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„The generation of short protein S1 variants during stress in *Escherichia coli*“

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This thesis is dedicated to my parents,

for their love, support and encouragement.

1. Abstract

During their lifetime bacteria are exposed to many stressful conditions, such as nutrient deprivation, changes in temperature or pH. It was shown that these stress conditions lead to activation of so-called toxin-antitoxin (TA) modules. These modules consist of two transcriptionally and translationally coupled genes, one encoding for a stable toxin and the other for a labile antitoxin. In *Escherichia coli* the first TA module that was described, was the *mazEF* module, consisting of the toxin MazF and the antitoxin MazE. It was shown, that MazF is an endoribonuclease that cleaves mRNA specifically at ACA sites, which besides degradation of bulk could result in formation of leaderless variants of specific transcripts, as it removes the 5'- UTR including the Shine and Dalgarno sequence.

One target mRNA for this MazF activity is the *rpsA* transcript coding for the ribosomal protein S1. This protein is essential for translation initiation in *E. coli* and most Gram-negative bacteria as it mediates the binding of the 30S ribosomal subunit to the translation initiation site at the mRNA and thus facilitates the formation of the initiation complex by recognizing a pyrimidine-rich region within the 5'-untranslated region of the mRNA. On the contrary, protein S1 is dispensable for translation initiation of leaderless mRNAs which start directly with an AUG start codon. Employing primer extension analyses performed on the *rpsA* mRNA upon induction of *mazF* expression we were able to show that the mRNA is cleaved by the toxin at an ACA sequence located directly upstream of the AUG start codon. Moreover, these analyses indicated that MazF likewise cleaves at an internal ACAUG motif. Since this AUG codon is in frame with the *rpsA* gene it potentially can serve as start codon for the translation of a 5'-truncated leaderless mRNA that codes for a short S1 protein variant that lacks the N-terminal domains. This 36,8 kDa protein termed protein $S1_{MazF}$ could play a significant role in growth recovery of *E. coli* cells from stress conditions. To test for this assumption, I first studied the effect of protein S1 and $S1_{MazF}$ overexpression on cell growth and protein synthesis. Growth of *E. coli* strain Tuner harboring either plasmid pET-S1 (*rpsA*) or pET-$S1_{MazF}$ (*rpsA**) was observed in LB and minimal medium respectively. The results showed that especially overexpression of $S1_{MazF}$ had stimulating effects on cell growth and thus the presence of the protein might be beneficial under adverse conditions.

Moreover mutations introduced at the internal ACAUG sequence of the *rpsA* gene revealed that the lack of selective expression of $S1_{MazF}$ affects cell growth

during recovery conditions. Taken together, my results indicate that the selective translation of $S1_{MazF}$ from the MazF generated *rpsA* mRNA contributes to the recovery of cells under non-permissive conditions.

2. Zusammenfassung

Während Ihres Lebenszyklus sind Bakterien vielen schwierigen Bedingungen ausgesetzt. Einige Studien haben gezeigt, dass diese Bedingungen zusätzlich zur Aktivierung von sogenannten Toxin – Antitoxin(TA) Modulen führen können. Diese Module bestehen aus zwei transkriptional und translational gekoppelten Genen. Das erste TA Modul das in *Escherichia coli* beschrieben wurde, war das *mazEF* Modul. Es kodiert für das Toxin MazF und das Antitoxin MazE. Viele Studien haben gezeigt, dass das Toxin MazF eine Endoribonuklease ist, die die mRNA spezifisch an ACA Sequenzen schneidet. Dadurch wird die Mehrzahl der mRNAs degradiert. Zusätzlich kann diese Aktivität aber auch zur Bildung von sogenannten „leaderless mRNAs“ führen, da MazF hier direkt vor dem Startkodon schneidet und somit die 5´-untranslatierten Region und somit auch die Shine-Dalgarno-Sequenz entfernt. Da MazF zusätzlich das Ribosom modifiziert, kommt es zu einer selektiven Translation dieser „leaderless mRNAs“ und das translationale Program wird geändert.

Eine Transkript, das durch MazF geschnitten wird, ist die *rpsA* mRNA, die für das ribosomale Protein S1 kodiert. In *Escherichia coli* und den meisten Gram-negativen Bakterien ist Protein S1 essenziell für die Translationsinitiation. Das Protein bindet an eine pyrmidinreichen Region in der 5‘- untranslatierten Region der mRNA in der Nähe der Ribosomenbindestelle und vermittelt dadurch die Ausbildung des Translationsinitiationskomplexes. Im Gegensatz dazu ist das Protein nicht essenziell für die Translation von „leaderless mRNAs“, die direkt mit einem AUG Startcodon beginnen. Wir konnten zeigen, dass die *rpsA* mRNA direkt vor dem AUG Startkodon durch MazF geschnitten wird. Zusätzlich haben wir einen weiteren Schnitt in der kodierenden Region beobachten. Da sich dieser zweite Schnitt direkt vor einem internen AUG Kodon im Leserahmen von *rpsA* befindet, liegt die Vermutung nahe, dass unter Stressbedingungen die Aktivierung des MazEF Systems zur Synthese einer verkürzten Variante des Proteins S1 kommt. Diesem 36,8 kDa Protein fehlt die N-terminale Domäne, die zur Bindung an das Ribosom essentiell ist. Da aber die RNA-bindenden Domänen vorhanden sind, könnte dieses verkürzte Protein eine signifikante, extraribosomale Rolle in der Regulation der Genexpression unter Stressbedingungen spielen.

Das Ziel dieser Arbeit war daher diese Hypothese zu testen. Mithilfe von Überexpressionsstudien konnte ich zeigen, dass Protein $S1_{MazF}$ einen positiven Einfluss auf das Zellwachstum und die Proteinsynthese des *E. coli* Stammes Tuner

hat. Somit könnte die Präsenz des $S1_{MazF}$ Proteins vorteilhalt für Zellen unter ungünstigen Bedingungen sein. Weiters konnte ich mithilfe von Mutationen an dem ACAUG Motiv, die einerseits den Schnitt durch MazF oder andereseits die Translation des kurzen S1 Proteins verhindern, feststellen, dass das Fehlen der Proteinvariante das Wachstum unter Stressbedingungen negativ beeinflußt. Zusammengefasst, weisen diese Resultate darauf hin dass, das Protein $S1_{MazF}$ eine wichtige Rolle für das Überleben unter Stressbedingungen und die nachfolgende Genesung der Zellen spielt.

3. Introduction

3.1 Translation initiation in prokaryotes

In bacteria transcription and translation are two tightly coupled processes, as translation starts before transcription is completed (Simonetti et al. 2009). The process of translation can be divided into four phases: initiation, elongation, termination and ribosome recycling. Translation initiation is the rate limiting process that can be modulated by different *cis*- or *trans*-acting regulators (Kaberdin and Blasi 2006; Marzi et al. 2008).

The components involved in bacterial translation initiation are the ribosome, the messenger RNA (mRNA), the initiator tRNA fMet-$tRNA_f^{Met}$ and the three initiation factors IF1, IF2 and IF3 (Figure 1) (Laursen et al. 2005). The ribosome is a large ribonucleoprotein complex composed of two subunits, the large 50S and the small 30S. It catalyzes the peptide bond formation and thus synthesizes the polypeptides according to the information encoded by the mRNA (Kozak 1999).

Translation initiation is mediated by a specific purine-rich sequence located upstream of the start codon, the so called Shine and Dalgarno (SD) sequence, which interacts with the complementary 3' terminal sequence, the so-called anti-SD sequence (aSD) of the 16S rRNA (Shine and Dalgarno 1974). Thereby the ribosome can identify the translation initiation region (TIR) and the AUG start codon is positioned in the ribosomal P-site (Yusupova et al. 2001). The efficiency of translation initiation complex formation is moreover dependent on the distance between the SD - sequence and the AUG start codon, which is optimal between 7-9 bases (Bram et al. 1980), and can be modulated by secondary structures of the ribosome binding site (rbs), which can prevent ribosome binding (de Smit and van Duin 1990; Chen et al. 1994; Shultzaberger et al. 2001). During initiation the 30S subunit protects the ribosome binding site from ribonucleolytic cleavage (Steitz 1969; Boni 2006), which corresponds to nucleotids from -20 to +15 related to the first nucleotide of the initiation codon (Hartz et al. 1988; Yusupova et al. 2001).

When the AUG start codon is adjusted and the initiator tRNA fMet-$tRNA_f^{Met}$ is bound in the P-site promoted by initiation factor 2, IF3 stabilizes binding of the correct initiator t-RNA in the ribosomal P-site (Laursen et al. 2005). Moreover, it confers proofreading activity by selectively destabilizing the complex harbouring a non-

cognate codon anti-codon base pairing (Risuleo et al. 1976; Gualerzi et al. 1977; Hartz et al. 1989).

This unstable complex undergoes a conformational change that promotes the codon - anticodon interaction and forms a more stable 30S initiation complex (Gualerzi et al. 1977; Pon and Gualerzi 1984). Thereby IF1 and IF3 are released. A structural rearrangement introduced by the IF2-dependent GTP-hydrolysis, stimulates the association of the 50S subunit and leads to the dissociation of IF2. Now, the translation initiation step is completed and the 70S initiation complex is ready to enter the elongation phase (Gualerzi and Pon 1990; Boelens and Gualerzi 2002; Laursen et al. 2005) (Figure 1).

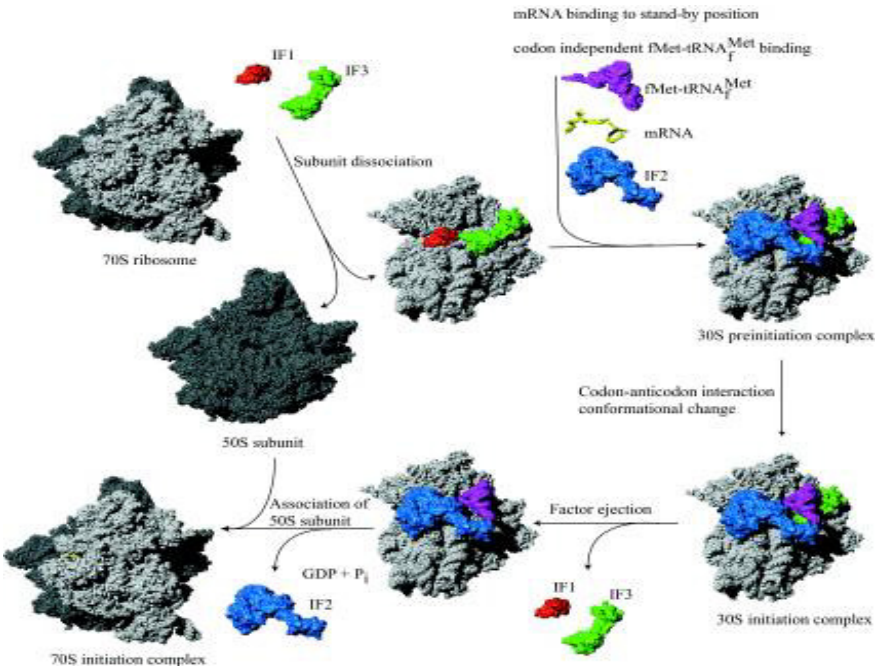

Figure 1: Translation initiation pathway in bacteria. Dissociation of the two ribosomal subunits 50S (indicated in dark grey) and 30S (indicated in light grey). Initiation factors IF1, IF2, IF3, mRNA and fMet-tRNA$_f^{Met}$ shown in red, blue, green, yellow and magenta respectively (Laursen et al. 2005). For details see text.

3.2 The role of ribosomal protein S1 in the regulation of translation initiation

The ribosomal protein S1 in *E. coli* and most Gram-negative bacteria plays an important role in mediating the primary binding of the 30S ribosomal subunit to the ribosome binding site (rbs) of mRNA (Sorensen et al. 1998). S1 is the largest ribosomal protein in *E. coli* and has a molecular weight of 61,000 (van Dieijen et al. 1976). It is involved in the formation of the initiation complex by recognizing a pyrimidine-rich region in the 5'-UTR of the mRNA (Laursen et al. 2005). The protein destabilizes mRNA secondary structures close to the translational start site, so that the 30S subunit can be positioned at the AUG start codon (de Smit and van Duin 1994).*Vice versa*, S1 is dispensable for translation of leaderless mRNAs which contain no 5'-UTR and start directly with an AUG start codon (Tedin et al. 1997; Moll et al. 2002a).

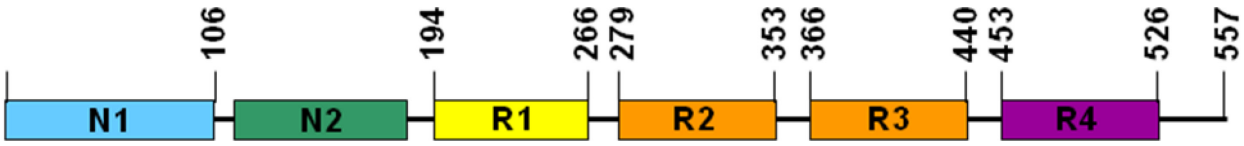

Figure 2: The 6 domains of protein S1. The first two are responsible for binding on the ribosome and the last four, for binding on mRNA (taken from Salah et al. 2009).

The protein consists of six domains (Figure 2) and 557 amino acid residues (Schnier and Isono 1982; Subramanian 1983; Schnier et al. 1986). Recently, several lines of evidence indicate that the N-terminal Domain (amino acids 1-106; $S1_{106}$) of protein S1 is sufficient for ribosome binding, since over production of $S1_{106}$ displaces native protein S1 from its binding site on the ribosome (Byrgazov et al. 2012). Moreover, it has been shown that protein S2 is required for binding of protein S1 on the ribosome (Moll et al. 2002a).

Cryo-electronmicroscopy indicated that the C-terminus of S1 is located on the solvent side on the small 30s ribosomal subunit (Figure 3).

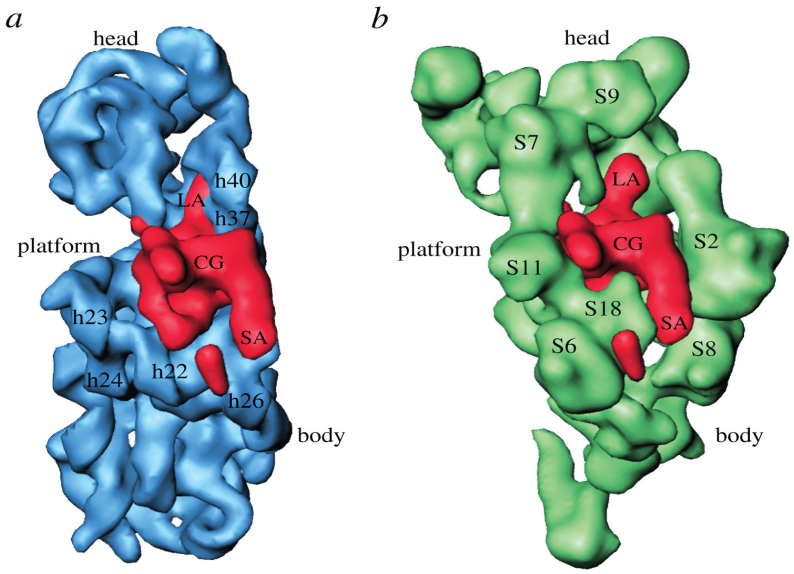

Figure 3: Structure and neighbourhood of protein S1 within the 30S small ribosomal subunit. (a) Position in respect to the 16S rRNA and (b) in respect to the other small subunit ribosomal proteins (taken from Sengupta et al. 2001).

3.3 Leaderless mRNAs

Leaderless mRNAs (lmRNAs) are present in all three kingdoms of life. Due to the absence of a 5´-UTR they lack a SD-sequence and start directly with a AUG start codon (Moll et al. 2002b). It was shown that the translation initiation complex formation on leaderless mRNAs can be performed by 30S ribosomal subunits that either lack protein S1 or IF3, as protein S1 is dispensable for the formation of the initiation complex in leaderless mRNAs (Tedin et al. 1997; Moll et al. 1998; Moll et al. 2002b). Moreover, there is no evidence of ribosomal recruitment downstream of the AUG start codon of leaderless mRNAs (Resch et al. 1996; Moll et al. 2001).

The 5´-terminal start codon of leaderless mRNAs can be recognized by a ribosome-initiator-tRNA-IF2 complex (Grill et al. 2000), and translation efficiency of

leaderless mRNAs is regulated by the ratio between translation initiation factors IF2 and IF3, whereby IF2 works as enhancer and IF3 works as repressor of leaderless mRNA translation (Tedin et al. 1999; Grill et al. 2001).

Furthermore, an alternative pathway for translation of leaderless mRNA was described. 70S monosomes can perform translation initiation on leaderless mRNAs starting with an AUG start codon (Balakin et al. 1992; Moll et al. 2004). It is intriguing to note that 70S ribosomes can perform selective translation of leaderless mRNAs even in the absence of proteins S1/S2 (Moll et al. 2004).

Another mechanism of leaderless mRNA translation in the presence of the antibiotic kasugamycin (Ksg) has been described. When E*. coli* cells are treated with the antibiotic Ksg *in vivo*, ribosomal particles lacking several proteins on the 30S small subunit are generated (Kaberdina et al. 2009). These 61S particles, in addition to proteins S2, S6, S12, and S18 lack the ribosomal protein S1, which is essential for translation initiation on canonical mRNAs (Boni et al. 1991). These protein-deficient ribosomes, selectively translate leaderless mRNAs *in vivo* and *in vitro* in the presence of kasugamycin (Ksg). These observations indicate that ribosomes lacking several essential proteins are fully functional in translation of leaderless mRNAs.

3.4 The toxin-antitoxin module *mazEF*

Most bacteria harbour toxin-antitoxin (TA) modules which are composed of two transcriptionally and translationally coupled genes, one encoding for a stable toxin and the other for a labile antitoxin. TA systems have initially been found in extra chromosomal elements like plasmids. These systems were identified as addiction modules that prevent growth of plasmid-free bacteria by a mechanism called post-segregational killing system (PSK). The antitoxin must be continually produced to prevent the toxin from killing the cell. Thus, the PSK system ensures that the cells remains plasmid-containing (Ogura and Hiraga 1983) and the effect of killing plasmid-free cells appears to “addict” the host to the plasmid (Cooper and Heinemann 2000).

In *E. coli* the *mazEF* module was first described as a prokaryotic addiction module. It is located downstream of the *relA* gene and consists of toxin gene *mazF* and antitoxin gene *mazE*. Besides *mazEF*, up to now several toxin-antitoxin (TA) systems are described in *E. coli* such as *chpBIK* (Masuda and Ohtsubo 1994), *relBE*

(Bech et al. 1985; Gotfredsen and Gerdes 1998; Christensen et al. 2001), and *yefM-yoeB* (Grady and Hayes 2003; Cherny and Gazit 2004; Christensen et al. 2004).

The toxin MazF is a sequence specific endoribonuclease that cleaves single-stranded mRNAs at ACA sequences (Zhang et al. 2003; Zhang et al. 2005). The expression of *mazEF* is regulated by cellular levels of ppGpp, which are synthesized by the product of the *relA* gene after amino acid starvation (Aizenman et al. 1996; Engelberg-Kulka et al. 1998; Kolodkin-Gal and Engelberg-Kulka 2006). It has been shown that stressful situations like oxidative stress, UV irradiation, nalidixic acid (Hazan et al. 2004) or thymin starvation (Sat et al. 2003) can trigger the activity of the *mazEF* module. Moreover, inhibition of transcription and translation mediated by antibiotics, like rifampicin, chloramphenicol and spectinomycin can trigger MazF activity (Sat et al. 2001).

Several studies revealed that the toxic effect of *mazF* overexpression can be reversed by the activity of the antitoxin MazE (Pedersen et al. 2002). The authors suggested that MazF induces a state of reversible bacteriostasis. In contrast, Amitai and colleges have shown that over-expression of *mazE* could reverse MazF lethality only for a short period of time. This time was depended on the growth conditions used during *mazF* over-expression, as during growth in M9 minimal medium, the "point of no return" occurred much faster than during growth in rich medium (Amitai et al. 2004).

3.5 Selective translation of leaderless mRNAs after *mazF* overexpression

As mentioned before the endoribonuclease MazF cleaves single stranded mRNAs specifically at ACA sites and thereby leads to degradation of bulk mRNA. As a consequence MazF activation results in death of the majority of cells of a bacterial population. However, it was shown that MazF not only causes cell death by inhibition of translation but in contrast can lead to selective synthesis of specific proteins (Amitai et al. 2009). The authors showed that *mazF* induction leads to synthesis of about 10% of proteins. They identified some of these small proteins by mass spectrometry and reported that some of these proteins are required for the death of most cells of a population and others for the survival of a small sub-population. This

small sub-population can re-grow when the conditions ameliorate and thus ensure the survival of the bacterial population.

Recently, Vesper and colleges reported that MazF cleaves specific mRNAs closely upstream of the AUG start codon thereby removing their 5'-UTR (Figure 4) (Vesper et al. 2011). In addition MazF targets the 16S rRNA of intact ribosomes at the decoding center. Thereby, a region of 43 nucleotids from the 3-' terminus of the 16S rRNA is removed, which harbors the aSD sequence required for translation initiation on canonical mRNAs. The authors showed that consequently this modified translation machinery generated by MazF selectively translates the leaderless mRNAs that are likewise formed by the toxin, resulting in the modulation of the translational program in *E. coli* (Vesper et al. 2011).

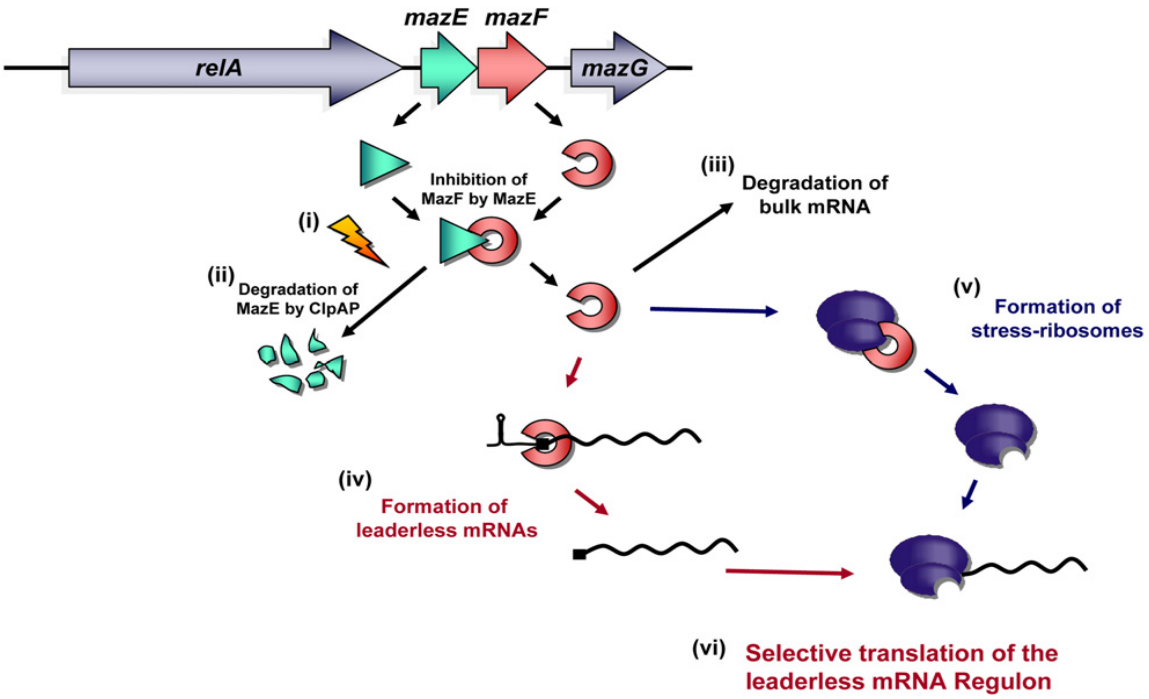

Figure 4: Model for the generation of leaderless mRNAs and stressed ribosomes by MazF. (i) Several stressful conditions trigger MazF activity by (ii) degradation of the anti-toxin MazE. (iii) Thereby most bulk mRNAs are degraded. (iv) In addition, MazF removes the 5'-UTR of specific mRNAs, thereby rendering them leaderless, and (v) concomitantly, it removes 43 nucleotides from the 3'-terminus of the 16S rRNA comprising helix 45 as well as the anti-Shine Dalgarno sequence. (vi) Together, this activities of MazF results in selective translation of the leaderless mRNA ("leaderless mRNA regulon") (taken from Vesper et al. 2011).

3.6 MazF cleaves the *rpsA* mRNA encoding ribosomal protein S1 directly upstream of an internal AUG codon

As mentioned before, the toxin MazF cleaves certain mRNAs specifically at ACA sites upstream of their AUG start codon thus making them leaderless. The *rpsA* open reading frame contains an internal ACAUG motif which could represent a potential target for MazF cleavage. As the AUG codon is in frame with the *rpsA* gene, we anticipate that MazF cleavage at this position could lead to the generation of a 5' truncated leaderless mRNA that codes for a short S1 protein variant that lacks the N-terminal domains D1 and D2 (Figure 6). To determine whether the *rpsA* mRNA encoding protein S1 is cleaved specifically by MazF directly upstream of the internal "A_{665}CAUG" sequence, a primer extension reaction was performed (Konstantin Byrgazov, unpublished data, see Figure 5). *E. coli* strain MC4100*relA*$^{+}$F´ harboring plasmid pSA1 that bears an IPTG-inducible *mazF* gene was grown in LB at 37°C. 30 minutes upon induction of *mazF* expression by the addition of 100µM IPTG, total RNA was purified and primer extension was performed employing specific primers for *rpsA* (E8, Z7; see materials and methods).

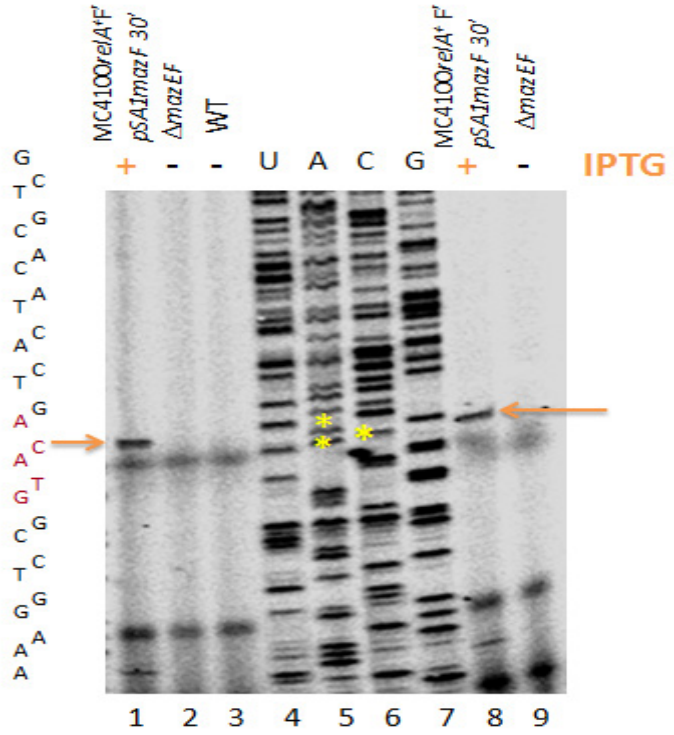

Figure 5: Primer extension analysis using total RNA purified from *E. coli* strain MC4100*relA*$^{+}$F´ pSA1 upon *mazF* overexpression (lanes 1 and 8), strain MC4100*relA*$^{+}$ (lane 3) and from strain MC4100*relA*$^{+}$*ΔmazEF* (lanes 2 and 9) employing primers E8 and Z7 specific for the *rpsA* mRNA. Signals corresponding to MazF cleavage are indicated by orange arrows and the ACA triplet in the left is indicated by yellow asterisks. MazF cleaves the *rpsA* mRNA directly upstream of the internal AUG start codon, resulting in the formation of a leaderless mRNA. U, A, C, and G (lanes 4, 5, 6, 7): sequencing reactions.

As anticipated, MazF activity resulted in cleavage of *rpsA* mRNA at the "A_{665}CA" sequence located directly upstream of the internal AUG codon. In contrast, no MazF cleavage signal was obtained employing total RNA prepared from strains MC4100*relA*$^+$ *ΔmazEF* and MC4100*relA*$^+$ (Figure 5, lanes 2 and 3, respectively).

3.7 The protein $S1_{MazF}$

The primer extension reaction shown above reveals that the *rpsA* mRNA is cleaved specifically at the "A_{665}CAUG" site by the bacterial toxin MazF. This cleavage results in the generation of a truncated variant of the *rpsA* mRNA, which lacks the coding sequence for domains N1 and N2 and starts directly with an AUG start codon and is thus leaderless. Vesper and colleges have shown that leaderless mRNAs are selectively translated by stressed ribosomes generated by MazF (Vesper et al. 2011). Therefore, we anticipate that the protein "$S1_{MazF}$", the product of the truncated and leaderless *rpsA* mRNA (from here on termed '*rpsA**'), might play a specific role under stress conditions. The N-terminally truncated protein has a mass of 36,8 kDa comprising amino acids 223-557 and contains only the RNA-binding domains R1, R2, R3 and R4 (Figure 6). Since the N-terminal domain is required for interaction with the ribosome (Byrgazov et al. 2012) (McGinness and Sauer 2004) we hypothesized that this short protein variant $S1_{MazF}$ could play a potential role in regulating mRNA stability and turn over.

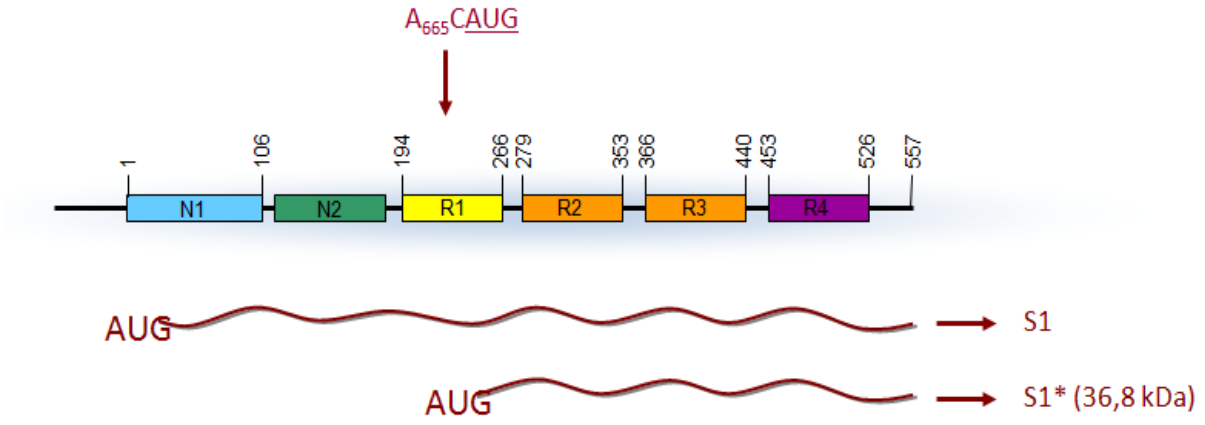

Figure 6: The structure of the ribosomal protein S1 and the N-terminally truncated variant $S1_{MazF}$. The red arrow indicates the signal corresponding to the cleavage directly upstream of the internal "A_{665}CAUG" start codon upon *mazF* overexpression.

The structural organization and interactions between the domains R1, R2 and R3 of protein S1 which are present in the variant $S1_{MazF}$ have been recently studied by Aliprandi and colleges (Aliprandi et al. 2008). In the absence of mRNA the domains R2 and R3 are associated and represent a continuous interface for RNA interaction, whereas domains R1 and R2 are in equilibrium between open (non-interacting) and closed (weakly interacting) forms (Figure 7). Moreover, a large perturbed area in the case of domain R1 and a much smaller area in the case of domain R2 have been observed, confirming a sum of interactions and dimerization surfaces in domain R1 (Aliprandi et al. 2008). The large perturbations are located within the linker regions between the domains R1-R2 and in the long loops (L3). These L3 loops are involved in interdomain interactions, which strongly suggest that RNA binding induces perturbations in the relative positioning of the domains. When RNA is present, there is a modification of the equilibrium between the R1 and R2 domain and a structural reorganization of the domains (Aliprandi et al. 2008).

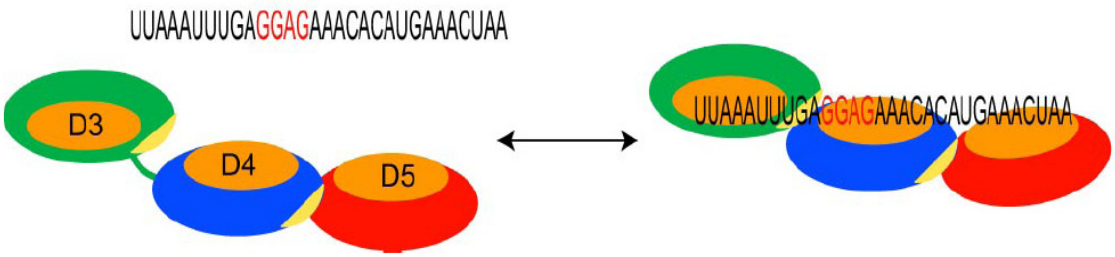

Figure 7: Structural organization of the RNA-binding domains. The D3 domain is shown in green, D4 in blue and D5 in red, respectively. Yellow indicates the positions affected by interdomain interactions and orange presents the continuous interface for RNA interactions. D3 is free positioned in the absence of RNA, whereas D4 and D5 are always associated (Aliprandi et al. 2008).

4. Aims of this study

As mentioned in the introduction, the *rpsA* gene encoding protein S1 is specifically cleaved at the $A_{665}CA$ site by the bacterial toxin MazF. As the cleavage site is directly upstream of an AUG codon, which is in-frame with the *rpsA* open reading frame, this cleavage consequently results in the formation of a shorter variant of the *rpsA* mRNA, which contains a 5' terminally start codon. Since leaderless mRNAs are selectively translated under stress conditions by *mazF*-processed stress-ribosomes, we anticipated that expression of the N-terminally truncated S1 protein, comprising amino acids 223-557 ($S1_{MazF}$), might have a physiological significance under these conditions.

Therefore, the aim of this study was to verify the expression of the protein $S1_{MazF}$ under stress conditions and to investigate its physiological role in cell growth and protein synthesis. To this end, I introduced alterations in the *rpsA* gene coding for protein S1 at the internal ACAUG motif and analyzed the effect of expression of the mutant genes under stress conditions. The internal ACAUG start codon of the *rpsA* gene was changed into ACAUC and AGAUG, with the rational first to follow MazF cleavage but interfere with translation of the protein $S1_{MazF}$ and second to prevent MazF cleavage. *E. coli* cells harboring these mutant *rpsA* genes were tested for cell growth and translation under stress conditions to determine a possible role of $S1_{MazF}$ under these physiological conditions.

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5. Results and Discussion

5.1 Effects of intact S1 and $S1_{MazF}$ overexpression on cell growth and translation

Many studies have shown that overexpression of S1 fragments can adversely affect cell growth and translation efficiency (Nishi and Schnier 1986; McGinness and Sauer 2004). To get insights into the potential activity of the protein $S1_{MazF}$, the following experiments were performed to study the effects of overexpression of proteins S1 and $S1_{MazF}$ on cell growth.

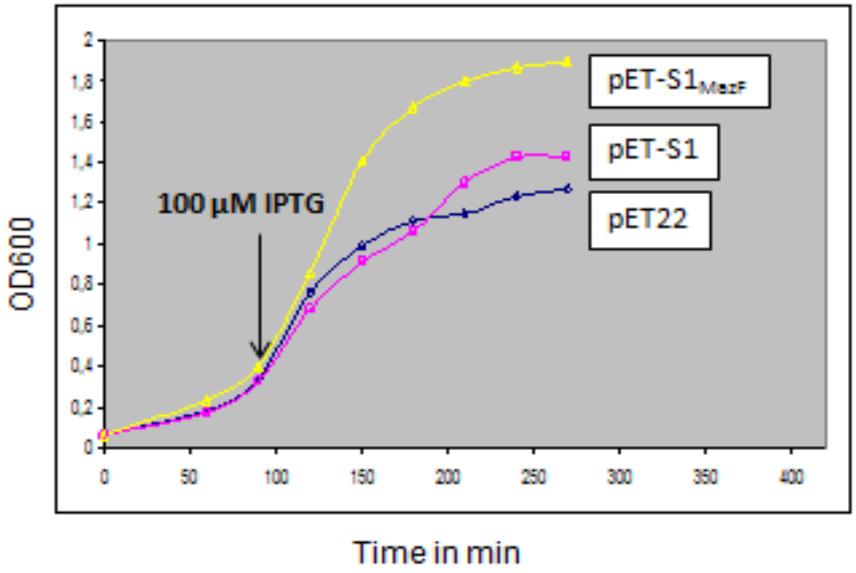


Figure 8: Effects of induced synthesis of S1 and $S1_{MazF}$ on cell growth. *E. coli* strain Tuner harboring plasmids pET-S1 encoding for protein S1 (shown in magenta) and pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$(shown in yellow) or harboring pET22 as vector control(shown in blue). Cells were grown in LB at 37°C. At OD_{600} of 0.3, 100 µM IPTG (indicated by a black arrow) was added to induce synthesis of protein S1 and its variant $S1_{MazF}$ pET22: vector control.

First, the coding sequences of the respective proteins were PCR amplified with primers H7, H9, G9, (see materials and methods) and cloned under control of the T7 RNA polymerase promoter between the NdeI and XhoI sites of vector pET22b (Novagen), resulting in a His-6 tag at the C-terminus of each protein (McGinness and Sauer 2004). The resulting plasmids containing the genes *rpsA* and *rpsA** are termed pET-S1 and pET-$S1_{MazF}$, respectively. *E. coli* strain Tuner was transformed with plasmids pET-S1 or pET-$S1_{MazF}$, and the cells were grown in LB broth at 37°C in the

presence of 100 µg/ml ampicillin. Growth was monitored by measuring the optical density at 600nm (OD_{600}). At OD_{600} of 0.3-0.4 the synthesis of protein S1 and $S1_{MazF}$ was induced by addition of 100µM (IPTG). Samples for protein analysis were taken before and after induction. As shown in Figure 8, cells containing plasmids for pET-S1 and pET-$S1_{MazF}$ grew at rates similar to the cells containing plasmid pET22b before addition of IPTG. After induction, overexpression of the truncated *rpsA** gene resulted in a stimulation of growth (Figure 8).

Since we anticipated that the truncated protein S1 might play a key role in regulation of leaderless mRNA translation during the MazF-mediated stress response, the same experiment was performed employing *E. coli* strain Tuner harboring plasmid pKTplaccI that encodes the leaderless *cI-lacZ* fusion gene, to allow monitoring of leaderless mRNA translation upon overexpression of $S1_{MazF}$ (Grill et al. 2000) (Figure 9). As shown in Figure 9, the effect of induction of synthesis of proteins S1 and $S1_{MazF}$ on cell growth is comparable to the results shown in Figure 8.

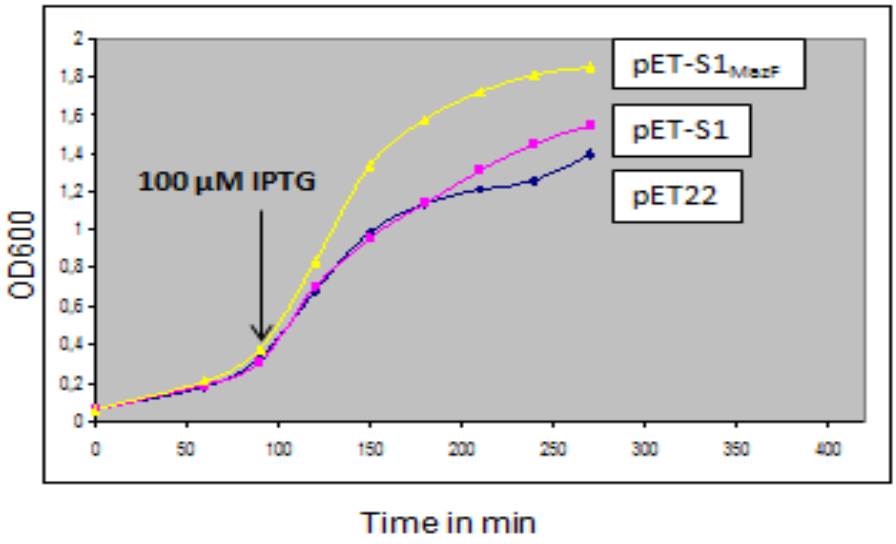


Figure 9: Effects of induced synthesis of S1 and $S1_{MazF}$ on cell growth. *E. coli* strain Tuner harboring plasmid pKTplaccI and either plasmid pET-S1 encoding for protein S1 (shown in magenta) ,pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$(shown in yellow) or harboring pET22 as vector control(shown in blue).Cells were grown in LB at 37°C. At OD_{600} of 0.3 100 µM IPTG (indicated by a black arrow) was added to the cultures to induce synthesis of protein S1 and its variant $S1_{MazF}$. pET22: vector control.

Next, the levels of S1 and $S1_{MazF}$ proteins synthesized under these conditions were determined. The protein samples, which were withdrawn before and two hours

after induction of *mazF* expression the growth experiments shown in Figures 8 and 9, were separated on a 12% SDS-page and visualized with Coomassie blue staining (Figure 10). In addition, three hours after induction the same cultures were harvested and the synthesized proteins S1 and $S1_{MazF}$ proteins were purified employing Ni-NTA agarose columns (see materials and methods). The proteins bound to the column were eluted with imidazol, separated on a 12% SDS-page and visualized with Coomassie blue staining (Figure 11).

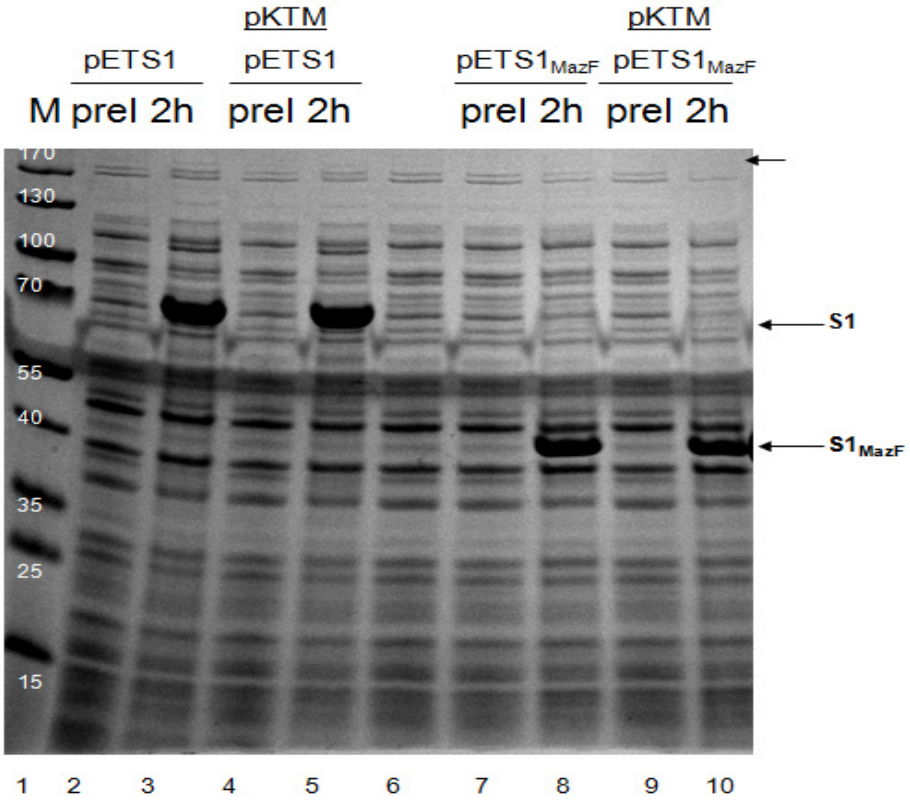


Figure 10: Validation of induced synthesis of proteins S1 and $S1_{MazF}$ after addition of IPTG. Lane 1: protein size marker; Lanes 2-5: *E. coli* strain Tuner (lanes 2 and 3) and strain Tuner harboring pKTplaccI (lanes 3 and 4), respectively harboring plasmid pET-S1 encoding for protein S1 before induction (lanes 2 and 4) and 2 hours after induction (lanes 3 and 5); Lanes 7-10: *E. coli* strain Tuner (lanes 7 and 8) and Tuner pKTplaccI (lanes 9 and 10), respectively harboring plasmid pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$, before induction (lanes 7 and 9) and 2 h after induction (lanes 8 and 10). The black arrows indicate the position of the proteins S1 and $S1_{MazF}$.pKTM: pKTplaccI

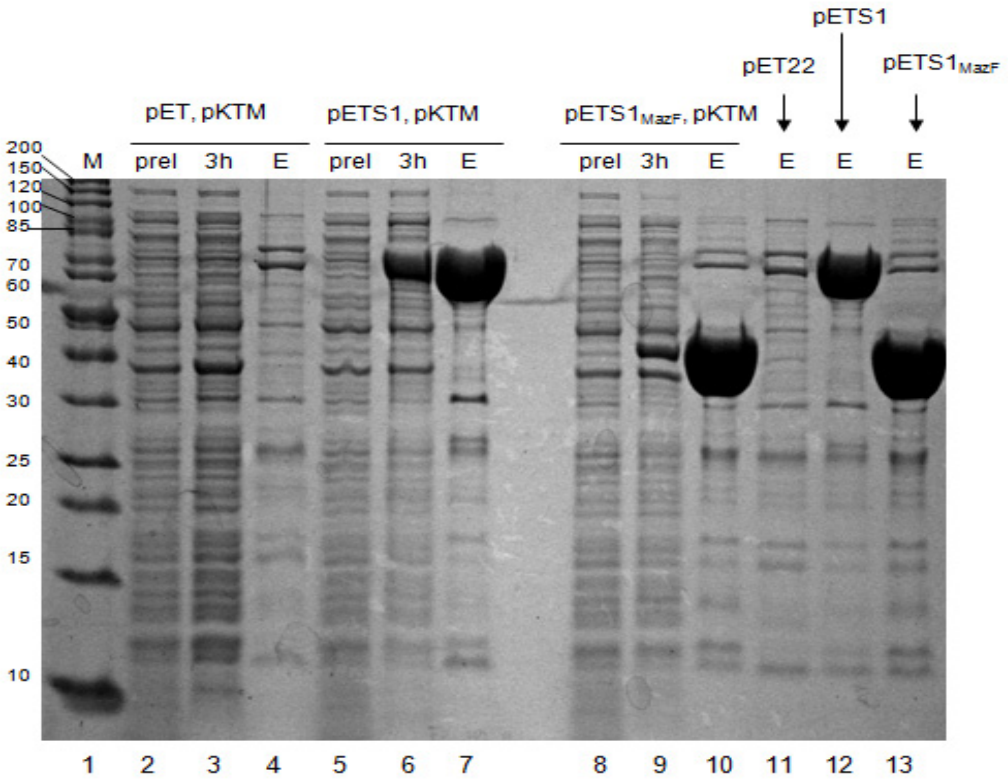


Figure 11: Purification of proteins S1 and $S1_{MazF}$ with Ni-NTA-agarose. Lane 1: protein size marker; Lanes 2-4: *E. coli* strain Tuner pKTplaccI harboring plasmid pET22b before induction (lane 2), 3h after induction (lane 3) and elution fraction (lane 4 and 11); Lanes 5-7: *E. coli* strain Tuner pKTplaccI harboring plasmid pET-S1 encoding for protein S1 before induction (lane 5), 3h after induction (lane 6) and fraction upon elution of the Ni-NTA-agarose with imidazol (lane 7 and 12); Lanes 8-10: *E. coli* strain Tuner pKTplaccI harboring plasmid pET-$S1_{MazF}$ before induction (lane 8) 3h after induction (lane 9) and elution fraction (lane 10 and 13); E: Elution fractions.

The results shown in Figure 10 surprisingly revealed that after induction of $S1_{MazF}$ synthesis in *E. coli* strain Tuner pKTplaccI (lane 10), the amounts of protein S1 as well as of a protein of about 170 kDa in size are reduced. To verify that the protein of about 70 kDa is native protein S1, a Western blot analysis was performed with anti-$S1_{106}$ specific antibodies. As shown in Figure 12, indeed induction of $S1_{MazF}$ synthesis results in a reduced amount of native protein S1 (lane 5). Moreover, in the presence of plasmid pKTplaccI the signal for native protein S1 disappeared almost completely (lane 9). As the investigation of this surprising observation would exceed the frame of this thesis, these results were not further scrutinized.

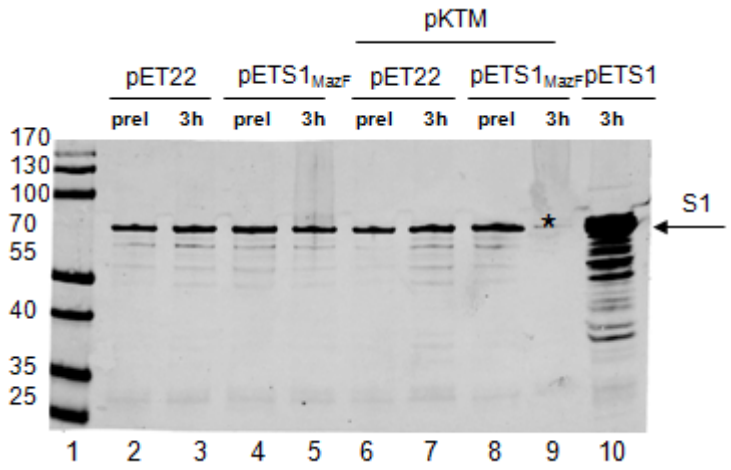
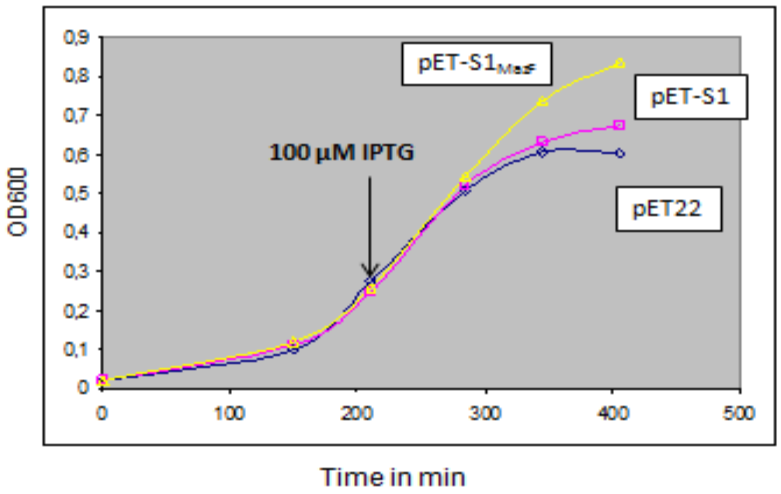


Figure 12: Western blot analysis to determine the amount of native protein S1 upon synthesis of $S1_{MazF}$ with anti-$S1_{106}$ specific antibodies. Lane 1: protein size marker; *E. coli* strain Tuner harboring plasmid pET22b before and 3h after induction respectively(lanes 2 and 3); *E. coli* strain Tuner harboring plasmid pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$ before (lane 4) and 3h after induction (lanes 5); *E. coli* strain Tuner pKTplaccI harboring plasmid pET22b before (lane 6) and 3h after induction (lane 7); *E. coli* strain Tuner pKTplaccI harboring plasmid pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$ before (lane 8) and 3h after induction (lane 9); *E. coli* strain Tuner harboring plasmid pET-S1 encoding for protein S1 3h after induction(lane 10). The black asterisk (lane 9) indicates the reduction of protein S1 upon $S1_{MazF}$ overexpression in the presence of plasmid pKTplaccI coding for the leaderless mRNA. As the antibody used is specific for the N-terminal domain of protein S1, overexpression of $S1_{MazF}$ cannot be determined here.

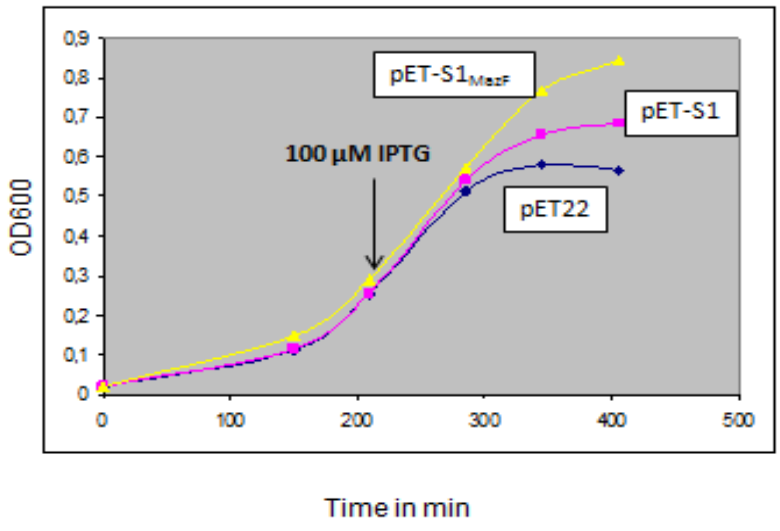
5.2 Effects of S1 and $S1_{MazF}$ synthesis on bacterial growth in minimal medium

As mentioned before, we hypothesized that $S1_{MazF}$ might play a significant physiological function under stress conditions. Thus, I studied growth of *E. coli* harboring plasmid $pETS1_{MazF}$ in M9 minimal medium on 37°C, as these conditions were shown to mimic stress (Vesper et al. 2011). *E. coli* strain Tuner und *E. coli* strain Tuner harboring plasmid pKTplaccI were transformed with plasmids pETS1 and pETS1MazF respectively, containing the genes *rpsA* and *rpsA**. Cells were grown in M9 minimal medium in the presence of 100 µg/ml ampicillin. Growth was monitored by measuring the optical density at 600nm (OD_{600}). At OD_{600} of 0.2-0.3 the

synthesis of protein S1 and $S1_{MazF}$ was induced by addition of 100µM IPTG (Isopropyl-b-D-thiogalactopyranosid) (Figure 13).



A.



B.

Figure 13: Induction of synthesis of proteins S1 and $S1_{MazF}$ in M9 minimal medium on 37°C. Growth curves of the *E. coli* strain Tuner **(A)** and *E. coli* strain Tuner containing plasmid pKTplaccI **(B)** transformed with plasmid pET-S1 encoding for protein S1(shown in magenta) and pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$(shown in yellow) or harboring pET22 as vector control(shown in blue). Cells were grown in M9 minimal medium containing 100mg/ml ampicilin. At OD_{600} of 0.2-0.3 100 µM IPTG was added to the cultures indicated by a black arrow, pET22: vector control.

As shown in Figure 13, growth of all strains was comparable before induction of proteins S1 and $S1_{MazF}$ synthesis. After addition of IPTG, growth of cells containing plasmid pETS1 which encodes for protein S1 and cells which contain plasmid pET22b respectively ceases, whereas growth of cells containing plasmid pETS1_{MazF} which encodes for protein $S1_{MazF}$ continues. Taken together, these results indicate

that the presence of protein $S1_{MazF}$ might be beneficial for growth under adverse conditions.

5.3 Effects of S1 and $S1_{MazF}$ synthesis on cell growth using *E. coli* NovaBlue strain

The results of the studies performed employing strain Tuner strongly indicate that synthesis of $S1_{MazF}$ might have a beneficial effect on cell growth during stress. However, induction of a variety of toxin-antitoxin systems by stressful conditions was shown to require the activity of the ATP-depended Lon protease (Williams and Hergenrother, 2012). As the *E. coli* strain Tuner, which is specifically designed for overexpression and protein purification, is deficient for the *lon* gene, I repeated the experiment employing the *E. coli* K12 strain NovaBlue. Like *E. coli* Tuner, the strain NovaBlue harbors the gene encoding the T7 RNA-Polymerase, thus the following experiment was performed employing the same set of plasmid as before. The *E. coli* K12 strain NovaBlue was transformed with plasmids pET-S1 and pET-$S1_{MazF}$ encoding for protein S1 and for protein $S1_{MazF,}$ respectively. Cells were grown in LB broth in the presence of 100 µg/ml ampicillin, 6µg/ml tetracycline and 20% glucose. Growth was monitored by measuring the optical density at 600nm (OD_{600}). At OD_{600} of 0.3-0.4 the synthesis of both proteins was induced by addition of 100µM IPTG (Figures 14 and 15). Samples for protein analysis were taken before and after induction (Figure 16).

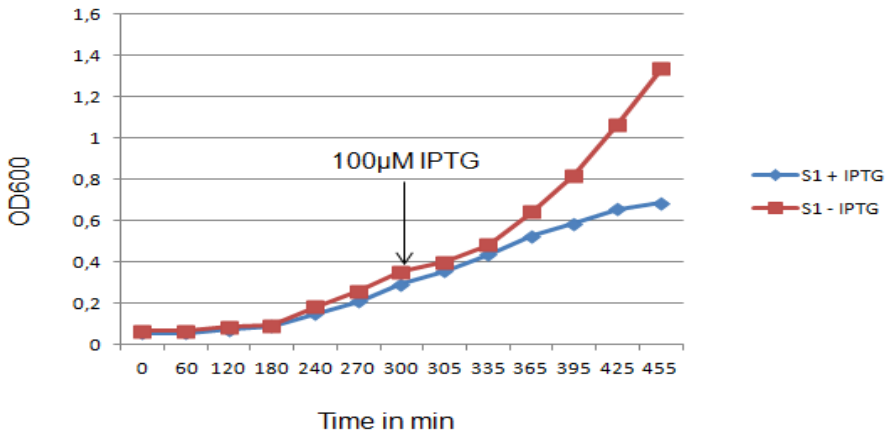


Figure 14: Effects of induced synthesis of S1 on cell growth. *E. coli* NovaBlue strain harboring plasmid pET-S1 was grown in LB medium containing 100mg/ml ampicilin, 6µg/ml tetracycline and 20% glucose. At OD_{600} of 0.3-0.4 100 µM IPTG was added indicated by a black arrow. Cultures grew similar before IPTG induction. 30' after induction, cells overexpressing S1 stopped growth, as overproduction of the N-terminal fragment of protein S1 inhibits general protein synthesis and slows growth.

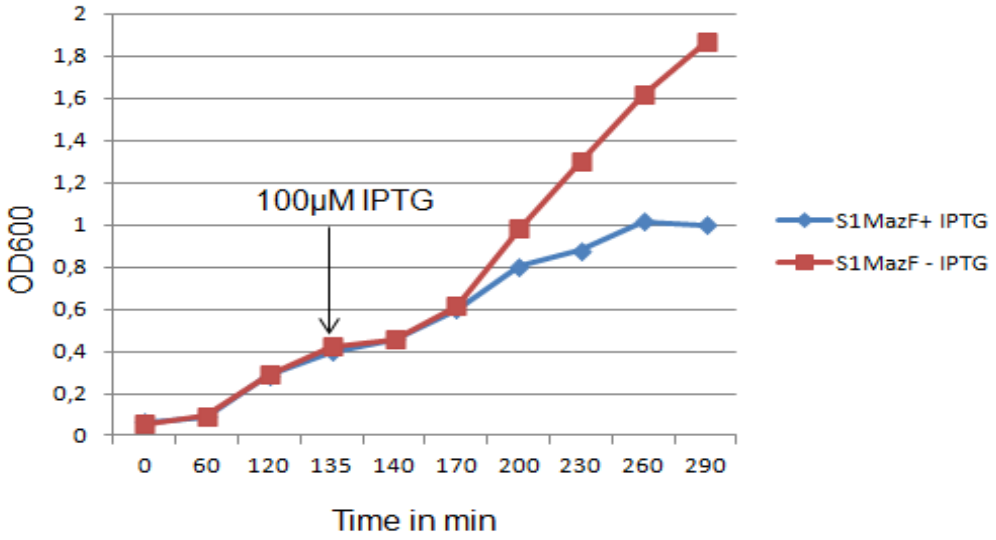


Figure 15: Effects of induced synthesis of $S1_{MazF}$ on cell growth. *E. coli* NovaBlue strain harboring plasmid pET-$S1_{MazF}$ was grown in LB medium containing 100mg/ml ampicilin, 6µg/ml tetracycline and 20% glucose. At OD_{600} of 0.3-0.4 100 µM IPTG was added indicated by a black arrow. Cultures grow similar before IPTG induction. After IPTG induction growth ceases.However when compared to the overexpression of the wild type *rpsA* gene, the growth rate is still higher (Figure 13).

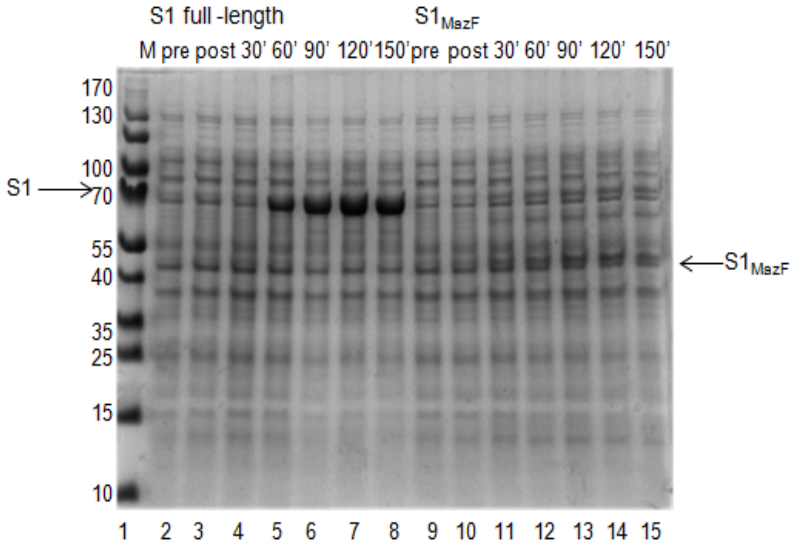


Figure 16: Validation of synthesis of proteins S1 and $S1_{MazF}$ after addition of IPTG (estimated position indicated by black arrows). Protein samples were loaded on a 12.5% SDS-polyacrylamide gel and stained with Coomassie blue staining. Lane 1: protein size marker; Lanes 2-8: *E. coli* strain Novablue harboring plasmid pET-S1 encoding for protein S1 before induction (lane 2) and after induction (lanes 3, 4, 5, 6, 7 and 8 respectively) at the time points indicated above; Lanes 9-15: *E. coli* strain Novablue harboring plasmid pET-$S1_{MazF}$ encoding for protein $S1_{MazF}$ before induction (lane 9) and after induction (lanes 10, 11, 12, 13, 14 and 15 respectively) at the time points indicated above.

5.4 Is $S1_{MazF}$ selectively synthesized after *mazF* overexpression?

As mentioned in the introduction, the endoribonuclease MazF cleaves the *rpsA*` mRNA sequence on its internal ACAUG site thus makes it leaderless, which could be selectively translated by stressed-*mazF* generated ribosomes (Vesper et al. 2011). Therefore, the next aim was to determine whether the product of this truncated *rpsA* mRNA, protein $S1_{MazF}$ 36.8kDa is selectively synthesized, after *mazF* overexpression. To unambiguously validate, whether the truncated protein $S1_{MazF}$ is generated under stress conditions, we chose plasmid pJS200, a pACYC184 derivative, that contains the transcription unit of *rpsA,* which is able to complement an *rpsA* amber mutation in *E. coli* strain MB3001 (Schnier et al. 1986). The strain MB3001 contains an S1 amber mutation which is suppressed by a temperature-sensitive suppressor and thus it is unable to grow above 28°C (see materials and methods) (Schnier et al. 1986).

To identify the S1 protein that originates from the *rpsA* gene encoded by the plasmid, the coding sequence of protein S1 was amplified by PCR with primers I10 and J10 (see materials and methods) and introduced between the BamHI sites of vector pJS200 resulting in plasmid pJSF7 encoding a C-terminally Flag-tagged S1 protein. *E. coli* strain MB3001, was transformed with plasmid pJS200-S1Flag. To check the viability of the cells, plates were incubated on 28°C (permissive conditions), 37°C and 42°C (non-permissive conditions) overnight. The cells survived at all three temperatures, because the vector pJSF7 was able to complement the S1 amber mutation and permitted growth of MB3001 at the non-permissive temperatures(37°C) at which the mutant is unable to grow (Schnier et al. 1986). To determine the expression of the *rpsA* gene containing the 3' terminal Flag-tag sequence, protein samples were taken and separated on a 12.5% SDS polyacrylamid gel. Cells were grown on 37°C in LB medium containing 30µg/ml chloramphenicol. Western blot analysis employing Flag-tag specific primary antibodies was performed (Figure 17) (see materials and methods).

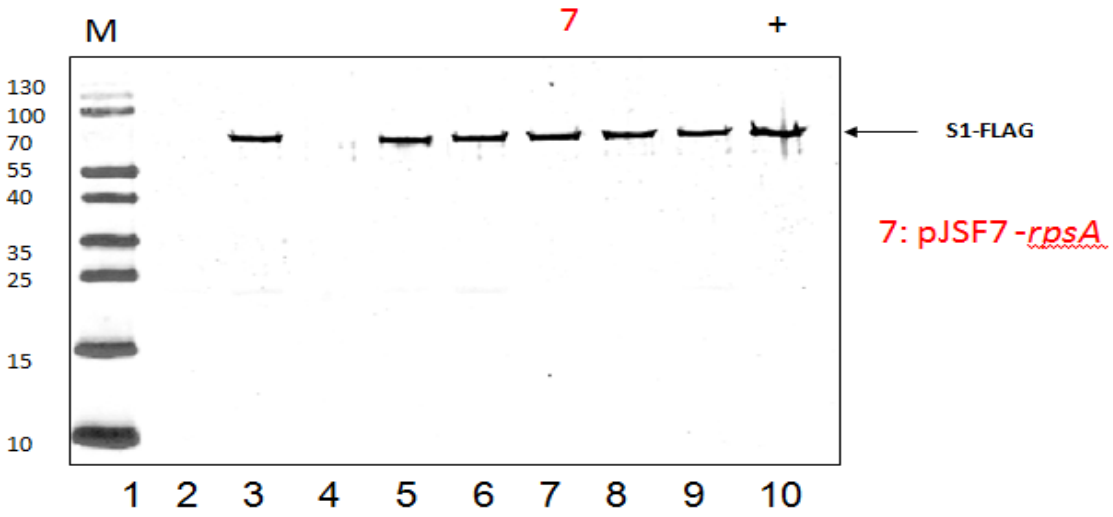


Figure 17: Western blot analysis employing anti-Flag antibodies reveals the presence of the Flag-tag at the C-terminus of S1 encoded by plasmid pJSF7 transformed in *E. coli* MB3001. Lane 1: protein size marker; Lanes: 2-9 *E. coli* strain MB3001 harboring plasmid pJSF7-*rpsA*Flag-tag which encodes for protein S1 (lanes 3, 5, 6, 7, 8 and 9 respectively, single colonies that contain the Flag-tag sequence; lanes 2 and 4, single colonies without a Flag-tag sequence); Lane 10: protein S1-Flag-tag positive control.

Next, *E. coli* strain MB3001 harboring plasmid pJSF7 encoding the *rpsA* gene was grown in LB broth at 37°C in the presence of 30μg/ml chloramphenicol (Figure 18). Growth was monitored by measuring the optical density at 600nm (OD_{600}) every hour. At OD_{600} of 0.3-0.4 cells were collected and competent cells with the CaCl method were prepared (see materials and methods). As shown in Figure 18 the strain has a long lag phase.

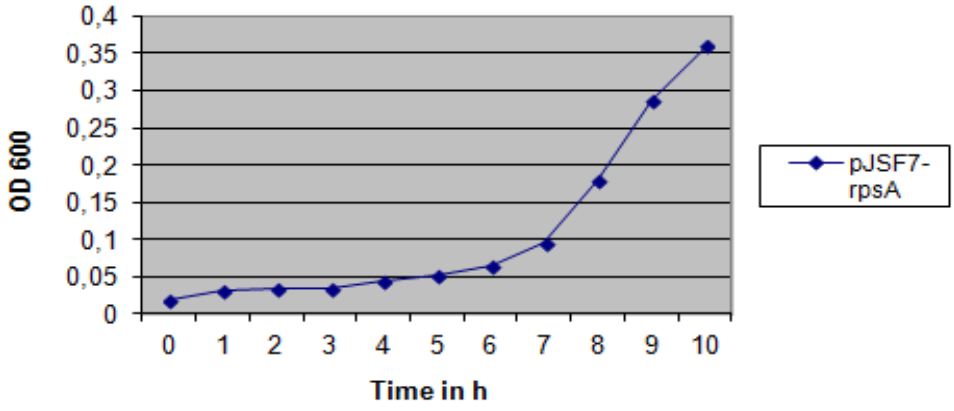


Figure 18: Growth of *E. coli* strain MB3001 on 37°C in LB Media and 30μg/ml chloramphenicol, transformed with plasmid pJSF7, which encodes for the *rpsA*-Flag gene.

Next *E. coli* strain MB3001-pJSF7*rpsA* was transformed with plasmid pSA1 which carries the IPTG-inducible *mazF* gene. The transformants were grown in LB broth on 37°C in the presence of 100mg/µl ampicillin, 30µg/ml chloramphenicol and 40% glucose. Growth was monitored by measuring the optical density at 600nm (OD_{600}). At OD_{600} of 0.3-0.4, 100µM IPTG was added to one culture to induce *mazF* overexpression (Figure 19). Protein samples were taken for Western blot analysis before and after induction. Western blot analysis with Flag-tag specific antibodies were performed (Figure 20).

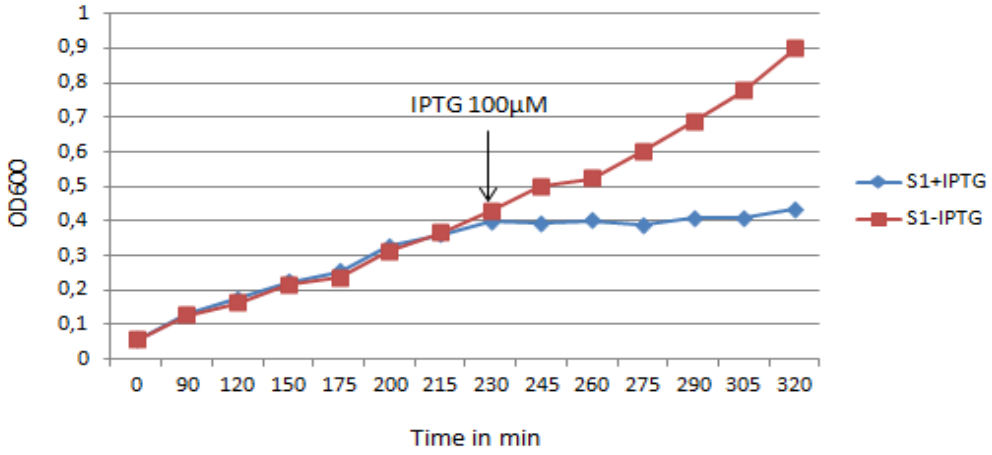


Figure 19: Growth of *E. coli* strain MB3001-pJSF7-*rpsA* transformed with plasmid pSA1 in rich media with 100mg/µl ampicillin and 30µg/ml chloramphenicol on 37°C, in the absence (red line) and upon induction (blue line) of IPTG (indicated by a black arrow). The growth of cells ceases upon *mazF* overexpression.

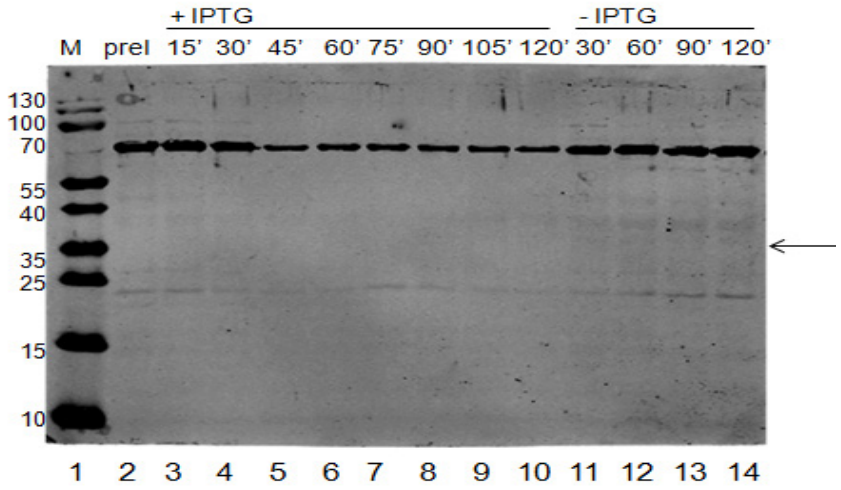


Figure 20: Western blot analysis with anti-Flag antibodies does not reveal expression of $S1_{MazF}$. Expression levels of protein $S1_{MazF}$ are probably too low (estimated position of $S1_{MazF}$ is indicated by a black arrow). Lane 1: protein size marker; *E. coli* strain MB3001-pJSF7-*rpsA* harboring plasmid pSA1, before IPTG induction (lane 2), after IPTG induction (lanes 3-10), and without IPTG induction (lanes 11-14) at the time points indicated above.

As expected, *mazF* overexpression reduced the growth rate of the cells containing the pJSF7 plasmid encoding for ribosomal protein S1. To determine the selective synthesis of the short variant of S1 containing a C-terminal Flag-tag, samples were withdrawn before and several time points after induction of *mazF* expression as indicated in Figure 19. However, the protein $S1_{MazF}$ with an estimated size of 36,8 kDa was not detected in the Western blot analysis. Since the lack of the corresponding signal could be attributed to the low amount of the protein synthesized, the double amount of the protein samples were employed in Western Blot analysis to test whether $S1_{MazF}$ would be selectively produced (Figure 21). Western blot analysis with Flag-tag specific antibodies was performed. However, as before the expression of the truncated protein $S1_{MazF}$ is not detectable by Western blot analysis, what might be attributed to the small amounts of protein synthesized from the leaderless *rpsA** mRNA.

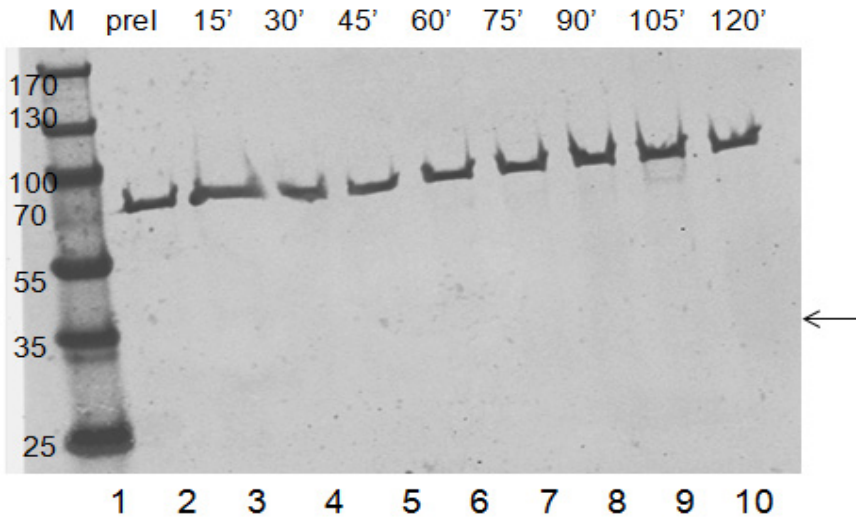


Figure 21: Western blot analysis employing anti-Flag antibodies with the double amount of protein loaded reveals no expression of protein $S1_{MazF.}$ Lane 1: protein size marker; *E. coli* strain MB3001-pJSF7-*rpsA* harboring plasmid pSA1 before IPTG induction (lane 2) and after IPTG induction (lanes 3-10) at time points indicated above. Estimated position of protein $S1_{MazF}$ is indicated by a black arrow.

5.5 Impact of $S1_{MazF}$ on bacterial growth under stress conditions

As the translation of the truncated *rpsA** mRNA was not detectable at the protein level we next aimed to determine the effect of the selective synthesis of the protein $S1_{MazF}$ on cell growth in order to validate the presence $S1_{MazF}$ under stress conditions. To this end, two mutants of the *rpsA* gene were designed, where the internal GAC-ATG (aspartate-methionine) codons of the *rpsA* gene were first changed to GAC-ATC (aspartate - isoleucine) to prevent translation initiation of the leaderless mRNA which is generated by MazF cleavage and second into GAG-ATG (glutamate- methionine) to prevent cleavage by MazF as the ACA-site was removed.

As the amino acids isoleucine and methionine are both hydrophobic and prefer not to be buried in protein hydrophobic cores they are expected to substitute for each other without affecting the overall structure of the protein and are thus anticipated not to interfere with protein S1 function. Aspartate and glutamate are both negatively charged and polar residues and are generally positioned at the surface of proteins facing an aqueous environment.

Taken together, the mutant *rpsA* genes were anticipated to give insights whether the lack of selective translation of $S1_{MazF}$ either introduced by the removal of the 5'-terminal start codon, generated in the wild type *rpsA* gene by MazF (mutant 1) or by removing the ACA-cleavage site for MazF (mutant 2) does affect growth behavior of *E. coli* upon *mazF* induction or under different stress conditions.

As a positive control to determine the effect of artificial expression of the truncated *rpsA** the following construct was designed: The coding sequence encoding protein $S1_{MazF}$ was PCR amplified with primers U11 and T11 (see materials and methods) and cloned between the BamHI and NcoI sites of vector pRB381cI resulting in a gene encoding protein $S1_{MazF}$ harboring a C-terminal Flag-tag. This plasmid is termed pRBS1_{MazF}Flag. To verify the successful cloning and to test the *rpsAmazF*-Flag gene expression, *E. coli* TOP10 strain was transformed with plasmid pRBS1_{MazF}Flag harboring the artificial $S1_{MazF}$ leaderless variant and cells were grown in LB medium containing 100mg/ml ampicilin. As this *E. coli* strain does not harbor the *lacI* repressor gene under these conditions *rpsAmazF*-Flag is constitutively expressed. Samples were withdrawn and separated on a 12 % SDS-page. The presence of the Flag-tag was checked by Western blot analysis using Flag-tag specific primary antibodies (Figure 22).

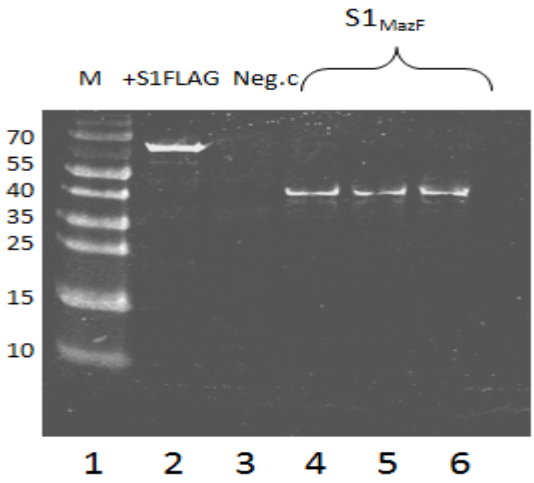


Figure 22: Western Blot analysis employing anti-Flag antibodies reveals the presence of the C-terminal Flag-tag on protein $S1_{MazF}$ encoded by the leaderless mRNA variant. Lane 1: protein size marker; Lane 2: S1-Flag-tag positive control; Lane 3: negative control; Lanes 4, 5, 6: $S1_{MazF}$-Flag-tag protein.

5.6 On 28°C pJSF7-*rpsA* and the two mutants can complement the S1 amber thermo-sensitive mutation in MB3001

The two different mutations were introduced in the *rpsA* gene within vector pJSF7 employing primers M10, N10 and O10 by inverted PCR (see materials and methods). The respective *rpsA* mutant genes harbor the Flag-tag sequence at the 3'-end and the sequencing analysis revealed that all mutant clones were positive. From here on, the plasmid containing the ACAUC mutant gene lacking the AUG codon was termed pOM3 and the plasmid harboring the AGAUG mutant gene was termed pON5. The numbers correspond to the positive clone numbers and these two clones were used for all further studies.

E. coli strain MB3001 was transformed on 28°C either with plasmid pJSF7 containing the WT *rpsA* gene encoding for protein S1, with plasmid pOM3 (ACAUC) or with plasmid pOM5 (AGAUG) encoding for the mutant *rpsA* genes respectively. Cells were grown in LB broth on 28°C in the presence of 30mg/µl chloramphenicol (Figure 23). Samples for Western blot analysis were collected every hour.

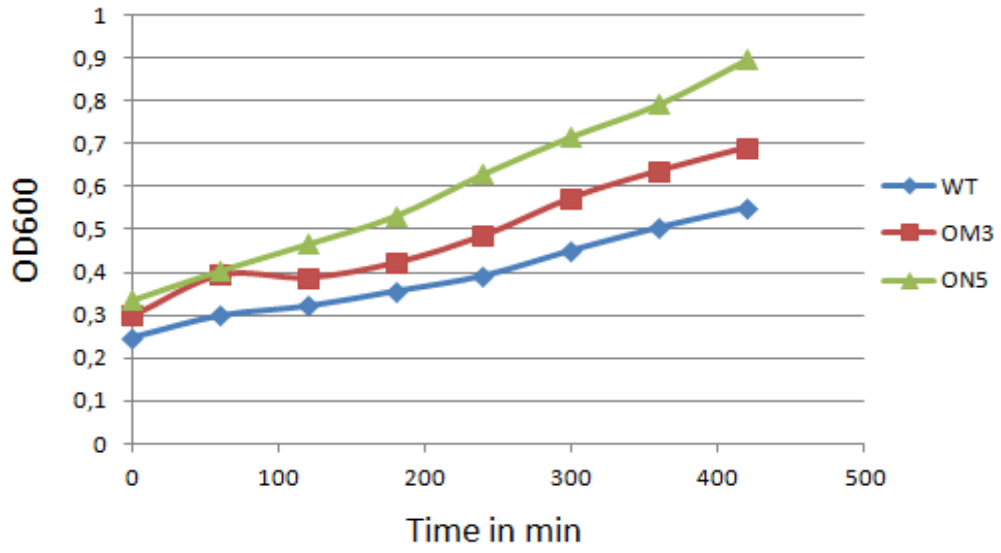


Figure 23: Growth of *E. coli* strain MB3001 in rich media and 30µg/ml chloramphenicol harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1(WT), with plasmid pOM3 which encodes for the ACAUC mutant *rpsA* gene and with plasmid pON5 which encodes for the AGAUG mutant *rpsA* gene respectively. On 28°C the growth of all three cultures is similar because of the expression of the chromosomal *rpsA* gene.

Next, these cells were grown in LB broth on 37°C in the presence of 30mg/µl chloramphenicol (Figure 24) to determine the growth of the cells harboring plasmids pOM3(ACAUC) and pON5(AGAUG) respectively under non-permissive conditions. Samples for protein analysis were taken every hour.

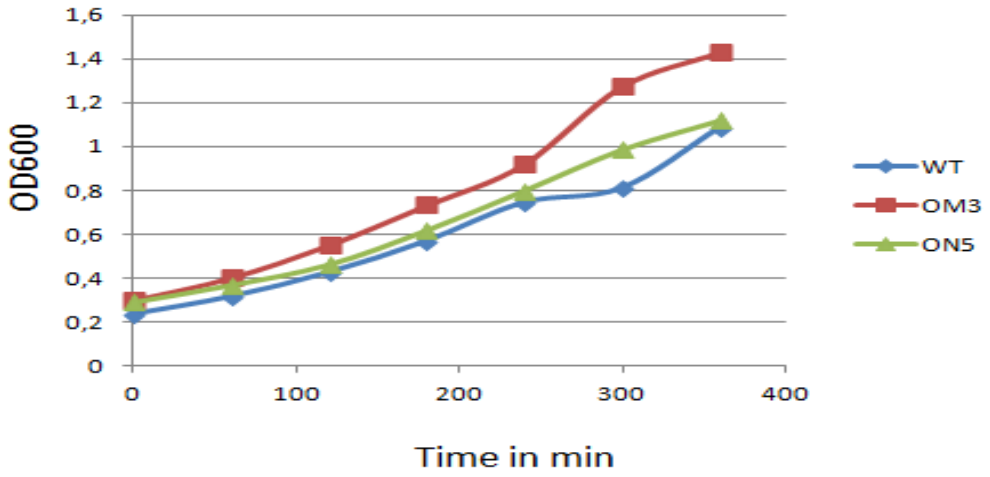


Figure 24: Growth of *E. coli* strain MB3001 (1st day) on 37°C in rich media and 30µg/ml chloramphenicol, harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3 which encodes for the ACAUC mutant *rpsA* gene and with plasmid pON5 which encodes for the AGAUG mutant *rpsA* gene respectively. The first day on 37°C, the wild type *rpsA* and the two mutants grow very similar, because of the expression of the chromosomal *rpsA* gene.

Although shifted to non-permissive temperature no difference in growth was observed between the strains harboring the wild type or the mutant *rpsA* genes. This unexpected phenomenon could be attributed to the fact that if generated the $S1_{MazF}$ protein would be important during the lag phase, when cells are recovering from the stationary phase. Upon shifting the cells from the overnight incubation at 28°C to the new media in 37°C one could assume that the wild type *rpsA* mRNA is still present and could thus be cleaved by MazF and would still contribute to the generation of the $S1_{MazF}$ protein. Thus, the growth experiment was expanded and the cultures were kept at 37°C for overnight (Figure 25). The next day, the cultures were diluted and regrown under the same conditions at 37°C. Surprisingly, the two cultures harboring the mutant *rpsA* genes, where generation of $S1_{MazF}$ is prevented either by inhibition of translation due to the lack of the AUG codon or by interfering with MazF cleavage, are severely impaired in growth recovery at 37°C. Taken together, these results strongly corroborate the notion that the selective translation of $S1_{MazF}$ from the truncated *rpsA* mRNA has indeed an effect on recovery from stress conditions.

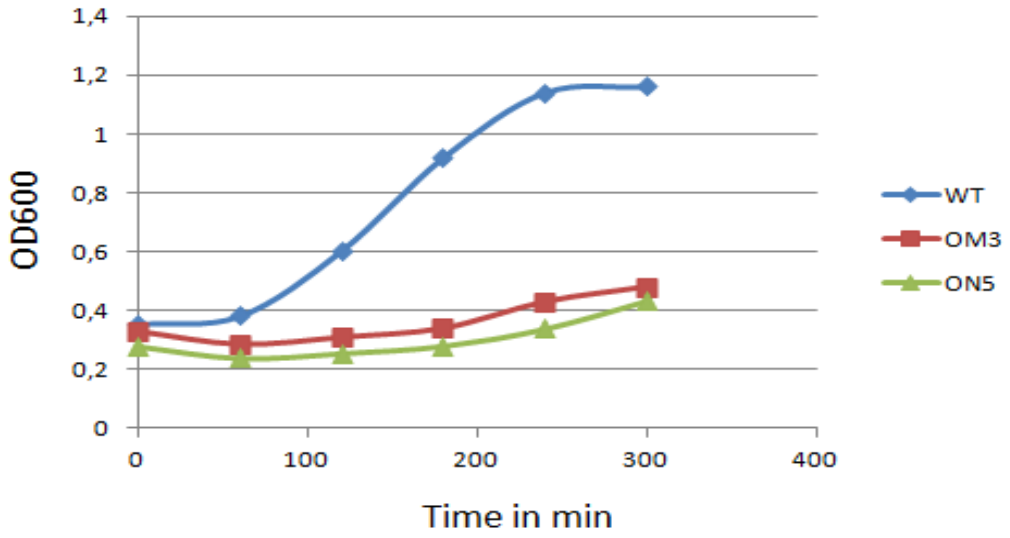


Figure 25: Growth of *E. coli* strain MB3001 (2nd day) on 37°C in rich media and 30µg/ml chloramphenicol, harboring plasmid which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3 which encodes for the ACAUC mutant *rpsA* gene and with plasmid pON5 which encodes for the AGAUG mutant *rpsA* gene respectively. Growth of the wild type differentiates from the strains harboring plasmids pOM3 and pON5.

To determine the formation of $S1_{MazF}$ during growth analysis shown above (Figures 23, 24, and 25), protein samples for Western blot analysis were taken.

Expression of both mutants and wild type S1 was observed on 28°C and 37°C. Protein samples were separated on 12.5% SDS polyacrylamid gels (see materials and methods), blotted and probed with anti-Flag antibodies to determine the amount of S1-Flag expressed from the plasmid encoded gene. However, as already observed before, the protein analysis did not reveal expression of protein $S1_{MazF}$ at 28°C and 37°C, respectively (Figures 26, 27, 28).

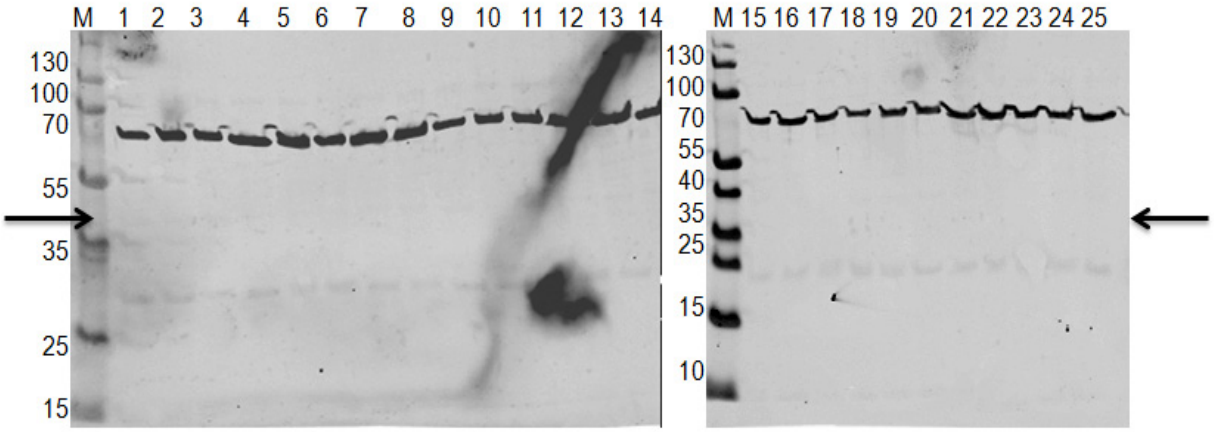


Figure 26: Protein $S1_{MazF}$ was not detectable by Western blot analysis of samples collected on 28°C and analyzed with anti-Flag antibodies (estimated position of the protein indicated by a black arrow). Cells were grown on 28°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lanes 1-9: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 10-16 samples from cells harboring plasmid pOM3 (ACAUC); Lanes 17-25: samples from cells harboring plasmid pON5 (AGAUG) at the time points indicated above.

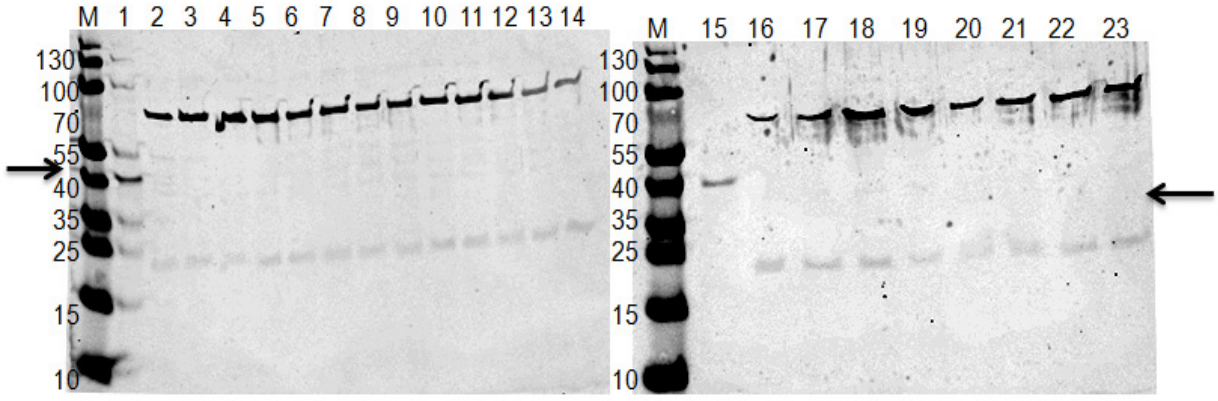


Figure 27: Protein $S1_{MazF}$ was not detectable by Western blot analysis of samples collected on 37°C (1st day) and analyzed with anti-Flag antibodies (position of the protein indicated by a black arrow). Cells were grown on 37°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lane 1: protein $S1_{MazF}$ (positive control); Lanes 2-8: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 9-14: samples from cells harboring plasmid pOM3 (ACAUC); Lanes 15-23: samples from cells harboring plasmid pON5 (AGAUG) at the time points indicated above.

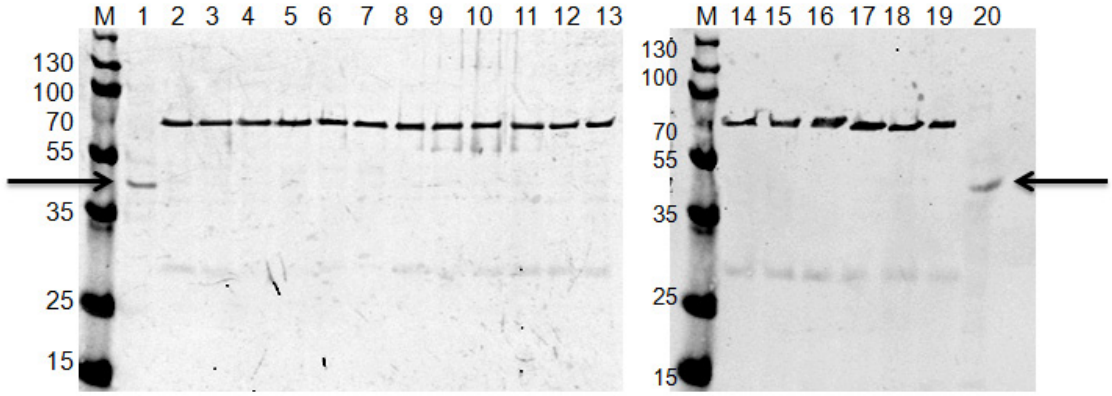


Figure 28: Protein $S1_{MazF}$ was not detectable Western blot analysis of samples collected on 37°C (2nd day) and analyzed with anti-Flag antibodies (position of the protein indicated by a black arrow). Cells were grown on 37°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lane 1: protein $S1_{MazF}$ (positive control); Lanes 2-7: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 8-13: samples from cells harboring plasmid pOM3 (ACAUC); Lanes 14-19: samples from cells harboring plasmid pON5 (AGAUG) and lane 20 protein $S1_{MazF}$ (positive control) at the time points indicated above.

As the lack of the signal for the $S1_{MazF}$-Flag protein determined by the anti-Flag antibody might be attributed potentially to the anti-Flag antibody, the Western blot analysis was repeated with anti-S1 antibody (Figures 29, 30, 31). However, no signal corresponding to the $S1_{MazF}$ protein was detected.

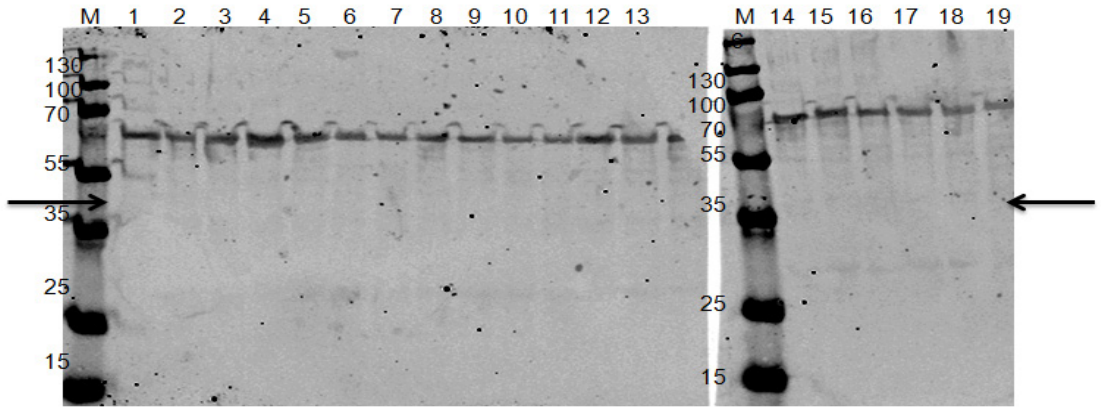


Figure 29: Western blot analysis of samples collected on 28°C and analyzed with anti-S1 antibodies (tentative position of the protein $S1_{MazF}$ indicated by a black arrow). Cells were grown on 28°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lanes 1-7: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 8-13: samples from cells harboring plasmid pOM3 (ACAUC); Lanes 14-19: samples from cells harboring plasmid pON5 (AGAUG) at the time points indicated above.

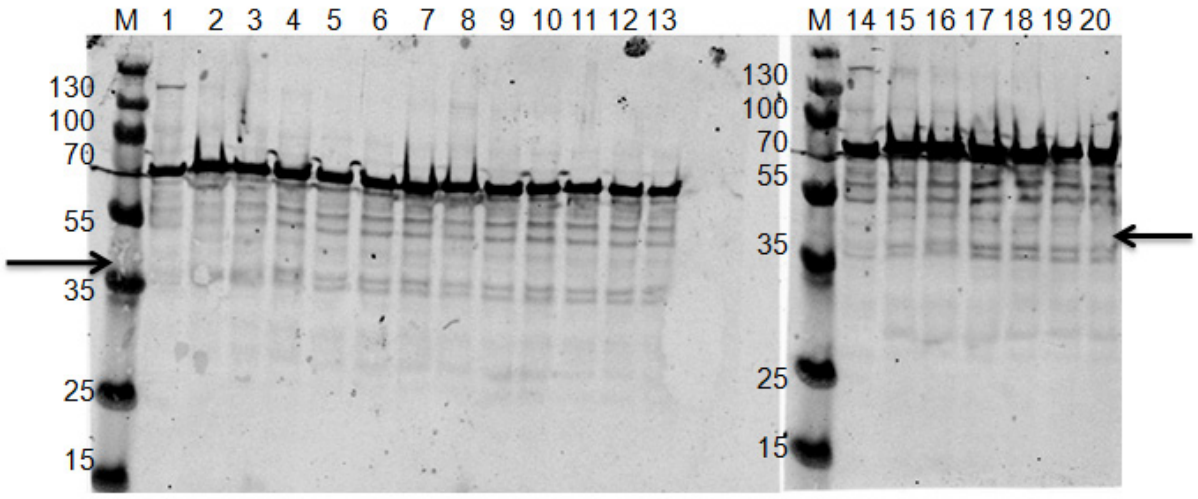


Figure 30: Western blot analysis of samples collected on 37°C (1st day) and analyzed with anti-S1 antibodies (tentative position of the $S1_{MazF}$ protein indicated by a black arrow). Cells were grown on 37°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lanes 1-7: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 8-13: samples from cells harboring plasmid pOM3 (ACAUC); Lanes 14-20: samples from cells harboring plasmid pON5 (AGAUG) at the time points indicated above.

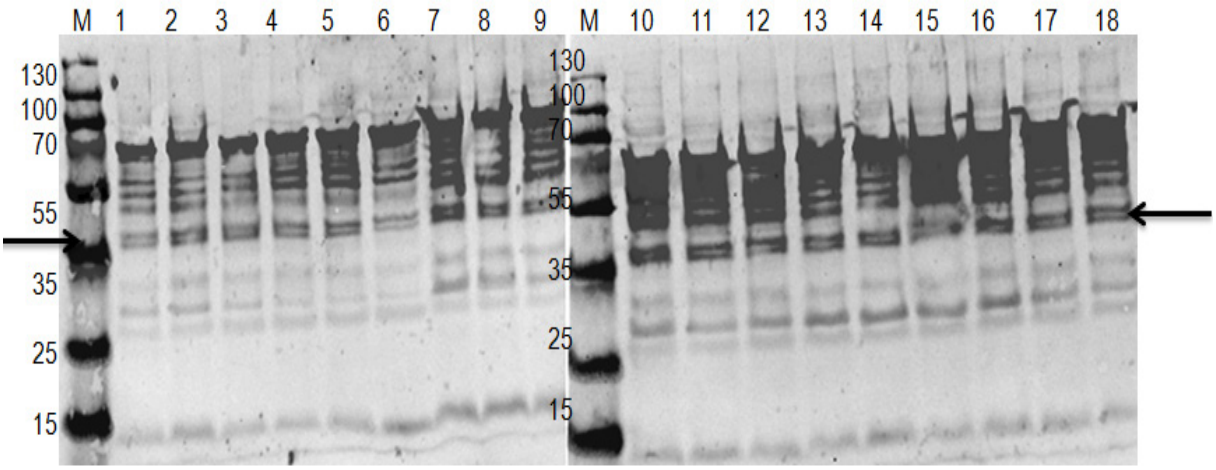


Figure 31: Western blot analysis of samples collected on 37°C (2nd day) and analyzed with anti-S1 antibodies (tentative position of the $S1_{MazF}$ protein indicated by a black arrow). Cells were grown on 37°C in LB broth and 30mg/ml chloramphenicol. M: protein size marker; Lanes 1-6: samples from cells harboring plasmid pJSF7-*rpsA*-WT; Lanes 7-12: samples from cells harboring plasmid pOM3 (ACAUC); Lanes 13-18: samples from cells harboring plasmid pON5 (AGAUG) at the time points indicated above.

5.7 The lack of $S1_{MazF}$ synthesis affects cell growth upon *mazF* overexpression

In the last experiment of this study, I addressed the question whether overexpression of *rpsA** affects cell growth. Thus, *E. coli* strain MB3001 harboring either plasmid pJSF7 encoding for the *rpsA* gene, or the plasmids pOM3(ACAUC) and pON5(AGAUG) encoding for the mutant *rpsA* genes respectively was grown on 28°C in LB broth in the presence of 30μg/ml chloramphenicol. Growth was monitored by measuring the optical density at 600nm (OD_{600}) every hour. At OD_{600} of 0.3-0.4 cells were collected, competent cells with the CaCl method were prepared (see materials and methods) and transformed with the plasmid $pRBS1_{MazF}$ harboring the *rpsA** gene coding for the $S1_{MazF}$ protein. These cells were grown in LB broth on 28°C in the presence of 30mg/μl chloramphenicol and 100mg/μl ampicillin. In parallel, cells that contain no $pRBS1_{MazF}$ plasmid were also grown in LB broth on 28°C in the presence of 30mg/μl chloramphenicol in 96 well plates; one plate was placed for shaking on 28°C and the other plate on 37°C. Growth was monitored by measuring the optical density at 595nm every hour with an I-Microplatte Absorbance Reader (595nm). Cell cultures were incubated overnight on 28°C and 37°C. The second day, cells were grown in fresh LB media plus 30mg/ml chloramphenicol and 100mg/μl ampicillin or only 30mg/ml chloramphenicol for cells which contain no $pRBS1_{MazF}$ plasmid (Figures 32, 33, 34, 35).

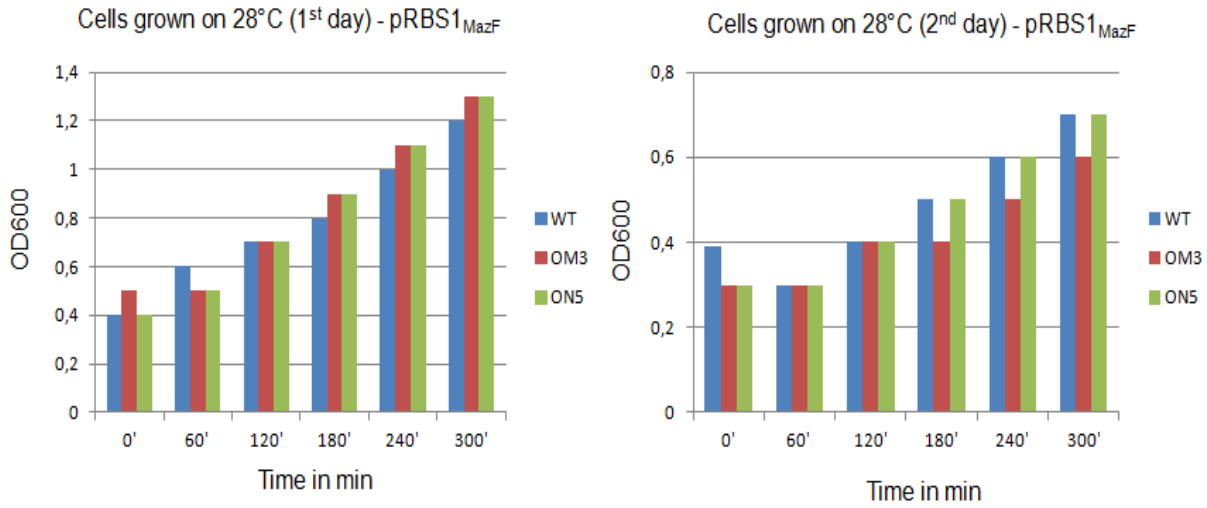


Figure 32: Growth rates of the *E. coli* MB3001 strain (- $pRBS1_{MazF}$) in rich media and 30μg/ml chloramphenicol on 28°C harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3(ACAUC) and with plasmid pON5 (AGAUG) encoding for the mutant *rpsA* genes.

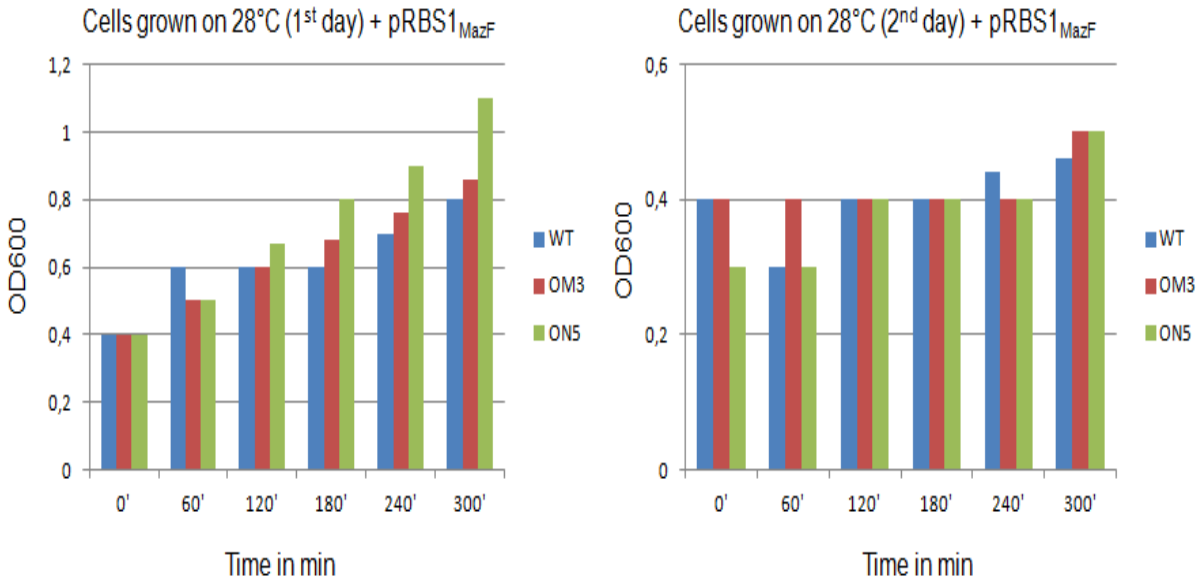


Figure 33: Growth rates of the *E. coli* MB3001 strain(+ pRBS1$_{MazF}$) in rich media and 30µg/ml chloramphenicol on 28°C, harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3(ACAUC) and with plasmid pON5(AGAUG) encoding for the mutant *rpsA* genes.

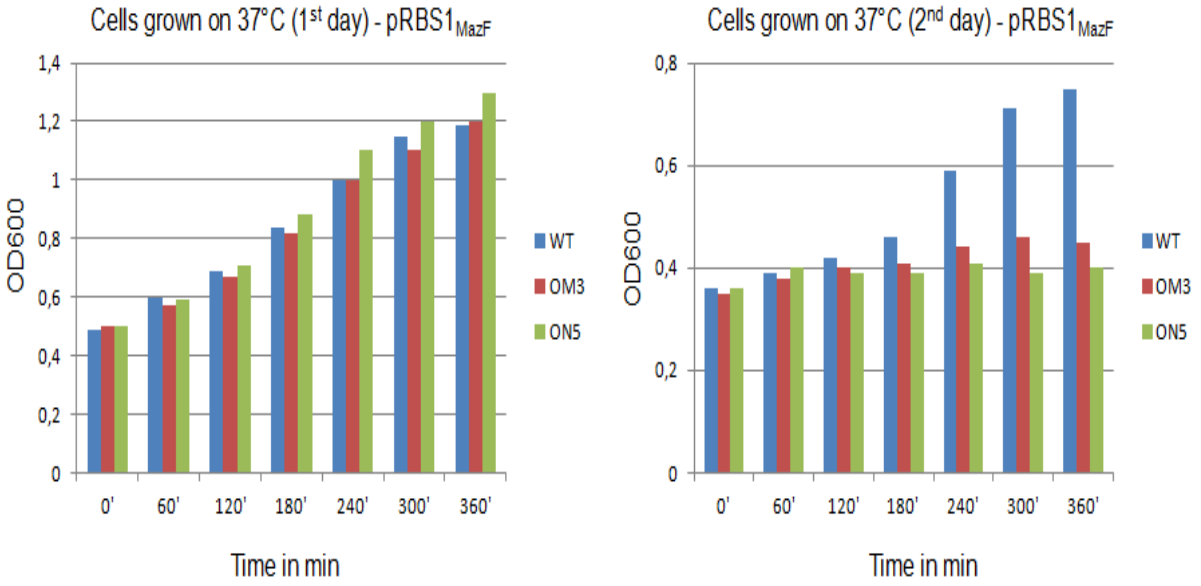


Figure 34: Growth rates of the *E. coli* MB3001(- pRBS1$_{MazF}$), strain in rich media and 30µg/ml chloramphenicol on 37°C, harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3(ACAUC) and with plasmid pON5(AGAUG) encoding for the mutant *rpsA* genes.

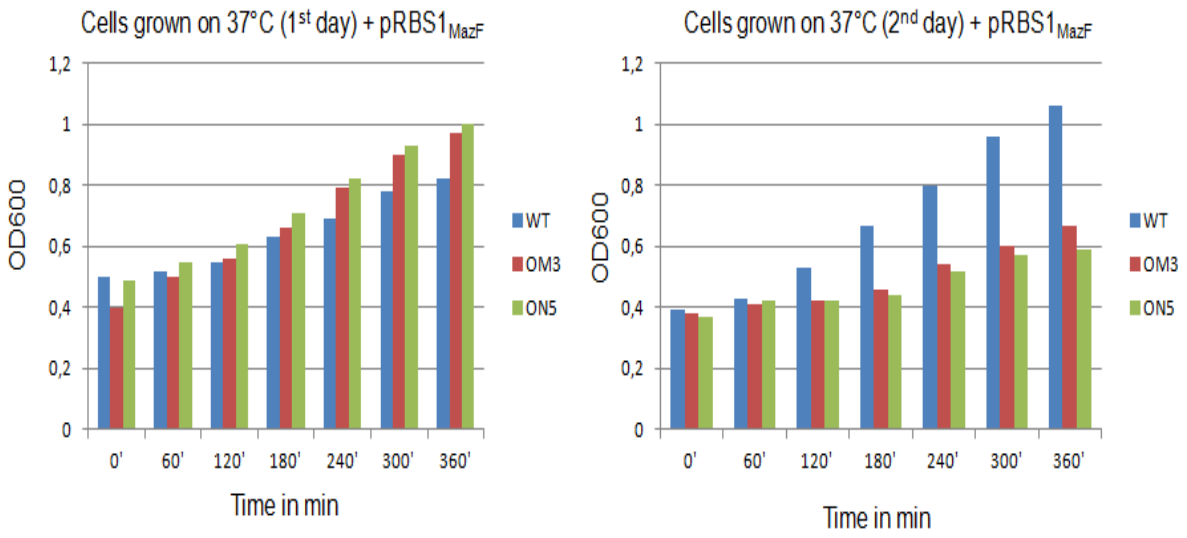


Figure 35: Growth rates of the *E. coli* MB3001 strain in rich media and 30µg/ml chloramphenicol on 37°C (+ pRBS1$_{MazF}$), harboring plasmid pJSF7 which encodes for the *rpsA* gene of protein S1 (WT), with plasmid pOM3(ACAUC) and with plasmid pON5(AGAUG) encoding for the mutant *rpsA* genes.

As expected, on 28°C the cells harboring plasmid pJSF7 which encodes protein S1 and the cells harboring the plasmids pOM3 (ACAUC) and pON5 (AGAUG) which harbor the mutant *rpsA* genes, show no growth difference on the 1st and the 2nd day on 28°C, because of the presence of the chromosomal *rpsA* gene under permissive conditions. The presence of the artificial pRBS1$_{MazF}$ plasmid harboring the S1$_{MazF}$ protein does not affect cell growth under these conditions (Figure 33).

When the same cultures were grown on 37°C, we observed the same phenomenon of cell growth at the first day on 37°C mirroring the observed growth shown in Figure 24. As the chromosomal *rpsA* mRNA is still present and would contribute to the generation of S1$_{MazF}$ protein, the wild type and cells harboring harboring the mutant *rpsA* variant genes grow very similar. Similar to growth on 28°C, the presence of the artificial S1$_{MazF}$ protein does not affect growth under these conditions. At the second day of growth at 37°C we observed that the wild type has clearly a higher growth rate than the cells containing plasmid pOM3 and pON5 because it still contains the WT *rpsA* gene on the pJSF7 vector. The growth rate of the mutants ceases after one day on 37°C. As they both contain mutations which prevent formation of S1$_{MazF}$ under stress conditions, again these results strongly point to the notion that the generation of S1$_{MazF}$ is physiologically relevant for growth

recovery. Moreover, concomitant expression of the plasmid born *rpsA** gene seems to stimulate growth of the cells harboring the plasmids encoding the mutant *rpsA** genes. Again these results are strongly in favor of the hypothesis that $S1_{MazF}$ plays an important role in recovery or growth under stress conditions.

6. Materials and Methods

6.1 Bacterial strains used in this study

TOP 10	*F–* mcr*A Δ(*mrr-hsd*RMS*-mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74* rec*A1* ara*D139 Δ(*ara leu*) 7697* gal*U* gal*K* rps*L (StrR)* end*A1* nup*G* (INIVITROGEN)
TUNER	*F– ompT hsdSB (rB– mB–) gal dcm lacY1*(DE3) (NOVAGEN)
NovaBlue	*endA1 hsdR17* (r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* F′[*proA*$^{+}$*B*$^{+}$ *lacI*^{q}*ZΔM15*::Tn*10*] (Tet^{R}) (NOVAGEN)
MB3001	*F-, leu*$_{am}$*, trp*$_{am}$*, lac2*$_{am}$*, galK*$_{am}$*, galE, tsx,relA, supD43,74, rpsL, sueA, sueB s ueC, rpsA*$_{am,}$*),* (Schnier et al,1985)

6.2 Plasmids used in this study

pET22b	Amp^R, T7 Promotor, C'Terminal His-Tag (Novagen)
pJS200 (pACYC184 derivative)	Cam^R, P1 and P2 constitutive Promotors, C'Terminal Flag-Tag (Schnier et al.1986)
pSA1	Amp^R T5 Promotor IPTG-inducible encodes for MazF (Amitai et al. 2009)
pRB381cI	Amp^R, lac Promotor, *cI-lacZ* C'Terminal Flag-Tag (Bruckner 1992)
pRBS1$_{MazF}$-Flag	Amp^R, lac Promotor, *cI-lacZ* C'Terminal Flag-Tag, artificial leaderless S1$_{MazF}$ variant (This study)
pKTplaccI	λ-cI, lac promoter, encoding the leaderless *cI-lacZ* fusion gene (Grill et al. 2002)

6.3 Primers used in this study

E8	fwd *rpsA* 746	GTCGAACTTCAGCAC
Z7	rev *rpsA* 100	CGTCTTTGTCGATAGCAAC
H7	fwd, *rpsA*	ggtggt*CATATG*ACTGAATCTTTTGC
H9	*rpsA* rev XhoI without STOP	TATACTCGAGGCCTTTAGCTGC
G9	*rpsA667* fwd NdeI	TATACATATGGCCTGGAAACGC
I10	fwd, Flag to *rpsA* on pJS200	[Phos]GACGATGAAAAATAATTCTCTGACTC
J10	rev, Flag to *rpsA* on pJS200	[Phos]ATCCTTATAGTCCTCGCCTTTAG
M10	fwd, 2nd *rpsA* ACAUG -> ACAUC	[Phos]GACATCGCCTGGAAACG
N10	fwd, 2nd *rpsA* ACAUG -> AGAUG	[Phos]GAGATGGCCTGGAAACG
O10	rev, 2nd *rpsA* ACAUG	[Phos]AGTGATGTGCAGCAG
T11	lmRNA *rpsA* mf-Flag into NcoI-BamHI of pRBcI fwd, NcoI	TATACCATGGCCTGGAAACGCG
U11	lmRNA *rpsAmf*-Flag into NcoI-BamHI of pRBcI rev, Flag-STOP-BamHI	TATAGGATCCTTATTTTTCATCGTCATC CTTATAGTCCTCGCCTTTAGCTGCTTTG

6.4 Media and buffers

LB (rich) Media

Bacto- Tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

In 1L ddH_2O adjust pH at 7.0

10 X Minimal (M9) Media

Na_2HPO_4	30g
KH_2PO_4	15g
NH_4Cl	5g

Adjust in 500ml ddH_2O

1 X M9 media

100ml 10 X M9

15ml 20% Glucose

1ml $CaCl_2$ 0.1M

1ml $MgSO_4$ 1M

1ml Thiamin 10mg/ml

1g NH_4Cl

Adjust In 1L ddH_2O

Antibiotics final concentrations

Ampcillin 100mg/µL

Chloramphenicol 30mg/µL

Tetracyclin 6mg/µl

10 X TBE

324g Tris Base

165g Boric Acid

120ml 0,5M EDTA (pH H_2O)

Adjust in 3L ddH_2O

Western blot Buffers

10 X PBS

400g NaCl

10g KCl

72g Na_2HPO_4

12g KH_2PO_4

In 1L ddH_2O adjust pH at 7.4

1 X PBS (5% milk)

100 ml 10 X PBS

50 g dry milk pulver

Adjust in 900 ml ddH_2O

10 X Transfer buffer

29g Glycin

58g Trizma

37ml 10% SDS

In 1 L ddH_2O adjust pH at 8,3

1 X Transfer Buffer

50ml 10X Transfer Buffer

400ml ddH_2O

100ml Methanol

Adjust in 500 ml ddH_2O

Destain Solution for Comassie Blue

40% Methanol

10% CH_3COOH

50% dd H_20

In dd H_2O

Comassie Stain Solution

40% Methanol

10% CH_3COOH

0,25% Comassie Blue

In dd H_2O

6.5 Methods used in this study

Competent cells (CaCl – method)

250ml LB were inoculated with 2.5 mL overnight culture and grown to OD_{600} 0.6 for 2 - 3h. Then the cells were transferred to appropriate centrifuge bottles and kept on ice for 20-30 min. Cells were centrifuged for 10 min on 4°C and 4000rpm and the cell pellet was resuspended in 15 ml cold 0.1 M $CaCl_2$. All further steps were preformed on ice. Again cells were centrifuged for 10 min on 4°C and 4000rpm and the cell pellet was resuspended in 15 ml cold 0.1 M $CaCl_2$. The third and last time cells were centrifuged for 10 min on 4°C and 4000rpm and the cell pellet was resuspended in 15 ml cold 0.1 M $CaCl_2$ plus 15% Glycerol. Cells were aliquot (50x 100µL) and freeze in liquid N_2. Cells were stored at -80°C until use.

Transformation

Competent cells were thawed on ice and 2-3 µl of plasmid were added. The mixture was kept on ice for 30 min. Cells were incubated on 42°C for 40 seconds and then on ice for 2 min. 1ml of LB media was added and cells were incubated on 37°C for 1h shaking. 100µl of the cell mixture were spread on the LB plates plus antibiotics and the rest was centrifuged for 4min on 3000rpm. Only 100µl of supernatant was kept to resuspend the cell pellet and spread it on the LB plates plus antibiotics. Cells were incubated overnight on 37°C.

SDS-Page protocol

Protein gel for a 12% SDS-PAGE:

Separation Gel (12%)	Stacking Gel (4,5%)
3,924ml dH2O	7,1ml dH2O
2,5ml 1M Tris-HCl, pH 8.8	1,25ml 1M Tris-HCl, pH 6.8
0,1ml 10% SDS	1,1ml 40% Acrylamide
2,976ml 40% Acrylamide	40µl 10% APS
50µl 10% APS 20µl	17µl TEMED
20µl TEMED	

APS and TEMED were added last and mixed carefully to avoid formation of bubbles. Polymerization begins as soon as APS is added to the mixture, so all subsequent were performed promptly. The separation gel solution was poured between the glass plates and about 1/4 of the space was left free for the stacking gel. The top of the resolving gel was covered with water until the gel polymerized (~30 min). Next, the water was discarded and the stacking gel solution was poured (prepared as described above, add APS and TEMED last). The combs were inserted and the stacking gel was left to polymerize for at least 60 min. After removing the combs carefully, the gel was placed into the electrophoresis tank, with fresh 1X Tris-glycine-SDS Buffer. The protein samples were heated on a 95° C heating block for 4 min then cooled quickly and shortly centrifuged. The protein samples and protein marker were loaded.

Western blot analysis

After transferring the protein to membrane by the standard procedure and rinsed it in 1X PBS the membrane was blocked in PBS/5% for one hour. No Tween-20 or BSA was added when using the Odyssey scanner. Next, membrane was washed 3x 5 minutes with 1X PBS+Tween20 with gentle shaking, using a generous amount of buffer. Then the membrane was incubated for one hour on room temperature with primary antibody diluted in PBST and washed 3x 5 minutes in PBST with gentle shaking. The fluorescently-labeled secondary antibody was diluted (1:10,000) in PBST and exposure to light was avoided. The membrane was incubated with the secondary antibody for one hour on room temperature and then washed 3x 5 minutes in PBST. Finally the membrane was rinsed 5x 5min in PBS to remove residual Tween. Membrane was scanned in Odyssey scanner.

7. References

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