Universität fur Bodenkultur Wien

Influence of the endoribonuclease MazF on cellular growth and recombinant protein production in *Escherichia coli*

MASTERARBEIT

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1 Abstract

Plasmid based production of recombinant proteins using *Escherichia coli*, is state of the art technology to achieve high yields. Taking in account growth associated product formation kinetics synthesis of recombinant protein occurs on the expense of total metabolic synthesis capacity. Therefore an alternative approach to increase recombinant protein production is, to develop gene constructs for product formation in a non growth associated manner. Thereby maximal resources can be directed to recombinant protein synthesis avoiding metabolic overload of host cell metabolism and enhancing efficiency of substrate utilization. Moreover, the production of toxic proteins can be enabled.

By utilizing the endoribonuclease MazF of *E. coli* which is able to inhibit cell growth, recombinant proteins derived from ACA free mRNA can be accumulated in high amounts in a so called single protein production system.

The goal of this diploma thesis was to create a MazF based *E. coli* expression strain suitable for application on industrial scale.

Due to controversial findings published in literature the influence of MazF on host cell metabolism had to be determined. Therefore MazF was overexpressed in *E. coli* HMS174(DE3)pBAD33_*mazF* in shake flask and bioreactor cultivations. The impact of MazF on cell growth was monitored by optical density and cellular dry weight, the influence on cell viability by flow cytometry. The expression level of a target protein was analysed by SDS-PAGE electrophoreses.

Expression of MazF in exponentially growing *E. coli* cells led to reduced or completely inhibited cell growth without negative impact on cell viability. Cells were in a reversible "quasi dormant" state and cell growth could be fully restored subsequent to repression of the *mazF* promoter. Protein expression of a target protein susceptible for MazF was completely inhibited.

For the construction of a production strain, the gene encoding for the model protein human superoxide dismutase (hSOD) was synthesized to be devoid of the triplet ACA and incorporated into an appropriate expression vector. The thus obtained vector pET30a*SOD_ACAfree* can be integrated into the expression host after modification of the disruptive ACA between the RBS and the MCS and thereafter used for construction of a two plasmid production strain.

2 Zusammenfassung

Die plasmidbasierte Herstellung rekombinanter Proteine mit *Escherichia coli* ist Stand der Technik und hohe Ausbeuten sind damit erzielbar. In Anbetracht der wachstumsassoziierten Produktbildungskinetik erfolgt die Produktion rekombinanter Proteine auf Kosten der Gesamtsyntheseleistung der Zelle. Ein alternativer Ansatz für gesteigerte Proteinsynthese ist deshalb, die Entwicklung von Genkonstrukten für eine wachstumsentkoppelte Produktbildung. Durch verbesserte Effizienz der Substratverwertung und gezielte Lenkung der Ressourcen in Richtung Proteinsynthese kann eine gesteigerte metabolische Belastung der Wirtszelle vermieden werden. Zusätzlich wird die Produktion toxischer Proteine ermöglicht.
Mit Hilfe der Endoribonuklease MazF aus *E. coli* ist es möglich das Zellwachstum zu inhibieren und rekombinante Proteine, die von ACA freier mRNA translatiert werden, spezifisch in einem Produktionsprozess anzureichern.
Ziel dieser Arbeit war die Herstellung eines auf MazF basierten *E. coli* Expressionsstammes für die Anwendung eines Produktionssystems im industriellen Maßstab.

Da kontroverse Ansichten über den Einfluss von MazF auf den Wirtszellmetabolismus publiziert sind, wurde dieser näher untersucht. Zu diesem Zweck wurde *E. coli* HMS174(DE3)pBAD33_*mazF* in Schüttelkolben und im Bioreaktor kultiviert und MazF überexprimiert. Durch Bestimmung der optischen Dichte und der Biomassetrockensubstanz wurde der Einfluss auf das Zellwachstum und mit Durchflusszytometrie der Einfluss auf die Zellviabilität untersucht. Auswirkungen auf das Expressionslevel eines Proteins wurden mit einer SDS PAGE Elektrophorese bestimmt.
Die Produktion von MazF führte in exponentiell wachsenden Zellen zu Wachstumsinhibierung jedoch ohne negative Auswirkung auf die Viabilität. Die Zellen befanden sich in einem reversiblen Ruhezustand und Zellwachstum konnte durch Repression des *mazF* Promoters wieder angeregt werden. Die Expression eines Zielproteins war vollständig inhibiert.
Für die Konstruktion des Expressionsstammes wurde das Gen der humanen Superoxid Dismutase (hSOD) synthetisiert und die nun ACA freie Sequenz in einen geeigneten Expressionsvektor integriert. Das entstandene Plasmid pET30a*SOD_ACAfree* kann nun nach Entfernung einer störenden ACA Sequenz zwischen RBS und MCS, in den Wirt transformiert und für die Konstruktion eines zweiplasmidigen Produktionsstammes verwendet werden.

3 Introduction

3.1 Growth associated product formation in *Escherichia coli*

Due to extensive knowledge on genetics and molecular biology, the gram negative bacteria *Escherichia coli* (*E. coli*) is a workhorse for biological studies and a model system for strategies that are even transferable to other, more complex organisms.

Especially the production of heterologous proteins with plasmid based systems, in *E coli* K12 strains, has become a standard technique with use in biotechnological and therapeutic implementations. Product formation is thereby growth associated and the accumulation of a target protein leads to a competition for the limited pool of cellular resources. Thus the synthesis of recombinant protein occurs on the expense of total metabolic synthesis capacity and leads to reduced cellular growth, reduced final biomass and eventually to breakdown of host cell metabolism. (Sang, 2009)

Plasmid based systems are well established, characterized and optimized. Drawbacks like the restricted period of protein production have been tolerated, also due to lack of alternatives. But during the last years bimolecular and engineering methods have been developed to overcome the disadvantages of growth associated product formation.

3.2 Decoupling of product formation and cell growth

One approach to avoid metabolic overload of host cell metabolism was development of plasmid based gene constructs for product formation in a non growth associated manner. By decoupling of product formation and cell growth, substrate can be channelled almost solely to product synthesis leading to enhanced substrate utilization with economic benefit for cultivation process and downstream operations.

Suzuki et al. developed such a bacterial expression system for non growth associated production of recombinant proteins in *E. coli*, the so called single protein production (SPP) system. Thereby the *E. coli* protein MazF is overexpressed to trigger cell growth arrest and exclusively produce a protein of interest in absence of other cellular protein production. (Suzuki et al. 2005, Suzuki et al. 2006)

The goal of this diploma thesis was to create a MazF based *E. coli* expression strain suitable for application on industrial scale.

3.2.1 MazF mediated growth arrest

The *E. coli* protein MazF is an ACA-sequence specific endoribonuclease that works in ribosome independent manner. (Zhang et al. 2003b) MazF cleaves mRNA preferentially at the 5'end of the first A in ACA sequences leading to a 2'3' cyclic phosphate at one side and a free 5'OH group at the other. In low yield the hydrolysis reaction can also occur at the 3' side of the phosphodiester linkage of the first A residue. (Figure 3.1) For efficient cleavage the 2'OH group of N in a NACA substrate is essential, all the other residues can be desoxyriboses. MazF can neither cleave in a RNA/DNA or RNA/RNA duplex, nor in single stranded DNA. It is highly specific for single stranded RNA and degradation is regardless of the position of ACA in or out of a reading frame. (Zhang et al. 2005)

In the expression strain *E. coli* K12 MazF cleaves almost all cellular mRNAs, as 99 % contain one or more ACA triplet. However, one example for mRNA resistant to MazF cleavage is *lpp* mRNA containing four ACA triplets. Three out of four ACA sequences are protected from cleavage by secondary structure and only the fourth ACA sequence leads to the remove of a four base fragment from the 5' end. This protection may be due to the high translation efficiency because the *lpp* product, lipoprotein, is the most abundant protein in *E. coli*. (Zhang et al. 2003b)
In *E. coli* and other prokaryotes the degradation of cellular mRNA leads to an efficiently blocked protein synthesis resulting in cell growth arrest. (Baik et al. 2009)

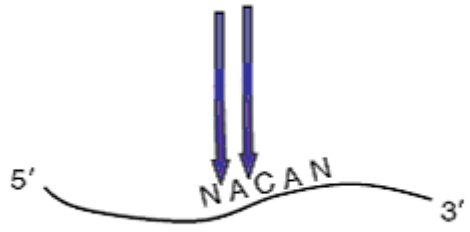


Figure 3.1 The mode of action of the ACA specific endoribonuclease MazF (Engelberg-Kulka et al. 2005)

MazF is naturally found on the chromosome of *Escherichia coli* and is .encoded by a so called toxin antitoxin (TA) locus. The *mazEF (chpA)* module is located in the *relA* operon downstream of the *relA* gene and consists of a pair of genes. The two genes *mazE* and the downstream located *mazF*, encode for the unstable antitoxin MazE (ChpAI) and the stable toxin MazF (ChpAK). The antitoxin MazE sequesters the lethal action of the toxin MazF by direct protein - protein interaction. (Zhang et al. 2003b) The co-expression of both genes is directed by two strong promoters (P_2 and P_3), who are negatively regulated by binding of the toxin and the antidote to a palindrome sequence in the promoter region resulting in decreased transcription of their own genes. (Marianovsky et al. 2001) By determination of the crystal structure (Figure 3.2) the MazEF complex was resolved as a 2:4 heterohexmer with alternating MazE and MazF homodimers. (Zhang et al. 2003b)

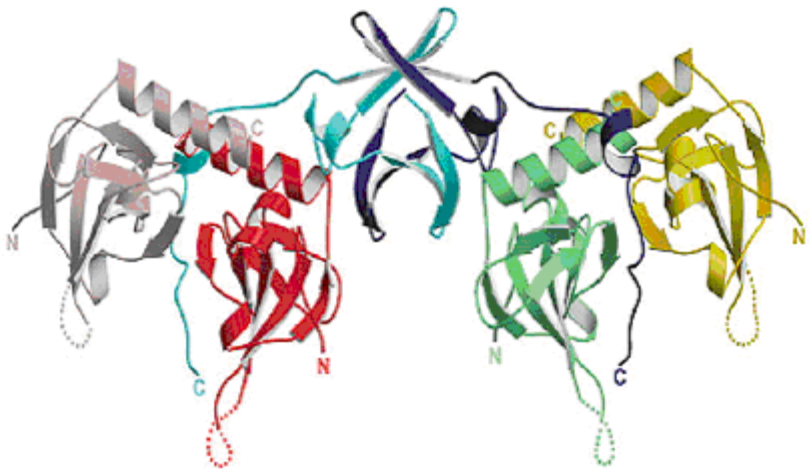


Figure 3.2 The MazEF complex

The MazEF complex consist of two MazE molecules (blue) and four MazF molecules (yellow, green, pink and red) (Engelberg-Kulka et al. 2005)

Protein synthesis can continue on mRNA free of ACA triplets

The basis for development of a single protein production system in *E. coli* was the finding that, despite reduced global rate of macromolecular synthesis, protein synthesis can continue on mRNA free of the recognition sequence ACA. Cells are fully me-

tabolically active because the noncoding RNA's, involved in protein synthesis and translocation, are protected from MazF cleavage by various mechanisms.

Escherichia coli K12 contains 87 tRNAs and ACA is the last frequent triplet, out of the possible 64 triplets, used in the coding sequences. Overall just 19 tRNA sequences contain ACA and out of this selection only three are possible targets for MazF cleavage because the other 16 are protected by secondary structure and methylation. The remaining three have redundant functions with other tRNAs.

Ribosomal RNAs are resistant, despite the presence of ACA, because of their extensive secondary structures and their association with ribosomal proteins. In the eight genes coding for 5 S rRNAs one ACA triplet is found in a double strand region and the endoribonuclease MazF possessing single strand activity cannot cleave. The 4.5 S rRNA, important for targeting of secretory and membrane proteins does not contain ACA triplets, neither does the tmRNA, that is responsible for the rescue of ribosomes stalled on damaged mRNAs. (Baik et al. 2009)

The characteristics of MazF as a sequence specific endoribonuclease which degrades mRNA to inhibit protein synthesis and thereby leading to inhibited cell growth have been consistently stated. Target for discussion has been the biological function of the *mazEF* module encompassing the consequences of ectopic overexpression of MazF. Two contradictory theories arose in the last 15 years.

3.2.2 *MazEF* as stringent response element

Stringent response compasses all physiological changes due to amino acid starvation. Within the stringent response the function of the *mazEF* module was supposed to be the modulation of the global rate of macromolecule synthesis. (Figure 3.3) Expression of the TA locus leads to accumulation of stable MazF due to degradation of the less stable antidote MazE by cellular proteases. Further, overproduction of toxic protein inhibits translation by cleavage of translated RNA's. (Christensen et al. 2003) Owing to thereby inducing reversible bacteriostatic conditions growth arrested cells are able to survive episodes of extreme nutritional stresses. (Buts et al 2005)

Most changes during stringent response are mediated by the increase of the effector molecules guanosine tetraphosphate (ppGpp) and pentaphoshate (pppGpp)

(Christensen et al. 2003). During nutritional stress uncharged tRNAs at the ribosomal A site activate the synthesis of ppGpp and pppGpp and by increasing the cellular concentration of these alarmones the synthesis of stable RNAs (rRNA, tRNA) is down regulated. Further ppGpp enhances the use of alternative σ factors leading to favoured transcription of stress related genes and accumulates polyphosphate (polyP). The signalling molecule polyP binds and activates Lon protease, which activates the expression of TA modules and degrades inactive ribosomal proteins. By proteolytic degradation an endogenous source of amino acids for protein synthesis is generated. (Gerdes et al. 2005)

Ectopic overproduction of MazF – "Quasi dormancy"

For ectopic overproduction of MazF it was equally stated that the toxin induces a bacteriostatic condition which can be fully reversed by overexpression of the cognate antitoxin MazE. (Pedersen et al. 2002) Cells are in novel physiological state called "quasi dormancy" under which cells are fully metabolically active and capable of synthesizing proteins in the absence of cell growth. (Christensen et al. 2003). Based on these results the single protein production system was developed by Suzuki et al.

Overview of the stringent response pathway

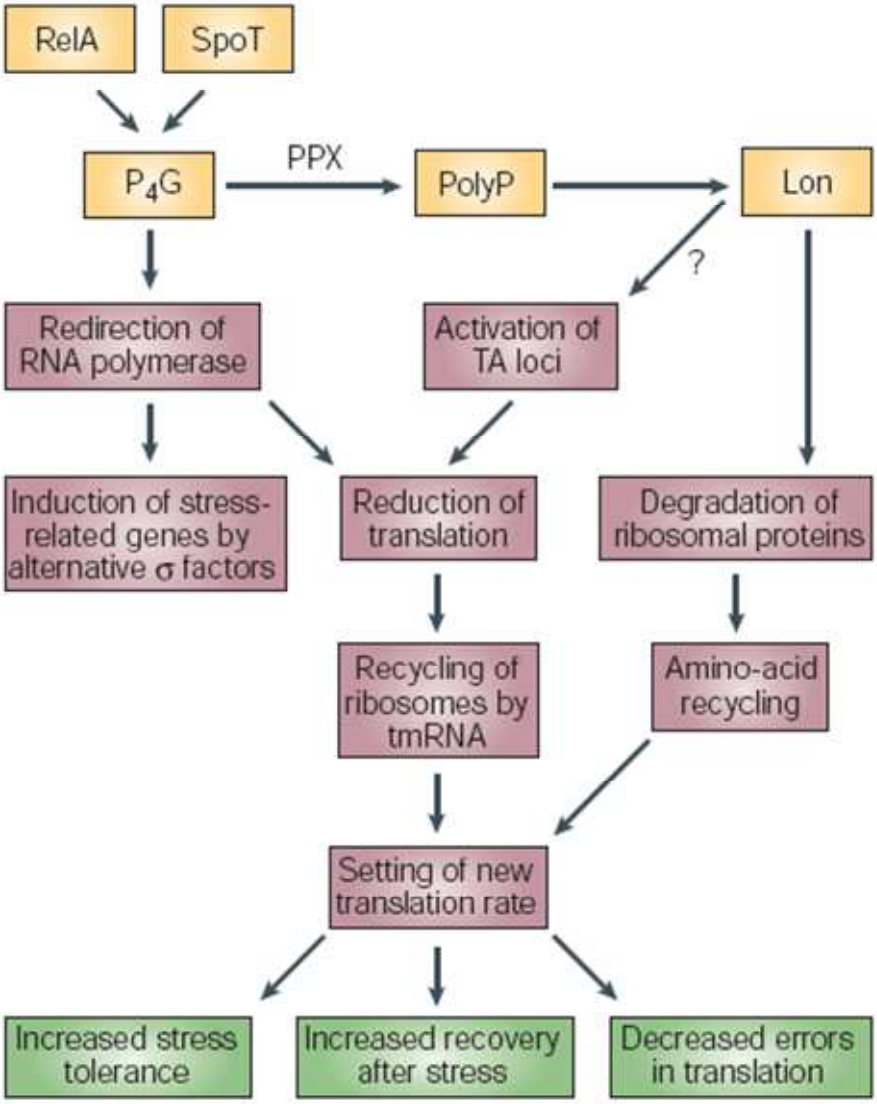


Figure 3.3 Suggested role of TA loci in the stringent response pathway

TA loci: In the genome of Escherichia coli K-12 at least five loci are found: *relBE, mazEF(chpA), chpB, yefM-yoeB and dinJ-yafQ*. (Zhang et al. 2005) **RelA**: ppGpp Synthetase I; **SpoT**: ppGpp synthetase II; **?** - TA locus activation might be passive with no external signal involved or an active process where nutrient starvation generates a signal that triggers Lon. (Gerdes et al. 2005)

3.2.3 Programmed cellular death (PCD)

In total contrast to MazF mediated “quasi dormancy” is the hypothesis that MazF plays a role in a so called programmed cellular death (PCD) mechanism, which is activated under various stressful conditions and leads to death of most of the cells with benefit in surviving cells. (Figure 3.4)

According to Engelberg-Kulka et al. the expression of the *mazEF* locus is inhibited during stressful conditions. *In vivo* the antidote MazE is thereby degraded by the ATP dependent serine protease clpPA and leaves the toxin MazF unimpeded to exercise mRNA degradation resulting in cell death. These stressful conditions include extreme amino acid starvation, inhibition of transcription and/or translation by antibiotics, lethal action of toxic phage products and DNA damage caused by thymine starvation, UV irradiation, oxidative stress and DNA damaging agents. Although these conditions are well known to trigger cell death it was stated that death is triggered by the action of the "suicide module" *mazEF* in a so called programmed cellular death mechanism. (Engelberg-Kulka et al. 2006)

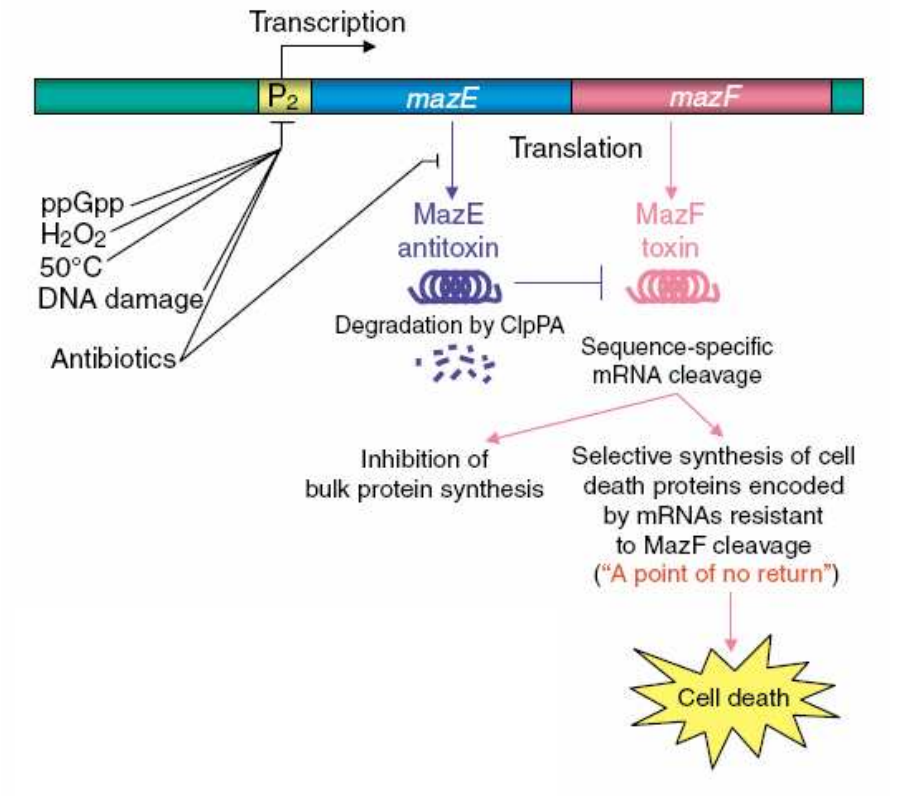


Figure 3.4 Overview of the proposed mechanism of programmed cellular death
(Engelberg-Kulka et al. 2005)

As a regulator of this programmed cell death a third gene product MazG encoded from the gene *mazG*, which is located in the same operon further downstream of the *mazEF* genes was introduced. *MazG* is being transcribed from the same polycistronic mRNA and the MazG protein has a pyrophosphohydrolase activity (Gross et al. 2006). It was suggested that MazG is involved in limiting cell death mechanisms as deletion of MazG decreases cell survival during nutritional stress. (Engelberg-Kulka et al. 2006)

A suicide module like *mazEF* is obviously counterproductive for an individual bacterium. Therefore an advantageous effect for the whole cell population and multi cellular like behaviour was suggested for a unicellular prokaryote like *E. coli*. Possible benefits of such an altruistic killing mechanism are preventing a population from spreading of phage infections, guarding the chromosome in cases of failed DNA repair systems and occurring mutations and in cases of nutrient limitation providing substrate for surviving cells. (Engelberg-Kulka et al. 2006)

It was further declared that during stationary phase *mazEF* mediated cell death is not observed due to the use of a different sigma factor - the stationary phase sigma factor σ^S. (Engelberg-Kulka and Kolodkin-Gal 2009a)

Ectopic overproduction of MazF – Cell death

For ectopic overproduction of MazF it was stated that the lethal action of MazF can be reversed by MazE, but only over a short period of time and that there is a "point of no return". After that point artificial overexpression of MazF or indirect induction of the PCD mechanism under various stressful conditions leads to an irreversible loss of viability and cell death is unavoidable. (Engelberg-Kulka et al. 2006)

On one hand it was stated that artificial overexpression of *mazF* leads sooner or later to cell death due to a programmed cellular death mechanism and on the other hand it was proposed that MazF does not kill the cell, it rather induces a reversible bacteriostatic state. However, for the aim of stable and prolonged protein production in a non growth associated manner the suggested PCD mechanism is counterproductive. Although the hypothesis is intriguing, a single protein production would not be feasible if MazF unavoidable causes cell death.

3.2.4 Single Protein Production (SPP) System

As mentioned previously Suzuki et al. developed a new bacterial expression system which is designed to produce a single protein of interest in living *E. coli* cells. By utilizing the properties of the endoribonuclease MazF ACA free mRNA of a target protein can be efficiently translated. With the SPP system theoretically any protein can be produced because any ACA triplet found in or out of an open reading frame can be altered without changing the original amino acid sequence. Cells are indeed converted to bioreactors for a single protein production. (Suzuki et al. 2006)

The protein production occurs virtually in the absence of background cellular protein synthesis. The level of protein production may be as high as 20 – 30 % of total cellular proteins. Thereby advantages of the SPP system are efficient and cost-effective protein production and a high signal-to-noise ratio for labelling of the produced protein. (Suzuki et al. 2007)

Components of a SPP system

A bacterial strain suitable for a single protein production system should contain two plasmids. The *mazF* gene should be cloned into a low copy vector under control of a tightly controllable promoter. The expression vector should contain the genetic information for the protein of interest encoded by ACA free mRNA. The expression vector DNA should be free of any ACA triplets, but at least the RNA transcript of the target protein has to be free of MazF cleavable sequences.

By co-expression of MazF and protein of interest, target protein can be exclusively produced in living cells in the absence of other cellular protein synthesis. (Suzuki et al. 2005, Suzuki et al. 2006, Suzuki et al. 2007)

Examples for use of the SPP system

One target for the SPP system is according to Suzuki et al. performance of labelling experiments for structural analysis of proteins by NMR and X-ray crystallography. Proteins of interest can therefore be labelled with toxic amino acids (selenomethionine and fluorophenylalanine) or with highly expensive isotope labelled amino acids (^{15}N), amino acid analogues or carbon sources. (^{13}C)

Using selenomethionine and fluorophenylalanine in a growing culture leads due to the toxicity of these amino acids to growth inhibition and further to decrease in product

yield. By use of an expression system where induction of MazF causes cell growth arrest, cytotoxic effects are no longer an issue. High efficient and cost effective ^{15}N incorporation in a condensed culture (cSPP) is also supported by such an expression system. (Suzuki et al. 2006)

Mao et al. used the condensed single protein production (cSPP) system for high level production of three prokaryotic inner membrane proteins, two prokaryotic outer membrane proteins and one human virus membrane protein for NMR studies. By using a 150-fold concentrated culture they achieved isotope enriched membrane proteins with cost savings of more than 99 % for isotopes. (Mao et al. 2009)

Other applications of MazF

The properties of the *mazEF* module in *E. coli* were also used for enhanced expression of β-1,6-N-Acetylglucosaminidase (Dispersin B); an enzyme usually encoded by *dspB* from *Aggregatibacter actinomycetemcomitans*. In contrast to the SPP system MazF was not overexpressed but the system relied on MazF accumulation as a stress response to metabolic burden due to overproduction of recombinant proteins in *E. coli*. (Yakandawala et al. 2009)

Shin and Noireaux used the mRNA interferase MazF to study the messenger inactivation and protein degradation in an *E. coli* cell-free expression system. MazF was therefore used to increase and adjust the mRNA inactivation of two model proteins, the *Firefly luciferase* (Luc) and the enhanced green fluorescent protein (eGFP).(Shin and Noireaux 2010)

Use of the SPP system in industrial scale

So far the SPP system has only been used for research in lab scale. Application of this method in industrial scale is difficult as the *mazEF* module and MazF are extensively studied and protected by several patents. Nevertheless it was the aim of this work to create a two plasmid expression strain for application on production scale. Additionally the designed expression system can be used as a model system for similar methods based on other genetic modules with the same goal of non growth associated product formation.

3.2.5 Alternatives to the SPP system

Two other strategies focus on protein production at a low background protein synthesis.

Cell free systems

Cell free systems are based on bacteriophage transcription for large scale production of proteins in *E. coli* (Shin and Noireaux 2010). Protein production is achieved in cytoplasmatic extracts containing mRNA for specific protein and by adding amino acids and ATP or GTP for energy production to the reaction. Major drawback for such a cell free system is high costs. (Suzuki et al. 2007)

Inhibition of *E. coli* RNA polymerase by rifampicin

The viral RNA polymerase from bacteriophage T7 has high processivity, is insensitive towards antibiotic rifampicin and recognizes only phage origin promoter regions and no promoter regions naturally found on the bacterial chromosome. (Schumann, Ferreira 2004)

Using T7 vector systems background cellular protein synthesis can be suppressed by addition of rifampicin. The antibiotic inhibits the bacterial RNA polymerase and further expression of genes located on the chromosome and on the plasmid is repressed. Under control of a T7 promoter only target protein expression is favoured. (Suzuki et al. 2007)

Drawbacks for application of this method are use of antibiotic, the strong promoter system leading to inclusion body formation and since all promoter systems are leaky this method is not applicable for toxic products.

4 Aim of work

The aim of work was to create an *Escherichia coli* production strain, by constructing a two vector system, which is capable of high level, non growth associated production of a single protein of interest by taking advantage of the mRNA "interferase" MazF.

5 Material and methods

5.1 Bacterial strains

For molecular cloning procedures strain *E. coli* DH5α was used [F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–] and as expression strain *E. coli* HMS174(DE3). [F- recA1 hsdR(rK12- mK12+) (DE3) (Rif R)]

Strain *E. coli* HMS174(DE3) is a lysogen of prophage λDE3 and carries a chromosomal copy of the gene encoding for the T7 polymerase under control of a lacUV5 promoter. This strain is suitable for production of recombinant proteins from genes cloned in pET vectors by induction with isopropyl β-D-thiogalactopyranoside (IPTG). By addition of IPTG repressor *lacI* is inactivated, as binding of inducer leads to dissociation of repressor from its operator. (http://openwetware.org/wiki/E._coli_genotypes; 2nd of august,2010).

Strains were from the strain collection of the Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna.

5.2 Plasmid vectors

5.2.1 pET30a

The pET system is a powerful and well established tool for cloning and expression of target genes in *E. coli*. Plasmids from the pET - family are characterized by high copy number (15 – 60 plasmid copies per chromosome) and are derivatives of colE1 type plasmids.

Genes encoding for recombinant proteins, cloned into a pET vector, are controlled by a strong bacteriophage T7 translational signal. After inducing expression by providing T7 – RNA polymerase encoded from the host genome, viral RNA polymerase works selectively and in a very active form. (http://www.merck-chemicals.com/chemdat/en_CA/Merck-US-Site/USD/ViewProductDocuments-File?ProductSKU=EMD_BIO-71867&DocumentType=USP&DocumentId=/emd/biosciences/userprotocols/en-US/TB055.pdf&DocumentSource=GDS, 09th of august, 2010)

During this work pET30a was used as a vector for cloning procedures with target protein superoxide dismutase (SOD). In table 5.1 important properties of vector pET30a are listed, a detailed plasmid map is supported in the Appendix.

pET30a	
Origin of replication	f1, pBR322
Marker	Kanamycin
PCN	~ 40
Characteristics	T7 promoter, MCS, lac operator, *lacI* coding sequence

Table 5.1: Properties of pET30a

5.2.2 pBAD33

pBAD vectors are a series of plasmid vectors containing promoter P_{BAD} of the *ara-BAD* (arabinose) operon and its regulatory gene *araC*. The *araC* gene product, AraC is a positive as well as a negative regulator. Transcription from promoter P_{BAD} is activated in presence of arabinose and in its absence transcription rate is very low. In presence of glucose or D-fucose, uninduced level is further reduced due to catabolite repression of promoter P_{BAD} with 3' 5' –cyclic AMP. By using a vector from the pBAD series, tightly controlled expression is possible and this tight control helps in avoiding harmful effects of uninduced expression of a toxic gene, like *mazF*. (Guzman et al. 1995)

pBAD33	
Origin of replication	pACYC184/p15a
Marker	Chloramphenicol
Characteristics	P_{BAD} promoter, *araC*

Table 5.2: Properties of pBAD33

During this work the low copy number plasmid pBAD33(Cm^R) was used which contains the pBR322 compatible origin of replication from the pACYC184 vector. Table 5.2 lists important properties of the vector and a detailed vector map is found in the Appendix.

Gene encoding for *mazF* was, together with a ribosomal binding site (rbs), cloned into the multiple cloning site of pBAD33 by using restriction endonucleases EcoRV and HindIII. Vector was subsequently transformed into competent *E. coli* HMS174(DE3) cells. Cloning procedures have been done by Sven Gross-Selbeck, a member of the microbial fermentation group of the Department of Biotechnology. (University of Natural Resources and Applied Life Sciences, Vienna)

5.3 MazF

MazF is an endoribonuclease which specifically cleaves single stranded RNA at ACA sequences. Overexpression of this toxic gene product, encoded by *mazF* leads, due to cleavage of whole cellular mRNA, to complete inhibition of protein synthesis and further to cell growth arrest. For more details see Introduction.

5.4 Model Protein for recombinant protein production

5.4.1 Recombinant human superoxide dismutase (rhSOD)

The copper and zinc containing metalloprotein human superoxide dismutase catalyzes the enzymatic conversion of radicals, formed during aerobic metabolism, to H_2O_2. The protein has a molecular mass of 32 kDa and is a dimmer with two identical, not covalently linked subunits, each subunit containing one copper and one zinc atom. *E. coli* expresses rhSOD into the cytoplasm in a very soluble form without toxic effects on host cell metabolism. (Kramer, W. et al. 1996)

5.5 Cell banks

5.5.1 Research Cell Bank (RCB)

A single colony from a fresh LB – Agar plate with an appropriate antibiotic was picked and transferred into 20 ml of liquid LB – media with same antibiotic. Culture was grown for 12 – 16 hours until it reached OD_{600} = 3. An equal volume of sterile glycerine (87 %) was added and 1.1 ml each were portioned into sterile 1.8 ml Cryo-Tube® Vials (Nunc) and immediately frozen at -80 °C.

5.5.2 Master Cell Bank (MCB)

All steps were accomplished in a laminar flow workbench. A RCB was used to plate out cells on fresh LB plates containing an appropriate antibiotic. A single colony was picked and used to inoculate 80 ml of M9ZB media in a sterile 250 ml baffle flask. After ten minutes in a tempered shaker (37 °C) at 180 rpm cell suspension was split in half and 40 ml each were transferred into fresh sterile baffle flasks. One flask contained cell suspension for the WCB; the other was used as a reference for optical density measurements to control bacterial growth. As soon as optical density of reference flask reached a value of OD_{600} = 3, flask containing the WCB was used for further steps. A sample for determination of colony forming units and optical density was drawn and cell suspension was diluted 1:2 with sterile 87 % glycerine. 1.1 ml each were portioned into sterile 1.8 ml CryoTube® Vials (Nunc) and immediately frozen at -80°°C.

5.5.3 Working Cell Bank (WCB)

All steps were accomplished in a laminar flow workbench. 80 ml of semi synthetic media were transferred into a 250 ml baffle flask and inoculated with 1 ml MCB. After ten minutes in a tempered shaker (37 °C) at 180 rpm, suspension was split in half and 40 ml each were transferred into fresh sterile baffle flasks. One flask contained cell suspension for the WCB; the other was used as a reference for optical density measurements to control bacterial growth. As soon as optical density of the reference flask reached a value of OD_{600} = 3, flask containing the WCB was used for further steps. A sample for determination of colony forming units and optical density was

drawn and the cell suspension was diluted 1:2 with sterile 87 % glycerine. 1.1 ml each were portioned into sterile 1.8 ml CryoTube® Vials (Nunc) and immediately frozen at -80 °C.

5.6 Characterization of MazF

To determine influence of MazF on bacterial growth, cell viability and protein expression, strain *E. coli* HMS174(DE3)pBAD33_*mazF* was used. Shake flask experiments and cultivations in a 20 l bioreactor were conducted. MCB was from the strain collection of the Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna. For further experiments a WCB was created.

5.6.1 Shake flask experiments

For shake flask experiments either LB media or M9ZB media was used. LB media contained tryptone (10 g/l) as a source for peptides and peptones, yeast extract (5 g/l) as a source for vitamins and trace elements and NaCl (5 g/l). Sodium chloride is important for transportation of ions and maintenance of osmotic balance. M9ZB media contained same amount of tryptone, yeast extract and NaCl. Additionally media comprises KH_2PO_4 (3 g/l) and Na_2HPO_4 (6 g/l) as a buffering agent and source for phosphor and potassium, NH_4Cl as N-source, $MgSO_4$ (1M) as Mg-source and glucose (4 g/l) as main C - source.

Overnight cultures were set up in 25 ml media containing appropriate antibiotic. Cultures were inoculated either with a single colony from an LB-agar plate or with 10 µl from a WCB. After incubation on an tempered (37 °C) orbital shaker for 12-16 hours cultures were diluted in fresh media and grown until they reached OD_{600} = 0.5 – 1.5. Cultures were induced with a 20 % arabinose stocksolution to reach final concentration of 0.2 % [w/w] or with 0.1 M IPTG stocksolution to reach final concentration of 0.1 mM.

Samples were drawn every 30 minutes or hourly. Biomass accumulation was controlled by measuring optical density at 600 nm in a spectrophotometer. Total and viable cell counts were determined by flow cytometry in a FACSCalibur flow cytometer (Becton Dickinson Bioscience). Determination of plasmid stability and colony forming units was done by agar plate counting. Analytical methods are further described in the next chapter.

5.6.2 Cultivation process

Media composition

All substances were purchased from Merck unless noticed otherwise. For distinct media composition and preparation see Appendix.

All minimal media used for cultivations contained K_2HPO_4 * 2 H_2O and $KHPO_4$ as a buffering agent and as source for potassium and phosphor. As a major carbon source glucose was added, as well as $MgSO_4$ * 7 H_2O, $CaCl_2$ * 2 H_2O and trace element solution in relation to cell dry mass. To supply nitrogen demand an ammonia solution (12.5 % v/v) was added, that concurrently was used for pH maintenance.

Batch media was additionally supplemented with the complex component yeast extract to ensure initial growth of population.

Cultivation set up

Bacterial cells were cultivated in a 20 l computer controlled bioreactor (MBR, Wetzikon, CH) with a working volume of 14 l. Batch volume was usually 4 l unless indicated otherwise. For online monitoring of processes and maintenance of defined cultivation conditions bioreactor was equipped with a standard control unit (Siemens PS7, Intellution iFIX).

Temperature was set to 37 °C ± 0.5 °C and monitored by a standard temperature probe measuring electric resistance (Pt_{100}), pH value was set to 7 ± 0.05 by addition of a 12.5 % ammonia solution. For calibration of pH probe (Mettler Toledo) a three step protocol (7 – 4 – 7) and commercially available buffer solutions (Merck) were used. By adjusting stirrer speed and aeration rate partial oxygen pressure was held above 30 % to ensure high enough dissolved oxygen concentration to avoid oxygen limitation. Partial oxygen pressure was determined with a Clark probe (Mettler Toledo). For calibration a two step protocol was used. Zero point was set by aeration with nitrogen (4 l/min), slope (100 %) was set by aeration with pressurized air (4 l/min) By addition of 0.5 ml antifoam solution (PPG200, Sigma) per litre media foam building was suppressed.

Batch cultivations were started by inoculating reactor under aseptic conditions with 1 ml of a deep frozen working cell bank (OD_{600}°=°1) diluted in 30 ml sterile physio-

logical NaCl – solution. Batch media was laid out for 22.5 g total biomass and addition of feed media was started as soon as bacterial growth reached stationary phase.
During Fed batch cultivation media was added with an exponential substrate feed to maintain a constant growth rate of $\mu = 0.1\ h^{-1}$. Duration of fed batch cultivation was 27.7 hours, equal to four doubling times. Media was laid of for 363.3 g of total biomass.
Cultures were induced by addition of 0.2 % [w/w] arabinose after one generation by adding appropriate amounts of arabinose stock solution to bioreactor and to remaining feed media as well.

On line analysis

Partial oxygen pressure and pH value were measured as described above.
The concentration of oxygen and carbon dioxide in the outlet air was detected with a Magnos 16 and Uras 14 Advanced Optima gas analyzer (Hartmann-Braun). Zero points for O_2 and CO_2 (0.03 %) were set with nitrogen and process air, respectively. End points were set with process air (20.9 % O_2) and a calibration cuvette. (CO_2)
Base consumption was determined by decrease in weight of ammonia stock solution.
Capacity and conductivity, for determination of cell diameter and biomass volume, were detected with a biomass monitor (BM220, ABERScan Software). A two dimensional multi-wavelength on-line fluorescence spectroscopy was performed with a BioView® spectrofluorometer (DELTA Light & Optics) which is capable of measuring a complete excitation-emission matrix in a range between 270 nm – 550 nm for excitation and 310 nm – 590 nm for emission. A near infrared (NIR) spectroscopy was performed with a TruCell® cell growth monitor, which allowed in - line monitoring of cultivation progress.

Off-line analysis

During fed batch cultivation samples for offline analytics, usually 15 – 20 ml of cell suspension, were drawn continuously. Sample volume was later subtracted from total volume.

Optical density

Optical density (OD_{600}) was determined by measuring extinction at 600 nm in a linear range between 0.1 – 0.6 in a spectrophotometer. (ULTRASPEC 500pro, Amersham Bioscience) During cultivation samples were diluted with PBS – buffer to reach linear range.

Cellular dry weight (CDW)

10 ml of cell suspension were separated by centrifugation. (10 min, 5000 rpm, 4°C) For chromatographic analysis 2 ml of supernatant were transferred into microcentrifuge tubes and stored at – 20 °C for further testing. Remaining supernatant was discarded. Pellet was completely resuspended with 10 ml RO – H_2O and whole supernatant was discarded after a second centrifugation step Pellets were transferred into pre – weighed beakers and dried at 105 °C for 24 h. After cooling beakers were weighed and biomass concentration, total amount of biomass and growth rate µ was calculated.

Total and Viable Cell Number (TCN / VCN)

Agar plate count

Once per generation an aseptic sample (5 ml) was drawn to determine colony forming units, plasmid stability and percentage of plasmid carrying cells with an agar plate count. In physiological NaCl solution a dilution series from 10^{-1} – 10^{-9} was created. For determination of colony forming units dilutions 10^{-7}, 10^{-8} and 10^{-9} were plated on non-selective nutrient agar plates. Plasmid stability and percentage of plasmid containing cells was determined by plating dilutions 10^{-7} – 10^{-9} on selective nutrient agar plates with suitable antibiotic. Additionally number of cells producing high amounts of recombinant protein was evaluated by plating dilutions 10^{-2} – 10^{-4} on selective agar plates supplemented with appropriate antibiotic and agent used for induction. Plates were incubated upside down at 37 °C for 24 h to 48 h and colony forming units were determined by counting.

Flow cytometry

Total (TCN) and viable (VCN) cell counts were determined by flow cytometry in a FACSCalibur four colour flow cytometer. (BD Bioscience) 25 µl of an OD_{600} sample were mixed with 500 µl staining solution in FACS tubes. Staining solution contained sterile filtered staining buffer (PBS), 0.01 % Tween20, propidium iodide stocksolution (1 mg/ml) and a known quantity of BD Liquid Counting Beads (BD Bioscience). Prepared samples were acquired in a cytometer equipped with a 488 nm laser excitation and analysed with BD CellQuestTM software.

Concentration of cell populations was determined with following equation

$$\frac{\#events\ \ in\,cell\,region}{\#events\ \ in\,bead\,region}\times\frac{beads/test}{test\,volume}\times diltuion\ \ factor = concentration\,of\ \ cell\ \ population$$

Number of dead cells was calculated with ratio of TCN and PI–positive cells (DC)

$$VCN = TCN - \frac{TCN * DC}{100}$$

Analysis of recombinant protein

A sample volume equal to approximately 1 mg of CDW was transferred into microcentrifuge tubes. After centrifugation (13 200 rpm, 10 min) supernatant was discarded and pellets were stored at – 20 °C for further analysis.

HPLC analysis

Analysis of residual substrate

Quantitative analysis of residual substrate for metabolites and substrate components including pyruvate, lactate, formate and glucose was performed with an ion exchange high performance liquid chromatography (1100 HPLC, Agilent Technologies) with an Aminex HPX-87H Ion Exclusion Column (BioRad)

Quantification of nucleotides

To stabilize nucleotides a 30 % perchloric acid extraction solution was prepared. 1 ml of ice cold extraction solution was mixed with 4 ml culture broth immediately after sample was drawn. Following incubation for 15 minutes reaction was stopped by ad-

dition of a 2 M K_2HPO_4/KH_2PO_4 buffer solution. (Cserjan-Puschmann et al. 1999) Subsequent to centrifugation (5000 rpm, 10 min) supernatant was drawn and stored at -80 °C for later analysis.

Analyzed nucleotides ppGpp, ATP, cAMP, GDP and GTP were separated and quantification was performed with an ion-pair reversed phase HPLC (1100 HPLC, Agilent Technologies) using a SupelcosilTM LC-18 T column in connection with a SupelguardTM LC-18T guard column. (Sulpeco)

Calculated values

Culture volume

Actual culture volume was estimated by summation of batch volume, added feed volume and base consumption. Sample volumes were subtracted.

Growth rate µ

Growth rate µ was calculated with total CDW and distinct time periods according to following equation

$$\mu = \frac{\ln\left(\frac{x_2}{x_1}\right)}{t_2 - t_1}$$

Respiration quotient (RQ)

The respiration coefficient is ratio of respired CO_2 to consumed O_2 and was calculated according to following equations

$$RQ = \frac{CO_{2\,produced}}{O_{2\,consumed}} = \frac{B}{A}$$

$$A = A' - \left(\frac{R_L}{R_L + A' - B'} - 1 \right) * \left(O_2 - A' \right)$$

$$B = B' - \left(\frac{R_L}{R_L + A' - B'} - 1 \right) * \left(CO_2 + B' \right)$$

$A' = O_2 - O_2'$

$B' = CO_2' - CO_2$

A'.....*actual* O_2 *uptake*

B'.....*actual* CO_2 *production*

O_2.....*oxygen in process air* (20.9%)

O_2'.....*oxygen in exhaust gas* (%)

CO_2..*carbondioxide in process air* (0.03%)

CO_2'..*carbondiocide in exhaust air* (%)

R_L.....*residual gas* (79.09%)

Specific oxygen consumption rate [g O_2/g CDW_{built} *h]

The specific oxygen consumption rate is of consumed oxygen referring to built CDW in a defined period of time, usually one hour. It was calculated according to subsequent equations

$$qO_2 = \frac{V_{O_2} * O_{2,consumed} * \frac{32}{24}}{\frac{(X_2 - X_1)}{(t_2 - t_1)}}$$

$$V_{O_2} = V_{air} * 60 * 0.209$$

$$O_{2,consumed}\,[\%] = \left(1 - \frac{A}{20.9} \right) * 100$$

V_{O_2}....*flow rate of* O_2 *in process air* $[l/h]$

V_{air}...*flow rate of process air* $[l/h]$

A....O_2 *consumed* (*see RQ*)

Specific carbon dioxide production rate [g CO2/g CDW_{built} *h]

The specific carbon dioxide production rate is respired carbon dioxide referring to built CDW in a defined period of time, usually one hour. It was calculated according to subsequent equations

$$qCO_2 = \frac{V_{CO_2} * CO_{2,produced} * \frac{44}{24}}{\frac{(X_2 - X_1)}{(t_2 - t_1)}}$$

$$V_{CO_2} = V_{air} * 60 * \frac{CO_{2,produced}}{100}$$

$$CO_{2,produced} = B[\%]$$

$V_{CO_2} \ldots flow\,rate\,of\,CO_2\,in\,\,exhaust\,gas[l/h]$

$V_{air} \ldots flow\,rate\,of\,\,process\,air\,[l/h]$

$B \ldots\ldots CO_2\,\,produced\,\,(see\,RQ)$

5.6.3 SDS Gel Electrophoreses

For analysis of changes in protein expression pattern after MazF overexpression a discontinuous SDS page electrophoreses system from Invitrogen was used.

During shake flask cultivations samples equal to 1 mg of CDW were drawn and supernatant was poured of subsequent to centrifugation (13 200 rpm, 10 min). Pellets were stored at -20 °C. For cell disintegration pellets were resuspended with 200 µl disintegration buffer (18 mM Tris-buffer, 5 mM EDTA-solution, 7 mM Mercaptoethanol, 1 mM $CuSO_4$, 0.1 mM $ZnSO_4$) and 50 µl lysozyme. (10 mg/ml) Following incubation for 10 min at 37 °C (350 rpm) 750 µl Triton-X were supplemented and samples again incubated. Suspensions were separated by centrifugation (10 min, 4 °C, 13 200 rpm) and supernatant was used for protein analysis.

In safe-lock microcentrifuge tubes 13 µl sample, 5 µl NuPAGE® LDS sample buffer (4 x) and NuPAGE® reducing agent (10 x) were mixed and kept on 70 °C for 10 min. 15 µl of each sample solution and 7 µl of ready to use MW-standard (Mark12) were loaded on NuPAGE® Bis-Tris Pre Cast Gels. Running buffer was 20 x MES running buffer from Invitrogen supplemented with NuPAGE® antioxidant. Running conditions were 200 V (max. 400 mA) for 45 min.

Subsequent to fixation for 30 min in fixing solution, gels were dyed for 10 min in staining solution (Coomassie brilliant blue R250) and destained for several hours in destaining solution.

5.7 Construction of a two plasmid system

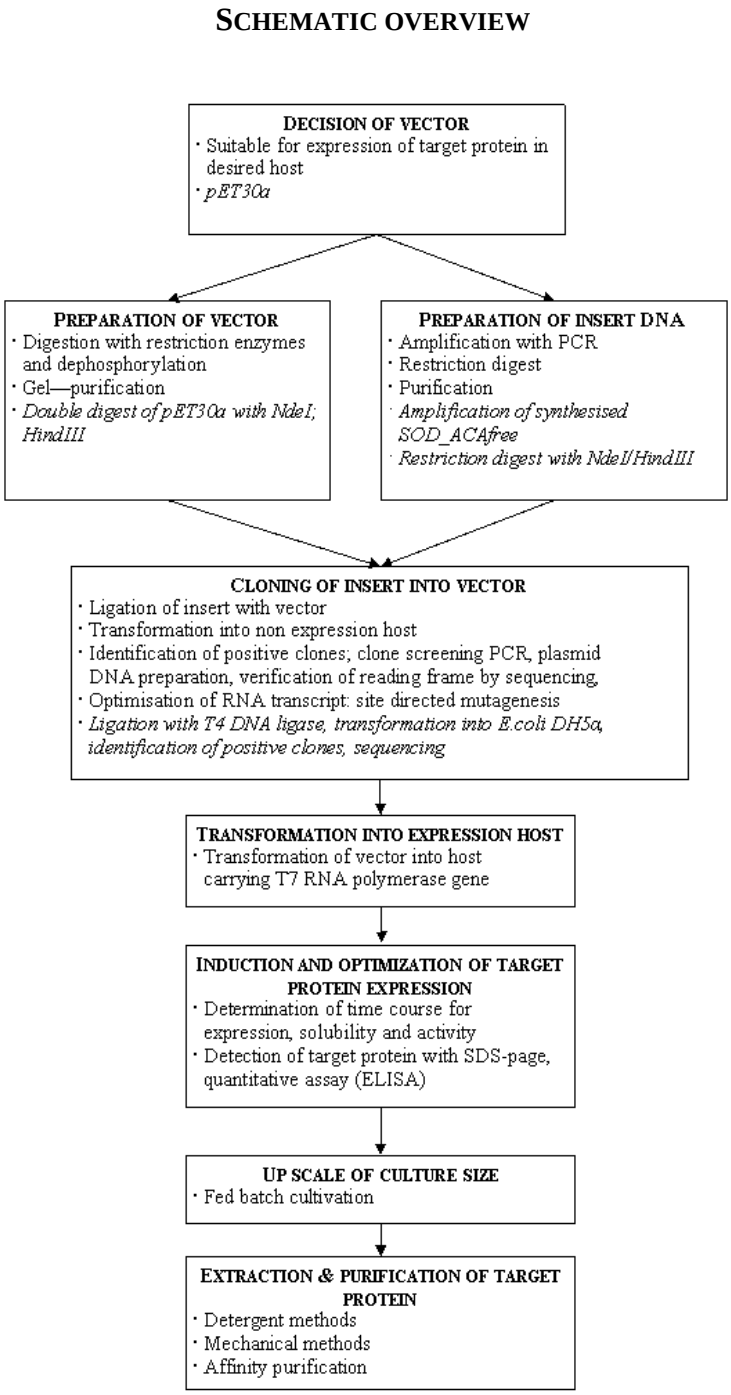


Figure 5.1: Schematic overview of steps for creation of target protein producing strain

Decisions made and steps done during this work are written italic.

For construction of a production strain that is able to produce recombinant protein (SOD) non growth associated, subsequent to overexpression of MazF, strain *E. coli* HMS174 (DE3) has to be co-transformed with two plasmids.
Plasmid pBAD33_*mazF* has the gene for the endoribonuclease MazF cloned into the multiple cloning site and under control of promoter P_{BAD}, expression was inducible with arabinose. Plasmid pBAD33_*mazF* was supported by the microbial fermentation group of the Institute of Applied Microbiology (IAM).
For construction of the expression plasmid, that harbours the gene for the recombinant protein SOD under control of promoter T_7, plasmid pET30a (Novagen) was used. Cloning methods were part of this diploma thesis.

For engineering the mRNA of superoxiddismutase (SOD) to be devoid of ACA, DNA sequence had to be altered at five positions. This was possible without changing original amino acid sequence. ACA free DNA was synthesized by GeneArt and delivered incorporated into plasmid pMK. Plasmid was linearized with PstI (New England Biolabs) and 5 ng were used to set up a PCR reaction for amplification.
With the primerpair used for PCR amplification two recognition sequences for restriction enzymes NdeI (upstream) and HindIII (downstream) were attached to the DNA sequence SOD_ACAfree. Length of PCR product (488 bp) was controlled with an agarose electrophoreses and pooled PCR products were digested with DpnI. The restriction endonuclease DpnI cleaves only when the recognition site is methylated and therefore only plasmid backbone is digested without negative effect on amplificated DNA. After a cleaning step with a "Wizard® SV Gel and PCR Clean-Up System" to get rid of plasmid backbone fragments, remaining DNA was template for a double digest with NdeI/HindIII.

Plasmid pET30a-c(+) was purchased from Novagen, transformed into electrocompetent *E. coli* DH5α and isolated with a "Pure Yield™ Plasmid Miniprep System" from Promega. A double digest reaction was set up with NdeI and HindIII. Subsequent to heat inactivation and addition of 6x loading dye 50 µl were applied on a 1.5 % preparative agarosegel and the 5249 bp plasmid backbone was separated from the 173 bp insert. Band equal to the linearized backbone was cut out under UV light and cleansing to get purified DNA was done with a "Wizard® SV Gel and PCR Clean-Up Sys-

tem" from Promega. Purified plasmid DNA and double digested SOD_ACAfree DNA were used for ligation.

Plasmid pET30a_*SOD_ACAfree* was transformed into competent *E. coli* DH5α cells with electroporation. Subsequent to plasmid isolation purified vector DNA was used for sequencing analysis to control if SOD_ACAfree DNA is free of mutations and correctly positioned in vector and under control of promoter T_7. Sequencing analysis was done by Agowa by using a T_7 promoter/T_7 terminator primer pair. Analysis results were controlled with a CLC Main Workbench 5.

5.7.1 Cloning methods

Polymerase chain reaction

Polymerase chain reactions (PCR) were conducted in a "Mastercycler gradient" (Eppendorf) with a Phusion ® High-Fidelity PCR Mastermix. Primers were designed with a CLC Main Workbench 5 and purchased from Sigma Aldrich.

For DNA amplification 50 µl reactions were set up with approximately 5 ng template DNA, 25 µl 2x Phusion Master Mix and 1 µl of each primer (10 µM). Nuclease free water was added up to 50 µl. For screening procedures 20 µl reactions were set up and either 1 µl of a resuspended clone isolated from an agar plate or 5 ng of purified plasmid DNA were used.

For cleaning PCR reactions a "Wizard® SV Gel and PCR Clean-Up System" from Promega was used. An equal volume of Membrane Binding Solution was added to a PCR reaction and PCR product was transferred to a minicolumn assembly. After one minute incubation at room temperature and centrifugation at 13 200 rpm for one minute, flowthrough was discarded. Following two purification steps with 700 µl and 500 µl Membrane Washing Solution the DNA was eluted with 50 µl in a clean microcentrifuge tube.

Agarose gel electrophoreses

For qualitative and quantitative electrophoreses 1.5 % gels were prepared. For a single quantitative agarosegel 1.8 g agarose (Genxpress) and 2.4 g 50x TAE buffer were weighed in, 120 ml water were added and solution was heated in the microwave until

it melted. After cooling to approximately 50 °C, 3µl ethidiumbromide (10 mg/ml) were added and gel was poured. For qualitative gels same amount was equal to two gels. As a running buffer 1x TAE buffer supplemented with ethidiumbromide was used. Running conditions were 130 V/45 min and 90 V/120 min for qualitative and quantitative gels, respectively

For sample preparations 6x Gel Loading Dye Blue from NEB was used and as molecular weight marker O´Gene Ruler DNA ladder mix (0,1 µg/µl) from Fermentas.

Restriction enzyme digest

Enzymes were from New England Biolabs and reactions were set up to their recommendations. For a 50 µl double digest reaction 1 µg template DNA was mixed with 5 µl 10x NEBuffer and1 µl of each restriction enzyme. Water was added up to 50 µl. Subsequent to incubation for 2 h at 37 °C reaction was stopped by incubation for 20 min at 65 °C. Digested templates were desalted before further use. Single digest reactions were set up similarly.

Ligation reactions were set up according to recommendations and technical references from New England Biolabs. The insert: vector molar ratio was 2:1 and T4 DNA Ligase was used for an overnight digestion at 16 °C. A 20 µl reaction was set up with 25 ng vector DNA, 11.4 ng insert, 2 µl T4 DNA reaction buffer and 1 µl T4 DNA ligase. Reaction was stopped by heat inactivation at 65 °C for 10 minutes.

DNA precipitation

To an inactivated ligation reaction 4 µl sodiumacetate (0.5 M) and 20 µl isopropanol (Sigma) were added followed by resuspension. Reaction was kept at – 20 °C for 30 min and on ice for 10 minutes. Subsequent to a centrifugation step (20 min, 13 200 rpm, 4 °C) supernatant was poured off and a hardly visible pellet was purified by addition of 200 µl ethanol (70 %) and centrifugation (15 min, 13 200 rpm, and 4 °C). The pellet was completely dried at 37 °C and then eluted in 10 µl water. A whole reaction was used for transformation.

Preparation of electrocompetent cells

A glycerine stock for preparation of electrocompetent cells was supported by the microbial fermentation group of the Institute of Applied Microbiology.

A preparatory culture was set up with cells of a glycerine stock suspended in 25 ml of LB media, culture was grown overnight at 37 °C and 180 rpm.

An overnight culture was diluted 1:100 twice in 400 ml LB media and grown until cultures reached an OD_{600} of 0.8. All solutions, buffers and tubes were kept on ice during following steps. Bacterial suspension was centrifuged for 5 min at 7500 rpm and 4 °C. Centrifugation conditions were the same for all centrifugation steps. Pellets were purified with 1 mM Hepes buffer and subsequent to another centrifugation step pellets were suspended in 1 mM Hepes buffer and combined in one tube. After centrifugation and a cleansing step with 1 mM Hepes buffer supplemented with 10 % (v/v) glycerine, final pellet was suspended in 10 % glycerine. After filling of 100 µl aliquots into pre-cooled microcentrifuge tubes, electrocompetent cells were frozen immediately in liquid nitrogen and stored until use at -80 °C.

Transformation

900 µl SOC media were pre warmed at 37 °C in a sterile microcentrifuge tube. Competent cells were kept on ice the whole time. Ready to use cuvettes for electroporation with a 0.2 cm gap were purchased from Sigma Aldrich. A whole ligation reaction or 10 ng DNA were mixed with 100 µl cellsuspension and transferred into cuvettes.

Transformation conditions:	2.5 kV
	1000 Ohm
	25 µF
Tine constant:	18 – 20 ms.

After the pulse cells were transferred immediately into prepared SOC media. Following 30 minute incubation at 37 °C, 200 µl and 800 µl were stroke out on selective agar plates and incubated overnight.

Plasmid Isolation

For plasmid isolations either a QIAprep® Spin Miniprep Kit from QIAGEN or a Wizard® Plus SV Miniprep system from Promega was used. Between these two kits are only slightly differences and both are based on principles for alkaline extraction procedures for screening recombinant plasmid DNA (Birnboim, H.C., Doly, J., 1979)

A single colony was picked from a selective LB agar plate and 5 ml of LB-media containing an appropriate antibiotic were inoculated. Culture was grown overnight (14 –

16 hours) to mid-logarithmic phase. Cells were harvested by centrifugation for ten minutes at 8000 rpm in an Eppendorf bench top centrifuge. Supernatant was poured off and excess media was removed by blotting inverted tube on a paper towel. Pellet was resuspended by pipetting in Cell Resuspension Buffer supplemented with RNase A to avoid contaminations with RNA fragments. Suspension was transferred to a sterile microcentrifuge tube and Cell Lysis Buffer was added. After inverting tubes four times and incubation for two minutes the now partial cleared lysate was neutralized with Neutralization Solution. Sodiumdodecylsulfate (SDS) in the Lysis Buffer degrades bacterial cell walls, NaOH leads to a denaturation of chromosomale and plasmid DNA. During the neutralization process only plasmid DNA is able to renaturate and cell fragments, proteins and genomic DNA fragments form a non soluble complex with potassiumdodecylsulfate. Following precipitation with a centrifugation step for one minute at 13 200 rpm, supernatant was transferred to a minicolumn where DNA was attached to a silica membrane. After two purification steps to remove endonucleases and salts plasmid DNA was eluted with 50 µl water.

DNA quantification

For determination of purity and quantification of nucleic acid samples a NanoDrop® ND-100 Spectrophotometer was used. The Spectrophotometer measured absorbance at 230 nm and 260 nm and calculated sample concentrations according to the Beer – Lambert equation with absorbance at 260 nm and an analysis constant (50 for DNA). The 260/230 ratio determined purity of nucleic acid samples. A sample volume of 1 µl was sufficient.

6 Results and discussion

6.1 Characterization of MazF in shake flask cultivations

Due to the controversial reports in the literature, prior to the establishment of a single protein expression system, the inhibitory effect of the endoribonuclease on cellular growth had to be confirmed. Therefore experiments were set up to investigate the impact of MazF on bacterial growth, cell viability and protein expression in different nutrient media and under different cultivation conditions. For an initial "proof of concept" shake flask experiments were set up to gain first insight into the physiological changes in *E. coli* due to overexpression of *mazF*.

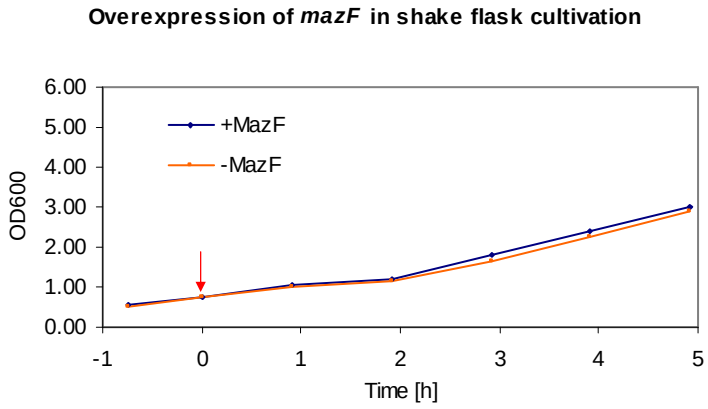
6.1.1 Influence of MazF on bacterial growth and cell viability

MazF production was induced in shake flask cultivation (37°C; 180 rpm) of *E. coli* HMS174 (DE3) pBAD33_*mazF* during exponential growth phase by pulsed addition of arabinose to gain a final concentration of 0.2 % [w/w].

Impact on biomass accumulation was determined by measuring optical density at 600 nm and by measuring cell number with a FACS flow cytometer. Cell viability was monitored by flow cytometry of propidium iodide stained cells.

Shake flask cultivation with semi-synthetic media

Strain HMS174 (DE3) pBAD33_*mazF* was grown to mid-logarithmic phase with semi-synthetic media. Production of MazF was induced at OD_{600} of 0.5 to 1.0. Control experiment was set up with non-induced cells. Optical density, cell density and dead cell ratio were determined hourly for both experiments. (Figure 6.1)



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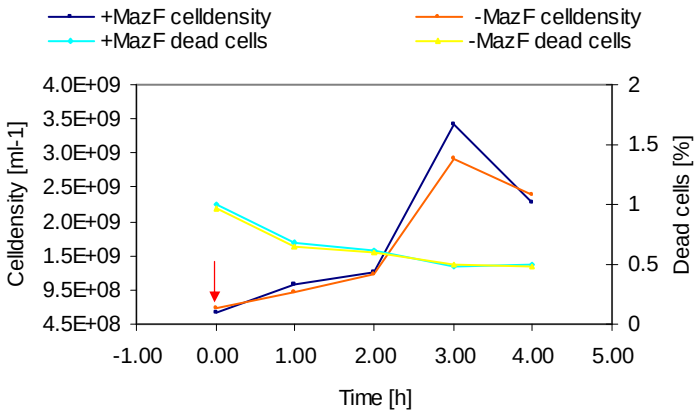


B:

Figure 6.1 Effect of MazF production on cell growth in semi synthetic media

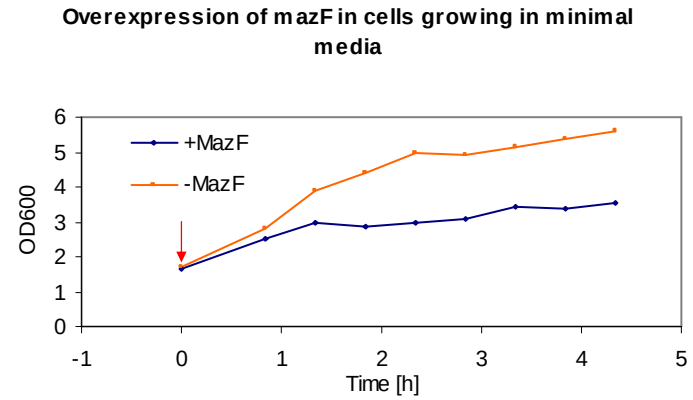
Induction of *mazF* expression by addition of 0.2 % arabinose at point zero (↓);

A: Optical density to observe impact on bacterial growth +MazF: Induced culture; -MazF: Reference culture **B:** Cell density (+MazF/-MazF) and cell viability (+MazF/-MazF) obtained with FACS flow measurements:

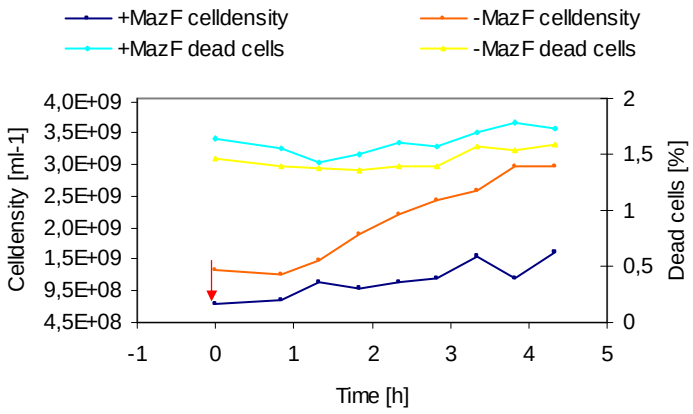
In semi synthetic media induction with arabinose to overexpress *mazF* had, during a time period of five hours, no effect on bacterial growth compared with control reaction. Single cell analysis revealed no effect on cell density or dead cell ratio**.** In semi-synthetic media high glucose concentration (9 g/l) inhibited the expression of *mazF* due to inducer exclusion of arabinose by elevated glucose concentration and catabolite repression by cyclic AMP. Therefore, semi synthetic media was not suitable for further experiments.

Shake flask cultivation with minimal media

To avoid catabolite repression of glucose *E. coli* HMS174(DE3)pBAD33_*mazF* was cultivated in shake flasks with a buffered minimal media (M9ZB) enriched with tryptone and yeast extract but with reduced glucose concentration (4 g/l). Nutrient medium was laid-out for high cell densities up to OD_{600} of 9.; Production of MazF was induced with arabinose at OD of 1.5. Reference experiment was set up without induction. Bacterial growth was controlled by measuring OD_{600} and cell density, cell viability was monitored by flow cytometry. Samples were drawn every 30 minutes for a time period of 4.5 hours after induction. (Figure 6.2)



A



B

Figure 6.2 Inhibited growth in minimal media

Induction of *mazF* expression by addition of 0.2 % arabinose at point zero (↓);

A: Optical density to observe impact on bacterial growth +MazF: Induced culture; -MazF: Reference culture **B:** Cell density (+MazF/-MazF) and cell viability (+MazF/-MazF) obtained with FACS flow measurements:

In minimal media growth of induced culture was clearly suppressed ninety minutes after addition of arabinose. Cell density showed same characteristics as optical density and negative effect on dead cell ratio could not be observed.

Shake flask cultivation with glucose free media

To determine whether the influence of MazF on cell growth and dead cell ratio is increased by a substrate devoid of repressing glucose, shake flask experiments were set up with lysogeny broth (LB) media composed of yeast extract, tryptone and sodium chloride. *E. coli* HMS174(DE3)pBAD33_*mazF* was grown in shake flasks to mid logarithmic phase. Production of MazF was induced at OD_{600} = 0.5 by addition of arabinose. Optical density, cell density and dead cell ratio were determined every 30 minutes. A reference culture was set up with non induced cells. (Figure 6.3)

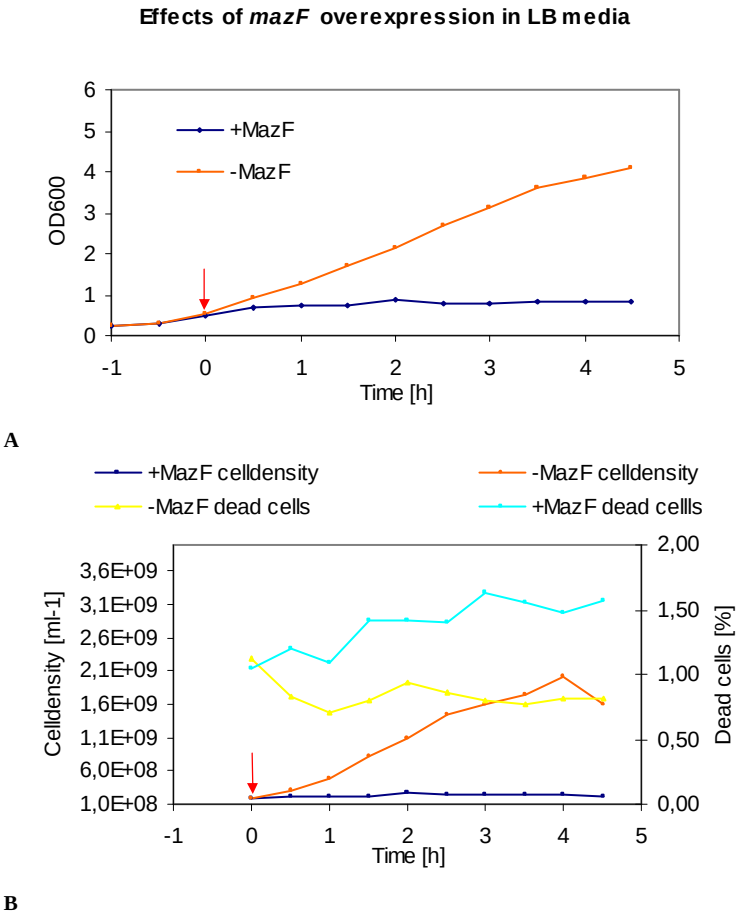


Figure 6.3: Cell growth arrest due to MazF production

Induction of *mazF* expression by addition of 0.2 % arabinose at point zero (↓);

A: Optical density to observe impact on bacterial growth +MazF: Induced culture; -MazF: Reference culture **B:** Cell density (+MazF/-MazF) and cell viability (+MazF/-MazF) obtained with FACS flow measurements:

In glucose free media cell growth of induced culture came to almost complete rest 30 minutes after addition of arabinose. Dead cell ratio slightly increased but at the end of cultivation almost 98.5 % of growth inhibited cells were still alive.

These first results indicated, that MazF does not confer cell killing during exponential growth phase. Growth of bacterial cells experiencing *mazF* expression was suppressed or almost completely stopped dependent on cultivation conditions. Cells were in a "quasi dormant" state that had no negative effect on cell viability during observed time period.

To evaluate the system stability *mazF* was overexpressed for seven hours in exponential growing *E. coli* cells. Subsequent to the induction period glucose was added to repress further *mazF* expression.

Evaluation of the system stability

In context with MazF – mediated cell death it was proposed that there is a "point of no return", where the lethal action of MazF can not be reversed. It was stated that cells experiencing overexpression of *mazF* within a period of six hours have undergone lethal transformation and cell death is unavoidable. (Amitai et al. 2004)

To determine whether this "point of no return" exists for the strain used during this work *E. coli* HMS174(DE3)pBAD33_*mazF* was grown in LB and in minimal media (M9ZB) to mid logarithmic phase. Expression of *mazF* was induced with 0.2 % arabinose [w/w] at time point zero and inhibited after seven hours by addition of 0.2 % glucose [w/w]. Cells were subsequently grown overnight. 24 hours subsequent to induction of MazF expression minimal media was supplemented with 0.4 % glucose [w/w]. as initial glucose supplementation had no inhibitory effect.

Optical density and dead cell ratio were determined. Reference cultures were set up for both reactions (LB media; minimal media).

Colony plate count was performed to evaluate the colony forming ability. Samples for plate count were drawn from the cultivation set up with LB media prior to induction, prior to repression and subsequent to overnight cultivation.

Influence of prolonged *mazF* expression on bacterial growth and cell viability is shown in Figures 6.4 and 6.5.

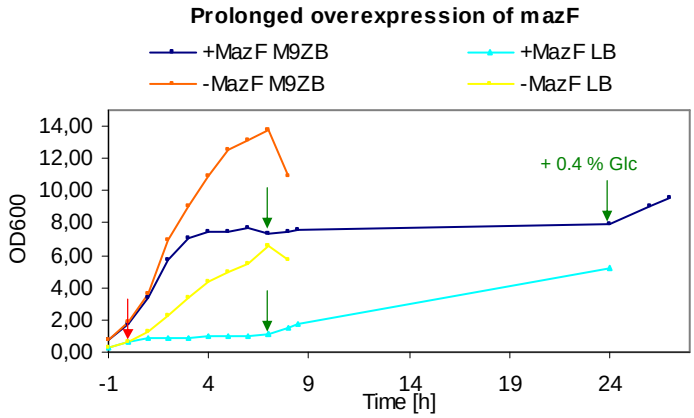


Figure 6.4 Restored cell growth subsequent to prolonged MazF production

LB media: MazF expression (+MazF) within a period of seven hours. Induction with arabinose point zero (↓); Addition of glucose (glc) to repress promoter P_{BAD} (↓) point seven

Minimal media (M9ZB): MazF expression (+MazF) within a period of 24 hours. Induction with arabinose point zero (↓); Addition of glucose (glc) to repress promoter P_{BAD} (↓): point seven and point 24. For reference cultures *E. coli* was grown without induction (-MazF) until end of exponential growth phase

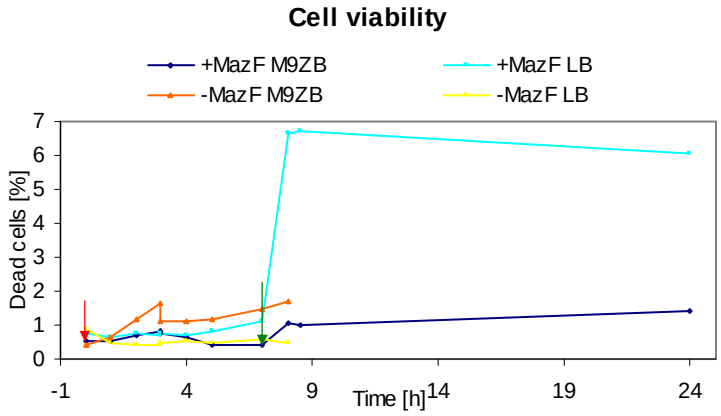


Figure 6.5 Dead cell ratio during prolonged *mazF* expression

Dead cell ratio was observed within a period of 24 hours. Induction at point zero (↓); Addition of repressing agent glucose point seven (↓)

LB media: At the end of cultivation more than 93 % of cell population (+MazF) were still viable.

M9ZB media: Strong impact on dead cell ratio could not be monitored.

E. coli was grown without induction (-MazF) until end of exponential growth phase

Ability to form colonies: Prior to induction count was $5.7*10^7$ CFUs/ml. Subsequent to *mazF* expression within a period of seven hours only 3.5 % of the cell population were able to form colonies as $2.0*10^6$ CFUs/ml could be detected.. Following overnight incubation, during which cell growth was no longer inhibited, the ability to form colonies was restored. $9.9*10^9$ CFU/ml could be determined 24 hours after induction point. With flow cytometry such a dramatic decrease in cell viability could not be observed.

Prolonged expression of *mazF* did not lead to an irreversible condition from which only a small subpopulation was able to recover while most of cells experienced cell death. An opposite effect was observed. In lysogeny broth and in minimal media it was possible to immediately restore bacterial growth subsequent to addition of appropriate amount of repressing agent glucose, while only a small subpopulation died. The ability to form colonies was lost during induction of MazF but fully restored as bacterial cells recovered. By comparison of dead cell ratio and colony forming units it becomes evident that, the ability to form colonies on agar plates is not a reliable indicator for cell viability in *E. coli* cultures experiencing *mazF* expression.

During this diploma thesis several shake flask experiments have been conducted and cell death in context with overexpression of *mazF* in exponentially growing *E. coli* cells has never been observed. These results support the theory that the endoribonuclease MazF leads to a so called "quasi dormant state", a reversible condition, where cells are still viable, but growth arrested.

6.1.2 Influence of MazF on protein expression

To evaluate the effect of MazF production on the protein expression pattern of a recombinant protein a discontinuous NuPAGE electrophoreses was conducted. Samples were drawn from shake flask experiments with strain *E. coli* HMS174(DE3)pBAD33*mazF*/pET30a*SODmut*. The target protein SODmut was a defective variant of the designed protein SOD_ACAfree. However, the mRNA transcript contained the triplet ACA due to a frame shift mutation and SODmut was therefore susceptible for MazF cleavage. Production of target protein was induced at OD_{600} = 2 with 1 mM IPTG. Overepxression of *mazF* was induced one hour after product formation by addition of 0.2 % arabinose [w/w]. In a reference experiment only product formation was induced.

Discontinuous NuPAGE electrophoreses

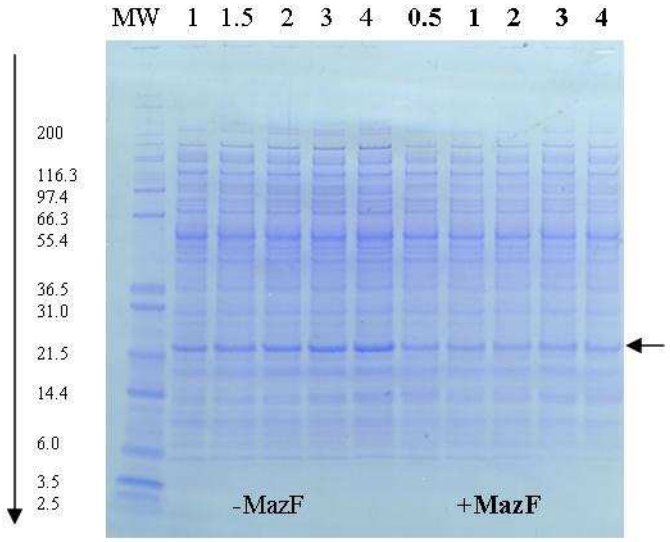


Figure 6.6 Protein expression pattern with and without *mazF* co – expression

MW: molecular weight marker; ← Position of target protein SODmut

Lane 1 to 4 (-MazF): Reference experiment; Numbers represent the period [h] of target protein production.

Lanes 0.5 to 4 (+MazF): Co-expression of MazF; Numbers represent the period [h] of co-expression of target protein SODmut and MazF

As shown in Figure 6.6, NuPAGE electrophoreses revealed influence of *mazF* on protein expression pattern of the recombinant protein. In induced culture, protein synthesis of target protein was inhibited due to degradation of mRNA at recognition sequence ACA. Degradation of cellular mRNA led to immediate growth arrest of induced culture (data not shown).

For an initial "proof of concept" shake flask experiments were well suited but only to gain first insight into the physiological changes in *E. coli* after overexpression of *mazF*. As cultivation conditions in shake flasks cannot be monitored or regulated cultivation process is not controlled e.g. regarding the pH value or the dissolved oxygen demand. For evaluating the consequences of MazF production under defined conditions, cultivation in a computer controlled bioreactor was necessary.

6.2 MazF production during cultivation of *E. coli* in bioreactor

6.2.1 Batch cultivation

To further evaluate the influence of *mazF* overexpression on cells growing under defined conditions, batch cultivation was set up in a 20 l bioreactor with a batch volume of four litres and concentrated LB media (5x) as substrate. *E coli* HMS174(DE3)pBAD33_*mazF* was grown to OD_{600} = 10 before expression of *mazF* was induced by aseptic addition of arabinose to reach a final concentration of 0.2 % [w/w] in batch media. Temperature was set to 37 °C, pH to 7 and oxygen partial pressure was controlled at 30 % by stirrer speed.
Samples were drawn hourly to monitor biomass accumulation by determination of cellular dry weight and OD_{600}. Cell viability was derived from cell density and dead cell number by flow cytometry. Process parameters were determined as described in Material and Methods.

Growth was soon inhibited after MazF production was induced but as the LB media was not buffered growth arrest resulted in increasing pH value. Ammonia used for pH control was no longer consumed. Increase of pH value and measures to stabilize pH value by addition of diluted hydrochloric acid led to increase of dead cell ratio and cell lysis. Continuing cultivation was not reasonable and cultivation process was aborted four hours after induction.

These results indicated that, simple upscale of shake flask experiments was not possible for characterization of MazF during batch cultivation. The problem of impeded induction due to excess glucose can be either solved by fed batch cultivation or by designing a media free of glucose, rich in yeast extract and tryptone and supplemented with buffering salts.
As the development of a batch cultivation process was not the ultimate aim for implementing single protein production further batch cultivations were not conducted.

6.2.2 Fed batch cultivation

For a single protein production system in *E. coli* using properties of MazF, a distinct fed batch cultivation strategy has to be developed to produce protein of interest non growth associated and in high yield. Taking in account the co-expression of MazF and of target protein a customized feed regime has to be developed to supply the substrate demand and concurrently avoid inhibition of *mazF* expression.
As prerequisite work for such a production process and for determination of influence of MazF production on biomass accumulation, glucose consumption and dead cell ratio, on cells growing at a constant growth rate, fed batch cultivation was conducted.

Cultivation was set up in a 20 l bioreactor, *E. coli* HMS174(DE3)pBAD33_*mazF* was initially grown in three litres batch media and substrate feed was started as soon as cell growth reached decline phase. Feed media was added exponentially providing a growth rate of $\mu = 0.1\ h^{-1}$ for 3.5 generations. Overexpression of *mazF* was induced after one doubling time at feed hour 7. To induce expression media in bioreactor and remaining substrate in feed tank were supplemented with 0.2 % arabinose [w/w]. Temperature was set to 37 °C, pH to 7 and oxygen partial pressure was controlled at 30 % by stirrer speed.
Subsequent to induction samples were drawn hourly to monitor biomass accumulation by determination of cellular dry weight and OD_{600}. Cell viability was derived from cell density and dead cell number by flow cytometry. Process parameters were determined as described in Material and Methods.

Subsequent to induction of *mazF* expression, cell growth was effectively inhibited. Despite the reduced substrate demand due to growth arrest, substrate was further added exponentially to determine the glucose demand and metabolic activity of "quasi dormant" cells and to evaluate the glucose concentration leading to inducer exclusion. For a period of almost two doubling times (13 h) cell growth came to complete rest. Subsequent to the dormant state *mazF* expression was steadily inhibited due to inducer exclusion of arabinose by elevated glucose concentrations and catabolite repression. Cell growth was restored slowly within a period of seven hours. (Figure 6.7)

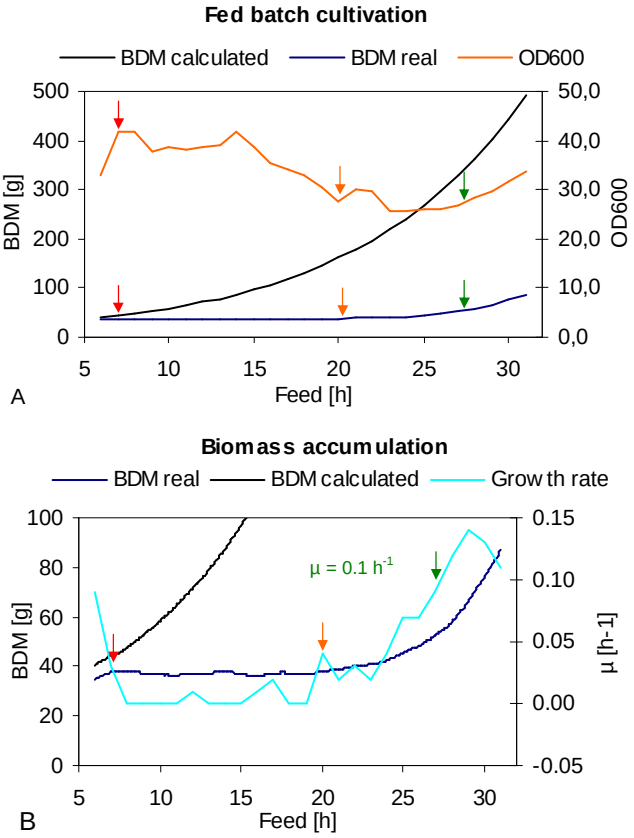


Figure 6.7 MazF production in *E. coli* during fed batch cultivation

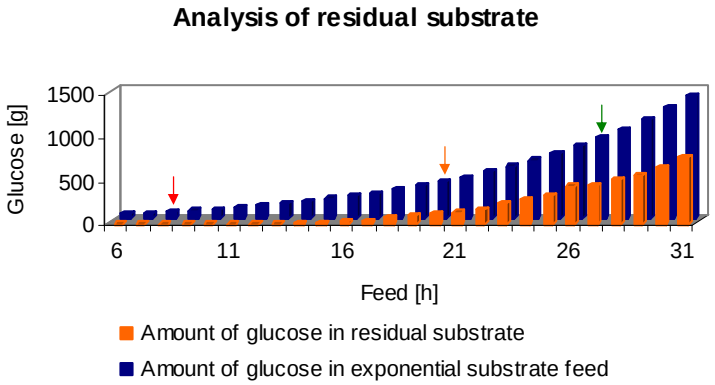
Feed hour 7 (↓): Induction of MazF production; Feed hour 20 (↓): Ongoing inducer exclusion; Feed hour 27(↓): Restored growth at a rate of μ=0.1 h^{-1};

A: Optical density and bacterial dry matter (BDM): Theoretical yield of total biomass (─) calculated with a yield coefficient of $Y_{X/S}$ = 0.33 compared with actual yield of total biomass (─) to demonstrate cell growth arrest.

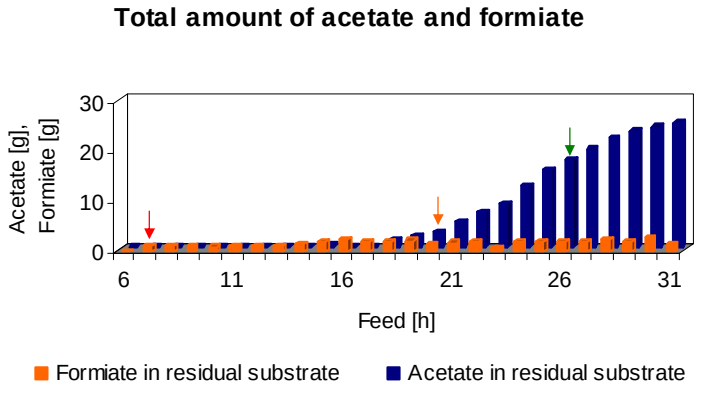
B: Detailed illustration of **A** and growth rate μ to elucidate growth arrest and restored cell growth

By analysis of the residual substrate the concentrations of the major carbon source glucose, the metabolites acetate, formiate, lactate, pyruvate and the inductor arabinose were determined. The concentration of arabinose was constant at approximately 1.6 g/l during the dormant state and rose to a constant value of 2.5 g/l subsequent to inducer exclusion. The concentrations of pyruvate and lactate were below the lower detection limit. The concentration of glucose and acetate increased throughout cultivation, the concentration of formiate was almost constant.

Figure 6.8 illustrates the total amount of glucose in the residual substrate and the total amounts of the metabolites acetate and formiate.



A



B

Figure 6.8 Glucose and acetate accumulation in residual substrate

Feed hour 7 (↓): Induction of MazF production; Feed hour 20 (↓): Ongoing inducer exclusion; Feed hour 27(↓): Restored growth at a rate of μ=0.1 h^{-1}

A. Glucose accumulation: Within a period of 31 hours a total amount of 1424 g glucose was added with the exponential substrate feed (■). As a consequence of repressed cell growth, glucose accumulated in residual substrate (■) and at the end of cultivation 763 g glucose was not metabolized.

B Formiate and Acetate: Production of formiate (■) was constant during fed batch cultivation. Acetate (■) accumulated in high yield (up to 25 g) in residual substrate

The analysis of the residual substrate showed that cell growth restored at a glucose concentration of 26 g/l (feed hour 20) and that half of the supplied glucose was metabolized throughout fed batch cultivation. Only one fifth of the theoretical bacterial dry matter was built up and glucose was channelled to non growth associated metabolic pathways. One major metabolite was acetate, which was secreted in high yield as cell growth slowly restored. Acetogenesis occurred because cells grew in excess glucose subsequent to dormant state, leading to the bacterial crabtree effect. Two other major metabolites were carbon dioxide and formiate.

But as illustrated in the carbon balance in Table 6.1 high amounts of glucose were apparently channelled to other metabolic pathways and futile cycles. During the dormant state over 50 % of C could not be assigned to biomass or the major metabolites. The value decreased as soon as cell growth was restored but at the end of cultivation almost 34 % of the total carbon amount could not be designated.

Table 6.1: Carbon Balance for feed phase of fed batch cultivation

Carbon Balance Feed [h]	0 to 31		0 to 7		7 to 20		20 to 27		27 to 31	
C in Metabolized Glucose [g]	264,2	100,0%	26,8	100,0%	91,5	100,0%	53,2	100,0%	71,8	100,0%
C in Biomass [g]	30,9	11,7%	7,3	27,2%	0,1	0,2%	7,1	13,3%	16,4	22,8%
C in Acetat [g]	10,1	3,8%	0,0	0,0%	1,2	1,3%	6,8	12,9%	2,1	3,0%
C in Formiat [g]	1,4	0,5%	0,4	1,3%	0,4	0,4%	0,4	0,7%	0,3	0,4%
C in Carbondioxide [g]	108,3	41,0%	17,1	63,7%	40,6	44,4%	21,9	41,2%	28,7	40,1%
Residual C [g]	113,5	43,0%	2,1	7,8%	49,2	53,8%	17,0	32,0%	24,2	33,8%

0 – 31: Exponential feed within a period of 3.5 generations
0 – 7: Cells growing uninduced for one generation
7 – 20: Cell growth arrest within a period of 13 hours
20 – 27: Slowly restored cell growth
27 – 31: Restored growth at a rate of $\mu = 0.1\ h^{-1}$

Residual carbon: Amount and percentage of carbon in metabolized glucose ($C_6H_{12}O_6$) which cannot be assigned to biomass ($CH_{1.77}O_{0.49}N_{0.24}$), acetate ($CH_3COO^-$), formiate ($HCOO^-$) and carbon dioxide ($CO_2$)

Chromatographic analysis confirmed the assumption of several other metabolites as three peaks found on the chromatogram could not be assigned. Taking in account the peak area and the dilution factor these metabolites may represent the amount of carbon which could not be assigned. (Data not shown) Subsequent to identification of these metabolites (e.g. MS) residual substrate will be further investigated to clarify the composition.

Acetate was not only one of the major metabolites it had supposably also negative influence on dead cell ratio. At the end of feed hour 20 1.6 % of cells were dead. But as cell growth restored and acetate was accumulated due to bacterial growth on excess glucose, dead cell ratio elevated until it reached maximum of 8 % at feed hour 30. (Figure 6.9)

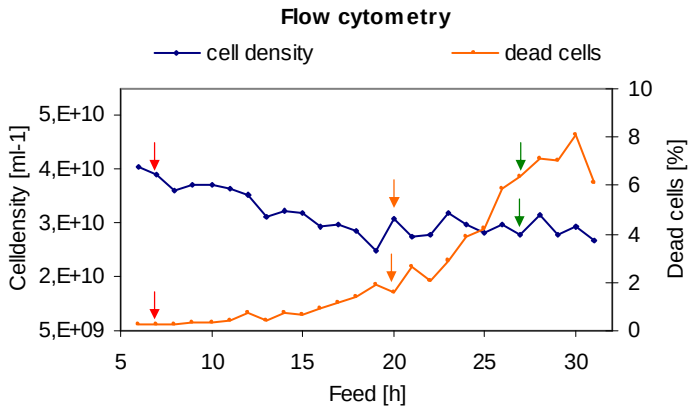


Figure 6.9: Cell viability during fed batch cultivation

Feed hour 7 (↓): Induction of MazF production; Feed hour 20 (↓): Ongoing inducer exclusion; Feed hour 27(↓): Restored growth at a rate of μ=0.1 h^{-1}; Cell viability (—) and cell density (—) obtained with FACS flow measurements

The level of the alarmone ppGpp detected by nucleotide analysis was very low throughout fed batch cultivation. It slightly increased from 0.01 µmol/g BDM to a final value of 0.15 µmol/g BDM as soon as cell growth was restored and acetogenesis occurred.

Ability to form colonies: Colony plate count revealed that cells experiencing *mazF* expression lost the ability to form colonies on nutrient agar plates and fully regained the ability as soon as growth was no longer inhibited. As these results are similar to

those results obtained with shake flask experiments it was confirmed that, for cells experiencing *mazF* expression, the ability to form colonies is no reliable indicator for cell viability.

With fed batch cultivation it was demonstrated that overexpression of mazF in *E. coli* HMS174(DE3)pBAD33_mazF leads to a bacteriostatic condition. Cells are in a "quasi dormant state" but still viable and metabolically active. The carbon balance revealed that not all of the metabolites could be defined with the applied analytic methods.
The dormant state was maintained as long as the expression of *mazF* was not repressed due to inducer exclusion of arabinose by elevated glucose concentrations and catabolite repression. The majority of cells were able to fully restore growth on the excess glucose. Cell growth was restored at a glucose concentration of 26 g/l and thereby acetate accumulated in high yield which had supposably negative influence on cell viability. The expression of *mazF* itself had no negative impact on the dead cell ratio.

With the above described cultivation it could be shown that growth of a bacterial culture can be controlled by expression and inhibition of the endoribonuclease MazF. Fed batch cultivation was also prerequisite work for establishment of a two plasmid single protein production system with *E. coli* HMS174(DE3)pBAD33_*mazF* and plasmid for expression pET30a_*SOD_ACAfree*. It illustrated need for development of a distinct feed regime for producing protein of interest non growth associated with cells in a quasi dormant state. For prolonged protein expression it is necessary to avoid accumulation of glucose finally leading to acetogenesis and formation of other non growth associated metabolites. It is crucial to determine, experimentally or by applying thermodynamic equations, demand in glucose, as major carbon and energy source, and nitrogen demand for maintenance and protein production during dormant state. Application of optimized feed regime would also have an economic benefit.

6.3 Single protein production system

For use in a single protein production system, to produce the model protein SOD in a non growth associated manner, strain *E. coli* HMS174(DE3) had to be co-transformed with pBAD33_*mazF* and an expression vector containing the genetic information for protein of interest encoded by ACA free mRNA.

DNA sequence encoding for SOD was modified at five positions by synthesizing whole gene sequence (GeneArt). DNA sequence devoid of any ACA triplets, in- or outside of coding frame, was termed SOD_ACAfree. (Sequence is provided in the Appendix.) With polymerase chain reaction, restriction digest and ligation, DNA was amplified and cloned into plasmid for expression pET30a. By sequence analysis integration of gene for target protein was confirmed at right position, under control of promoter T7, and free of any mutations. (Figure 6.9)

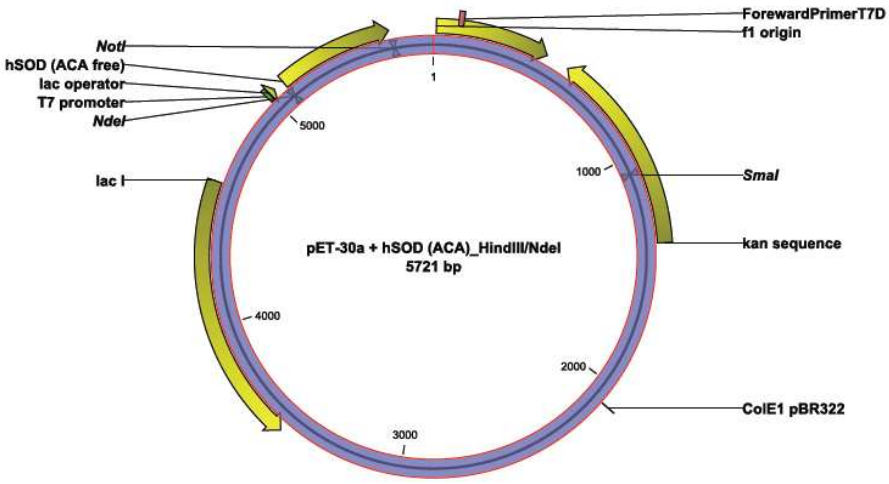


Figure 6.10 SOD_ACAfree integrated into the expression vector pET30a

Amplified SOD_ACAfree was incorporated into multiple cloning site of pET30a between recognition sequences of enzymes NdeI and HindIII. No mutation was found and gene was under control of promoter T7.

However, sequence analysis of T7 promoter region revealed localization of a disruptive ACA sequence in the non coding region between ribosomal binding site and start codon for translation (ATG). (Figure 6.11)

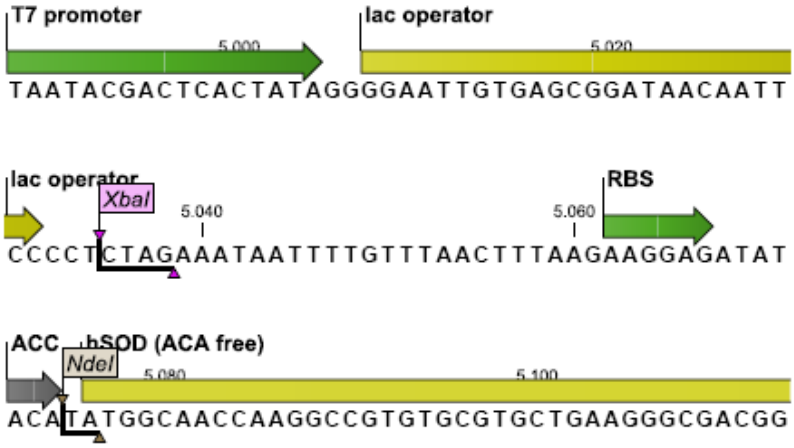


Figure 6.11 Section of pET30aSOD_ACAfree sequence

→ ACA triplet located in the non coding region between RBS and start codon

Thereby the obtained RNA transcript is susceptible for MazF degradation and co-transformation of plasmid pBAD33_*mazF* and pET30a_*SOD_ACAfree* into *E. coli* HMS174(DE3) was not accomplished. Before creation of the two plasmid production strain the triplet ACA has to be changed into a non cleavable sequence (e.g. ACC) with a site directed mutagenesis reaction.

The molecular cloning procedures for creating a plasmid for expression with desired properties were a complex task. A frame shift mutation, in DNA of target protein SOD, led to a defective production strain. This strain was used to demonstrate the influence of MazF on the protein expression pattern, but was otherwise not suitable for use in a SPP system because the mRNA encoding for the recombinant protein was not free of the MazF recognition sequence ACA.

In a second attempt the DNA encoding for SOD_ACAfree was cloned into pET30a and confirmed to be free of mutations but nevertheless plasmid could not be used for a production system. A disruptive ACA triplet is located in a non coding region of the RNA transcript and thereby a site directed mutagenesis reaction is necessary to change the triplet into a non cleavable sequence.

Thus the applicability of an expression vector for the SPP system is dependent on the number of disruptive ACA triplets found at the integration site of the desired vector. Several site directed mutagenesis reactions may be needed for RNA transcript optimization. Therefore the high sequence specifity of MazF is not only an advantage it is also a limitation for use of the single protein production system.

Nevertheless with shake flask and bioreactor cultivations the proof of concept for the non growth associated protein production system was provided. *Escherichia coli* cells experiencing *mazF* expression were in a dormant state under which cells were fully metabolically active in the total absence of cell growth. With the carbon balance drawn for the fed batch cultivation the basis for development of a distinct feed regime for a production process was provided. Furthermore the obtained vector pET30a*SOD_ACAfree* can be integrated into the expression host after modification of the disruptive ACA in the non coding region between the RBS and the MCS and thereafter used for construction of a two plasmid production strain.

7 Conclusion

The consequences of MazF induced cell growth arrest for bacteria like *E. coli* have been widely discussed and were stated to range between loss of viability leading to programmed cellular death and a reversible bacteriostatic condition termed "quasi dormant" state. Due to the controversial reports in the literature prior to the establishment of a single protein expression system on industrial scale, the inhibitory effect of the endoribonuclease had to be confirmed. By using *E. coli* HMS174(DE3) the influence of MazF overexpression on bacterial growth, cell viability and protein expression was determined.

With shake flask experiments, where overexpression of *mazF* was induced in exponentially growing *E. coli* HMS174(DE3)pBAD33_*mazF*, it was demonstrated that enhanced production of MazF does not lead to cell death during exponential growth phase. Cells were in a "quasi dormant" state and this condition was maintained for seven and 24 hours in lysogeny broth and minimal media, respectively without drastic increase of dead cell ratio.
Cell growth came to almost complete stop indicating that MazF efficiently cleaved cellular mRNA and led to inhibited cellular protein synthesis, which was also confirmed by analysing the protein expression pattern with discontinuous SDS gel electrophoreses. Even though cells were still viable, the ability to form colonies on agar plates was lost during the period of *mazF* expression. This loss was dramatically, as over 95 % of cells, which were able to form colonies before hand, lost this feature after addition of inductor arabinose. Measurements of dead cell ratio in a FACS flow cytometer showed that majority of cells was still alive and only a small sub population lost viability as soon as cell growth was restored. Most of the cells were able to fully restore cell growth and the ability to form colonies. However, these first results showed that the ability to form colonies on agar plates is rather an indicator for cell proliferation than an indicator for cell viability and overexpression of *mazF* does not lead to cell killing during exponential growth phase, it rather induces a reversible bacteriostatic condition.

Under defined conditions of fed batch cultivation equivalent results were obtained. Cell growth was almost completely inhibited for a time period of 13 hours, equal to

almost two doubling times at a constant growth rate of $\mu=0.1\ h^{-1}$. Despite the reduced substrate demand due to growth arrest, substrate was further added exponentially to determine the glucose demand and metabolic activity of “quasi dormant” cells.
However, as feed regime was not changed subsequent to induction glucose accumulated in residual substrate and *mazF* expression was inhibited due to inducer exclusion of arabinose. The majority of cells were able to fully restore growth within a period of seven hours and bacterial growth on excess glucose led to accumulation of acetate. Acetogenesis was confirmed with chromatographic analysis of residual substrate and had supposably negative influence on cell viability as dead cell ratio elevated during acetate accumulation. By chromatographic analysis and carbon balancing it was demonstrated that dormant cells were fully metabolically active as glucose was metabolized in high yield and channelled to non growth associated pathways. Not all of the metabolites could be defined with the applied analytical methods but the metabolite formation will be further investigated by the Microbial Fermentation Group of the Department of Biotechnology.

Fed batch cultivation confirmed that overexpression of *mazF* does not result in cell killing, as cells were in a reversible bacteriostatic condition and death of a small sub-population subsequent to restored cell growth was supposably influenced by acetate accumulation.
Fed batch cultivation was also prerequisite work for establishing a single protein production system where protein of interest is produced non growth associated in *E. coli* cells experiencing *mazF* expression. Results illustrated that a distinct feed regime has to be developed for such a production process to inhibit glucose accumulation, repression of *mazF* expression and acetogenesis on one hand and support carbon, nitrogen and energy source for protein production and maintenance of cell population and on the other hand.

Construction of a strain for a single protein production system was a complex task. For the desired two plasmid system, vector pBAD33_*mazF* was readily available and physiological changes in production strain subsequent to *mazF* expression have been sufficiently examined. For construction of the expression vector, DNA of target protein SOD was modified to be resistant to MazF cleavage and integrated into plasmid

pET30a. However the thus obtained vector could so far not be used for transformation into *E. coli* HMS174(DE3) as sequence analysis revealed a disruptive ACA triplet in the non coding region of the RNA transcript of SOD.

Future work will therefore encompass a site directed mutagenesis to change triplet ACA into a non cleavable sequence, sequence analysis to confirm alteration, co-transformation of both plasmids into production strain and shake flask experiments for first proof of concept.

The applicability of an expression vector for the SPP system is dependent on the number of disruptive ACA triplets found at the integration site of the desired vector. Therefore further optimization would be to perform multiple site directed mutagenesis reactions to create a plasmid suitable for expression without any cleavable ACA triplets in the DNA sequence or to synthesize the plasmid DNA. Both methods may turn out to be time and cost consuming with risk of frame shift or deleting mutations.

The complexity of creating the expression vector and the protection of MazF by several patents limit the applicability of the SPP system on industrial scale. Nevertheless subsequent to creation of the two plasmid production strain with the obtained expression vector the system should be well suitable in lab scale. Possible applications are the determination of the maximal capacity of a production system with the benefit of a detailed insight into product formation kinetics. Furthermore the expression system is a suitable model system for similar methods based on other genetic modules with the same goal of non growth associated protein formation.

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9 Abbreviations

ATP	Adenosine Tri Phosphate
cAMP	cyclic Adenosine Mono Phosphate
BDM	Bacterial Dry Matter
bp	base pairs
CDW	Cellular Dry Weight
cfu	colony forming units
cam	Chloramphenicol
ΩCam	Chloramphenicol resistance
CO_2	Carbon dioxide
DC	Dyed Cells
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
GDP	Guanosin Di Phosphate
GTP	Guanosin Tri Phosphate
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
hSOD	human superoxiddismutase
IAM	Institute of Applied Microbiology
IPTG	Isopropyl β-D thiogalactopyranosid
kan	Kanamycin
ΩKan	Kanamycin resistance
kDa	kilo Dalton
LB	Lysogeny Broth
MCB	Master Cell Bank
MCS	Multiple Cloning Site
mRNA	messenger RNA
NA	Nutrient Agar
NaOH	Sodium hydroxide
O_2	Oxygen
OD_{600}	Optical density at a wavelength of 600 nm

ORF	Open Reading Frame
PBS	Phosphored Buffer Saline
PCA	Perchloric Acid
PCD	Programmed Cellular Death
PCN	Plasmid Copy Number
PCR	Polymerase Chain Reaction
ppGpp	guanosine tetraphosphate
pppGpp	guanosine pentaphoshate
RCB	Research Cell Bank
RO-H_2O	water purified by reverse osmosis
ROS	Reactive Oxygen Species
rpm	radiation per minute
SDS	Sodiumdodecylsulfate
TA	Toxin - Antitoxin
TCN	Total Cell Number
VCN	Viable Cell Number
WCB	Working Cell Bank

10 Appendix

10.1 Media

All chemicals were purchased from Merck, Tryptone and Arabinose was purchased from Sigma and Amresco respectively. Water used was always RO-H_2O unless otherwise noticed. Subsequent to sterilization all liquid media were, if not used immediately, stored at 4 °C. LB Agar plates were stored upside down at 4 °C; NA Agar for agar plates was prepared shortly before use and kept in liquid stage in a water bath at 60 °C.

LB Media:

NaCl	10 g/l
Tryptone	10 g/l
Yeastextract	5g/l

LB Agar:

LB media supplemented with 20 g/l Agar Agar

M9ZB Media:

Tryptone	10 g/l
Yeastextract	5 g/l
NaCl	5 g/l
NH_4Cl	1 g/l
KH_2PO_4	3 g/l
Na_2HPO_4	6 g/l

After sterilization 1 ml of a 1M $MgSO_4$ solution and 4 g/l glucose were added.

Nutrient Agar:

Nutrient agar was weighed in (20 g/l), RO-H_2O was added. After sterilization agar was stored in a water bath at 60 C.

SOC media:

NaCl	0.5 g/l
Tryptone	20 g/l
Yeastextract	5 g/l
KCl solution (250 mM)	1 ml/l

pH was set to 7 and media was sterilized (20 min, 121°°C). 5 ml $MgCl_2$ solution (2 M) and 20 ml glucose solution (1 M) were added per litre media. Media was filtered through a 0.2 µm filter unit before storage.

Semi synthetic media for shake flask cultivations:

	SUBSTANCE	AMOUNT
1	KH_2HPO_4	3.00 g/l
	K_2HPOP_4	4.58 g/l
2	Tryptone	0.10g/g BTS
	Yeastextract	0.05 g/g BTS
3	Na_3citratdihydrate	0.25g/g BTS
	$MgSO_4 * 2 H_2O$	0.10 g/g BTS
4	$CaCl_2 * 2 H_2O$	0.01 g/g BTS
5	Traceelementsolution	50.00 µl/g BTS
6	$(NH)_4SO_4$	0.45 g/g BTS
	NH_4Cl	0.37 g/g BTS
7	Glucose	3.30 g/g BTS

Semi synthetic media was used for shake flask cultivations. Usually one litre was prepared and stored for further experiments. Media was laid out for a biomass concentration of 3 g/l.

Chemicals were weighed in and dissolved in RO-H_2O separately according to their numbers in first row. Solutions 1 to 6 were combined. After filling up to an appropri-

ate amount, (Approximately 7/8th of total weight) media and glucose solution (1/8th of total weight) were sterilized separately and assembled after cooling to give final volume.

Semi synthetic media for batch cultivations:

	SUBSTANCE	AMOUNT
1	KH_2HPO_4 $K_2HPOP_4 * 3 H_2O$	3.00 g/l 6.00 g/l
2	Yeastextract	0.15g/g BTS
3	Na_3citratdihydrate $MgSO_4 * 2 H_2O$	0.25g/g BTS 0.10 g/g BTS
4	$CaCl_2 * 2 H_2O$	0.02 g/g BTS
5	Antifoam solution (Sandoz)	0.5 ml/l
6	Traceelementsolution	50.00 µl/g BTS
7	$(NH)_4SO_4$ NH_4Cl	2.5 g/l 2.1 g/l
8	Glucose	3.00 g/g BTS

This semi synthetic media was used for batch cultivations. Batch volume was usually 4 litres (4 kg), unless mentioned otherwise and media was laid out for 22.5 g total biomass.

Chemicals were weighed in and dissolved in RO-H_2O separately according to their numbers in first row. Solutions 1 to 4 and 7 were combined; antifoam solution and traceelementsolution were added. After filling up to an appropriate amount, (Approximately 7/8th of total weight) media and glucose solution (1/8th of total weight) were sterilized separately and assembled after cooling to give final volume.

Synthetic media:

	SUBSTANCE	AMOUNT
1	KH_2HPO_4 K_2HPOP_4	3.00 g/l 6.00 g/l
2	Na_3citratdihydrate $MgSO_4 * 2 H_2O$	0.25g/g BTS 0.10 g/g BTS
4	$CaCl_2 * 2 H_2O$	0.02 g/g BTS
5	Traceelementsolution	0.02 g/g BTS
6	Antifoam solution (Sandoz)	50.00 µl/g BTS
7	Glucose	3.00 g/g BTS

Synthetic media was used as feed media for fed batch cultivations. Fed batch volume was usually 7.87 litres (8.5 kg), unless mentioned otherwise. Fed batch media was laid out for 363.3 g of total biomass.

Chemicals were weighed in and dissolved separately in RO-H_2O according to their numbers in first row. The solutions 1 to 5 were combined and filled up to an appropriate amount ($7/8^{th}$ of total weight). Media and glucose solution ($1/8^{th}$ of total weight) were sterilized separately and assembled after cooling.

Traceelementsolution:

	SUBSTANCE	AMOUNT
1	$FeSO_4 * 7 H_2O$	40.00 g/l
2	$MnSO_4 H_2O$	10.00 g/l
3	$AlCl_3 * 6 H_2O$	10.00 g/l
4	$CoCl_2 * 6 H_2O$	7.30 g/l
5	$ZnSO_4 * 7 H_2O$	2.00 g/l
6	$Na_2MoO_4 2 H_2O$	2.00 g/l
7	$CuCl_2 * 2 H_2O$	1.00 g/l
8	H_3BO_3	0.50 g/l

Substances were weighed in, dissolved in 5 M HCl (414 ml 37 % HCl diluted with 586 ml RO-H_2O) and filled up to 1000 ml. Traceelementsolution was stored at room temperature.

10.2 Buffers and stock solutions:

Ammonia stocksolution [12.5 % v/v]

Ammonia solution (Merck, 25 % v/v) diluted 1:2 with RO-H_2O

Arabinose stocksolution [20 % w/w]

20 g Arabinose (Sigma) dissolved in 80 g RO-H_2O, Final concentration in media 0.2 % w/w

Chloramphenicol stocksolution [25 mg/ml]

25 mg chloramphenicol dissolved in 1 ml 96 % ethanol. Final concentration in media 25 µg/ml

Cu-Zn Solution

1.7048 g $CuCl_2$*2 H_2O and 0.2875 g $ZnSO_4$*7 H_2O dissolved in 50 ml RO-H_2O to give final concentration of 0.2 M $CuCl_2$ and 0.02 M $ZnSO_4$

Destaining solution

250 ml ethanol, 80 ml acetic acid filled up to 1000 ml with RO-H_2O

Disintegration buffer

Had to made freshly before use; 5 ml were sufficient for 25 samples

4.5 ml Tris-buffer, 250 µl EDTA(KOH), 12.5 µl Mercaptoethanol and 125 µl Cu-Zn solution

EDTA stocksolution [0.5 M]

18.61 g EDTA dissolved in 100 ml H_2O; pH was either set to 8.0 with 5 M NaOH or to 8.2 with KOH

Ethanol [70 % v/v]

73 ml ethanol (96 %) diluted with 27 ml RO-H_2O

FACS staining solution

For 10 ml staining solution: 9946 µl PBS buffer (sterile filtrated), 5 µl Tween20 (ACROS Chemicals), 50 µl propidium iodide stocksolution and 1.1 ml Counting Beads (BD Bioscience)

Fixing solution

500 ml ethanol and 100 ml acetic acid filled up to 1000 ml with RO-H_2O

Glycerine [10 % v/v]

10 ml glycerine (87 % v/v) diluted with 75 ml RO-H_2O, sterilization before use

HEPES buffer [1 M]

23.83 g HEPES dissolved in 100 ml RO-H_2O

HEPES buffer [1 mM]

1:1000 dilution of 1 M HEPES stocksolution

HEPES glycerine buffer

75 ml HEPES buffer (1 mM) diluted with 10 ml glycerine (87 %)

IPTG stocksolution [0.1 M]

0.238 g Isopropyl-β-D-thiogalactopyranoside (therapeutic grade, BioVectra) dissolved in 10 ml RO-H_2O, sterile filtration before use (pore size: 0.22 µm) Final concentration in media: 1 mM IPTG

Kanamycin stocksolution [50 mg/ml]

50 mg Kanamycin (Merck) dissolved in 1 ml RO-H_2O, sterile filtration before use (pore size: 0.22 µm), final concentration: 50 µg/ml

PBS buffer (1x)

9.55 g PBS DULBECCO (Biochrom) dissolved in 1 l RO-H_2O, pH = 7.3

Perchloric acid extraction solution

10 ml extraction solution contained 2.98 ml PCA (70 %, Merck), 1.6 ml sterile filtrated EDTA solution (0.5 M, pH 8.3) and 5.42 ml sterile filtrated RO-H_2O

Physiological NaCl solution [0.9 % w/w]

9 g NaCl (Merck) dissolved in 9991 g RO-H_2O, sterilization before use

Propidium iodide stocksolution [1mg/ml]

500 µl of 4.3 mM propidium iodide (BD Biosciences) diluted with 940 µl RO-H_2O

Running buffer

200 ml 50x TAE buffer and 300 µl ethidiumbromide stocksolution filled up with RO-H_2O to a total volume of 10 litres.

Sodium acetate [0.5 M]

4.1 g Sodium acetate (Merck) dissolved in 100 ml RO-H_2O; pH was set to 5.2 with glacial acetic acid

Staining solution

1.16 g Coomassie brilliant blue R250 diluted with 250 ml ethanol, 80 ml acetic acid filled up to 1000 ml H_2O

TAE buffer [50x]

242 g Tris base, 57.1 ml glacial acetic acid and100 ml of 0.5 M EDTA (pH 8.0)

Tris-buffer [20 mM]

1.2114 g Tris-HCl dissolved in 500 ml RO-H_2O, pH was set to 8.2 with HCl

Triton X [0.5 %]

Triton X-100 dissolved in Tris-buffer

10.3 Polymerase chain reaction:

10.3.1 DNA Polymerisation

Table 10.1: Three step protocol for DNA Polymerisation

	TEMPERATURE	TIME	CYCLES
Initial denaturation	98°C	30 s	
Denaturation	98°C	5 s	
Annealing	68°C	20 s	
Extension	72°C	15 s	30
Final extension	72°C	8 min	

Primer sequences:

SOD_back_NdeI: 5´GGGAATTCCATATGGCAACCAAGGCCGTGTG 3´

SOD_for_HindIII: 5´CCGAAGCTTCTACTATTGGGCGATCCCAATT 3'

10.3.2 Screening PCR

Table 10.2: Screening PCR

	TEMPERATURE	TIME	CYCLES
Initial denaturation	98°C	30 s	
Denaturation	98°C	5 s	
Annealing	70°C	20 s	
Extension	72°C	30 s	30
Final extension	72°C	10 min	

Primer sequences:

Upper Primer: 5´GCTTCCTTTCGGGCTTTGTTAG 3´

Lower Primer: 5´CGATGCGTCCGGCGTAGAG 3´

10.4 DNA ladder

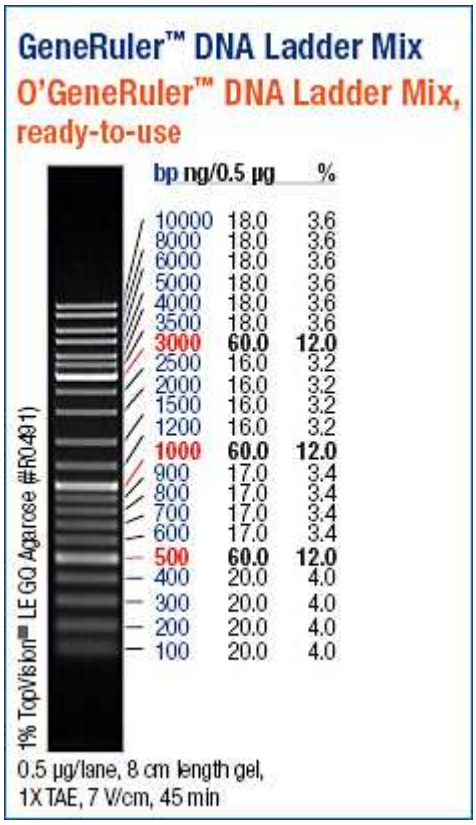


Figure 10.1: GeneRuler DNA ladder

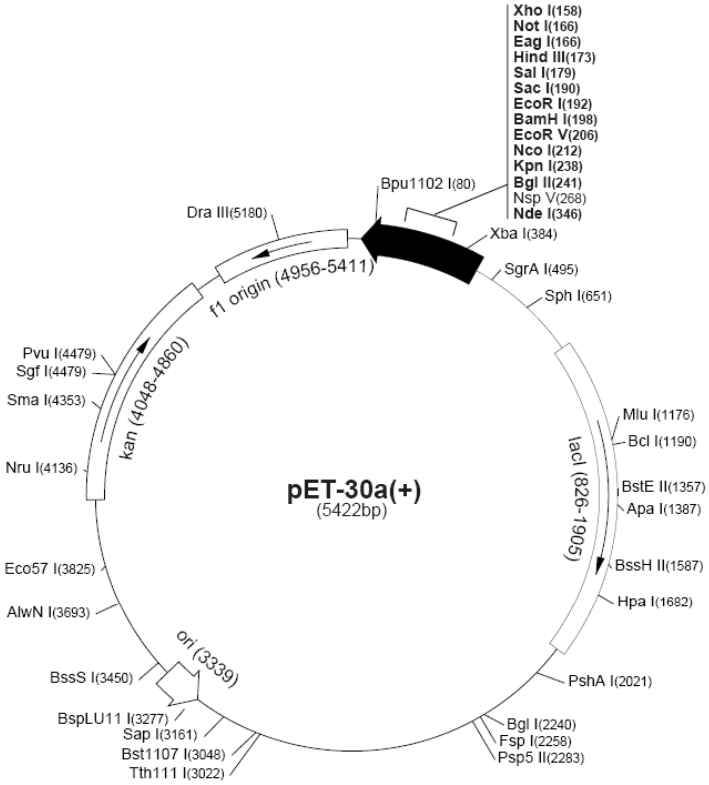
(http://www.fermentas.com/en/products/all/dna-electrophoresis/ogeneruler-dna-ladders/sm117-ogeneruler-mix, 4th of august, 2010)

10.5 Vector maps and sequences

10.5.1 pET30a

pET-30a(+) sequence landmarks	
T7 promoter	419-435
T7 transcription start	418
His• Tag coding sequence	327-344
S• Tag coding sequence	249-293
Multiple cloning sites (*Nco* I - *Xho* I)	158-217
His• Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	826-1905
pBR322 origin	3339
Kan coding sequence	4048-4860
f1 origin	4956-5411

The maps for pET-30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-30c(+) is a 5423bp plasmid; add 1bp to each site beyond *Bam*H I at 198.



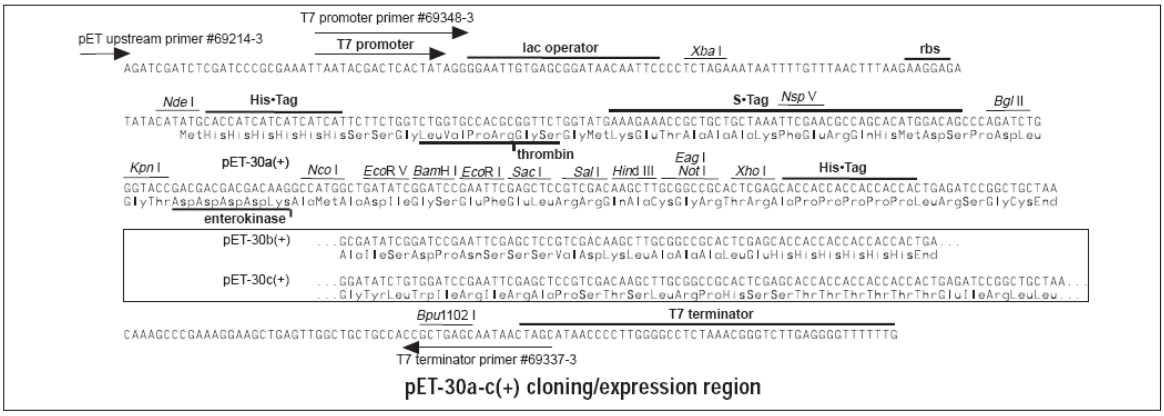


Figure 10.2: pET30a vector map

(http://www.merck-chemicals.de/chemdat/en_CA/Merck-US-Site/USD/ViewProductDocuments-File?ProductSKU=EMD_BIO 69909&DocumentType=VMAP&DocumentId=/emd/biosciences/vectormaps/en-US/TB095VM.pdf&DocumentSource=GDS; 3rd of august 2010)

10.5.2 pBAD33

ATCC 87402

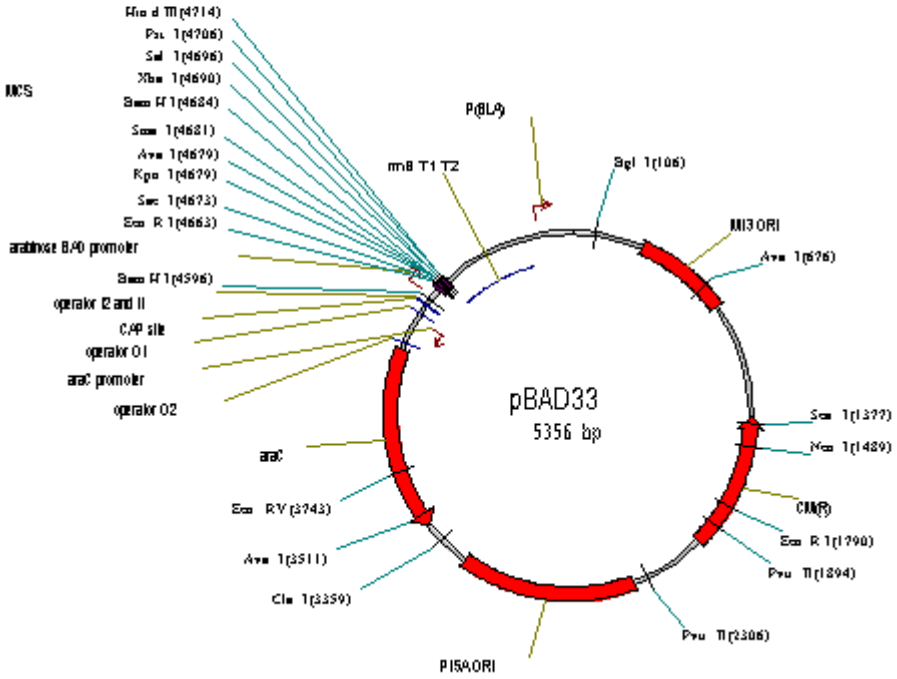


Figure 10.3: pBAD33 vector map

(http://www.atcc.org/attachments/1648.gif, 4th of august, 2010)

10.5.3 SOD_ACAfree

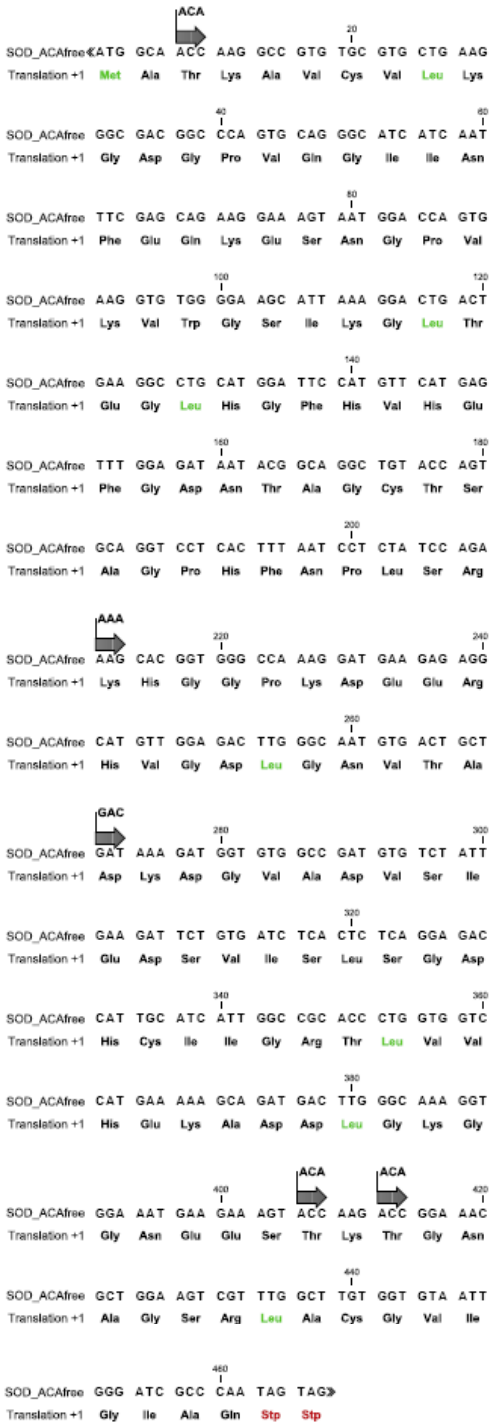


Figure 10.4 SOD_ACAfree sequence

→ DNA sequence had to be altered at five positions to be devoid of triplet ACA