Processing and turn-over of small non-coding RNA OxyS in *E.coli*
&
Post-transcriptional regulation of RpoS levels by small non-coding RNAs OxyS and DsrA and the Hfq protein in *E.coli*

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Dedicated to

My beloved Parents

And

My beloved Sisters and Brothers

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Madhugiri R, Basineni SR, Klug G (2010) Turn-over of the small non-coding RNA RprA in *E. coli* is influenced by osmolarity (*in press*) Molecular Genetics and Genomics 101

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PUBLICATIONS

The following publication is based on this work:

Basineni SR, Madhugiri R, Kolmsee T, Hengge R, Klug G. **(2009)** The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target rpoS in *E. coli* is growth phase dependent. **RNA Biology, 6(5):584-594.**

1. INTRODUCTION

Escherichia coli is the organism in which researchers first identified and studied regulatory proteins, worked out most metabolic pathways, clearly recognized "regulons" and the concept of global regulatory networks, and documented regulatory degradation of proteins (Willetts 1967; Willetts 1967; Gold 1988; Zheng, Wang et al. 2001; Chang, Smalley et al. 2002; Weber, Polen et al. 2005; Durfee, Hansen et al. 2008). Decades of genetics, biochemistry, and, more recently, global analysis of gene expression have been documented for this organism (Zheng, Wang et al. 2001; Weber, Polen et al. 2005; Durfee, Hansen et al. 2008). In the last few years, *E. coli* has once again been in the forefront of a new field of interest, the discovery and study of many new and exciting regulators – "**Small non-coding RNAs**". Small RNA regulators are proving to be multifunctional and have provided explanations for a number of previously mysterious regulatory effects. Not surprisingly, these sorts of regulators are not only confined to *E. coli* but also present in other bacterial species such as *Salmonella sp*., *Vibrio sp.*, *Mycobacterium sp.*, *Bacillus sp.*, *Rhodobacter sp.*, *Sinorhizobium sp.*, and also in Archaea (Lenz, Mok et al. 2004; Tang, Polacek et al. 2005; Silvaggi, Perkins et al. 2006; del Val, Rivas et al. 2007; Pfeiffer, Sittka et al. 2007; Arnvig and Young 2009; Berghoff, Glaeser et al. 2009; Jager, Sharma et al. 2009; Straub, Brenneis et al. 2009). In bacteria, RNA molecules that act as regulators were known years before the first microRNA (miRNA)s and short interfering RNAs (siRNA) were discovered in eukaryotes. In 1981, the 108 nucleotide RNA I was found to block ColE1 plasmid replication by base pairing with the RNA that is cleaved to produce the replication primer (Stougaard, Molin et al. 1981; Tomizawa, Itoh et al. 1981). This work was followed by the 1983 discovery of a 70 nucleotide RNA that is transcribed from the pOUT promoter of the Tn10 transposon and represses transposition by preventing translation of the transposase mRNA (Simons and Kleckner 1983). The first chromosomally encoded small RNA regulator, reported in 1984, was the 174 nucleotide *E.coli* MicF RNA, which inhibits translation of the mRNA encoding the major outer membrane porin OmpF (Mizuno, Chou et al. 1984). These discoveries have led others to identify and characterize small non-coding RNAs in various bacterial species in recent years by various methods.

1.1 Discovery of sRNAs in bacteria

Gene regulation was long thought to be controlled almost entirely by proteins that bind to DNA and RNA. Most of these regulatory proteins have been identified by mutational screens that hindered the regulation of a particular gene. Further additional putative protein regulators in different bacterial species were identified by their similarity to known regulatory proteins. Over the last years, it has become evident that small non-coding RNAs (sRNAs) also play an important role in gene regulation. But for many years small regulatory RNAs were largely overlooked because they were hard to find in biochemical assays or by mutational screens may be due to their smaller size.

RNA molecules with regulatory functions in bacteria were known for years before the first microRNA (miRNA) and short interfering RNAs (siRNA) were discovered, but until 2001 only ten genes were known in *E.coli* (Wassarman, Zhang et al. 1999). Most of these RNAs were discovered accidentally, using genetic screens or through radio-labelling of total RNA and subsequent isolation from gels (Wassarman, Zhang et al. 1999). RNAs such as the 4.5S (part of the secretion machinery), RNase P (catalytic part of the ribozyme), Spot 42, 6S and tmRNA (transfer messenger RNA) were detected on gels by using metabolic radio-labelling (Hindley 1967; Griffin 1971; Ikemura and Dahlberg 1973). Small RNAs such as MicF, DicF, DsrA, OxyS and CsrB were identified subsequently and have been assigned to have important regulatory and housekeeping functions (Mizuno, Chou et al. 1984; Bouche and Bouche 1989; Sledjeski and Gottesman 1995; Altuvia, Weinstein-Fischer et al. 1997; Romeo 1998).

In *E.coli* **OxyS**, was detected as transcript made divergently from the genes for the LysR family regulatory proteins OxyR in transcription studies (Altuvia, Weinstein-Fischer et al. 1997; Urbanowski, Stauffer et al. 2000). The synthesis of these sRNAs is regulated by the regulator protein in a manner analogous to other LysR family proteins that regulate divergent protein-encoding genes (Schell 1993). **DsrA**, was identified during studies of capsule regulation as a gene capable of increasing capsule synthesis when present on a multicopy plasmid being studied for other reasons (Sledjeski and Gottesman 1995). Another sRNA, **RprA**, was identified in a screen of a multicopy plasmid library for plasmids that suppressed a phenotype of a *dsrA* mutant (Majdalani, Chen et al. 2001).

Several groups have identified approximately 80 non-coding RNAs in *E.coli* and many more throughout the bacterial kingdom by different methods such as, computational identification, RNomics, comparative genomics and microarrays and by using Hfq to identify sRNA and mRNA targets (Argaman, Hershberg et al. 2001; Rivas, Klein et al. 2001; Wassarman, Repoila et al. 2001; Chen, Lesnik et al. 2002; Tjaden, Saxena et al. 2002; Vogel, Bartels et al. 2003; Zhang, Wassarman et al. 2003; Kawano, Reynolds et al. 2005; Altuvia 2007). In recent years by using above mentioned methods small regulatory RNAs have been discovered not only in *E.coli* but also in other bacteria such as *Bacillus subtilis, Vibrio cholera, Pseudomonas aeruginosa, Staphylococcus aureus, Listeria monocytogene, and Rhodobacter sphaeroides* (Lenz, Mok et al. 2004; Wilderman, Sowa et al. 2004; Pichon and Felden 2005; Livny, Brencic et al. 2006; Silvaggi, Perkins et al. 2006; Mandin, Repoila et al. 2007; Berghoff, Glaeser et al. 2009).

1.2 Regulatory roles of RNAs

The discovery of catalytic RNAs in the early 1980s (T. Cech and S. Altman, Nobel Prize in Chemistry 1989) entirely changed our views about the roles of RNA molecules (Kruger, Grabowski et al. 1982; Guerrier-Takada, Gardiner et al. 1983). Further, RNAs such as RNase P (Gopalan, Vioque et al. 2002), 4.5S RNA (Herskovits, Bochkareva et al. 2000) and tmRNA (Lee, Bailey et al. 1978) have been studied in detail. The involvement of these non-coding RNAs with the translation apparatus has lead to the hypothesis that many other sRNAs also play a key role in translation quality control and translational regulation. Some of other non-coding RNAs that function as regulatory molecules such as 6S RNA, CsrB and CsrC that regulate proteins have also been studied in detail in *E.coli*. These sRNAs are known to regulate the proteins by direct binding (Fig 1.1C) (Wassarman and Storz 2000). A significant number of sRNAs that have been discovered so far are believed to act as antisense regulators, these sRNAs work by pairing to their target messenger RNAs. This pairing affects the stability or translation of the message. A few antisense RNA regulators are encoded on the opposite strand of the DNA from the regulated mRNA (cis-acting), resulting in the potential for complete pairing, there by activating or repressing the protein expression (Fig 1.1 D & E). The majority of the known bacterially encoded anti-sense RNAs are encoded far from their targets (trans-encoded); these trans-encoded RNAs can base-pair imperfectly with mRNA targets and either repress or activate the translation (Fig 1.1 A & B). The vast majority of sRNAs also binds to and requires the RNA chaperone Hfq. Furthermore, recent genome wide searches and deep sequencing analysis for Hfq-binding RNAs may have come close to saturating the search for this class of RNAs (Zhang, Wassarman et al. 2003; Sittka, Lucchini et al. 2008; Liu and Camilli 2010).

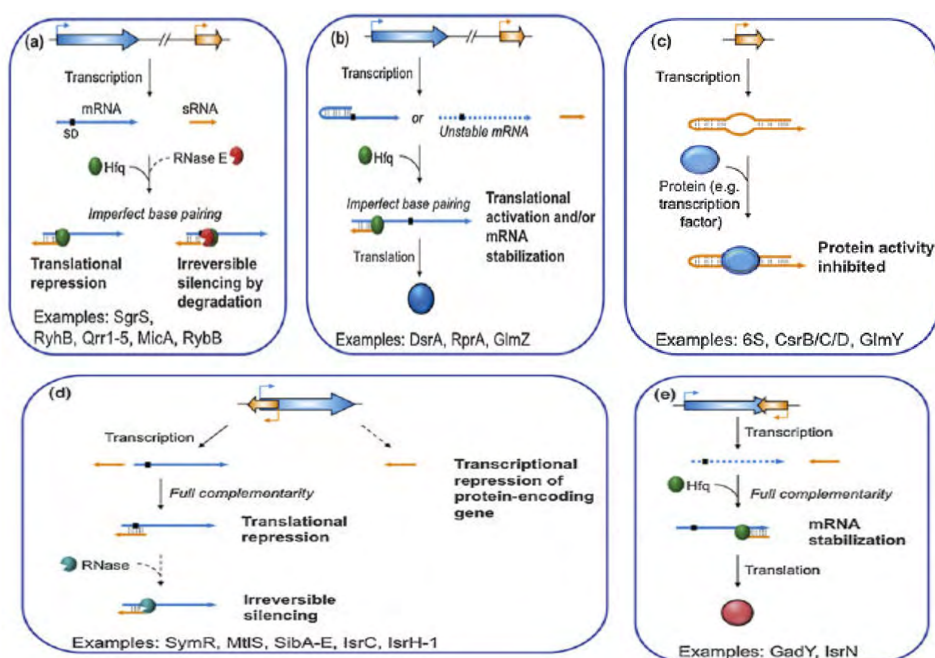


Figure 1.1 Different types of sRNAs based on their mechanism of action. Trans-encoded sRNAs can basepair imperfectly with mRNA targets and either **(a)** repress or **(b)** activate translation. Alternatively, **(c)** some trans-encoded sRNAs interact with proteins, including transcription factors, and inhibit their activity. Cis-encoded antisense sRNAs can also either **(d)** activate or **(e)** repress protein expression. Colored arrows represent RNA transcripts; black boxes indicate Shine-Dalgarno (SD) sequences. Dashed color arrows represent unstable transcripts. Dashed black arrows represent hypothetical mechanistic steps of sRNA-mediated regulatory pathways. **Taken from Jane M Liu and Andrew Camilli, Current Opinion in Microbiology, 2010, 13:18-23.**

1.3 Role of Hfq in sRNA function

Hfq is one the most abundant RNA-binding protein in *E.coli*. Hfq was first identified as a host factor, required for the replication of the RNA phage Q*β* ~40 years ago (Blumenthal and Carmichael 1979). Hfq is known to have an important physiological role in many bacteria (Vogel and Wagner 2007). Half of all sequenced Gram-Positive and Gram-Negative bacteria and at least one archaeon encode an Hfq homologue (Sun, Zhulin et al. 2002; Nielsen, Boggild et al. 2007). It was found to bind specifically to AU-rich single-stranded RNA regions (Moll, Leitsch et al. 2003). The RNA chaperon Hfq is required for the effective regulation of many ncRNAs that act by base-pairing with target mRNAs (Moller, Franch et al. 2002; Zhang, Wassarman et al. 2002; Zhang, Wassarman et al. 2003; Lease and Woodson 2004; Mikulecky, Kaw et al. 2004; Kawamoto, Koide et al. 2006). However, Hfq can also act alone as a translational repressor of mRNA (Vytvytska, Moll et al. 2000; Urban and Vogel

2008) and can modulate mRNA decay by stimulating polyadenylation (Hajnsdorf and Regnier 2000; Mohanty, Maples et al. 2004).
The properties of the *hfq* mutants led to recognition that Hfq was necessary for translation of *rpoS* mRNA, encoding the major stress sigma factor of σ^S of *E. coli* (Muffler, Fischer et al. 1996). Hfq was shown to be important for overcoming an inhibitory hairpin upstream of *rpoS* (Brown and Elliott 1997). The hairpin sequesters ribosome binding to the RpoS translation start site; the bypass mutants abolish the hairpin so that translation is constitutive. Independently, two sRNAs, DsrA and OxyS, were found to regulate *rpoS* translation (Sledjeski and Gottesman 1995; Altuvia, Weinstein-Fischer et al. 1997). Both sRNAs require Hfq for their activity (Zhang, Altuvia et al. 1998; Sledjeski, Whitman et al. 2001). These sRNAs act by pairing with complementary sequences in their mRNA targets, suggesting that Hfq is important for this pairing. This was demonstrated *in vitro* for OxyS (Zhang, Wassarman et al. 2002). In a test of 46 known sRNAs found in various searches, 15 were found to bind Hfq tightly; at least 5 other sRNAs were defined by their binding to Hfq, bringing the total to at least 22, and possibly as many as 36 (Zhang, Wassarman et al. 2003). Five small Hfq-binding RNAs, DsrA, OxyS, RprA, Spot 42, and RyhB, have been studied in detail in *E.coli*. Experiments with each of them provide different insights into how the Hfq-binding RNAs can act.

1.4 Regulation of RpoS Translation

Bacteria in response to changing environmental conditions usually grow slowly as there will be limited resources to uphold and to survive under stress. In order to survive such drastic conditions *E.coli* triggers the expression of many genes that are involved in both transient emergency response and in long-term adaptation. Many of these genes are dependent on the stationary phase/stress sigma factor, RpoS (σ^S or σ^{38}) for their transcription. RpoS, which is an alternative sigma factor, that orchestrates the expression of a number of stress-response genes with different physiological functions such as cell envelope integrity, morphology, carbon metabolism, stress resistance and stationary phase (Hengge-Aronis 2002). Expression of the RpoS regulon depends primarily on the levels of RpoS protein, with global regulatory factors (HNS, Lrp, CRP or IHF) modulating or fine tuning the expression (Hengge-Aronis 2002). RpoS levels are very tightly fine-tuned at the levels of transcription, translation and proteolysis, so one can say that the regulation of RpoS levels is a very intricate mechanism. The default state for *rpoS* translation is normally “off;” but even in cells that grow in relative absence of stress there will be a basal level expression of RpoS. This low level of RpoS in optimally growing cells is due to the active degradation by ClpXP protease (Lange and Hengge-Aronis 1994; Schweder, Lee et al. 1996). Once cells reach stationary growth phase, RpoS - the master regulator of stationary phase becomes stable in stationary phase or under starvation conditions, allowing a rapid accumulation of RpoS in the cells (Hengge-Aronis 2002). The inhibition of the *rpoS* mRNA translation depends on the upstream structure of the

rpoS start codon. A long 5'untranslated (UTR) region can fold into a hairpin that inhibits ribosome binding. RpoS translation rapidly increases after stress treatments; this increase requires Hfq and, subsequently it has been shown that sRNAs (DsrA and RprA) are also involved in the translational activation. Two sRNAs, DsrA and RprA, are complementary to the upstream stem of the hairpin and both sRNAs can activate the translation of *rpoS* by pairing with the help of Hfq. Mutations in DsrA or RprA that disrupt pairing can be restored to function by compensating mutations in the *rpoS* RNA pairing target. Finally, a negative regulation of RpoS induction by sRNAs also occurs. OxyS, regulated by OxyR and induced in response to oxidative stress, negatively regulates RpoS as well as a number of other targets, including *fhlA* (Altuvia, Weinstein-Fischer et al. 1997).

1.4.1 DsrA, a translational activator of RpoS – DsrA, is a 85 nucleotide (nt) sRNA that is expressed under various stress conditions such as low temperature (<30^0C), osmotic stress and even under oxidative stress (Fig 1.3) (Repoila and Gottesman 2001; Repoila and Gottesman 2003; Basineni, Madhugiri et al. 2009). DsrA RNA acts like a translation activator of RpoS expression by binding to the 5' untranslated region (UTR) with the help of the RNA chaperon Hfq (Fig 1.2 A). The binding of DsrA to *rpoS* mRNA not only helps in the translational activation but also increases the stability of *rpoS* mRNA. The amounts of DsrA are normally 30 fold higher at 25^0C than at 42^0C, this in turn leads to higher translation of RpoS. So RpoS is more abundant at 25^0C than at 42^0C, and RpoS dependent promoters are expressed at low temperatures, even in exponential phase. This thermoregulation of RpoS occurs at the level of translation and is completely abolished in cells lacking DsrA (Sledjeski, Gupta et al. 1996; Repoila and Gottesman 2001). Transcription initiation of the *dsrA* gene and the stability of DsrA are dependent on temperature. This regulation of transcription initiation and the changes in the stability of DsrA show the contribution of DsrA-dependent temperature regulation of RpoS translation.

1.4.2 OxyS, a negative regulator of RpoS translation – OxyS, is a 109 nucleotide RNA strictly expressed under oxidative stress (Fig 1.2 B) and plays a key role in the adaptation to oxidative stress in bacteria. This sRNA was initially detected in Northern blots when cells were treated with hydrogen peroxide (Altuvia, Weinstein-Fischer et al. 1997). OxyS is regulated by, and synthesized divergently from the *oxyR* gene encoding OxyR, one of the major regulatory proteins activated upon oxidative stress (Christman, Morgan et al. 1985; Altuvia, Weinstein-Fischer et al. 1997). The signals that lead to OxyS induction (activation of OxyR) also lead to induction of a set of genes that deal with oxidative stress. It has been suggested that this negative regulation of RpoS synthesis may provide a mechanism to fine tune the expression of antioxidant activities and prevent the redundant induction of *katG*, *gorA* and *dps*, by both the hydrogen peroxide specific OxyR transcription factor and the

general stress factor σ^S (Zhang, Altuvia et al. 1998). The sRNA OxyS has been shown to directly repress *rpoS* translation through base pairing (Zhang, Wassarman et al. 2002).

(A)

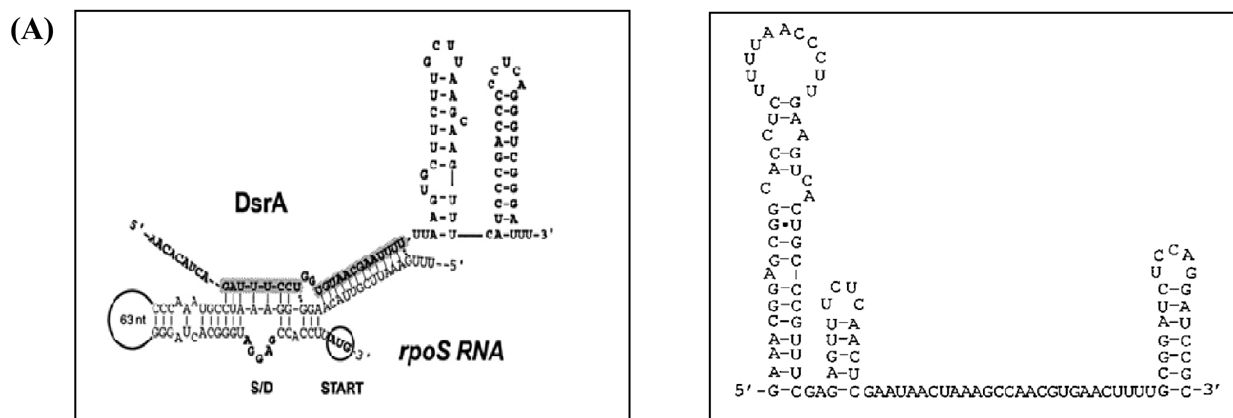


Figure 1.2 (A) A model of DsrA-*rpoS* mRNA hybrid. Paring of DsrA with the upstream message should block the inhibitory paring with the *rpoS* ribosome-binding region, allowing high levels of translation. **Taken from Lease et al., 2000, PNAS, 97:9919-24. (B)** OxyS, secondary structure based on structural probing analysis (Altuvia et al., 1997). **Taken from Jörg Vogel and Cynthia Mira Sharma, 2005, Biol.Chem, 386:1219-1238.**

It has also been shown that OxyS acts like an antimutator by repressing the *rpoS* translation, because RpoS increases the mutation rate by upregulating transcription by error-prone RNA polymerase (Layton and Foster 2003; Ponder, Fonville et al. 2005). It has been suggested that OxyS may modulate Hfq availability to repress RpoS translation or by some other manner by blocking the access of the positively acting sRNAs such a DsrA or RprA to the leader (Zhang, Altuvia et al. 1998). But the mechanism by which OxyS inhibits the translation of RpoS is not very well understood. OxyS also down regulates the translation of FhlA, a transcriptional activator of formate hydrogenlyase complex and an antimutator (Altuvia, Weinstein-Fischer et al. 1997). The regulation of *fhlA* by OxyS is direct, translation is repressed through a kissing complex pairing that blocks the ribosome binding site (Altuvia, Zhang et al. 1998). A schematic overview of the external factors affecting RpoS expression through OxyS and DsrA is shown in Fig 1.3.

1.5 Consequences of ncRNA/mRNA pairing

The majority of ncRNAs thus far characterized exert their control of gene expression through anti-sense mechanisms, mainly by base-pairing to mRNAs. Typically, ncRNA/mRNA hybrids involve short stretches of sequence complementarity between ncRNA and their cognate mRNA with or without the help of Hfq. Some non-coding RNAs base-pair to the sequences that overlap or adjacent to the Shine-Dalgarno sequence (SD), and/or translation initiation codon within the TIR (Translation Initiation Region) of the target mRNA and some

ncRNAs base-pair upstream to the TIR. The base-pairing of ncRNA to its cognate mRNA can lead to, translation inhibition or translation activation or coupled degradation of the ncRNA/mRNA duplex by RNases.

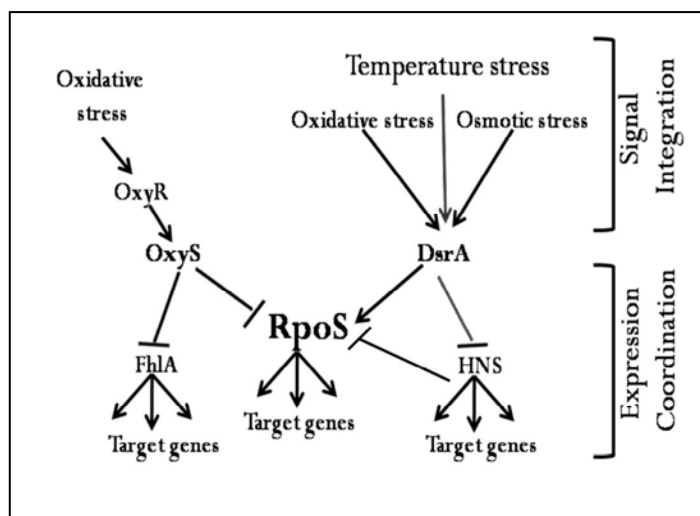


Figure 1.3 Environmental signals as shown above may act either directly or through signaling proteins to stimulate the levels and activities of the two small RNAs OxyS and DsrA that affect RpoS translation. **Adapted and modified from Repoila et al., 2003.**

1.5.1 Translation inhibition - The formation of RNA duplex is generally sufficient to prevent binding of the ribosome and there by inhibiting the translation by inducing the structural changes within the TIR. Translation inhibition is supposed to occur due to the competition between ribosome and ncRNA to the same binding region. However, the exact mechanism is much more complex. Several groups have studied in detail the role of ncRNAs in translation inhibition of their respective targets, yet the mechanism by which some ncRNAs (eg: OxyS/rpoS) inhibit the translation of their target is not very well understood (Zhang, Altuvia et al. 1998; Aiba 2007; Heidrich, Moll et al. 2007; Sharma, Darfeuille et al. 2007).

1.5.2 Translation activation - In contrast to inhibition, translation activation by ncRNAs has been studied in only few cases (Majdalani, Cunning et al. 1998; Majdalani, Chen et al. 2001; Kalamorz, Reichenbach et al. 2007; Sharma, Darfeuille et al. 2007; Urban, Papenfort et al. 2007). The best studied examples are DsrA and RprA RNAs that activate *rpoS* translation with the help of Hfq under stress conditions (Majdalani, Cunning et al. 1998; Majdalani, Chen et al. 2001). The only direct mechanism described so far involves the pairing of ncRNA to 5′ UTR of an mRNA, upstream to the TIR. This pairing induces structural changes, rendering the SD sequence accessible to ribosomes. But recently Resch et al., have demonstrated the exact mechanism of activation of *rpoS* mRNA translation by DsrA, which is depicted in Fig 1.4 (Resch, Afonyushkin et al. 2008).

1.5.3 Coupled degradation of ncRNA/mRNA duplex - In addition to above effects on the target mRNA, pairing can also lead to rapid degradation of ncRNA/mRNA duplex by RNases such as RNase E or RNase III. This indicates that ncRNAs might act stoichiometrically and might be degraded as they exert their effect (Masse, Escorcia et al. 2003; Morita, Maki et al. 2005). This means that these ncRNAs are degraded upon pairing with their mRNA targets. Formation of ncRNA/mRNA duplexes is sufficient for the translation inhibition, but the RNase E- or RNase III-dependent degradation is the subsequent step that renders inhibition irreversible.

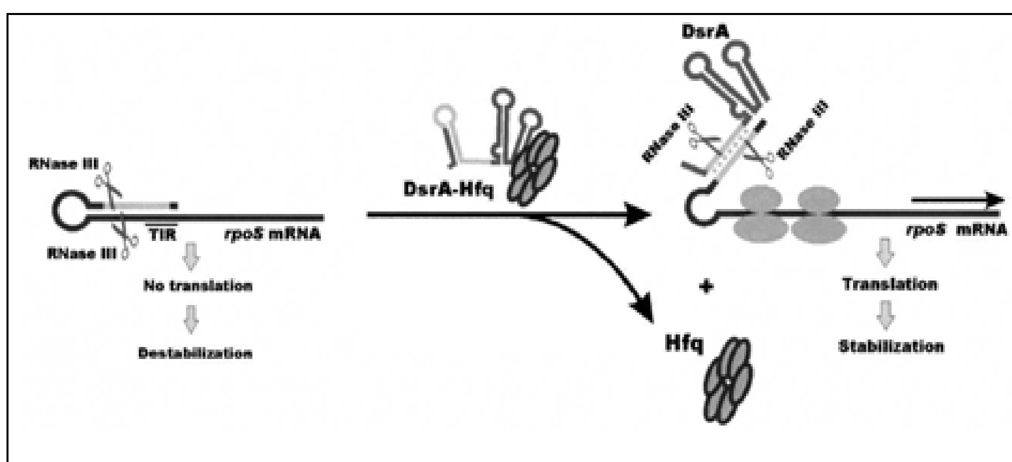


Figure1.4 Model for post-transcriptional regulation of *rpoS* expression by RNase III. The translation initiation region (TIR) of the *E. coli rpoS* mRNA is embedded into a complex secondary structure, thereby preventing efficient ribosome binding. RNase III can cleave within the double-stranded segment of this structure, which results in destabilization of the transcript in the wild-type *E. coli* strain when compared to its isogenic counterpart lacking functional RNase III. In contrast to limited synthesis of RpoS under normal growth conditions, the level of RpoS is up-regulated in the presence of increasing amounts of DsrA, which accumulate at low temperature. By base-pairing with the complementary region of the *rpoS* leader, DsrA disrupts the inhibitory secondary structure, thereby facilitating ribosome loading and subsequent translation of RpoS (Repoila et al. 2003). DsrA/*rpoS* duplex formation, which is facilitated by the RNA chaperone Hfq, not only abrogates RNase III cleavage within the *rpoS* leader at positions (-94/-15), but also creates a new RNase III cleavage site within the DsrA/ *rpoS* duplex. RNase III cleavage at this site prevents reuse of DsrA for multiple cycles of *rpoS* activation. Moreover, the main body of the DsrA-activated *rpoS* mRNA is covered by translating ribosome and therefore protected from degradation by *E. coli* ribonucleases. **Taken from Resch et al., 2008, NAR, 14:454-459.**

1.6 The role of Ribonucleases in Post-Transcriptional Regulation

In addition to the efficiency of transcription and/or translation, the level of gene expression can be affected at the level of the stability of an individual transcript (mRNA or ncRNA). So to understand the action of regulatory RNAs (ncRNAs) it is very important to study the processing and turn-over of these molecules. In general the processing and turn-over of RNAs is carried out by a class of cellular enzymes called ribonucleases (RNases). Bacterial RNA processing, turn-over and the RNases that are involved have been best studied in *E.coli*. More than twenty RNases have been described in *E. coli* and importance of RNases in the post-transcriptional regulation has been studied by several groups. Several groups have also characterized the mutant strains deficient in RNases and the role of the RNases in the RNA decay mechanisms (Arraiano, Yancey et al. 1988; Amblar, Viegas et al. 2004; Viegas, Fernandez De Palencia et al. 2004; Viegas, Schmidt et al. 2005). In general, *E. coli* mRNAs decay is initiated by a primary endonucleolytic cleavage often by RNase E or less commonly by RNase III (Apirion 1973; Ehretsmann, Carpousis et al. 1992). This cleavage is followed by 3'-to-5' exonucleolytic RNA decay. Two enzymes, polynucleotide phosphorylase (PNPase) and RNase II are involved in this process. The limiting step of degradation in the majority of *E. coli* transcripts is considered to be the cleavage by RNase E (Kushner 2002), with minor roles attributed to other more specialized endonucleases, such as its paralog RNase G, RNase III, RNase P and, the more recently characterized, RNase Z (Perwez and Kushner 2006).

1.6.1 Endoribonucleases

1.6.1.1 RNase E - RNase E (*rne*) is a single-strand specific endoribonuclease responsible for the processing and turn-over of mRNAs (Arraiano, Yancey et al. 1988; Ow, Liu et al. 2000; Bernstein, Lin et al. 2004), small regulatory RNAs (Suzuki, Babitzke et al. 2006), stable RNAs (Apirion and Lassar 1978; Ow, Liu et al. 2000) and in the maturation of many tRNA precursors (Li and Deutscher 2002; Ow, Liu et al. 2002; Li 2007). Although RNase E cleaves mRNAs internally, its catalytic activity is determined by the 5'-end of the substrate. RNase E requires at least four nucleotides of single stranded RNA at the 5' end in order to be able to bind efficiently. RNase E has a significant preference for 5'-monophosphorylated extremities over the 5'-triphosphate ends of primary transcripts (Mackie 1998; Callaghan, Marcaida et al. 2005). Belasco and co-workers have found that a pyrophosphatase acts on mRNAs to enable RNase E to launch its initial attack, perhaps in analogy with the decapping step of eukaryotic mRNAs (Celesnik, Deana et al. 2007). RNase E is a large protein of 1,061 amino acids, with an N-terminal catalytic region and a C-terminal non-catalytic region (Chauhan and Apirion 1991; Kaberdin, Miczak et al. 1998; Callaghan, Marcaida et al. 2005). Because RNase E is responsible for many decay and maturation processes, its activity must be regulated tightly. Indeed, RNase E auto regulates its own synthesis by repressing the

concentration of the transcript through increased decay rates (Jain and Belasco 1995). The non-catalytic C-terminal half acts as a scaffold region for the association of other proteins with RNase E, in an RNA degrading complex called the "degradosome". This complex facilitates the efficient degradation of structured RNAs due to the cooperative activity of different enzymes (Carpousis, Van Houwe et al. 1994; Miczak, Kaberdin et al. 1996; Py, Higgins et al. 1996; Kaberdin, Miczak et al. 1998; Vanzo, Li et al. 1998). Its main components are the 3'-to-5' exoribonucleases polynucleotide phosphorylase (PNPase), an ATP-dependent RNA-helicase (RhlB) and a glycolytic enzyme (enolase). The association of RNase E and PNPase in a complex provides direct evidence for their cooperation in the degradation of mRNAs.

1.6.1.2 RNase III - RNase III (*rnc*) was first detected in *E.coli* as an endoribonuclease that cleaves double stranded RNA molecules. It is a ubiquitous endoribonuclease that participates in the maturation of ribosomal RNA from precursors and processing and decay of mRNAs such as *pnp* (Burgin, Parodos et al. 1990; Babitzke, Granger et al. 1993; Evguenieva-Hackenberg and Klug 2000).The enzyme is also responsible for the cleavage of several bacterial and phage messages. However the stability of most cellular mRNAs is not changed in mutants defective in RNase III (Nicholson 1999). Perfect antisense/sense RNA duplex formed in ncRNA/mRNA interactions constitute an optimal substrate for this enzyme. In bacteria, RNase III exists in a form characterized by a classical RNA binding domain and a nuclease domain (Nicholson 1999). It is a dimer of two identical 25.4 KDa polypeptides (Li and Nicholson 1996). The expression of *rnc* is autoregulated (Dasgupta, Fernandez et al. 1998).

1.6.2 Exoribonucleases

1.6.2.1 PNPase – *E.coli* PNPase (*pnp*) acts as a 3'-5' exoribonuclease. It catalyzes the processive 3'-5' phosphorolytic degradation of RNA releasing nucleoside diphosphates from the 3' end. It is generally described as a homotrimer of 78-kDa subunits (Regnier, Grunberg-Manago et al. 1987). Expression of *pnp* is negatively auto-regulated at the posttranscriptional level by the PNPase and RNase III (Robert-Le Meur and Portier 1992). In an RNase III deficient strain there is a stabilization of the *pnp* mRNA leading to the 10-fold increased levels of PNPase (Portier, Dondon et al. 1987). Strains lacking *pnp* do not show any growth defects at optimal temperatures. However, *pnp* mutants of *E.coli* are unable to grow below 30^0C (Zangrossi, Briani et al. 2000). *In vivo* this enzyme is essentially devoted to the processive degradation of RNA, but is responsible for adding polynucleotide tails observed in *E .coli* mutants lacking polyadenylating enzyme PAP I (Mohanty and Kushner 2000).

1.6.2.2 RNase II – RNase II (*rnb*) a single-strand specific, sequence-independent, 3'-exoribonuclease like PNPase, that participates in the terminal stages of mRNA degradation. It processively hydrolyzes the RNA in 3'-5' direction, yielding 5'-nucleoside monophosphates. It is a single polypeptide composed of 644 amino acids with a calculated molecular mass of 72.3-kDa. RNase II levels are inversely correlated with those of PNPase. The expression of *rnb* is increased 2- to 2.5-fold in PNPase deficient strains by post-transcriptional regulation. Conversely, PNPase overproducing strains contain reduced levels of *rnb* message and RNase II. This explains the possibility that each enzyme can replace the other to degrade mRNA in vivo (Donovan and Kushner 1986). RNase II expression is also affected at the post-transcriptional level by the endonuclease RNase III and RNase E. In RNase III mutant there is a reduced RNase II level and activity. However, this effect seems to be indirect, due to the increased levels of PNPase which in turn affects the *rnb* levels (Zilhao, Regnier et al. 1995).

1.6.2.3 RNase R – RNase R was initially identified as an enzyme responsible for hydrolytic activity in a mutant for RNase II (Kasai, Gupta et al. 1977). RNase R is a 92kDa protein, a product of *rnr* gene (Cheng, Zuo et al. 1998). RNase R is very effective against structured RNA and also plays a major role in the RNA quality control (Cheng and Deutscher 2003; Richards, Mehta et al. 2006). RNase R belongs to the RNase II family of exoribonucleases (Grossman and van Hoof 2006). RNase R acts as a monomer and requires a monovalent cation and Mg^{2+} for the activity (Cheng and Deutscher 2002). RNase R is a processive, sequence independent nonspecific 3'-5' exoribonuclease. RNase R levels change according to the environmental stimuli (Cairrao, Cruz et al. 2003). RNase R expression is controlled at the posttranscriptional level by RNase E, although RNase G might also participate (Cairrao and Arraiano 2006).

1.7 Polyadenylation and Poly (A)-mediated decay

Polyadenylation is a posttranscriptional modification of RNAs that occur in prokaryotes, eukaryotes and in organelles. For long time it was regarded as a special feature of eukaryotic mRNAs. The function and extent of bacterial polyadenylation first appeared in marked contrast to those of eukaryotic mRNAs (Dreyfus and Regnier 2002). It is now established that polyadenylation facilitates the exonucleolytic degradation of structured mRNA by providing single-stranded tails for PNPase attack. Secondary structures constitute an important determinant of RNA stability, since they protect the 3' end of the transcripts from degradation. Poly(A) Polymerase I (PAPI) a 53 kDa polypeptide, encoded by the *pcnB* gene, is the main enzyme responsible for RNA polyadenylation in *E.coli*, which can destabilize bacterial transcripts by facilitating the 3' to 5' exo-mediated decay of RNAs with a structured 3' end. When the endonucleolytic cleavages are hindered, the poly(A) degradation pathway becomes more effective (Marujo, Braun et al. 2003). In vitro results have demonstrated protein-protein interaction of PAP I with RNase E, as a component of the degradosome

(Raynal and Carpousis 1999). Some evidences suggest that endonucleolytic cleavage by RNase E could provide the signal for polyadenylation of fragments (Haugel-Nielsen, Hajnsdorf et al. 1996). PAPI has also been found to interact with RNase R (Khemici and Carpousis 2004; Cheng and Deutscher 2005) and Hfq (Hajnsdorf and Regnier 2000; Mohanty, Maples et al. 2004). It has been suggested that PAPI requires the participation of Hfq in the polyadenylation of the 3' end of transcripts that contain *Rho*-independent transcription terminators. Hfq destabilizes the terminator stems, which are then direct targets for PAPI activity and are ultimately subjected to exonuclease degradation. In the absence of PAP I still polynucleotide tails were detected in *E.coli*. Later it has been suggested that PNPase synthesizes heterogeneous tails *in vivo* in the absence of PAP I and occasionally incorporates U and C residues in poly (A) tails, even when PAP I is active (Mohanty and Kushner 2000).

1.8 The Role of RNases in small Non-Coding RNA processing

Some ncRNAs represent primary transcripts, whereas others are generated from longer RNA transcripts by processing. Regarding processing, ncRNAs seem to be more similar to rRNAs and other stable RNAs. The biological significance of sRNA processing is largely unexplored but it often appears to have an important role in creating an active RNA species. While some sRNAs occur as multiple size species others undergo a growth-condition dependent processing (Argaman, Hershberg et al. 2001; Viegas, Pfeiffer et al. 2007). It remains unclear whether the different RNA species are functional, or whether the conversion of an inactive precursor to a mature form is part of the regulatory circuit. For example, DsrA and GadY are each expressed from a single promoter, yet multiple active sRNA species can be detected for both (Majdalani, Cunning et al. 1998; Repoila and Gottesman 2001; Opdyke, Kang et al. 2004). In the case of SraC, SraF and SraJ (now GlmZ) sRNAs, there are indications that RNase III is involved in the processing of the primary transcript (Argaman, Hershberg et al. 2001; Brantl 2007). RNase III and RNase E are responsible for the maturation from a polycistronic transcript of DicF, a sRNA whose target is the cell-division gene *ftsZ* (Faubladier, Cam et al. 1990). RNase III processing generates a 190 nucleotide precursor, and further processing by RNase E generates the functional 53 nts DicF-sRNA. Another example is MicX, an OMP regulatory sRNA. Full-length MicX appears to have some biological activity, although it is unstable and relatively rare. It is processed by RNase E to a shorter form that was seen to retain the biological activity (Davis and Waldor 2007). Maturation of ssrA/tmRNA has been reported to result from endonucleolytic processing by RNase P, RNase III and RNase E, followed by exonucleolytic trimming (Kaberdin, Miczak et al. 1998; Lin-Chao, Wei et al. 1999). The 6S RNA, modulator of RNA polymerase/σ^{70} holoenzyme activity, is transcribed as part of a large primary transcript, and two different 6S RNA precursors are generated. The larger precursor is transcribed from a distal promoter P2 (both σ^{70} and σ^{S} dependent) and is then processed exclusively by RNase E; whereas the shorter

precursor is expressed via a proximal promoter P1 (σ^{70} dependent) and processed by both RNase E and RNase G. 6S RNA primary transcripts are subjected to both 5' and 3' processing but the exoribonucleases involved in 3' final trimming have not been specifically identified (Li, Pandit et al. 1998).

The vast majorities of sRNA are expressed under specific stress conditions, most probably related to their function, and play a central role in the adaptability of bacteria to new environmental conditions (Argaman, Hershberg et al. 2001; Wassarman, Repoila et al. 2001; Viegas, Pfeiffer et al. 2007). So to know how sRNA amounts are controlled intracellularly is an integral part of understanding sRNA regulation. The steady-state level of RNA in the cell is determined by a balance between its rate of synthesis and degradation. Half-life determination of an individual RNA is a critical parameter in understanding the function of regulatory RNAs. Even if the sRNA does not influence the turnover of its target mRNA(s), its own decay rate may strongly influence its regulatory impact. The decay rate of a sRNA is a critical parameter. Decay rate can directly influences its cellular concentration and there by determines for how long it can be part of the regulation and how strong it may act. It was seen that sRNAs can cover the entire range, from very unstable (turnover in few minutes) to very stable (half-lives over 30 minutes) (Vogel, Bartels et al. 2003). The half-life of several small non-coding RNAs also depends on its interacting target(s) mRNA. If a sRNA half-life is analyzed when it is in excess over its target, the predominant value is that of its "independent half-life." However, when it is interacting with the target (not free in the cell anymore) this value can be changed. The results of a typical stability experiment (transcription arrest by the addition of rifampicin) can be misinterpreted in cases where the decay of a sRNA is coupled to that of its mRNA target(s) (Masse, Escorcia et al. 2003). In fact, it was seen for some sRNAs, that if the stability is measured in the absence of ongoing transcription they appear much more stable than mRNA; if the stability is measured while transcription of mRNAs proceeds they are rapidly turned-over (Masse, Escorcia et al. 2003). However, direct biological effects of the action of ribonucleases and other posttranscriptional regulatory factors on sRNA function are not straightforward. There are examples of sRNAs whose function and decay mechanisms have been characterized. For example, the expression of RyhB sRNA is induced under iron starvation. This sRNA pairs with the mRNAs of at least five operons encoding iron-binding proteins and leads to the degradation of their messages. For instance, RyhB acts on the trans-encoded *sodB* mRNA (encoding superoxide dismutase) and triggers its decay in an RNase E dependent fashion. As a result, the synthesis of the iron-binding protein is ceased, leaving the iron available for the essential proteins until the iron starvation condition is overcame. In an RNase E deficient mutant, RyhB and *sodB* mRNA accumulate. The implies that alterations in the half-lives of sRNAs can directly lead to changes in their cellular amount, which in turn can activate or repress the target mRNA.

1.9 Objectives of this work

The aim of the work was to determine the stabilities of the OxyS sRNA and *rpoS* mRNA in exponential and stationary growth phase to address a possible impact of processing of these RNAs on growth phase-dependent regulation. To learn more about the RNases involved in the processing, strains lacking RNases such as endo- or exoribonucleases that have major roles in mRNA and sRNA turn-over in *E. coli* were analyzed. From previous studies it has become evident that the RNA binding protein Hfq influences the stability of sRNA as well as target mRNA (Sledjeski, Whitman et al. 2001; Viegas, Pfeiffer et al. 2007). In order to reveal the role of Hfq on OxyS and *rpoS* mRNA stabilities were determined in strains lacking Hfq. Since DsrA is also expressed at high levels in the presence of hydrogen peroxide a plausible influence of DsrA on OxyS stability can be expected, so OxyS stability was analyzed in DsrA lacking strain backgrounds. Another objective of the work was to elucidate a possible interplay of the two sRNAs and Hfq on cellular RpoS levels in late exponential and stationary growth phase. As OxyS, DsrA and Hfq co-exist under oxidative stress condition, it is very tempting to speculate that there can be competition between the sRNAs and Hfq for the regulation of *rpoS* mRNA translation. To understand this complex mechanism, RNA was isolated at different time points after addition of hydrogen peroxide and quantified OxyS and DsrA levels by Northern blots. In parallel the amount of RpoS protein was also quantified on Western blots by applying a specific antibody.

2 MATERIALS

2.1 Chemicals and Reagents

Name	**Supplier**
2-propanol	Roth
3-(N-morpholino) propanesulfonic acid (MOPS)	Roth
Acetic acid	Roth
Acrylamide/Bisacrylamide (Rotiphorese Gel 30)	Roth
Agarose, *low melt*	Biozym
Ammoniumpersulphate (APS)	Aldrich
Bacto-Agar	Difco
Bromophenolblue	Merck
Calcium chloride	Merck
Chloroform	Roth
Diethylpyrocarbonate (DEPC)	Roth
Dithiothretol (DTT)	Roth
Dimethylsulfoxide (DMSO)	Sigma
Dimethylsuphate	Aldrich
Ethanol (Rotisol)	Roth
Ethidiumbromide	Roth
Ethylene-diamine-tetracetate (EDTA)	Roth
Formaldehyde	Roth
Formamide	Roth
Glucose	Roth
Glycerol	Roth
Glycine	Roth
Isoamylalcohol	Roth
Magnesium chloride	Roth
Magnesium sulphate	Merck
Methanol	Roth
Phenol-Chloroform-Isoamylalcohol	Roth
Phenol-Water	Roth
Polyvinylpyrrolidone	Aldrich
Sodium acetate	Roth
Sodium chloride	Roth
Sodiumdihydrogenphospahte	Roth
Di-Sodiumhydrogenphospahte	Roth
Sodiumdodecylsulphate (SDS)	Roth
Sodium hydroxide	Roth
Sulfuric acid	Roth

Standard-I-Naehrboullion	Merck
N, N, N′, N′- Tetramethylendiamine (TEMED)	Roth
Tris-(hydroxymethyl)-amino methane (Tris)	Roth
Tryptone	Difco
Urea	Roth
Xylene cyanol	Serva
Yeast extract	Roth

All other reagents were purchased either from Roth or Applichem

2.2 Antibiotics

Table 2.1 Antibiotics (sterilized by 0.22 µm filter)

Antibiotic	Stock solution	Concentration in Medium
Ampicillin, Roth	100 mg/ ml	200 µg/ml
Kanamycin , Sigma-Aldrich	10 mg/ml	25 µg/ml
Streptomycin, Sigma-Aldrich	100 mg/ml	100 µg/ml
Tetracycline, Sigma-Aldrich	10 mg/ml	20 µg/ml
Chloramphenicol,Sigma-Aldrich	17 mg/ml	34 µg/ml

2.3 Plasmids

Table 2.2 Palsmids

Plasmid	Markers	Reference
pDrive	*lacZα, Ap^r, Km^r*	Qiagen

2.4 Oligonucleotides

Table 2.3 Oligonucleotides All oligonucleotide were purchased from Operon

Oligonucleotides	Sequence
OxyS	5' GCAAAAGTTCACGTTGG 3'
DsrA	5' GCACTTAAAAAATTCGTTACACCAGG 3'
5S rRNA	5' CGTTTCACTTCTGAGTTCGGAATGG 3'
16S rRNA	5' CAGAAAGGAGGTGATCC 3'
RpoS-Fw	5' ATGAGTCAGAATACGCTGAAAG 3'
RpoS-Rv	5' GCTTACTTACTCGCGGAACAG 3'

2.5 Bacterial Strains

Table 2.4 Bacterial strains

Strain	Markers	Source
MG1655	*F⁻, λ⁻, ilvG-, rfb-50, rph-1*	Blattner, et al., 1977
JVS 2001	Δ*hfq*:: km^R	Urban and Vogel 2007
BL322	*RNaseIII$^+$,nadB$^+$, purI$^+$*	Studier, et al., 1975
BL321	*RNaseIII$^-$,nadB$^+$, purI$^+$*	Studier, et al., 1975
N3433	*lacZ43, relA1, spoT1, thi-1*	Goldblum and Apririon 1981
N3431	*lacZ43, relA1, spoT1, thi-1, rne-3071 (ts)*	Goldblum and Apririon 1981
MG1693	*thyA 715, λ-*	Kushner, et al 1986
SK5726	*thyA 715, λ-, Km^r , pnp-7, rnb-500*	Arraianoet al., 1988
SK5704	*thyA 715, λ-, pnp-7, rnb-500, rne-1*	Arraianoet al., 1988
SK8901	Δ*pcnB, pnp-7, rnb-500, rne-1, thyA715*	O’ Hara, et al., 1995
TK38	MC4100 *hfq1*:: Ω *dsrA1*::cat	Kolmsee T, Freie Univesity, Berlin, 2008
TK39	N3431 *dsrA1*::cat	Kolmsee T, Freie Univesity, Berlin, 2008
TK40	N4333 *dsrA1*::cat	Kolmsee T, Freie Univesity, Berlin, 2008
TK41	BL321 *dsrA1*::cat	Kolmsee T, Freie Univesity, Berlin, 2008
TK42	BL322 *dsrA1*::cat	Kolmsee T, Freie Univesity, Berlin, 2008
TK43	BL321 *hfq1*::Ω	Kolmsee T, Freie Univesity, Berlin, 2008
TK44	BL322 *hfq1*::Ω	Kolmsee T, Freie Univesity, Berlin, 2008
TK46	N3431 *hfq1*::Ω	Kolmsee T, Freie Univesity, Berlin, 2008
TK47	N3433 *hfq1*::Ω	Kolmsee T, Freie Univesity, Berlin, 2008

2.6 Radioactive nucleotides used for labeling

Name	**Supplier**
[γ-^{32}P]-ATP, 3000 Ci/mmol	Hartman Analytic
[α-^{32}P]-dCTP, 3000 Ci/mmol	Hartman Analytic

2.7 Enzymes

Name	**Supplier**
DNase I (RNase-free)	Fermentas
T4-Polynucletidekinase (PNK)	Fermentas
Proteinase K (PCR *grade*)	Boehringer
Restriction endonucleases	NEB/Fermentas
RNasin (RNase inhibitor)	NEB/Promega
RNase A	Roche
Taq-DNA polymerase	Qiagen
BIO-X-ACT-*Long* DNA-Polymerase	Bioline

DeepVent DNA-Polymerase	NEB

2.8 Molecular weight standards

Name	**Supplier**
Prestained Protein Marker, *Broad Range*	NEB
Low Range Protein Marker	Bio-rad
GeneRuler 1kb DNA *Ladder* Plus	Fermentas

2.9 Molecular biological reagents and kits

Name	**Supplier**
Desoxyribonukleosidtriphosphate (dNTPs)	Qiagen/Fermentas
Lumi-Light Western Blotting Substrate I und II	Roche
Ponceau S	Sigma
Nick Translation Kit	GE Healthcare
QIAEX II Gel Extraction Kit	Qiagen
QIAGEN PCR Cloning Kit	Qiagen
QIAquick PCR purification Kit	Qiagen

2.10 Antibodies

RpoS-specific antibody (Rabbit, Anti-RpoS Serum)	Prof. Dr. Regine Hengge, Freie University, Berlin
Anti-Rabbit IgG-Peroxidase conjugate	Sigma

2.11 Equipment and devices

Name	**Supplier**
Biodyne® Transfer membrane	Pall
E.coli Pulser (electro-transformation)	Bio-Rad
Flour-S^{TM} *MultiImager*	Bio-Rad
Gel blotting papers (Whatman papers)	Carl-Roth
UV/VIS spectrometer	Analytic-Jena
Liquid scintillation counter LS6500	Amersham Pharmacia
Phosphoimaging Screens	Bio-rad/Fuji
Phospho-Imager: *Molecular Imager*® FX	Bio-Rad
ProbeQuant^{TM} G-50 and G-25 micro-column	Amersham Pharmacia
Protran® Nitrocellulose Transfer Membrane	Schleicher & Schuell
Semidry-Blot Apparatus	PeQ-lab
UV-Stratalinker 1800 (UV-*crosslink*-Apparatus)	Stratagene
Vacuum-Blot Apparatus	Appligene
Glaswolle (silanized)	Serva
Membrane filter (RC 55, 50 mm, 0,45 µm)	Schleicher & Schuell
Membrane filter (RC 58, 50 mm, 0,2 µm)	Schleicher & Schuell
Screen Eraser K	Bio-rad
Thermocycler Primus 96 plus	MWG
Thermal Cycler S1000	Bio-rad
Video documentations system	Herolab/Decon

3 METHODS

3.1 *E.coli* cultivation

Standard I medium (ST I)	25 g Standard I nutrient broth in 1l H_2O, autoclaved
Agar plates	1.6% (w/v) Bacto-Agar in the media above, autoclaved

3.1.1 *E.coli* plating culture

Bacteria were grown on the STI-agar medium in 9 cm diameter Petri dish at 37 ^{0}C. Antibiotics were added when required.

3.1.2 *E.coli* liquid culture

Bacteria were grown in a conical flask filled to 1/4th of total volume with STI-medium at 37 ^{0}C on a shaker at a speed of 180 rpm. Antibiotics were added when required.

3.1.3 Preparation of glycerol stocks for the -80 ^{0}C strain collection

Collect the cells from liquid overnight culture of *E.coli* (4 ml) from late exponential growth phase by centrifugation at 4 ^{0}C. Resuspend the pellet by 2 ml relevant medium without antibiotics and 1 ml 80% glycerol, transfer the suspensions into two cryo-tubes, freeze them in liquid nitrogen and store at -80 ^{0} C.

3.2 Plasmid minipreparation by alkaline lysis

Lysis solution I	50mM glucose
	25mM Tris-HCl (pH 8.0)
	10mM EDTA (pH 8.0)
	Autoclaved and stored at 4 ^{0} C
Lysis solution II	0.2 M NaoH
	1% (w/v) SDS
	Prepared freshly
Lysis solution III	60 ml 5 M potassium acetate
	11.5 ml glacial acetic acid
	28.5 ml Autoclaved H_2O
	Stored at 4^0 C

Perform plasmid minipreparation by alkaline lysis according to standard protocol (Sambrook and Russell, 2001). Inoculate 3 ml of STI-medium containing the appropriate antibiotic with a single colony of bacteria. Incubate the culture overnight at 37 ^{0} C with vigorous shaking.

Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 60 sec in a microfuge. After centrifugation, remove the medium. Resuspend the bacterial pellet in 200µl of ice-cold lysis solution I by vigorous vortexing and incubate at room temperature for 5 min. Add 200µl of lysis solution II to each bacterial suspension. Close the tube tightly, and gently mix the contents by inverting the tube approximately five times. Store the tube at room temperature for 5 min. Add 200µl of lysis solution III. Close the tube and gently disperse lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 15 min. Centrifuge the bacterial lysate at maximum speed for 20 min in a microfuge. Transfer the supernatant to a fresh tube. Add an equal amount of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 10 min in a microfuge. Transfer the aqueous upper layer to a fresh tube. Precipitate the nucleic acids from the supernatant by adding 2.5 volumes of ethanol. Mix the solution and then allow the mixture to stand at -80 ^{0}C for 30 min. Collect the precipitated nucleic acids by centrifugation at maximum speed for 30 min in a microfuge. Remove the supernatant. Add 1 ml of 70% ethanol to the pellet and then collect the DNA by centrifugation at maximum speed for 10 min in a microfuge. Dissolve the nucleic acids in 50µl of H_2O containing 20µg/ml DNase-free RNase. Store the DNA solution at -20 ^{0} C.

3.3 Chromosomal DNA isolation from *E.coli*

TE buffer: 10 mM Tris-HCl pH8.0, 1 mM EDTA

10% (w/v) SDS
20 mg/ml Proteinase K (stored in small single-use aliquots at -20°C)
5 M NaCl
CTAB/NaCl solution
24:1 chloroform/isoamyl alcohol
Isopropanol
70% ethanol

Collect the cells (20 ml over night liquid culture) by centrifugation at 7000 rpm (using Sorvall® SS-34 rotor) for 10 min at 4 ^{0}C. Resuspend the cells in 567 µl TE buffer and add 3 µl of 20 mg/ml Proteinase K. Mix and incubate at 37°C for 1 hr. Add 100 µl of 5 M NaCl and mix thoroughly. Add 80 µl of CTAB/NaCl solution. Mix and incubate at 65 ^{0}C for 10 min. Add an equal volume of chloroform/Isoamylalcohol and spin 5 min at 13000 rpm in micro-centrifuge. Transfer aqueous phase to a fresh tube. Then add an equal volume of phenol/chloroform/isoamyl alcohol and spin 5 min at 13000 rpm in micro-centrifuge. Transfer aqueous phase to a fresh tube. Precipitate the DNA by adding 0.6 volumes Isopropanol. Wash precipitate with 70% ethanol. Remove supernatant and briefly dry pellet. Resuspend pellet in 100 µl TE buffer.

3.4 Gel electrophoresis of DNA

TAE buffer	0.04 M Tris-acetate
	0.001 M EDTA, pH 8.0
DNA blue marker	4 M urea
	50% saccharose
	50 mM EDTA (pH 8.0)
	0.1% (w/v) bromophenol blue
	0.1% (w/v) xylene cyanol
10% polyacrylamide gel	0.6% ml 10X TBE buffer
(For DNA)	2.0 ml RotiphoreseR
	24 µl 10% (w/v) APS
	6.0 µl TEMED
	3.4 ml H_2O

Perform the gel electrophoresis in TAE buffer according to standard protocol (Protocol 5.1, Sambrook and Russell, 2001).

3.4.1 Gel extraction:

Extract DNA fragment from the agarose gel by using QIAEX II Gel Extraction Kit or QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instruction.

3.5 Molecular cloning

3.5.1 Polymerase chain reaction (PCR)

Standard PCR

Reaction components	100 ng template DNA
	5.0 µl 10X amplification buffer
	3.0 µl dNTP mix (4 mM each)
	2.0 µl primers mix (50 pmol/µl each)
	1 to 2 units thermo stable DNA polymerase
	Add H_2O to 50 µl

Programme	96 ^{0}C	5min	
	96 ^{0}C	1min	35 cycles
	Annealing temperature	45 sec	35 cycles
	72 ^{0}C	1 min/kb	35 cycles
	72 ^{0}C	7 min	

Perform standard PCR either by *Vent* polymerase (New England Biolabs) or by *Taq* DNA polymerase (QIAGEN) according to the manufacturer's instruction.
Precipitate the PCR products by ethanol precipitation. Precipitate the nucleics acids by adding 1/10 volumes of sodium acetate (pH 4.5) and 5 volumes of ethanol. Mix the solution and then allow the mixture to stand at-20 ^{0}C overnight. Collect the precipitated nucleic acids by centrifugation at maximum speed for 30 min in a microfuge. Remove the supernatant and add 1 ml of 70% ethanol to the pellet and then collect the DNA by centrifugation at maximum speed for 10 min in a microfuge. Dissolve the nucleic acids in 50 µl of deionized-H_2O containing 20 µg/ml DNase-free RNase. Store the DNA solution at -20 ^{0}C.

3.6 Preparation of *E. coli* competent cells for electroporation

Inoculate the fresh overnight culture *E. coli* cells into I l STI-medium with 1:100 dilution and grow at 37 ^{0}C with vigorous shaking until the optical density at 600 nm reaches 0.5 to 1.0. After chilling the cells on ice for 15 to 30 min, harvest the cells by centrifugation in a cold rotor at 4,000xg for 15 min. Resuspend the pellet by 1 l cold sterile water, spin down; resuspend in 0.5 l cold sterile water, spin down; and resuspend in 50 ml of 10% ice-cold sterile glycerol, spin down; and resuspend in 6 ml of 10% ice-cold sterile glycerol. Freeze the suspension in aliquots in liquid Nitrogen and store at -80 ^{0}C.

3.6.1 Transformation by electroporation

Mix 10-50 pg plasmid or 25 ng ligated DNA with 40 µl competent cells on ice. Sterilize the Gene Pulser® cuvette (Bio-rad) by UV-stratalinker 1800 (Stratagene) using programme "Auto Crosslink" three times and chill on ice before use. Apply a pulse of electricity (2.4 KV, 5 milliseconds) to the mixture using *E.coli* Pulser (Bio-rad). Afterwards transfer DNA/cell mixture to 1 ml STI-medium without any antibiotics. After incubation at 37 ^{0}C with vigorous shaking for 1 h, plate 25 µl, 100 µl or 900 µl mixture on STI-agar plate containing appropriate antibiotics and incubate the agar-plates at 37 ^{0}C overnight.

3.7 Extraction, purification and analysis of mRNA from *E. coli*

3.7.1 RNA ISOLATION

Hot-phenol extraction

Solution I	0.3 M saccharose 0.01 M NaOAc, pH 4.5
Solution II	0.01 M NaOAc, 2% (w/v) SDS
RNA storage buffer	500 mM Na_2HPO_4 / NaH_2PO_4 250 mM EDTA, pH 6.5
10x DNase buffer	200 mM Tris-HCl pH 8.3 20 mM $MgCl_2$ 500 mM KCl

Resuspend cell pellet by 250 µl RNA extraction solution I and 250 µl solution II. After vigorous mixing, incubate the suspension at 65 ^{0}C for 1.5 min, add 500 µl Roti-Aqua®-Phenol, vortex, incubate at 65 ^{0}C for 3 min, freeze in liquid nitrogen for 2 min, centrifuge for 10 min at 13,000 x g. Repeat hot-phenol extraction three times. Then add 80 µl 3 M NaoAc (pH 4.5) and then add 1200 µl of ethanol to the upper phase and keep the mixture in liquid nitrogen for 1 h or at -20 ^{0}C overnight. After centrifugation (4 ^{0}C, 13,000 x g, 30 min), wash the pellet with 70% ethanol, dry the pellet by using speed vac for 5 min and dissolve it in 180 µl of DEPC H_2O. Add 20 µl 10x DNase buffer and then add DNase I enzyme (15 unit per 1 µg RNA), and incubate the mixture at 37 ^{0}C for 30 min. Then add 200 µl Roti - Phenol/Chloroform/Isoamylalcohol, vortex and centrifuge (13,000x g for 5 min). Treat the upper phase again with Roti - Phenol/Chloroform/Isoamylalcohol. Add 25 µl 3 M NaOAc (pH 7.0) and 1 ml ethanol (96%) to the upper phase and freeze the mixture in liquid nitrogen for 1 h or at -20 ^{0}C overnight. After centrifugation (4°C,13,000 x g, 30 min), wash the pellet with 200µl of 70% ethanol, dry it in Speed vac for 5 min and dissolve the pellet in 50 µl of DEPC-H_2O. Calculate the concentration of the total RNA by using the following formula: C (mg/ml) = A_{260} x dilution factor x 40

3.7.2 Northern Blot

Formaldehyde agarose gel	1.43 g agarose (1% final)
	104 ml DEPC-H_2O
	14.3 ml 10x MOPS (1x final)
	26 ml formaldehyde (2.2 M final)
10% 7 M Urea Polyacrylamide gel	4.5 ml 10x TBE (final 1x)
	11.25 ml Acrylamide/Bisacrylamide
	Make up the volume to 45 ml
RNA loading buffer	50 µl 10x MOPS
	250 µl formamide
	89 µl formaldehyde
	111 µl DEPC-H_2O
RNA blue marker	625 µl 80% glycerol
	25 µl 250 mM EDTA (pH 8.0)
	375 µl 1% bromophenol blue
Fu-mix (Formamide-Urea buffer)	6 M Urea
	80% deionized formamide
	10% 10xTBE
	0.1% Bromophenolblue
	0.1% Xylenecyanol
Denaturing buffer	10 ml 1 N NaoH
	6 ml 5 M NaCl
	184 ml DEPC-H_2O
Neutralizing buffer	20 ml 1 M Tris-HCl
	6 ml 5 M NaCl
	174 ml DEPC-H_2O
50x Denhardt’s reagent	1% (w/v) polyvinylpyrrolidone
	1% (w/v) ficoll 400
	1% (w/v) BSA

10x MOPS	41.8 g/L MOPS 10 mM EDTA 50 mM NaOAc 100 mM NaOH pH 7.0
10x TBE buffer	0.09 M Tris-borate 0.001 M EDTA, pH 8.0
20x SSC	1.5 M NaCl 150 mM Sodium citrate pH 7.0
20x SSPE	3 M NaCl 20 mM EDTA 200 mM NaH_2PO_4 pH 7.0
Prehybridization buffer (for DNA probes, 42^0C for 5 h)	250 mg glycine 2.8 ml DEPC-H_2O 12.5 ml formamide 7.5 ml 20x SSPE 250 µl 10% SDS 1.93 ml 50x Denhardt's 1.25 ml 5 mg/ml LSD
Hybridization buffer (For DNA probes)	2.75 g dextransulphate 13.75 ml formamide 7.5 ml 20x SSPE 0.5 ml 0.2 M NaPPi 250µl 10% (w/v) SDS 550 µl 50x Denhardt's 500 µl 5 mg/ml LSD

Prehybridization buffer	15.5 ml DEPC-H_2O 7.5 ml 20x SSC 500 µl 10% (w/v) SDS 1.25 ml 50x Denhardt's 500 µl 5 mg/ml LSD
Hybridization buffer (For Oligonucleotides)	16.5 ml DEPC-H_2O 7.5 ml 20x SSC 500 µl 10% (w/v) SDS 500 µl 5 mg/ml LSD
Membrane wash buffer	5x SSC 0.01% (w/v) SDS (For OxyS, DsrA RNAs and *rpoS* mRNA) 5x SSC 0.1% (w/v) SDS (For 5S and 16S rRNA)
Nick translation	4.5 µl dATP/dGTP/dTTP mixture 5.0 µl DNA fragment (~ 500 ng) 3.0 µl [α-^{32}P]-dCTP 3.0 µl enzyme 14.5 µl H_2O Incubated at room temperature for 1 h
5'-end labeling of Oligonucleotide	3.0 µl Oligonucleotide (10 pmol/ µl) 1.0 µl 10x T4 PNK buffer 3.0 µl [γ-^{32}P]-ATP 1.0 µl T4 PNK 2.0 µl H_2O Incubated at 37 ^{0}C for 1 h

30 µg per lane total RNA was dissolved in 9 µl RNA loading buffer or in Fu-mix, incubate at 65 ^{0}C for 10 min, and RNA was separated on a 1% (w/v) agarose, 2.2 M formaldehyde gel in 1x MOPS at 100 V for around 3 h or in 7 M Urea-10% PAGE in 1x TBE buffer at 300 V for around 3 h. Afterwards denature RNA by denaturing buffer (30-60 min) and transfer it on to a nylon membrane (Biodyne® B membrane; Pall) by vacuum pressure blotting (under 60-75 mbar for 1h) with transfer solution: 10x SSC in DEPC-H_2O or by semi dry electro-blotting

(300 mA for 3 h). Fix the RNA on the membrane by UV crosslinking (UV-Stratalinker 1800; Stratagene). Membranes were hybridized with specific [α-^{32}P]-dCTP labeled DNA fragments from *E.coli* or with [γ-^{32}P]-ATP end-labeled oligonucleotide. Purify the labeled probe fragments by ProbeQuant^{TM} G-50 (DNA fragments) or G-25 (Oligonucleotides) Micro-column. Denature $2x10^6$ c.p.m (labeled DNA probe and/or oligonucleotide probe) radiolabelled probe together with LSD at 95°C for 5 min before adding to hybridization tube. Incubate the membrane in the prehybridization buffer and then in the hybridization buffer as described above. Wash the membrane by the membrane wash buffer I and II (1-2 min). Quantify the signals using a phosphoimaging system (*Molecular Imager*® FX; Bio-Rad) and the appropriate software (QUANTITY ONE; Bio-Rad).

3.8 SDS-polyacrylamide gel electrophoresis

Resolving gel (30 ml)	7.5 ml 1.5 M Tris-HCl (pH 8.8) Various amount of Rotiphorese® gel 30* 0.3 ml 10% (w/v) SDS 0.3 ml 10% (w/v) APS 12 µl TEMED Add H_2O to 30 ml
Stacking gel (8 ml)	1.0 ml 1 M Tris-HCl (pH 6.8) 1.3 ml Rotiphorese® gel 30 80 µl 10% (w/v) SDS 80 µl 10% (w/v) APS 8 µl TEMED 5.5 ml H_2O
Running buffer	3 g Tris 14.4 g glycine 10 ml 10% (w/v) SDS Add 1 ml H_2O
4x SDS gel-loading buffer	15% (v/v) glycerol 4% (w/v) SDS 200 mM β-mercaptoehanol 125 mM Tris-HCl (pH 6.8) 0.2% (w/v) bromophenol blue

Mix the protein sample with 4x SDS gel-loading buffer in a ratio 4:1 (v/v) and denature the protein at 95 ^{0}C for 5 min. Load the sample in an SDS-polyacrylamide gel of appropriate percentage and run the gel at 150 to 200 volts for 3-5 hours. Stain the gel either with silver salt or with Coomassie brilliant blue.

3.9 Western Blot

Transfer buffer	5% (v/v) methanol 50 mM Tris (pH 7.9) 40 mM glycine 0.045 (w/v) SDS
TBS	50 mM Tris (pH 7.4) 0.2 M NaCl
Blocking agent	5% (w/v) milk powder in TBS

Separate the protein samples by SDS-polyacrylamide gel electrophoresis and transfer them to Protran® nitrocellulose transfer membrane (Carl-Roth). Perform the electrophoretic transfer using western blotting machine (PeQlab biotechnology GmbH) at 1.1mA/cm^2 for 45 min. Use the Whatman paper saturated with transfer buffer as a reservoir. Rinse the blot briefly in TBS and soak it in blocking agent at 4 ^{0}C overnight. Incubate the blot with specific antibody in TBS containing 5% (w/v) milk powder on a shaker at room temperature for 2 hours, and then wash it by shaking in TBS three times, 5 min each. Then incubate the blot with the second antibody conjugated with enzyme (*e.g.* alkaline phosphatase or peroxidase) in TBS containing 5% (w/v) milk powder on a shaker at room temperature for 2 hours, and then wash the membrane by shaking in TBS three times, 5 min each. For detection of peroxidase, apply lumi-light western blotting substrate 1 and 2 (v:v = 1:1) on the blot, and capture the chemiluminiscence on an X-ray film. Quantify the signals by software QUANTITY ONE (Bio-Rad).

3.10 Transcription inhibition

Stability of an individual RNAs can be determined by inhibiting the ongoing transcription by the addition of rifampicin. Rifampicin binds to β subunit of RNA polymerase and blocks the entry of first nucleotide which leads to the arrest of the transcription. Inoculate 5 ml of STI-medium containing the appropriate antibiotic with a single colony of *Escherichia coli* K12 cells. Incubate the culture overnight at 37 ^{0}C under constant shaking. *Escherichia coli* K12 cells from overnight cultures were diluted 1/100 in Std-I medium and subsequently grown at 37 ^{0}C. Stability of OxyS, DsrA sRNAs and *rpoS* mRNA were determined in exponential (OD_{600}= 0.5) and/or in stationary growth phase (OD_{600}= 3.5). Once the cultures reached an optical density OD_{600}= 0.5 or/and OD_{600}= 3.5, cultures were stressed with 1mM H_2O_2 for 7min and then the RNA half-lives under stress were determined by inhibiting the transcription with Rifampicin (final concentration: 500 µg/ml, SERVA Electrophoresis GmbH. Co.). Total RNA was isolated before (0 min) and 2, 4 8, 16, 32 and 64 min after Rifampicin addition by hot-phenol method and Northern blot analysis was performed. Half-lives were calculated from linear-log graphs of time after Rifampicin addition against RNA signal intensity. The intensity of the sRNA and mRNA bands was normalized to the intensity of the 5S or 16S rRNA.

The effect of RNase III was studied in the *rnc*$^-$ mutant (BL321) derived from strain BL322 (Studier 1975) and the effect of PNPase, RNase II and RNase E was studied in the strain *pnp*-7, *rne*^{ts} and *rnb*-500 derived from strain SK5726 (Arraiano, Yancey et al. 1988). The effect of RNase E was studied in the *rne*^{ts} mutant (N3431) derived from strain N3433 (Goldblum and Apririon 1981), In the case of strains N3431 and N3433, the cultures were shifted to 42 ^{0}C for 10min for the inactivation of RNase E in strain N3431 and then cells were treated with 1mM H_2O_2 for 7min and transcription inhibition was performed as described above.

3.11 Translation inhibition

Translation inhibition was performed to determine the stability of RpoS in various strain backgrounds. Inoculate 5 ml of STI-medium containing the appropriate antibiotic with a single colony of *Escherichia coli* K12 cells. Incubate the culture overnight at 37 ^{0}C under constant shaking. *Escherichia coli* K12 cells from overnight cultures were diluted 1/100 in Std-I medium and subsequently grown at 37 ^{0}C. Once the cells reached mid-exponential phase (OD_{600} - 0.5) cells were first treated with H_2O_2 for 112 min and then translation was inhibited by the addition of Chloramphenicol (final concentration of 175µg/ml, SERVA Electrophoresis GmbH. Co.) to a growing culture. In stationary phase (OD_{600}: 3.5) translation was inhibited after 7 min of H_2O_2 treatment. Translation was inhibited by the addition of Chloramphenicol to the growing culture and 3 ml cells were collected at regular intervals such as 0, 5, 10, 20, 30, 40 and 60 min after the addition. Cells were suspended in 1ml of 50mM glucose, 25mM Tris-HCl (pH 8.0) and 10mM EDTA (pH 8.0) as SDS – PAGE and immunoblot was performed as described above.

3.12 Oxidative Stress

Inoculate 5 ml of STI-medium containing the appropriate antibiotic with a single colony of *Escherichia coli* K12 cells. Incubate the culture overnight at 37 ^{0}C under constant shaking. *Escherichia coli* K12 cells from overnight cultures were diluted 1/100 in Std-I medium and subsequently grown at 37 ^{0}C. Once the cultures reached the required optical density OD_{600}= 0.5 (exponential) and/or OD_{600}= 3.5 (stationary growth phase), *E.coli* cultures were treated with 1mM H_2O_2 for 7min and then the stabilities of RNAs (OxyS, DsrA and *rpoS* mRNA) and/or Protein (RpoS) under stress was determined by inhibiting the transcription by Rifampicin and/or inhibition of translation by Chloramphenicol.

4 RESULTS

4.1 Effect of growth rate on OxyS turn-over in *E. coli* MG1655

To elucidate the role of ribonucleases and growth phase on the processing of the small non-coding RNA OxyS, first we determined the stability of OxyS in wild type *E.coli* strain MG1655 (*E.coli* K12 derivative). Previously, in two publications the half life of OxyS was reported, in *E.coli* strain K12 to be 12-15 min (Altuvia, Weinstein-Fischer et al. 1997), and in MC4100 to be 30 min (Zhang, Wassarman et al. 2002). In both cases the stabilities were determined only in exponential growth phase (OD_{600}: 0.4). It was also shown that OxyS represses the synthesis of RpoS (Altuvia, Zhang et al. 1998), which is an alternative sigma factor required for the transition of *E.coli* cells into stationary growth phase as well as in the stress adaptation. So it is very tempting to speculate a possible role of OxyS processing in RpoS expression in different growth phases. So we have determined the stabilities of OxyS in *E.coli* MG1655, in early exponential (OD_{600}:0.5) as well as in late stationary growth phase (OD_{600}: 3.5). To induce the chromosomally encoded OxyS expression, *E.coli* strain MG1655 was treated with 1 mM H_2O_2 for 7 min and then rifampicin was added to stop further transcription either in early exponential (OD_{600}: 0.5) or in late stationary growth phase (OD_{600}: 3.5) to determine the stabilities. We observed average half-lives of 22 ± 2.3 min in early exponential phase and 21.2 ± 6.2 min in stationary phase, respectively, for the K12 derived strain MG1655 (Fig 4.1, Fig 4.9A and Table 4.1- Page No: 50) indicating no significant changes in OxyS stability in different growth phases. In both growth phases the accumulation of smaller processing products of approximately 40-50 nt in size was observed, which was reported earlier too (Altuvia, Weinstein-Fischer et al. 1997)

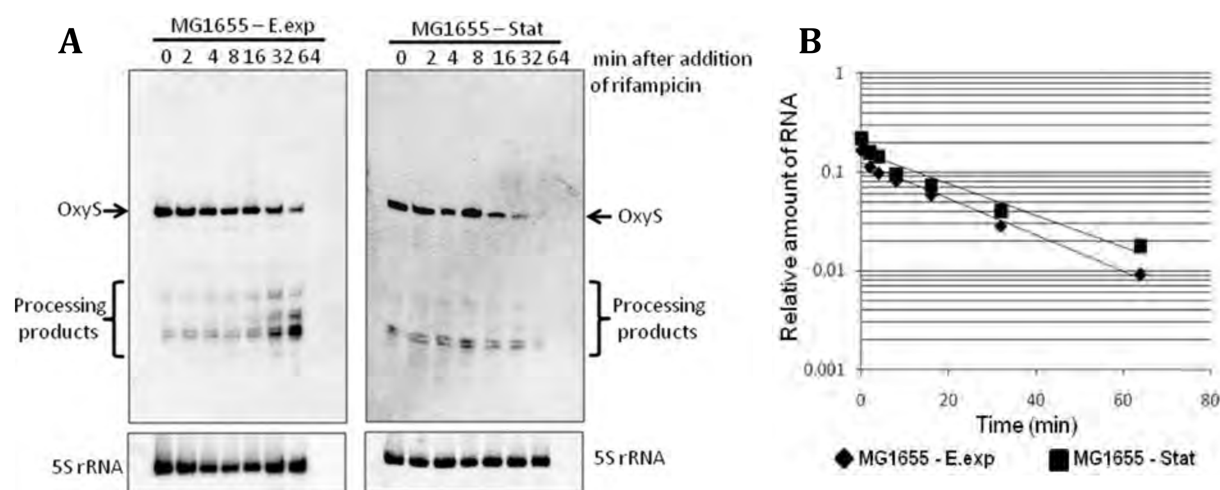
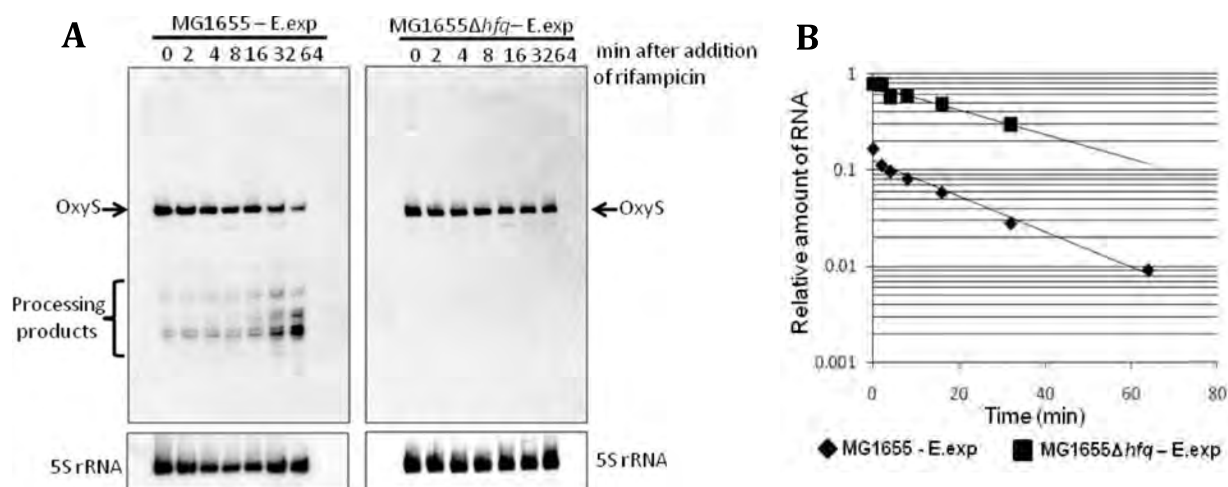


Figure 4.1 Stability determination of OxyS in *E.coli* MG1655, under oxidative stress in early exponential and in stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** Northern blot analysis of the isolated total RNA from early exponential and stationary phases of *E.coli*

MG1655. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** Half life determination of the OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.exp** – Early exponential growth phase: The half life of OxyS in early exponential phase is about 22 min. **Stat** – Stationary phase: The half life of OxyS in stationary phase is about 21 min.

4.2 The effect of the RNA chaperone, Hfq on OxyS stability

It has been shown that Hfq facilitates the base pairing of sRNA to its target mRNA and thereby influences the stability and/or translation directly and/or indirectly (Moll 2003; Storz, Opdyke et al. 2004; Valentin-Hansen, Eriksen et al. 2004). It has been shown to decrease the stabilities of certain mRNAs (Tsui, Feng et al. 1997; Vytvytska, Moll et al. 2000) and to increase the stabilities of certain sRNAs (Sledjeski, Whitman et al. 2001; Masse, Escorcia et al. 2003; Geissmann and Touati 2004). Previously Zhang et al., (2002) have shown that the RNA chaperone Hfq facilitates the base pairing of OxyS to its target mRNAs. However, an effect of Hfq on the turn-over of OxyS in *E.coli* strain MC4100 was not observed in exponential growth phase (Zhang, Wassarman et al. 2002).We were not able to observe any changes in the turn-over rate of OxyS in *E.coli* strain MG1655Δ*hfq* in exponential phase, confirming the earlier observations. In early exponential growth phase the half-life of OxyS in a strain lacking Hfq is almost similar to that in the isogenic wild type strain MG1655 (27.7 ± 6.8 min compared to 22.0 ± 2.3 min; Fig 4.2, Fig 4.9A and Table 4.1- Page No: 50). However, a strong effect of Hfq on OxyS turn-over was observed in stationary phase in strain MG1655. In strain MG1655Δ*hfq*, OxyS stability was significantly decreased in stationary phase (9.3 ± 0.3 min) when compared to its isogenic wild type strain MG1655 (21.2 ± 6.2) (Fig 4.9A and Table 4.1- Page No: 50). We were also not able to observe any smaller processing products in the *hfq*$^{-}$ strain when compared to the isogenic wild type strain as described earlier (Zhang, Wassarman et al. 2002).



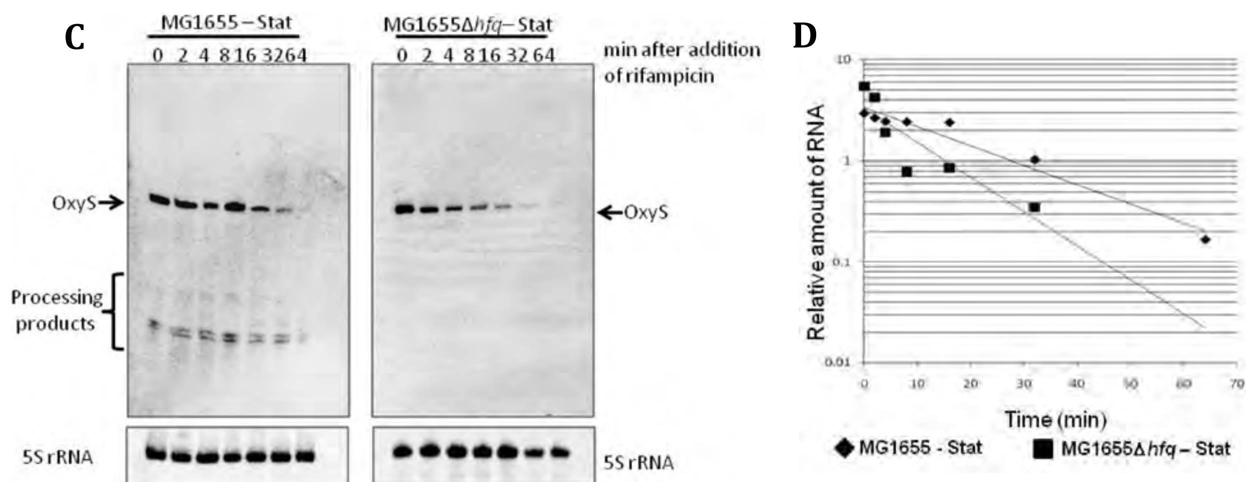


Figure 4.2 Stability determination of OxyS in *E.coli* MG1655 and MG1655Δ*hfq*, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* MG1655 and MG1655::Δ*hfq*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp** – Early exponential growth phase: The half life of OxyS in *E.coli* MG1655 is about 22 min and in MG1655Δ*hfq* about 27 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* MG1655 is about 21 min and in MG1655Δ*hfq* about 9 min.

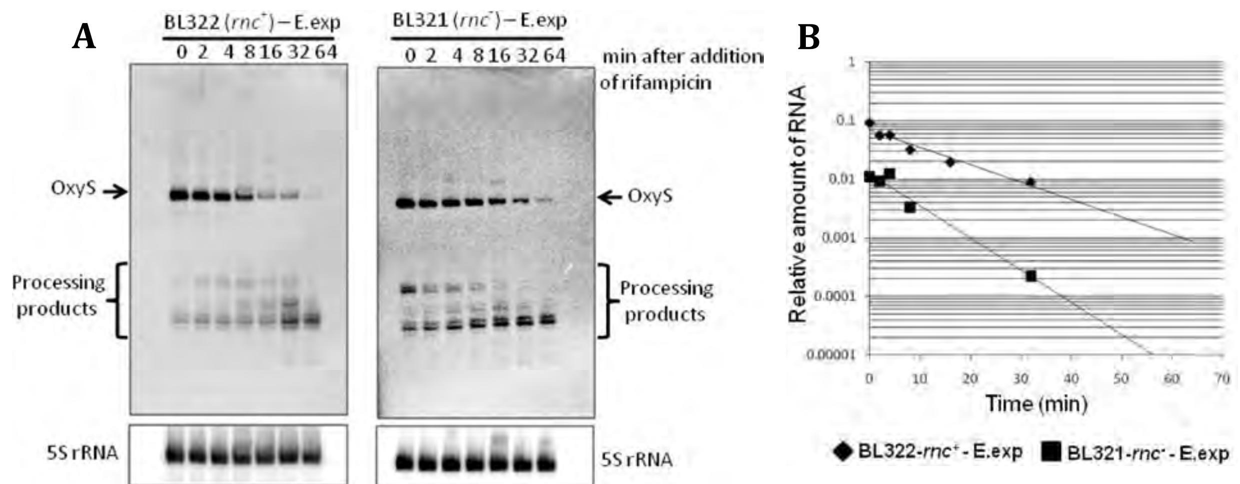
4.3 Influence of the endoribonucleases RNase E and RNase III on OxyS turnover

Some sRNAs represent a primary transcript, while some are generated from longer RNA transcripts by processing (Faubladier, Cam et al. 1990; Argaman, Hershberg et al. 2001; Gottesman, Storz et al. 2001). These sRNAs can occur in multiple sized species and others undergo a growth phase- dependent processing (Majdalani, Cunning et al. 1998; Argaman, Hershberg et al. 2001; Repoila and Gottesman 2001; Opdyke, Kang et al. 2004; Viegas, Pfeiffer et al. 2007). It has also been shown in different bacterial species that the stability of sRNAs differ greatly, some are very stable whereas, others are turned over faster. Since ribonucleases play a key role in RNA decay and processing, we wanted to analyze the role of ribonucleases (endo- and exoribonucleases) in the processing of OxyS RNA in different growth phases.

It is known for some sRNAs that their turn-over is catalyzed by endoribonuclease E and/or III (Faubladier, Cam et al. 1990; Lin-Chao, Wei et al. 1999; Vogel, Argaman et al. 2004; Morita, Maki et al. 2005; Rasmussen, Eriksen et al. 2005; Davis and Waldor 2007; Viegas, Pfeiffer et al. 2007). It has also been shown by several groups that the base pairing of sRNA to the target mRNA can create new RNase cleavage sites (Masse, Escorcia et al. 2003; Afonyushkin,

Vecerek et al. 2005) or can stabilize its target by protecting from cleavage (Moll 2003; Reichenbach, Maes et al. 2008). In a recent publication the authors showed that the paring of sRNA to the target mRNA led to an alternate RNase III cleavage site within the sRNA/mRNA duplex, thereby altering the processing (Resch, Afonyushkin et al. 2008). Endoribonuclease RNase III preferentially cleaves double stranded RNA regions, while RNase E recognizes single stranded AU rich segments (Li, Chelladurai et al. 1993; Mackie and Genereaux 1993; McDowall, Lin-Chao et al. 1994).
To address the role of endoribonuclease in OxyS turn-over, we analyzed OxyS stabilities in strains lacking RNases and compared to the isogenic wild type backgrounds. Since the established mutants that we have used in this work to determine the role of different RNases on OxyS stability were derived from different parental strains, we have determined the half-lives in the corresponding isogenic parental strains too (Figure 4.9A and Table 4.1- Page No: 50).

To discern the role of RNase III in the turn-over of OxyS, half-lives of OxyS in the *rnc*$^-$ strain (BL321) and in the corresponding wild type strain were analyzed (BL322). OxyS turn-over rates did not differ significantly in *rnc*$^-$ strain when compared to the *rnc*$^+$ strain (BL322) and an significant effect of growth phase was also not observed (Fig 4.3, Fig 4.9A and Table 4.1- Page No: 50).



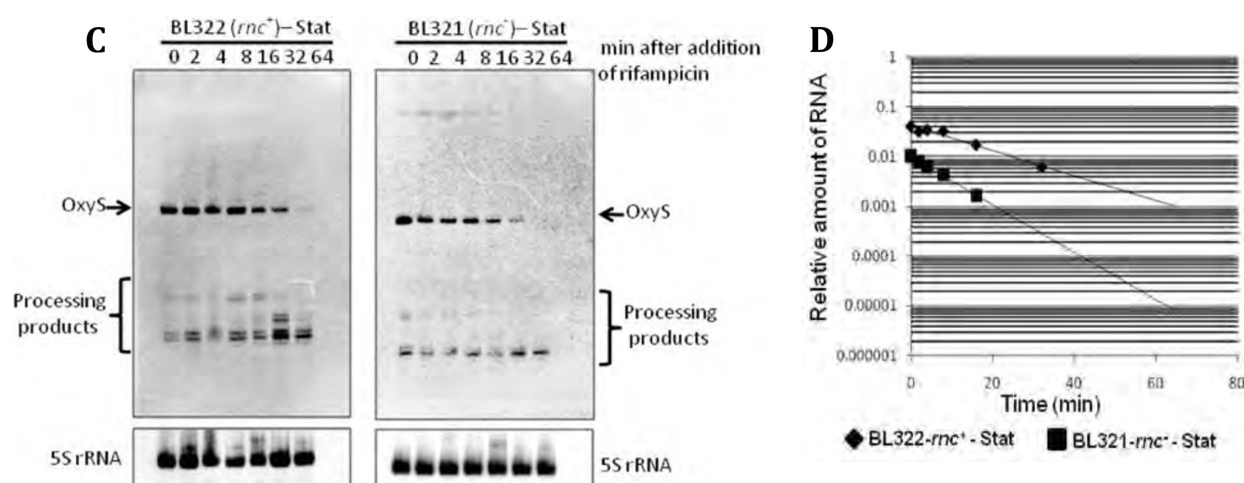


Figure 4.3 Stability determinations of OxyS in *E.coli* BL322-*rnc*$^+$ and *E.coli* BL321-*rnc*$^-$, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* BL322 and BL321. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp** – Early exponential growth phase: The half life of OxyS in *E.coli* BL322 is about 14 min and in BL321 is about 16 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* BL322 is about 16 min and in BL321 is about 16 min.

In previous studies it has been shown that RNase E plays an important role in the turn-over of RyhB, a small RNA that is expressed under iron starvation and it was also shown that the RNA chaperone Hfq protects RyhB against RNase E cleavage (Masse, Escorcia et al. 2003). Hfq as well as RNase E are known to bind to identical sequences. Hfq preferentially binds to AU-rich single stranded regions of RNA (Moller, Franch et al. 2002; Schumacher, Pearson et al. 2002) and the recognition sequence for RNase E cleavage is also single stranded AU sequences (Mackie and Genereaux 1993; McDowall, Lin-Chao et al. 1994). It was shown in the case of OxyS that Hfq binds to AU-rich linker region (Zhang, Wassarman et al. 2002). As we observed decreased stability of OxyS in the absence of Hfq in stationary phase (Fig 4.2, Fig 4.9A and Table 4.1- Page No: 50), we speculated that RNase E might play a role in the degradation of OxyS in stationary growth phase. To reveal a possible role of RNase E in the OxyS turn-over, we determined the stabilities of OxyS in RNase E temperature sensitive strain (N3431-*rne*^{ts} : RNase E can be inactivated by shifting the growing cultures from 32 ^{0}C to 42 ^{0}C) and in the corresponding isogenic wild type strain (N3433) in both growth phases. In early exponential phase when RNase E was inactivated at the non-permissive temperature, we did not observe any significant change in the stability of OxyS when compared to the isogenic wild type strain.

But we observed an increased stability of OxyS in stationary phase when RNase E was inactivated (Fig 4.4, Fig 4.9A and Table 4.1- Page No: 50). This increased stability of OxyS in the absence of RNase E activity might explain the protective role of Hfq in the stabilization of OxyS in stationary phase as it was shown in the case of MicA sRNA (Viegas, Pfeiffer et al. 2007).

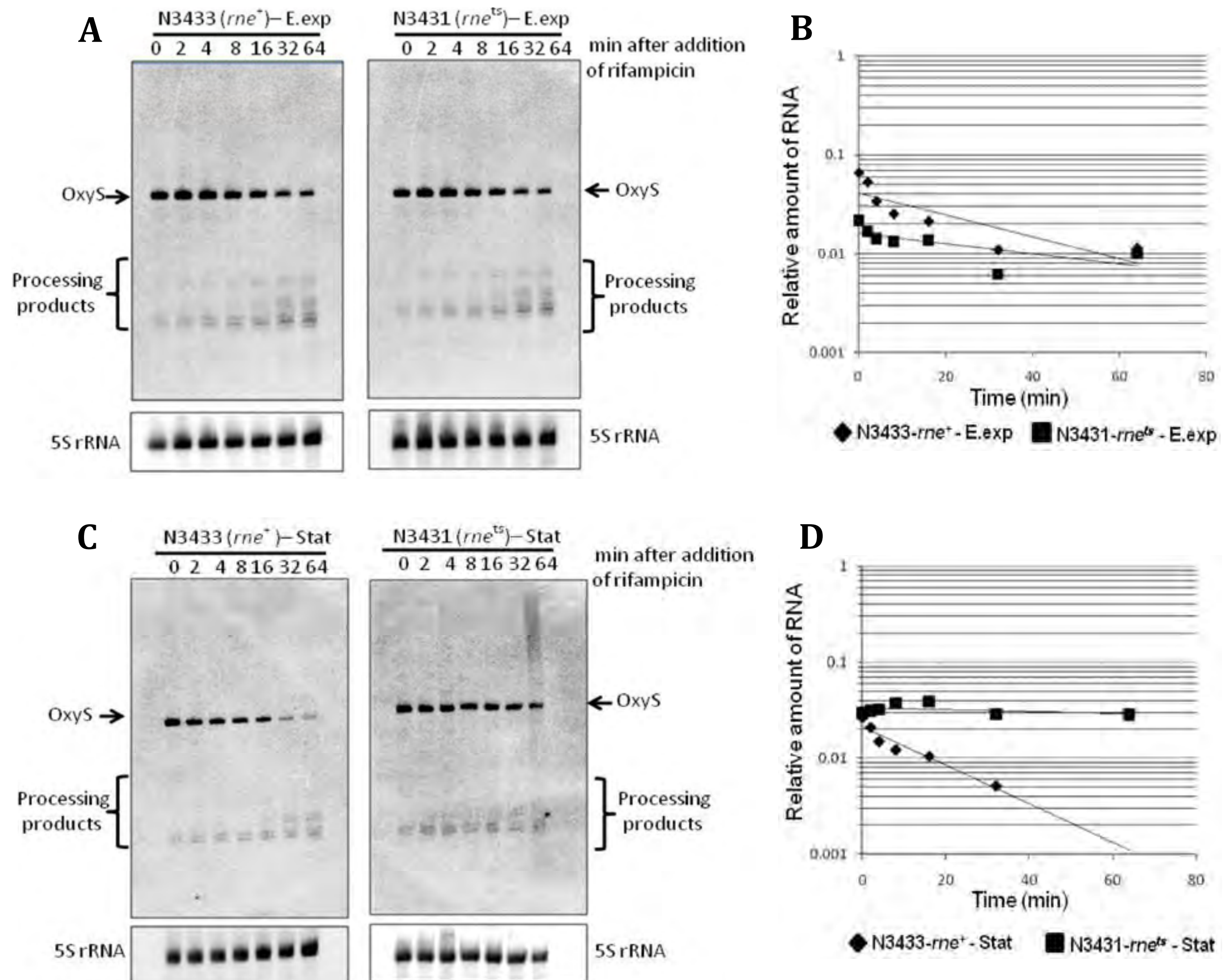


Figure 4.4 Stability determination of OxyS in *E.coli* N3433-*rne*$^{+}$and *E.coli* N431-*rne*^{ts}, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* N3433 and N3431. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp –** Early exponential growth phase: The half life of OxyS in *E.coli* N3433 is about 24 min and in N3431 is about 21 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* N3433 is about 17 min and in N3431 is >70 min.

We were able to observe the accumulation of smaller OxyS processing products in the strain lacking RNase III (BL321) (Fig 4.3) as well as in a strain when RNase E (N3431) (Fig 4.4) was inactivated and in their corresponding isogenic wild type strains. These results indicate that neither of these endoribonucleases (RNase E or RNase III) plays a role in the generation of the OxyS processing products or their decay.

4.4 Influence of the exoribonucleases PNPase, RNase II and endoribonuclease RNase E on OxyS turnover

It was shown earlier that exoribonucleases along with endoribonucleases take part in the maturation of small stable RNA molecules such as 4.5S, 6S and tmRNAs (Li, Pandit et al. 1998). Subsequently the regulatory role of exoribonuclease, polynucleotide phosphorylase (PNPase) in controlling the levels of MicA and RyhB sRNAs was shown (Andrade and Arraiano 2008). Since neither of endoribonucleases (RNase E or RNase III) had a significant effect on OxyS turn-over in early exponential growth phase, we wanted to evaluate the roles of exoribonucleases PNPase and RNase II. We used a strain lacking both exoribonucleases; PNPase and RNase II (SK5726) and a strain lacking PNPase and RNase II along with RNase E activity at non-permissive conditions (SK5704). We have determined the stabilities of OxyS in SK5726, SK5704 and in their isogenic wild type strain MG1693 in order to reveal their role in the processing of OxyS. We chose a double mutant SK5726 in our studies while, no change in mRNA turn-over can be observed in single mutants, so significant *in vivo* effects can only be observed when both are missing (Kivity-Vogel and Elson 1967; Lennette, Gorelic et al. 1971; Donovan and Kushner 1983; Arraiano, Yancey et al. 1988).

In strain SK5726, which lacks RNase II and PNPase the turn-over rate of OxyS is similar when compared to the isogenic wild type MG1693 in both growth phases (Fig 4.5, Fig 4.9B and Table 4.1- Page No: 50). Furthermore, we have analyzed the decay rates of OxyS in strain SK5704, in this strain in addition to PNPase and RNase II, endoribonuclease RNase E can be inactivated when grown at non-permissive temperature. Comparison of OxyS decay rates in exponential growth phase of triple mutant (SK5704: 34.3 ± 1.0 min) to that in the parental double mutant (SK5726: 15.5 ± 0.5 min) and to the isogenic wild type MG1693 strain (MG1693: 16.5 ± 0.5 min) indicate that all three RNases together act in the OxyS turn-over (Fig 4.5, Fig 4.9B and Table 4.1- Page No: 50). The fact that OxyS in SK5704 strain is still less stable in exponential phase when compared to the stationary phase (no significant turn-over is detected in stationary phase, Fig 4.5B) suggests that additional ribonucleases might contribute to turn-over in exponential phase. The strong stabilization of OxyS that is observed in stationary growth phase of SK5704 can be ascribed to the lack of RNase E, since the same effect was observed in the *rne*^{ts} strain N3431 (Fig 4.9A). In the *pnp*$^{-}$*rne*^{ts} *rnb*$^{-}$ strain also we were able to observe OxyS processing products, but the accumulation of processing products were less abundant when compared to the other strains in the presence of Hfq.

Our results disclose that the influence of different RNases as well as the dependence of growth phase on OxyS turn-over. We were able to show that the processing of OxyS cannot be assigned to the action of a single ribonuclease.

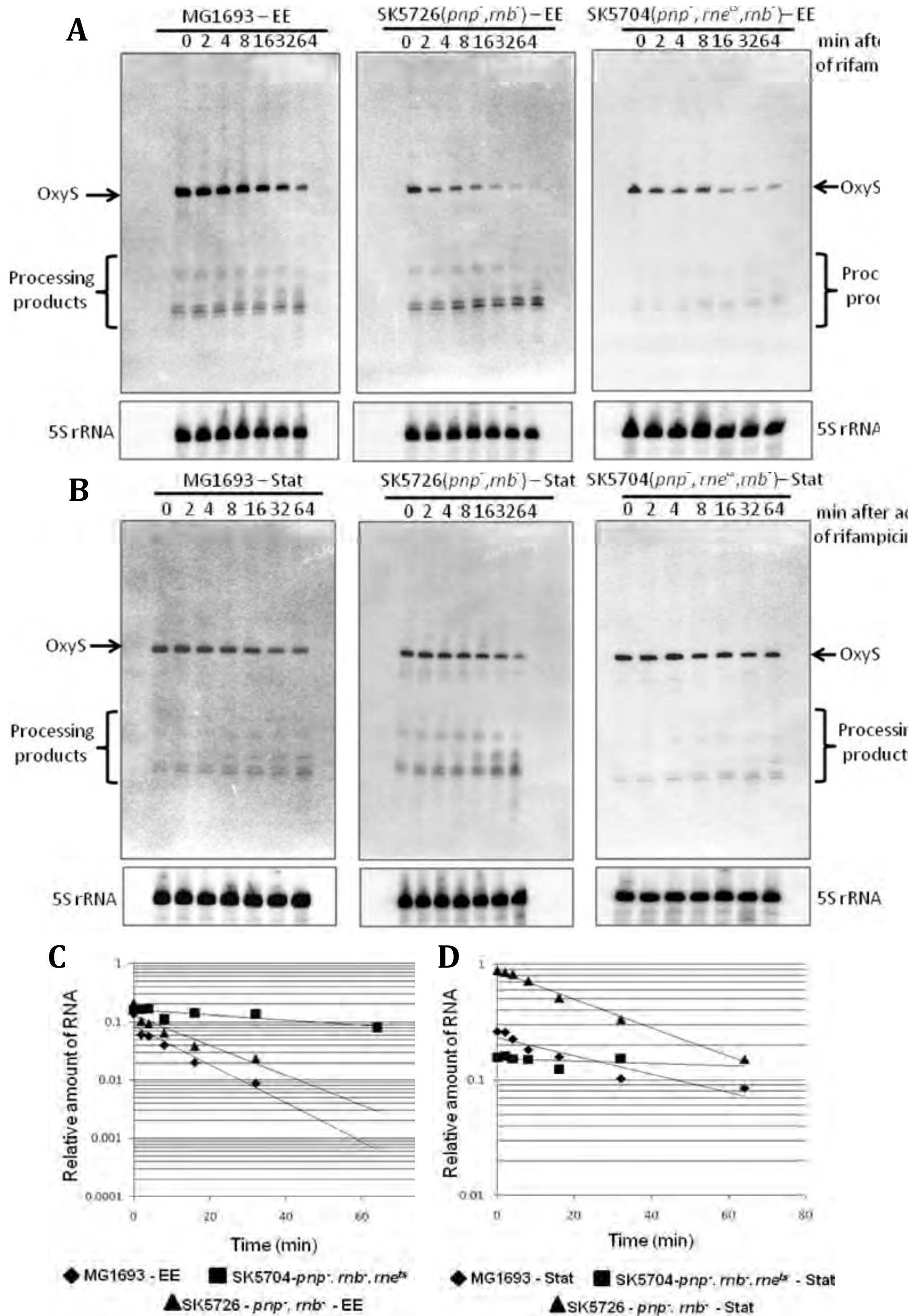


Figure 4.5 Stability determination of OxyS in *E.coli* MG1693, SK5726 (*pnp*-7,*rnb*-500) and *E.coli* SK5704 (*pnp*-7,*rnb*-500 & *rne*^{ts}), under oxidative stress in early exponential

and in stationary growth phases. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(B)** Northern blot analysis of the isolated total RNA from *E.coli* MG1693, *E.coli* SK5726 (*pnp*-7,*rnb*-500) and *E.coli* SK5704 (*pnp*-7,*rnb*-500 & rne^{ts}). Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and then the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(C)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp –** Early exponential growth phase: The half life of OxyS in *E.coli* MG1693 is about 16 min, in *E.coli* SK5726 is about 15 min and in SK5726 is about 34 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* MG1692 is about 26 min, *E.coli* SK5726 is about 22 and in SK5704 is >70 min.

4.5 The influence of DsrA on the decay rate of OxyS

Massé and co-workers in 2003 have reported that sRNA/mRNA complexes can be recognized by endoribonuclease RNase E, which will lead to the coupled degradation of both sRNA and target mRNA. It was also shown that binding of sRNA to the target mRNA will lead to the generation of new RNase cleavage sites (Masse, Escorcia et al. 2003; Vogel, Argaman et al. 2004; Afonyushkin, Vecerek et al. 2005). *rpoS* mRNA translation is regulated by at least two other small RNAs besides OxyS, DsrA and RprA (Repoila, Majdalani et al. 2003). The mechanism by which DsrA and RprA activate the *rpoS* mRNA translation was analyzed in detail, but the mechanism by which OxyS represses *rpoS* mRNA translation is still elusive (Majdalani, Cunning et al. 1998; Majdalani, Hernandez et al. 2002; Updegrove, Wilf et al. 2008). We were able to detect DsrA but not RprA under our experimental conditions (cells were grown at 37^0C and oxidative stress) along with OxyS in both growth phases (Supplementary Fig 9.1). Therefore we speculated that there can be competition between OxyS and DsrA in pairing to *rpoS* mRNA and/or binding to Hfq and thus on the turn-over rates of each other.

Under our experimental conditions (see Methods) DsrA was more abundant in stationary phase than in exponential phase, about 10 fold difference was observed in all tested strains (Supplementary Fig 9.1). We were not able to determine the stabilities of DsrA in any of the wild type strains, since there was no significant turn-over of DsrA observed. These higher stabilities of DsrA were observed in the presence or absence of hydrogen peroxide. But we observed reduced stability of DsrA in the absence of Hfq in strain MG1655 (21 min) confirming earlier published data (Sledjeski, Whitman et al. 2001). Like OxyS, reduced stability of DsrA in the absence of Hfq was observed only in stationary phase, but not in early exponential growth phase (see Fig 4.9A and Table 4.1- Page No: 50).
If there is a competition between OxyS and DsrA for binding to *rpoS* mRNA, OxyS stability should be influenced in the absence of DsrA. To test this hypothesis, we have analyzed an existing DsrA mutant. In strain MG1655 the lack of DsrA had no significant effect on OxyS

turn-over in exponential phase but we were able to observe faster turn-over of OxyS in stationary phase (1.4 fold faster turn-over) (Fig 4.6, Fig 4.9A and Table 4.1- Page No: 50)

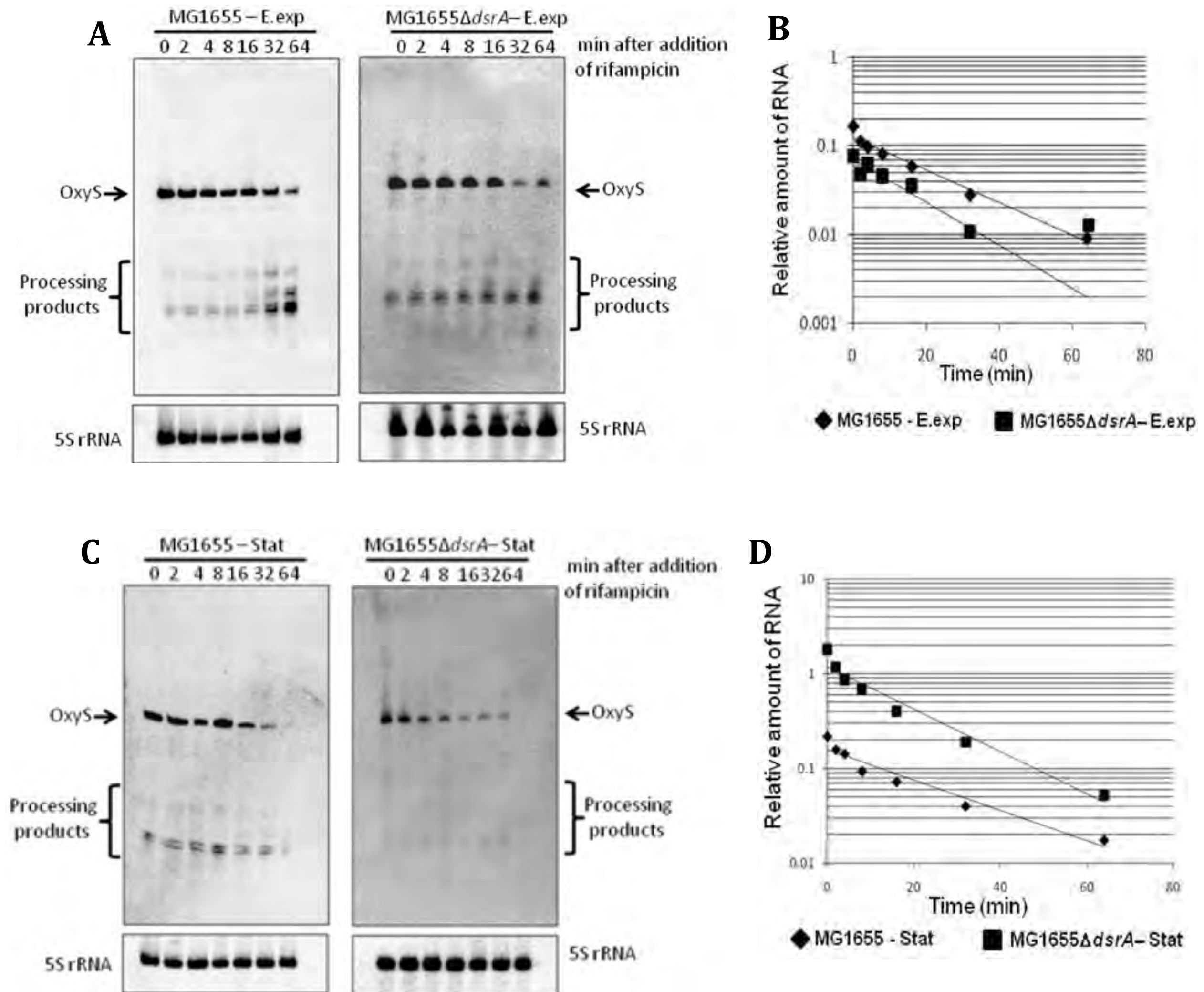


Figure 4.6 Stability determination of OxyS in *E.coli* MG1655 and *E.coli* MG1655Δ*dsrA*, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* MG1655 and MG1655Δ*dsrA*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp** – Early exponential growth phase: The half life of OxyS in *E.coli* MG1655 is about 22 min and in MG1655Δ*dsrA* is about 22 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* MG1655 is about 21 min and in MG1655Δ*dsrA* is about 16 min.

4.6 The decay rate of OxyS in double mutants – N3431Δ*hfq*, BL321Δ*hfq*, N3431Δ*dsrA*, BL321Δ*dsrA*

To further analyze the roles of the RNA chaperone Hfq, DsrA, endoribonucleases (RNase E and RNase III) and exoribonucleases, we constructed mutants lacking Hfq and/or DsrA with different genetic backgrounds such as, N3431(*rne*^{ts})Δ*hfq*, BL321(*rnc*$^{-}$)Δ*hfq*, N3431(*rne*^{ts})Δ*dsrA*, BL321 (*rnc*$^{-}$) Δ*dsrA* and *hfq-dsrA* double mutants (Fig 4.9A and Table 4.1- Page No: 50) (in collaboration with Dr. Regine Hengge, Freie University of Berlin, Germany). We were able to observe differential effects in the turn-over of OxyS in the absence of Hfq in different strain backgrounds. In strains N3433 and N3431 a little increase in the stability of OxyS was observed in early exponential phase when Hfq was missing (*rne*$^{+}$Δ*hfq*, *rne*^{ts}Δ*hfq*) (Fig 4.7, Fig 4.9A and Table 4.1- Page No: 50).

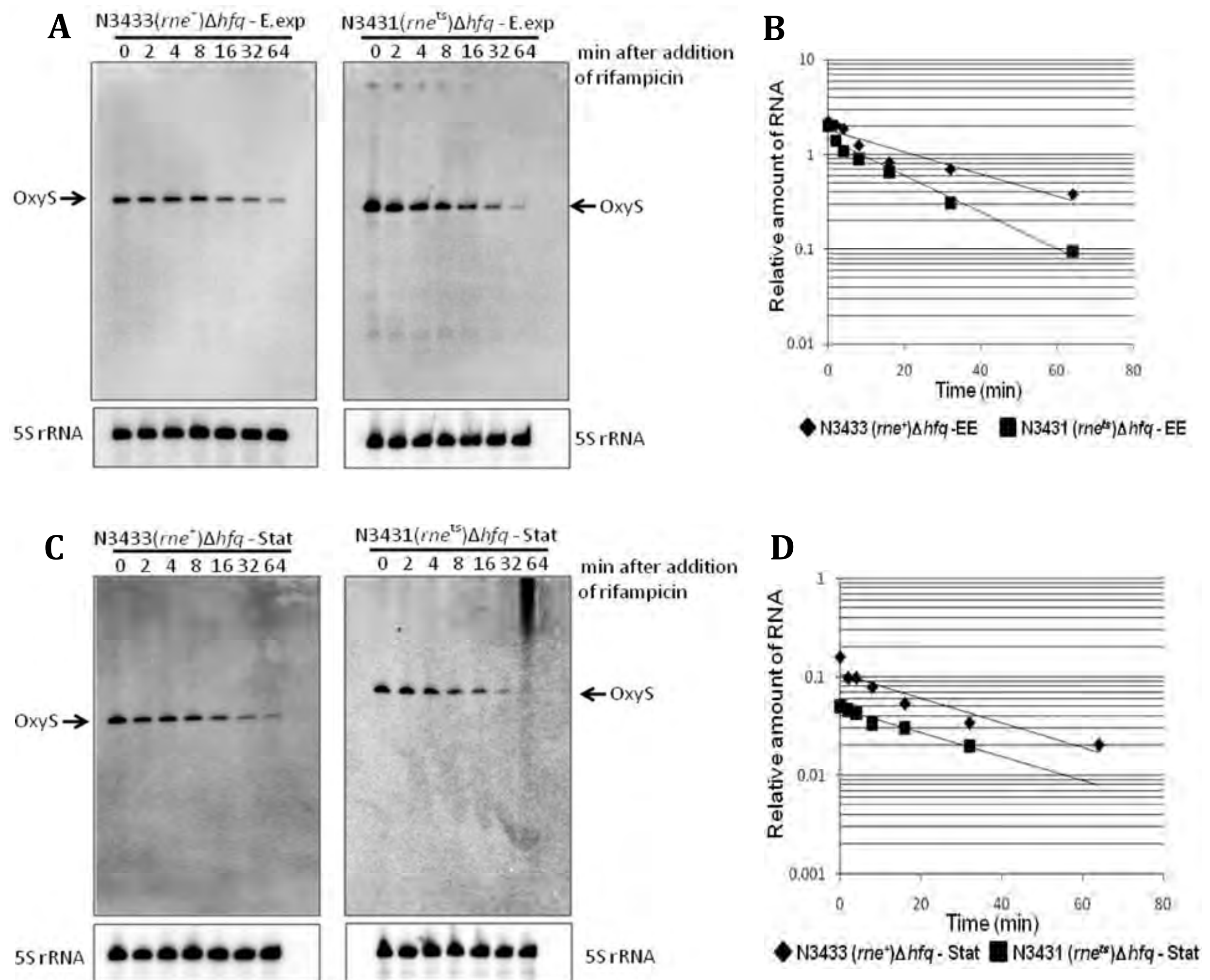
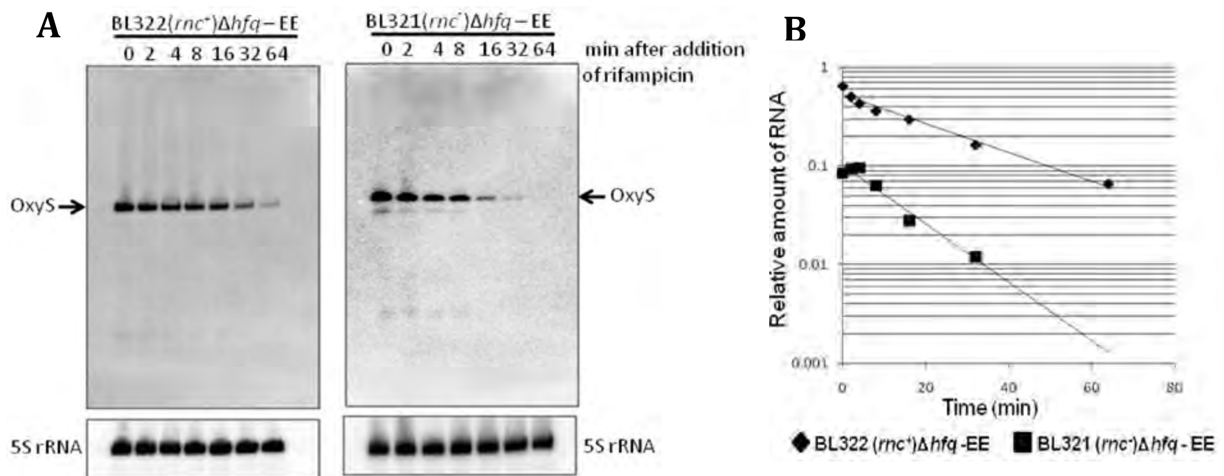


Figure 4.7 Stability determination of OxyS in *E.coli* N3433Δ*hfq* and *E.coli* N3431Δ*hfq*, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)**

& **(C)** Northern blot analysis of the isolated total RNA from *E.coli* N3433*Δhfq* and N3431Δ*hfq*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp –** Early exponential growth phase: The half life of OxyS in *E.coli* N3433Δ*hfq* is about 29 min and in N3431Δ*hfq* is about 26 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* N3433*Δhfq* is about 19 min and in N3431Δ*hfq* is about 20 min.

We were not able to observe any change in decay pattern in strain BL322 (*rnc*$^+$) Δ*hfq* when compared to the isogenic strain harboring Hfq (BL322) (Fig 4.9A and Table 4.1- Page No: 50). However, lack of Hfq in strain BL321 (*rnc*$^-$Δ*hfq*) has led to the decreased stability of OxyS in exponential phase (1.4 fold decrease) when compared to its isogenic strain containing Hfq (BL321) (Fig 4.8). In contrast, lack of Hfq in strains N3433 (*rne*$^+$) and in BL322 (*rnc*$^+$) did not have any significant effect on OxyS turn-over in stationary phase, however, the effects were surprisingly strong in stationary phase when Hfq was absent in strain N3431 (*rne*^{ts}Δ*hfq*) or BL321 (*rnc*$^-$Δ*hfq*) backgrounds (Fig 4.7 and 4.8, Fig 4.9A and Table 4.1- Page No: 50)



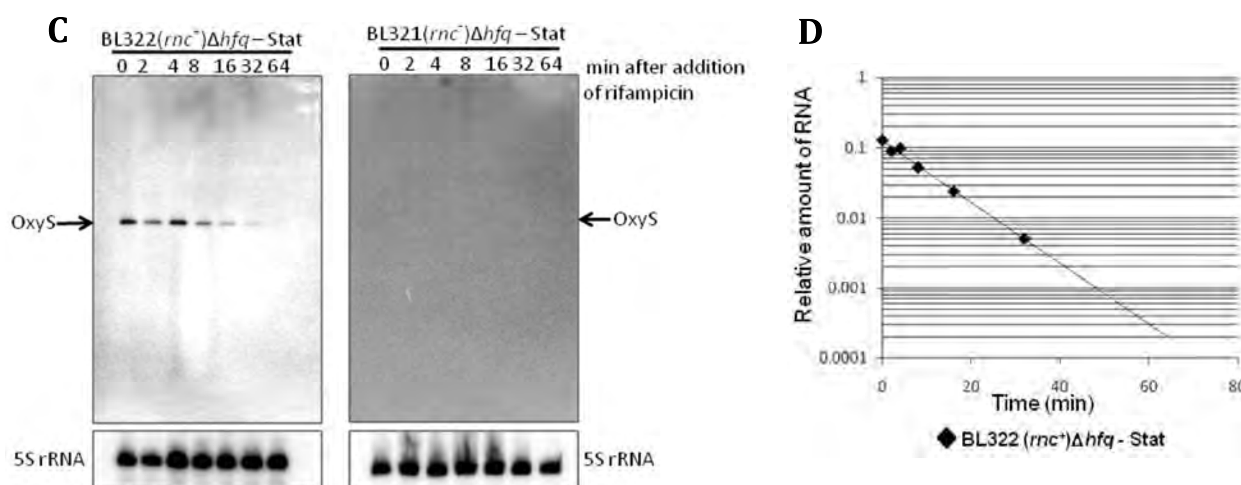


Figure 4.8 Stability determination of OxyS in *E.coli* BL322Δ*hfq* and *E.coli* BL321Δ*hfq*, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* BL322Δ*hfq* and BL321Δ*hfq*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp** – Early exponential growth phase: The half life of OxyS in *E.coli* BL322Δ*hfq* is about 18 min and in BL321Δ*hfq* is about 11 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* BL322Δ*hfq* is about 15 min and in BL321Δ*hfq* OxyS signal was not detectable.

Increased stability of OxyS RNA was observed when RNase E was inactive in stationary phase compared to exponential phase at non-permissive growth conditions (Fig 4.4, Fig 4.9A and Table 4.1- Page No: 50), but this increased stability was not observed when Hfq was absent (N3431 (*rne^{ts}*): > 60 min and N3431 (*rne^{ts}*) Δ*hfq*: 20.8 ± 2.9) (Fig 4.9A and Table 4.1- Page No: 50). OxyS was not detectable in stationary growth phase when both RNase III and Hfq were missing (BL321 (*rnc^{-}*)Δ*hfq*). We were not able to detect OxyS processing products in any of the strains lacking Hfq; this effect is the same as observed in the MG1655 background. Our results once again suggest that the effect of Hfq in the turn-over of OxyS does not only depend on the presence or absence of certain ribonucleases but also on growth phase. Indicating that, turn-over of OxyS in different growth phases is carried out by different factors and by different mechanisms. We have also determined the stability of OxyS in a strain lacking Hfq and DsrA. Surprisingly, faster turn-over of OxyS in the *hfq-dsrA* double mutant when compared to the *dsrA* single mutant in stationary growth phase was observed. The determined half-life for OxyS in stationary phase in the *hfq-dsrA* double mutant is similar to the half-life that was determined

in *hfq* single mutant (*hfq-dsrA* double mutant: 11.0 ± 1.0 min and *hfq* single mutant: 9.3 ± 0.6 min), suggesting that in the absence of Hfq there is no influence of DsrA on OxyS turn-over (Fig 4.9A).

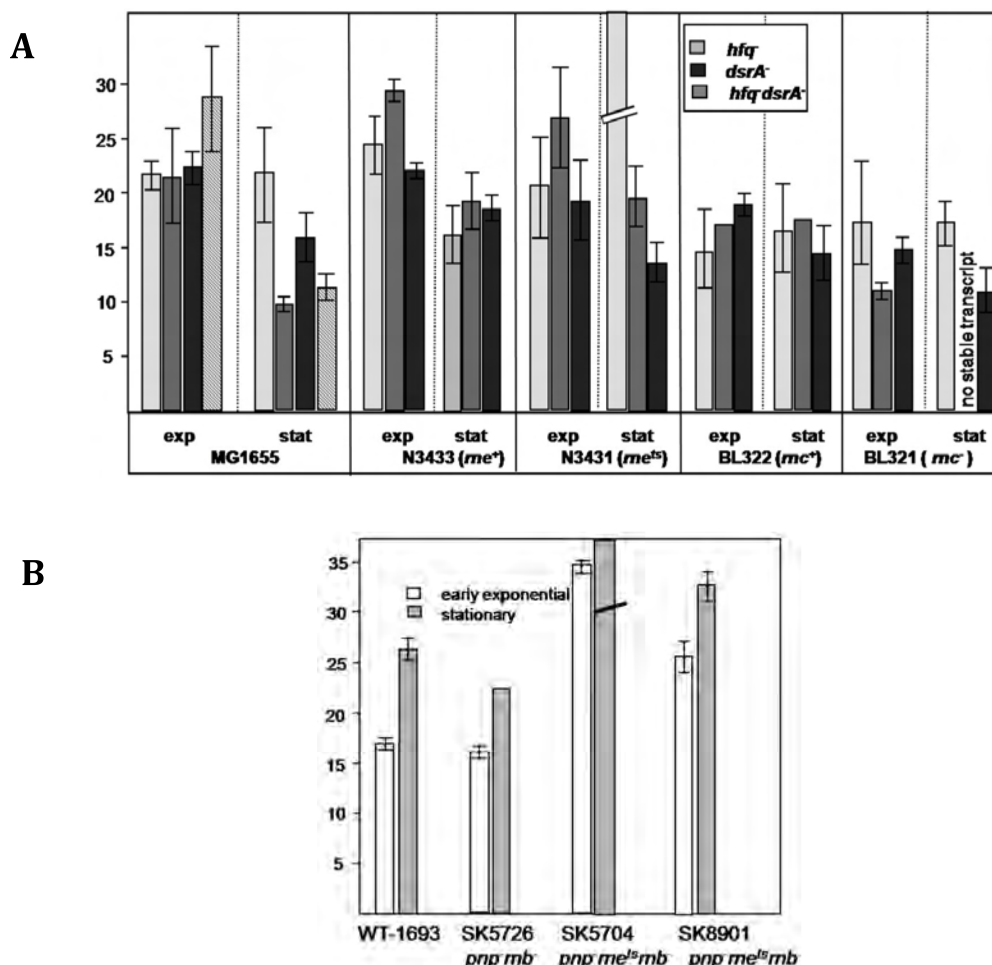


Figure 4.9 Graphical representation of the stability determination of OxyS sRNA: (A) Stability determination in wild type strains and in strains lacking RNase E or RNase III, and in mutants derived from these strains, in early exponential (exp) and stationary (stat) growth phases. Panels 1, the mutants lacking Hfq and/or DsrA were derived from the strain MG1655. Panels 2, the mutants lacking Hfq or DsrA were derived from the strain N3433 (*rne*$^{+}$). Panels 3, the mutants lacking Hfq or DsrA were derived from the strain N3431 (*rne*^{ts}). Panel 4, the mutants lacking Hfq or DsrA were derived from the strain BL322 (*rne*$^{+}$) Panel 5, the mutants lacking Hfq or DsrA were derived from BL321 (*rne*$^{-}$). (B) Stability determination of OxyS sRNA in strain lacking PNPase-RNase II (SK5726) and in a strain lacking PNPase-RNase II-RNase E^{ts} (SK5704) and the isogenic wild type background MG1693. OxyS RNA half-lives were calculated from at least 3 independent biological repeats and the standard deviation is given.

However, strong effects were observed when either RNase E was inactive (N3431 (*rne*^{ts}) Δ*dsrA*) or RNase III (BL321 (*rnc*$^{-}$) Δ*dsrA*) was missing along with DsrA, but this effect was

visible only in stationary phase. The strong OxyS stabilization that was observed in stationary growth phase of strain N3431 (*rne*^{ts}) was no longer noticed, when DsrA was missing. The half life of OxyS was even reduced in stationary phase (13.0 ± 2.0 min) compared to exponential phase (19.3 ± 4.2 min) in the *rne*^{ts}-*dsrA*$^{-}$ strain. Whereas, OxyS stability was almost identical in the BL321 (*rnc*$^{-}$) as well as in the BL321 (*rnc*$^{-}$) Δ*dsrA* double mutant in exponential phase (about 16 min), but the stability of OxyS in the *rnc*$^{-}$*dsrA*$^{-}$ double mutant was reduced to 10.3 ± 2.1 min in stationary phase when compared to the half-life that was determined in the case of BL321 (16.0 ± 2.1) (Fig 9B and Table 4.1- Page No: 50).
Our data demonstrates that DsrA influences the turn-over rates of OxyS RNA in the absence of RNase E or RNase III activity, but this effect can only be observed in stationary growth phase.

4.7 The effect of growth phase on the stability of the *rpoS* mRNA

The σ^S (RpoS) subunit of RNA polymerase is considered to be the master regulator of the general stress response (Lange and Hengge-Aronis 1991; McCann, Kidwell et al. 1991). Regulation of σ^S occurs at the levels of transcription, translation, proteolysis and protein activity (Lange and Hengge-Aronis 1994). *rpoS* was among the first genes reported to be regulated by small non-coding RNAs (Altuvia, Weinstein-Fischer et al. 1997; Majdalani, Cunning et al. 1998; Majdalani, Hernandez et al. 2002). The *rpoS* mRNA is regulated by 3 small non-coding RNAs in response to different stimuli. OxyS inhibits *rpoS* mRNA translation, while DsrA and RprA activate translation in presence of RNA chaperone Hfq (Zhang, Altuvia et al. 1998; Sledjeski, Whitman et al. 2001). As it was shown earlier that the translation inhibition leads to the rapid degradation of the small RNA/mRNA hybrids by RNases (RNase E or RNase III) (Masse, Escorcia et al. 2003; Morita, Maki et al. 2005), we asked, whether growth phase-dependent turn-over contributes to differential *rpoS* expression under oxidative stress (in presence of OxyS). We were also concerned in knowing, the influence of growth phases on *rpoS* mRNA turn-over and we wanted to know whether growth phase affects *rpoS* turn-over in a similar way as OxyS turn-over.
It was reported that several accessory factors are required in the regulation of RpoS levels at post-transcriptional level, one of the important factors being the Hfq protein (Brown and Elliott 1996; Muffler, Fischer et al. 1996). *rpoS* mRNA folds into a complex structure in which the translational initiation region is base-paired to a region further upstream in the long 5'-untranslated region which leads to the inhibition of ribosomal binding. Subsequently it was demonstrated that Hfq protein plays a role in the unfolding of this inhibitory structure and then stimulates the translation of *rpoS* mRNA (Muffler, Fischer et al. 1996; Brown and Elliott 1997; Sledjeski, Whitman et al. 2001). Brown and Elliott, also predicted that Hfq might influence the stability of *rpoS* mRNA (Brown and Elliott 1996). In order to know whether this is true, we determined the turn-over rates of *rpoS* mRNA in a strain lacking Hfq along with its isogenic strain MG1655 in exponential and stationary growth phases. In strain MG1655 we determined a half-life about 7 min in exponential and 5 min in stationary growth phase (Table 4.1- Page

No: 50), however 1.5 and 2 fold decreased stability was observed in exponential and stationary phase, respectively, when Hfq was absent (Fig 4.10 and Table 4.1- Page No: 50). This implies that Hfq not only helps in unfolding the inhibitory structure in order to activate the translation but also plays a role in determining the stability of the transcript. As we know that Hfq and RNase E share a common binding sequence, we tried to know if there is any role of RNase E in *rpoS* turn-over. In order to know the role of RNase E, we determined the stabilities of *rpoS* in N3433 (*rne*$^{+}$) and in the derived N3431 (*rne*^{ts}) strains. We did not observe any change in the stability of *rpoS* in the presence or absence of RNase E activity in exponential growth phase, however an increased stability of *rpoS* was observed when RNase E was inactive in stationary growth phase (N3431: 2.3 ± 0.2 compared to N3433: 1.5 ± 1.0) (Fig. 4.10 and Table 4.1- Page No: 50). This shows that RNase E also plays a role in the turn-over of *rpoS* in stationary phase.

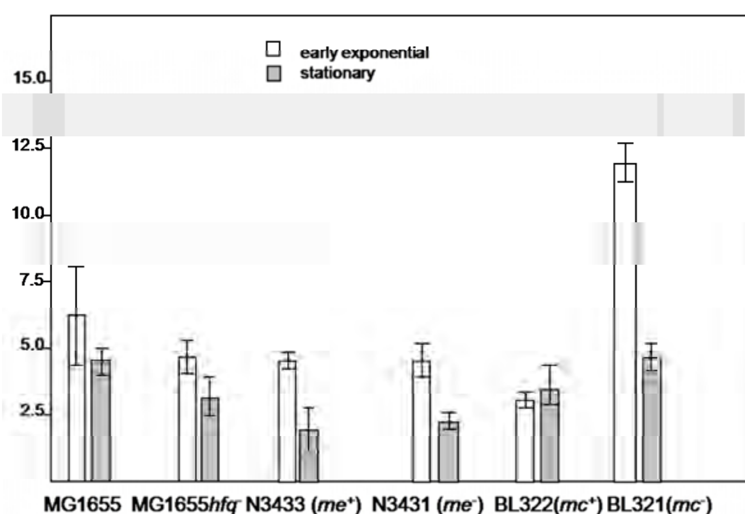


Figure 4.10 Graphical representation of the stability determination of *rpoS* mRNA: Stability determination in wild type strains and in strains lacking RNase E or RNase III or Hfq, in early exponential (exp) and stationary (stat) growth phases. *rpoS* mRNA half-lives were calculated from at least 3 independent biological repeats and the standard deviation is given.

We also tested the role of RNase III in the turn-over of *rpoS* mRNA, as we know that RNase III plays a role in the turn-over of sRNA/mRNA hybrids. We observed an increased stability of *rpoS* mRNA in a strain lacking RNase III (BL321) when compared to the isogenic strain BL322 (*rnc*$^{+}$) in exponential phase (BL321: 12.0 ± 1.0 and BL322: 2.8 ± 0.2) (Fig 4.10 and Fig 4.11), however we did not observe any changes in half-lives in stationary phase in the presence or absence of RNase III activity (Fig 4.11, Table 4.1- Page No: 50). The observations that we have made in this study are in agreement with earlier findings (Resch, Afonyushkin et al. 2008), the absence of RNase III had a strong effect on *rpoS* stability, however only in

exponential growth phase. These data show that the effect of Hfq and RNases on *rpoS* stability is growth phase dependent, as we observed similar effect on OxyS.

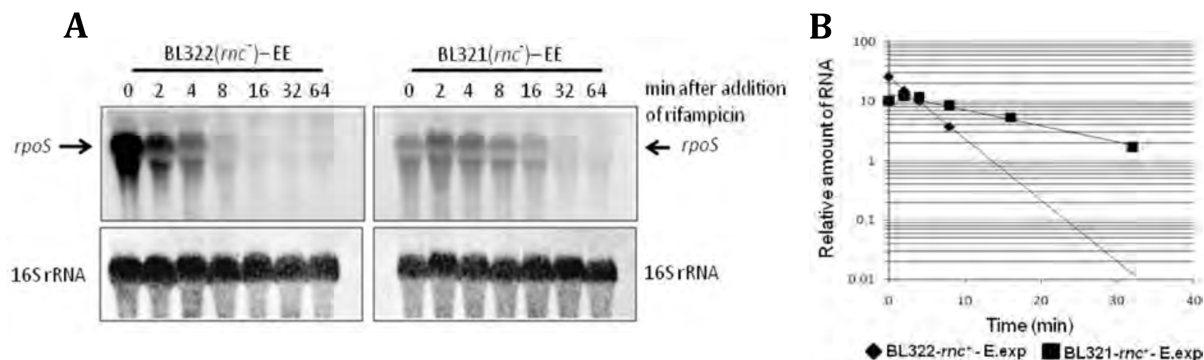


Figure 4.11 Stability determination of *rpoS* mRNA in *E.coli* BL322 and strain BL321-*rnc*$^-$, under oxidative stress in exponential growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** Northern blot analysis of the isolated total RNA from *E.coli* BL322 and strain BL321-*rnc*$^-$ as described above. Membranes were first hybridized with *rpoS* mRNA specific radioactively labeled probe (upper panel) and then the same membrane was re-hybridized with a 16S rRNA specific probe as an internal loading control (lower panel). **(B)** Half life determination of the *rpoS* mRNA transcript. Band intensities of the transcript were normalized against the intensity of the 16S rRNA and plotted against the time to calculate the half-life in this representative experiment. The half-life of *rpoS* mRNA in *E.coli* BL322 is about 3 min and in *E.coli* BL321 half-life about 12 min. **EE** – Early exponential phase.

Table 4.1: Summary of OxyS RNA and *rpoS* mRNA half-lives in different strain back grounds (Averages of at least three different biological repeats with standard deviation)

Strain	Half-life OxyS [min]		Half-life *rpoS* [min]	
	Early exp	Stat	Early exp	Stat
MG1655	22.0 ± 2.3	21.2 ± 6.2	6.5 ± 2.0	4.6 ± 0.5
MG1655*hfq*$^-$	27.7 ± 6.8	9.3 ± 0.3	4.7 ± 0.7	2.7 ± 0.4
MG1655*dsrA*$^-$	22.0 ± 1.0	16.0 ± 2.0		
MG1655*hq*$^-$ *dsrA*	29.0 ± 5.6	11.0 ± 1.0		
N3433 (*rne*$^+$)	24.2 ± 2.9	16.8 ± 2.9	3.4 ± 0.2	1.5 ± 1.0
N3433*hfq*$^-$	29.0 ± 1.4	19.5 ± 3.0		
N3433*dsrA*$^-$	21.7 ± 0.6	18.3 ± 1.2		
N3431 (*rne*^{ts})	20.6 ± 5.1	Stable	3.5 ± 0.7	2.3 ± 0.2
N3431*hfq*$^-$	26.7 ± 5.5	20.8 ± 2.9		
N3431*dsrA*$^-$	19.3 ± 4.2	13.0 ± 2.0		
BL322 (*rnc*$^+$)	14.5 ± 3.7	16.8 ± 4.1	2.8 ± 0.2	3.0 ± 1.0
BL322*hfq*$^-$	18.0 ± 2.6	15.0 ± 4.4		
BL322*dsrA*$^-$	19.0 ± 1.0	14.7 ± 2.9		
BL321 (*rnc*$^-$)	16.0 ± 6.5	16.0 ± 2.1	12.0 ± 1.0	3.5 ± 0.5
BL321*hfq*$^-$	11.3 ± 1.0	-		
BL321*dsrA*$^-$	15.0 ± 1.0	10.3 ± 2.1		
MG1693	16.5 ± 0.5	26.0 ± 1.0		
1693*pnp*$^-$*rnb*$^-$ SK5726	15.5 ± 0.5	22.0. ± 0.5		
1693*pnp*$^-$*rne*$^{ts-}$*rnb*$^-$ SK5704	34.3 ± 1.0	Stable		

4.8 The effect of hydrogen peroxide and growth phase on OxyS, DsrA and RpoS levels in an *E. coli* wild type strain

Throughout its life cycle, *E. coli* is faced with different environmental challenges and regulates gene expression accordingly. In order to face environmental challenges, the master regulator RpoS (σ^S) plays an important role (Loewen and Hengge-Aronis 1994; Hengge-Aronis 2002). σ^S regulation is a quite complex mechanism, with the induction of both positive and negative regulators after stress. One such example is the induction of OxyS – an inhibitor of RpoS translation (Zhang, Altuvia et al. 1998) and DsrA – an activator of RpoS translation (Majdalani, Cunning et al. 1998) under oxidative stress. Activation of RpoS translation by DsrA has been demonstrated experimentally (Resch, Afonyushkin et al. 2008), but the mechanism by which OxyS represses RpoS translation is not yet clear. However, Zhang et al., in 1998 proposed a model to explain the inhibition of RpoS translation by OxyS. The authors predicted that OxyS induced under oxidative stress binds to Hfq and prevents the binding of Hfq to *rpoS* mRNA and thereby inhibits *rpoS* translation. It was shown earlier that Hfq is essential for the translation of *rpoS* mRNA (Muffler, Fischer et al. 1996).

As we observed DsrA along with OxyS under oxidative stress conditions we asked the question - “To which extent *rpoS* translation is inhibited when both the sRNAs co-exists along with the RNA chaperone Hfq”. Being an important regulator in controlling stress related genes, we expect that there will be a competition between the two small RNAs in regulating *rpoS* translation in different growth phases under oxidative stress. The effects of oxidative stress on OxyS as well as on RpoS levels have been studied in detail by several groups (Altuvia, Weinstein-Fischer et al. 1997; Gonzalez-Flecha and Demple 1999; Bougdour, Cunning et al. 2008; Merrikh, Ferrazzoli et al. 2009). In spite of all the earlier observations, detailed *in vivo* quantification of RpoS protein levels in parallel with the two sRNAs that co-exists under oxidative stress along with role of RNA chaperone and their influence on *rpoS* translation was not performed till now. In order to reveal a plausible interplay between the sRNAs (OxyS and DsrA) and RNA chaperone Hfq in regulating the translation of *rpoS* mRNA we have performed the following experiments.

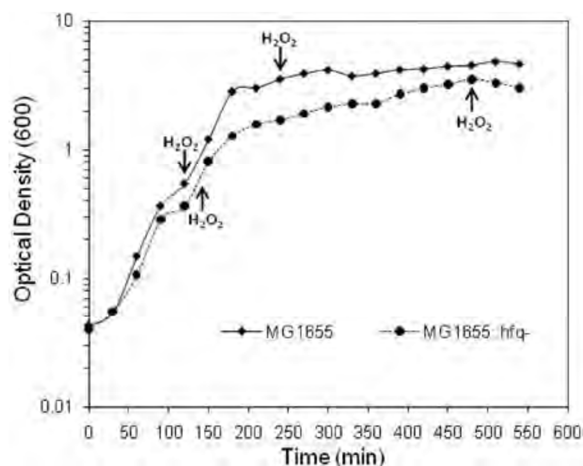


Figure 4.12 Growth curves of *E.coli* MG1655 and the isogenic *hfq*⁻ strain. Cultures were grown with shaking at 37^0C to exponential and stationary phase, respectively. Once cells reached an OD_{600} - 0.5 or 3.5 (indicated with arrows) cells were stressed with 1mM H_2O_2 and collected at regular intervals. Cells were collected before the addition of H_2O_2 (0*) and 0, 7, 14, 28, 56, 112 and 142 min after the addition of H_2O_2.

Escherichia coli K12 cells from overnight cultures were diluted 1/100 in LB medium and subsequently grown at 37 ^{0}C. Once the cells reached an OD_{600} of 0.5 (exponential growth phase) or an OD_{600} of 3.5 (stationary phase) (Fig 4.12) oxidative stress was induced by the addition of 1 mM of H_2O_2. Care was taken to start with equal amounts of cells by normalizing with optical density of the samples in order to determine the RpoS levels by western blot analysis.

We were able detect higher amounts RpoS in stationary growth phase in all tested *E.coli* K12 strains, which is agreement with the earlier observations (Hengge-Aronis 1993). In our experimental conditions RpoS protein was clearly detectable upon entry in stationary phase (OD_{600}: 3.5) even before the hydrogen peroxide addition and further incubation led to increased RpoS protein levels in strain MG1655 (Fig 4.13, Fig 4.15). However, RpoS was not detectable before or after the addition of hydrogen peroxide in exponential growth phase. We were able to detect RpoS at 112 min after the addition hydrogen peroxide, by this time cells were already in transition to stationary phase (OD_{600}: 2.4) (Fig 4.12). These data indicate that growth phase determines the expression of RpoS rather than stress induction by the H_2O_2. In order to verify this, RpoS levels were also determined in untreated *E.coli* MG1655 in both growth phases (no addition of H_2O_2). Identical patterns of RpoS expression were observed in stressed as well as in non-stressed cells in exponential growth phase (Fig 4.14, Fig 4.15). During transition to stationary growth phase in the presence of H_2O_2, surprisingly reduced levels of RpoS were observed when compared to untreated cultures (Fig 4.15). These data further substantiate that growth phase has a strong effect on RpoS levels, rather oxidative stress.

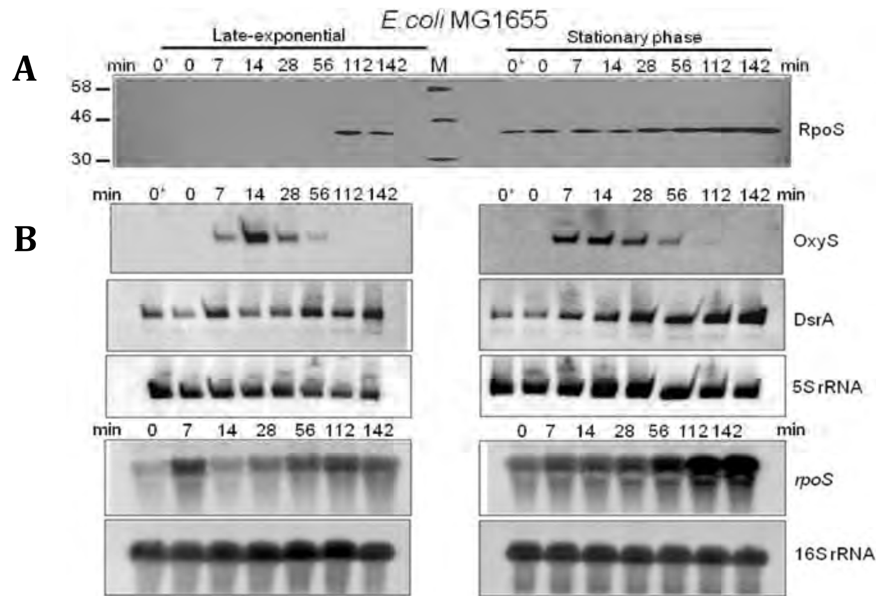


Figure 4.13 Expression analysis of RpoS, OxyS, DsrA and *rpoS* mRNA under oxidative stress condition in *E.coli* MG1655. Cells were harvested for the detection of RpoS levels and for the isolation of RNA at the indicated time points before (0*) and 0, 7, 14, 28, 56, 112 and 142 min after the addition of H_2O_2. **(A)**: Western blot analysis of RpoS levels. Poly-clonal antiserum specific to RpoS was used for the detection. Total protein from equal amounts of cells was loaded. Size standard proteins (58, 46 and 30 KDa) are shown in lane M and indicated on the left side of the western blot **(B)**: Northern blot analysis of OxyS, DsrA and *rpoS* mRNA. 30µg of total RNA was loaded per lane. The membrane was first hybridized with a specific 5'- end labeled probe (OxyS or DsrA) or with nick translated *rpoS* specific probe and the same membrane was re-hybridized with a 5S or 16 S rRNA specific probes as an internal loading control. **LE** – Late-exponential phase and **Stat** – Stationary phase

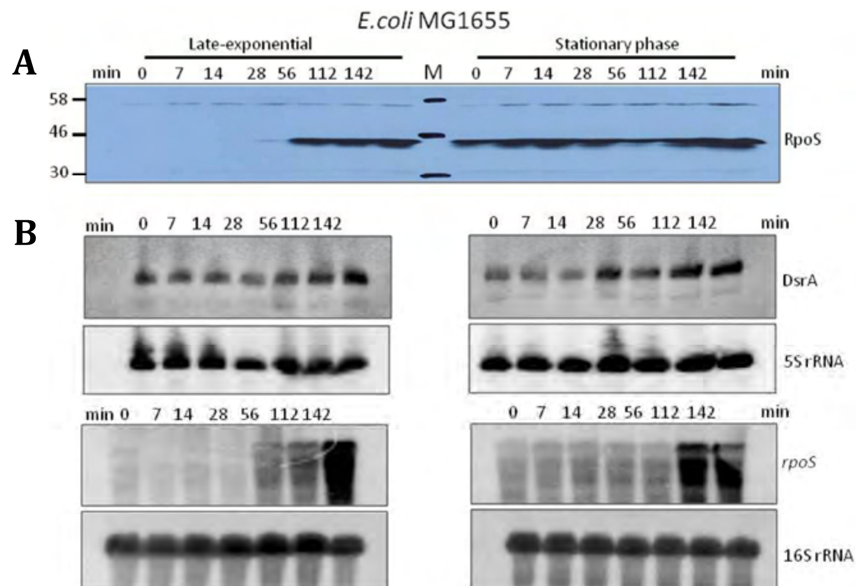


Figure 4.14 Expression analysis of RpoS, DsrA and *rpoS* mRNA levels in *E.coli* MG1655 (without H_2O_2). Cells were harvested for the detection of RpoS levels and for the isolation of RNA at the indicated time points 0, 7, 14, 28, 56, 112 and 142 min once the cells reached the required

OD_{600}. **(A)**: Western blot analysis of RpoS levels. Poly-clonal antiserum specific to RpoS was used for the detection. Total protein from equal amounts of cells was loaded. Size standard proteins (58, 46 and 30 KDa) are shown in lane M and indicated on the left side of the western blot **(B)**: Northern blot analysis of DsrA and *rpoS* mRNA. 30µg of total RNA was loaded per lane. The membrane was first hybridized with a specific 5'- end labeled probe (DsrA) or with nick translated *rpoS* specific probe and the same membrane was re-hybridized with a 5S or 16 S rRNA specific probes as an internal loading control.

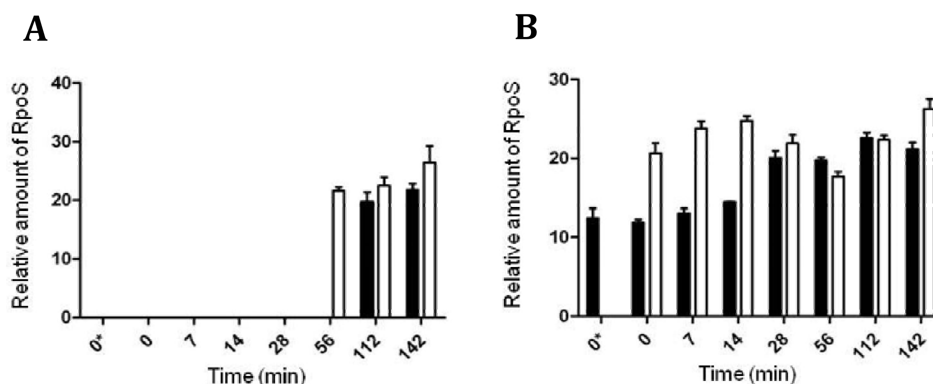


Figure 4.15 Graphical representations of the RpoS levels. Relative amount of RpoS expression in untreated *E.coli* MG1655 was compared to the H_2O_2 treated *E.coli* MG1655 in exponential **(A)** and stationary phase **(B)**. For **(A)** and **(B)** cultures were grown in triplicates, and error bars denote the standard deviations of the mean. Densitometric values for the bands on Western blot were quantified (FluorS, Biorad). **Black bars**: RpoS-MG1655-H_2O_2, **White bars**: RpoS-MG1655- No H2O2.

In parallel to the RpoS protein levels, *rpoS* mRNA as well as OxyS and DsrA sRNA levels were determined in both growth phases. Increased levels *rpoS* mRNA were observed as the cells steadily entered into the stationary phase and further increase of *rpoS* mRNA in stationary phase was observed (Fig 4.17 A & B). From the earlier experiments it has become evident that OxyS RNA can only be detected when cells are treated with hydrogen peroxide in both growth phases. We were able to detect OxyS 7 min after the addition of hydrogen peroxide and OxyS accumulated to its maximal amount at 14 min after the onset of oxidative stress and then reduction of OxyS was observed.

In contrast to OxyS, DsrA was expressed in exponential as well as in stationary growth phases in the presence or absence of hydrogen peroxide. The DsrA amount gradually accumulated over the time of the experiment and reached maximal amounts in stationary phase compared to exponential growth phase (Fig 4.13, Fig 4.16 C & D)

4.9 Hfq affects the levels of RpoS protein and of OxyS and DsrA

In an earlier study it has been shown that Hfq is essential for the translation of *rpoS* mRNA and it was also described that Hfq is necessary for OxyS and/or DsrA to interact with their target mRNA *rpoS* (Muffler, Fischer et al. 1996; Zhang, Altuvia et al. 1998; Sledjeski, Whitman et al. 2001). It is also clear that the RNA chaperone Hfq binds to OxyS and DsrA and thereby influences the stability of these sRNAs in stationary phase (Fig 4.9A and Table 4.1- Page No: 50). In earlier part of the results we have shown that Hfq stabilizes OxyS and DsrA in the MG1655 strain background in stationary growth phase but not in exponential phase (Fig 4.9A and Table 4.1- Page No: 50). Hfq also had a slight stabilizing effect on the *rpoS* mRNA in both growth phases (Fig 4.10). We wanted to analyze the expression levels of RpoS protein in parallel to the OxyS and DsrA sRNAs in the absence of Hfq

In the absence of Hfq, we observed reduced expression of RpoS in both growth phases when compared to the isogenic strain MG1655 under oxidative stress (Fig 4.19). This observation goes hand in hand with earlier observations made under osmotic stress conditions (Muffler, Fischer et al. 1996). This implies that irrespective of the stress RpoS translation is very much dependent on Hfq.

We observed higher levels of RpoS protein accumulation in stationary phase compared to the exponential phase in the absence of Hfq (Fig 4.16), which is identical to the observation made in the isogenic MG1655 strain (Fig 4.13). However, the amounts of RpoS that we detected were very low and the kinetics of RpoS accumulation was different in the presence or absence of Hfq (Fig 4.19). In the absence of Hfq, 4 fold lower levels in late-exponential phase and 5-25 fold lower levels in stationary phase when compared to the parental strain MG1655 were determined. We were able to detect RpoS at much earlier time points (28 min) in exponential phase after hydrogen peroxide addition and in the absence of Hfq the maximal amounts were reached much earlier in stationary phase (Fig 4.19, Fig 4.16). We were able to observe the accumulation of *rpoS* mRNA at earlier time points in the strain lacking Hfq. This is in agreement with the kinetics observed in the case of RpoS accumulation in a strain lacking Hfq. In the absence of Hfq *rpoS* mRNA levels dropped after the addition of hydrogen peroxide in exponential as well as in stationary phase (Fig 4.16). About 2.5 fold decreased levels of *rpoS* mRNA were determined after the hydrogen peroxide addition in both growth phases (Fig 4.17 A and B). The OxyS RNA was accumulated in an identical manner after the hydrogen peroxide addition in exponential phase in the absence or presence of Hfq (Fig 4.13, Fig 4.16).

A

B

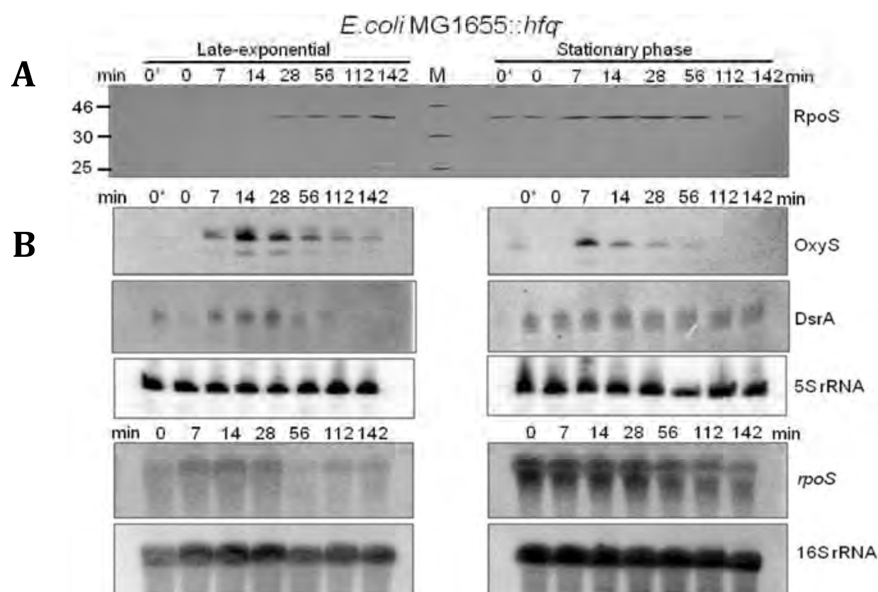


Figure 4.16 Expression analysis of RpoS, OxyS, DsrA and *rpoS* mRNA under oxidative stress condition in *E.coli* MG1655::Δ*hfq*. Cells were harvested for the detection of RpoS levels and for the isolation of RNA at the indicated time points before (0*) and 0, 7, 14, 28, 56, 112 and 142 min after the addition of H_2O_2. **(A)** Western blot analysis of RpoS levels. Poly-clonal antiserum specific to RpoS was used for the detection. Total protein from equal amounts of cells was loaded. Size standard proteins (46, 30 and 25 KDa) are shown in lane M and indicated on the left side of the western blot **(B)** Northern blot analysis of OxyS, DsrA and *rpoS* mRNA. 30µg of total RNA was loaded per lane. The membrane was first hybridized with a specific 5'- end labeled probe (OxyS or DsrA) or with nick translated *rpoS* specific probe and the same membrane was re-hybridized with a 5S or 16 S rRNA specific probes as an internal loading control. **LE** – Late-exponential phase and **Stat** – Stationary phase

However, in stationary phase OxyS levels were reduced faster and the maximal amount of OxyS was observed at 7 min after stress exposure rather 14 min that was observed in exponential phase. The reduced levels of OxyS in stationary phase in the absence of Hfq can attribute to the reduced stability of OxyS (Fig 4.2 and Fig 4.9).

In the absence of Hfq, DsrA levels were also clearly diminished in both growth phases (Fig 4.16). About 15 fold decreased levels of DsrA in exponential growth phase and about 10 fold reduced levels in stationary growth phase compared to the parental wild type strain were observed (Fig 4.17 C and D). This reduced expression can be assigned to the lower stability of DsrA in the absence of Hfq (Sledjeski, Whitman et al. 2001).

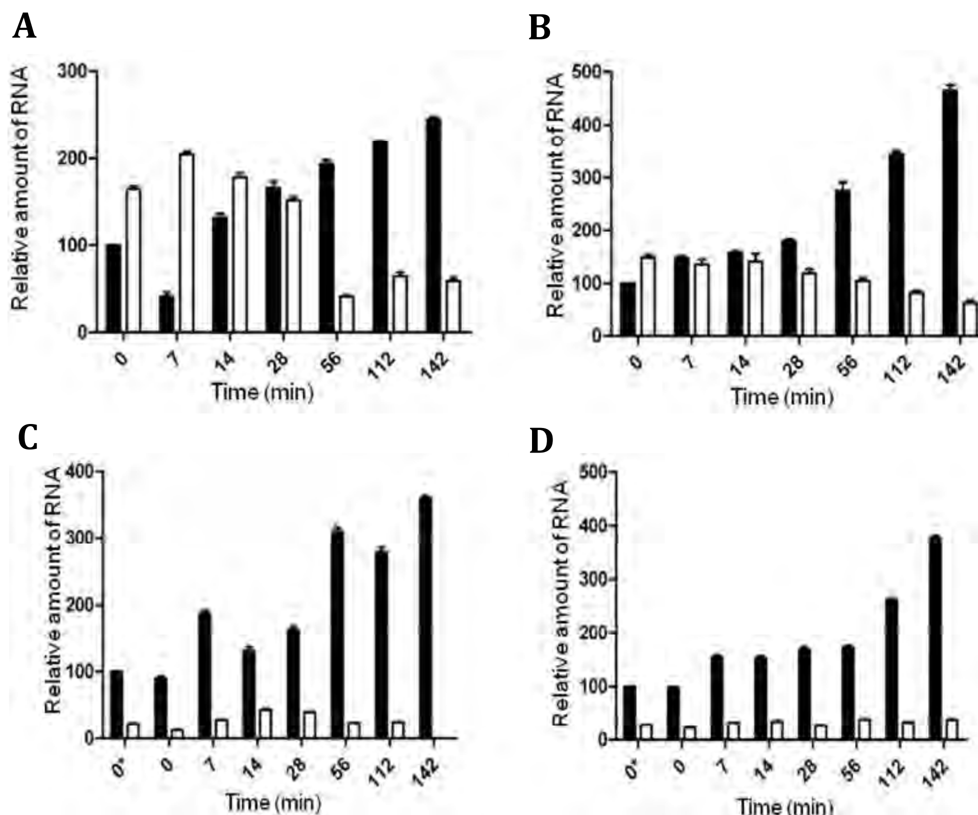


Figure 4.17 Graphical representation of the levels of *rpoS* mRNA and of DsrA under oxidative stress: Levels of *rpoS* mRNA in *E.coli* MG1655 and in the isogenic strain lacking Hfq, in early exponential **(A)** and in stationary **(B)** growth phases. Relative amount of DsrA expressed in MG1655 *E.coli* was compared to the expression levels in isogenic strains lacking Hfq at early exponential **(C)** and stationary phase **(D)**. For (A), (B), (C) and (D), cultures were grown in triplicates, and error bars denote the standard deviations of the mean. RNA levels were detected by Northern Blots and the band intensity as quantified using phoshoimaging (Molecular Imager, Biorad). **Black bars**: *rpoS* or DsrA-MG1655-H_2O_2, **White bars**: *rpoS* or DsrA-MG1655::Δ*hfq*-H_2O_2.

4.10 Effect of DsrA on the levels of RpoS protein and on OxyS levels

We showed that DsrA, a small non-coding RNA that activates *rpoS* translation is also expressed under oxidative stress along with the *rpoS* translation inhibitor OxyS. We also have observed decreased stability of OxyS in the absence of DsrA in stationary phase (Fig 4.9A, Table 4.1- Page No: 50), implying a collective influence of the sRNAs at least at the level of stability. It is plausible that the formation of a hybrid with *rpoS* mRNA influences the stability of OxyS and/or DsrA and both sRNAs might compete with each other in order to bind to their target mRNA. It was proposed that under oxidative stress when OxyS is abundant, it titrates out the RNA chaperone Hfq and inhibits the *rpoS* translation. But the DsrA that is expressed under these conditions will induce the *rpoS* translation.

No change in the pattern of accumulation of RpoS protein in exponential as well as in stationary growth phase was observed in the presence or absence of DsrA (Fig 4.13, Fig 4.18).

We were able to observe slightly reduced expression levels of RpoS in a strain lacking DsrA (Fig 4.19). It was shown earlier that DsrA activates RpoS translation under low temperature and osmotic stress (Majdalani, Cunning et al. 1998; Repoila, Majdalani et al. 2003), the slight reduction in the RpoS levels in the absence of DsrA under oxidative stress supports the earlier published data.

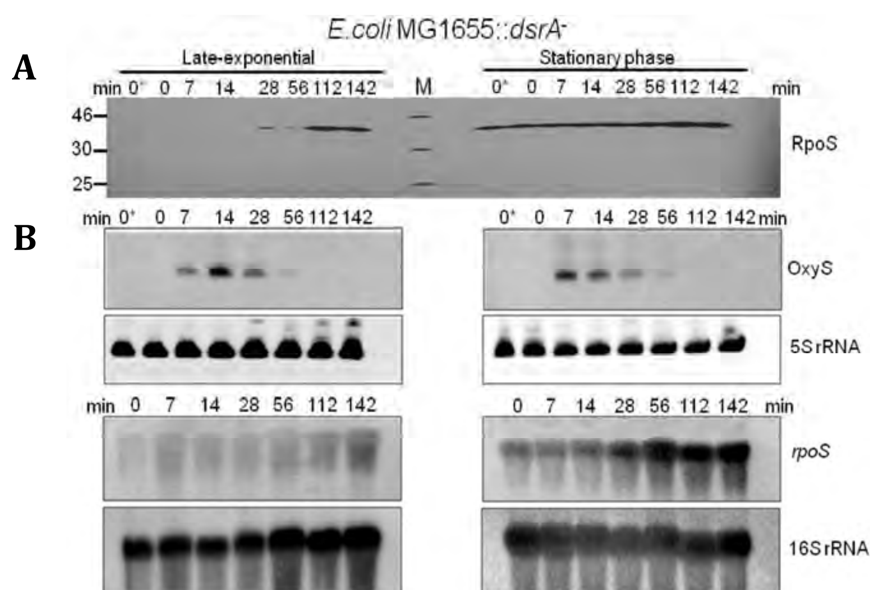


Figure 4.18 Expression analysis of RpoS, OxyS and *rpoS* mRNA under oxidative stress condition in *E.coli* MG1655::Δ*dsrA*. Cells were harvested for the detection of RpoS levels and for the isolation of RNA at the indicated time points before and after the addition of H_2O_2. **(A)**: Western blot analysis of RpoS levels. Poly-clonal antiserum specific to RpoS was used for the detection. Total protein from equal amounts of cells was loaded. Size standard proteins (46, 30 and 25 KDa) are shown in lane M and indicated on the left side of the western blot **(B)**: Northern blot analysis of OxyS and *rpoS* mRNA. 30µg of total RNA was loaded per lane. The membrane was first hybridized with a specific 5'- end labeled probe (OxyS) or with nick translated *rpoS* specific probe and the same membrane was re-hybridized with a 5S or 16 S rRNA specific probes as an internal loading control. **LE** – Late-exponential phase and **Stat** – Stationary phase

The OxyS RNA was accumulated in exponential phase with similar kinetics in the presence or absence of DsrA: OxyS was detectable at 7 min after hydrogen peroxide addition and maximal amounts were observed at 14 min after the addition of hydrogen peroxide. However, faster disappearance of OxyS was observed in a strain lacking DsrA when compared to the isogenic wild type strain in stationary phase. In contrast to exponential phase, maximal levels of OxyS in stationary phase were observed after 7 min of hydrogen peroxide treatment. This is in agreement with a decreased OxyS half-life in stationary phase when DsrA is lacking (Fig 4.9A and Table 4.1- Page No: 50).

In order to see the effect of oxidative stress and OxyS on *rpoS* translation, the RpoS protein levels were determined in a strain lacking both Hfq and DsrA (positive regulators of *rpoS* translation). Only a negligible amount of RpoS was detected at 142 min after the hydrogen

peroxide addition in exponential growth phase. Even in stationary growth phase reduced expression of RpoS was observed before and at early time points after exposure to stress. But RpoS levels were increased an hour later after the onset of the stress. Accumulation of OxyS was also greatly affected when Hfq and DsrA were missing in exponential phase (Supplementary Fig 9.2). OxyS levels reached maximal levels around 30 min after the addition of hydrogen peroxide and did not decrease until 142 min after exposure. The reduced level of RpoS observed is in contrary to the higher levels of OxyS accumulated in the absence of both Hfq and DsrA. This suggests that in the absence of both the positive regulators OxyS has higher inhibitory effect on *rpoS* translation in exponential growth phase. In stationary growth phase we were able to observe faster reduction of OxyS in the absence of Hfq and DsrA. The faster reduction in the absence of Hfq and DsrA can be assigned to the destabilization of OxyS in stationary phase (Fig 4.9A).

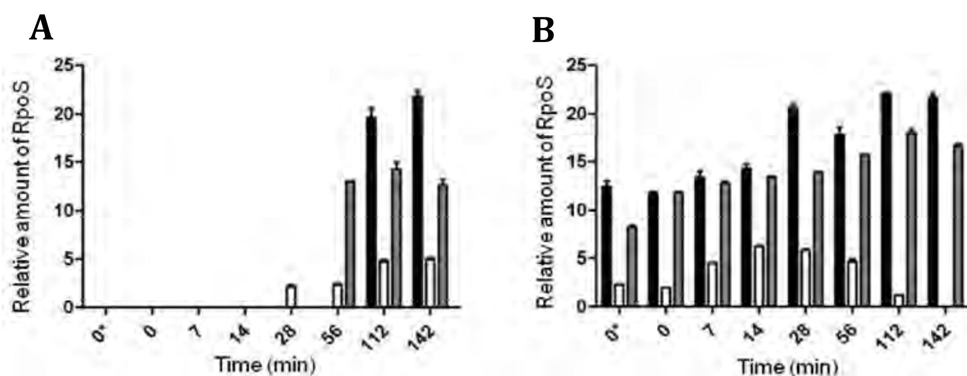


Figure 4.19 Graphical representation of the RpoS levels. Levels of RpoS in *E.coli* MG1655 and in isogenic strains lacking Hfq or DsrA, in early exponential **(A)** and **(B)** in stationary growth phases under oxidative stress. For (A) and (B), cultures were grown in triplicates, and error bars denote the standard deviations of the mean. Densitometric values for the bands on Western blot were quantified (FluorS Biorad). **Black bars**: RpoS-MG1655-H_2O_2, **Grey bars**: RpoS-MG1655::Δ*dsrA*-H_2O_2, **White bars**: RpoS-MG1655::Δ*hfq*-H2O2.

4.12 Hfq, DsrA and OxyS influence turn-over of RpoS

Sigma S (σ^S), the master of general stress response and stationary phase Sigma factor expression, stability and activity is controlled by complex regulatory network (Hengge-Aronis 2002; Bougdour, Lelong et al. 2004; Gaal, Mandel et al. 2006). In exponentially growing cells RpoS is maintained at a low level due to the active degradation by ClpXP proteases (Lange and Hengge-Aronis 1994; Schweder, Lee et al. 1996; Becker, Klauck et al. 1999). Once the *E.coli* cells enter in to stationary growth phase or under starvation condition RpoS rapidly accumulates due to the increased stability (Hengge-Aronis 2002). Recently it has been shown in detail about the factors that influence the RpoS levels and stability under various stress conditions such as Magnesium starvation, Phosphate starvation and under DNA damaging conditions (Bougdour, Cunning et al. 2008).

As we observed differential expressions of RpoS in the absence of Hfq, DsrA and both Hfq-DsrA, we asked a question whether stability of RpoS will be influenced in these strain backgrounds under oxidative stress. Therefore we tested the RpoS stability in Hfq, DsrA, Hfq-DsrA and OxyS lacking strain backgrounds. Chloramphenicol was added to the growing cultures in order to stop the ongoing translation and to follow the disappearance of RpoS. In wild type strain MG1655, RpoS was degraded with a half-life of about 30 min in late-exponential phase and a half-life of about 23 min in stationary growth phase was determined (Fig 4.20A). In the absence of Hfq or DsrA we were not able to observe any turn-over of RpoS in Western blots. For both strains and both growth phases the half-lives of RpoS were more than 60 min indicating a significantly reduced turn-over when compared to the parental strain MG1655 (Fig 4.20B and C). This implies that reduced levels of RpoS in the above mentioned backgrounds can be compensated by the increased stability under stress condition. The same data were obtained for a strain lacking both, Hfq and DsrA (Supplementary Fig 9.3). In a strain lacking OxyS, RpoS was turned over even more quickly than in the parental strain after blocking translation (Fig 4.20D) in both growth phases. We observed a half-life of about 9 min in both growth phases. Thus, the Hfq protein and the two sRNAs DsrA and OxyS have strong but differential effects on RpoS turn-over.

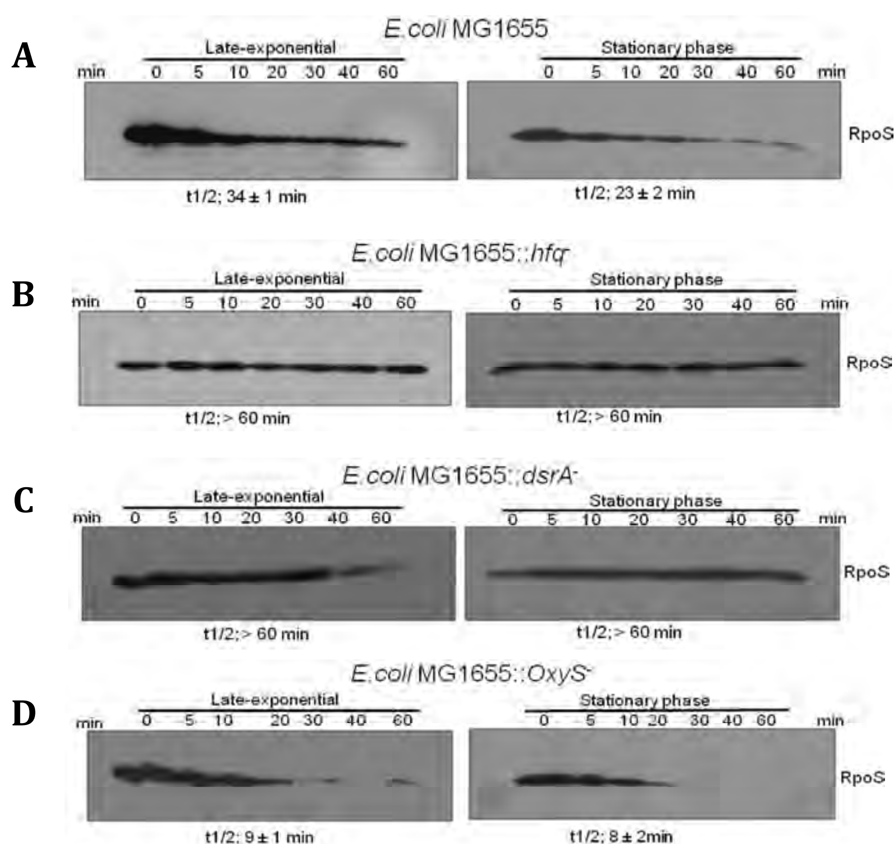


Figure 4.20 Stability determination of RpoS in *E.coli* MG1655 and isogenic strains lacking Hfq, DsrA or OxyS under oxidative stress in late-exponential and in stationary growth phase. Cells were harvested for detection of RpoS by western blot at the indicated

time points after the addition of Chloramphenicol as described in Material and Methods. **(A)**: The half life of RpoS in *E.coli* MG1655 is about 34 ± 1 min in late-exponential and 23 ± 2 in stationary phase. **(B)** and **(C)**: The half life of RpoS in *E.coli* MG1655::Δ*hfq* and *E.coli* MG1655::Δ*dsrA* is > 60 min in late-exponential and stationary phase. **(D)**: The half life of RpoS in *E.coli* MG1655:: Δ*oxyS* is about 9 ± 1 min in late-exponential and 8 ± 2 in stationary phase.

5 Discussion

Bacteria have developed a variety of complex and sophisticated mechanisms in order to compete and survive in their natural environments. Bacteria can get exposed to a variety of signals comprising limitations of nutrients, a variety of stress signals such as temperature, osmolarity, oxidative stress and pH in natural habitats. In order to tackle the above mentioned challenges *Escherichia coli* has to induce the general stress response. In *E.coli* this depends on the sigma factor σ^S (RpoS) subunit of RNA polymerase (Hengge-Aronis 2000). Cellular levels of RpoS, the master regulator of general stress response is regulated by a delicate balance between the activators and repressors at the levels of *rpoS* transcription, *rpoS* mRNA turn-over and translation as well as proteolysis (Hengge-Aronis 2002). At post-transcriptional level *rpoS* mRNA translation is regulated by several modes (Brown and Elliott 1996; Muffler, Fischer et al. 1996), one of the modes of regulation of *rpoS* translation is by small non-coding RNA (Sledjeski, Gupta et al. 1996; Zhang, Altuvia et al. 1997; Majdalani, Hernandez et al. 2002). So far, three small non-coding RNAs that are expressed under various stress conditions, DsrA, RprA and OxyS have been found to regulate *rpoS* translation. The DsrA (85 nt) and RprA (105 nt) sRNAs have been implicated in the activation of *rpoS* translation under low temperature and under hyperosmotic condition respectively (Sledjeski, Gupta et al. 1996; Majdalani, Cunning et al. 1998; Majdalani, Chen et al. 2001; Majdalani, Hernandez et al. 2002). However, the OxyS sRNA (109 nt) induced under oxidative stress has been shown to inhibit the *rpoS* translation (Altuvia, Weinstein-Fischer et al. 1997; Zhang, Altuvia et al. 1997; Zhang, Altuvia et al. 1998). Even though *rpoS* translation is repressed by OxyS, RpoS is very essential for bacterial survival under oxidative stress (Barth, Gora et al. 2009). Activation of *rpoS* translation by DsrA and RprA has been shown experimentally by different groups (Resch, Afonyushkin et al. 2008; Updegrove, Wilf et al. 2008) however, the inhibition of *rpoS* translation by OxyS is not clear. Here, I describe the influence of growth phase, different RNases, the RNA chaperone Hfq and DsrA in the turn-over of OxyS and *rpoS* RNAs as well as on the expression of RpoS in *Escherichia coli*.

5.1 Growth phase dependent turn-over of OxyS sRNA

The OxyS RNA (109 nt) is almost one of the first small non-coding RNA that has been identified and characterized when *E.coli* cells were exposed to hydrogen peroxide (Altuvia, Weinstein-Fischer et al. 1997). It was shown that within 1 min of exposure to hydrogen peroxide OxyS accumulated approximately to as much as 4500 copies per cell. Altuvia et al., also proposed that OxyS RNA is a quite stable RNA with a half-life of 12-15 min in *E.coli* K12 strain in exponential growth phase. OxyS was also implicated in the activation and repression of almost 40 genes, including the *fhlA* encoded transcriptional activator of formate hydrogenlyase complex and the *rpoS* encoded σ^S subunit of RNA polymerase and protection against DNA damage. It was predicted that OxyS might act at multiple levels including

transcription, stability of the target mRNA, translation and even at the level of protein stability. Subsequently, it was demonstrated experimentally that OxyS represses the translation of *fhlA* by blocking the Shine-Dalgarno sequence, the ribosomal binding site (Altuvia, Zhang et al. 1998). Later Zhang et al., in 1998 showed that OxyS also represses the RpoS synthesis at post-transcriptional level. In order to achieve inhibition of RpoS synthesis, authors proposed that OxyS intervenes with binding of Hfq to *rpoS* mRNA and thereby leads to the translation inhibition. The RNA binding protein Hfq was shown to be essential for the translational activation of *rpoS* (Brown and Elliott 1996; Muffler, Fischer et al. 1996). It was also shown that Hfq increases the interaction of OxyS RNA to its target mRNAs and also determined the stability of OxyS in *E.coli* strain MC4100 as 30 min in exponential growth phase (Zhang, Wassarman et al. 2002). In an earlier study it has been demonstrated that transcription of *rpoS* increases during transition to stationary growth phase (Lange and Hengge-Aronis 1994; Loewen and Hengge-Aronis 1994). RpoS being an important transcriptional regulator under stress as well in stationary growth phase and OxyS being an inhibitor of RpoS synthesis, we investigated, whether turn-over rates of OxyS and *rpoS* vary in different growth phases which in turn might have an impact in gene regulation (Fig 4.9 and Fig 4.10). The OxyS turn-over rates were determined only in exponential growth phase in earlier studies and only in *Salmonella dublin* growth phase dependent turn-over of *rpoS* was reported (Paesold and Krause 1999).

In *E.coli* strain MG1655 (a *E.coli* K12 derivative), we determined an half-life of 22.0 ± 2.3 min in exponential growth phase and 21.0 ± 6.2 min in stationary growth phase (Fig 4.9 and Table 4.1- Page No: 50). The half-lives that we determined in strain MG1655 were in the range of earlier published half-lives which is 10-30 min (Altuvia, Weinstein-Fischer et al. 1997; Zhang, Wassarman et al. 2002). Nevertheless, we were not able to observe any significant change in the turn-over rates in different growth phases in strain MG1655. Interestingly we were able to observe growth phase dependent turn-over of OxyS when Hfq was absent in strain MG1655. In exponential growth phase no change in the stability was observed in the presence or absence of Hfq, which is in agreement with the earlier finding (Zhang, Wassarman et al. 2002). However, a 3 fold decrease in stability was observed in stationary phase when Hfq was absent (Fig 4.9 and Table 4.1- Page No: 50). From our results we conclude that Hfq is not only essential for increasing the interaction of OxyS with target mRNA but also plays a vital role in determining the stability in different growth phases.

In order to reveal a growth phase dependent processing or turn-over of OxyS we determined the turn-over rates of OxyS in various strains lacking endo- and/or exoribonucleases and in their isogenic wild type strains. Like in strain MG1655, we were not able to observe any meaningful differences in the turn-over rates of OxyS in exponential growth phase in N3433 (*rne*$^{+}$) and in N3431 (*rne*^{ts}) (Fig 4.9 and Table 4.1- Page No: 50). Nonetheless we observed a 1.4 fold faster turn-over of OxyS in stationary growth phase of N3433 (Fig 4.9A and Table 4.1- Page No: 50) and we observed a drastic increase in the stability of OxyS when RNase E

activity was inactivated at non-permissive conditions in stationary growth phase (Fig 4.9A and Table 4.1- Page No: 50). Faster turn-over that is observed in N3433 could also be due to the mutations in *relA* and *spoT*, which may affect the levels of *rpoS* mRNA through altered ppGpp levels in a growth-phase dependent manner (Gentry, Hernandez et al. 1993; Lange, Fischer et al. 1995) In N3431 we were not able to determine the half-life of OxyS within 60 min of transcription inhibition by rifampicin when RNase E activity was missing, implying that RNase E plays a decisive role in turn-over of OxyS in the absence of Hfq in stationary growth phase. We also have analyzed the stabilities of OxyS in a strain lacking RNase III (BL321 *rnc*$^-$) and in the isogenic wild type strain BL322, another important endoribonuclease in *E.coli*. We were not able to observe any change in the turn-over rates of OxyS either in exponential or in stationary growth phases, similar to the observations made in MG1655. In this study OxyS stabilities have also been determined in strains lacking exoribonucleases: PNPase, RNase II (SK5726) and in a strain lacking PNPase, RNase II along with missing of RNase E activity at non-permissive conditions (SK5704) and in their isogenic wild type strain MG1693. In strain MG1693, slightly increased stability of OxyS was observed in stationary growth phase when compared to the exponential growth phase (1.6 fold increase in stationary phase) (Fig 4.9B and Table 4.1- Page No: 50). In strain SK5726 we observed similar kind of kinetics as observed in the isogenic wild type strain (Fig 9B and Table1). However, we observed overall increase in the stability of OxyS in triple mutant (SK5704), this increase could be due to the lack of exo- and endoribonucleolytic activity (see below).
In overall OxyS showed the same or slightly increased stability in stationary growth phase for most strain backgrounds with the exception of strain N3433. As we did not observe any significant changes in the OxyS half-lives in the different parental strains either in exponential or stationary phase growth phase, we conclude that growth phase-dependent turn-over of OxyS is not expected to have a major impact on regulation if Hfq, RNase E, RNase III, PNPase and RNase II are present at regular levels. However, the contributions of several of these factors in maintaining a constant OxyS turn-over vary between exponential and stationary phase.

5.2 Influence of endo- and exoribonucleases on the turn-over of OxyS

In an earlier study it was shown that Hfq protects RyhB and MicA sRNAs against RNase E cleavage (Masse, Escorcia et al. 2003; Viegas, Pfeiffer et al. 2007). It was demonstrated that Hfq and RNase E preferentially bind and cleave single stranded AU rich sequences, respectively (Mackie and Genereaux 1993; McDowall, Lin-Chao et al. 1994; Moller, Franch et al. 2002; Schumacher, Pearson et al. 2002). As we observed reduced stability of OxyS in a strain lacking Hfq, we speculated that principle endoribonuclease - RNase E might be involved in the turn-over of OxyS in stationary phase in the absence of Hfq, while the AU rich linker region (Fig 1.2) in OxyS will be no more protected. We determined the stabilities of OxyS in RNase E temperature sensitive strain (N3431 *rne*^{ts}) as well as in it isogenic wild type strain N3433. In strain N3433 we did not observe any significant changes in the turn-

over in exponential as well as in stationary growth phase. However, there was dramatic increase in the stability of OxyS was observed in stationary growth phase of RNase E temperature sensitive strain (N3431 *rne*^{ts}) at non-permissive conditions. We were not able to observe the turn-over of OxyS in strain N3431 even after the arrest of ongoing transcription in stationary growth phase. These results confirmed our hypothesis, that RNase E plays an important role in the turn-over of OxyS in stationary growth phase in the absence of the RNA binding protein Hfq.
The OxyS RNA turn-over rates were also determined in strain lacking another principle endoribonuclease; double strand specific RNase III (BL321 *rnc*$^{-}$) and in the isogenic wild type strain BL322. We were not able to detect any observable changes in the turn-over rates OxyS in strain BL321 (*rnc*$^{-}$) compared to the strain harboring RNase III (BL322). From these observations we were able to rule out a role of the RNase III in the turn-over of OxyS in either of the growth phases.
In almost all strain backgrounds irrespective of presence or absence of RNase E or RNase III we observed smaller processing products of sizes of 40-50 nt, except in the strain MG1655Δ*hfq*. These results are in agreement with the earlier published results (Zhang, Wassarman et al. 2002). Since neither RNase E nor RNase III had a distinct role in the generation of these smaller processing products, we assumed exoribonucleases supposed to be playing a role in the generation of aforementioned processing products. In order to verify our assumption, we determined stabilities of OxyS in a strain lacking exoribonucleases (PNPase and RNase II – SK5726) and in strain lacking exoribonucleases along with lack of RNase E activity at non-permissive conditions (SK5704). We also have determined the stability of OxyS in strain MG1693, isogenic strain of SK5726. We were unable to observe any considerable changes in the in the turn-over rates of OxyS in both growth phases when both PNPase and RNase II (SK5726 *pnp*$^{-}$ *rnb*$^{-}$) were absent when compared to the isogenic strain MG1693 (Fig 9B and Table 4.1- Page No: 50). Nevertheless, a detectable increase in the stability of OxyS was observed in strain SK5704 (*pnp*$^{-}$, *rnb*$^{-}$, *rne*^{ts}) in exponential growth phase (Fig 4.9B and Table 4.1- Page No: 50) and there was no turn-over of OxyS were observed in stationary growth phase after the transcription inhibition at non-permissive conditions. Not only changes in the turn-over of OxyS was observed but also a visible reduction in the processing products in strain SK5704 (*pnp*$^{-}$, *rnb*$^{-}$, *rne*^{ts}), implying both exo- and endoribonucleases are involved in the generation of smaller processing products of OxyS in exponential as well in stationary growth phases. The increased stability that is observed in stationary growth phase of strain SK5704 is due to the lack of RNase E activity, since we observed identical turn-over of OxyS in strain N3431(*rne*^{ts}). In this study we were unable to assign a major function to any of the individual RNases in OxyS turn-over in both growth phases. This study also illustrates a combined action of exo- and endoribonucleases in the turn-over of OxyS and in maintaining appropriate OxyS levels in exponential and in stationary

growth phase. This assumption can be supported by the slower turn-over of OxyS in exponential growth phase in a strain lacking PNPase, RNase II and RNase E (SK5704).

5.3 Role of Hfq on the turn-over of OxyS

Hfq was identified as a bacterial host factor required for replication of the Qβ RNA bacteriophage (Franze de Fernandez, Eoyang et al. 1968). The *hfq* gene inactivation in *E.coli* leads to a variety of phenotypes and alters the expression of many proteins (Tsui, Leung et al. 1994; Muffler, Traulsen et al. 1997), indicating that Hfq acts as a pleiotropic regulator. Hfq destabilizes the *mutS*, *miaA*, and *hfq* mRNAs (Tsui, Feng et al. 1997), and the *ompA* mRNA decay is stimulated by interfering with ribosome binding, thus exposing the mRNA for endonuclease cleavage (Vytvytska, Jakobsen et al. 1998; Vytvytska, Moll et al. 2000). Hfq is shown to be very important for both target interaction and for intracellular stability. It has been described by several studies that Hfq enhances the longevity of sRNAs *in vivo* by protecting against ribonucleases (Masse, Escorcia et al. 2003; Rasmussen, Eriksen et al. 2005). It has also been shown that Hfq protects MicA sRNA against ribonucleolytic cleavage in stationary growth phase (Viegas, Pfeiffer et al. 2007). Zhang et al., in 2002 have shown that Hfq facilitates the base pairing of OxyS to its target mRNA. Nonetheless, Zhang and co-workers were not able observe the influence of Hfq on the turn of OxyS in exponential growth phase. In our study we were able to observe faster turn-over (3 fold) of OxyS in the absence of Hfq in stationary growth phase but not in exponential phase which is in agreement with the earlier finding. In the absence of Hfq we were also not able to detect the smaller processing products when compared to the isogenic wild type strain harboring Hfq (MG1655) in both growth phases. It has been described earlier that Hfq binds to OxyS in the "AU" rich linker region (Zhang, Wassarman et al. 2002), and we were able to show that RNase E might be involved the turn-over of OxyS in stationary growth phase, while RNase E and Hfq share a identical binding sequences (Moll, Afonyushkin et al. 2003) (discussed above). From our results we can say that Hfq protects OxyS against RNases in stationary growth phase like observed in the case of MicA (Viegas, Pfeiffer et al. 2007).

The OxyS processing products were detectable in strains N3431 (*rne*^{ts}) and in BL321 (*rnc*$^{-}$) harboring Hfq and we were not able to assign a role to any of the endoribonuclease in the generation of OxyS processing products. We also observed decreased but not the complete disappearance of processing products in strain lacking PNPase, RNase II and lack of RNase E activity at non-permissive conditions (SK5704). In order to identify the other factors involved in the generation of aforementioned processing products we have inactivated the *hfq* gene in strains N3431 (*rne*^{ts}), BL321 (*rnc*$^{-}$) and in their isogenic wild type strains (N3433 and BL322) (in collaboration with Dr. Regine Hengge, Freie University of Berlin, Germany). We were not able to observe any significance change in the turn-over rates of OxyS in strain N3433 in the presence or absence of Hfq in both growth phases (Table 4.1- Page No: 50). Interestingly in strain N3431 (*rne*^{ts}), we observed a faster turn-over of OxyS in stationary

phase in the absence of Hfq (N3431Δ*hfq*). We were surprised by the above result, while we did not observe the turn-over of OxyS after inhibiting the transcription in strain N3431(*rne*^{ts}) harboring Hfq (Table1 and Fig 9A). The reduced stability that we observed in strain N3431Δ*hfq* could also be due to the other RNases. In the absence of Hfq in strain N3431, RNase E attack may no longer be rate-limiting, since other RNases will also gain an access for cleavage. From our results we conclude that Hfq not only protects OxyS against RNase E cleavage but also from other RNases in stationary growth phase.

Like in strain N3433, even in strain BL322 we were not able to observe any change in the turn-over rates of OxyS in the presence or absence of Hfq in exponential as well as in stationary growth phase. Surprisingly, the OxyS transcript was not detectable in a strain lacking both RNase III and Hfq in stationary phase (BL321Δ*hfq*). I was also able to observe an enhanced turn-over of OxyS in exponential growth phase (reduced half life by a factor of about 1.4) when both RNase III and Hfq were absent when compare to the strain harboring Hfq (BL321- *rnc*$^{-}$) (Table 4.1-Page No: 50 and Fig 4.9A). These data indicate that OxyS is expressed in strain N3431Δ*hfq* with a half-life of about 11 min in exponential phase, strongly suggesting that OxyS in stationary phase is not detected due to the faster turn-over. The drastic destabilization of OxyS that we observed in the absence RNase III in stationary growth phase indicates RNase III might have an indirect role in the turn-over of OxyS. The lack of RNase III most likely stabilizes another transcript, which consequently affects OxyS stability. RNase III is known to be involved in *pnp* mRNA turnover and higher levels of PNPase are present in the absence of RNase III (Robert-Le Meur and Portier 1992). These results once again confirm that OxyS turn-over is carried out by a combined action of exo- and endoribonuclease.

The observed faster turn-over of OxyS in strains lacking Hfq could also be due to the lower amount of OxyS-mRNA hybrids. While it was shown that Hfq facilitates OxyS to interact with its target mRNAs (Zhang, Wassarman et al. 2002). So we determined the half-lives of OxyS in a strain lacking *rpoS* and in the isogenic wild type strain. The OxyS stability in an *rpoS*$^{-}$ strain did not differ significantly when compared to the isogenic wild type (Supplementary Fig 9.4). We conclude that the level of *rpoS* mRNA has no influence on OxyS stability.

5.4 Role of DsrA on the turn-over of OxyS

DsrA, a 85 nt sRNA expressed under low temperature and osmotic stress has been implicated in the activation of *rpoS* mRNA translation (Repoila and Gottesman 2001; Repoila and Gottesman 2003). In this study we were able to show that DsrA is also expressed under oxidative stress along with the *rpoS* translation inhibitor OxyS (Supplementary Fig 9.1). We expected a competition between these two sRNAs in the regulation of *rpoS* mRNA translation and influence of DsrA on OxyS turn-over rates. No detectable changes in OxyS turn-over was observed in exponential phase however, in stationary growth phase 1.4 fold faster turn-over of OxyS was observed in strain lacking DsrA when compared to the isogenic wild type strain MG1655 (Table 4.1- Page No: 50). Furthermore we have also inactivated *dsrA* gene in strains

N3431 (rne^{ts}), BL321 (rnc^{-}) and in their isogenic wild type backgrounds (N3433 and BL322) (in collaboration with Dr. Regine Hengge, Freie University of Berlin, Germany). In strains N3433 and BL322 we did not observe any visible differences in the half-lives in the presence or absence of DsrA in both growth phases. However, increased turn-over of OxyS was observed in strains N3431 (rne^{ts}) and BL321 (rnc^{-}) in the absence of DsrA in stationary growth phase but not in the exponential growth phase when compared to the isogenic strain (Table 4.1- Page No: 50 and Fig 4.9A). Increased stability of OxyS in the absence of RNase E activity in stationary phase was no more observable in the absence DsrA (N3431Δ*dsrA*). These results are identical to the observation made in strain N3431Δ*hfq*. The reduced stabilities of OxyS in the absence of DsrA in strains lacking RNase III or RNase E activity might hint a possible competition between negative regulator - OxyS and positive regulators - DsrA and Hfq in order to regulate *rpoS* translation. It was predicted that OxyS inhibits *rpoS* translation by titrating out Hfq (Zhang, Wassarman et al. 2002). We speculate that in the absence of DsrA in stationary phase, Hfq gets dissociated from OxyS (by unknown mechanism) in order to activate the translation of stationary phase sigma factor S (σ^S). This dissociation might result in accessibility of RNase targets on OxyS in the absence of the protector Hfq. From our results we conclude that OxyS processing is performed by a complex multi-factorial system of RNA-RNA and RNA-protein interactions.
To further validate our data and exclude effects by general inhibition of transcription we have also determined the half-lives of OxyS without the addition of rifampicin. *E. coli* cultures were treated with hydrogen peroxide to induce OxyS expression and collection of samples for RNA isolation was started 14 min later. The OxyS half-lives determined by this approach were in general shorter than those determined by the addition of rifampicin (Supplementary Fig 9.5) but we observed the same effects as in the presence of rifampicin: i) growth-phase did not influence OxyS half-life in strain MG1655, ii) the lack of Hfq reduced OxyS half-life about two fold, iii) lack of RNase E activity resulted in significantly increased stability in stationary phase.

The results presented here reveal that processing of a transcript as short as OxyS (109 nt) involves different RNases and is influenced by multiple factors. These complex processes cannot be analyzed by *in vitro* systems. These findings imply that control of OxyS turn-over is crucial for maintaining important biological functions in the cell.

5.5 Growth phase dependent turn-over of *rpoS* mRNA

The synthesis of RpoS, the master regulator of the general stress response is a tightly regulated mechanism (Lange and Hengge-Aronis 1994). It was demonstrated that several contributory factors such as the RNA binding protein Hfq, small non-coding RNAs – DsrA and RprA assist in the translational activation of *rpoS* mRNA (Brown and Elliott 1996; Muffler, Fischer et al. 1996; Majdalani, Cunning et al. 1998; Sledjeski, Whitman et al. 2001; Majdalani, Hernandez et al. 2002). *rpoS* mRNA folds in to a complex structure, the 5' – untranslated region base pairs

with the translation initiation region, which results in weak accessibility of the ribosomal binding site and poor translation (Brown and Elliott 1997; Cunning and Elliott 1999; Hengge-Aronis 2002). Brown and co-workers in 1996 showed that Hfq plays an important role in the unfolding and in the stimulation of translation of *rpoS* mRNA and they also suggested that Hfq might influence the stability of this mRNA.

As predicted we were able to observe the influence of Hfq on the turn-over of *rpoS* mRNA. We determined 1.4 and 1.7 fold decrease in the stability in the absence of Hfq in exponential and stationary growth phase respectively when compared to the isogenic wild type strain MG1655. As we observed that OxyS turn-over in part is carried out by RNase E in stationary growth phase, we analyzed the role of RNase E in the turn-over of *rpoS* mRNA. We did not observe any significant change in the turn-over rates in exponential growth phase in the presence or absence of RNase E activity. However there was a 1.5 fold increase in the stability of *rpoS* mRNA was observed in the absence of RNase E activity at non-permissive conditions. These results conclude that Hfq not only plays role in the unfolding of *rpoS* mRNA to stimulate translation but also protects it against RNases like observed in the case of OxyS.

We have also analyzed the role of RNase III, a double stranded specific RNase in the turn-over of *rpoS* mRNA. In the isogenic wild type strain BL322 (rnc^{+}) half-lives of *rpoS* mRNA did not vary in exponential as well as in stationary growth phase, however enhanced stability of *rpoS* mRNA by a factor of 3 was observed in the absence of RNase III (BL321 - rnc^{-}). Surprisingly the influence of RNase III on the *rpoS* mRNA turn-over was not visible in stationary growth phase of strain BL321 (rnc^{-}). This indicates that RNase III plays an important role in the turn-over of *rpoS* mRNA in exponential growth phase rather in stationary phase, which is in agreement with the earlier published results (Resch, Afonyushkin et al. 2008).

In all strains we tested in this study with the exception of BL322 *rpoS* was somewhat more stable in exponential phase than in stationary phase. The results that we have discussed in this study is in contrast to an earlier report on the *rpoS* stability in *Salmonella dublin*, which determined a half-life of 2.5 min for *rpoS* in early exponential growth phase and 3.5 min in stationary phase (Paesold and Krause 1999). From our result we conclude that the reduction of *rpoS* mRNA turn-over does not contribute to increase of RpoS levels.

5.6 Influence of OxyS, DsrA and Hfq on RpoS synthesis

rpoS mRNA was among the first target mRNAs shown to be regulated by at least three sRNAs (DsrA, RprA and OxyS) at post-transcriptional level (Majdalani, Cunning et al. 1998; Zhang, Altuvia et al. 1998; Majdalani, Hernandez et al. 2002). DsrA and RprA sRNAs with the help of the RNA chaperone Hfq activates the translation under low temperature and osmotic stress conditions (Majdalani, Cunning et al. 1998; Lease and Belfort 2000; Majdalani, Hernandez et al. 2002; Arluison, Hohng et al. 2007; Updegrove, Wilf et al. 2008). By contrast, the third small RNA OxyS inhibits the translation by an unexplained mechanism (Zhang, Altuvia et al. 1998). OxyS RNA is under the control of one of the major regulatory proteins activated upon

oxidative stress, OxyR (Christman, Morgan et al. 1985). The OxyR locus is central to the adaptive response of exponentially growing cells to H_2O_2 (Gonzalez-Flecha and Demple 1997) and increased resistance to oxidants of stationary growth phase is linked to *rpoS* (Jamieson et al 1997). It was predicted that *rpoS* translation repression by OxyS might help to prevent redundant usage of transcriptional regulators (Altuvia, Weinstein-Fischer et al. 1997). Several studies have shown that both OxyR and RpoS regulate several genes under oxidative stress in exponential and stationary growth phase respectively (Altuvia, Almiron et al. 1994; Ivanova, Miller et al. 1994; Becker-Hapak and Eisenstark 1995). Altuvia et al., also suggested that OxyR is expressed in log-phase under oxidative stress which further leads to activation of the genes that scavenge hydrogen peroxide and RpoS is not needed in log-phase and inhibition of RpoS synthesis by OxyS prevents the activation of genes that are already activated by OxyR.

In our study OxyS was not only detected in exponential growth phase but also in stationary growth phase under oxidative stress. I was also able detect 10 fold higher expression of DsrA, the *rpoS* translation activator in stationary growth phase along with OxyS when cells were treated with hydrogen peroxide. From our studies for the first time we could show that not only DsrA is expressed along with OxyS but also influences the stability of OxyS like Hfq. We also speculated that *rpoS* mRNA translational repression by OxyS will not be 100%, while the repression of RpoS synthesis can profoundly affect cell viability and growth under stress conditions such as oxidative stress in stationary growth phase. So we expected interplay among the regulators in regulating *rpoS* translation.

Our data confirm the earlier reported higher abundance of RpoS in stationary growth phase (Hengge-Aronis 1993). I was able to observe a comparatively lower impact of oxidative stress on RpoS levels. Even though an increase of RpoS levels upon entry into stationary phase was observed in our study, RpoS levels were slightly reduced in the presence of H_2O_2 compared to the untreated *E.coli* MG1655 cells (Fig 4.15). Decreased expression of RpoS under oxidative stress is due to expression of OxyS RNA, which is in agreement with the earlier results (Fig 4.13) (Zhang, Altuvia et al. 1998). I was able to detect OxyS only when cells were treated with H_2O_2. The OxyS RNA was induced after 7 min of exposure to H_2O_2 and reached its maximal levels by 14 min in both growth phases. In exponential as well as in stationary growth phases, during the course of the experiment OxyS RNA levels declined, and in contrast RpoS protein levels accumulated. These results once again confirm that OxyS represses RpoS synthesis. In strain MG1655 accumulation of RpoS levels was observed during transition to stationary phase which correlates with increased levels of *rpoS* mRNA. I also observed RpoS protein levels along with *rpoS* mRNA levels were steadily increased after the addition of H_2O_2 in stationary phase. These increased RpoS levels correlated with increased levels of DsrA, an sRNA activating translation of *rpoS* (Repoila, Majdalani et al. 2003). I have also analyzed RpoS expression in a strain lacking DsrA. I was able to observe identical accumulation pattern of RpoS as observed in the isogenic wild type strain MG1655. However I observed slightly

reduced levels of RpoS in the absence of DsrA, which is in agreement with its activating effect on *rpoS* translation (Repoila, Majdalani et al. 2003). We conclude that DsrA is not a major player in growth phase-dependent activation of *rpoS* translation in *E.coli*.

Hfq is known to be a very important accessory factor to activate *rpoS* translation (Muffler, Fischer et al. 1996). It was shown that Hfq is essential for sRNA/mRNA interaction and for their stability (Sledjeski, Whitman et al. 2001; Zhang, Wassarman et al. 2002). So I have analyzed the role to Hfq on RpoS levels under oxidative stress in both growth phases. We observed a strong influence of the Hfq protein on RpoS levels. In the absence of Hfq OxyS RNA was induced after 7 min of exposure to hydrogen peroxide and reached maximal levels by 14 min however, disappearance of OxyS was very slow when compared to the isogenic strain MG1655 in exponential growth phase. So the reduced levels of RpoS in a strain lacking Hfq in exponential growth phase could also be due to the presence of OxyS RNA. Not only RpoS protein levels but also DsrA levels were strongly reduced in a strain lacking Hfq. The reduced DsrA levels are a consequence of reduced DsrA stability in the absence of Hfq (Sledjeski, Whitman et al. 2001). I also did not observe an accumulation of *rpoS* mRNA either during transition to stationary growth phase or in stationary growth phase, but *rpoS* mRNA levels steadily decreased when compared to the other strains. These reduced levels are most likely the consequence of the reduced stability of *rpoS* mRNA in the absence of Hfq (Table 4.1- Page No: 50). Supporting the view that RpoS protein levels are mainly determined by *rpoS* mRNA levels. We also suggest that Hfq not only activates the translation of *rpoS* mRNA by unfolding but also determines stability which in turn affects RpoS levels. Since a strain lacking DsrA has only slightly reduced RpoS levels the reduced DsrA levels do not account for strongly reduced RpoS protein levels in the *hfq*$^-$ strain.

RpoS levels are not only regulated at post-transcriptional level by sRNAs but also at the level proteolysis. The half-life of the RpoS protein was determined to be 2 min in early exponential phase and about 25 min in stationary phase (Lange and Hengge-Aronis 1994; Loewen and Hengge-Aronis 1994). It was also shown by previously and recently that RpoS is stabilized under various stress conditions such as: carbon starvation, reduced phosphorous sources, and/or under oxidative stress condition induced by hydrogen peroxide (Muffler, Fischer et al. 1996; Merrikh, Ferrazzoli et al. 2009; Merrikh, Ferrazzoli et al. 2009). In strain MG1655 we observed a similar half-life of RpoS in stationary phase (23 ± 2) under oxidative stress as described previously, however we observed a higher stability of RpoS in exponential phase. The observed difference in the half-life to the earlier published data might be due to the fact that we did not perform the experiment during early exponential growth but the translation was inhibited at mid-exponential growth phase.

I also have analyzed the stability of the RpoS protein in strains lacking DsrA and/or Hfq, while we observed reduced levels of RpoS in the absence of DsrA and Hfq. We did not observe any turn-over within 60 min after stopping translation when Hfq and /or DsrA was missing. The high protein stability is in agreement with an increase of RpoS when *rpoS* mRNA levels are

already decreasing in strain lacking Hfq. The RNA chaperone Hfq acts like a global regulator, which is largely due to its role in the expression of RpoS (Muffler, Traulsen et al. 1997). The deletion of Hfq leads to a variety of pronounced pleiotropic phenotypes, including altered growth rates, largely reduced supercoiling of plasmid DNA in stationary-phase cells, altered cell division, osmosensitivity, altered patterns of protein synthesis, alterations and multicopy suppression of *hns* phenotype (Shi and Bennett 1994; Valentin-Hansen, Eriksen et al. 2004). It has been shown in *Vibrio cholera* and in *Salmonella enterica*, a close relative of *E.coli*, that RpoE, an alternative sigma factor involved in stress responses, is constitutively expressed in the absence of Hfq (Ding, Davis et al. 2004; Figueroa-Bossi, Lemire et al. 2006). This induction of RpoE consequently leads to the induction of other stress related genes that are under its own control. In *E.coli* Hfq also modulates RpoH-mediated heat shock response (Guisbert, Rhodius et al. 2007; Yura, Guisbert et al. 2007). The RpoH-mediated cytoplasmic stress response was repressed in cells lacking Hfq due to an increased expression of the DnaK chaperone. It has been shown that DnaK influences the stability of RpoS in stationary phase by Muffler et al (1997) and Rockabrand et al., (1998). In a wild type strain RpoS showed a half-life of 30 min, while a reduce half-life of 20 min was observed in the absence of DnaK (Rockabrand, Livers et al. 1998). It was also suggested that DnaK might indirectly affect the levels of heat shock responsive protease ClpXP or response regulator RssB (Rockabrand, Livers et al. 1998) . Recently it was shown that anti-adaptor proteins of RssB also play a role in the proteolysis of RpoS under various stress conditions (Bougdour, Cunning et al. 2008). It was shown by Merrikh and co-workers that IraD, an anti-adaptor protein of RssB increases the stability of RpoS when cell were treated with hydrogen peroxide in exponential growth phase. However the factors that are involved in stabilizing RpoS in stationary phase under oxidative stress are yet to be identified. Thus we speculate that the increased stability of RpoS observed in the strain lacking Hfq is most likely the consequence to the higher expression of DnaK (Muffler, Barth et al. 1997; Rockabrand, Livers et al. 1998). This complex network of regulation may guarantee the maintenance of the RpoS response under a variety of conditions. We presently have no clue, how the lack of DsrA affects stability of RpoS but it is likely that it is an indirect effect as in the case of Hfq. It is not known whether DsrA has target mRNAs in addition to RpoS.

Like Hfq and DsrA, OxyS also affected RpoS levels as well as its turn-over but in a different way. We observed a rapid turn-over of RpoS under oxidative stress in a strain lacking OxyS. We also observed higher levels of RpoS as well as *rpoS* mRNA throughout all growth stages we tested. It is conceivable that an increased turn-over of RpoS in the absence of OxyS prevents the accumulation of RpoS to harmful levels, but the underlying mechanisms are unknown.

6 SUMMARY

rpoS mRNA is one of the first target RNA shown to be regulated by at least 3 small non-coding RNAs under various stress conditions. Two out of three sRNAs are involved in the activation of *rpoS* mRNA translation. By contrast the third sRNA, OxyS has been implicated in the repression of RpoS synthesis. OxyS RNA is strictly induced under oxidative stress and reduces the levels of the stationary phase sigma factor RpoS. In order to reveal a possible impact of processing on growth-dependent regulation, we analyzed the turn-over rates of OxyS and *rpoS* mRNA in early exponential and in stationary growth phase in different *E. coli* strains. We were not able to assign a major role to any of the individual endo- or exoribonucleases (RNase E, RNase III, PNPase or RNase II) on OxyS turn-over in exponential growth phase. However, the simultaneous lack of RNase E, PNPase and RNase II activity resulted in some stabilization of OxyS in exponential growth phase, indicating the combined action of both endo- and exoribonucleases on OxyS turn-over. A greater impact of RNase E on OxyS turn-over was observed in stationary phase and this in turn was dependent on the presence of the RNA chaperone Hfq and of DsrA sRNAs (activator of *rpoS* mRNA translation). Our data also confirm a role of RNase III in *rpoS* turn-over, however, only in exponential growth phase. We conclude that OxyS and *rpoS* mRNA turn-over rates are influenced by different RNases and additional factors such as Hfq and DsrA and that the impact of these factors is strongly dependent on growth phase.

Furthermore, we analyzed the influence of OxyS – inhibitor RpoS synthesis and Hfq and DsrA – activators of RpoS synthesis, while all three regulators co-exist under oxidative stress. Our data reveal that growth phase has a strong effect on RpoS levels, while no major influence of oxidative stress generated by H_2O_2 is observed. The lack of DsrA resulted in a small decrease of RpoS levels, which is in agreement with the activating effect of this sRNA on RpoS translation. A lack of Hfq resulted in significantly reduced levels of RpoS, although the turn-over of RpoS was reduced compared to the parental wild type. DsrA and OxyS influenced RpoS turn-over in opposite way by yet unknown mechanisms. These findings illustrate that main control of growth-phase dependent RpoS expression is at level of *rpoS* transcription and that growth phase has a stronger impact on RpoS levels than oxidative stress.

7 ZUSAMMENFASSUNG

RpoS ist ein bedeutender Sigmafaktor der stationären Wachstumsphase in *E. coli*. Die *rpoS* mRNA ist eine der ersten RNAs für die unter verschiedenen Stressbedingungen eine Regulation durch mindestens drei *small non-coding* RNAs gezeigt wurde. Zwei dieser drei sRNAs sind an der Aktivierung der *rpoS* mRNA Translation beteiligt. Im Gegensatz dazu reprimiert die dritte sRNA die RpoS Synthese. Diese OxyS RNA wird unter oxidativem Stress induziert und reduziert die Menge an RpoS.

Um einen möglichen positiven Einfluss der RNA-Prozessierung auf deren Wachstumsphasen-abhängige Regulation nachzuweisen, wurden in dieser Arbeit die Umsatzraten von OxyS und *rpoS* in der frühen exponentiellen sowie in der stationären Wachstumsphase in verschiedenen *E. coli* Stämmen untersucht. Hierbei war es zwar nicht möglich einer einzelnen Endo- oder Exoribonukleasen (RNase E, RNase III, PNPase oder RNase II) eine Hauptrolle im OxyS-Umsatz zuzuweisen, das gleichzeitige Fehlen der Aktivitäten von RNase E, PNPase und RNase II resultierte jedoch in einer Stabilisierung von OxyS in der exponentiellen Wachstumsphase. Dies lässt auf eine kombinierte Wirkung der Aktivitäten von Endo- und Exoribonukleasen auf den OxyS-Umsatz schließen. Ein größerer Einfluss von RNase E auf den OxyS-Umsatz konnte in der stationären Wachstumsphase nachgewiesen werden, der außerdem von der Anwesenheit des RNA-Chaperons Hfq und von der DsrA sRNA (Aktivator der *rpoS* mRNA-Translation) abhängig ist. Des Weiteren konnte ausschließlich in der exponentiellen Wachstumsphase eine Beteiligung von RNase III am OxyS-Umsatz nachgewiesen werden. Die OxyS und *rpoS* mRNA-Umsätze werden demnach sowohl von verschiedenen RNasen als auch von weiteren Faktoren wie Hfq und DsrA reguliert. Der Einfluss der einzelnen Faktoren ist wiederum stark von der Wachstumsphase abhängig.

Da alle drei Regulatoren von RpoS, OxyS, DsrA und Hfq, unter oxidativem Stress existieren aber sowohl aktivierenden wie inhibierenden Einfluss auf RpoS haben, wurde in dieser Arbeit ebenfalls deren Synthese untersucht. Oxidativer Stress, hervorgerufen durch H_2O_2, zeigte keinen starken Einfluss, wogegen ein starker Effekt der Wachstumsphase auf die RpoS-Menge beobachtet werden konnte.

Mutationsanalysen zeigten, dass in Abwesenheit von DsrA eine geringe Verminderung der RpoS-Menge stattfindet, was in Einklang mit dem aktivierenden Effekt dieser sRNA auf die RpoS-Translation steht. Das Fehlen von Hfq resultierte sowohl in einer signifikant reduzierten Menge an RpoS als auch in einem reduzierten RpoS-Umsatz im Vergleich zum entsprechenden Wildtyp. DsrA und OxyS beeinflussen den RpoS-Umsatz in der entgegengesetzten Richtung durch bisher unbekannte Mechanismen.

Die gezeigten Ergebnisse verdeutlichen, dass die Regulation der Wachstumsphasen-abhängigen Expression von RpoS auf Ebene der *rpoS*-Transkription stattfindet und sich die Wachstumsphase stärker auf RpoS auswirkt als oxidativer Stress.

8 REFERENCES

Afonyushkin, T., B. Vecerek, et al. **(2005)**. "Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB." Nucleic Acids Res **33**(5): 1678-1689.

Aiba, H. **(2007)**. "Mechanism of RNA silencing by Hfq-binding small RNAs." Curr Opin Microbiol **10**(2): 134-139.

Altuvia, S. **(2007)**. "Identification of bacterial small non-coding RNAs: experimental approaches." Curr Opin Microbiol **10**(3): 257-261.

Altuvia, S., M. Almiron, et al. **(1994)**. "The *dps* promoter is activated by OxyR during growth and by IHF and sigma S in stationary phase." Mol Microbiol **13**(2): 265-272.

Altuvia, S., D. Weinstein-Fischer, et al. **(1997)**. "A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator." Cell **90**(1): 43-53.

Altuvia, S., A. Zhang, et al. **(1998)**. "The *Escherichia coli* OxyS regulatory RNA represses *fhlA* translation by blocking ribosome binding." EMBO J **17**(20): 6069-6075.

Amblar, M., S. C. Viegas, et al. **(2004)**. "Homologous and heterologous expression of RNase III from *Lactococcus lactis*." Biochem Biophys Res Commun **323**(3): 884-890.

Andrade, J. M. and C. M. Arraiano **(2008)**. "PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins." RNA **14**(3): 543-551.

Apirion, D. **(1973)**. "Degradation of RNA in *Escherichia coli*. A hypothesis." Mol Gen Genet **122**(4): 313-322.

Apirion, D. and A. B. Lassar **(1978)**. "A conditional lethal mutant of *Escherichia coli* which affects the processing of ribosomal RNA." J Biol Chem **253**(5): 1738-1742.

Argaman, L., R. Hershberg, et al. **(2001)**. "Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*." Curr Biol **11**(12): 941-950.

Arluison, V., S. Hohng, et al. **(2007)**. "Spectroscopic observation of RNA chaperone activities of Hfq in post-transcriptional regulation by a small non-coding RNA." Nucleic Acids Res **35**(3): 999-1006.

Arnvig, K. B. and D. B. Young **(2009)**. "Identification of small RNAs in *Mycobacterium tuberculosis*." Mol Microbiol **73**(3): 397-408.

Arraiano, C. M., S. D. Yancey, et al. **(1988)**. "Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12." J Bacteriol **170**(10): 4625-4633.

Babitzke, P., L. Granger, et al. **(1993)**. "Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III." J Bacteriol **175**(1): 229-239.

Barth, E., K. V. Gora, et al. **(2009)**. "Interplay of cellular cAMP levels, {sigma}S activity and oxidative stress resistance in *Escherichia coli*." Microbiology **155**(Pt 5): 1680-1689.

Basineni, S. R., R. Madhugiri, et al. **(2009)**. "The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target *rpoS* in *E. coli* is growth phase dependent." RNA Biol **6**(5): 584-594.

Becker-Hapak, M. and A. Eisenstark **(1995)**. "Role of *rpoS* in the regulation of glutathione oxidoreductase (*gor*) in *Escherichia coli*." FEMS Microbiol Lett **134**(1): 39-44.

Becker, G., E. Klauck, et al. **(1999)**. "Regulation of RpoS proteolysis in *Escherichia coli*: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS." Proc Natl Acad Sci U S A **96**(11): 6439-6444.

Berghoff, B. A., J. Glaeser, et al. **(2009)**. "Photooxidative stress-induced and abundant small RNAs in *Rhodobacter sphaeroides*." Mol Microbiol **74**(6): 1497-1512.

Bernstein, J. A., P. H. Lin, et al. **(2004)**. "Global analysis of *Escherichia coli* RNA degradosome function using DNA microarrays." Proc Natl Acad Sci U S A **101**(9): 2758-2763.

Blumenthal, T. and G. G. Carmichael **(1979)**. "RNA replication: function and structure of Qbeta-replicase." Annu Rev Biochem **48**: 525-548.

Bouche, F. and J. P. Bouche **(1989)**. "Genetic evidence that DicF, a second division inhibitor encoded by the *Escherichia coli dicB* operon, is probably RNA." Mol Microbiol **3**(7): 991-994.

Bougdour, A., C. Cunning, et al. **(2008)**. "Multiple pathways for regulation of sigmaS (RpoS) stability in *Escherichia coli* via the action of multiple anti-adaptors." Mol Microbiol **68**(2): 298-313.

Bougdour, A., C. Lelong, et al. **(2004)**. "Crl, a low temperature-induced protein in *Escherichia coli* that binds directly to the stationary phase sigma subunit of RNA polymerase." J Biol Chem **279**(19): 19540-19550.

Brantl, S. **(2007)**. "Regulatory mechanisms employed by cis-encoded antisense RNAs." Curr Opin Microbiol **10**(2): 102-109.

Brown, L. and T. Elliott **(1996)**. "Efficient translation of the RpoS sigma factor in *Salmonella typhimurium* requires host factor I, an RNA-binding protein encoded by the *hfq* gene." J Bacteriol **178**(13): 3763-3770.

Brown, L. and T. Elliott **(1997)**. "Mutations that increase expression of the *rpoS* gene and decrease its dependence on *hfq* function in *Salmonella typhimurium*." J Bacteriol **179**(3): 656-662.

Burgin, A. B., K. Parodos, et al. **(1990)**. "The excision of intervening sequences from Salmonella 23S ribosomal RNA." Cell **60**(3): 405-414.

Cairrao, F. and C. M. Arraiano **(2006)**. "The role of endoribonucleases in the regulation of RNase R." Biochem Biophys Res Commun **343**(3): 731-737.

Cairrao, F., A. Cruz, et al. **(2003)**. "Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA." Mol Microbiol **50**(4): 1349-1360.

Callaghan, A. J., M. J. Marcaida, et al. **(2005)**. "Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover." Nature **437**(7062): 1187-1191.

Carpousis, A. J., G. Van Houwe, et al. **(1994)**. "Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation." Cell **76**(5): 889-900.

Celesnik, H., A. Deana, et al. **(2007)**. "Initiation of RNA decay in *Escherichia coli* by 5' pyrophosphate removal." Mol Cell **27**(1): 79-90.

Chang, D. E., D. J. Smalley, et al. **(2002)**. "Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model." Mol Microbiol **45**(2): 289-306.

Chauhan, A. K. and D. Apirion **(1991)**. "The *rne* gene is the structural gene for the processing endoribonuclease RNase E of *Escherichia coli.*" Mol Gen Genet **228**(1-2): 49-54.

Chen, S., E. A. Lesnik, et al. **(2002)**. "A bioinformatics based approach to discover small RNA genes in the *Escherichia coli* genome." Biosystems **65**(2-3): 157-177.

Cheng, Z. F. and M. P. Deutscher **(2002)**. "Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II." J Biol Chem **277**(24): 21624-21629.

Cheng, Z. F. and M. P. Deutscher **(2003)**. "Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R." Proc Natl Acad Sci U S A **100**(11): 6388-6393.

Cheng, Z. F. and M. P. Deutscher **(2005)**. "An important role for RNase R in mRNA decay." Mol Cell **17**(2): 313-318.

Cheng, Z. F., Y. Zuo, et al. **(1998)**. "The *vacB* gene required for virulence in *Shigella flexneri* and *Escherichia coli* encodes the exoribonuclease RNase R." J Biol Chem **273**(23): 14077-14080.

Christman, M. F., R. W. Morgan, et al. **(1985)**. "Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*." Cell **41**(3): 753-762.

Cunning, C. and T. Elliott **(1999)**. "RpoS synthesis is growth rate regulated in *Salmonella typhimurium*, but its turnover is not dependent on acetyl phosphate synthesis or PTS function." J Bacteriol **181**(16): 4853-4862.

Dasgupta, S., L. Fernandez, et al. **(1998)**. "Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III--the effect of dsRNA binding on gene expression." Mol Microbiol **28**(3): 629-640.

Davis, B. M. and M. K. Waldor **(2007)**. "RNase E-dependent processing stabilizes MicX, a *Vibrio cholerae* sRNA." Mol Microbiol **65**(2): 373-385.

del Val, C., E. Rivas, et al. **(2007)**. "Identification of differentially expressed small non-coding RNAs in the legume endosymbiont *Sinorhizobium meliloti* by comparative genomics." Mol Microbiol **66**(5): 1080-1091.

Ding, Y., B. M. Davis, et al. **(2004)**. "Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression." Mol Microbiol **53**(1): 345-354.

Donovan, W. P. and S. R. Kushner **(1983)**. "Amplification of ribonuclease II (*rnb*) activity in *Escherichia coli* K-12." Nucleic Acids Res **11**(2): 265-275.

Donovan, W. P. and S. R. Kushner **(1986)**. "Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in *Escherichia coli* K-12." Proc Natl Acad Sci U S A **83**(1): 120-124.

Dreyfus, M. and P. Regnier **(2002)**. "The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria." Cell **111**(5): 611-613.

Durfee, T., A. M. Hansen, et al. **(2008)**. "Transcription profiling of the stringent response in *Escherichia coli*." J Bacteriol **190**(3): 1084-1096.

Ehretsmann, C. P., A. J. Carpousis, et al. **(1992)**. "mRNA degradation in procaryotes." FASEB J **6**(13): 3186-3192.

Evguenieva-Hackenberg, E. and G. Klug **(2000)**. "RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the alpha subclass of Proteobacteria." J Bacteriol **182**(17): 4719-4729.

Faubladier, M., K. Cam, et al. **(1990)**. "*Escherichia coli* cell division inhibitor DicF-RNA of the *dicB* operon. Evidence for its generation *in vivo* by transcription termination and by RNase III and RNase E-dependent processing." J Mol Biol **212**(3): 461-471.

Figueroa-Bossi, N., S. Lemire, et al. **(2006)**. "Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*." Mol Microbiol **62**(3): 838-852.

Franze de Fernandez, M. T., L. Eoyang, et al. **(1968)**. "Factor fraction required for the synthesis of bacteriophage Qbeta-RNA." Nature **219**(5154): 588-590.

Gaal, T., M. J. Mandel, et al. **(2006)**. "Crl facilitates RNA polymerase holoenzyme formation." J Bacteriol **188**(22): 7966-7970.

Geissmann, T. A. and D. Touati **(2004)**. "Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator." EMBO J **23**(2): 396-405.

Gentry, D. R., V. J. Hernandez, et al. **(1993)**. "Synthesis of the stationary-phase sigma factor sigma S is positively regulated by ppGpp." J Bacteriol **175**(24): 7982-7989.

Gold, L. **(1988)**. "Posttranscriptional regulatory mechanisms in *Escherichia coli*." Annu Rev Biochem **57**: 199-233.

Goldblum, K. and D. Apririon **(1981)**. "Inactivation of the ribonucleic acid-processing enzyme ribonuclease E blocks cell division." J Bacteriol **146**(1): 128-132.

Gonzalez-Flecha, B. and B. Demple **(1997)**. "Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*." J Bacteriol **179**(2): 382-388.

Gonzalez-Flecha, B. and B. Demple **(1999)**. "Role for the *oxyS* gene in regulation of intracellular hydrogen peroxide in *Escherichia coli*." J Bacteriol **181**(12): 3833-3836.

Gopalan, V., A. Vioque, et al. **(2002)**. "RNase P: variations and uses." J Biol Chem **277**(9): 6759-6762.

Gottesman, S., G. Storz, et al. **(2001)**. "Small RNA regulators of translation: mechanisms of action and approaches for identifying new small RNAs." Cold Spring Harb Symp Quant Biol **66**: 353-362.

Griffin, B. E. **(1971)**. "Separation of ^{32}P-labelled ribonucleic acid components. The use of polyethylenimine-cellulose (TLC) as a second dimension in separating oligoribonucleotides of '4.5 S' and 5 S from *E. coli*." FEBS Lett **15**(3): 165-168.

Grossman, D. and A. van Hoof **(2006)**. "RNase II structure completes group portrait of 3' exoribonucleases." Nat Struct Mol Biol **13**(9): 760-761.

Guerrier-Takada, C., K. Gardiner, et al. **(1983)**. "The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme." Cell **35**(3 Pt 2): 849-857.

Guisbert, E., V. A. Rhodius, et al. **(2007)**. "Hfq modulates the sigmaE-mediated envelope stress response and the sigma32-mediated cytoplasmic stress response in *Escherichia coli*." J Bacteriol **189**(5): 1963-1973.

Hajnsdorf, E. and P. Regnier **(2000)**. "Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I." Proc Natl Acad Sci U S A **97**(4): 1501-1505.

Haugel-Nielsen, J., E. Hajnsdorf, et al. **(1996)**. "The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation." EMBO J **15**(12): 3144-3152.

Heidrich, N., I. Moll, et al. **(2007)**. "*In vitro* analysis of the interaction between the small RNA SR1 and its primary target *ahrC* mRNA." Nucleic Acids Res **35**(13): 4331-4346.

Hengge-Aronis, R. **(1993)**. "Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in E. coli." Cell **72**(2): 165-168.

Hengge-Aronis, R., Ed. **(2000)**. The general stress response in *Escherichia coli*. Bacterial Stress Responses Washington, DC, ASM Press.

Hengge-Aronis, R. **(2002)**. "Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase." Microbiol Mol Biol Rev **66**(3): 373-395, table of contents.

Herskovits, A. A., E. S. Bochkareva, et al. **(2000)**. "New prospects in studying the bacterial signal recognition particle pathway." Mol Microbiol **38**(5): 927-939.

Hindley, J. (1967). "Fractionation of ^{32}P-labelled ribonucleic acids on polyacrylamide gels and their characterization by fingerprinting." J Mol Biol **30**(1): 125-136.

Ikemura, T. and J. E. Dahlberg **(1973)**. "Small ribonucleic acids of *Escherichia coli*. I. Characterization by polyacrylamide gel electrophoresis and fingerprint analysis." J Biol Chem **248**(14): 5024-5032.

Ivanova, A., C. Miller, et al. **(1994)**. "Role of *rpoS* (*katF*) in oxyR-independent regulation of hydroperoxidase I in *Escherichia coli*." Mol Microbiol **12**(4): 571-578.

Jager, D., C. M. Sharma, et al. **(2009)**. "Deep sequencing analysis of the *Methanosarcina mazei* Go1 transcriptome in response to nitrogen availability." Proc Natl Acad Sci U S A **106**(51): 21878-21882.

Jain, C. and J. G. Belasco **(1995)**. "RNase E autoregulates its synthesis by controlling the degradation rate of its own mRNA in *Escherichia coli*: unusual sensitivity of the rne transcript to RNase E activity." Genes Dev **9**(1): 84-96.

Kaberdin, V. R., A. Miczak, et al. **(1998)**. "The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis sp*. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly." Proc Natl Acad Sci U S A **95**(20): 11637-11642.

Kalamorz, F., B. Reichenbach, et al. **(2007)**. "Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*." Mol Microbiol **65**(6): 1518-1533.

Kasai, T., R. S. Gupta, et al. **(1977)**. "Exoribonucleases in wild type *Escherichia coli* and RNase II-deficient mutants." J Biol Chem **252**(24): 8950-8956.

Kawamoto, H., Y. Koide, et al. **(2006)**. "Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq." Mol Microbiol **61**(4): 1013-1022.

Kawano, M., A. A. Reynolds, et al. **(2005)**. "Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli*." Nucleic Acids Res **33**(3): 1040-1050.

Khemici, V. and A. J. Carpousis **(2004)**. "The RNA degradosome and poly(A) polymerase of *Escherichia coli* are required in vivo for the degradation of small mRNA decay intermediates containing REP-stabilizers." Mol Microbiol **51**(3): 777-790.

Kivity-Vogel, T. and D. Elson **(1967)**. "On the metabolic inactivation of messenger RNA in *Escherichia coli*: ribonuclease I and polynucleotide phosphorylase." Biochim Biophys Acta **138**(1): 66-75.

Kruger, K., P. J. Grabowski, et al. **(1982)**. "Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena." Cell **31**(1): 147-157.

Kushner, S. R. **(2002)**. "mRNA decay in *Escherichia coli* comes of age." J Bacteriol **184**(17): 4658-4665; discussion 4657.

Lange, R., D. Fischer, et al. **(1995)**. "Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*." J Bacteriol **177**(16): 4676-4680.

Lange, R. and R. Hengge-Aronis **(1991)**. "Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*." Mol Microbiol **5**(1): 49-59.

Lange, R. and R. Hengge-Aronis **(1994)**. "The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability." Genes Dev **8**(13): 1600-1612.

Lange, R. and R. Hengge-Aronis **(1994)**. "The *nlpD* gene is located in an operon with *rpoS* on the *Escherichia coli* chromosome and encodes a novel lipoprotein with a potential function in cell wall formation." Mol Microbiol **13**(4): 733-743.

Layton, J. C. and P. L. Foster **(2003)**. "Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in *Escherichia coli*." Mol Microbiol **50**(2): 549-561.

Lease, R. A. and M. Belfort **(2000)**. "A trans-acting RNA as a control switch in *Escherichia coli*: DsrA modulates function by forming alternative structures." Proc Natl Acad Sci U S A **97**(18): 9919-9924.

Lease, R. A. and S. A. Woodson **(2004)**. "Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA." J Mol Biol **344**(5): 1211-1223.

Lee, S. Y., S. C. Bailey, et al. **(1978)**. "Small stable RNAs from *Escherichia coli*: evidence for the existence of new molecules and for a new ribonucleoprotein particle containing 6S RNA." J Bacteriol **133**(2): 1015-1023.

Lennette, E. T., L. Gorelic, et al. **(1971)**. "An *Escherichia coli* mutant with increased messenger ribonuclease activity." Proc Natl Acad Sci U S A **68**(12): 3140-3144.

Lenz, D. H., K. C. Mok, et al. **(2004)**. "The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and Vibrio cholerae." Cell **118**(1): 69-82.

Li, H. **(2007)**. "Complexes of tRNA and maturation enzymes: shaping up for translation." Curr Opin Struct Biol **17**(3): 293-301.

Li, H. and A. W. Nicholson **(1996)**. "Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants." EMBO J **15**(6): 1421-1433.

Li, H. L., B. S. Chelladurai, et al. **(1993)**. "Ribonuclease III cleavage of a bacteriophage T7 processing signal. Divalent cation specificity, and specific anion effects." Nucleic Acids Res **21**(8): 1919-1925.

Li, Z. and M. P. Deutscher **(2002)**. "RNase E plays an essential role in the maturation of *Escherichia coli* tRNA precursors." RNA **8**(1): 97-109.

Li, Z., S. Pandit, et al. **(1998)**. "3' exoribonucleolytic trimming is a common feature of the maturation of small, stable RNAs in *Escherichia coli*." Proc Natl Acad Sci U S A **95**(6): 2856-2861.

Lin-Chao, S., C. L. Wei, et al. **(1999)**. "RNase E is required for the maturation of *ssrA* RNA and normal *ssrA* RNA peptide-tagging activity." Proc Natl Acad Sci U S A **96**(22): 12406-12411.

Liu, J. M. and A. Camilli **(2010)**. "A broadening world of bacterial small RNAs." Curr Opin Microbiol **13**(1): 18-23.

Livny, J., A. Brencic, et al. **(2006)**. "Identification of 17 *Pseudomonas aeruginosa* sRNAs and prediction of sRNA-encoding genes in 10 diverse pathogens using the bioinformatic tool sRNAPredict2." Nucleic Acids Res **34**(12): 3484-3493.

Loewen, P. C. and R. Hengge-Aronis **(1994)**. "The role of the sigma factor sigma S (KatF) in bacterial global regulation." Annu Rev Microbiol **48**: 53-80.

Mackie, G. A. **(1998)**. "Ribonuclease E is a 5'-end-dependent endonuclease." Nature **395**(6703): 720-723.

Mackie, G. A. and J. L. Genereaux **(1993)**. "The role of RNA structure in determining RNase E-dependent cleavage sites in the mRNA for ribosomal protein S20 *in vitro*." J Mol Biol **234**(4): 998-1012.

Majdalani, N., S. Chen, et al. **(2001)**. "Regulation of RpoS by a novel small RNA: the characterization of RprA." Mol Microbiol **39**(5): 1382-1394.

Majdalani, N., C. Cunning, et al. **(1998)**. "DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription." Proc Natl Acad Sci U S A **95**(21): 12462-12467.

Majdalani, N., D. Hernandez, et al. **(2002)**. "Regulation and mode of action of the second small RNA activator of RpoS translation, RprA." Mol Microbiol **46**(3): 813-826.

Mandin, P., F. Repoila, et al. **(2007)**. "Identification of new noncoding RNAs in *Listeria monocytogenes* and prediction of mRNA targets." Nucleic Acids Res **35**(3): 962-974.

Marujo, P. E., F. Braun, et al. **(2003)**. "Inactivation of the decay pathway initiated at an internal site by RNase E promotes poly(A)-dependent degradation of the *rpsO* mRNA in *Escherichia coli*." Mol Microbiol **50**(4): 1283-1294.

Masse, E., F. E. Escorcia, et al. **(2003)**. "Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*." Genes Dev **17**(19): 2374-2383.

McCann, M. P., J. P. Kidwell, et al. **(1991)**. "The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*." J Bacteriol **173**(13): 4188-4194.

McDowall, K. J., S. Lin-Chao, et al. **(1994)**. "A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage." J Biol Chem **269**(14): 10790-10796.

Merrikh, H., A. E. Ferrazzoli, et al. **(2009)**. "A DNA damage response in *Escherichia coli* involving the alternative sigma factor, RpoS." Proc Natl Acad Sci U S A **106**(2): 611-616.

Merrikh, H., A. E. Ferrazzoli, et al. **(2009)**. "Growth phase and (p)ppGpp control of IraD, a regulator of RpoS stability, in *Escherichia coli*." J Bacteriol **191**(24): 7436-7446.

Miczak, A., V. R. Kaberdin, et al. **(1996)**. "Proteins associated with RNase E in a multicomponent ribonucleolytic complex." Proc Natl Acad Sci U S A **93**(9): 3865-3869.

Mikulecky, P. J., M. K. Kaw, et al. **(2004)**. "*Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs." Nat Struct Mol Biol **11**(12): 1206-1214.

Mizuno, T., M. Y. Chou, et al. **(1984)**. "A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (mic RNA)." Proc Natl Acad Sci U S A **81**(7): 1966-1970.

Mohanty, B. K. and S. R. Kushner **(2000)**. "Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in *Escherichia coli*." Proc Natl Acad Sci U S A **97**(22): 11966-11971.

Mohanty, B. K., V. F. Maples, et al. **(2004)**. "The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*." Mol Microbiol **54**(4): 905-920.

Moll, I., T. Afonyushkin, et al. **(2003)**. "Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs." RNA **9**(11): 1308-1314.

Moll, I., D. Leitsch, et al. **(2003)**. "RNA chaperone activity of the Sm-like Hfq protein." EMBO Rep **4**(3): 284-289.

Moll, R. G. **(2003)**. "Protein-protein, protein-RNA and protein-lipid interactions of signal-recognition particle components in the hyperthermoacidophilic archaeon *Acidianus ambivalens*." Biochem J **374**(Pt 1): 247-254.

Moller, T., T. Franch, et al. **(2002)**. "Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction." Mol Cell **9**(1): 23-30.

Morita, T., K. Maki, et al. **(2005)**. "RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs." Genes Dev **19**(18): 2176-2186.

Muffler, A., M. Barth, et al. **(1997)**. "Heat shock regulation of sigmaS turnover: a role for DnaK and relationship between stress responses mediated by sigmaS and sigma32 in *Escherichia coli*." J Bacteriol **179**(2): 445-452.

Muffler, A., D. Fischer, et al. **(1996)**. "The RNA-binding protein HF-I, known as a host factor for phage Qbeta RNA replication, is essential for *rpoS* translation in *Escherichia coli*." Genes Dev **10**(9): 1143-1151.

Muffler, A., D. D. Traulsen, et al. **(1997)**. "The RNA-binding protein HF-I plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the sigmaS subunit of RNA polymerase in *Escherichia coli*." J Bacteriol **179**(1): 297-300.

Nicholson, A. W. **(1999)**. "Function, mechanism and regulation of bacterial ribonucleases." FEMS Microbiol Rev **23**(3): 371-390.

Nielsen, J. S., A. Boggild, et al. **(2007)**. "An Hfq-like protein in archaea: crystal structure and functional characterization of the Sm protein from *Methanococcus jannaschii*." RNA **13**(12): 2213-2223.

Opdyke, J. A., J. G. Kang, et al. **(2004)**. "GadY, a small-RNA regulator of acid response genes in *Escherichia coli*." J Bacteriol **186**(20): 6698-6705.

Ow, M. C., Q. Liu, et al. **(2000)**. "Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly." Mol Microbiol **38**(4): 854-866.

Ow, M. C., Q. Liu, et al. **(2002)**. "RNase E levels in *Escherichia coli* are controlled by a complex regulatory system that involves transcription of the *rne* gene from three promoters." Mol Microbiol **43**(1): 159-171.

Paesold, G. and M. Krause **(1999)**. "Analysis of rpoS mRNA in *Salmonella dublin*: identification of multiple transcripts with growth-phase-dependent variation in transcript stability." J Bacteriol **181**(4): 1264-1268.

Perwez, T. and S. R. Kushner **(2006)**. "RNase Z in *Escherichia coli* plays a significant role in mRNA decay." Mol Microbiol **60**(3): 723-737.

Pfeiffer, V., A. Sittka, et al. **(2007)**. "A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the Salmonella core genome." Mol Microbiol **66**(5): 1174-1191.

Pichon, C. and B. Felden **(2005)**. "Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains." Proc Natl Acad Sci U S A **102**(40): 14249-14254.

Ponder, R. G., N. C. Fonville, et al. **(2005)**. "A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation." Mol Cell **19**(6): 791-804.

Portier, C., L. Dondon, et al. **(1987)**. "The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end." EMBO J **6**(7): 2165-2170.

Py, B., C. F. Higgins, et al. **(1996)**. "A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome." Nature **381**(6578): 169-172.

Rasmussen, A. A., M. Eriksen, et al. **(2005)**. "Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control." Mol Microbiol **58**(5): 1421-1429.

Raynal, L. C. and A. J. Carpousis **(1999)**. "Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation." Mol Microbiol **32**(4): 765-775.

Regnier, P., M. Grunberg-Manago, et al. **(1987)**. "Nucleotide sequence of the *pnp* gene of *Escherichia coli* encoding polynucleotide phosphorylase. Homology of the primary structure of the protein with the RNA-binding domain of ribosomal protein S1." J Biol Chem **262**(1): 63-68.

Reichenbach, B., A. Maes, et al. **(2008)**. "The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*." Nucleic Acids Res **36**(8): 2570-2580.

Repoila, F. and S. Gottesman **(2001)**. "Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA." J Bacteriol **183**(13): 4012-4023.

Repoila, F. and S. Gottesman **(2003)**. "Temperature sensing by the *dsrA* promoter." J Bacteriol **185**(22): 6609-6614.

Repoila, F., N. Majdalani, et al. **(2003)**. "Small non-coding RNAs, co-ordinators of adaptation processes in *Escherichia coli*: the RpoS paradigm." Mol Microbiol **48**(4): 855-861.

Resch, A., T. Afonyushkin, et al. **(2008)**. "Translational activation by the noncoding RNA DsrA involves alternative RNase III processing in the *rpoS* 5'-leader." RNA **14**(3): 454-459.

Richards, J., P. Mehta, et al. **(2006)**. "RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner." Mol Microbiol **62**(6): 1700-1712.

Rivas, E., R. J. Klein, et al. **(2001)**. "Computational identification of noncoding RNAs in *E. coli* by comparative genomics." Curr Biol **11**(17): 1369-1373.

Robert-Le Meur, M. and C. Portier **(1992)**. "*E.coli* polynucleotide phosphorylase expression is autoregulated through an RNase III-dependent mechanism." EMBO J **11**(7): 2633-2641.

Rockabrand, D., K. Livers, et al. **(1998)**. "Roles of DnaK and RpoS in starvation-induced thermotolerance of *Escherichia coli*." J Bacteriol **180**(4): 846-854.

Romeo, T. **(1998)**. "Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB." Mol Microbiol **29**(6): 1321-1330.

Schell, M. A. **(1993)**. "Molecular biology of the LysR family of transcriptional regulators." Annu Rev Microbiol **47**: 597-626.

Schumacher, M. A., R. F. Pearson, et al. **(2002)**. "Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA complex: a bacterial Sm-like protein." EMBO J **21**(13): 3546-3556.

Schweder, T., K. H. Lee, et al. **(1996)**. "Regulation of *Escherichia coli* starvation sigma factor (sigma S) by ClpXP protease." J Bacteriol **178**(2): 470-476.

Sharma, C. M., F. Darfeuille, et al. **(2007)**. "A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites." Genes Dev **21**(21): 2804-2817.

Shi, X. and G. N. Bennett **(1994)**. "Plasmids bearing hfq and the hns-like gene *stpA* complement hns mutants in modulating arginine decarboxylase gene expression in *Escherichia coli*." J Bacteriol **176**(21): 6769-6775.

Silvaggi, J. M., J. B. Perkins, et al. **(2006)**. "Genes for small, noncoding RNAs under sporulation control in *Bacillus subtilis*." J Bacteriol **188**(2): 532-541.

Simons, R. W. and N. Kleckner **(1983)**. "Translational control of IS10 transposition." Cell **34**(2): 683-691.

Sittka, A., S. Lucchini, et al. **(2008)**. "Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq." PLoS Genet **4**(8): e1000163.

Sledjeski, D. and S. Gottesman **(1995)**. "A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*." Proc Natl Acad Sci U S A **92**(6): 2003-2007.

Sledjeski, D. D., A. Gupta, et al. **(1996)**. "The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli*." EMBO J **15**(15): 3993-4000.

Sledjeski, D. D., C. Whitman, et al. **(2001)**. "Hfq is necessary for regulation by the untranslated RNA DsrA." J Bacteriol **183**(6): 1997-2005.

Storz, G., J. A. Opdyke, et al. **(2004)**. "Controlling mRNA stability and translation with small, noncoding RNAs." Curr Opin Microbiol **7**(2): 140-144.

Stougaard, P., S. Molin, et al. **(1981)**. "RNAs involved in copy-number control and incompatibility of plasmid R1." Proc Natl Acad Sci U S A **78**(10): 6008-6012.

Straub, J., M. Brenneis, et al. **(2009)**. "Small RNAs in haloarchaea: identification, differential expression and biological function." RNA Biol **6**(3): 281-292.

Studier, F. W. **(1975)**. "Genetic mapping of a mutation that causes ribonucleases III deficiency in *Escherichia coli*." J Bacteriol **124**(1): 307-316.

Sun, X., I. Zhulin, et al. **(2002)**. "Predicted structure and phyletic distribution of the RNA-binding protein Hfq." Nucleic Acids Res **30**(17): 3662-3671.

Suzuki, K., P. Babitzke, et al. **(2006)**. "Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E." Genes Dev **20**(18): 2605-2617.

Tang, T. H., N. Polacek, et al. **(2005)**. "Identification of novel non-coding RNAs as potential antisense regulators in the archaeon *Sulfolobus solfataricus*." Mol Microbiol **55**(2): 469-481.

Tjaden, B., R. M. Saxena, et al. **(2002)**. "Transcriptome analysis of *Escherichia coli* using high-density oligonucleotide probe arrays." Nucleic Acids Res **30**(17): 3732-3738.

Tomizawa, J., T. Itoh, et al. **(1981)**. "Inhibition of ColE1 RNA primer formation by a plasmid-specified small RNA." Proc Natl Acad Sci U S A **78**(3): 1421-1425.

Tsui, H. C., G. Feng, et al. **(1997)**. "Negative regulation of *mutS* and *mutH* repair gene expression by the Hfq and RpoS global regulators of *Escherichia coli* K-12." J Bacteriol **179**(23): 7476-7487.

Tsui, H. C., H. C. Leung, et al. **(1994)**. "Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in *Escherichia coli* K-12." Mol Microbiol **13**(1): 35-49.

Updegrove, T., N. Wilf, et al. **(2008)**. "Effect of Hfq on RprA-*rpoS* mRNA pairing: Hfq-RNA binding and the influence of the 5' *rpoS* mRNA leader region." Biochemistry **47**(43): 11184-11195.

Urban, J. H., K. Papenfort, et al. **(2007)**. "A conserved small RNA promotes discoordinate expression of the *glmUS* operon mRNA to activate GlmS synthesis." J Mol Biol **373**(3): 521-528.

Urban, J. H. and J. Vogel **(2008)**. "Two seemingly homologous noncoding RNAs act hierarchically to activate *glmS* mRNA translation." PLoS Biol **6**(3): e64.

Urbanowski, M. L., L. T. Stauffer, et al. **(2000)**. "The *gcvB* gene encodes a small untranslated RNA involved in expression of the dipeptide and oligopeptide transport systems in *Escherichia coli*." Mol Microbiol **37**(4): 856-868.

Valentin-Hansen, P., M. Eriksen, et al. **(2004)**. "The bacterial Sm-like protein Hfq: a key player in RNA transactions." Mol Microbiol **51**(6): 1525-1533.

Vanzo, N. F., Y. S. Li, et al. **(1998)**. "Ribonuclease E organizes the protein interactions in the *Escherichia coli* RNA degradosome." Genes Dev **12**(17): 2770-2781.

Viegas, S. C., P. Fernandez De Palencia, et al. **(2004)**. "Development of an inducible system to control and easily monitor gene expression in *Lactococcus lactis*." Plasmid **51**(3): 256-264.

Viegas, S. C., V. Pfeiffer, et al. **(2007)**. "Characterization of the role of ribonucleases in Salmonella small RNA decay." Nucleic Acids Res **35**(22): 7651-7664.

Viegas, S. C., D. Schmidt, et al. **(2005)**. "Effect of the increased stability of the penicillin amidase mRNA on the protein expression levels." FEBS Lett **579**(22): 5069-5073.

Vogel, J., L. Argaman, et al. **(2004)**. "The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide." Curr Biol **14**(24): 2271-2276.

Vogel, J., V. Bartels, et al. **(2003)**. "RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria." Nucleic Acids Res **31**(22): 6435-6443.

Vogel, J. and E. G. Wagner **(2007)**. "Target identification of small noncoding RNAs in bacteria." Curr Opin Microbiol **10**(3): 262-270.

Vytvytska, O., J. S. Jakobsen, et al. **(1998)**. "Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability." Proc Natl Acad Sci U S A **95**(24): 14118-14123.

Vytvytska, O., I. Moll, et al. **(2000)**. "Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding." Genes Dev **14**(9): 1109-1118.

Wassarman, K. M., F. Repoila, et al. **(2001)**. "Identification of novel small RNAs using comparative genomics and microarrays." Genes Dev **15**(13): 1637-1651.

Wassarman, K. M. and G. Storz **(2000)**. "6S RNA regulates *E. coli* RNA polymerase activity." Cell **101**(6): 613-623.

Wassarman, K. M., A. Zhang, et al. **(1999)**. "Small RNAs in *Escherichia coli*." Trends Microbiol **7**(1): 37-45.

Weber, H., T. Polen, et al. **(2005)**. "Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity." J Bacteriol **187**(5): 1591-1603.

Wilderman, P. J., N. A. Sowa, et al. **(2004)**. "Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis." Proc Natl Acad Sci U S A **101**(26): 9792-9797.

Willetts, N. S. (1967). "Intracellular protein breakdown in growing cells of *Escherichia coli*." Biochem J **103**(2): 462-466.

Willetts, N. S. (1967). "Intracellular protein breakdown in non-growing cells of Escherichia coli." Biochem J **103**(2): 453-461.

Yura, T., E. Guisbert, et al. **(2007)**. "Analysis of sigma32 mutants defective in chaperone-mediated feedback control reveals unexpected complexity of the heat shock response." Proc Natl Acad Sci U S A **104**(45): 17638-17643.

Zangrossi, S., F. Briani, et al. **(2000)**. "Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*." Mol Microbiol **36**(6): 1470-1480.

Zhang, A., S. Altuvia, et al. **(1997)**. "The novel oxyS RNA regulates expression of the sigma s subunit of *Escherichia coli* RNA polymerase." Nucleic Acids Symp Ser(36): 27-28.

Zhang, A., S. Altuvia, et al. **(1998)**. "The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein." EMBO J **17**(20): 6061-6068.

Zhang, A., K. M. Wassarman, et al. **(2002)**. "The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs." Mol Cell **9**(1): 11-22.

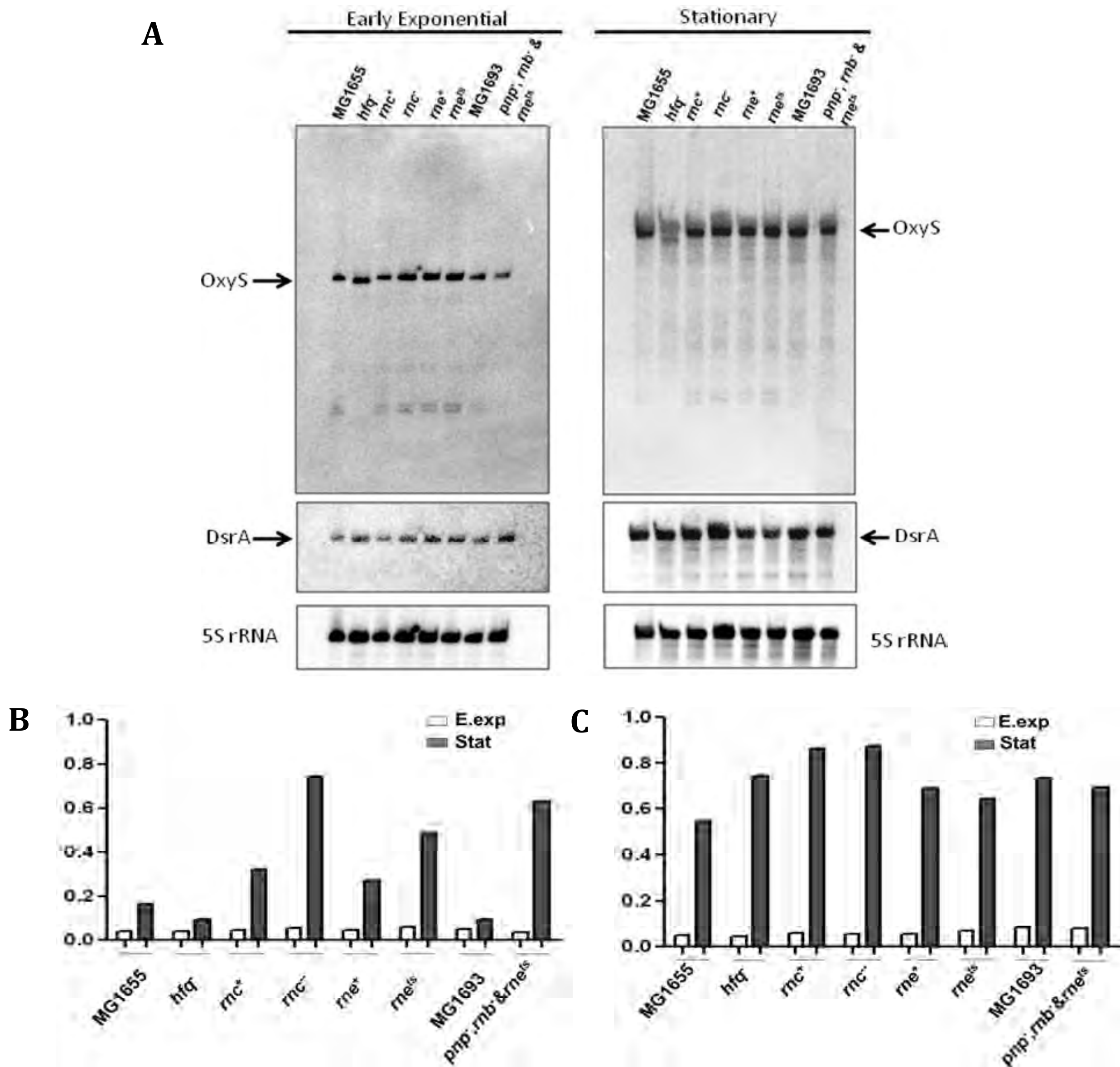
Zhang, A., K. M. Wassarman, et al. **(2003)**. "Global analysis of small RNA and mRNA targets of Hfq." Mol Microbiol **50**(4): 1111-1124.

Zheng, M., X. Wang, et al. **(2001)**. "DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide." J Bacteriol **183**(15): 4562-4570.

Zilhao, R., P. Regnier, et al. **(1995)**. "The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*." FEMS Microbiol Lett **130**(2-3): 237-244.

9 SUPPLEMENTARY DATA

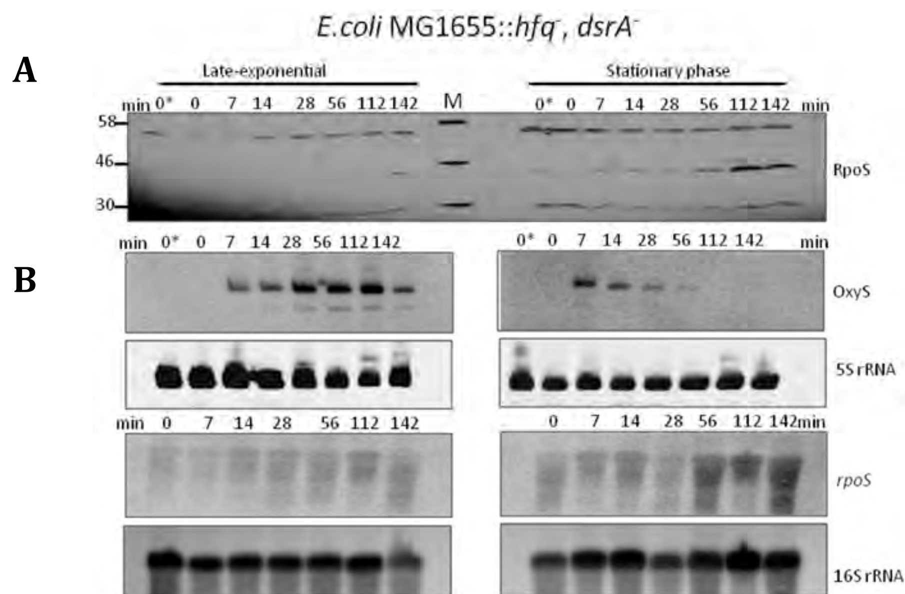
9.1 Expression analyses of OxyS, and DsrA sRNAs under oxidative stress condition in all studied *E. coli* strains



Supplementary Fig 9.1 Expression analyses of OxyS, and DsrA sRNAs under oxidative stress condition in all studied *E.coli* strains: Cells were harvested for the detection of OxyS and DsrA RNAs by isolating the total RNA at the indicated time points 0, 2, 4, 8, 16, 32 and 64 min after the addition of rifampicin. And 0 time point RNA from all strains was used in order to compare the levels of OxyS and DsrA in early exponential and stationary growth phase. **(A)** Northern blot analysis of the isolated total RNA from all *E.coli* strains. Membranes were first hybridized with OxyS and/or DsrA specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** and **(C)** Graphical representation of the levels of OxyS and DsrA RNAs respectively under oxidative stress in different *E.coli* strains in early exponential (**white bars**) and in stationary growth phase (**grey bars**). Relative amount of OxyS and/or DsrA expressed in early exponential phase was compared to the

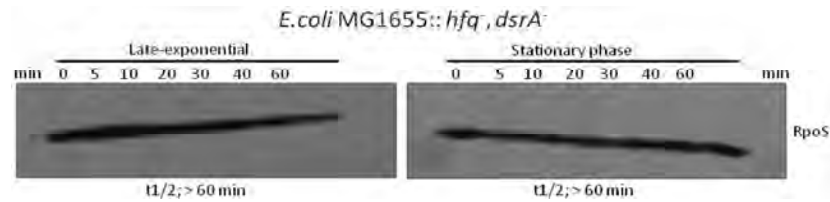
expression levels in stationary growth phase. RNA levels were detected by Northern Blots and the band intensity as quantified using phosphoimaging (Molecular Imager, Bio-rad).

9.2 Expression analysis of RpoS, OxyS and *rpoS* mRNA under oxidative stress condition in *E. coli* MG1655:: Δ*hfq*, Δ*dsrA*



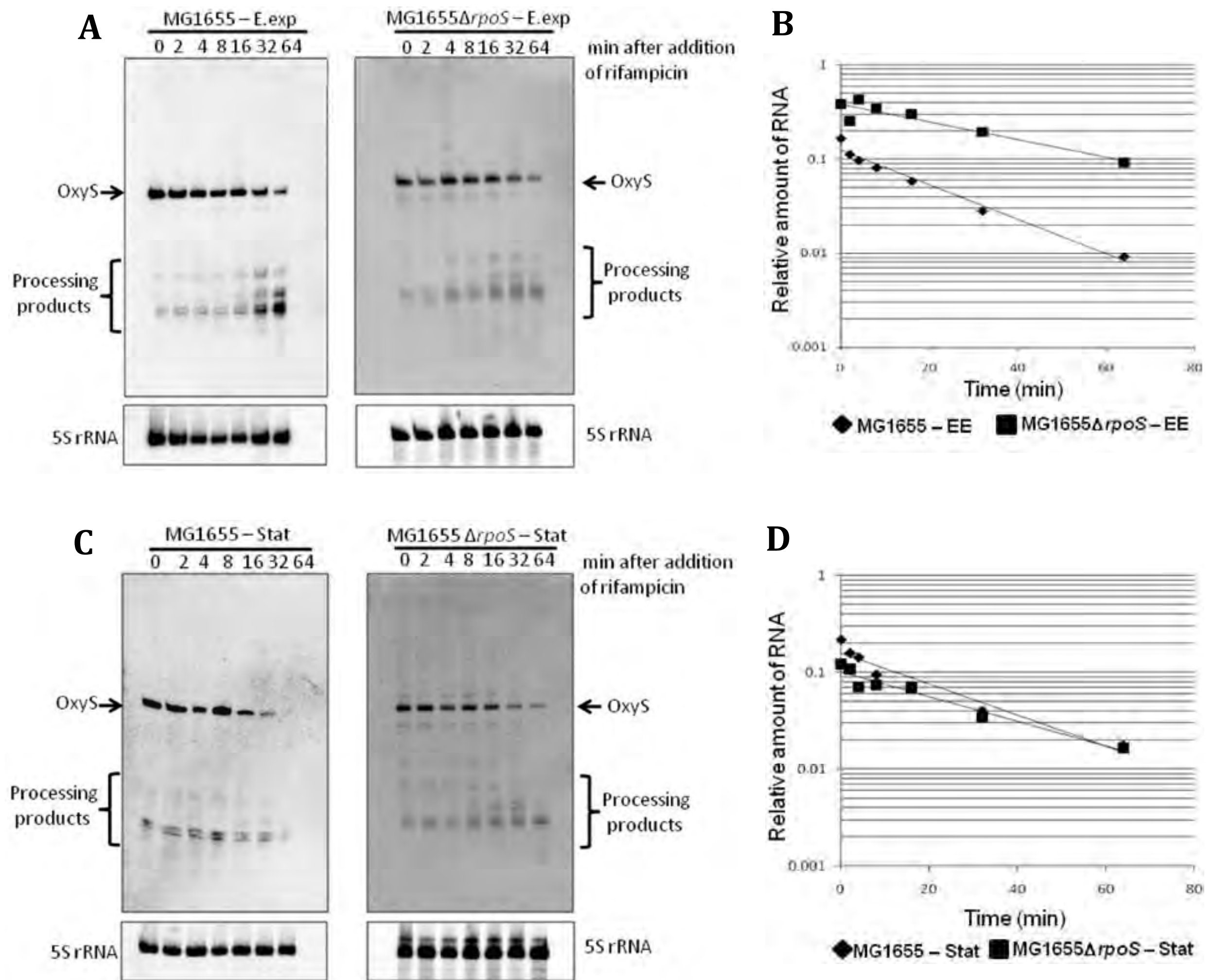
Supplementary Figure 9.2 Expression analysis of RpoS, OxyS and *rpoS* mRNA under oxidative stress condition in *E.coli* MG1655:: Δ***hfq***Δ***dsrA***. Cells were harvested for the detection of RpoS levels and for the isolation of RNA at the indicated time points before and after the addition of H_2O_2. (A): Western blot analysis of RpoS levels. Poly-clonal antiserum specific to RpoS was used for the detection. Total protein from equal amounts of cells was loaded. Size standard proteins (58, 46, and 30 KDa) are shown in lane M and indicated on the left side of the western blot (B): Northern blot analysis of OxyS and *rpoS* mRNA. 30µg of total RNA was loaded per lane. The membrane was first hybridized with a specific 5'- end labeled probe (OxyS) or with nick translated *rpoS* specific probe and the same membrane was re-hybridized with a 5S or 16 S rRNA specific probes as an internal loading control. **LE** – Late-exponential phase and **Stat** – Stationary phase

9.3 Stability determination of RpoS in *E. coli* MG1655 lacking Hfq and DsrA under oxidative stress in late-exponential and in stationary growth phase



Supplementary Figure 9.3 Stability determination of RpoS in *E.coli* MG1655 lacking Hfq and DsrA under oxidative stress in late-exponential and in stationary growth phase. Cells were harvested for detection of RpoS by western blot at the indicated time points after the addition of Chloramphenicol as described in Material and Methods. The half life of RpoS in *E.coli* MG1655::Δ*hfq,* Δ*dsrA* is > 60 min in late-exponential and stationary phase.

9.4 Stability determination of OxyS in *E. coli* MG1655 and *E. coli* MG1655Δ*rpoS*, under oxidative stress in early exponential and in stationary growth phase



Supplementary Figure 9.4 Stability determination of OxyS in *E. coli* MG1655 and *E. coli* MG1655Δ*rpoS*, under oxidative stress in early exponential and in stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** & **(C)** Northern blot analysis of the isolated total RNA from *E.coli* MG1655 and MG1655::Δ*rpoS*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **(B)** & **(D)** Half life determination of OxyS transcript. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp** – Early exponential growth phase: The half life of OxyS in *E.coli* MG1655 and in MG1655Δ*hfq* about 27 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* MG1655 is about 21 min and in MG1655Δ*rpoS* about 20 min.

9.5 Stability determination of OxyS in *E. coli* MG1655 & strain MG1655Δ*hfq* and *E. coli* N3433 (*rne*+) & N3431 (*rne*ts) under oxidative stress in early exponential and in stationary growth phase without transcription inhibition

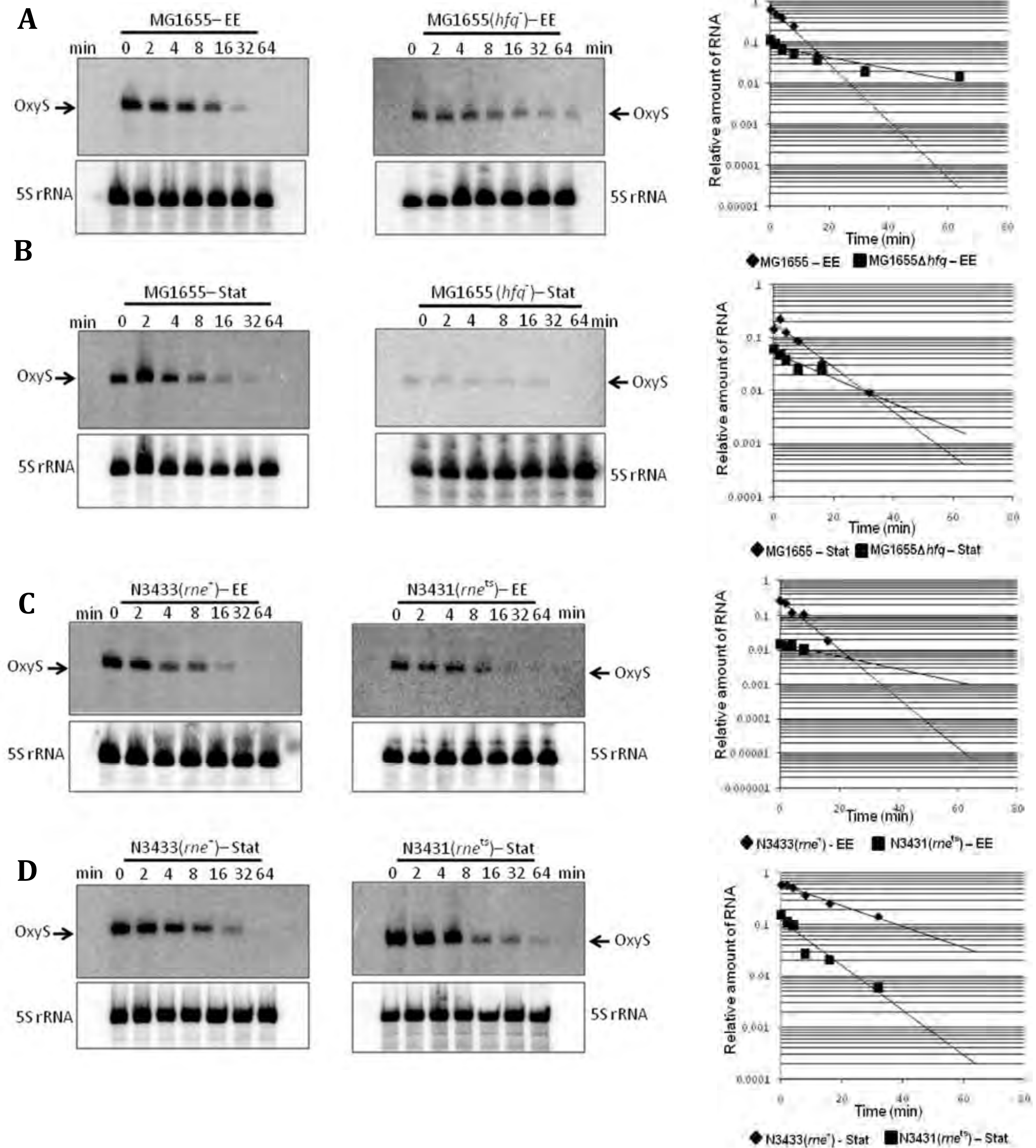
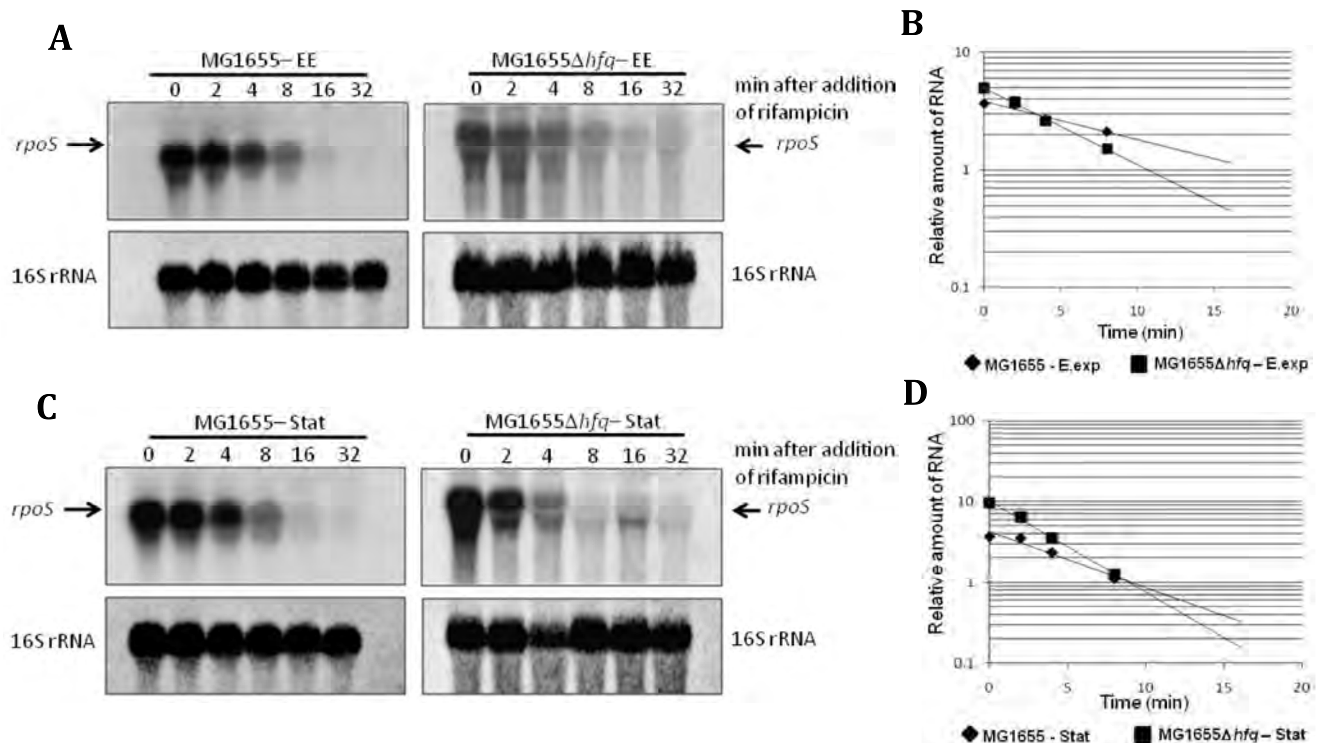


Figure 9.5 Stability determination of OxyS in *E. coli* MG1655 & MG1655Δ*hfq* and *E. coli* N3433 (*rne*$^{+}$) & N3431 (*rne*^{ts}) under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after 14 min of H_2O_2 treatment. **(A)** & **(B)** Northern blot analysis of the isolated total RNA from *E.coli* MG1655 and MG1655::Δ*hfq*. Membranes were first hybridized with OxyS specific 5' end labeled probe (upper panel) and the same membrane was re-hybridized with a 5S rRNA specific probe as an internal loading control (lower panel). **E.Exp –** Early exponential growth phase: The half life of OxyS in *E.coli* MG1655 is about 8 min and in MG1655Δ*hfq* about 8 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* MG1655 is about 10 min and in MG1655Δ*hfq* about 7 min **(C)** & **(D)** Northern blot analysis of the isolated total RNA from *E.coli* N3433(*rne*$^{+}$) & N3431 (*rne*^{ts}) as described above. Band intensities of the transcript were normalized against the intensity of the 5S rRNA and plotted against the time to calculate the half-life in this representative experiment. **E.Exp –** Early exponential growth phase: The half life of OxyS in *E.coli* N3433 (*rne*$^{+}$) is about 5 min and in N3431 (*rne*^{ts}) about 16 min. **Stat** – Stationary phase: The half life of OxyS in *E.coli* N3433 (*rne*$^{+}$) is about 10 min and in N3431 (*rne*^{ts}) about 16 min.

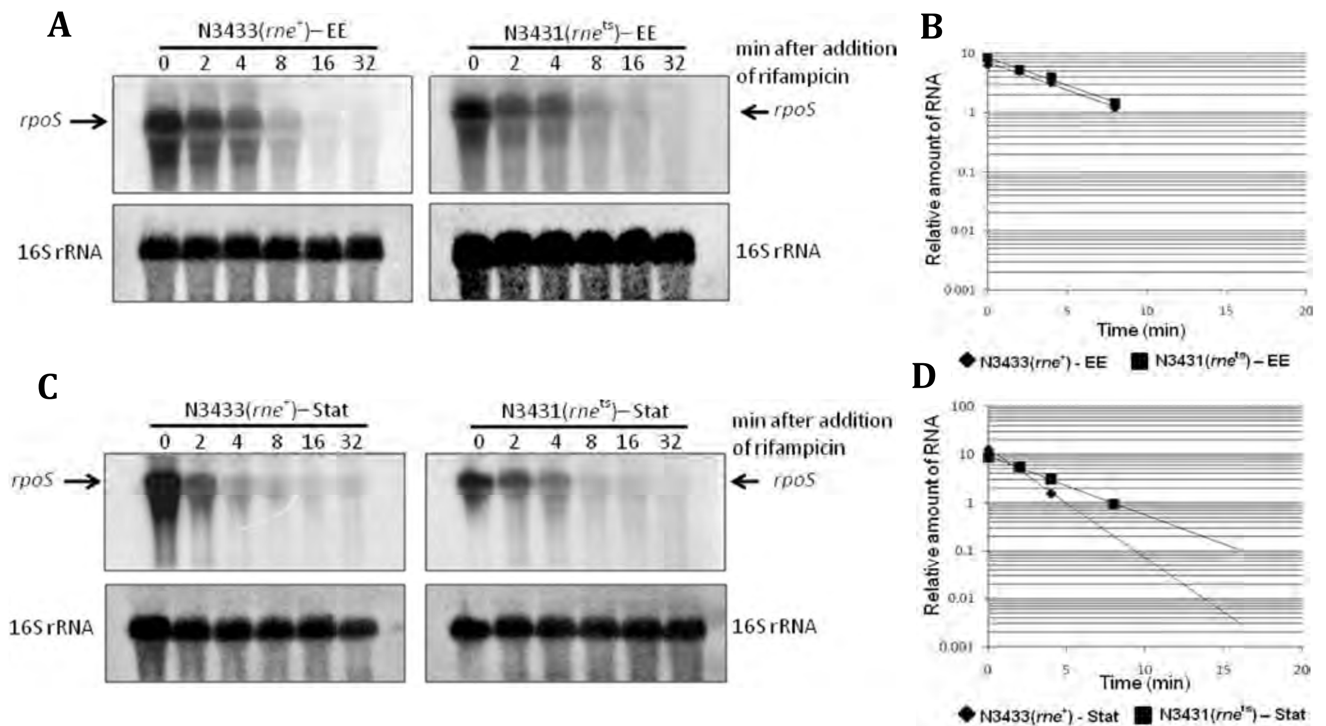
9.6 Stability determination of *rpoS* mRNA in *E. coli* MG1655 and strain MG1655Δ*hfq*, under oxidative stress in early exponential and in stationary growth phase



Supplementary Figure 9.6 Stability determination of *rpoS* mRNA in *E. coli* MG1655 and strain MG1655-*hfq*$^{-}$, under oxidative stress in early exponential and stationary growth phases. Cells were harvested for RNA isolation at the indicated time points after

the addition of rifampicin. **(A) and (C)** Northern blot analysis of the isolated total RNA from *E.coli* MG1655 and strain MG1655-*hfq*⁻ as described in materials and methods. Membranes were first hybridized with *rpoS* mRNA specific radioactively labeled probe (upper panel) and then the same membrane was re-hybridized with a 16S rRNA specific probe as an internal loading control (lower panel). **(B) and (D)** Half life determination of the *rpoS* mRNA transcript. Band intensities of the transcript were normalized against the intensity of the 16S rRNA and plotted against the time to calculate the half-life in this representative experiment. The half-life of *rpoS* mRNA in *E.coli* MG1655 is about 6 min and in *E.coli* MG1655-*hfq*⁻ half-life about 4 min. **EE** – Early exponential phase. The half-life of *rpoS* mRNA in *E.coli* MG1655 is about 4 min and in *E.coli* MG1655-*hfq*⁻ half-life about 3 min. **Stat** – Stationary phase.

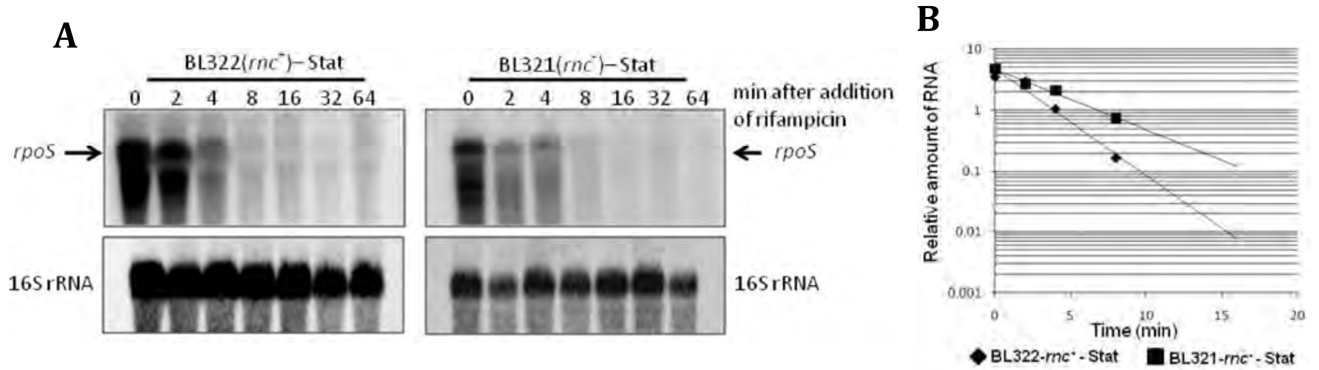
9.7 Stability determination of *rpoS* mRNA in *E. coli* N3433 and strain N3431-*rne^{ts}*, under oxidative stress in early exponential and in stationary growth phase



Supplementary Figure 9.7 Stability determination of *rpoS* mRNA in *E.coli* N3433 and strain N3431-*rne^{ts}*, under oxidative stress in early exponential and stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A) and (C)** Northern blot analysis of the isolated total RNA from *E.coli* N3433 and strain N3431-*rne^{ts}* as described in Materials and Methods. Membranes were first hybridized with *rpoS* mRNA specific radioactively labeled probe (upper panel) and then the same membrane was re-hybridized with a 16S rRNA specific probe as an internal loading control (lower panel). **(B) and (D)** Half life determination of the *rpoS* mRNA transcript. Band intensities of the transcript were normalized against the intensity of the 16S rRNA and plotted against the time to calculate the half-life in this representative experiment. The half-life of *rpoS* mRNA in *E.coli* N3433 is about 3 min and in *E.coli* N3431 half-life about

3 min. **EE** – Early exponential phase. The half-life of *rpoS* mRNA in *E.coli* N3433 is about 1 min and in *E.coli* N3431 half-life about 2 min. **Stat** – Stationary phase.

9.8 Stability determination of *rpoS* mRNA in *E. coli* BL322 and strain BL321-*rnc*⁻, under oxidative stress in stationary growth phase



Supplementary Figure 9.8 Stability determination of *rpoS* mRNA in *E.coli* BL322 and strain BL321-*rnc*⁻, under oxidative stress in stationary growth phase. Cells were harvested for RNA isolation at the indicated time points after the addition of rifampicin. **(A)** Northern blot analysis of the isolated total RNA from *E.coli* BL322 and strain BL321-*rnc*⁻ as described above. Membranes were first hybridized with *rpoS* mRNA specific radioactively labeled probe (upper panel) and then the same membrane was re-hybridized with a 16S rRNA specific probe as an internal loading control (lower panel). **(B)** Half life determination of the *rpoS* mRNA transcript. Band intensities of the transcript were normalized against the intensity of the 16S rRNA and plotted against the time to calculate the half-life in this representative experiment. The half-life of *rpoS* mRNA in *E.coli* BL322 is about 3 min and in *E.coli* BL321 half-life about 3 min. **Stat** – Stationary phase.

10 ABBREVIATIONS

A	Adenine
Ap	Ampicillin
APS	Ammoniumpersulfat
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
β-Me	β-Mercaptoethanol
bp	base pair(s)
C	Cytosine
°C	centigrade
Ci	Curie
cpm	counts per minute
CTP	Cytosine triphosphate
Da	Dalton
dATP	2´ Deoxyriboadinosine-triphosphate
dCTP	2´ Deoxyribocytosine-triphosphate
dGTP	2´ Deoxyriboguanosine-triphosphate
dTTP	2´ Deoxyribothymidine-triphosphate
dH_2O	distilled water
ddH_2O	double distilled water
DEPC	Diethyl-pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribo nuclease
dNTP	2´ Deoxyribonucleotide-triphosphate
ds	double stranded
***E**.coli*	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
E. exp	Early exponential growth phase
e.g.	*exempli gratia* (for example)
et al.	*et alli* (and others)
etc.	*et cetera* (and other things)
EtOH	ethanol
Fig	Figure
g	Gram
G	Guanine
GTP	Guanosine triphosphate
h	Hour(s)
H_2O	Water
H_2O_2	Hydrogen peroxide
***i**.e*	*id est* (that is)
IPTG	Isopropyl-β-D-Thiogalactopyranoside
k	Kilo
kb	Kilo base pairs
kDa	Kilodalton
Km	Kanamycin
l	Liter
LSD	salmon sperm DNA
m	Milli
M	Molar (mol/l)
µ	Micro
mA	Milliampere
min	Minute(s)
miRNA	microRNA
mmol	millimol
mM	millimolar
MOPS	3-Morpholino-1-Propansulfonic acid
mRNA	messenger RNA
Mut	Mutant
MW	Molecular weight
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar
n	Nano
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
ncRNA	non coding RNA
nm	Nanometer
nt	Nucleotide(s)
OD	optical density

p	Pico
PAA	Polyacrylamide
PAGE	Polyacrylamide-gel electrophoresis
PAP I	Poly (A) polymerase I
PCR	Polymerase chain reaction
Pi	Pyrophosphate
PNK	Polynucleotide kinase
PNPase	Polynucleotide phosphorylase
RhlB	RNA-helicase B
Rif	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
RNase E	Ribonuclease E
RNase R	Ribonuclease R
RNase II	Ribonuclease II
RNase III	Ribonuclease III
RNP	Ribonucleoprotein
rRNA	ribosomal RNA
rpm	revolutions per minute
RT	Room temperature
SDS	sodium dodecyl sulfate
Sec	Second(s)
siRNA	small interfering RNA
sRNA	small non-coding RNA
ss	single stranded
Stat	Stationary growth phase
Str	Streptomycin
T	Thymine
TAE	Tris-Acetate-EDTA-Buffer
TBE	Tris-Borate-EDTA-Buffer
TBS	Tris-Base-Sodium-Buffer
Tc	Tetracycline
TEMED	N,N,N',N'-Tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)v-aminomethane
tRNA	transfer RNA
ts	temperature sensitive
TTP	Thymidine triphosphate
U	Uracil or Unit
UV	Ultraviolet
UTP	Uridine triphosphate
V	Volt
v/v	Volume/Volume
W	Watt
wt	Wild-type
w/v	Weight/Volume
X-Gal	5-Bromo-4-chloro-3-indol-β-D-galactopyranoside

OTHER CONTRIBUTIONS

Madhugiri R, **Basineni SR**, Klug G **(2010)** Turn-over of the small non-coding RNA RprA in *E. coli* is influenced by osmolarity **(*in press*)** **Molecular Genetics and Genomics**

Poster presentations

Processing and degradation of regulatory small RNA OxyS and its target mRNA *rpoS* in *E.coli*. 3th-6th June 2009, Conference on "**Regulatory RNA in prokaryotes", Berlin, Germany.**

Processing and degradation of regulatory small RNA OxyS and its target mRNA *rpoS* in *E.coli*. **SPP 1258: Sensory and Regulatory RNAs in Prokaryotes,** 16th-18th September 2008, Kassel, Germany.

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zitate sind als solche gekennzeichnet.

Giessen, den 13. August 2010

Sobha Rani Basineni