::TN7<T7-RNAI>pUC19*ΔRNAI*	35 ng/µl
HMS174(DE3)::TN7<T7-RNAI>pUC19*ΔRNAI*+IPTG	**17 ng/µl**

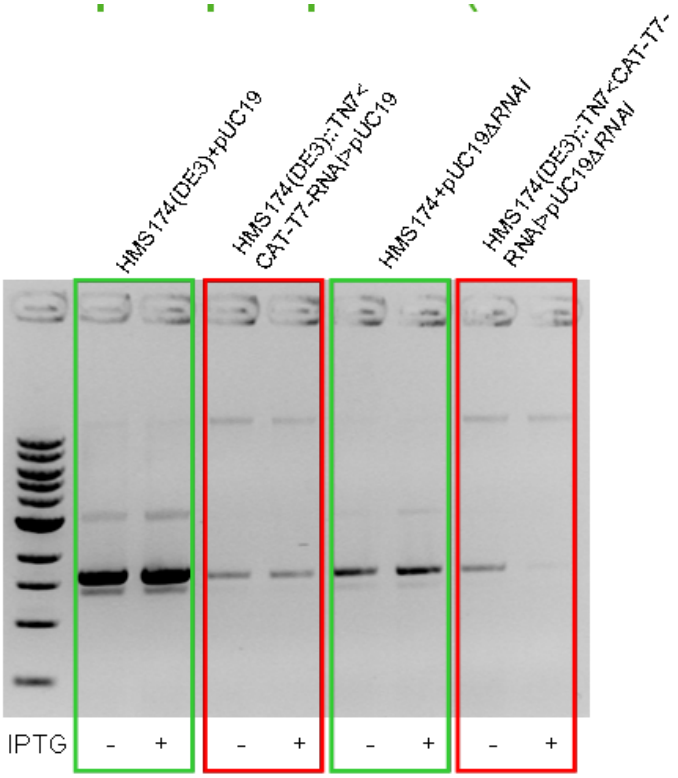

Fig.4 3: Extracted plasmid DNA after growth in LB-medium with and without IPTG. 2 µl of extracted plasmids were loaded onto an agarose gel. Plasmids extracted from control cells not carrying the T7-RNAI fragment in their chromosome are highlighted in green whereas plasmids extracted from cells carrying the T7-RNAI fragment are pointed out in red.

The results of the plasmid preparation assay showed clearly, that the pUC19 plasmid is much more stable in the HMS174(DE3) strain than the pUC19*ΔRNAI* plasmid. Only approximately a third of the amount of plasmid DNA was extracted from the HMS174(DE3)+pUC19*ΔRNAI* when compared to the HMS174(DE3)+pUC19 strain. It could be further shown that IPTG per se had no influence on plasmid concentration, as the addition of IPTG to the RNAI-free strains did not change plasmid concentration. Concerning the HMS174(DE3) strains, where RNAI was integrated into the genome, plasmid concentration decreased dramatically. As the T7-system is a very leaky one, sufficient RNAI seemed to be produced for replication inhibition even in the absence of IPTG. The addition of IPTG led to only a further slight

decrease in plasmid concentration. With these experiments, a clear “proof of concept” was demonstrated.

As it was shown that a down regulation of plasmid replication could be achieved by an over expression of RNAI, the next step was to make the expression of RNAI tightly controllable. This was done by changing the T7-promoter to a repressible, phagal pL_{lacO-1} promoter as described in the next chapter.

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4.1.2 Regulation of plasmid copy number via inducible expression of RNAI under control of the pL_{lacO-1} promoter

In order to be able to regulate RNAI expression, the pBSK::TN7<CAT-T7-RNAI> vector was modified and the T7 promoter was substituted with the pL_{lacO-1} promoter. The modified plasmid was transformed into a JM109 strain because this strain already owned the lac-repressor (lacIq). The effect of IPTG on plasmid replication was investigated by plasmid preparation assays.

In a next step the linear pL_{lacO-1}-RNAI fragment was integrated into the genome of JM109. The influence of RNAI overexpression on the plasmids pUC19, pUC19*ΔRNAI* and pBR322 in the JM109 strain was investigated. Plasmid preparation assays were carried out and the PCN per cell was determined by using the Bioanalyzer.

4.1.2.1 Construction of the pBSK::TN7<CAT-pL_{lacO-1}-RNAI> vector

The pBSK::TN7<CAT-T7-RNAI> vector carrying the RNAI gene under the control of the T7 promoter was digested with the enzymes *Pst*I and *Nco*I. With this enzymatic restriction, a fragment was cut out of the vector constituting the T7-RNAI part of the vector.

In order to separate the vector backbone from the DNA fragment, the restriction reaction mix was loaded onto a preparative agarose gel. The fragment was cut out from the gel and the DNA was purified according to section 3.3.4. This fragment was used in the next step as PCR template. For this PCR, the Phusion polymerase was used together with primers proms_RNAI_for, which carried a *Xho*I restriction site, and the primer placO_back, which carried a *Blp*I restriction site. With this PCR reaction, a linear DNA fragment was constructed, with the RNAI gene under the control of the

pL_{lacO-1} promoter. In order to substitute the T7-RNAI fragment with the pL_{lacO-1}-RNAI fragment, the pBSK::TN7<CAT-T7-RNAI> vector, as well as the pL_{lacO-1}-RNAI fragment were cut with the enzymes *Xho*I and *Blp*I. As both enzymes produce sticky ends at defined restriction sites, a directed ligation could be carried out where the pL_{lacO-1}-RNAI fragment was ligated into the linearized pBSK::TN7 vector (figure 4.4.). The DNA of the ligation mix was precipitated with isopropanol and the pL_{lacO-1}-RNAI plasmid was electrotransformed into competent NEB10ß cells. Cells were plated onto LB-cm agar plates and incubated over night at 37°C. Growing cells were screened using the primers placO_back and TN7/2-HO-for and plasmids were sequenced.

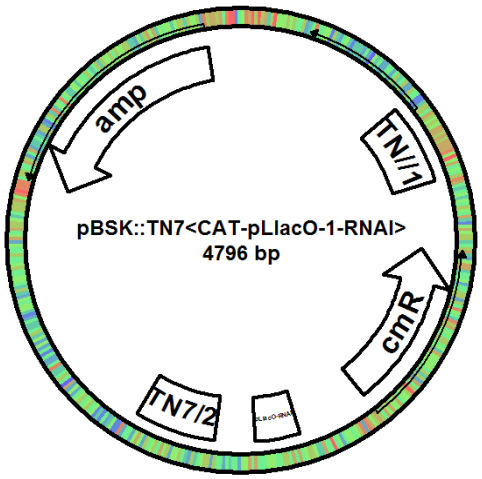

Fig.4 4: pBSK::TN7<CAT-pL_{lacO-1}-RNAI> plasmid. The T7 promoter was therefore substituted by PCR with the pL_{lacO-1} promoter.

4.1.2.2 Impact of IPTG induction on replication of pBSK::TN7<CAT-pL$_{lacO-1}$-RNAI> plasmid

The pBSK::TN7<CAT-pL$_{lacO-1}$-RNAI> plasmid was purified using a Miniprep kit and transferred into competent JM109 cells by electroporation. After electroporation cells were plated onto LB-cm agar plates and incubated over night at 37°C. The next day, colonies were picked from the plate and 10 ml of LB-cm medium with and without IPTG were inoculated. OD_{600} of these cell cultures was measured the next day and plasmid preparation assays were performed. Therefore, same cell numbers were pelleted and the plasmids were extracted from the cell pellets according to the manufactures instructions. The eluted plasmid DNA was measured using the Nanophotometer and 2 µl of each plasmid solution were loaded onto an agarose gel. The same procedure was carried out with an over night culture grown in LB-cm medium that was diluted 1:100 the next day and freshly induced with IPTG. After 5 hours of induced growth, minipreps with same cell numbers were carried out and the extracted plasmid concentration was again determined by the Nanophotometer and by loading 2 µl of plasmid solution onto an agarose gel. Measured values as well as the gel photo are depicted in figure 4.5.

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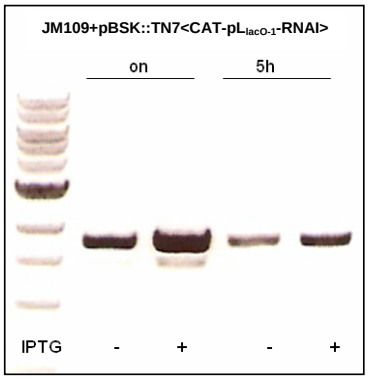
JM109+pBSK::TN7<CAT-pL$_{lacO-1}$-RNAI>	**on**	**5h**
uninduced	83 ng/µl	71,5 ng/µl
induced with 100µM IPTG	178 ng/µl	76,5 ng/µl

a)



b)

Fig.4 5: Plasmid concentration of JM109 hosting the pBSK::TN7<CAT-pL$_{lacO-1}$-RNAI> plasmid. (a) Cells were grown in LB-medium with and without IPTG for 5 hours and over night. Plasmids were isolated according to a Miniprep Kit and concentrations were measured. (b) 2 µl of each plasmid solution were loaded onto an agarose gel.

Surprisingly, the results of the plasmid preparation assay showed that the induction of RNAI expression by IPTG led to an increased concentration of plasmid DNA. This was true for plasmid preparations after 5 hours as well as for those from the overnight cultures. The expression of RNAI might have a controversial effect in this case. On the one hand it inhibits replication and thereby decreases plasmid concentration by binding to the primer RNAII. But as the pBSK::TN7<CAT-pL_{lacO-1}-RNAI> vector is a high copy number plasmid, the reduced replication may simultaneously lead to a greater stability of the plasmid. Fewer plasmid molecules get lost during cell division. Although there are lower numbers of plasmid DNA in one JM109 cell, more cells of the population still own the pBSK::TN7<CAT-pL_{lacO-1}-RNAI> vector, finally resulting in an increase in the total amount of plasmid DNA.

4.1.2.3 Integration of pL_{lacO-1}-RNAI fragment into JM109 genome

In order to investigate the effect of RNAI expression from a chromosomal copy of the RNAI gene, a linear pL_{lacO-1}-RNAI fragment was constructed and integrated into the JM109 genome by using the pRED/ET plasmid.

The linear pL_{lacO-1}-RNAI fragment was obtained using PCR with the Phusion DNA polymerase and the primers TN7/2-HO-for for and TN7/2-HO-long-back. The emerging fragment (figure 4.6.) was purified from the PCR mix according to the Qiagen QIAEXII Kit.

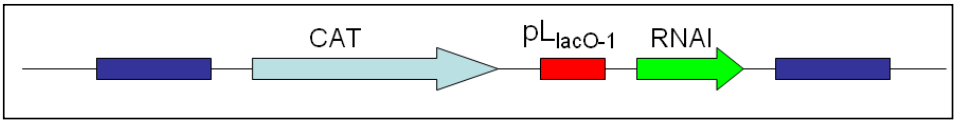


Fig.4 6: Linear pL_{lacO-1}-RNAI fragment consisting of the RNAI gene under the control of the pL_{lacO-1} promoter and a chloramphenicol resistance gene. The fragment is flanked by homologous sequences (indicated by dark blue bars) necessary for the later integration into the JM109 genome

For integrating the linear DNA fragment into the genome of JM109, the Red/ET Kit from Gene Bridges was used as described in section 3.4.1.2. Therefore competent JM109 cells were transformed with the pRED/ET plasmid. These cells were then made competent and 400 ng of the linear pL_{lacO-1}-RNAI fragment were added to 40 µl of JM109 cells by electroporation. Cells were plated on LB-cm agar plates and incubated over night at 37°C. Growing colonies were used for shake flask experiments.

4.1.2.4 Bacterial growth under induced and uninduced conditions

In order to check the impact of RNAI expression on plasmid copy number, JM109::TN7<CAT-pL$_{lacO-1}$-RNAI> cells were made competent and transformed with the plasmids pUC19, pUC19Δ*RNAI* and pBR322. Over night cultures were inoculated in LB-cm medium with and without IPTG. The next day, OD_{600} was measured and over night cultures, grown without IPTG, were adjusted to the same OD_{600} value by diluting them with fresh medium with and without IPTG and grown at 37°. Cell growth was observed by measuring OD_{600} hourly (table 4.5.).

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Table 4. 5.: Bacterial cell growth of different JM109 strains with and without IPTG

Host/Vector pair	**o/n**	**2h**	**3h**	**4h**
JM109+pUC19	2,610	0,535	1,040	1,397
JM109+pUC19+IPTG	**1,860**	**0,518**	**0,987**	**0,936**
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pUC19	2,2	0,522	1,239	1,470
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pUC19+IPTG	**1,240**	**0,485**	**0,759**	**1,084**
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pBR322*	2,630	0,526	0,952	1,567
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pBR322+IPTG	**2,495**	**0,565**	**0,985**	**1,507**
JM109+pUC19*ΔRNAI*	2,630	0,745	0,936	1,122
JM109+pUC19*ΔRNAI*+IPTG	**1,735**	**0,803**	**0,107**	**1,396**
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pUC19*ΔRNAI*	2,145	0,289	0,464	0,711
JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pUC19*ΔRNAI*+IPTG	**1,815**	**0,270**	**0,414**	**0,611**

*As there was no difference in plasmid concentration or cell growth between the induced (+IPTG) and the uninduced (without IPTG) state, controls were disregarded in the case of JM109::TN7<CAT-pL$_{lacO-1}$-RNAI>+pBR322.

Comparison of the different OD_{600} values showed that there were no big differences in cell growth between the JM109::TN7<CAT-pL_{lacO-1}-RNAI> strains and their controls. In every case, the addition of IPTG led to a slower cell growth. Concerning the pUC19 and the pUC19Δ*RNAI* hosting cells, grown over night with IPTG, the OD_{600} value was even only half of that measured for uninduced cells. The addition of IPTG to JM109::TN7<CAT-pL_{lacO-1}-RNAI>+pBR322 cells had nearly no effect on cell growth.

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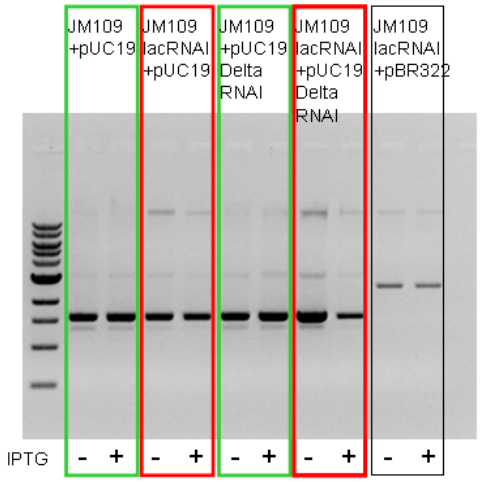
4.1.2.5 Determination of pDNA concentration by plasmid preparation assays

For the quantification of plasmid concentration, same cell numbers were pelleted and plasmid preparations were carried out according to the manufactures instructions. Plasmid concentrations were determined by the Nanophotometer and by loading of 2 µl of plasmid DNA onto an agarose gel (fig. 4.7.).



JM109::TN7<CAT-pL_{lacO-1}-RNAI>	**pDNA yield**
+pUC19	89 ng/µl
+pUC19+IPTG	**71 ng/µl**
+pUC19*ΔRNAI*	198 ng/µl
+pUC19*ΔRNAI*+IPTG	**59 ng/µl**
JM109 (control)	
+pUC19	102 ng/µl
+pUC19+IPTG	**115 ng/µl**
+*ΔRNAI*	107 ng/µl
+*ΔRNAI* +IPTG	**119 ng/µl**

Fig.4 7: Extracted plasmid DNA after cell growth in LB / LB-cm medium, with and without IPTG. Plasmid concentrations were determined by using the Nanophotometer and by loading of 2 µl of plasmid DNA onto an agarose gel. Plasmids extracted from control cells not carrying the pLlacO-1-RNAI fragment in their chromosome are highlighted in green whereas plasmids extracted from cells carrying the pLlacO-1-RNAI fragment are pointed out in red.

Irrespective of pBR322 hosting cells, the expression of RNAI, induced by IPTG addition, led to a reduction of plasmid concentration. Especially, the impact on the pUC19Δ*RNAI* plasmid was rather impressing. Plasmid concentration was reduced about approximately two third when RNAI was expressed. In contrast to the T7 promoter, the pL_{lacO-1} promoter could be tightly regulated, and RNAI expression was only achieved in the presence of IPTG. As the controls showed very clearly, both plasmids, pUC19 and pUC19Δ*RNAI*, were stable in the JM109 cells in the presence and absence of IPTG.

With these results, the hypothesis that RNAI leads to a reduction of plasmid concentration by inhibiting RNAII, was clearly confirmed. In the next step, the plasmid copy number per cell was determined by the Agilent 2100 expert bioanalyzer.

4.1.2.6 Determination of PCN by Agilent 2100 expert bioanalyzer

For the calculation of the plasmid copy number per cell, two parameters had to be determined in advance; the dried biomass and the total amount of DNA. 10 ml of the over night cultures, listed in table 4.6., were used for the determination of the biomass dry matter (BDM).

Table 4.6.**:** Dried biomass from 10 ml of cell suspension

JM109	dried biomass (mg)
+pUC19	10,7
+pUC19+IPTG	9,6
+pUC19ΔRNAI	10,6
+pUC19ΔRNAI+IPTG	9,1
JM109::TN7<CAT-pL_{lacO-1}-RNAI>	
+pUC19	10,5
+pUC19+IPTG	8,6
+pUC19ΔRNAI	10,8
+pUC19ΔRNAI+IPTG	11,1

The amount of total DNA was determined by fluorimetric measurement. The standard curve was obtained by the dilution of calve thymus DNA.

Samples were prepared for the bioanalyzer as described in section 3.5.1. and loaded to the chip.

The results of the dried biomass and the total amount of DNA were combined with those obtained by the bioanalyzer and the plasmid copy number per cell was calculated and is depicted in the diagrams below (figure 4.8.).

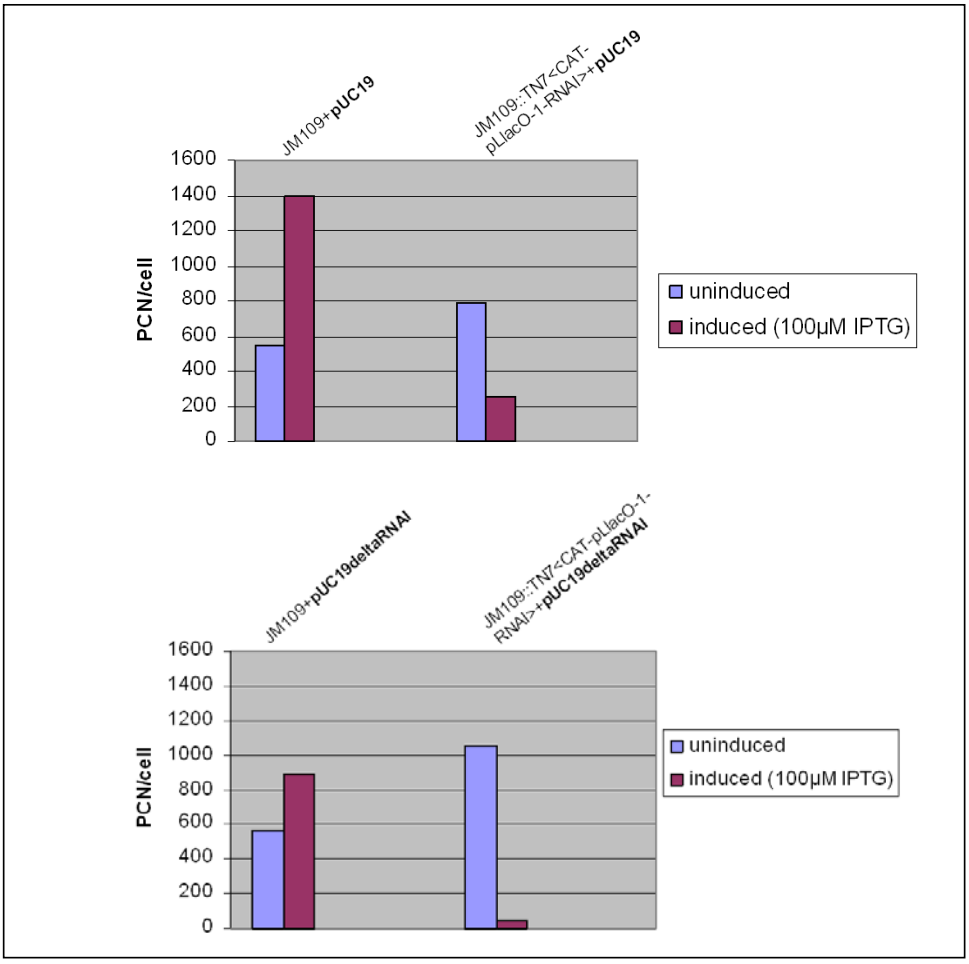


Fig.4 8: PCN/cell calculated by using the Agilenta 2100 expert bioanalyzer. PCNs were determined for the strains JM109+pUC19, JM109::TN7<CAT-pL_{lacO-1}-RNAI>pUC19,, JM109+pUC19Δ*RNAI* and JM109::TN7<CAT-pL_{lacO-1}-RNAI>pUC19Δ*RNAI* grown in LB / LB-cm with (pink column) and without (blue column) IPTG.

The results obtained by the bioanalyzer clearly showed that the plasmid copy number per cell of both plasmids (pUC19 and pUC19Δ*RNAI*) did decrease in the presence of RNAI. Whereas the PCN of the pUC19 plasmid was decreased from 800 to 300

plasmids/cell, the PCN of the pUC19Δ*RNAI* plasmid was even reduced about 900 plasmids/cell. When RNAI was present in the host genome, PCNs of pUC19 as well as of pUC19Δ*RNAI* were higher even in the absence of IPTG when compared to cells without RNAI in their genome. This might be due to a slight basal activity of the pL_{lacO-1} promoter, leading to low levels of RNAI inside the cell. As both plasmids are high copy number plasmids, these low levels of RNAI may have a stabilizing effect leading to a slight increase in PCN. The PCN of the pUC19Δ*RNAI* plasmid in strain JM109::TN7<CAT-pL_{lacO-1}-RNAI> was thereby 1000 compared to the pUC19 plasmid with a PCN of 800. As the pUC19Δ*RNAI* plasmid itself does not produce any RNAI, the stabilizing effect of low basal transcription levels of the integrated copy of RNAI might be even more impressive resulting in this high PCN per cell.

4.2 Up regulation of plasmid copy number via inducible expression of uncharged tRNAs

For the proper charging of a tRNA with its corresponding amino acid an enzyme called aminoacyl-tRNA synthetase is necessary. Regarding the *E. coli* alanyl-tRNA synthetase, the $tRNA^{Ala}$ acceptor stem is sufficient for the right recognition (38).
In the following experiments a vector was designed, carrying the $tRNA^{Ala}$ gene under the control of the T7 promoter. The sequence of the $tRNA^{Ala}$ was thereby mutated twice in order to inhibit proper charging with alanin (fig. 4.9.). Based on this $tRNA^{Ala}$ vector a linear DNA fragment was amplified for the integration into the MG1655 genome. Via P1 transduction the T7-tRNA fragment was transferred into the HMS174(DE3) chromosome, the JM108 chromosome and into the DH5α(DE3) chromosome by homologous recombination.

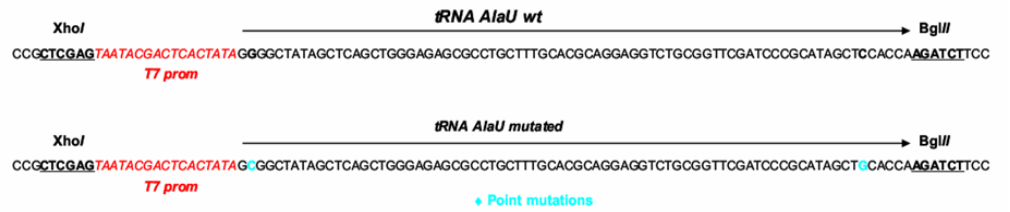


Fig.4 9: Wt- and mutated $tRNA^{Ala}$. The two point mutations (blue) were incorporated by oligonucleotide synthesis and responsible for the presence of uncharged tRNAs (picture taken from Mairhofer et al., unpublished).

The strains were transformed with the plasmids pUC19 and pBR322 by electroporation and shake flask experiments, for determining the impact of tRNA overexpression, were conducted.

4.2.1 Construction of MCS-vector

Using PCR the pBSK::TN7<CAT-T7-GFP> vector was modified to contain a Multiple Cloning Site (MCS) instead of the GFP gene. By using the MCS_TN7_GFP_1500bp_for and MCS_TN7_GFP_2435bp_back primer together with the Phusion DNA Polymerase, a linearized vector was produced with both ends consisting of parts of a multiple cloning site. The linearized vector was loaded onto a gel and purified using a gel extraction Kit. The purified fragment was later on treated

with the enzymes *BamH*I, encoded on both primers, and *Dpn*I to get rid of template contaminations. The fragment was ligated afterwards, giving rise to the circular pBSK::TN7<CAT-MCS> as shown in figure 4.10. This vector was used in a later step for inserting the tRNA fragment.

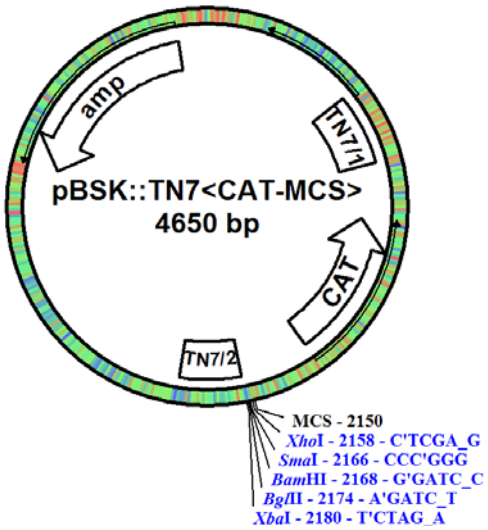


Fig.4 10: pBSK vector carrying a multiple cloning site (MCS). For MCS insertion, a PCR was carried out with the primers MCS _TN7_GFP_1500bp_for and MCS_TN7_GFP_2435bp_back.

Competent NEB10ß cells were transformed with the vector by electroporation and cells were plated on LB-cm agar plates. As control, the linearized vector was also transferred into the cells, to check for template carry-over. The next day, growing colonies were picked and inoculated in LB-medium. Plasmids were extracted by a miniprep Kit and a control digestion was done using the restriction enzyme *Hind*III. In the case of the pBSK::TN7<CAT-MCS> vector, the linearized plasmid should appear on the gel with a length of 4650 bp. Vector sequence was verified by sequencing.

4.2.2 Construction of T7-tRNA plasmid

The T7-tRNA$_{mut}$ fragment was obtained by annealing the two oligonucleotides *Xho*-pT7-AlaU-back (carried the T7-promoter) and *Bgl*II-tRNA-AlaU-for (carried the tRNA sequence). Therefore, the oligos were mixed in equal amounts and cooled down slowly from 99°C to 36°C. During this drop of temperature, the oligos bound due to

their homologous sequences. Using the Klenow polymerase the remaining single strand ends were filled up with nucleotides and the double stranded T7-tRNA$_{mut}$ fragment was obtained. As the two oligos carried a defined restriction site, the fragment, as well as the pBSK::TN7<CAT-MCS> vector, were cut with the restriction enzymes *Bgl*II and *Xho*I. Vector and fragment were purified from the restriction reaction and were ligated afterwards. Competent DH10ß cells were transformed with the pBSK::TN7<CAT-T7-tRNA$_{mut}$> by electroporation and cells were plated on LB-cm agar plates. Plasmids were extracted from the growing colonies and a control restriction was carried out by using the enzyme *BseY*I. In the case of a positive result, two fragments, ~1000 bp and ~3733 bp long, should be obtained. As these fragments were actually visible on an agarose gel, plasmids were sequenced. In this way, the pBSK::TN7<CAT-T7-tRNA$_{mut}$> plasmid, depicted in figure 4.11. was constructed.

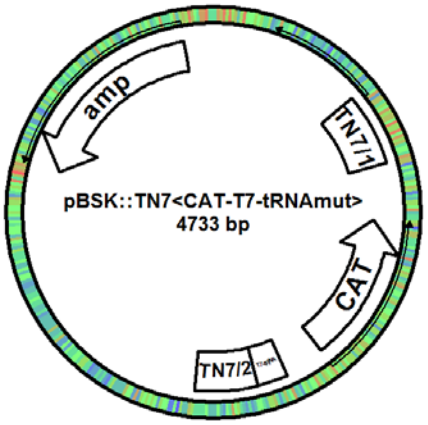


Fig.4 11: pBSK::TN7<CAT-T7-tRNA$_{mut}$> plasmid obtained by cloning the annealed T7- and tRNA$_{mut}$- oligonucleotides into the MCS of the pBSK::TN7<CAT-MCS> vector.

4.2.3 Construction of linear T7-tRNA$_{mut}$ fragment for homologous recombination into *E. coli* chromosome

The T7-tRNA plasmid was extracted from DH10ß cells and the T7-tRNA$_{mut}$ fragment was cut out of the vector using the enzymes *Kpn*I and *Sac*I. The obtained fragment did carry a chloramphenicol resistance gene, the tRNA gene under the control of the

T7 promoter and was flanked by homologous sequences (figure 4.12.). The fragment was purified from the restriction mix and competent MG1655 cells carrying the pKD46 plasmid were transformed with 300 ng of the linear fragment.

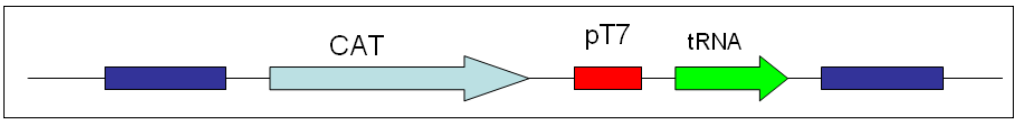


Fig.4 12: T7-tRNA fragment for integration into MG1655 genome. The fragment was transferred to a HMS(DE3)174 strain by P1 transduction and inserted into it's chromosome via homologous recombination. Homologous sequences are indicated by dark blue bars.

Cells were plated on LB-cm agar plates. Growing colonies were screened with plasmid ori primers (RNAII 115 back, RNAII 625 for) on the one hand and primers that bound to the cm-resistance gene (cat begin for) and in the MG1655 genome (TN7/2-HO-for) on the other hand. Positive colonies were cured from the pKD46 plasmid by placing them to 42°C. The T7-tRNA$_{mut}$ fragment was amplified in a PCR from the MG1655 genome by using the primers cat begin for and TN7/2 extern. The obtained product was sequenced to check for integrity of the T7-tRNA$_{mut}$ expression element.

4.2.4 Integration of T7-tRNA$_{mut}$ fragment into HMS(174)DE3 genome

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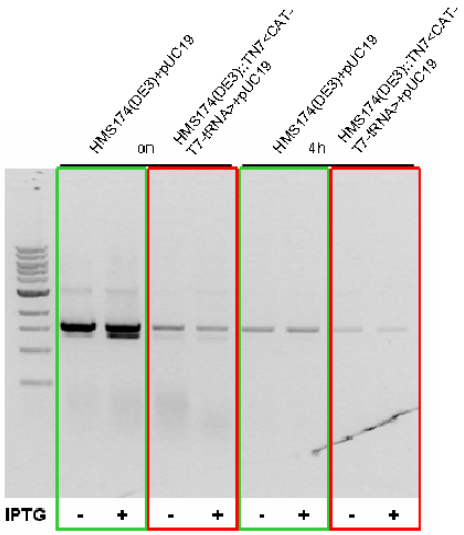
In the next step, a P1 phage transduction was carried out and the T7-tRNA$_{mut}$ fragment was integrated into the HMS174(DE3) genome by homologous recombination. Growing colonies were screened with the primers cat begin for and TN7/2 extern and the amplified fragments were sequenced. Using this approach HMS174(DE3)::TN7<CAT-T7-tRNA$_{mut}$> cells were obtained.

4.2.4.1 Shake flask experiments and determination of plasmid concentration

In order to investigate the effect of tRNA overexpression on plasmid concentration, cells of the HMS174(DE3)::TN7<CAT-T7-tRNA$_{mut}$> strain were made competent and transformed with the pUC19 plasmid by electroporation. 10 ml of LB-cm medium with

and without IPTG were inoculated with HMS174(DE3)::TN7<CAT-T7-$tRNA_{mut}$>+pUC19 cells. As control, HMS174(DE3)+pUC19 were also grown induced and uninduced over night. The next day, same cell numbers were centrifuged and plasmids were extracted from the cell pellets. Additionally, uninduced over night cultures were diluted 1:100 and induced again with 100 µM IPTG. After 4 hours of growth at 37°C shaking, plasmids from equal amounts of cells were extracted again. Plasmid concentrations were measured by the Nanophotometer and 2 µl of the eluted plasmids were loaded onto an agarose gel (figure 4.13.).



HMS174(DE3)	Minipreps (o/n)
+pUC19	214 ng/µl
+pUC19+IPTG	**296 ng/µl**
HMS174(DE3)::TN7 <CAT-T7-tRNA>	
+pUC19	128 ng/µl
+pUC19+IPTG	**85 ng/µl**

HMS174(DE3)	Minipreps (4h)
+pUC19	34 ng/µl
+pUC19+IPTG	**31 ng/µl**
HMS174(DE3)::TN7 <CAT-T7-tRNA>	
+pUC19	18 ng/µl
+pUC19+IPTG	**28 ng/µl**

Fig.4 13.: Concentrations of extracted plasmid DNA after over night cell growth and 4 hours of growth in LB-cm medium and in LB-cm medium supplied with 100 µM IPTG. Plasmid concentrations were determined by the Nanophotometer and by loading of 2 µl of eluted plasmids onto an agarose gel. Plasmids extracted from control cells not carrying the T7-$tRNA_{mut}$ fragment in their chromosome are highlighted in green whereas plasmids extracted from cells carrying the T7-$tRNA_{mut}$ fragment are pointed out in red.

Surprisingly, the results showed the very opposite of the expected effect. The over expression of uncharged tRNAs led to a massive decrease in plasmid concentration instead of increasing it. This phenomenon was observed in both cases, after 4 hours of cell growth as well as after over night growth. The strong reduction of plasmid concentration might be caused by the activation of the stringent response, as the strain used in this experiment is a *relA*$^+$ one. The stringent response possibly leads to a down regulation of RNAI and RNAII expression which in turn is responsible for the inhibition of plasmid replication. Further, it was again clearly apparent, that the T7-system is a very leaky one. Even in the absence of IPTG, tRNA expression seemed to be induced and nearly no difference in plasmid concentration could be detected between the induced and the uninduced state. When the tRNA producing cells were inspected under the microscope, cells showed a phenotype that is normally observed under starvation like conditions. Instead of being short and rod shaped, cells were very long and filamentous.

4.2.5 Integration of T7-tRNA$_{mut}$ fragment into genome of *relA*$^-$ strains

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4.2.5.1 Shake flask experiments and determination of plasmid concentration

It was reported that up regulation of plasmid copy number by the action of tRNAs, might not be that efficient in *relA*$^+$ *E. coli* strains .As this was the case for the HMS174(DE3) strain used, all experimental set up was repeated with the *relA* mutant strains DH5α(DE3) and a JM108(DE3).

Therefore the two new strains were made competent and transformed with the pTSA29 plasmid by electroporation. Cells were infected with the already existing P1 phage lysate, and the T7-tRNA$_{mut}$ was integrated in their chromosome by homologous recombination. The tRNA-containing strains were transformed in the next step with the plasmids pUC19 and with the low copy number plasmid pBR322. Cells of both strains, as well as controls, consisting of cells only carrying the plasmids but not the tRNA copy in their chromosome, were grown in LB-cm medium, with and without IPTG over night. The next day, OD_{600} was measured and same cell numbers were centrifuged. Plasmids were extracted from cell pellets and plasmid concentration was determined by the Nanophotometer and by loading 2 µl of the eluted plasmids onto an agarose gel (fig. 4.14.).

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	Minipreps o.n.		**Minipreps o.n.**
DH5α(DE3)+pBR322	51,5 ng/µl	**JM108(DE3)+**pBR322	50 ng/µl
DH5α(DE3)+pBR322+IPTG	36,5 ng/µl	**JM108(DE3)+**pBR322+IPTG	49 ng/µl
DH5α(DE3)::TN7<T7-tRNA$_{mut}$>+pBR322	55 ng/µl	**JM108(DE3)-T7-tRNA**+pBR322	38 ng/µl
DH5α(DE3)::TN7<T7-tRNA$_{mut}$>+pBR322+IPTG	35 ng/µl	**JM108(DE3)-T7-tRNA**+pBR322+IPTG	35 ng/µl
DH5α(DE3)+pUC19	71 ng/µl	**JM108(DE3)+**pUC19	112 ng/µl
DH5α(DE3)+pUC19+IPTG	77,5 ng/µl	**JM108(DE3)+**pUC19+IPTG	97 ng/µl
DH5α(DE3)::TN7<T7-tRNA$_{mut}$>+pUC19	75,5 ng/µl	**JM108(DE3)-T7-tRNA**+pUC19	68 ng/µl
DH5α(DE3)::TN7<T7-tRNA$_{mut}$>+pUC19+IPTG	76,5 ng/µl	**JM108(DE3)-T7-tRNA**+pUC19+IPTG	66 ng/µl

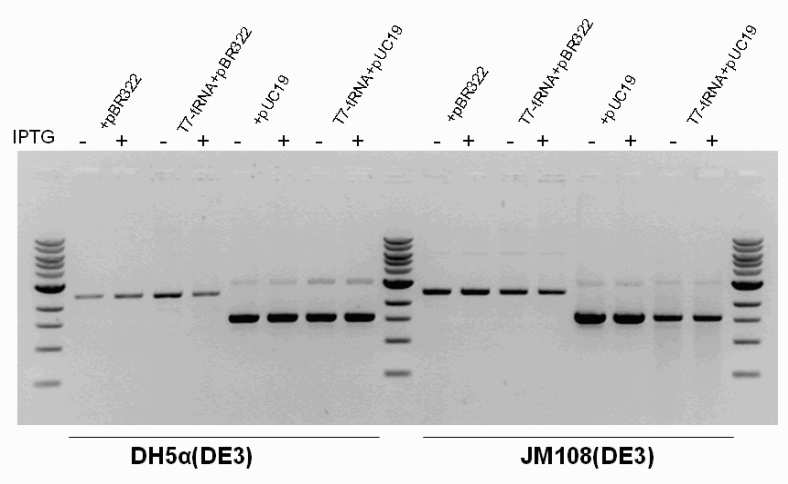

Fig.4 14.: Plasmid concentrations of listed strains, determined by using the Nanophotometer and by loading 2 µl of eluted plasmids onto an agarose gel. Plasmids were extracted from cells that were grown in LB-cm / LB medium, with and without IPTG over night.

In general one could say, that plasmid concentrations were rather unaffected by tRNA expression. Concerning the DH5α(DE3)-strain, this was true for both plasmids, the pBR322 and the pUC19. In the case of JM108(DE3), plasmid concentration was slightly reduced regarding the pBR322 plasmid. The effect of tRNA expression in this strain on the pUC19 plasmid was more impressive, as plasmid concentration was

decreased about approximately 40 ng/µl. The same effect was observed before in the HMS174(DE3) strain.

For all the experiments described above, a mutated form of the tRNA gene was used. This mutation included two point mutations as pictured in fig. 4.9. These mutations should inhibit the efficient charging of the tRNA with its corresponding amino acid. At the same time it is possible, that due to the alteration of the tRNA sequence, the enzymatic cleavage of the bound RNAI was disturbed since the accurate mechanism has not been elucidated yet. The original sequence might be important for the proper action of tRNAs as nucleases.

5 Conclusion and Outlook

The idea of using plasmid DNA for the treatment of genetic disorders and for the development of vaccines has become a major issue for the pharmaceutical industry. As more and more DNA-based drugs have already reached phase III of clinical trials or even are available on the market, there is a steadily increasing demand for high quality pDNA.
A big problem of excessive plasmid production is the high metabolic load that is exerted on the host cell by sole plasmid replication, leading to a decrease in growth rate and biomass yield and finally to decreased overall pDNA yield.

The main goal of this work was to develop a system that allows for the control of plasmid replication in *E.coli*, and therefore enables the decrease of metabolic load. By the insertion of a chromosomal copy of the RNAI gene under the control of the pL_{lacO-1} promoter the major objective of the current studies was fulfilled, since PCN could be decreased by addition of the inducer IPTG. It was shown in shake flask experiments, that the developed regulation system allows precise and impressive downregulation of PCN of ColE1-type plasmids by RNAI overexpression. As a successful application of this system was already proved in a small experimental set up, a large scale feasibility study, employing fed batch fermentations, has to be conducted in the near future. By the use of this regulation mechanism, it will be possible to keep the metabolic burden on the host cell low at the beginning of the bioprocess. Once biomass has accumulated, downregulation of the PCN can be stopped since a metabolizable inducer (e.g. lactose) is used and excessive production of pDNA is induced in the absence of the inducer.
Additionally, the feasibility of another extra high copy variant of the ColE1 ori could be shown. By chromosomal complementation of the Δ*RNAI* mutation of pUC19Δ*RNAI* by TN7<CAT-pL_{lacO-1}-RNAI> the pUC19Δ*RNAI* plasmid could be stabilized and it is therefore anticipated that higher pDNA yields are possible using this novel ori. Another interesting approach of this work was the active upregulation of PCN by the overexpression of uncharged tRNAs. As already published, interaction of uncharged tRNAs with RNAI should result in an increase in plasmid replication initiation, since RNAI is degraded. Contrarily, the opposite effect was observed throughout these studies. Massive downregulation of PCN, was achieved in *relA*$^+$ strains. In the case

of *relA*$^-$ cells, PCN was unaffected by tRNA overexpression. As tRNAs used in these experiments did carry two point mutations to inhibit tRNA charging, it is possible that the RNAI-degrading activity of the tRNAs was disturbed thereby. Repetition of experiments with wt tRNAs might bring the expected results and lead to an increase in plasmid replication initiation.

Consequently, it could be shown that negative regulation of PCN is possible, by the overexpression of RNAI from a chromosomal gene copy. Tight regulation of RNAI expression can thereby be achieved by the use of the pL_{lacO-1} promoter. This newly developed regulation system might lead to a dramatic increase in the amount of pDNA yield during the fermentation process.

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7 Appendix

Primersequences:

Sma_tetR_TN10_back	TCCcccgggCCAATGCGATCTTTGTCGAAC
	ccgCTCGAGgtagtTaatacgactcactataGGGGCTATAGCTCAG
XhoI_pT7_AlaU_ORG_back	CTGGGAGAGCGC
	GGAagatctTGGTGgAGCTATGCGGGATCGAACCGCAGACC
	TCCTG
BglII_tAlaU_ORG_for	CGTGCAAAGCaggcgctctcccagctgagcta
tRNA_seq_primer	GTTGTATGTCTTCGCCGATC
Xba_CAT_back	gcTCTAGAagccactggagcacctc
XbaI_rrnBT12_for_new	ggTCTAGAaaaaggccatccgtcagg
SpeI_rrnBT12_back_new	gcACTAGTataaaacgaaaggctcagtc
Xba_rrnBT12_back_new	ggTCTAGAaaaaggccatccgtcagg
SpeI_rrnBT12_for_new	gcACTAGTataaaacgaaaggctcagtc
pN25_part1	gcgcaacgcaattaatgtaagttagcgcgaATTGTCGAGGGA
pN25_part2	TTCCTGAAAGCAAATaaatttttatgattTCCCTCGACAAT
pN25_part3	ATTTGCTTTCAGGAAaatttttCTGTAtaatagattcataaa
	ATGATGcgtctcaTCATatttaaacTCTTCTctcaaaTTTATGAATCT
BsmBI_pN25_part4	ATTA
SmaI_pN25_back	tccCCCGGGgcgcaacgcaattaatgtaag
SpeI_rrnBT12_back	gACTAGTataaaacgaaaggctcagtcg
XbaI_rrnBT12_for	gcTCTAGAcatccgtcaggatggcc
HindIII_lacIq_back	cccAAGCTTgacaccatcgaatggtgc
EcoRI_lacIq_for	CCGgaattcTCACTGCCCGCTTTCCAG
BsmBI_tetR_back	GATGATcgtctctATGATGTCTAGATTAGATAAAAG
BamHI_tetR_for	GCTGCTggatccTTAAGACCCACTTTCACATTTAAG
XhoI_proms_RNAI_for	CCGctcgagAACAAAAAAACCACCGCTACC
	ATGATGgctaagcTCCCTATCAGTGATAGAGATTGACATCCCTAT
BlpI_pLtetO_back	CAGTGATAGAGATACTGAGCACacagtatttggtatctgcgc
	GGAagatctTGGTGcAGCTATGCGGGATCGAACCGCAGACCTCC
	TGCGTGCAAAGCAGgcgctctcccagctgagctaTAGATT
BlpI_placara_back	Cacagtatttggtatctgcgc
	ATGATGgctaagcATAAATGTGAGCGGATAACATTGACATTG
	TGAGCGGATAACAAGATACTGAGCAC
BlpI_pLlacO_back	acagtatttggtatctgcgc
	ccgCTCGAGTaatacgactcactataGcGGCTAtagctcagctgggag
Xho_pT7_AlaU_back	agcgcatctgcgc
	GGAagatctTGGTGcAGCTATGCGGGATCGAACCGCAG
BglII_tRNA_AlaU_for	ACCTCCTGCGTG

	CAAAGCAGgcgctctcccagctgagcta
MCS_TN7_GFP_1500bp_for	gcgcGGATCCcGGGctcgagGCCACTGGAGCACCTCAAAAAC
MCS_TN7_GFP_2435bp_back	gatgGGATCCagatctTCTAGAgcatccatttattactcaaccg
	TTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACC
T7prom-RNAI	AAATACTGTCTATAGTGAGTCGTATTAAAGCTT
	GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGTTGG
T7term-RNAI	TAGCGGTGGTTTTTTTGTTCTAGCATAACCCCTTGGGGC
RNAI-10promΔback	CTACGGCATGTGATCTTGAACAGTATTTGGTATCTGCGC
RNAI-35promΔfor	TTAGGCCACGTGAAGTTGAACTCTGTAGCACCGCCTAC
m13-20	5‘ tgtaaaacgacggccagtg 3‘
TN7/2-HO-for	5‘ tga aga agt tcg cgc gcg 3‘
cat begin for	5‘ caacggtggtatatccagtg 3‘
RNAII 115 back	5‘ TCTAGTGTAGCCGTAGTTAG 3‘
RNAII 652 for	5‘ GGTTATCCACAGAATCAGGG 3‘
TN7/2 extern	5‘tggcgctaattgatgccg 3‘
TN7/1-HO-long-back	5' gttgcgacggtggtacgc 3'

8 Abbreviations

aa	aminoacid
amp	ampicillin
Amp^R	ampicillin resistance
bp	base pairs
cfu	colony forming unit
cm	chloramphenicol
Cm^R	chloramphenicol resistance
DMSO	dimethyl-sulfoxide
DNA	desoxyribonucleic acid
dNTPs	nucleotide triphosphates
E. coli	*Escherichia coli*
GFP	Green Fluorescent Protein
h	hours
IPTG	isopropyl □-D-thiogalactopyranoside
kan	kanamycin
Kan^R	kanamycin resistance
kbp	kilo base pairs
min	minutes
mRNA	messenger RNA
OD_{600nm}	optical density at a wavelength of 600 nm
Ori	origin of replication
PCN	plasmid copy number
PCR	polymerase chain reaction
pDNA	plasmid DNA
ppGpp	guanosine tetraphosphate
RNA	ribonucleic acid
ssDNA	single stranded DNA
tet	tetracycline
T_m	melting temperature

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11 Curriculum Vitae

General Information

Name	Esther Egger
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Education

2003 - 2008	University of Vienna: Molecular Biology 2008: diploma thesis at the University of Natural Resources and Applied Life Sciences Vienna, Institute of Applied Microbiology (IAM), Group “Baculo” (Reingard Grabherr)

Employment History

2003 (July)	Nycomed Linz, Pharmaceutical Production
2004 – 2007 (1-2 months during summer)	Nycomed Linz, Quality Control, Laboratory of Microbiology
2006 (November, December)	University of Vienna, Max F. Perutz Laboratories, Institute of Molecular Cell Biology, Group of Gerhard Wiche
2007 (February, March)	University of Vienna, Max F. Perutz Laboratories, Institute of Molecular Medicine, Group of Karl Kuchler
2007 (September)	University of Veterinary Medicine Vienna, Institute of Bacteriology, Mycology, Hygiene, Group of Hans-Jürgen Busse
2007 (October)	University of Natural Resources and Applied Life Sciences Vienna, Institute of Applied Microbiology, Group of Reingard Grabherr