Osmoregulation of the *proU* operon at a post-transcriptional level in *Escherichia coli*

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von
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Köln
2011

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Tag der mündlichen Prüfung: 28. Juni 2011

Contents

Abbreviations

5′ RACE	rapid amplification of cDNA 5′ ends
Amp	ampicillin
Cm	chloramphenicol
FRT (FRT site)	Flp recombinase target sites
Km	kanamycin
Rif	rifampicin
σ^{70}	RpoD, primary sigma factor, encoded by gene *rpoD*
σ^{S}	RpoS, stationary phase sigma factor, encoded by gene *rpoS*
SNP	single-nucleotide polymorphism
Spec	spectinomycin
sRNA	small regulatory RNA
TAP	tobacco acid pyrophosphatase
Tet	tetracycline

Zusammenfassung

Das *E. coli proU*-Operon besteht aus den drei Genen *proV*, *proW* und *proX*. Diese kodieren einen ABC-Transporter für die Aufnahme der kompatiblen Solute Prolin und Glyzinbetain bei hyperosmotischen Stressbedingungen. Die Expression des *proU*-Operons ist osmoreguliert: Das Operon ist bei niedriger Osmolarität reprimiert und wird durch hyper-osmotischen Stress induziert. Frühere Publikationen zeigten, dass die Osmoregulation des *proU*-Operons auf Ebene der Transkriptionsregulation erfolgt. Der *proU*-Promotor wird bei hoher Osmolarität stärker. Zusätzlich wird die Transkription durch das Nukleoid-assozierte Protein H-NS bei niedriger Osmolarität reprimiert. Nicht publizierte Ergebnisse der Arbeitsgruppe deuteten auf eine zusätzliche Post-transkriptionelle Ebene der Osmoregulation hin.

In dieser Arbeit wurde die post-transkriptionelle Osmoregulation von *proU* charakterisiert. Ergebnisse dieser Arbeit zeigen, dass die *proU*-mRNA durch RNase III an den Positionen +217/+218 und +281/+282 (relativ zum Transkriptionsstart) prozessiert wird. Die Prozessierung erfolgt innerhalb eines doppelsträngigen Bereichs einer in *Enterobacteriaceae* hoch konservierten RNA-Sekundärstruktur von *proU*. Diese Prozessierung durch RNase III ist osmoreguliert. RNase III prozessiert die *proU*-mRNA effizient bei Wachstum in Medium niedriger Osmolarität. Durch hohe Osmolarität wird die Prozessierung inhibiert. Die Prozessierung der *proU*-mRNA durch RNase III verursacht die post-transkriptionelle Osmoregulation von *proU*, da die Hemmung der RNase III-Prozessierung durch Mutation die post-transkriptionelle Osmoregulation aufhebt. Außerdem zeigen die Daten, dass die Stabilität der *proU*-mRNA durch die RNase III kontrolliert wird. Die *proU*-mRNA ist bei hoher Osmolarität relativ stabil. Die Halbwertszeit der mRNA an der Prozessierungsstelle beträgt 65 bis 75 Sekunden. Bei hypo-osmotischem Stress führt die Prozessierung durch RNase III zu einem schnellen Abbau der *proU*-mRNA mit einer Halbwertszeit von 4 bis 7 Sekunden. Diese Ergebnisse deuten darauf hin, dass die primäre Rolle der Prozessierung durch RNase III die schnelle Degradation der *proU*-mRNA bei hypo-osmotischen Stressbedingungen ist.

Die Ergebnisse dieser Arbeit werden in ein generelles Modell der Osmoregulation von *proU* integriert. Hyperosmotischer Stress bewirkt die Derepression der Transkription von *proU* und die Stabilisierung der *proU*-mRNA durch Hemmung der RNase III Prozessierung. Hypo-osmotischer Stress führt zur Repression der *proU* Transkription und zum schnellen Abbau der *proU*-mRNA, der durch RNase III initiiert wird.

Die hohe Konservierung der *proU*-Sequenz im Bereich der mRNA Sekundärstruktur um die RNase III-Prozessierungsstelle deutet darauf hin, dass die RNase III Prozessierung auch in anderen *Enterobacteriaceae* stattfindet. Der molekulare Mechanismus, welcher der Osmoregulation der RNase III Prozessierung unterliegt, ist noch offen.

Summary

The *E. coli proU* operon consists of three genes, *proV*, *proW* and *proX*. These encode an ABC transporter for uptake of osmoprotectants which help the cell to survive hyperosmotic stress. The expression of the *proU* operon is osmoregulated: it is repressed under low osmolarity conditions and induced by hyperosmotic stress. Previous studies demonstrated that the *proU* operon is osmoregulated at the transcriptional level. The *proU* promoter is osmoresponsive. In addition, transcription is repressed by the abundant nucleoid-associated protein H-NS under low osmolarity conditions. A recent study indicated that *proU* is also osmoregulated at the post-transcriptional level.

In this work post-transcriptional osmoregulation of *proU* was characterized. Results presented here demonstrate that the *proU* mRNA is processed by RNase III at positions +217/+218 and +281/+282 relative to the transcription start site. These processing sites are located within a highly conserved stem-loop secondary structure, which is cleaved by RNase III in a double-stranded part. RNase III-mediated processing of the *proU* mRNA is osmoregulated. RNase III processes *proU* mRNA efficiently under low osmolarity conditions, while under high osmolarity conditions processing is inhibited. Processing of *proU* RNA by RNase III is the cause for post-transcriptional osmoregulation of *proU*, as blocking of RNase III processing by mutations cancels post-transcriptional osmoregulation. Further, *proU* mRNA stability is largely dependent on the activity of RNase III processing. The *proU* mRNA is relatively stable at high osmolarity with an estimated half-life of the mRNA next to the processing site of 65 to 75 seconds. After hypoosmotic stress RNase III rapidly processes the *proU* mRNA which leads to quick mRNA degradation with an estimated half-life of 4 to 7 seconds. These results suggest that the primary function of RNase III in *proU* osmoregulation is to initiate rapid degradation of *proU* mRNA under hypoosmotic stress conditions.

The data obtained in the presented work are integrated in the general model of *proU* osmoregulation. Hyperosmotic stress leads to derepression of *proU* transcription and stabilisation of *proU* transcript due to inhibition of RNase III processing. Hypoosmotic stress leads to repression of *proU* transcription and rapid degradation of *proU* mRNA initiated by RNase III.

The high conservation of the *proU* sequence and *proU* mRNA secondary structure around the RNase III processing site suggests that RNase III processing is involved in *proU* osmoregulation in other enterobacterial species. The molecular mechanism underlying osmoregulation of RNase III processing of the *proU* mRNA requires further investigation.

1. Introduction

Escherichia coli inhabits the small intestine of humans and other vertebrates as a commensal organism. Some *E. coli* strains are pathogenic causing intestinal diseases. In addition, *E. coli* can colonize other parts of the human body and cause various extra-intestinal diseases, including urinary tract infections. *E. coli* can also survive outside of their host in soil, water or on plants surface. It is therefore not surprising that *E. coli*, as other bacteria, has to cope with different environmental stresses, such as temperature variations (cold and heat shock), pH variation (acid and alkaline stress), starvation, oxidative stress, action of antibiotics, heavy metals etc. *E. coli* employs various stress response systems to withstand these stresses. Each stress leads to the activation of expression of a specific set of stress response genes. Upon the stress *E. coli* RNA polymerase dissociates from the house-keeping transcriptional sigma factor σ^{70} and associates with one of the alternative transcriptional sigma factors. RNA polymerase associated with the alternative sigma factor recognizes the promoters and activates the transcription of stress response genes. In addition, transcription of stress response genes is often regulated by two-component signaling systems. The changes in extracellular environment are sensed by the membrane bound component of two-component systems. The signal is further transferred to the response regulator that activates the transcription of the target genes. Expression of stress response genes can also be regulated at a post-transriptional level, e.g. by stress responsive small regulatory RNAs. In addition, activity and degradation of stress response proteins can be regulated. In this study, the response of *E. coli* to osmotic stress was analyzed. Specifically, I analyzed osmoregulation of expression of the *proU* operon at a post-transcriptional level.

1.1 Adaption of *E. coli* to osmotic stress and the role of the *proU* operon in osmoadaptation

One of the most common stresses *E. coli* face is the osmotic stress. Osmotic stress is caused by a change in the concentration of solutes in the intracellular environment. The cellular membranes and the peptidoglycan cell wall are relatively permeable to water, but the membrane is normally not permeable to inorganic ions or organic molecules. Therefore change of the solute concentration outside of the cell leads to water efflux out of the cell during hyperosmotic stress or to water influx during hypoosmotic stress. Hyperosmotic stress leads to cell dehydration and fall in turgor pressure, cytoplasm shrinkage, overcrowdedness of the cytoplasm with macromolecules, inhibition of enzyme activities, disruption of transmembrane transport and protein synthesis, and cell growth arrest (Altendorf *et al.*, 2009). Hypoosomotic stress leads to massive influx of water and similarly to disruption of normal physiological processes. Severe hypoosomotic stress leads such a great increase of cell turgor that the cell wall can not hold the internal pressure and the cells burst (Altendorf *et al.*, 2009).

To withstand osmotic stresses *E. coli* have to control the fluxes of water and solutes in and out of the cell. Both outer and cytoplasmic membranes are semipermeable and allow the passage of water and uncharged small molecules like glycerol or ethanol. The outer membrane in addition carries porins, such as OmpF and OmpC, that allow the passage of larger molecules like sugars and aminoacids (Nikaido, 2003). The peptidoglycan layer allows passage of even larger molecules including small globular proteins (Dijkstra and Keck, 1996). Therefore, water, inorganic ions and small organic molecules can relatively easily move between the external environment and the periplasmic space. The principal regulation of solutes and water transport occurs with transporters localized in the cytoplasmic membrane. Aquaporin, AqpZ, and aquaglyceroporin, GlpF, both facilitate the passage of water across the cytoplasmic membrane (Stroud *et al.*, 2003). However, *E. coli* can not actively transport water across the membrane and instead controls water fluxes indirectly by transport of solutes.

Several systems are responsible for the adaptation of *E. coli* to osmotic stress (Fig. 1). The main mechanism that helps *E. coli* to cope with hypoosmotic stress involves mechano-sensitive channels belonging to three classes MscL, MscS and MscM (Booth *et al.*, 2007). These channels have low selectivity and are permeable to different solutes including inorganic ions and small organic molecules. Hypoosmotic stress leads to increase of turgor and as a result to the deformation of the cytoplasmic membrane. Msc channels sense this deformation, open and release solutes to the periplasm (Booth *et al.*, 2007). This lowers the internal pressure and saves cell from burst.

The response to hyperosmotic stress is more complex. The first response of *E. coli* to hyperosmotic stress is the accumulation of potassium ions (Sleator and Hill, 2002; Altendorf *et al.*, 2009). The uptake of K^+ is principally achieved by two homologous transporters TrkG and TrkH (Schlosser *et al.*, 1995), and by the transporter KdpFABC (Greie and Altendorf, 2007). Expression of the latter is regulated by the two-component signalling system KdpDE (Heermann and Jung, 2010). In addition, the Kup transporter (TrkD) plays a minor role in K^+ uptake during hyperosmotic stress (Trchounian and Kobayashi, 1999). Accumulation of K^+ is counterbalanced by the simultaneous accumulation of glutamate anions that are synthesized inside the cell (McLaggan *et al.*, 1994). This increase in the potassium glutamate concentration in the cytoplasm prevents water loss and in addition draws some water back from the external environment. However the increase in the concentration of inorganic ions has a detrimental effect on cell physiology, inhibiting protein biosynthesis and cell growth (Altendorf *et al.*, 2009). Therefore, as a second step of hyperosmotic stress response, these inorganic ions are substituted by organic osmoprotectants called compatible solutes.

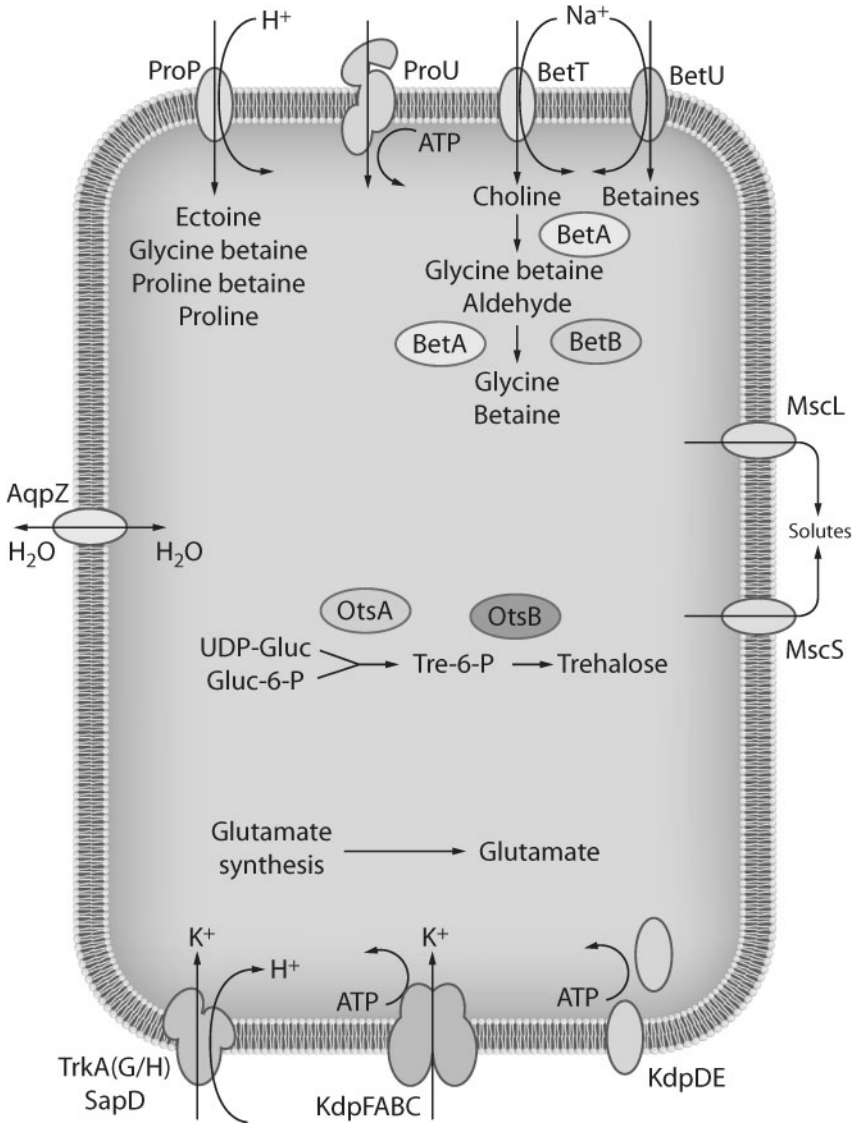


Figure 1. Osmoregulation systems of *E. coli*. Transporters ProP, ProU, BetU and BetT import compatible solutes; transporters TrkG/H and KdpFABC (expression controlled by two-component signaling system KdpDE) import potassium; AqpZ is a water channel; OtsA/B and BetA/B synthesize osmoprotectants trehalose and glycine betaine, respectively; glutamate is synthesized to counterbalance K^+ uptake; mechanosensitive channels MscL/S/M release osmolytes after hypoosmotic stress. Figure taken from Wood (2006).

Compatible solutes are organic substances that are relatively inert and do not interfere with the cell physiology. *E. coli* uses as compatible solutes amino acids and derivatives (like proline and glycine betaine) and some carbohydrates (mainly trehalose). The main mechanism of compatible solutes accumulation is the uptake from the external environment. *E. coli* possesses four principal transporters of compatible solutes: ProP, ProU, BetT and BetU (the latter is found only in one third of *E. coli* isolates) (Wood, 2006; Sleator and Hill, 2002; Kempf and Bremer, 1998; Altendorf *et al.*, 2009). BetT transports choline into the cell, which is then converted to glycine betaine. BetU is responsible for uptake of betaines like glycine betaine. ProU has a rather broad substrate specificity. It transports glycine betaine and proline betaine with high affinity and also a number of other substrates with lower affinity including proline, ectoine, taurine, choline, carnitine, dimethyl glycine, homobetaine, and γ-butyrobetaine (Lucht and Bremer, 1994; Kempf and Bremer, 1998). ProP also has a broad

substrate specificity and transports a similar set of substrates as ProU (Kempf and Bremer, 1998). Compatible solutes are usually present in the natural external environment, where they are released after organisms death or excreted by plants or microorganisms. *E. coli* can use different transporters to take up any compatible solute available. If no compatible solutes are present in the medium, *E. coli* can synthesize trehalose from glucose and use it as an osmoprotectant (Kempf and Bremer, 1998). Accumulation of compatible solutes helps the cell to rehydrate the cytoplasm and leads to transport of potassium out of the cell. Additionally, compatible solutes in contrast to ions are excluded from the surface of proteins which leads to the hydration of the protein surface and stabilization of protein conformation (Kempf and Bremer, 1998; Sleator and Hill, 2002).

1.2 Osmoregulation of *proU* transcription

The activity of osmoregulatory systems in *E. coli* is tightly regulated. The regulation of function of transporters occurs on two levels. Firstly, the activity of transporters is regulated in response to osmolarity change. For example ProP, the most studied example in *E. coli*, functions itself as an osmosensor and is activated by hyperosmotic stress (Wood *et al.*, 2001; Wood, 2006). Secondly, the expression of transporters is regulated. Several studies showed that hyperosmotic stress leads to repression of rRNA transcription and simultaneously to upregulation of expression of up to 400 genes (Weber *et al.*, 2005). Many genes induced by hyperosmotic stress are thought to be transcribed by polymerase associated with the alternative stress sigma factor σ^S (Weber *et al.*, 2005). It was proposed that accumulation of potassium glutamate serves as a primary signal leading to blockage of transcription from the majority of σ^{70} promoters (including rRNA transcription) and stimulation of transcription from a majority of σ^S promoters and selected σ^{70} promoters (Gralla and Huo, 2008; Gralla and Vargas, 2006; Lee and Gralla, 2004).

The regulation of expression of the *proU* operon has been intensively studied in *Salmonella enterica serovar* Typhimurium and in *E. coli*. The *proU* operon (Fig. 2) consists of three structural genes *proV*, *proW*, and *proX* (Dattananda and Gowrishankar, 1989; Gowrishankar, 1989). Proteins encoded by these genes form a transport system that belongs to the family of ATP-binding cassette transporters (ABC-transporters). ProW is an integral cytoplasmic membrane transporter, consisting of seven transmembrane segments, with a periplasmic N-tail and short cytoplasmic C-tail (Whitley *et al.*, 1994; Haardt and Bremer, 1996). ProV is a hydrophylic cytoplasmic ATPase associated with ProW at the cytoplasmic membrane (Mimmack *et al.*, 1989; May *et al.*, 1989). ProX is a periplasmic protein that has high affinity to glycine betaine and proline betaine and lower affinities to some other osmoprotectants (see above). The activity of the ProVWX transporter was shown to be osmoregulated (May *et al.*, 1986; Faatz *et al.*, 1988).

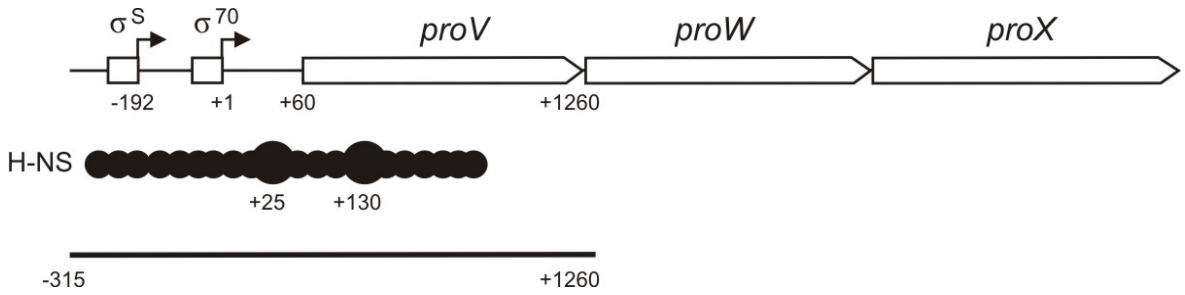


Figure 2. *E. coli proU* operon. Structural genes *proV*, *proW*, *proX* transcribed from σ^S and σ^{70} promoters. Coordinates indicate the position of σ^S promoter (-192), *proV* translation start (+60) and 3′ end of *proV* gene (+1260) in comparison to transcription start (+1) from σ^{70} promoter. H-NS was shown to bind *proU* at least between -230 to +270 region (Badaut *et al.*, 2002; Lucht *et al.*, 1994), H-NS binding nucleation sites +25 and +130 are shown. The -315 to +1260 *proU* region used in this work to study *proU* osmoregulation is indicated in the lower part of the figure.

The *proU* operon is expressed at an extremely low level under low osmolarity conditions. The expression is elevated up to several hundred times by hyperosmotic stress (Gowrishankar, 1985) and remains high in hyperosmotic medium (Lucht and Bremer, 1994). Transcription of *proU* is lead by two promoters. The main promoter is located 60 bp upstream of the start codon of the first structural gene *proV*, and the activity of this promoter is osmoregulated (Gowrishankar, 1989; May *et al.*, 1989; Stirling *et al.*, 1989; Lucht and Bremer, 1991; Park *et al.*, 1989; Mellies *et al.*, 1994). The transcription from this promoter was experimentally shown to be σ^{70}-dependent (Jovanovich *et al.*, 1989; Yim *et al.*, 1994). The second promoter is σ^S-dependent and maps 250 nt upstream of the *proV* translational start site (Gowrishankar, 1989; Dattananda *et al.*, 1991; Manna and Gowrishankar, 1994; Rajkumari *et al.*, 1996). However, this σ^S promoter seems to be not important for *proU* osmoregulation, and transcription from this promoter could only be activated under specific conditions (cold shock or after mutation of *hns*, *hfq*, *rho* genes) (Rajkumari and Gowrishankar, 2001).

Two different mechanisms were proposed to be responsible for osmoinduction of *proU* promoter activity. Firstly, the promoter was proposed to be induced by K^+ ions that influx into the cell during the initial phase of hyperosmotic stress response (see above). Indeed, induction of *proU* depends on the concentration of K^+ in growth medium during hyperosmotic stress (Sutherland et al., 1986). Also *proU* expression was shown to be induced by potassium glutamate in cell-free *in vitro* assays, although the degree of *proU* osmoinduction in these assays was lower than that observed *in vivo* (Ramirez *et al.*, 1989; Jovanovich *et al.*, 1989; Prince and Villarejo, 1990; Ueguchi and Mizuno, 1993). Secondly, *proU* was found to be repressed by the global regulator H-NS (heat-stable nucleoid structuring protein). No specific regulators were found to be involved in osmoregulation of *proU* transcription. Initially, it was proposed that H-NS induced changes in DNA supercoiling might be responsible for *proU* promoter osmoregulation (Higgins *et al.*, 1988). However, in another study supercoiling was found to have no effect (Ramirez and Villarejo, 1991). In the meantime it was shown that H-NS forms extended complexes on the DNA which cause repression of transcription as detailed below.

H-NS is a nucleoid-associated protein, which presumably regulates the structure of the bacterial chromosome and which functions as a global transcriptional repressor (Dillon and Dorman, 2010; Dorman, 2004). The mutation of the *hns* gene was found to affect *proU* expression and osmoregulation (Higgins *et al.*, 1988; May *et al.*, 1990; Lucht and Bremer, 1991). In fact, the *proU* operon serves as a key model system for the analysis of H-NS-mediated repression and regulation. H-NS represses *proU* by binding both within the coding sequence of *proV* (Dattananda *et al.*, 1991; Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992) and within the promoter region and upstream of the promoter (Lucht *et al.*, 1994). For repression of *proU* by H-NS its binding within the coding region is crucial (Overdier and Csonka, 1992; Owen-Hughes *et al.*, 1992; Nagarajavel *et al.*, 2007). At the *proU* locus H-NS covers AT-rich DNA stretches located next to the promoter (Lucht *et al.*, 1994). In addition, two identical 10 bp sequences located around +25 and +130 relative to the transcription start of the *proU* locus were found to be high affinity H-NS binding sites (Bouffartigues *et al.*, 2007). H-NS presumably first binds to these two high affinity (nucleation) sites and then laterally oligomerizes along the DNA by occupying lower affinity sites which results in an extended repression complex at the *proU* promoter (Bouffartigues *et al.*, 2007). The H-NS repression complex spreads as far as at least 230 nt upstream of the transcription start site (Lucht *et al.*, 1994) and up to 270 nt downstream of transcription start (Badaut *et al.*, 2002; Lucht *et al.*, 1994). H-NS binding leads to repression of *proU* transcription (Ueguchi and Mizuno, 1993) presumably by inhibiting binding of RNA polymerase (Jordi and Higgins, 2000; Nagarajavel *et al.*, 2007). H-NS repression of *proU* is more effective under low osmolarity conditions and depends on the promoter activity (Nagarajavel *et al.*, 2007).

Results of two studies also suggested that *proU* expression might be regulated by an H-NS homologue, StpA (Free *et al.*, 1998; Sonden and Uhlin, 1996). As H-NS, StpA is a nucleoid-associated protein that functions a transcriptional repressor, and in addition as an RNA chaperone (Dorman, 2004). In one study, overexpression of StpA was shown to repress *proU* expression in both wild type and *hns* mutant cells (Sonden and Uhlin, 1996). In another study, *stpA* deletion did not affect *proU* expression neither in wild type cells nor in an *hns* null mutant, however upregulated *proU* expression in cells expressing only the N-terminal part of H-NS (Free et al., 1998). StpA might potentially affect *proU* expression either at the transcriptional level or at post-transcriptional level as a RNA chaperone. Two other nucleoid-associated proteins HU (Manna and Gowrishankar, 1994) and IHF (Lucht and Bremer, 1991) were also found to have some effect on *proU* expression, however the effect was minor and probably indirect. In contrast Fis, another important nucleoid-associated protein, has no effect on *proU* expression (Lucht and Bremer, 1991).

1.3 Post-transcriptional regulation of gene expression and potential mechanisms of post-transcriptional regulation of *proU*

The only two mechanisms that were clearly demonstrated to contribute to *proU* osmoregulation are the osmoresponsiveness of the promoter activity and repression by H-NS. However, a recent study suggested that *proU* expression is still osmoregulated even when the native *proU* promoter is replaced by the constitutive *lacUV5* promoter and when expression was analyzed in *hns* mutant cells (Madhusudan S., 2007). Also osmoregulation of translational *proU-lacZ* fusions was stronger than that of the respective transcriptional *proU-lacZ* fusions. These results suggested that in addition to osmoregulation of transcription, *proU* might also be osmoregulated at a post-transcriptional level. Further results by Madhusudan (2007) suggest that the *proU* mRNA is processed by RNase III and degraded by RNase E.

Post-transcriptional regulation of gene expression includes the regulation of mRNA maturation, mRNA stability and the translation of the mRNA. The expression can further be regulated post-translationally by regulation of protein stability and post-translational protein modifications. RNases are the enzymes responsible for mRNA maturation and degradation. In bacteria the maturation of mRNA is carried out by endonucleases and includes the processing of polycistronic mRNA in intergenic regions, and the processing in 5′ untranslated regions. Degradation of mRNA is a multistep process including the action of different RNases. The degradation is usually initiated by endonucleases, and then the mRNA is degraded by exonucleases. Short oligonucleotide products are finally degraded by oligonucleases (Arraiano *et al.*, 2010; Condon, 2007; Deutscher, 2006; Kushner, 2007; Li and Deutscher, 2004).

RNase E is a principal endonuclease in *E. coli*, that takes part in maturation of rRNAs and tRNAs and also in degradation of many mRNAs (Condon, 2007; Deutscher, 2006). RNase E is the central component of a multienzyme complex, the degradasome. In addition to RNase E, the degradasome consists of exonuclease PNPase, helicase RhlB and enolase. Degradosome plays a central role in mRNA degradation (Carpousis, 2007).

RNase III is an endoribonuclease which primary role is maturation of rRNAs and some tRNAs (Arraiano *et al.*, 2010; Drider and Condon, 2004; Conrad and Rauhut, 2002). RNase III also takes part in mRNA processing. RNase III facilitates degradation of some mRNAs by specific cleavage within untranslated regions or within coding sequences. RNase III can also process polycistronic mRNA to generate separate mRNAs and process 5′ untranslated region to activate mRNA translation. RNase III cleaves double-stranded RNA: either double-stranded regions formed by single RNA molecules or complexes of a small regulatory RNA with mRNA target. RNase III cleavage is site-specific. No conserved sequences recognized by RNase III were found. However, efficiency of processing and enzyme binding depends on sequences located at the cleavage site and within 10 bp from the cleavage site (Pertzev and Nicholson, 2006; Zhang and Nicholson, 1997). In *E. coli*,

RNase III is encoded by a single gene *rnc* and its expression is autoregulated (Bardwell *et al.*, 1989)

RNase III is involved in stress response in *E. coli*. RNase III together with the sRNA DsrA regulates the expression of the stress-response sigma factor σ^S (Resch *et al.*, 2008). Therefore, RNase III should indirectly affect the expression of σ^S-dependent genes involved in stress response. RNase III together with the sRNA GadY regulates expression of the acid response genes *gadX* and *gadW* (Opdyke *et al.*, 2010). RNase III also regulates expression of *bolA*, encoding a protein involved the regulation of cell morphology and the general adaptation to stress (Freire et al., 2006).

RNase III processing is affected by some stress conditions. Activity of RNase III is inhibited by the stress-responsive regulator YmdB under cold stress conditions (Kim *et al.*, 2008). YmdB directly binds to RNase III and inhibits RNase III activity. RNase III processing is also downregulated during stationary-phase growth (Kim *et al.*, 2008). Further, the *rnc*, *pnp*, and *bdm* transcripts are all processed by RNase III (Bardwell *et al.*, 1989; Regnier and Hajnsdorf, 1991; Sim *et al.*, 2010). Also, the expression of these three genes is upregulated by hyperosmotic stress (Sim *et al.*, 2010). It was therefore proposed that RNase III activity is downregulated by hyperosmotic stress and this downregulation leads to the osmotical upregulation of *rnc*, *pnp* and *bdm* expression (Sim *et al.*, 2010).

Regulatory RNAs play an active role in post-transcriptional regulation of gene expression in bacteria (Waters and Storz, 2009). Many regulatory RNAs belong to the class of small regulatory RNAs (sRNAs) and usually have a size of 50-400 nucleotides (Frohlich and Vogel, 2009; Liu and Camilli, 2010; Thomason and Storz, 2010). Most sRNAs have homology to their target mRNA and carry out their function by basepairing to the target. Some of these sRNAs, termed cis-encoded sRNAs or antisense sRNA, are encoded in the same locus as the target mRNA (Thomason and Storz, 2010). Cis-encoded sRNAs are transcribed from the DNA strand opposite its target mRNA in the opposite direction. Other sRNAs, termed trans-encoded sRNAs, are transcribed from their own locus separately from the target gene. In contrast to cis-encoded sRNAs, trans-encoded sRNAs have only limited homology to their targets. Basepairing of sRNAs to mRNA targets can lead to activation or inhibition of mRNA translation, to initiation of mRNA processing and degradation by RNases, or on the contrary to stabilization of mRNA (Frohlich and Vogel, 2009; Waters and Storz, 2009; Liu and Camilli, 2010; Thomason and Storz, 2010). Binding of cis-encoded sRNA to mRNA target can also trigger termination of transcription of this mRNA (Thomason and Storz, 2010). Some trans-encoded sRNAs do not have homology to mRNA targets and instead indirectly affect gene expression via binding to regulatory proteins (Waters and Storz, 2009; Liu and Camilli, 2010). These sRNAs can bind to and inactivate negative regulatory proteins stabilizing the target mRNA. Alternatively they can bind to RNA polymerase or transcription factors inhibiting the transcription of target genes (Waters and Storz, 2009; Liu and Camilli, 2010).

Another class of regulatory RNAs in bacteria are riboswitches (Waters and Storz, 2009; Smith *et al.*, 2010). These are RNA elements located in 5′ untranslated region of some mRNAs. These elements form a secondary structure that can be changed in response to different environmental stimuli. Riboswitches can bind various ligands, including some uncharged tRNA species, different small organic molecules (amino acids, nucleotides and derivatives) and inorganic ions. Interaction with the ligand leads to a conformational change of riboswitch. This conformational change results in activation or inhibition of translation, or alternatively in termination or antitermination of transcription (Waters and Storz, 2009; Smith *et al.*, 2010).

The regulation of mRNA stability and the translation efficiency generally involves RNA chaperones. RNA chaperones change the tertiary structure or secondary structure of the RNA, and thus disrupt or on the contrary promote basepairing within single RNA molecules or between two separate RNA molecules (Rajkowitsch *et al.*, 2007). The RNA chaperone Hfq is a global regulator which regulates mRNA translation, polyadenylation and mRNA stability (Brennan and Link, 2007; Chao and Vogel, 2010). Hfq also facilitates the binding of sRNAs to their mRNA targets. This sRNA-mRNA binding can lead to the inhibition or activation of translation or to the degradation of mRNA. One well studied example is the activation of *rpoS* mRNA translation by Hfq together with sRNA DsrA and RNase III (Resch *et al.*, 2008). Mutation of the gene coding for the RNA chaperone Hfq was reported to affect *proU* expression (Rajkumari and Gowrishankar, 2001; Madhusudan S., 2007). StpA, the H-NS homologue, also has RNA chaperone activity and might potentially regulate *proU* expression at the transcriptional or post-transcriptional level.

Some recent data indicate that protein ProQ might affect *proU* expression. ProQ consists of two domains that have homology to RNA chaperones Hfq and FinO (Chaulk *et al.*, 2011). FinO is a plasmid-encoded RNA chaperone that together with the sRNA FinP inhibits translation of *traJ* mRNA and consequently blocks the transfer of F plasmid by conjugation (Arthur *et al.*, 2011). RNA chaperone activity of ProQ was demonstrated by *in vitro* assays (Chaulk *et al.*, 2011). *In vivo*, mutation of *proQ* gene leads to the downregulation of expression of *proP* encoding the ProP transporter (Chaulk *et al.*, 2011). However, the mutation of *proQ* does not affect ProP expression when *proU* operon is also deleted, suggesting the possible interaction between *proQ* and *proU* (J. Wood, personal communication).

1.4 Objectives of the PhD project

The general aim of the PhD project was to study the post-transcriptional osmoregulation of the *proU* operon. Specifically, the objectives of the current study were:

1) to elucidate the molecular mechanisms of osmoregulation of *proU* expression at post-transcriptional level

2) to examine the possible role of RNase III, Hfq, ProQ and StpA in post-transcriptional osmoregulation of *proU*

3) to build a model of *proU* osmoregulation including both transcriptional and post-transcriptional mechanisms

2. Results

2.1 Osmoregulation of *proU* at post-transcriptional level

Previous analysis in the lab suggested that the *proU* operon might be osmoregulated at a post-transcriptional level (Madhusudan, 2007). In these studies repression of the *proU* operon was analyzed using a fragment of *proU* from position -315 to +303 relative to transcription start site of the σ^{70} promoter (Fig. 2). This *proU* fragment was assumed to include all DNA elements of the *proU* locus known to be important for repression by H-NS (Nagarajavel *et al.*, 2007). In addition, Madhusudan (2007) compared the osmoregulation of a transcriptional fusion of *proU*(-315 to +315) with the *lacZ* reporter gene and a reporter in which *lacZ* was translationally fused to the *proU*(-315 to +303) fragment. Osmoregulation of the translational fusion was higher, indicative of a post-transcriptional level of regulation. However, with these reporter constructs the degree of osmoregulation was significantly lower than what was observed in the literature where *lacZ* was fused further downstream of the transcription site of *proU* (Dattananda *et al.*, 1991)

In my thesis I analyzed osmoregulation of the *proU* operon using a longer *proU* sequence from position -315 to +1260 relative to transcription start site of the σ^{70} promoter (Fig. 2). This fragment includes the full-length sequence of the *proV* gene, extending up to position *proU*(+1260), and the regulatory region 315 bp upstream of the transcription start site (Fig. 2). As shown below osmoregulation of this longer *proU*(-315 to +1260) constructs was significantly stronger than that of the short *proU*(-315 to +315) fragment and in addition allowed detection of the ProV protein with Western blotting using a fusion with HA-tag. In this work I use the term *proV* without coordinates to indicate the full-length *proV* gene including the untranslated 5′ mRNA region, and the term *proV* with coordinates [e.g. *proV*(+1 to +303)] to indicate the corresponding truncations of the *proV* gene. The term *proU* refers to the *proU* operon in general. Term *proU* mRNA is used to designate mRNA transcript even when it is transcribed not from full-length *proU* but from a short *proU* fragment (e. g. *proV*). The term *proU* promoter refers to the *proU*(-315 to +1) sequence, which includes both σ^{70} and σ^{S} promoters. In some constructs the *proU* promoter was substituted by the constitutive *lacUV5* promoter. In all experiments *E. coli* strains were used in which the native *proU* operon was deleted.

In this work the expression of the *proU* operon was determined under different osmolarity conditions. Cells were grown in a standard LB medium and osmolarity was varied by adjusting the concentration of NaCl in the medium. Standard LB medium used in the laboratory contains 0.085M NaCl. LB supplemented with 0.3M of NaCl was used as the high osmolarity medium, and LB supplemented with 0.01M of NaCl was used as the low osmolarity medium.

2.1.1 Transcriptional and post-transcriptional osmoregulation of *proU*

In a first set of experiments the new constructs which include the full-length *proV* gene were used to determine the contribution of the promoter region and of H-NS-mediated repression to osmoregulation. In addition, they were used to find out whether any additional factors are involved in osmoregulation. To determine the expression level of *proV*, translational and transcriptional fusions of the *lacZ* reporter gene to the 3' end of *proV* were constructed and integrated in the *E. coli* chromosome (Fig. 3). The strains were grown under different osmolarity conditions and expression of the *proV-lacZ* fusions was determined with β-galactosidase assay. Then the osmoinduction of expression of each construct was calculated as a ratio of the expression value in high osmolarity medium and the expression value under low osmolarity conditions.

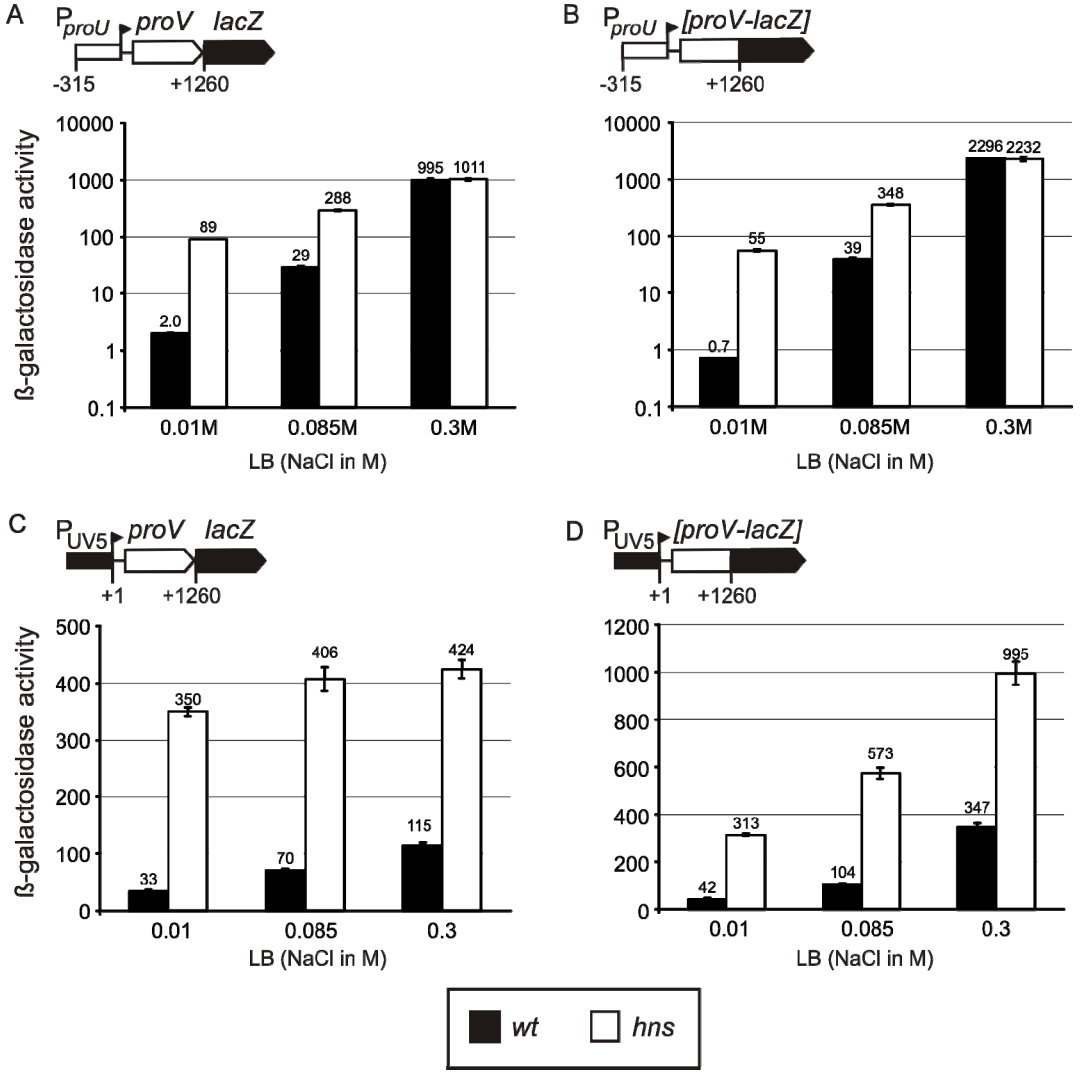


Figure 3. Contribution of *proU* promoter, H-NS repression and post-transcriptional mechanisms to *proU* osmoregulation. Transcriptional (A, C) and translational (B, D) fusions of *proV* with the *lacZ* reporter gene are integrated in the chromosome. Expression of these reporter constructs from the original *proU* promoter (A, B) and *lacUV5* promoter (C, D) was determined in *wt* and *hns* mutant cells using β-galactosidase assay. Cells were grown in LB with indicated NaCl concentrations. Bars show the determined expression level (black bars for *wt* and white bars for *hns* mutant). Values are the mean of at least three calculations. Error bars represent standard error of the mean. Strains used: A, *wt* S4066, *hns* S4113; B, *wt* S4068, *hns* S4115; C, *wt* S4064, *hns* S4111; D, *wt* S4070, *hns* S4117. Note that different scales are used to indicate β-galactosidase activity.

To estimate the contribution of H-NS repression to osmoregulation, expression was compared in *hns* mutants and wild type strains. To estimate the contribution of *proU* promoter and upstream H-NS binding sites to osmoregulation, constructs expressing *proV* from the native *proU* promoter and from a constitutive *lacUV5* promoter variant were used. In contrast to the native *proU* promoter, the activity of *lacUV5* promoter is not affected by osmolarity conditions (Csonka *et al.*, 1994).

The translational fusion of *lacZ* reporter to *proV* gene under control of the *proU* promoter should represent the behavior of the native *proU* locus with all the regulatory mechanisms involved (Fig. 3B). This construct was expressed at an extremely low level in wild type cells grown under low osmolarity conditions (Fig. 3B). The expression was induced more than 2000-fold in cells grown under high osmolarity conditions (Fig. 3B). As expected, both the *proU* promoter and H-NS repression contributed to this high degree of osmoinduction. The *proU* promoter was induced by hyperosmotic stress only 3.4-fold in wild type cells and 2-fold in *hns* mutant cells (Nagarajavel et al., 2007). However, substitution of the *proU* promoter by the constitutive *lacUV5* promoter lead to a drop in osmoinduction of the translational *proV-lacZ* fusion from more than 2000-fold to only 8-fold (compare black bars in Fig.3B versus 3D). This large drop in osmoinduction reflects the importance of the *proU* promoter and possibly also of upstream H-NS binding sites for *proU* osmoregulation.

Comparison of osmoregulation of translational *proV-lacZ* fusion from the *proU* promoter in *hns* mutants versus wild type cells confirmed the role of H-NS repression in osmoregulation (Fig. 3B). In the *hns* mutant the expression was more than 50-fold increased under low osmolarity conditions in comparison to wild type cells (Fig. 3B). In contrast, no difference between expression in wild type and in *hns* mutants (and therefore no H-NS repression) was detected under high osmolarity conditions. As the result, the osmoregulation dropped from more than 2000-fold in wild type cells to only 41-fold in the *hns* mutant.

Interestingly, expression was still osmoregulated even when the *proU* promoter was substituted by the constitutive *lacUV5* promoter and when simultaneously H-NS repression was abrogated. Namely, the expression of the translational *proV-lacZ* fusion from the *lacUV5* promoter was still 3.2-fold osmoregulated in *hns* mutant cells (Fig. 3D). These results support the idea that in addition to osmoregulated transcription initiation at the *proU* promoter and to H-NS repression some other mechanism contributes to *proU* osmoregulation. In contrast to the translational fusion, the transcriptional *PlacUV5-proV-lacZ* fusion was not osmoregulated anymore in the *hns* mutant (Fig. 3C). Thus, this putative additional mechanism of osmoregulation of *proU* expression operates at a post-transcriptional level. In conformity with this, osmoinduction of the transcriptional *proV-lacZ* fusions expressed from both the *proU* and the *lacUV5* promoters was uniformly lower than osmoinduction of the corresponding translational fusions in both the wild type and the *hns* mutants (Fig. 3A vs. B, and Fig. 3C vs D).

2.1.2 Osmoregulation of ProV protein expression

To ensure that the *proV-lacZ* translational fusion reflects regulation of native *proU*, osmoregulation was independently analyzed with Western blots. The *proV* gene was fused with a sequence coding for a C-terminal HA-tag. This *proV*-HA fusion was expressed from the *proU* promoter on low copy plasmids pKEKK11 (Fig. 4A) and from the *lacUV5* promoter on plasmid pKEKK12 (Fig. 4B). The expression level of ProV-HA was determined in wild type and *hns* mutant cells that were either grown constantly under low and high osmolarity conditions, respectively, or first grown under low osmolarity conditions and then subjected to hyperosmotic stress by addition of NaCl to the medium. In conformity with the results of the β-galactosidase assays, only minimal levels of ProV-HA were detected under low osmolarity conditions. Expression was significantly higher in cells constantly grown in hyperosmotic medium. Strong osmoinduction and similar levels of ProV-HA were observed already ten minutes after hyperosmotic upshift. As expected, expression from the *proU* promoter was more strongly osmoregulated than from the *lacUV5* promoter. Importantly, expression of ProV-HA was still osmoregulated when expressed from *lacUV5* promoter in *hns* mutant cells (Fig. 4B). These results confirmed that in addition to H-NS repression and *proU* promoter activity some additional mechanism contributes to osmoregulation of the *proU* locus.

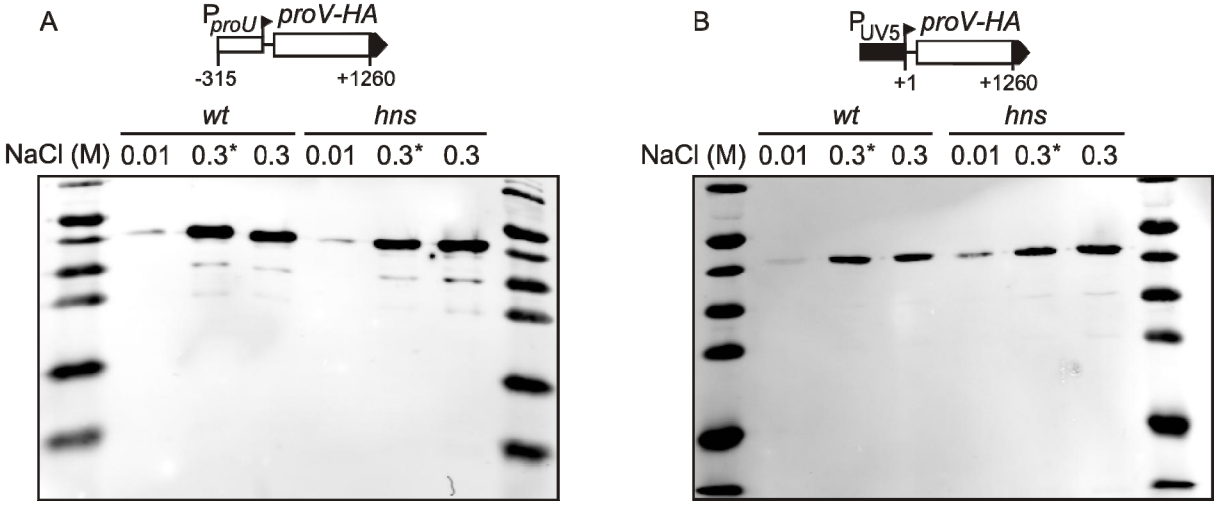


Figure 4. Western blot analysis of osmoregulation of ProV-HA expression. The *proV* gene fused with HA-tag sequence was expressed from the *proU* promoter (plasmid pKEKK11) and the *lacUV5* promoter (plasmid pKEKK12) in *wt* cells (S3460) and *hns* mutant cells (S4162). Cells were grown constantly in LB supplemented with 0.01M or 0.3M NaCl (labelled 0.01 and 0.3 respectively). Alternatively cells were first grown in LB with 0.01M NaCl to OD_{600} of 0.5, and then NaCl concentration was increased to 0.3M, and samples were taken 10 minutes later (labelled 0.3*). Total cell lysates were analyzed with antibodies to HA-tag.

2.1.3 ProV protein stability under different osmolarity conditions

The results of above described experiments showed that *proU* is osmoregulated at a post-transcriptional level. Different mechanisms might be responsible for this post-transcriptional induction of *proU* by hyperosmotic stress:

1. *proU* mRNA might be more stable under high osmolarity conditions
2. *proU* translation might be more efficient under high osmolarity conditions
3. protein ProV might be more stable under high osmolarity conditions

The latter possibility was examined by comparing the stability of ProV-HA under low and high osmolarity conditions. Cells carrying the high copy plasmid pKEM78 expressing ProV-HA from *lacUV5* promoter were grown under different osmolarity conditions. Protein synthesis was inhibited by addition of chloramphenicol (200 µg/ml) and samples were taken immediately before as well as five, ten, and twenty minutes after chloramphenicol addition. The experiment showed that protein ProV is more stable under low osmolarity conditions (Fig. 5). Therefore the higher amount of ProV under high osmolarity conditions can not be explained by the increased ProV stability. The possibility of osmoregulation of *proU* mRNA stability and *proU* translation were examined in further experiments.

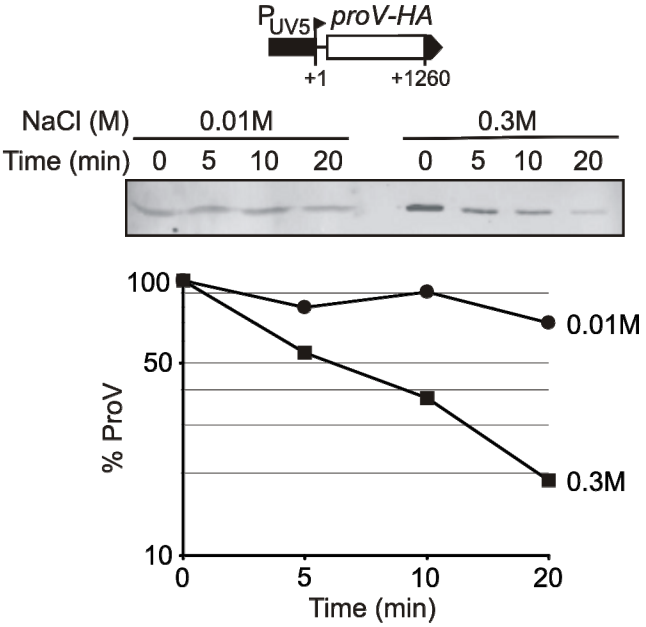


Figure 5. Stability of protein ProV-HA under different osmolarity conditions. Western blot analysis of expression of ProV-HA under control of *lacUV5* promoter from plasmid pKEM78. Cells (strain S3460) were grown in LB medium supplemented with 0.01M or 0.3M NaCl. Protein synthesis was inhibited by the addition of chloramphenicol to final concentration of 200 µg/ml. Samples were taken before, as well as 5, 10 and 20 minutes after chloramphenicol addition. ProV-HA protein was visualized with anti-HA antibodies. Four-times more lysate of cells grown at low osmolarity was loaded on a gel. The Western blot analysis was done twice with two independently obtained sets of samples. One representative Western blot image is shown. The corresponding chart below shows the relative amount of ProV-HA protein remaining at each time point as determined by Odyssey Infrared Imaging System software and plotted as a function of time. Each value is the average of four measurement done for two independently obtained sets of samples.

2.2 Osmoregulation of RNase III processing of *proU* mRNA

2.2.1 RNase III processing of *proU* mRNA in the conserved region

RNase E and RNase III were shown to process *proU* mRNA, with one RNase III processing site reported between positions +216 and +217 (Madhusudan S., 2007). In this study, RNase processing of *proU* mRNA was further analyzed and the possibility of osmoregulation of RNase processing was tested.

Processing of *proU* mRNA was examined with RACE (rapid amplification of cDNA ends) analysis of 5′ transcript ends (Wagner and Vogel, 2005). This method is a modification of the standard RACE protocol and includes an additional step of the treatment with tobacco acid pyrophosphatase (TAP) that removes diphosphate residue from the primary 5′ end of mRNA. The method enables mapping of both primary 5′ ends and 5′ ends formed after RNase processing. 5′ RACE was used to analyze the 5' region of *proU* mRNA extending from the known transcription site to position +496. The analyzed *proU* part covers a region between positions +203 and +293 that is highly conserved in *Enterobacteriaceae* species (Table 1) and therefore might be important for the regulation of *proU*.

Table 1. Conservation of the *proV(+203 to +293)* region in *Enterobacteriaceae*

Genus (number of strains)	Homology of *proV* sequence	
	full *proV*(+1 to +1263)	*proV*(+203 to +293) region
Escherichia (with *Shigella*) (33)	96-100%	99-100%
Salmonella (17)	82-85%	99-100%
Citrobacter (1)	86%	98%
Enterobacter (2)	82%	95-96%
Klebsiella (2)	82%	92%
Erwinia (2)	76%	93%
Sodalis (1)	75%	97%
Photorhabdus (1)	75%	91%

The sequence homology of full *proV*(+1 to +1263) sequence and *proV*(+203 to +293) sequence in *Enterobacteriaceae* species. Species are grouped in genera. Number of strains analyzed for each genus is provided in brackets. Note that *Shigella* strains belong to the species *E. coli*. The percent homology was determined by performing a BLAST search of full *proV*(+1 to +1263) sequence and *proV*(+203 to +293) sequence of *E. coli* K-12 substr. MG1655 against bacterial genome sequences.

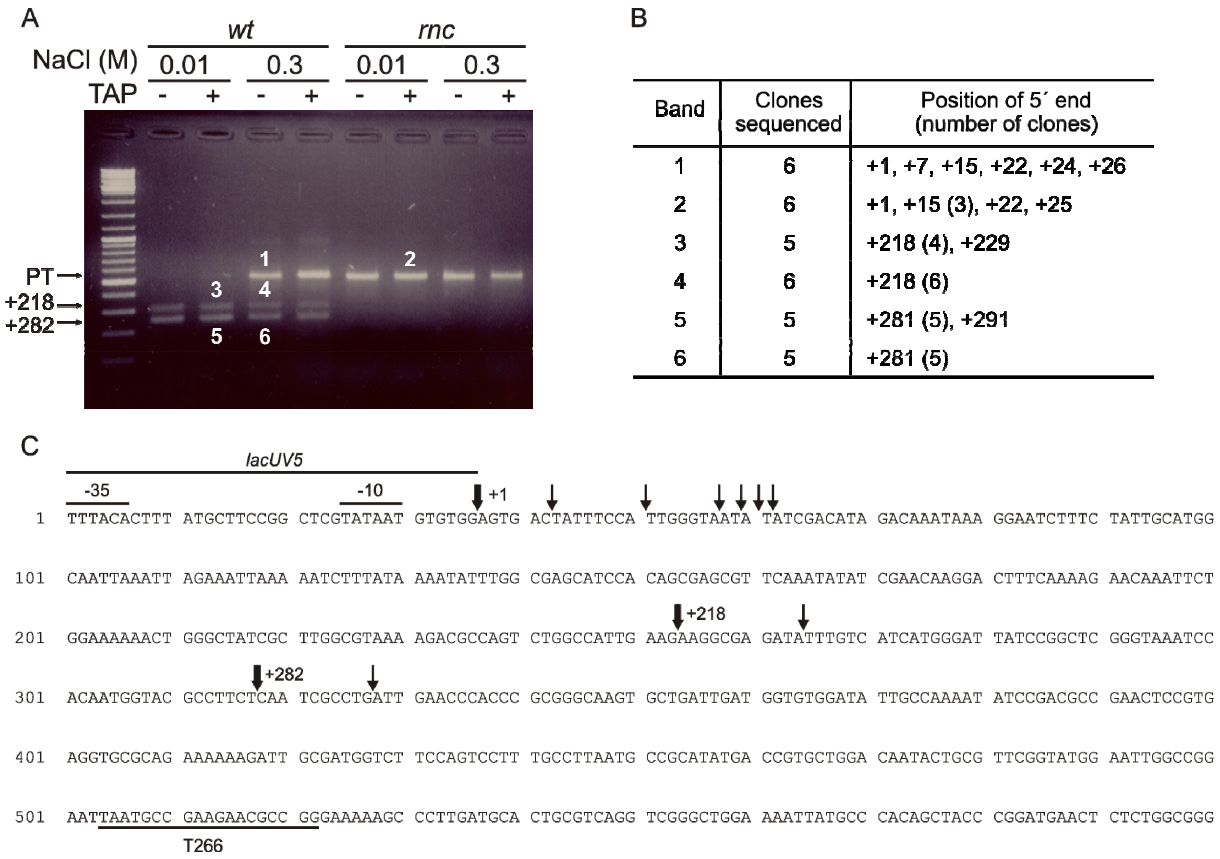
To perform 5′ RACE, *proV* was expressed from the low copy plasmid pKEKK12 under control of the *lacUV5* promoter. Cells carrying the plasmid were grown under high and low osmolarity conditions and total RNA was extracted. Part of each RNA sample (6 µg of RNA) was treated with TAP to remove diphosphate from 5′ primary end of mRNA. Another part of samples (also 6 µg of RNA) were not treated with TAP. RNA samples were then ligated to RNA adapter by incubation with T4 RNA ligase. T4 RNA ligase ligates RNA adapter only to

the transcripts that carry 5′ monophosphate but not 5′ triphosphate. As a result, the RNA adapter is expected to be ligated to 5′ primary ends of mRNA only in samples treated with TAP. In contrast, the adapter is ligated to processing products in both TAP-treated samples and samples not treated with TAP. Therefore, comparison of TAP-treated and not treated samples should allow to distinguish 5′ primary ends from 5′ ends generated by processing.

RNA ligation products were purified and used as a template for cDNA synthesis with the gene-specific DNA oligonucleotide complementary to +467 to +486 region of *proU*. The resulting cDNA was amplified by PCR with gene-specific and 5′-adapter-specific primers, and the PCR products were analyzed with agarose gel electrophoresis (Fig. 6A). PCR products with the size corresponding to the expected size of primary transcript (530 bp), as well as two shorter products were detected in wild type cells (Fig. 6A). While both primary transcript and processing products were detected in cells grown under high osmolarity conditions, almost no primary transcript was detected under low osmolarity conditions. These results suggest that RNase processing of *proU* mRNA is more efficient under low osmolarity conditions. The same 5′ RACE analysis was also carried out with RNA isolated of a *rnc* mutant expressing a non-functional RNase III. Only the large PCR product corresponding to the primary transcript was detected in the *rnc* mutant. This suggests that the two shorter bands detected in the RNA isolated from wild type cells correspond to the products of RNase III processing.

To map the RNase III processing sites, individual DNA bands were purified from the agarose gel, cloned in vector pUC12 and sequenced (Fig. 6B, C). For cloning of each DNA band, sequences of five to six inserts in pUC12 were analyzed, since both primary and processed 5′ ends may vary by a few nucleotides. Sequencing confirmed that the larger DNA fragment (Fig. 6A, bands 1 and 2) corresponds to the primary transcript, although most of the inserts sequenced corresponded to transcripts which are truncated by a few bases at the 5' end (Fig. 6B, C). It is not clear whether these truncations is based on RNase processing *in vivo* or later during experimental procedures. The presence of truncated primary transcripts explains why, in contrast to our expectations, the primary transcript was detected in both TAP-treated samples and samples not treated with TAP (Fig. 6A). More importantly, sequencing of shorter DNA bands showed that the 5' ends of processing products corresponded to positions +218 and +282 relative to *proU* transcription start (Fig. 6B, C). Therefore RNase III processes the *proU* mRNA at positions +217/+218 and +281/+282.

RNase III is known to process RNA in double-stranded regions. Therefore the secondary structure of *proU* mRNA was modeled using *mfold* software (Zuker, 2003) and RNase III processing sites were mapped on the predicted secondary structure. The *proU* mRNA was found to form an intensive stem-loop structure in conserved +203 to +293 region (Fig.7). The RNase III cleavage sites are located within the double-stranded region. Processing in these sites would lead to the formation of two-nucleotide overhang at the 3′ ends of mRNA, a typical feature of RNase III processing.



Band	Clones sequenced	Position of 5′ end (number of clones)
1	6	+1, +7, +15, +22, +24, +26
2	6	+1, +15 (3), +22, +25
3	5	+218 (4), +229
4	6	+218 (6)
5	5	+281 (5), +291
6	5	+281 (5)

Figure 6. 5′ RACE analysis of *proU* mRNA processing by RNases. (A) Gel electrophoresis of amplification products of cDNA. Each DNA band on a gel corresponds to the separate RNA species (primary *proU* transcript or processing product). Analysis of processing of *proU* mRNA in wild type cells and in *rnc* mutant cells is shown. The *proU*(+1 to +1260) mRNA was expressed from *lacUV5* promoter on low copy plasmid pKEKK12. Wild type (S3460) and *rnc* mutant cells (S4023) carrying pKEKK12 were grown in LB supplemented with either 0.01M or 0.3M of NaCl. RNA was extracted and further analyzed as described in text. (+) and (-) refers to TAP treatment prior to ligation with RNA adapter. PT - primary transcript. Numbers indicate the DNA bands that were subsequently purified from the gel, cloned in pUC12 and analyzed by sequencing. (B) and (C) Mapping of RNase III processing sites by sequencing. (B) The results of DNA bands sequencing. The band numbers in the table correspond to the band numbers in (A). Most DNA bands are a mixture of fragments with different 5′ ends. Therefore, five to six clones were sequenced per cloning of each band. The number of clones sequenced, the positions of 5′ ends determined by sequencing and the number of clones with corresponding 5′ ends (in brackets) are shown. (C) Sequence of the plasmid pKEKK12 used for 5′ RACE analysis with RNase III processing sites. Sequence includes *lacUV5* promoter and upstream part of *proU* analyzed by 5′ RACE. Primer T266 was used for PCR amplification of cDNA, and therefore corresponds to the 3′ end of the analyzed *proU* sequence. The location of 5′ ends of clones determined by sequencing are taken from the Fig. 6B and indicated by arrows. For each cloning, the most upstream position taken as the true 5′ end and depicted as a fat arrow with indication of position in comparison to transcription start (+1).

Not only the primary sequence but also the secondary structure of the stem-loop is highly conserved among *Enterobacteriaceae* species (Fig. 7). SNPs found in this region of *proU* in other species are predominantly located in single stranded parts of the mRNA (bulges and loops). SNPs found in double-stranded regions of mRNA in most cases also do not disrupt base pairing (e.g. G-C base pare changes to A-U or non-canonical G-U) (Fig. 7). Conservation of both the primary sequence and the putative secondary structure of *proU* mRNA around the RNase III cleavage site suggested that RNase III processing might be important for *proU* osmoregulation. Indeed, the results of the 5′ RACE analysis suggested that RNase III processes the *proU* mRNA more efficiently under low osmolarity conditions and the processing is inhibited under high osmolarity conditions (Fig. 6B).

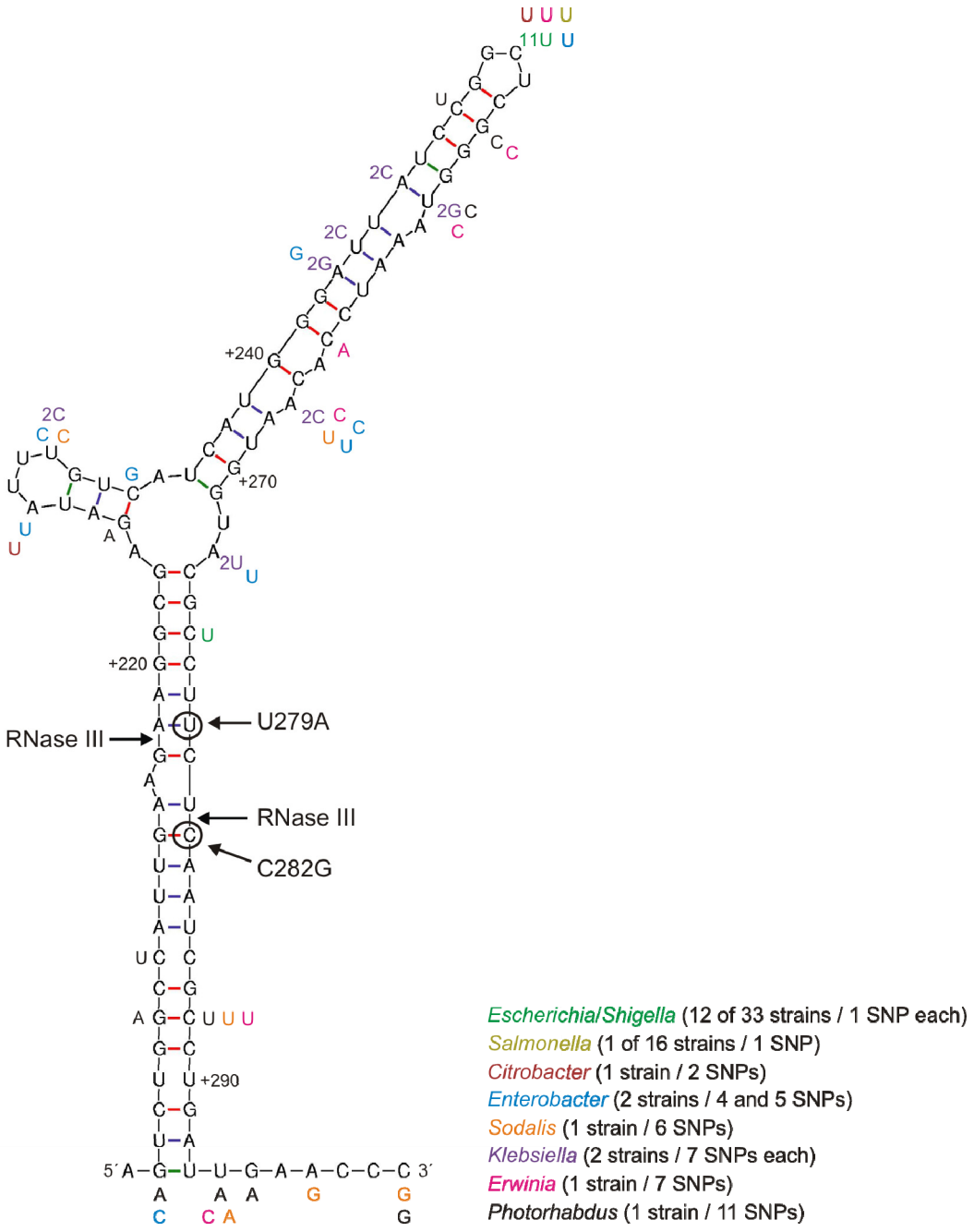


Figure 7. RNase III processes *proU* mRNA in a conserved region. Secondary structure of *proU* mRNA +202 to +300 region from *E. coli* K-12 substr. MG1655 was predicted using *mfold* software (Zuker, 2003). Numbers indicate the coordinates in the relation to the transcription start (+1). BLAST search of *proV* sequence of *E. coli* K-12 substr. MG1655 against the bacterial genomes was performed (see Table 1 for details) and SNPs found in 57 *Enterobacteriaceae* strains were mapped. SNPs are shown as single nucleotides, numbers in front of SNP indicate the number of strains this SNP was found. The list of *Enterobacteriaceae* genera with corresponding number of strains analyzed and number of SNPs is provided. The colors of font used in the list match the colors of the corresponding SNPs on the figure. RNase III cleavage sites mapped by 5´ RACE are shown with arrows. Nucleotides mutated to disrupt RNase III processing are circled and shown with arrows.

2.2.2 Effect of *rnc* mutation on *proU* osmoregulation

The possible involvement of RNase III in *proU* osmoregulation was further investigated by comparing the osmoregulation of *proU* in *rnc* mutants and the wild type. The *rnc* allele was transferred by transduction to strains which carry chromosomal copies of translational and transcriptional fusions, respectively, of *proU* with the reporter *lacZ* gene under control of *lacUV5* promoter. Expression of *proV-lacZ* fusions in *rnc* and double *rnc hns* mutants was compared to the expression in wild type and the *hns* mutant under different osmolarity conditions (Fig. 8). Mutation of *rnc* lead to slight 1.1- to 1.3-fold downregulation of the expression of the control *PlacUV5-lacZ* construct (Fig. 8A, B) that might be due to the indirect effect of *rnc* mutation (see below). Importantly, the expression of *proV-lacZ* fusions under low osmolarity conditions was 1.9- to 2.3-fold higher in *rnc* mutants than in cells with the intact *rnc* gene, both in the wild type and in the *hns* mutant background (Fig. 8C to F). In contrast, expression of these constructs under high osmolarity conditions was not significantly affected by *rnc* mutation. These data further support the results of the 5´ RACE analysis that RNase III processing of the *proU* mRNA is inhibited under high osmolarity conditions.

The effect of the *rnc* mutation on *proU* expression was also analyzed using the *proV-lacZ* fusions expressed from the native *proU* promoter. Unexpectedly, mutation of *rnc* gene lead to the noticeable 1.4- to 1.8-fold downregulation of the expression of a control *PproU-lacZ* fusion (which lacks the *proV* coding sequence and RNase III processing sites) (Fig. 9A, B). The negative effect of *rnc* mutation on the expression of *PlacUV5-lacZ* and *PproU-lacZ* fusions may be partly non-specific. *rnc* mutant cells grow significantly slower than wild type cells and as a result the *rnc* mutation indirectly affects the expression of several hundreds of growth rate-dependent genes (Sim *et al.*, 2010). However, the constructs expressed from the *proU* promoter might be further downregulated by mutation of *rnc* because RNase III is involved in activation of σ^S expression (Resch *et al.*, 2008). Therefore, mutation of *rnc* might lead to lower level of σ^S and as the result to the lower expression from the σ^S-dependent *proU* promoter.

Mutation of *rnc* had differential effects on the expression of the *PproU-proV-lacZ* fusions under different osmolarity conditions (Fig. 9C-F). Expression of all fusions was upregulated in the *rnc* mutant under low osmolarity conditions but downregulated under high osmolarity conditions. This probably results from the combination of two opposing effects: upregulation of expression due to blocking of RNase III processing and downregulation of the *proU* promoter.

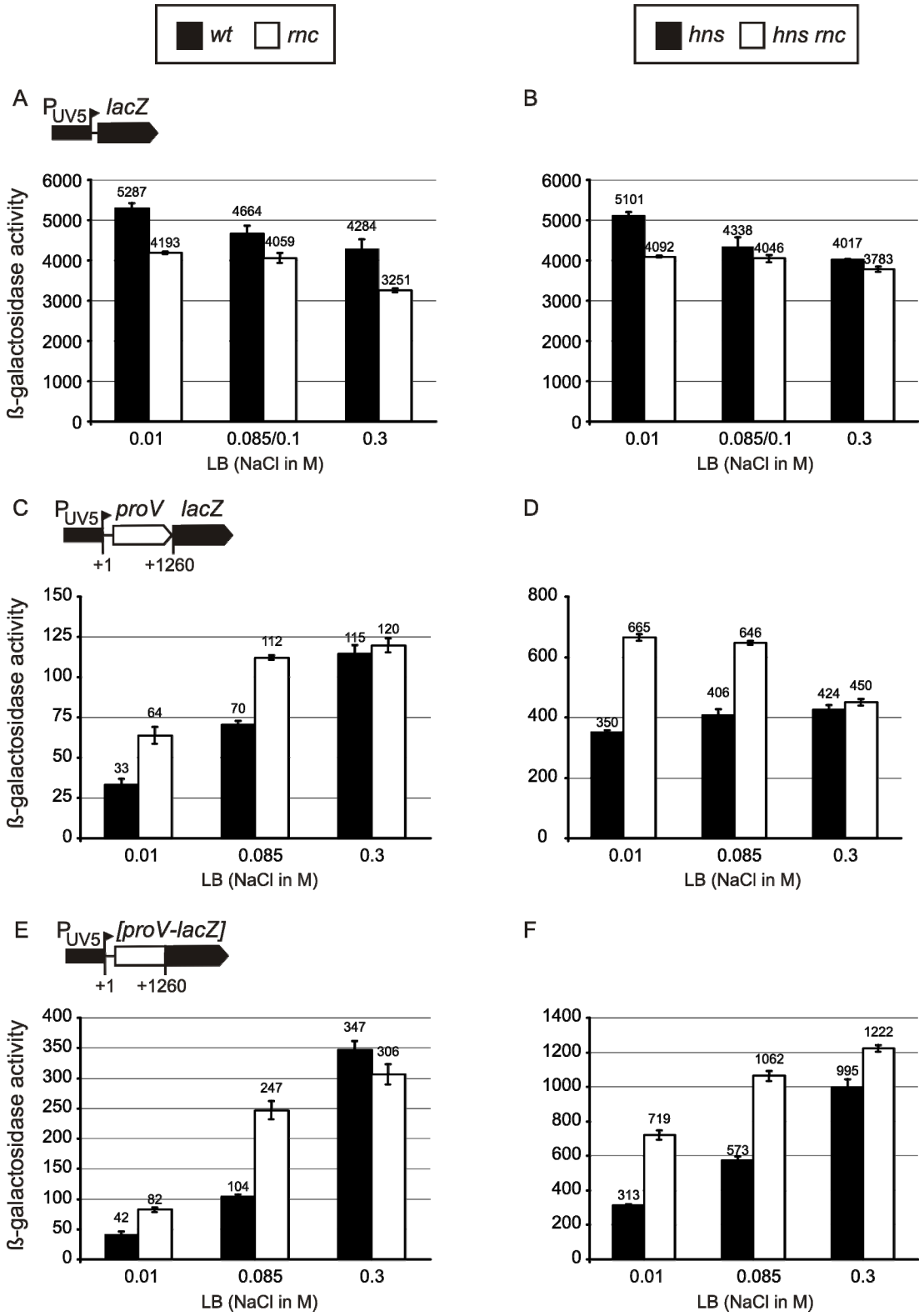


Figure 8. Effect of *rnc* mutation on the osmoregulation of *proV* expressed from the constitutive *lacUV5* promoter. Transcriptional (C, D) and translational (E, F) fusions of *proV* with the *lacZ* reporter gene, as well as *lacZ* gene alone (A, B) are integrated in the chromosome. Expression of these reporter constructs from the *lacUV5* promoter was determined with β-galactosidase assay. Expression of constructs was compared in *wt* versus *rnc* mutant cells (A, C, E) and in *hns* versus *hns rnc* double mutant cells (B, D, F). Cells were grown in LB supplemented with the indicated NaCl concentrations. Bars show the determined expression level (black bars for *wt* and *hns* mutants, and white bars for *rnc* and *hns rnc* mutants respectively). Values are the mean of at least three calculations. Error bars represent standard error of the mean. Strains used: A, *wt* S1979, *rnc* T214; B, *wt* S3122, *hns rnc* T216; C, *wt* S4064, *rnc* T97; D, *hns* S4111, *hns rnc* T99; E, *wt* S4070, *rnc* T100; F, *hns* S4117, *hns rnc* T102. Note that different scales are used to indicate β-galactosidase activity. Expression values for strains S1906 and S3122 are taken from (Madhusudan S., 2007).

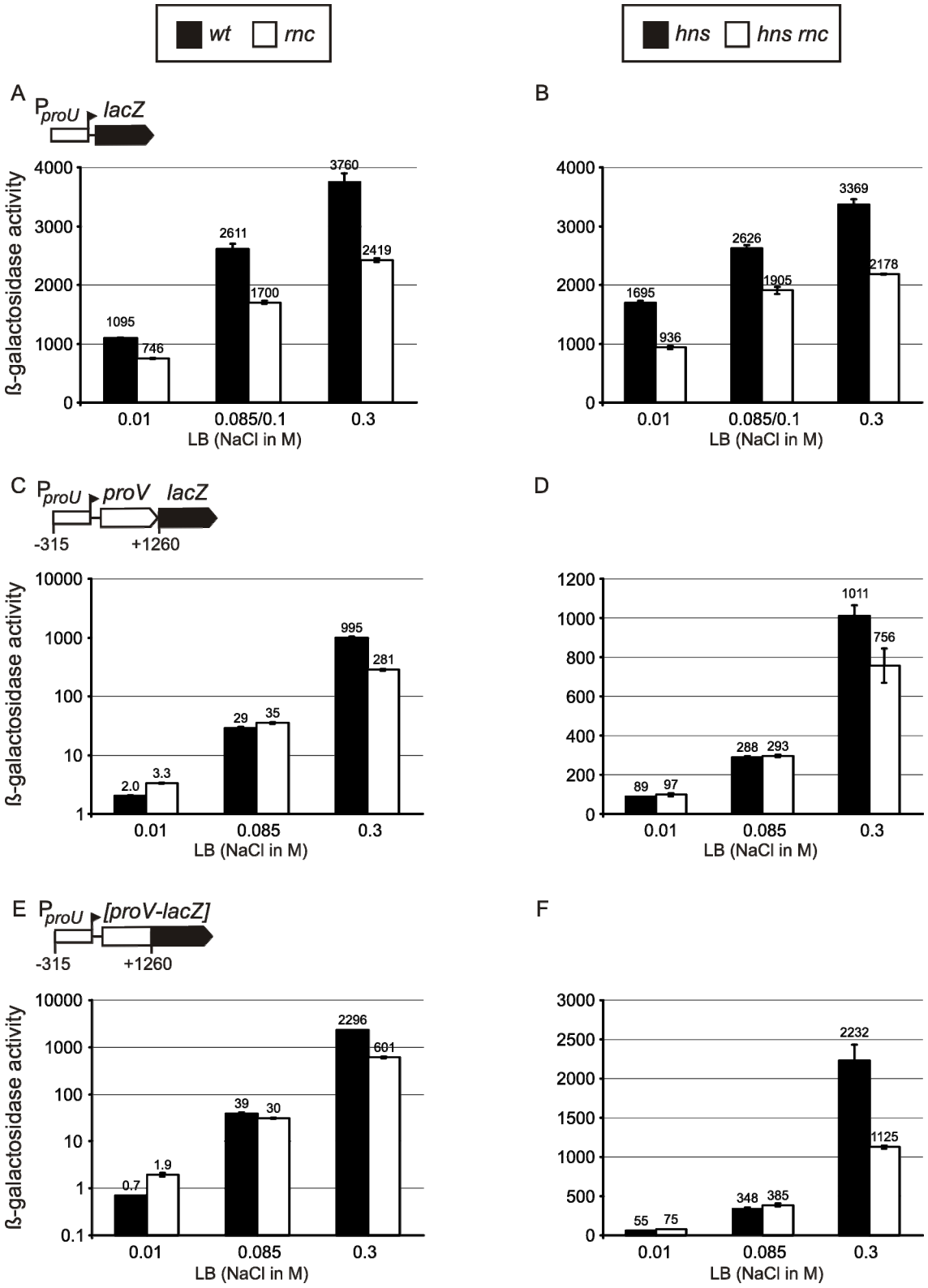


Figure 9. Effect of *rnc* inactivation on the osmoregulation of *proV* expressed from the native *proU* promoter. Transcriptional (C, D) and translational (E, F) fusions of *proV* with the *lacZ* reporter gene, as well as *lacZ* gene alone (A, B) are integrated in the chromosome. Expression of these reporter constructs from the original *ProU* promoter was determined with β-galactosidase assay. Expression of constructs was compared in *wt* versus *rnc* mutant cells (A, C, E) and in *hns* versus *hns rnc* double mutant cells (B, D, F). Cells were grown in LB supplemented with the indicated NaCl concentrations. Bars show the determined expression level (black bars for *wt* and *hns* mutants, and white bars for *rnc* and *hns rnc* mutants respectively). Values are the mean of at least three calculations. Error bars represent standard error of the mean. Strains used: A, *wt* S2048, *rnc* T380; B, *wt* S3124, *hns rnc* T382; C, *wt* S4066, *rnc* T90; D, *hns* S4113, *hns rnc* T92; E, *wt* S4068, *rnc* T94; F, *hns* S4115, *hns rnc* T95. Note that different scales are used to indicate β-galactosidase activity. Expression values for strains S2048 and S3124 are taken from (Madhusudan S., 2007).

2.2.3 Effect of RNase III processing site mutation on *proU* osmoregulation

For 5′ RACE analysis of *proU* mRNA processing, RNase III activity was abolished by mutation of the *rnc* gene. However, the *rnc* mutation may have pleiotropic effects and may indirectly affect the expression of the *lacZ* reporter constructs. Therefore a different strategy of blocking RNase III processing was required to measure the effect of RNase III processing on *proU* expression. Instead of using the *rnc* mutant, the processing was blocked by mutation of the RNase III processing site of *proU* mRNA. Two single nucleotide mutations C282G and T279A that do not change the encoded amino acid sequence of the ProV protein were introduced at the RNase III processing site by site-specific mutagenesis (Fig. 7). The resulting *proV* mutants (*proVC282G* and *proVT279A*, respectively) were cloned into various reporter plasmids. Wild type *proV* sequence in plasmid pKEKK12, which carries *proV*-HA construct under control of the *lacUV5* promoter, was substituted by the *proV* mutants resulting in plasmids pKEKK36 expressing *proVT279A*-HA and pKEKK38 expressing *proVC282G*-HA. Using these plasmids 5′ RACE analysis was performed under the same conditions as described above for wild type *proV* expressed from pKEKK12. After gel electrophoresis only one large fragment corresponding to the primary transcript was detected for both of the *proV* processing site mutants (Fig. 10). Therefore both mutations completely abolished RNase III processing of *proU* mRNA.

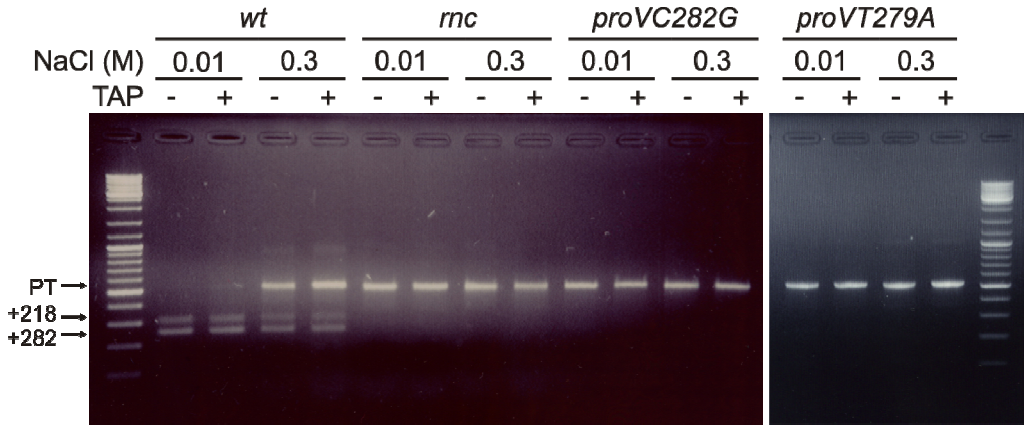


Figure 10. 5′ RACE analysis of *proU* mRNA processing by RNases. Gel electrophoresis of amplification products of cDNA is shown. Each DNA band on a gel corresponds to the separate RNA species (primary *proU* transcript or processing product). Analysis of processing of wild type *proU* in both wild type cells (indicated *wt*) and in *rnc* mutant cells (indicated *rnc*), as well as processing of *proU* mutants in wild type cells (indicated *proUC282G* and *proUT279A*) is shown. Wild type and mutant forms of *proU*(+1 to +1260) transcripts were expressed from *lacUV5* promoter from low copy plasmids: pKEKK12 for wild type *proU*, pKEKK38 for *proUC282G*, pEKK36 for *proUT279A*. Wild type (S3460) and *rnc* mutant cells (S4023) carrying these plasmids were grown in LB supplemented with either 0.01M or 0.3M of NaCl. RNA was extracted and further analyzed as described in text. (+) and (-) refers to TAP-treatment prior to ligation with RNA adapter. PT - primary transcript.

To estimate the effect of RNase III processing on *proU* osmoregulation, the expression of wild type and mutant *proV* genes was measured with Western blot and with β-galactosidase assays under different osmolarity conditions. The plasmids pKEKK12, pKEKK36 and pKEKK38 expressing wild type and mutant *proV-HA* genes and used for 5′ RACE were now also used for Western blot analysis. Expression was determined in wild type and *hns* mutant cells carrying these plasmids under low osmolarity conditions and ten minutes after hyperosmotic upshift. Both mutations of the RNase III processing site lead to significantly

higher expression levels of ProV-HA in cells grown under low osmolarity conditions (Fig. 11). This effect was evident in both wild type and *hns* mutant cells. In contrast, there was no significant effect of processing site mutations on ProV-HA expression under high osmolarity conditions. This supports the conclusion that RNase III processing of *proU* mRNA is effective under low osmolarity conditions and inhibited by hyperosmotic stress.

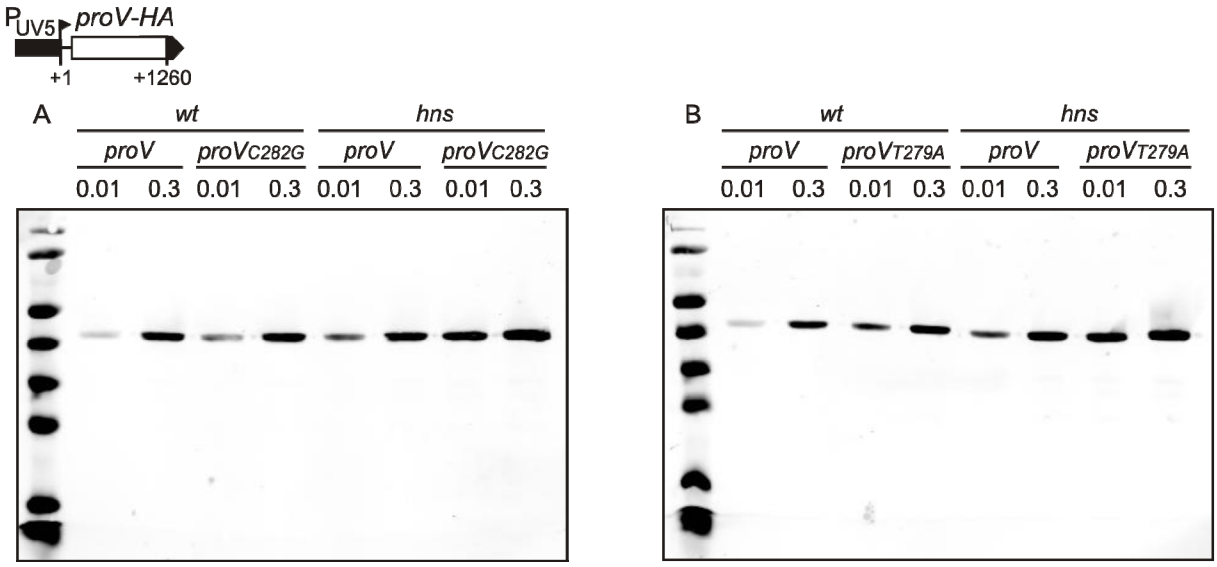


Figure 11. Western blot analysis of osmoregulation of *proV* with mutated RNase III processing site. Wild type *proV* and mutant *proVC282G* and *proVT279A* fused with HA-tag were expressed from *lacUV5* promoter from the plasmids pKEKK11 (wild type *proV*, A and B), pKEKK38 (*proVC282G*, A), and pKEKK36 (*proVT279A*, B) respectively. Wild type (S3460) and *hns* mutant cells (S4162) carrying these plasmids were grown in LB supplemented with 0.01M NaCl to OD_{600} of 0.5, NaCl was added to the final concentration of 0.3M and samples were taken immediately before and 10 minutes after this hyperosmotic stress. Total cell lysates were analyzed with antibodies to HA-tag.

Osmoregulation of the *proV* mutants was further studied using the *proV-lacZ* reporter fusions which were integrated into the chromosome (Fig. 12). Expression of the translational and transcriptional *proU-lacZ* fusions under the control of *lacUV5* and *proU* promoter was measured in wild type and *hns* mutant cells grown under different osmolarity conditions. Both mutations of the RNase III processing site lead to similar higher expression level of all the constructs tested (Fig. 12). The effect of abolishing RNase III processing was more pronounced under low than under high osmolarity conditions for all constructs expressed from *lacUV5* and *proU* promoters in wild type as well as in *hns* mutant cells. Specifically, mutations of processing site lead to only 1.3- to 1.9-fold increase of expression of constructs under high osmolarity conditions. In contrast, under low osmolarity conditions mutations of processing site lead to 2.4- to 4.4-fold increase in expression of transcriptional *proV-lacZ* fusions (Fig. 12A, B, E, F) and 5.0- to 10.0-fold increase of expression of translational *proV-lacZ* fusions (Fig. 12C, D, G, H). The latter difference between the translational and transcriptional fusions is expected, because RNase III processing should affect the translational *proV-lacZ* fusions stronger than the transcriptional fusions. To summarize, the results of the β-galactosidase assays and Western blot analysis of expression of *proV* mutants together with the results of 5′ RACE experiments clearly show that RNase III processing of *proU* mRNA is much more efficient under low osmolarity than under high osmolarity conditions.

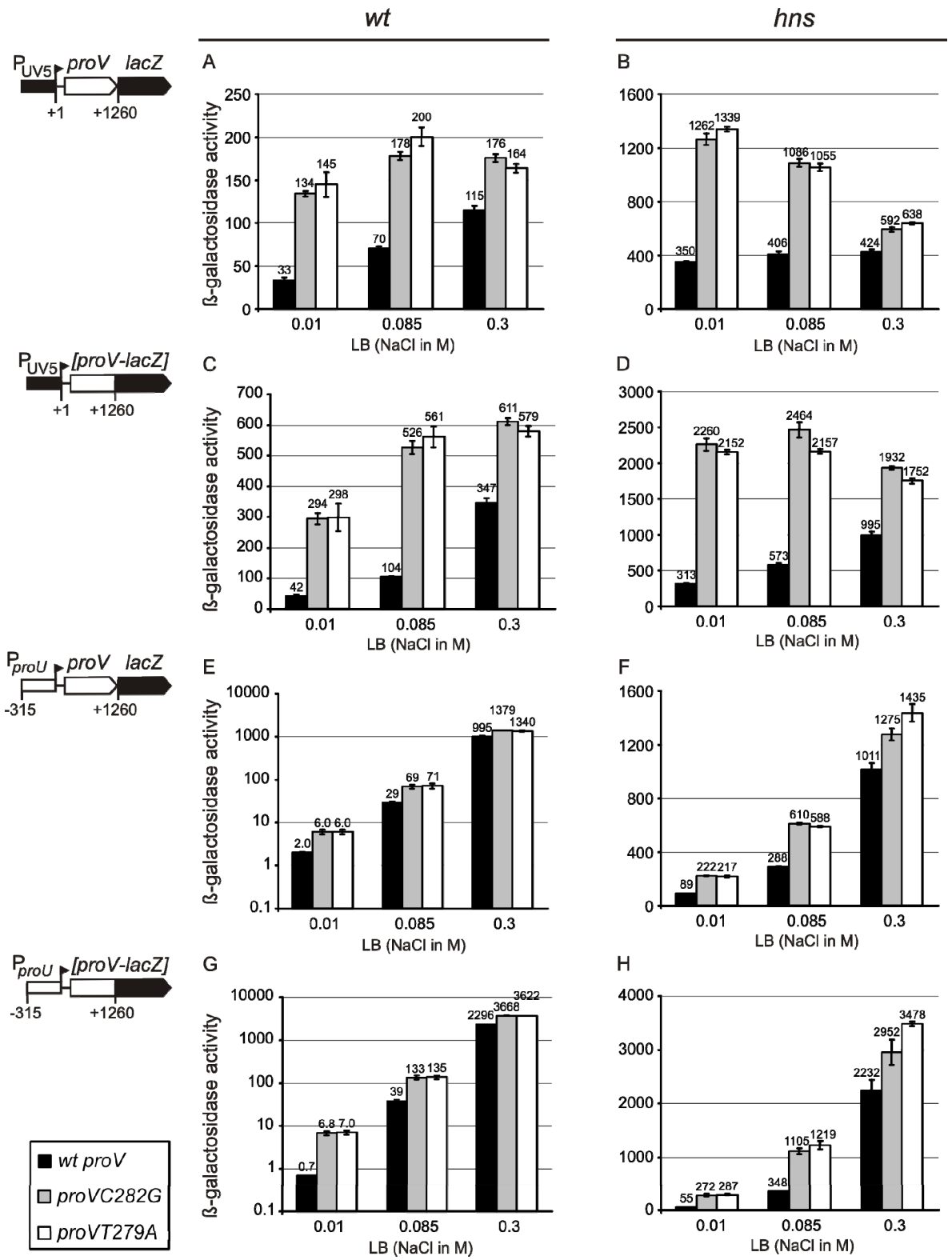


Figure 12. β-galactosidase assay of osmoregulation of *proV* with mutated RNase III processing site. Transcriptional (A, B, E, F) and translational (C, D, G, H) fusions of *proV* with the *lacZ* reporter gene are integrated in the chromosome. Expression of these reporter constructs from the *lacUV5* promoter (A, B, C, D) and the *ProU* promoter (E, F, G, H) was determined in *wt* and *hns* mutant cells using β-galactosidase assay. Cells were grown in LB with indicated NaCl concentrations. Bars show the determined expression level (black bars for wild type *proV*, grey bars for *proVC282G* and white bars for *proVT279A* respectively). Values are the mean of at least three calculations. Error bars represent standard error of the mean. Note that different scales are used to indicate β-galactosidase activity. Strains used: A, *wt proV* S4064, *proVC282G* T809, *proVT279A* T889; B, *wt proV* S4111, *proVC282G* T831, *proVT279A* T907; C, *wt proV* S4070, *proVC282G* T811, *proVT279A* T887; D, *wt proV* S4117, *proVC282G* T833, *proVT279A* T909; E, *wt proV* S4066, *proVC282G* T885, *proVT279A* T883; F, *wt proV* S4113, *proVC282G* T897, *proVT279A* T895; G, *wt proV* S4068, *proVC282G* T881, *proVT279A* T879; H, *wt proV* S4115, *proVC282G* T893, *proVT279A* T891.

The data obtained with β-galactosidase assays were also used to estimate the effect of RNase III processing on osmoinduction of *proU* expression. Osmoinduction was calculated as the ratio of *proU* expression under high osmolarity conditions and of expression under low osmolarity conditions. The osmoinduction of the translational *proV-lacZ* fusions under control of *proU* and *lacUV5* promoters in wild type and *hns* mutant cells was calculated (Fig. 13). Mutation of RNase III processing site lead to a 3.4- to 4.4-fold drop in osmoinduction of all constructs tested in both wild type and *hns* mutant cells. These uniform results suggest that RNase III processing contributes to *proU* osmoregulation independently of H-NS repression and promoter activity.

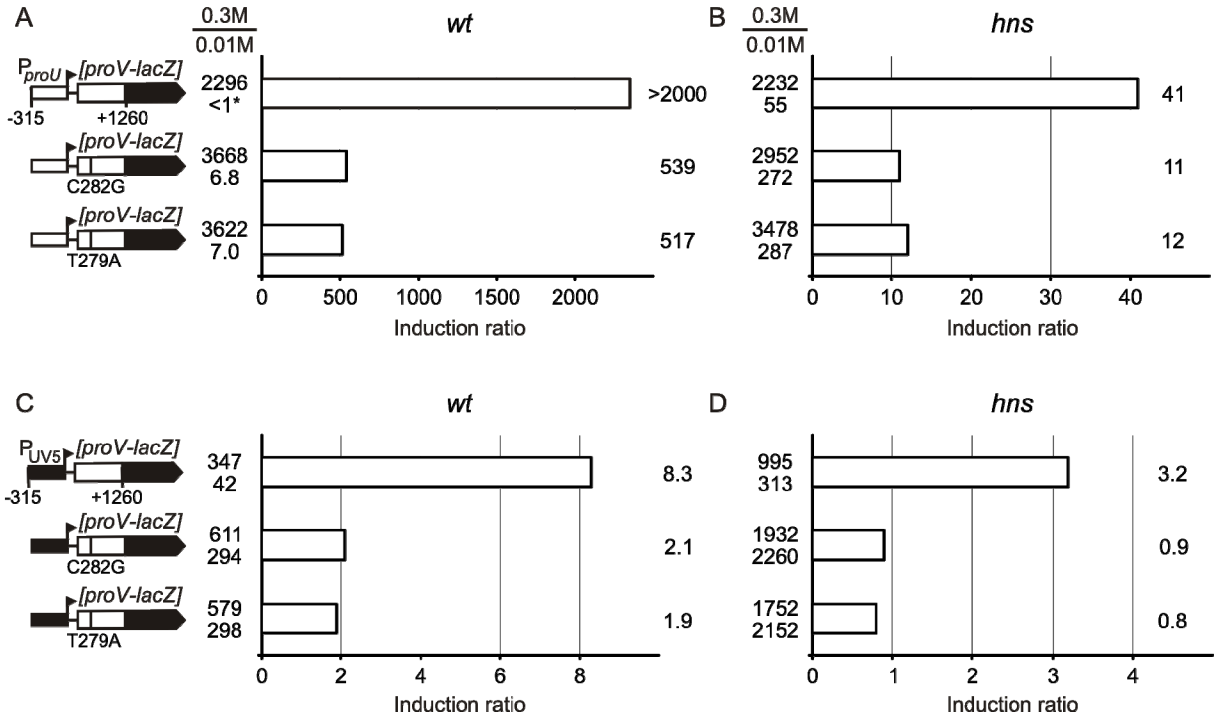


Figure 13. Effect of RNase III processing site mutation on induction of *proV* expression by hyperosmotic stress. Translational fusions of *proV* with the *lacZ* reporter gene were integrated in the chromosome. The osmoinduction of expression of the fusions from the *proU* promoter (A and B) and *lacUV5* promoter (C and D)) in *wt* cells (A and C) and *hns* mutant cells (B and D) is shown. Bars show the osmoinduction ratio that is calculated as the ratio of expression level in LB with 0.3M NaCl to expression level in LB with 0.01M NaCl. The expression values are taken from Figure 11. Strains used: A, *wt proV* S4068, *proVC282G* T881, *proVT279A* T879; B, *wt proV* S4115, *proVC282G* T891, *proVT279A* T891; C, *wt proV* S4070, *proVC282G* T811, *proVT279A* T887; D, *wt proV* S4117, *proVC282G* T833, *proVT279A* T909.

It was shown here, that *proV* expression is still osmoregulated when expressed from *lacUV5* promoter in *hns* mutants (Fig. 3C and D, Fig. 4B). This osmoregulation, together with the difference in expression between transcriptional and translational *proV-lacZ* fusions (Fig. 3), suggested the presence of osmoregulation of *proU* at a post-transcriptional level. Interestingly, both Western blot analysis (Fig. 11) and β-galactosidase assay (Fig. 13D) demonstrated that the RNase III processing site mutants *proVC282G* and *proVT279A* are not osmoregulated when expressed from *lacUV5* promoter in *hns* mutant cells. These results demonstrate that the observed post-transcriptional osmoregulation of *proU* is based on the osmoregulation of RNase III processing of *proU* mRNA.

In the end, the effect of the *rnc* mutation on *proU* expression was directly compared to the effect of one of the RNase III processing site mutants. The expression of the fusions of wild type *proV* and *proVC282G* with *lacZ* was measured in *rnc* mutants and wild type cells grown under different osmolarity conditions. Both *rnc* mutation and *proVC282G* mutation lead to the upregulation of *proV* expression (Fig. 14). However the upregulation effect was stronger when RNase III processing was blocked by mutation of the processing site. Also, the expression of *proVC282G* construct was significantly lower in *rnc* cells than in wild type cells. These results are consistent with the previous observations (Fig. 8 and Fig. 9) that the *rnc* mutation not only leads to *proU* upregulation by blocking RNase III processing, but also non-specifically downregulates the expression of the reporter constructs.

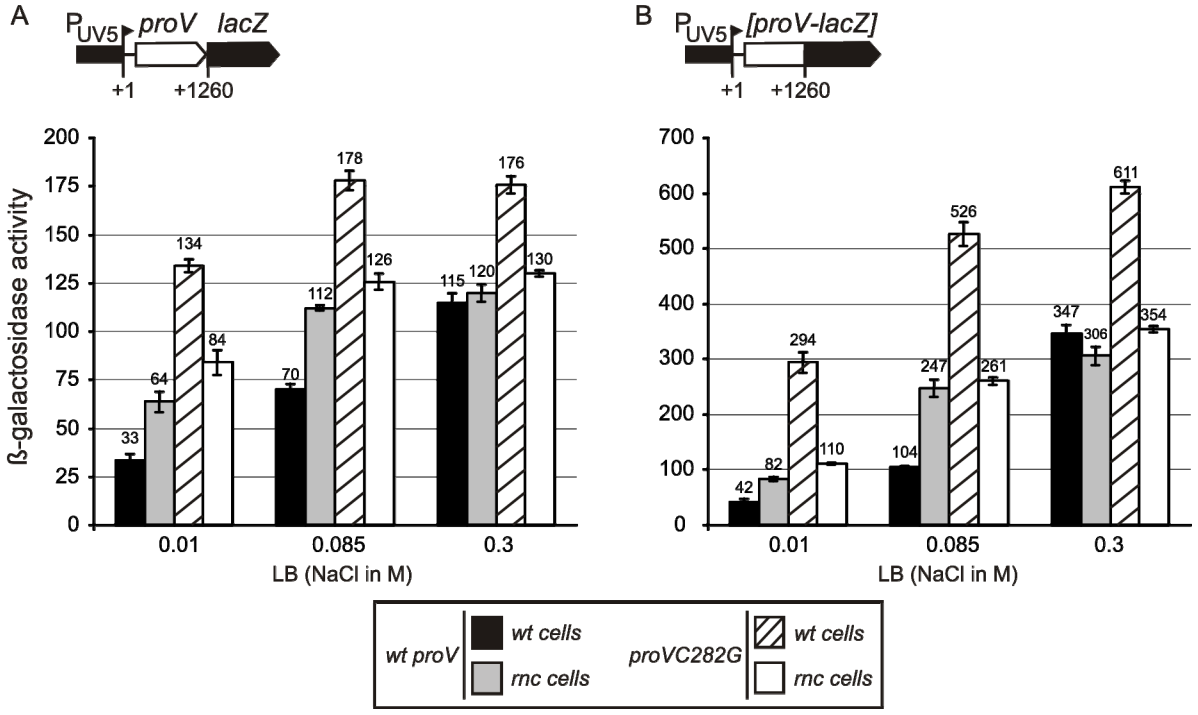


Figure 14. Comparison of the effects of RNase III processing site mutation and *rnc* mutation on *proU* expression. Transcriptional (A) and translational (B) fusions of wild type *proV* and *proVC282G* with the *lacZ* reporter gene are integrated in the chromosome. Expression of the reporter constructs from the *lacUV5* promoter was determined in *wt* and *rnc* mutant cells using β-galactosidase assay. Cells were grown in LB with indicated NaCl concentrations. Bars show the determined expression level. Values are the mean of at least three calculations. Error bars represent standard error of the mean. Strains used: A, *wt* cells / *wt proV* S4064, *rnc* cells / *wt proV* T97, *wt* cells / *proVC282G* T809, *rnc* cells / *proVC282G* T823; B, *wt* cells / *wt proV* S4070, *rnc* cells / *wt proV* T100, *wt* cells / *proVC282G* T811, *rnc* cells / *proVC282G* T825.

2.2.4 RNase III initiates rapid degradation of *proU* mRNA after hypoosmotic stress

The *proU* mRNA is processed by RNase III. This processing is efficient under low osmolarity conditions and inhibited under high osmolarity conditions. I therefore hypothesized that the function of RNase III in *proU* osmoregulation is to initiate the fast degradation of the *proU* mRNA after decrease of osmolarity. To test this hypothesis the stability of *proU* mRNA was determined with real-time RT-PCR under high osmolarity conditions and upon application of hypoosmotic stress. Also the effect of RNase III processing on the *proU* mRNA stability under different osmolarity conditions was determined. The *proU* mRNA was expressed from the native chromosomal *proU* locus in *E. coli* K12 wild type strain BW30270 (S3839). In addition, RNase III processing was blocked with C282G mutation in *proU* locus. The C282G mutation was therefore introduced into the chromosomal *proU* locus, and the stability of wild type *proU* mRNA and *proUC282G* mutant was compared.

The protocol of introduction of C282G mutation was based on the protocols described in (Tischer *et al.*, 2006) and (Lee *et al.*, 2009). Details of this two-step protocol are provided in the methods section of this thesis. Briefly, a PCR product was generated that carries the *proUC282G* mutation flanked by wild type *proU* sequences followed by the endonuclease I-SceI cleavage site, a kanamycin marker and a second stretch of DNA encompassing the *proUC282G* mutation (Fig. 21). Amplification was carried out from the pKD4 plasmid with primers T524 and T525 that carry *proU* sequences and the I-SceI cleavage site. The PCR product was then introduced via λ-Red recombination into the target strain S3839. The λ-Red recombinase promoted the integration of kanamycin marker together with mutated *proU* sequences and I-SceI site into the chromosomal *proU* locus. In the second step, the kanamycin selection marker was recombined out of the chromosome via another round of λ-Red recombination. The strain obtained on the previous step was transformed with the pACBSCE plasmid expressing λ-Red recombinase and I-SceI endonuclease. I-SceI cuts the chromosome next to the kanamycin marker within the *proU* locus and λ-Red recombinase carries out the recombination between chromosomal *proUC282G* sequences resulting in the loss of the marker. Plasmid pACBSCE is simultaneously lost due to I-SceI cleavage. The resulted strain T1001 carried the single point mutation C282G in the chromosomal *proU* locus. Strain T1001, expressing *proUC282G* mRNA, and strain S3839, expressing wild type *proU* mRNA, were further used for the real-time RT-PCR experiment.

To collect RNA samples for the real-time RT-PCR experiment, strains T1001 (*proUC282G*) and S3839 (wild type *proU*) were grown in LB with 0.3M NaCl. When cultures reached OD_{600} of 0.5, cells were harvested by centrifugation and then resuspended in a small volume of the LB with 0.3M NaCl. Part of the suspension was transferred to LB with 0.3M NaCl again to a final OD_{600} of ~0.5. Another part of the suspension (the same volume) was transferred to LB containing no NaCl, so that the final concentration of NaCl in medium

became 0.01M (hypoosmotic stress). Immediately samples were taken and then rifampicin was added to a final concentration of 100 μg/ml to block transcription. Further samples were taken 30 seconds, 1 min, 2.5 min, 5 min and 10 min after rifampicin addition. Total RNA was isolated and 1 μg of RNA samples was used for cDNA synthesis with random hexamer primers. cDNA was then used for real-time RT-PCR. Two pairs of *proU* primers were used for amplification (Fig. 15A):

1) primers T520 and T521 to amplify *proU*(+171 to +338) region including the *proU*(+203 to +293) stem-loop structure with the RNase III processing site

2) primers T522 and T523 to amplify *proU*(+1071 to +1212) region (3′ end of *proV* gene sequence).

In addition 16S rRNA used as the internal reference gene was amplified with primers T528 and T529.

SYBR Green was added to amplification reactions to quantify the amount of double-stranded DNA. On each PCR plate, PCR reactions were performed with the samples taken from the cultures grown under high osmolarity conditions and after hypoosmotic stress, using one pair of primers. This allowed to directly compare the amount of the *proU* mRNA under different osmolarity conditions. All the cDNA samples were analyzed in triplicate. In addition, equal volumes of all cDNA samples used in PCR reactions on one plate were mixed together and used as series of dilutions to build the standard curves. These standard curves were then used to determine the relative quantity of *proU*(+1071 to +1212) mRNA, *proU*(+171 to +338) mRNA, and 16S rRNA in each sample. The relative quantity of *proU* RNAs was then normalized to the level of 16S rRNA. The normalized amount of RNA was plotted against the time after the rifampicin addition to build the degradation curves. In addition, the half-life of RNA was estimated using the formula for half-life in exponential decay (see methods section for the details).

As already mentioned, the degradation of *proU* mRNA was monitored using primers amplifying two parts of *proU*. The degradation data obtained for region +171 to +338 reflect the efficiency of RNase III processing. The half-life of the *proU* mRNA in this region under high osmolarity conditions was 65 seconds (Fig. 15B). The speed of *proU* mRNA degradation increased dramatically after the hypoosmotic stress: the half-life decreased to 4 seconds (Fig. 15B). Moreover, samples taken after hypoosmotic stress contained ten times less RNA then samples taken from high osmolarity medium (Fig. 15B). This demonstrates that the majority of RNA was already degraded during the first few seconds elapsed between the addition of cells to low osmolarity medium and taking the culture sample for the RNA purification.

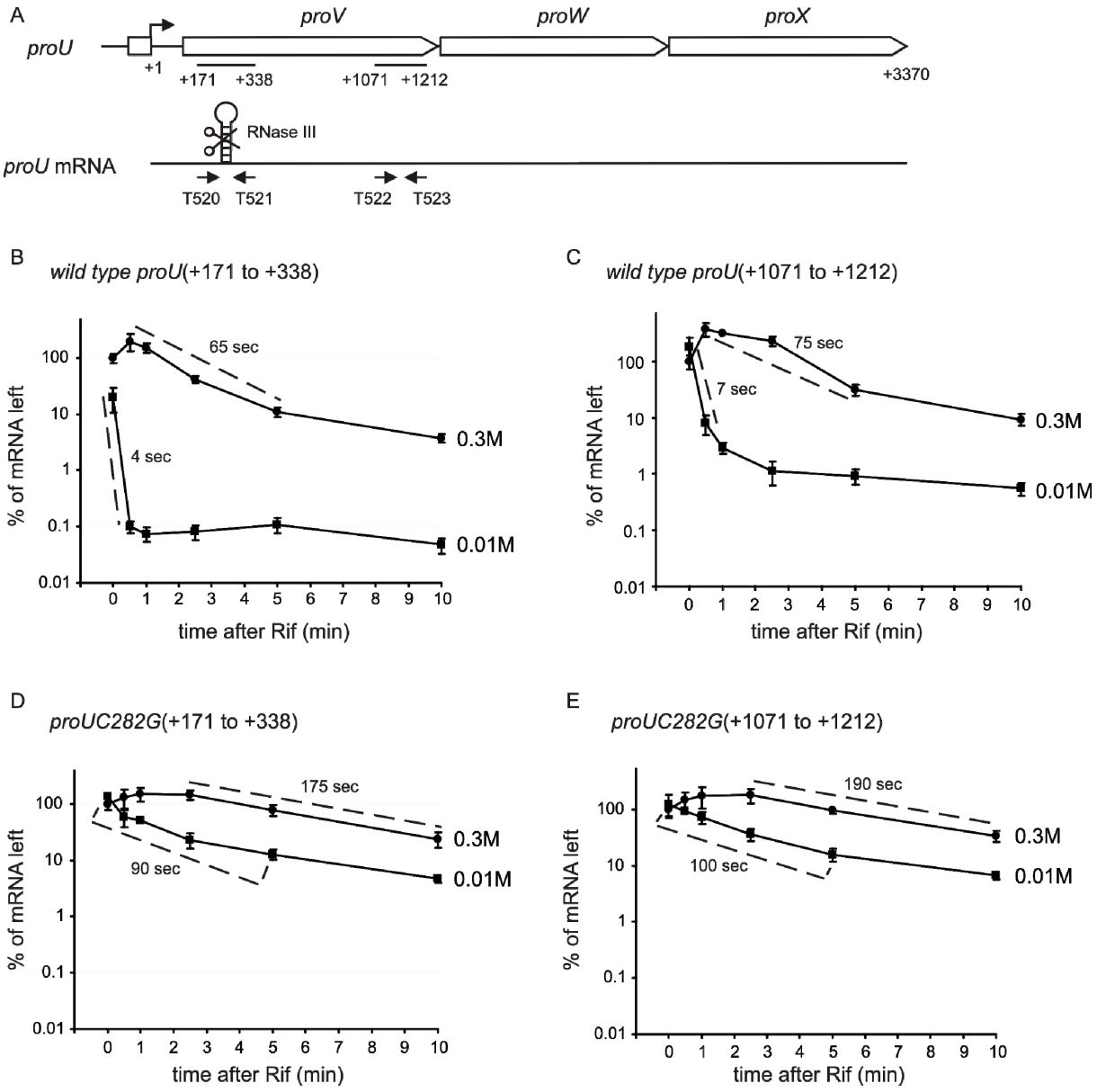


Fig. 15. RNase III initiates rapid degradation of *proU* mRNA after hypoosmotic stress. Stability of *proU* mRNA was determined with real-time RT-PCR. (A) Schematic representation of the *proU* locus and *proU* mRNA. Coordinates of the amplified regions relative to the transcription start site and primers used for this amplification are shown. (B) to (D). Stability of wild type *proU* mRNA (B, C) and *proUC282G* mRNA (D, E). The relative amount of RNA was determined with real-time RT-PCR as described in text. The relative amount of *proU* mRNA before rifampicin addition in cells in LB with 0.3M NaCl was taken as 100%. The amount of *proU* mRNA was plotted versus the time after transcription was blocked with rifampicin. Each plot represents the data obtained on one PCR plate with one pair of primers and therefore allows direct comparison of *proU* mRNA amount in samples taken from high osmolarity and low osmolarity medium. Dashed lines represent the part of degradation curve that was used for the estimation of RNA half-life in seconds (see methods section for details).

The blockage of RNase III processing by the C282G mutation lead to a dramatic increase of *proU* mRNA half-life in hypoosmotic stress conditions from 4 seconds to 90 seconds (Fig. 15D). The half-life under high osmolarity conditions was increased from 65 seconds to 175 seconds.

The dynamics of degradation of *proU*(+1071 to 1212) region was almost identical to the degradation of *proU*(+171 to +338) region (compare Fig. 15C vs B, and E vs D). Half-life of *proU* mRNA was only slightly increased in this more downstream region.

These results confirm the hypothesis that the function of RNase III processing is to initiate a rapid degradation of *proU* mRNA after hypoosmotic stress. RNase III processes *proU* mRNA in the upstream region and the rest of mRNA is probably rapidly degraded afterwards.

2.2.5 RNase III processing of minimal *proU* substrates

It was established in the above shown experiments that RNase III processing of *proU* mRNA is osmoregulated. There can be at least two general mechanisms of this osmoregulation:

1) RNase III processing itself might be inhibited under high osmolarity conditions, as it was proposed by Sim (2010);

2) initiation of *proU* mRNA translation might be more efficient under high osmolarity conditions and ribosomes might protect the *proU* mRNA from RNase III action under high osmolarity conditions.

Previous experiments showed that a *lacZ* reporter fusion including a *proV*(+1 to +303) fragment is 1.7-fold osmoregulated when expressed from *lacUV5* promoter in *hns* mutant cells (Madhusudan S., 2007). In contrast, a similar reporter with a *proV*(+1 to +93) fragment which does not include the stem-loop with RNase III processing site was not osmoregulated when expressed from *lacUV5* promoter in *hns* mutants (Madhusudan S., 2007). These results indicated that the translation initiation region alone is not enough for *proU* osmoregulation.

To further analyze the mechanism of osmoregulation of RNase III processing, a set of constructs carrying *proV*(+199 to +303), *proV*(+142 to +303) and *proV*(+64 to +1260*)* fragments was designed (Fig. 16). All *proU* fragments included the *proV*(+292 to +303) sequence coding for the stem-loop structure with RNase III processing site. The aim was to find out the minimal *proU* fragment required for RNase III processing and to determine whether the processing of this minimal substrate is osmoregulated. The *proV* fragments were fused to the *lacZ* reporter gene and *lacUV5* promoter (Fig. 16). Three different synthetic ribosome binding sites, designated SD145, SD205 and SD61, were used instead of the native *proU* ribosome binding site. These ribosome binding sites originate from the artificial *gfp* genes and ensure different levels of translation initiation efficiency (Kudla *et al.*, 2009). This excluded possible effects of the native *proU* ribosome binding site on RNase III processing and allowed to determine whether translation initiation efficiency affects processing. The final constructs were integrated into the chromosome of wild type and *rnc* mutant strains. The osmoregulation of the constructs was examined with β-galactosidase assay (Fig. 16). In addition, 5′ RACE analysis was used to study the RNase III processing of the constructs (Fig. 17).

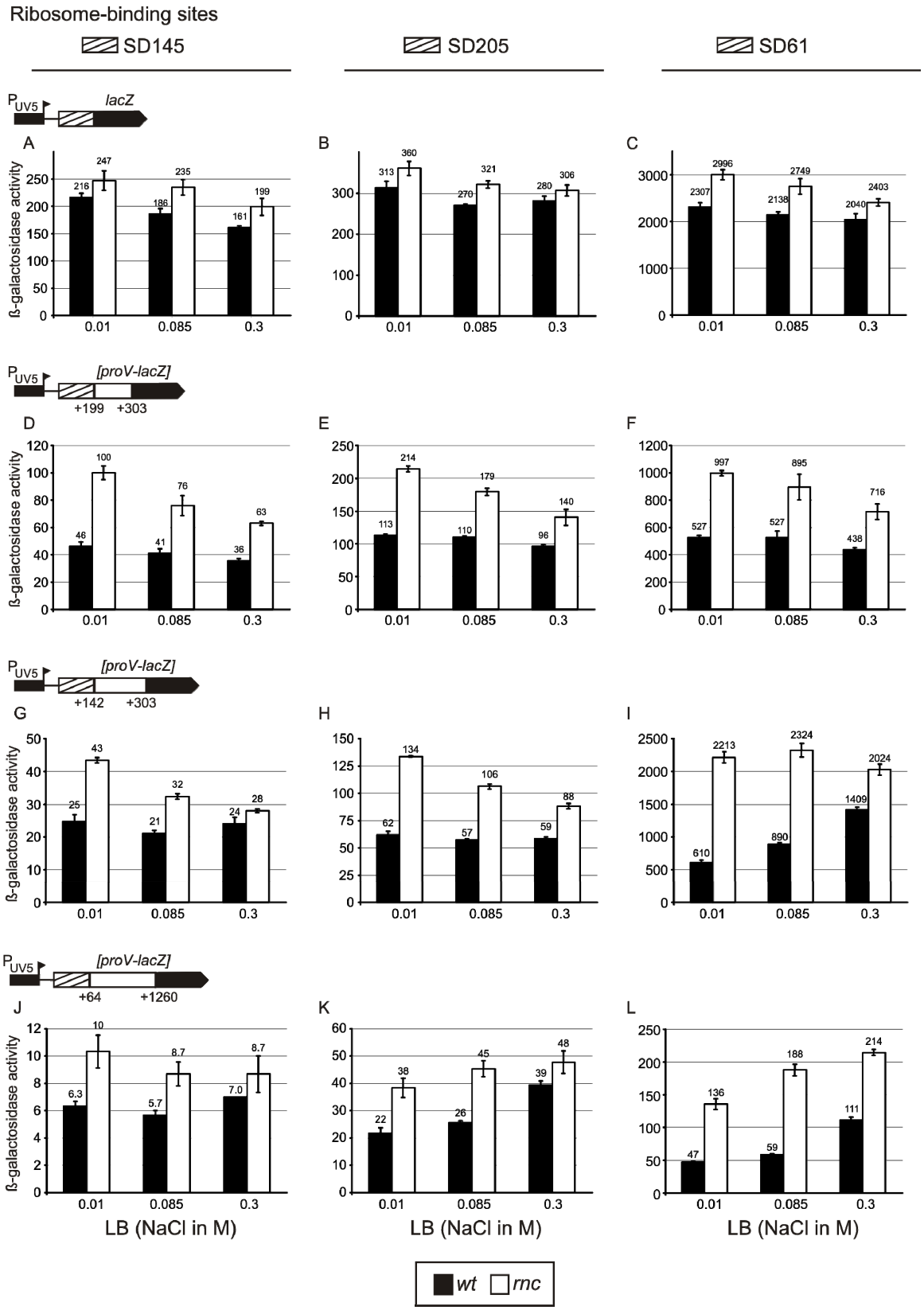


Figure 16. Osmoregulation of the *proV* fragments in wild type and *rnc* mutant cells. Expression of integrated in the chromosome translational fusions of *proV*(+199 to +303) (D, E, F*), proV*(+142 to +303) (G, H, I), *proV*(+64 to 1260) (J, K, L) fragments and *lacZ* gene, as well as *lacZ* gene alone (A, B, C) was determined in *wt* and *rnc* mutant cells. Expression is directed by *lacUV5* promoter and three synthetic ribosome binding sites (GFP145, GFP205, GFP61). Cells were grown in LB with indicated NaCl concentrations. Bars show the determined expression level (black bars for *wt* and white bars for *rnc* mutants respectively). Values are the mean of at least three calculations. Error bars represent standard error of the mean. Strains used: A, *wt* T613, *rnc* T681; B, *wt* T617, *rnc* T685; C, *wt* T615, *rnc* T683; D, *wt* T625, *rnc* T693; E, *wt* T629, *rnc* T697; F, *wt* T627, *rnc* T695; G, *wt* T619, *rnc* T687; H, *wt* T623, *rnc* T691; I, *wt* T621, *rnc* T689; J, *wt* T631, *rnc* T699; K, *wt* T635, *rnc* T703; L, *wt* T633, *rnc* T701.

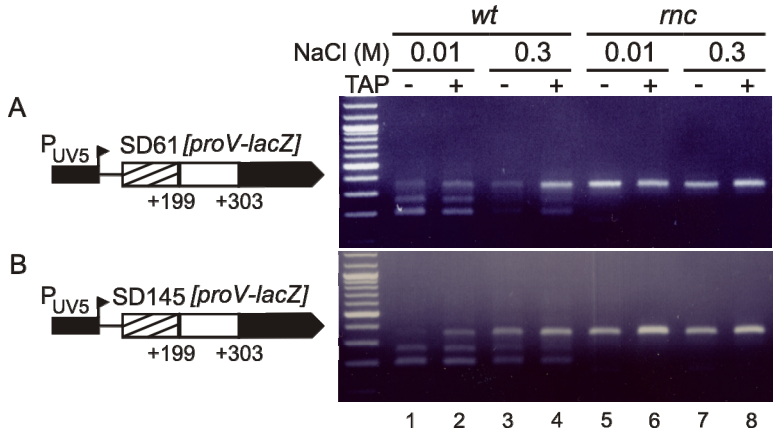


Figure 17. RNase III processing of the *pro*V(+199 to +303) mRNA fragment. 5′ RACE analysis of RNase III processing of *pro*V(+199...+303)-*lacZ* mRNA. Gel electrophoresis of PCR amplification products of cDNA is shown. For 5′RACE analysis, total RNA was extracted from *wt* and *rnc* mutant cells expressing chromosomally integrated *PlacUV5*-*pro*V(+199 to +303)-*lacZ* fusions carrying ribosome binding sites SD61 (A) or SD145 (B). Cells were grown in LB supplemented with either 0.01M or 0.3M of NaCl. RNA was extracted and further analyzed as described in the text. (+) and (-) refers to TAP-treatment prior to RNA adapter ligation. Strains used: A, *wt* T627, *rnc* T695; B, *wt* T625, *rnc* T693.

Control constructs without *proV* fragments demonstrated varying level of expression, with constructs carrying ribosome binding sites SD205 and SD61 expressing at 1.5-fold and 12-fold, respectively, higher levels than the SD145 construct (Fig. 16A-C). Processing of the constructs carrying the *proV*(+199 to +303) fragment and ribosome binding sites SD61 and SD145, respectively, was analyzed with 5' RACE (Fig. 17). Wild type and *rnc* mutant strains carrying these constructs in the chromosome were grown under low and high osmolarity conditions. Total RNA was extracted and one half part of the RNA samples was treated with TAP enzyme, prior to ligation of the RNA adapter, as described above. Purified RNA ligation products were used as a template for cDNA synthesis with primer S100 complementary to +270 to +292 region of the *lacZ* gene. The resulting cDNA was amplified by PCR with primer S118 complementary to +114 to +133 region of the *lacZ* gene and a 5'-adapter-specific primer T265. The PCR products were analyzed by agarose gel electrophoresis (Fig. 17). Three PCR products were observed in wild type cells. The size of the largest PCR product corresponded to the expected size of the primary transcript (347 bp). The size of two shorter PCR products corresponded to the expected size of RNase III processing products (260 bp and 194 bp). Only the PCR product corresponding to primary transcript was detected in *rnc* cells. Therefore the *proV*(+199 to +303) fragment is sufficient for RNase III processing.

More PCR product corresponding to the primary transcript and less PCR products corresponding to the processing products was detected under high osmolarity conditions than under low osmolarity conditions (Fig. 17). This indicates that RNase III processing of the *proV*(+199 to +303) fragment is osmoregulated. The difference in RNase III processing under different osmolarity conditions was, however, not as apparent as in the case of processing of the *proV*(+1 to 1260) mRNA (Fig. 6A).

The effect of *rnc* mutation on expression of the minimal *proU-lacZ* constructs was further analyzed with β-galactosidase assay (Fig. 16). The expression of the control constructs

without *proV* fragment was not significantly different in wild type and *rnc* mutant cells (Fig. 16A, B, and C). In contrast, constructs carrying the *proV*(+199 to +303) fragment were upregulated 1.9- to 2.2-fold under low osmolarity conditions and 1.5- to 1.8-fold under high osmolarity conditions in *rnc* cells (Fig. 16D, E, F). Therefore, only a minor difference in RNase III processing efficiency of *proV*(+199 to +303) mRNA under different osmolarity conditions was detected.

The *rnc* mutation had a similar effect on the expression of the *proV*(+199 to +303)-*lacZ* constructs carrying different ribosome binding sites (Fig. 16D, E, F). In agreement with this, 5' RACE showed that constructs with SD61 and SD145 ribosome binding sites were processed by RNase III with similar efficiency (Fig. 17). Therefore, no significant effect of the translation initiation efficiency on RNase III processing was observed.

Constructs with the longer *proV*(+142 to +303) and *proV*(+64 to +1260*)* fragments were also upregulated in *rnc* mutants in comparison to wild type cells, and this upregulation was more evident under low osmolarity conditions (Fig. 16G-L). These constructs were expressed generally at a lower level than the constructs with *proV*(+199 to +303) fragment. This might be explained by the fact that longer *proV* fragments are repressed more efficiently by H-NS, especially *proV*(+64 to +1260) fragment carrying +25 and +130 H-NS nucleation sites. Different efficiency of H-NS repression may also explain the varying degree of osmoinduction observed for different *proV* constructs.

To conclude, minimal *proV*(+199 to +303) fragment is sufficient for RNase III processing. However, some features of *proU* mRNA important for osmoregulation of processing may not be included in this minimal fragment. Translation initiation rate is not important for the osmoregulation of RNase III processing of *proU* mRNA.

2.3 RNA chaperones Hfq, StpA, ProQ and *proU* osmoregulation

The above experiments demonstrated that RNase III processing of the *proU* mRNA is osmoregulated and that this osmoregulation could not be explained by a variation of the translation initiation efficiency. One of the possible mechanisms of osmoregulation of RNase III processing is the involvement of RNA chaperones. RNA chaperones might potentially bind to *proU* mRNA and modulate the efficiency of RNase III processing in response to different osmolarity conditions. Indeed, RNA chaperone Hfq was shown to affect *proU* expression (Rajkumari and Gowrishankar, 2001; Madhusudan S., 2007). Also, the results of some studies suggested that RNA chaperones StpA (Sonden and Uhlin, 1996; Free *et al.*, 1998; Madhusudan S., 2007) and ProQ (J. Wood, personal communication) may affect *proU* expression. Therefore, osmoregulation of *proU* expression was studied in *hfq*, *stpA* and *proQ* mutant cells in the described below experiments.

2.3.1 Osmoregulation of *proU* in *hfq* mutant cells

Mutation of *hfq* leads to 1.7- to 7.3-fold downregulation of *proV-lacZ* fusions expression levels depending on osmolarity conditions (Fig. 18; also Madhusudan, 2007). This effect of Hfq on *proU* expression can be either direct or indirect. Hfq together with the sRNA DsrA blocks translation and lead to faster degradation of *hns* mRNA (Papenfort and Vogel, 2009). Mutation of *dsrA* leads to upregulation of *proU* in wild type but not in *hns* mutant cells (Lease *et al.*, 1998; Sledjeski and Gottesman, 1995), suggesting that DsrA affects *proU* expression indirectly by regulating H-NS. In this work I therefore tested whether Hfq affects *proU* indirectly via downregulation of H-NS together with DsrA.

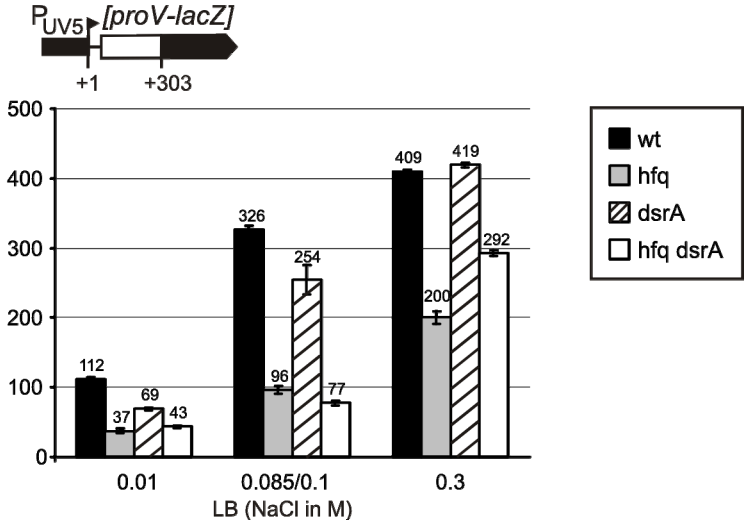


Figure 18. Osmoregulation of *proV* in *hfq* and *dsrA* mutant cells. Expression of translational *proV*(+1 to +303)*-lacZ* fusion from the *lacUV5* promoter was determined in the *wt* cells as well as in *hfq*, *dsrA* and double *hfq dsrA* mutants. Cells were grown in LB with indicated NaCl concentrations. Bars show the determined expression level (black bars for *wt*, grey bars for *hfq* mutant, hatched bars for *dsrA* mutant, and white bars for *hfq dsrA* double mutant). Values are the mean of at least three calculations. Error bars represent standard error of the mean. The expression values for *wt* and *hfq* mutants are taken from (Madhusudan, 2007). Strains used: *wt* S3252, *hfq* S3354, *dsrA* T399, *dsrA hfq* T445.

A mutant *dsrA* allele was introduced into the wild type and *hfq* mutant strains carrying chromosomally integrated translational *proV*(+1 to +303)-*lacZ* fusion under control of *lacUV5* promoter (the same construct was used by Madhusudan, 2007). Expression of this construct was measured with β-galactosidase assays under different osmolarity conditions. Mutation of *dsrA* lead to 1.6-fold downregulation of the reporter construct under low osmolarity conditions, 1.3-fold downregulation in a standard LB medium, and no *dsrA* effect was observed under high osmolarity conditions (Fig. 18). Hfq had stronger effect on *proV* expression under all conditions tested: *hfq* mutation lead to 3.0-fold downregulation of *proV* under low osmolarity conditions, 3.4-fold in a standard LB medium and 2.1-fold downregulation under high osmolarity conditions (Fig. 18). Expression in the double *dsrA hfq* mutant was at roughly the same level as in the *hfq* mutant. These data demonstrate that the Hfq effect on *proV* expression is mainly *dsrA*-independent. However it still remains to be investigated whether this Hfq effect is due to direct binding to *proU* mRNA. Under low osmolarity conditions Hfq probably also affects *proU* expression indirectly by repressing the H-NS expression together with DsrA.

2.3.2 Osmoregulation of *proU* in *stpA* mutant cells

Contradictory data regarding the effect of StpA on *proU* expression were reported previously (Free *et al.*, 1998; Sonden and Uhlin, 1996). Therefore it was tested again whether mutation of *stpA* has any effect on *proU* expression and osmoregulation, and whether it plays a role in post-transcriptional control by RNase III processing. Expression of wild type *proV* was compared in *hns* mutant and in *hns stpA* double mutant cells under different osmolarity conditions. Cells with deleted *hns* were used because *stpA* phenotypes can only be observed in an *hns* mutant background. Wild type *proV* fused with HA-tag was expressed from *lacUV5* promoter from the low copy plasmid pKEKK12. In addition, expression of the mutant *proVC282G*-HA from *lacUV5* promoter was analyzed in *hns* and *hns stpA* mutants. Western blot was used to detect ProV-HA expression under different osmolarity conditions.

The *stpA hns* double mutant cells were not able to grow in low osmolarity medium (LB with 0.01M NaCl) and high osmolarity medium (LB with 0.3M NaCl). Therefore, all cultures were first grown in a standard LB medium supplemented with 0.085M NaCl, then NaCl concentration was increased to 0.3M, and samples were taken immediately before and 10 minutes after hyperosmotic stress. Expression of neither wild type *proV* nor *proVC282G* were significantly affected by such an hyperosmotic stress (Fig. 19). This result was partially expected, because the expression of the corresponding *proV-lacZ* fusions in *hns* mutant cells was only 1.4- to 1.7-fold higher under high osmolarity conditions than in standard LB medium. Importantly, the mutation of *stpA* did not have any effect on expression of neither wild type *proV* nor mutant *proVC282G* under both osmolarity conditions tested.

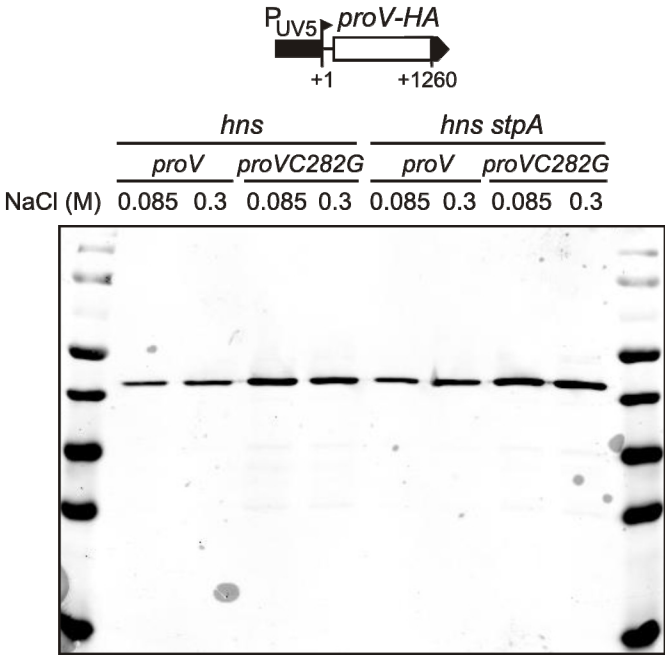


Figure 19. Osmoregulation of *proV* in *stpA* mutant cells. The *proV* fused with HA-tag sequence was expressed from *lacUV5* promoter from the plasmids pKEKK12 (wild type *proV*) and pKEKK38(*proVC282G*). *hns* (S4162) and *hns stpA* (T839) mutant cells carrying these plasmids were grown in LB supplemented with 0.085M NaCl (standard LB medium) to OD_{600} of 0.3, NaCl was added to the final concentration of 0.3M and samples were taken immediately before and 10 minutes after hyperosmotic stress. Total cell lysates were analyzed with antibodies to HA-tag.

2.3.3 Osmoregulation of *proU* in *proQ* mutant cells

ProQ is an RNA chaperone that affects the expression of the osmoprotectant transporter ProP (Chaulk *et al.*, 2011). Mutation of *proQ* normally leads to downregulation of *proP* expression. However, mutation of *proQ* does not affect *proP* expression in cells with the *proU* operon deleted, suggesting a possible interaction between *proQ* and *proU* (J. Wood, personal communication). It was therefore tested whether *proQ* mutation would affect *proU* expression and osmoregulation.

Expression of *proV*-HA fusion from *lacUV5* and *proU* promoters (using plasmids pKEKK11 and pKEKK12) was compared in wild type and *proQ* mutant cells under different osmolarity conditions. Western blot analysis was used to detect ProV-HA expression.

No ProV-HA expression from the native *proU* promoter was detected in wild type and the *proQ* mutant cells in low osmolarity medium and in standard LB medium (Fig. 20A). Expression of ProV-HA was strongly upregulated in high osmolarity medium and ten minutes after hyperosmotic stress. The expression of ProV-HA was, however, the same in wild type and *proQ* mutant cells. Similarly, a *proQ* mutation did not have any significant effect on ProV-HA expression directed by the *lacUV5* promoter under any osmolarity conditions tested (Fig. 20B). These data suggest that the RNA chaperone ProQ does not influence RNase III-based post-transcriptional osmoregulation of *proU*.

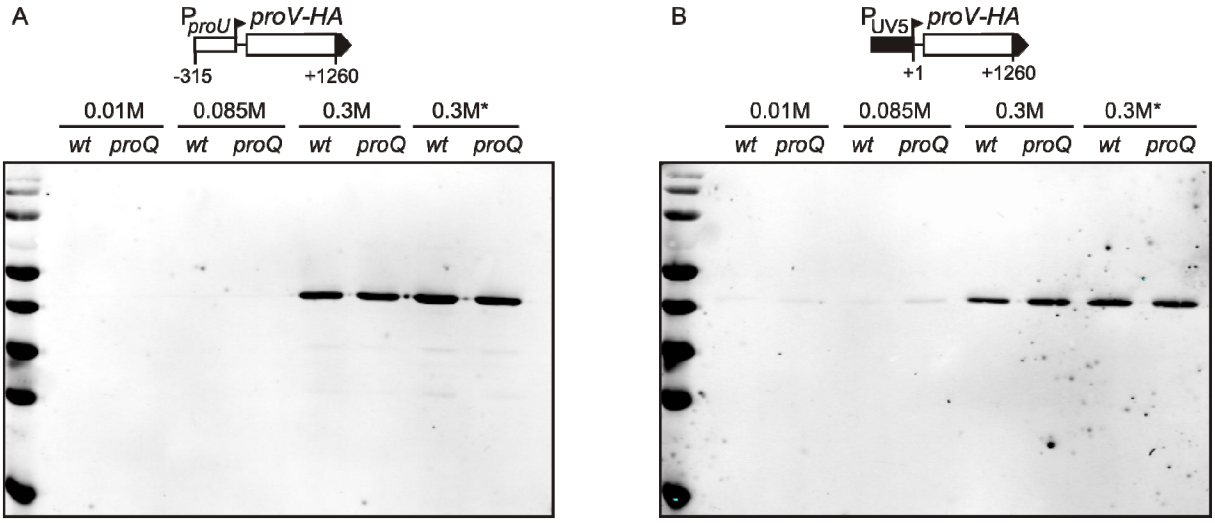


Figure 20. Osmoregulation of *proV* in *proQ* mutant cells. The *proV* fused with HA-tag sequence was expressed from *proU* promoter (plasmid pKEKK11, A) and *lacUV5* promoter (plasmid pKEKK12, B,) in *wt* (T948) and *proQ* mutant (T951) cells. Cells were constantly grown in LB supplemented with 0.085M or 0.3M NaCl to OD_{600} of 0.5 and samples were taken. Additionally, cells were constantly grown in LB supplemented with 0.01M NaCl to OD_{600} of 0.5, then NaCl concentration was increased to 0.3M and samples were taken immediately before (0.01M) and 10 minutes after hyperosmotic stress (0.3M*). Total cell lysates were analyzed with antibodies to HA-tag.

3. Discussion

The *E. coli proU* operon is osmoregulated at the transcriptional level (Gowrishankar and Manna, 1996). Previous analysis in the laboratory indicated that the *proU* operon might be also osmoregulated at the post-transcriptional level (Madhusudan, 2007). The presented study confirms that *proU* is osmoregulated at the post-transcriptional level and further demonstrates that post-transcriptional osmoregulation is based on osmoregulation of RNase III processing of the *proU* mRNA. RNase III processes the conserved stem-loop structure that is predicted to be formed in the upstream region of *proU* mRNA. RNase III processing is inhibited under high osmolarity conditions but activated under hypoosmotic stress conditions. The primary role of RNase III in *proU* osmoregulation seems to be the initiation of the rapid degradation of *proU* mRNA under hypoosmotic stress. The possible mechanism of RNase III processing of *proU* mRNA is discussed below. Translation efficiency did not have a major effect on RNase III processing. RNase III processing might be regulated by some osmostress-responsive protein inhibitor, similar to stress-responsive regulator YmdB, which directly binds to and inhibits RNase III. Alternatively, a protein binding to the *proU* mRNA might regulate RNase III processing. No effect of RNA chaperones ProQ and StpA on the *proU* expression or RNase III processing was detected. RNA chaperone Hfq affects *proU* expression and potentially can affect RNase III processing either independently or together with a small regulatory RNA. Further, predicted stem-loop structure formed by the *proU* mRNA and cleaved by RNase III might work as an osmosensor. In the end, the results obtained in this study are used to build a model of *proU* osmoregulation, integrating both transcriptional and post-transcriptional levels of osmoregulation.

3.1 Post-transcriptional osmoregulation of *proU* is based on osmoregulation of RNase III processing of the *proU* mRNA

Different mechanisms could be responsible for the post-transcriptional induction of *proU* by hyperosmotic stress. Osmoinduction at the post-transcriptional level could be due to increased *proU* mRNA stability, more efficient *proU* mRNA translation or increased stability of the ProV protein. Experiments showed that this post-transcriptional osmoinduction of *proU* cannot be explained by the increased stability of the ProV protein, because ProV is instead less stable under high osmolarity conditions (Fig. 5). Therefore it was next examined whether the *proU* mRNA stability is osmoregulated.

Analysis of *proU* mRNA processing with 5′ RACE showed that RNase III processes the *proU* mRNA at positions +217/+218 and +281/+282 relative to the transcription start site. (Fig. 6A, Fig. 10). These RNase III processing sites are located in a region of *proV* that is conserved in *Enterobacteriaceae* species (Table 1). The *proU* mRNA in this region forms a secondary structure that is also conserved (Fig. 7). This conservation indicated that RNase III

processing is important for *proU* osmoregulation. The results of 5′ RACE experiments suggested that RNase III processing of *proU* mRNA is osmoregulated (Fig. 6). Whereas both the primary transcript and the processing products were detected in cells grown under high osmolarity conditions, only the processing products were detected in cells grown under low osmolarity conditions (Fig. 6). I therefore tried to block the RNase III processing of *proU* mRNA and observe whether this blocking of processing would affect the osmoregulation of *proU*. First, RNase III processing was blocked by mutation of the *rnc* gene coding for RNase III. Experiments with *proV-lacZ* fusions showed that *proU* osmoregulation is diminished in *rnc* mutants in comparison to wild type cells (Fig. 8, Fig. 9). However, mutation of *rnc* was previously reported to have a non-specific effect on expression of several hundreds of genes in *E. coli* (Sim 2010). Similarly, in the *rnc* mutant I observed an indirect negative effect on expression of *lacZ* reporter expressed from the native *proU* promoter (Fig. 9A, B) and on expression of *proV-lacZ* fusions (Fig. 9C-F and Fig. 14). Therefore, I decided to block RNase III processing by mutation of the RNase III processing site on *proU* mRNA. Two mutations C282G and T279A that do not change the amino acid sequence of the ProV protein were introduced (Fig. 7). Both mutations completely blocked the processing of *proU* mRNA by RNase III (Fig. 10). Mutations of RNase III processing site lead to 1.3- to 1.9-fold upregulation of expression of *proV-lacZ* fusion under high osmolarity conditions and to the stronger 2.4- to 10-fold upregulation of expression under low osmolarity conditions (Fig. 12). Similarly, the T279A and C282G mutations lead to the upregulation of ProV-HA protein expression under low osmolarity conditions, whereas no significant upregulation under high osmolarity conditions was detected (Fig. 11). These results together with the results of 5′ RACE experiments showed that RNase III processing of *proU* mRNA is osmoregulated. RNase III processing is efficient under low osmolarity conditions and inhibited by hyperosmotic stress.

Interestingly, expression of ProV protein from *proVC282G* and *proVT279A* genes was not osmoregulated when expressed from *lacUV5* promoter in *hns* mutant cells, in contrast to expression from the wild type *proV* gene (Fig. 11). Likewise, fusions of *proVC282G* and *proVT279A* with *lacZ* reporter were not osmoregulated when expressed from *lacUV5* promoter in *hns* mutant cells, in contrast to wild type *proV-lacZ* fusions (Fig. 13D). These results showed that osmoregulation of *proU* at the post-transcriptional level is based on the osmoregulation of RNase III processing of *proU* mRNA.

3.2 The role of RNase III processing in *proU* osmoregulation

RNase III processing of *proU* mRNA is significantly more efficient under low osmolarity conditions then under high osmolarity conditions. This suggested that the role of RNase III processing is to quickly degrade *proU* mRNA after hypoosmotic stress. To test this hypothesis, I used real-time RT-PCR to determine the half-life of *proU* mRNA under high osmolarity conditions and after hypoosmotic stress. To determine the effect of RNase III processing on the *proU* mRNA stability, the degradation of both wild type *proU* mRNA and *proU* mRNA with C282G mutation were compared. Full-length *proU* and *proUC282G* mRNA were expressed from the native chromosomal locus.

The stability of *proU* mRNA in two regions were measured: the +171 to +338 region including the secondary structure cleaved by RNase III, and more downstream the +1071 to +1212 region located at the 3' end of the *proV* gene (Fig. 15). In the region including the RNase III processing site, the half-life of the *proU* mRNA was 65 seconds under high osmolarity conditions. The *proU* mRNA was almost instantly degraded after cells were subjected to hypoosmotic stress, with estimated half-life of less than 4 seconds. The mutation of RNase III processing site lead to stabilization of *proU* mRNA under high osmolarity conditions with a half-life of 175 seconds. More importantly, the half-life under hypoosmotic stress conditions was dramatically increased from 4 seconds to 90 seconds (Fig. 15). These results show that RNase III rapidly processes *proU* mRNA at hypoosmotic stress.

The degradation speed of *proU* mRNA more downstream at the +1071 to +1212 *proU* region was almost identical to that of the RNase III processing region. After hypoosmotic stress, the *proU* mRNA was quickly degraded with a half-life in this region of approximately 7 seconds. The mutation of RNase III processing site increased the half-life to roughly 100 seconds. This suggests that once RNase III processes the *proU* mRNA, the rest of the mRNA is then rapidly degraded by other RNases. Data obtained by Madhusudan (2007) indicate that the *proU* mRNA is processed by RNase E. Activity of RNase E is largely inhibited by the presence of 5′ terminal triphosphate, and activated when 5′ monophosphate is present instead (Mackie, 1998). Therefore RNase III processing in the upstream *proU* mRNA region might trigger RNase E processing by producing an RNA substrate with 5′ monophosphate.

To conclude, the presented results strongly suggest that the primary function of RNase III processing in *proU* osmoregulation is to initiate the rapid degradation of *proU* mRNA after the cells are subjected to hypoosmotic stress.

3.3 Possible mechanisms of osmoregulation of RNase III processing of *proU* mRNA

The mechanism of osmoregulation of RNase III processing of *proU* mRNA is not yet clear. Various mechanisms that can be potentially responsible for the osmoregulation of RNase III processing are discussed below.

RNase III activity was recently proposed to be inhibited under hyperosmotic stress conditions by some protein regulator (Sim *et al.*, 2010). Expression of *rnc*, *pnp*, and *bdm* genes are induced by hyperosmotic stress, and the corresponding mRNA transcripts are processed by RNase III (Bardwell *et al.*, 1989; Regnier and Hajnsdorf, 1991; Sim *et al.*, 2010). This lead Sim *et al.* (2010) to propose that the RNase III activity in the cell is inhibited by hyperosmotic stress. The authors further found that expression of YmdB, the cold-shock stress-responsive inhibitor of RNase III, is not affected by osmolarity conditions. They therefore suggested that RNase III activity might be inhibited during hyperosmotic stress by some uncharacterized protein regulator. Kim *et al.* (2008) who identified the cold-shock RNase III modulator YmdB reported that other putative RNase III regulators may exist, however, the data were never published.

The RNA stem-loop structure formed by the +203 to +293 region of *proU* mRNA might potentially function as an osmosensor. Some RNA structures function as riboswitches that change their conformation in response to the binding of small organic molecules or inorganic ions (Smith *et al.*, 2010). Also some RNA structures function as RNA thermometers or pH-responsive elements that change their conformation in response to temperature or pH change respectively (Narberhaus, 2010; Nechooshtan *et al.*, 2009). The conformational change leads to activation or inhibition of translation. Similarly, the *proU* stem-loop might potentially change the conformation in response to different osmolarity conditions that would lead to different efficiency of RNase III processing. I therefore tested whether the RNase III processing of *proU*(+199 to +303) stem-loop alone without other parts of *proU* mRNA would be osmoregulated. 5′ RACE experiment suggested that the processing of *proU*(+199 to +303) fragment is not as efficient as the processing of the longer *proU*(+1 to +1260) mRNA (compare Fig. 6 versus Fig. 17). This may be simply due to the different experimental setup. The *proU*(+199 to +303) mRNA was expressed from the chromosome as fusion with a long 3075 nt *lacZ* mRNA, in contrast to *proU*(+1 to +1260) mRNA that was expressed from the plasmid together with the short HA-tag sequence. The results of this 5′ RACE experiment also indicated that the processing of this *proU*(+199 to +303) fragment is still osmoregulated (Fig. 16). The results of β-galactosidase assays with the *proU*(+199 to +303)-*lacZ* reporter were inconclusive (Fig. 16D, E, F). While the expression of reporters was upregulated in *rnc* mutant cells, the degree of upregulation was quite similar under different osmolarity conditions (Fig. 16D, E, F). The more direct way to determine whether *proU* stem-loop structure functions as osmosensor is to perform *in vitro* or *in vivo* structure probing

experiments under different osmolarity conditions. This might reveal whether the stem-loop formed by *proU* mRNA indeed changes the conformation in response to osmotic stresses.

Processing of some mRNAs by RNases is known to be affected by translation (Deana and Belasco, 2005; Kaberdin and Blasi, 2006). It is therefore conceivable that RNase III processing itself is not osmoregulated, but instead *proU* mRNA translation is osmoregulated. The higher translation initiation rate could lead the ribosomes to protect the *proU* mRNA from RNase III processing under hyperosmotic stress conditions. In this work, I tested the effect of translation initiation efficiency on the RNase III processing of *proU* mRNA. In the above mentioned experiments with the short *proU*(+199 to +303) fragment, the native *proU* ribosome binding site was substituted by artificial ribosome binding sites (Fig. 16 and 17). These artificial ribosome binding sites provided different efficiency of translation initiation. Translation initiation from the strongest SD61 site is twelve-times more efficient then from the weakest SD145 site (Fig. 16 A, B, C). 5′ RACE experiment showed that the *proU* mRNA is still processed by RNase III even when translation is directed by the strongest ribosome binding site (Fig. 17). Similarly no clear effect of translation initiation efficiency on RNase III processing was detected with *proU*(+199 to +303)-*lacZ* fusions (Fig. 16D, E, F). In addition, previous experiments showed that a short *proU*(+1 to +93) fragment containing the *proU* ribosome binding site was not able to ensure osmoregulation of translationally fused *lacZ* reporter in the absence of osmoregulated *proU* promoter and H-NS repression (Madhusudan S., 2007). To conclude, it seems unlikely that the translation efficiency has a significant effect on osmoregulation of RNase III processing of the *proU* mRNA.

Another potential mechanism of osmoregulation of RNase III processing of *proU* mRNA is the modulation of the processing by proteins binding to *proU* mRNA. In this work I tested the effect of RNA chaperones StpA, ProQ, and Hfq on *proU* expression. Mutation of the *stpA* gene did not have any significant effect on expression of neither wild type *proV* gene nor the *proVC282G* mutant, when these genes where expressed from the constitutive *lacUV5* promoter (Fig. 19). Likewise, mutation of *proQ* gene did not affect the expression of wild type *proV* from neither the constitutive *lacUV5* promoter nor the native *proU* promoter under the different osmolarity conditions tested (Fig. 20). Mutation of *hfq* gene was previously reported to downregulate *proU* expression (Madhusudan S., 2007). Here I tested whether this effect is indirect due to inhibition of H-NS expression by Hfq together with sRNA DsrA. Hfq effect on *proU* was only partially DsrA-dependent under low osmolarity conditions and completely DsrA-independent under high osmolarity conditions (Fig. 18). It is therefore possible that Hfq affects RNase III processing by direct binding to *proU* mRNA. This, however, remains to be tested experimentally. Alternatively, the fact that Hfq affects *proU* expression suggests that RNase III processing of *proU* mRNA might be modulated by some stress-responsive sRNA. Hfq is the primary RNA chaperone which facilitates the action of many trans-acting sRNAs . The activity of some sRNAs is reported to be affected by osmotic stress. RprA is involved in induction of *rpoS* expression by osmotic stress (Majdalani *et al.*, 2001). Expression of MicF, the regulator of outer membrane porins OmpF and OmpC, is

induced by some environmental stresses including hyperosmotic stress (Delihas and Forst, 2001). I searched the sRNAMap database of *E. coli* small RNAs (Huang *et al.*, 2009; http://srnamap.mbc.nctu.edu.tw) for possible sRNA candidates that have homology to the *proU* sequence around the RNase III processing site. No sRNA candidate with high homology to *proU* sequence was found (data not shown). This outcome is not surprising, because most trans-encoded sRNAs have only limited homology with their mRNA targets and purely bioinformatic search for sRNA candidates is therefore problematic. Potential sRNA candidates might be found by screening known sRNAs or using a genome library.

3.4 The integrative model of *proU* osmoregulation

Osmoregulation of *proU* at the transcriptional level was intensively studied previously (for review, see Gowrishankar and Manna, 1996; Lucht and Bremer, 1994). The data presented in this work add an additional level of *proU* osmoregulation. A model integrating both transcriptional and post-transcriptional osmoregulation of *proU* is presented in Figure 21.

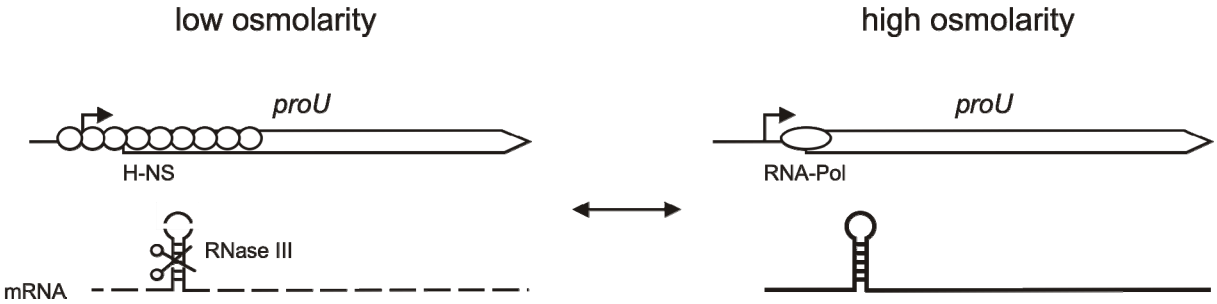


Fig. 21. Integrative model of *proU* osmoregulation. Under low osmolarity conditions, activity of the promoter (bent arrow) is low and transcription is completely blocked by H-NS (ovals). The minor amount of mRNA that is still transcribed is processed by RNase III and rapidly degraded. Under high osmolarity conditions, activity of the promoter is increased, H-NS does not repress transcription. This leads to the production of a large amount of mRNA transcript. In addition, RNase III processing is inhibited by hyperosmotic stress that leads to the stabilization of the mRNA transcript. Hypoosmotic stress leads to repression of transcription by H-NS and rapid degradation of *proU* mRNA initiated by RNase III.

Under low osmolarity conditions the activity of the *proU* promoter is low and transcription initiation is blocked by H-NS. H-NS binds to DNA more efficiently under low osmolarity conditions *in vitro* (Amit *et al.*, 2003). Also H-NS repression of *proU* is more efficient under low osmolarity conditions even when transcription is directed by the constitutive *lacUV5* promoter (Fig. 3C and D). These results suggest that H-NS binding to DNA within the *proU* locus is stronger under low osmolarity conditions. In addition, H-NS repression is affected by transcription rate (Nagarajavel et al., 2007). Under low osmolarity conditions, RNA polymerase cannot displace H-NS molecules because of the lower promoter activity and concomitantly lower transcription rates (Nagarajavel et al., 2007). As a result, H-NS efficiently represses transcription. If any mRNA is still produced by transcription, it is immediately processed by RNase III and then rapidly degraded by other RNases. Together

transcriptional repression and RNase III processing ensure complete shutdown of *proU* expression under low osmolarity conditions.

Under high osmolarity conditions, H-NS binds to DNA more weakly. In addition, the *proU* promoter activity is increased and RNA polymerase now can displace H-NS from DNA as the transcription rate is higher. As a result, *proU* transcription is completely derepressed. Hyperosmotic stress also inhibits RNase III processing of *proU* mRNA and the stability of *proU* mRNA dramatically increases. All these events result in rapid increase in expression of the ProVWX proteins after hyperosmotic stress.

When osmolarity in the external environment drops, *proU* expression is shut down quickly. Promoter activity decreases and H-NS represses transcription. RNase III activity increases and *proU* mRNA is rapidly degraded. RNase III processing ensures that the cell does not waste resources on production of ProVWX proteins when they are not required and protects the cell from excessive accumulation of solutes under hypoosmotic stress.

Whereas data presented here were obtained using *E. coli*, the *proU* mRNA might be similarly processed by RNase III in an osmosensitive manner in other bacterial species. Indeed, the *proU* sequence around the RNase III processing site and the putative secondary structure of the *proU* mRNA in this region are conserved in *Enterobacteriaceae*. This conservation suggests that the RNase III might be also involved in *proU* osmoregulation in other enterobacterial species.

4. Materials and Methods

4.1 Media and antibiotics

LB medium (for 1000 ml volume): 10 g of bacto trypton, 5 g of yeast extract, and various concentrations of NaCl: 5 g for normal LB (final concentration 0.085M), 0.585 g for low osmolarity media (final concentration 0.01M), 17.55 g for high osmolarity media (final concentration 0.3M). For plates 15 g of bacto agar was added.

SOB medium (for 1000 ml): 20 g of bacto tryptone, 5 g of bacto yeast extract, 0.5 g of NaCl, 1.25 ml of 2 M KCl. pH adjusted to 7.0 with NaOH. After autoclaving 10 ml of 1M $MgCl_2$ per liter added.

SOC medium: 19.8 ml of 20% Glucose added to 1000 ml of SOB.

X-gal was used at final concentration 40 μg/ml.

Antibiotics were used at the following concentrations: ampicillin 50 μg/ml, chloramphenicol 15 μg/ml, kanamycin 10 μg/ml or 25 μg/ml, rifampicin 100 μg/ml, spectinomycin 50 μg/ml, tetracyclin 12 μg/ml.

4.2 Standard molecular biology techniques

Standard molecular biology techniques like restriction enzyme digestions, ligations and other enzymatic reactions, PCR, plasmid purification were performed as described in (Sambrook and Russell, 2001) or according to the manufacturer's instructions.

4.3 Plasmids

Large scale preparations of plasmid DNAs were performed using the plasmid maxiprep or midiprep kits (Promega) according to the manufacturer's instructions. A list of plasmids used in the study with a relevant description is given in the Table 2. Details of the plasmid constructions are documented in the lab records and sequences are compiled in Vector NTI (Invitrogen). Plasmids used for ProV-HA-tag expression for Western blot and 5' RACE analysis are low copy plasmids that carry p15A origin of replication (except for pKEM78 high copy number plasmid that caries pBR322 *ori* region). Plasmids used for the integration of *proV-lacZ* reporter constructs in chromosomal *attB* site (Diederich et al., 1992) carry p15A *ori* region, kanamycin and spectinomycin resistance genes and *attP* attachment site; pLDR8 was used as a helper plasmid expressing integrase. Plasmids pCP20, pKD3, pKD4, pKD46 were used for chromosomal gene inactivation, as described (Datsenko and Wanner, 2000). Integration of reporter constructs in chromosome and gene inactivation are described below. *PproU* is a native *proU* promoter region including *proU*(-315 to +1) sequence (i.e. the sequence from position -315 to the transcription start site). Translational fusions of *proV* gene and *lacZ* reporter gene are designated with the symbol φ [for example, φ(*proV-HA-lacZ*)] to distinguish them from transcriptional *proV-lacZ* fusions. Symbol ts means that replication of plasmid is thermosensitive.

Table 2. Plasmids used in this study

Name	Relevant structure and description	Source
pKEKK5	PlacUV5-proV-HA-lacZ, KmR, SpecR, P15Aori	This study
pKEKK6	PproU-proV-HA-lacZ, KmR, SpecR, P15Aori	This study
pKEKK7	PproU-φ(proV-HA-lacZ), KmR, SpecR, P15Aori	This study
pKEKK8	PlacUV5-φ(proV-HA-lacZ), KmR, SpecR, P15Aori	This study
pKEKK11	PproU-proV-HA, KmR, P15Aori	This study
pKEKK12	PlacUV5-proV-HA, KmR, P15Aori	This study
pKEKK21	PlacUV5-GFP145-lacZ, SpecR, KmR, P15Aori	This study
pKEKK22	PlacUV5-GFP61-lacZ, SpecR, KmR, P15Aori	This study
pKEKK23	PlacUV5-GFP205-lacZ, SpecR, KmR, P15Aori	This study
pKEKK24	PlacUV5-GFP145-proV(+142 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK25	PlacUV5-GFP61-proV(+142 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK26	PlacUV5-GFP205-proV(+142 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK27	PlacUV5-GFP145-proV(+199 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK28	PlacUV5-GFP61-proV(+199 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK29	PlacUV5-GFP205-proV(+199 to +303)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK30	PlacUV5-GFP145-proV(+64 to +1260)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK31	PlacUV5-GFP61-proV(+64 to +1260)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK32	PlacUV5-GFP205-proV(+64 to +1260)-lacZ, SpecR, KmR, P15Aori	This study
pKEKK33	PlacUV5-φ(proVT279A-HA-lacZ), SpecR, KmR, P15Aori	This study
pKEKK34	PlacUV5-φ(proVC282G-HA-lacZ), SpecR, KmR, P15Aori	This study
pKEKK35	PlacUV5-proVT279A-HA-lacZ, SpecR, KmR, P15ori	This study
pKEKK36	PlacUV5-proVT279A-HA, KmR, P15Aori	This study
pKEKK37	PlacUV5-proVC282G-HA-lacZ, SpecR, KmR, P15Aori	This study
pKEKK38	PlacUV5-proVC282G-HA, KmR, P15Aori	This study
pKEKK39	PproU-φ(proVT279A-HA-lacZ), SpecR, KmR, P15Aori	This study
pKEKK40	PproU-φ(proVC282G-HA-lacZ), SpecR, KmR, P15Aori	This study
pKEKK41	PproU-proVT279A-HA-lacZ, SpecR, KmR, P15Aori	This study
pKEKK42	PproU-proVC282G-HA-lacZ, SpecR, KmR, P15Aori	This study
pKEM78	PlacUV5-proV-HA, ApR, pBRori	Lab collection
pLDR8	λ integrase, λ repressor cI857-ts, KmR, pSC101ori-ts	(Diederich et al., 1992)
pUC12	lacZα-peptide, AmpR, pBRori	Standard cloning vector
pCP20	FLP recombinase (thermal induction), AmpR, ori-ts	(Datsenko and Wanner, 2000)
pKD3	template plasmid for gene deletion, FRT-flanked CmR	(Datsenko and Wanner, 2000)
pKD4	template plasmid for gene deletion, FRT-flanked KmR	(Datsenko and Wanner, 2000)
pKD46	λ-Red recombinase, AmpR, repA101-ts	(Datsenko and Wanner, 2000)
pACBSCE	λ-Red recombinase, endonuclease I-Sce-I, I-SceI site, CmR	(Lee *et al.*, 2009)

4.4 Bacterial strains

The bacterial strains used in this study and their description are listed in Table 3. Strains are derivatives of CSH50 (Miller, 1972), N3433 (CGSC# 6976, *E. coli* genetic stock center maintained by Molecular, Cellular and Development Biology Department, Yale University, New Haven, Connecticut) or BW30270 (MG1655 rph+, CGSC # 7925). The relevant genotype of the strains refers to the *proU*, *bgl*, *lac*, *hns*, *hfq*, *dsrA*, *rnc*, *proQ* and *stpA* loci. Construction of strains was performed as described previously by the integration of *lacZ* fusions into the *attB* site of chromosome (Diederich et al., 1992), inactivation of chromosomal genes (Datsenko and Wanner, 2000) or by transduction with T4GT7 (Wilson *et al.*, 1979); all the construction techniques are also described below. *rnc* mutants were constructed by co-transduction of mutant *rnc105* allele together with *nadB51::Tn10(tet)* marker from the strain S3701 (N3433 *rnc105 nadB51::Tn10(tet)*). *rnc105* allele carries point mutation in *rnc* resulting in G44D substitution and inactivation of RNase III (Regnier and Hajnsdorf, 1991).

Table 3. *E. coli* K-12 strains used in this study

Strain	Relevant genotype	Source
N3433	CGSC# 6976, wild type isogenic to N3431 (rne-3071)	CGSC# 6976
S159	M182 stpA::tet	(Zhang et al., 1996)
S541	CSH50 Δbgl-AC11 ΔlacZ	(Dole et al., 2002)
S1979	S541 attB::[SpecR PlacUV5-lacO-lacZ]	Lab collection
S2048	S541 attB::[SpecR PproU-lacZ]	(Nagarajavel *et al.*, 2007)
S2212	(DDS1201) MG1655 dsrA1::cm	(Sledjeski and Gottesman, 1995)
S2608	S541 attB::[SpecR PproU-φ(proV(+1 to +303)-lacZ)]	Lab collection
S2979	S541 attB::[SpecR PproU-φ(proV(+1 to +303)-lacZ)] hfq1::km	Lab collection
S3010	S541 Δhns::kmKD4	(Nagarajavel *et al.*, 2007)
S3066	S541 ΔproU::cmKD3	Lab collection
S3077	S541 ΔproUfrt	Lab collection
S3122	S541 attB::[SpecR PlacUV5-lacO-lacZ] Δhns::kmKD4	(Nagarajavel *et al.*, 2007)
S3124	S541 attB::[SpecR PproU-lacZ] Δhns::kmKD4	(Nagarajavel *et al.*, 2007)
S3252	S541 attB::[SpecR PlacUV5-φ(proV(+1to+303)-lacZ)]	Lab collection
S3354	S541 attB::[SpecR PlacUV5-φ(proV(+1to+303)-lacZ)]	Lab collection
S3460	N3433 ΔproU::cmKD3	Lab collection
S3701	(IBPC633) N3433 rnc105 nadB51::Tn10(tet)	(Regnier and Hajnsdorf, 1991)
S3839	(BW30270) MG1655 rph+	CGSC # 7925
S4007	N3433 ΔproU::cmKD3 Δhns::kmKD4	S3460 x T4GT7 (S3010)
S4023	N3433 ΔproU::cmKD3 rnc105 nadB51::Tn10(tet)	S3460 x T4GT7 (S3701)
S4064	S541 ΔproUfrt attB::[SpecR PlacUV5-proV-HA-lacZ]	S3077 x pKEKK5
S4066	S541 ΔproUfrt attB::[SpecR PproU-proV-HA-lacZ]	S3077 x pKEKK6
S4068	S541 ΔproUfrt attB::[SpecR PproU-φ(proV-HA-lacZ)]	S3077 x pKEKK7
S4070	S541 ΔproUfrt attB::[SpecR PlacUV5-φ(proV-HA-lacZ)]	S3077 x pKEKK8
S4111	S541 ΔproUfrt attB::[SpecR PlacUV5-proV-HA-lacZ] Δhns::kmKD4	S4064 x T4GT7 (S3010)

Table 3. *E. coli* K-12 strains used in this study

Strain	Relevant genotype	Source
S4113	S541 ΔproUfrt attB::[SpecR PproU-proV-HA-lacZ] Δhns::kmKD4	S4066 x T4GT7 (S3010)
S4115	S541 ΔproUfrt attB::[SpecR PproU-φ(proV-HA-lacZ)] Δhns::kmKD4	S4068 x T4GT7 (S3010)
S4117	S541 ΔproUfrt attB::[SpecR PlacUV5-φ(proV-HA-lacZ)] Δhns::kmKD4	S4070 x T4GT7 (S3010)
S4162	N3433 ΔproUfrt Δhnsfrt	S4007/pCP20
T90	S541 ΔproUfrt attB::[SpecR PproU-proV-HA-lacZ] rnc105	S4066 x T4GT7 (S3701)
T92	S541 ΔproUfrt attB::[SpecR PproU-proV-HA-lacZ] Δhns::kmKD4 rnc105	S4113 x T4GT7 (S3701)
T94	S541 ΔproUfrt attB::[SpecR PproU-φ(proV-HA-lacZ)] rnc105	S4068 x T4GT7 (S3701)
T95	S541 ΔproUfrt attB::[SpecR PproU-φ(proV-HA-lacZ)] Δhns::kmKD4 rnc105	S4115 x T4GT7 (S3701)
T97	S541 ΔproUfrt attB::[SpecR PlacUV5-proV-HA-lacZ] rnc105	S4064 x T4GT7 (S3701)
T99	S541 ΔproUfrt attB::[SpecR PlacUV5-proV-HA-lacZ] Δhns::kmKD4 rnc105	S4111 x T4GT7 (S3701)
T100	S541 ΔproUfrt attB::[SpecR PlacUV5-φ(proV-HA-lacZ)] rnc105	S4070 x T4GT7 (S3701)
T102	S541 ΔproUfrt attB::[SpecR PlacUV5-φ(proV-HA-lacZ)] Δhns::kmKD4 rnc105	S4117 x T4GT7 (S3701)
T214	S541 attB::[SpecR PlacUV5-lacO-lacZ] rnc105	S1979 x T4GT7 (S3701)
T216	S541 attB::[SpecR PlacUV5-lacO-lacZ] Δhns::kmKD4 rnc105	S3122 x T4GT7 (S3701)
T380	S541 attB::[SpecR PproU-lacZ] rnc105	S2048 x T4GT7 (S3701)
T382	S541 attB::[SpecR PproU-lacZ] Δhns::kmKD4 rnc105	S3124 x T4GT7 (S3701)
T397	S541 attB::[SpecR PproU-φ(proV(+1 to +303)-lacZ)] dsrA1::cm	S2608 x T4GT7 (S2212)
T399	S541 attB::[SpecR PlacUV5- φ(proV(+1 to +303)-lacZ)] dsrA1::cm	S3252 x T4GT7 (S2212)
T443	S541 attB::[SpecR PproU-φ(proV(+1 to +303 lacZ)] dsrA1::cm hfq1::km	T397 x T4GT7 (S812)
T445	S541 attB::[SpecR PlacUV5-φ(proV(+1to +303)-lacZ] dsrA1::cm hfq1::km	T397 x T4GT7 (S812)
T613	S541 ΔproU attB::[SpecR PlacUV5-GFP145-lacZ]	S3077 x pKEKK21
T615	S541 ΔproU attB::[SpecR PlacUV5-GFP61-lacZ]	S3077 x pKEKK22
T617	S541 ΔproU attB::[SpecR PlacUV5-GFP205-lacZ]	S3077 x pKEKK23
T619	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+142 to +303)-lacZ]	S3077 x pKEKK24
T621	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+142 to +303)-lacZ]	S3077 x pKEKK25
T623	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+142 to +303)-lacZ]	S3077 x pKEKK26
T625	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+199 to +303)-lacZ]	S3077 x pKEKK27
T627	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+199 to +303)-lacZ]	S3077 x pKEKK28
T629	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+199 to +303)-lacZ]	S3077 x pKEKK29
T631	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+64 to +1260)-lacZ]	S3077 x pKEKK30
T633	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+64 to +1260)-lacZ]	S3077 x pKEKK31
T635	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+64 to +1260)-lacZ]	S3077 x pKEKK32
T681	S541 ΔproU attB::[SpecR PlacUV5-GFP145-lacZ] rnc105	T613 x T4GT7 (S3701)
T683	S541 ΔproU attB::[SpecR PlacUV5-GFP61-lacZ] rnc105	T615 x T4GT7 (S3701)
T685	S541 ΔproU attB::[SpecR PlacUV5-GFP205-lacZ] rnc105	T617 x T4GT7 (S3701)
T687	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+142 to +303)-lacZ] rnc105	T619 x T4GT7 (S3701)
T689	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+142 to +303)-lacZ] rnc105	T621 x T4GT7 (S3701)
T691	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+142 to +303)-lacZ] rnc105	T623 x T4GT7 (S3701)

Table 3. *E. coli* K-12 strains used in this study

Strain	Relevant genotype	Source
T693	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+199 to +303)-lacZ] rnc105	T625 x T4GT7 (S3701)
T695	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+199 to +303)-lacZ] rnc105	T627 x T4GT7 (S3701)
T697	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+199 to +303)-lacZ] rnc105	T629 x T4GT7 (S3701)
T699	S541 ΔproU attB::[SpecR PlacUV5-GFP145-proV(+64 to +1260)-lacZ] rnc105	T631 x T4GT7 (S3701)
T701	S541 ΔproU attB::[SpecR PlacUV5-GFP61-proV(+64 to +1260)-lacZ] rnc105	T633 x T4GT7 (S3701)
T703	S541 ΔproU attB::[SpecR PlacUV5-GFP205-proV(+64 to +1260)-lacZ] rnc105	T635 x T4GT7 (S3701)
T809	S541 ΔproU attB::[SpecR PlacUV5-proVC282G-HA-lacZ]	S3077 x pKEKK37
T811	S541 ΔproU attB::[SpecR PlacUV5-φ(proVC282G-HA-lacZ)]	S3077 x pKEKK34
T823	S541 ΔproU attB::[SpecR PlacUV5-proVC282G-HA-lacZ] rnc105	T809 x T4GT7 (S3701)
T825	S541 ΔproU attB::[SpecR PlacUV5-φ(proVC282G-HA-lacZ)] rnc105	T811 x T4GT7 (S3701)
T831	S541 ΔproU attB::[SpecR PlacUV5-proVC282G-HA-lacZ] Δhns::kmKD4	T809 x T4GT7 (S3010)
T833	S541 ΔproU attB::[SpecR PlacUV5-φ(proVC282G-HA-lacZ)] Δhns::kmKD4	T811 x T4GT7 (S3010)
T839	N3433 ΔproUfrt Δhnsfrt stpA::tet	S4162 x T4GT7 (S159)
T879	S541 ΔproU attB::[SpecR PproU-φ(proVT279A-HA-lacZ)]	S3077 x pKEKK39
T881	S541 ΔproU attB::[SpecR PproU-φ(proVC282G-HA-lacZ)]	S3077 x pKEKK40
T883	S541 ΔproU attB::[SpecR PproU-proVT279A-HA-lacZ]	S3077 x pKEKK41
T885	S541 ΔproU attB::[SpecR PproU-proVC282G-HA-lacZ]	S3077 x pKEKK42
T887	S541 ΔproU attB::[SpecR PlacUV5-φ(proVT279A-HA-lacZ)]	S3077 x pKEKK33
T889	S541 ΔproU attB::[SpecR PlacUV5-proVT279A-HA-lacZ]	S3077 x pKEKK35
T891	S541 ΔproU attB::[SpecR PproU-φ(proVT279A-HA-lacZ)] Δhns::kmKD4	T879 x T4GT7 (S3010)
T893	S541 ΔproU attB::[SpecR PproU-φ(proVC282G-HA-lacZ)] Δhns::kmKD4	T881 x T4GT7 (S3010)
T895	S541 ΔproU attB::[SpecR PproU-proVT279A-HA-lacZ] Δhns::kmKD4	T883 x T4GT7 (S3010)
T897	S541 ΔproU attB::[SpecR PproU-proVC282G-HA-lacZ] Δhns::kmKD4	T885 x T4GT7(S3010)
T907	S541 ΔproU attB::[SpecR PlacUV5-proVT279A-HA-lacZ] Δhns::kmKD4	T889 x T4GT7(S3010)
T909	S541 ΔproU attB::[SpecR PlacUV5-φ(proVT279A-HA-lacZ)] Δhns::kmKD4	T887 x T4GT7(S3010)
T932	S3839 ΔproQ::kmKD4	S3839/pKD46 x T472/T473, pKD4
T934	N3433 ΔproU::KD3cm ΔproQ::kmKD4	S3460 x T4GT7 (T932)
T948	N3433 ΔproUfrt	S3460/pCP20
T951	N3433 ΔproUfrt ΔproQfrt	T934/pCP20
T1000	S3839 proU::C282G I-SceI Frt-neo-Frt C282G with duplication of 37bp (from +264 to +300) flanking the I-SceI Frt-neo-Frt cassette	S3839/pKD46 x T524/T525, pKD4
T1001	S3839 proU-C282G	T1000 x pACBSCE

4.5 Transformation by $CaCl_2$ method

Cells were grown in 25 ml LB to an OD_{600} of 0.3 and pelleted by centrifugation at 3000 rpm for 10 minutes at 4°C. The pellets were resuspended in 12.5 ml of ice cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes, followed by centrifugation for 10 minutes at 3000 rpm. The resulting pellet was resuspended in 1 ml of 0.1M $CaCl_2$. For transformation 1 to 20 ng of plasmid DNA or 10 µl of ligations in 50 µl of TEN buffer (20mM Tris-HCl pH 7.5, 1mM EDTA, 50mM NaCl) was mixed on ice with 100 µl of competent cells. The cells were incubated on ice for 20 minutes followed by heat shock at 42°C for 2 minutes and additional 10 minutes incubation on ice. The competent cells were transferred to 1 ml of LB medium, incubated for 1 hour at 37°C and 100 µl of the culture was plated on suitable selection medium. Alternatively, 1 ml of culture was centrifuged for 1 minute at 5000 rpm, supernatant was decanted, the pellet resuspended in 100 µl LB and plated.

4.6 Electrocompetent cells and electroporation

Cells were grown overnight in 3 ml SOB medium with appropriate antibiotics and at appropriate temperature. Aliquot of 200 µl of culture was used to inoculate 50 ml of SOB medium with appropriate antibiotics, and this culture was grown to OD_{600} of 0.6. The flask with the culture was kept on ice for 1 hour. The culture was transferred to prechilled tubes and centrifuged at 4°C for 15 minutes at 3000 rpm. The pellet was resuspended in 50 ml of ice-cold water and centrifuged at 4°C for 15 minutes at 3000 rpm. The pellet was resupended in 25 ml of ice-cold water and centrifuged at 4°C for 15 minutes at 3000 rpm. The cells were resuspended in 2 ml of ice-cold 10% glycerol and pelleted by centrifugation at 4°C for 15 minutes at 6000 rpm. Cell pellet were resuspended in 200 µl of ice-cold 10% glycerol. The cells were either immediately used for electroporation or, for long term storage, further incubated for 1 hour on ice and stored as 40 µl aliquots at -80°C. For transformation 40 µl of competent cells were mixed with 1 µl of DNA (50-100 ng/µl solution in water) and incubated for 10 minutes on ice. The mixture was transferred to prechilled electroporation cuvette (Biorad). The cuvettes were placed in the electroporator (BioRad Gene Pulser) and the electric shock was given for 3 seconds at 1.8 kV. Then 1 ml of SOC medium was immediately added to the cuvettes, and the cells were transferred to glass tubes and incubated at 37°C for 1 hour. After incubation 100 µl of culture was plated on suitable selection medium. Alternatively, 1 ml of culture was centrifuged for 1 minute at 5000 rpm, supernatant was decanted, pellet resuspended in 100 µl SOC and plated.

4.7 Integration of plasmids into the *attB* site of the chromosome

Integration of *proV-lacZ* reporter constructs into the chromosomal lambda attachment site *attB* of *E. coli* was performed as described (Diederich et al., 1992). In brief, plasmids carrying the *attP* site, the *proV-lacZ* fusions, and the spectinomycin resistance cassette were digested with enzyme BamHI. The origin-less fragments were gel purified and eluted using the Qiagen Gel Extraction kit or 5 PRIME Agarose GelExtract Kit. Ten nanogrammes of the origin-less fragment was self-ligated and half of the ligation mixture was used to transform the strain of interest.

Target strain was first transformed with integrase-expressing temperature-sensitive plasmid pLDR8, transformants were selected at 28°C. Overnight culture of transformants was diluted twenty-fold and grown at 37°C for 90 minutes to induce the expression of integrase and to arrest the replication of pLDR8. These cells were transformed with the self-ligated origin-less fragments carrying the *proV-lacZ* fusions and the spectinomycin resistance gene. Integrase promotes recombination between the λ *attB* site in chromosome and *attP* sites resulting in integration of the circularized DNA fragment into the chromosome. The transformants were selected at 42°C on LB spectinomycin plates to select for the cells carrying the *proV-lacZ* DNA fragment integrated into attB site. In addition, the replication of pLDR8 is blocked at 42°C. The colonies were analyzed for kanamycin sensitivity (loss of pLDR8), and the correct integration was verified by PCR analysis. Two independent integrants were stored in the strain collection and used in further experiments.

4.8 Transduction with phage T4GT7

The technique is based on generalized transduction, which makes use of the bacteriophage T4GT7 to transfer DNA between bacteria (Wilson *et al.*, 1979). Briefly, 100 µl of the overnight culture to be transduced was incubated with 10 µl, 5 µl, and 2 µl of T4GT7 lysate prepared from the cells, which carried the allele of interest (donor strain). The incubation was carried out for 20 minutes at room temperature and 100 µl was plated on respective selection plates. The tranductants were restreaked at least four to five times to get rid of the contaminating phages. The transfer of the alleles was verified by PCR. The transfer of the mutant *rnc105* allele was also verified by sequencing.

4.9 Deletion of chromosomal genes

Deletion of chromosomal genes was performed according to Datsenko and Wanner (2000). This system is based on the λ-Red mediated recombination between linear DNA fragment and the chromosomal gene. The basic strategy is to replace the chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR and by using primers with 30 to 50 nt homology extensions of the gene to be deleted. Briefly, the cells were transformed with the temperature sensitive plasmid (pKD46) which encoded λ-Red system under the control of inducible arabinose promoter. The PCR product for deletion of a target gene was generated using primers carrying homology to the target chromosomal region and to antibiotic resistance cassettes of plasmids pKD3 and pKD4. This PCR generates a fragment carrying the chloramphenicol or kanamycin resistance genes, flanked by a short homology to upstream and downstream sequences of the target gene. In addition, the resistance genes are flanked by FRT sites (Flp recombinase target sites), that allow the deletion of the resistance gene by the Flp recombinase after gene replacement. Gel purified PCR products (50-100 ng/µl solution in water) were used to electro-transform cells harboring the helper plasmid pKD46 expressing λ-Red recombinase. Competent cells were prepared from cultures grown in LB 10mM L-arabinose for induction of λ-Red recombinase. The recombinants were selected at 37°C on LB chloramphenicol or kanamycin plates, respectively. The loss of the helper plasmid was confirmed by sensitivity to ampicillin and the deletion of the target gene was confirmed by PCR. Two independent colonies were stored in the laboratory strain collection and used in further experiments.

4.10 β-galactosidase assay

The β-galactosidase assays were performed as described (Miller, 1972). Cells were grown in LB medium with various NaCl concentrations as stated in the figure legends. Fresh overnight cultures were diluted with the same media to an OD_{600} of 0.1 and grown at 37°C (in case of induction IPTG was added to concentration of 1 mM). Cells were harvested on ice at an OD_{600} of 0.5. From every culture at least six dilutions were set up in Z-buffer on ice. To make the cells permeable, the dilutions were mixed with 15 µl of 0.1% SDS and 30 µl of chloroform. Samples were pre-incubate for 10 minutes at 28°C, 0.2 ml ONPG were added and incubated at 28°C. Reactions were stopped by adding 0.5 ml of 1M Na_2CO_3. Samples were centrifuged for 10 minutes at room-temperature and OD_{420} was measured (only values in range of 0.1 to 1.0 were considered). The activity of the enzyme was calculated in units according to the following formula:

1 unit = [OD_{420} x dilution factor x 1000]/[OD_{600} x time (minutes)]

4.11 SDS-PAGE, Western blotting and the determination of protein stability

Fresh overnight cultures grown in LB supplemented with 0.01M, 0.085M or 0.3M of NaCl at 37°C were diluted to OD_{600} of 0.1 in the same medium, grown under the same conditions to OD_{600} of 0.5, and samples were collected. To perform salt upshift, cultures were grown in LB with 0.01M NaCl to an OD_{600} of 0.5, then NaCl was added to the final concentration of 0.3M, and samples were taken immediately before, as well as 10 and 30 minutes after addition of NaCl. To collect samples, part of culture was taken in an Eppendorf tube on ice, cells were harvested by centrifugation and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (10% glycerol, 62.5mM Tris-HCl pH 6.8, 2% SDS, 0.05% Bromophenol blue) at a concentration of 0.05 OD_{600} per 10 µl sample buffer. Five µl (corresponding to OD_{600} of 0.025) were separated on 12% SDS-PAGE. The gel was blotted onto a 0.45 µm pore size polyvinylidene difluoride (PVDF) or nitrocellulose transfer membrane using a TE70 semidry blotting apparatus (GE Healthcare). The blot was handled according to the standard Western blotting protocol. Monoclonal rat antiserum directed against the HA-tag (Roche Diagnostics) were used as the primary antibodies. Alexa Fluor 680 goat anti-rat immunoglobulin G (Molecular Probes) was used as the secondary antibodies. Visualization and quantification was done using the Odyssey Imaging System (Li-Cor Biosciences) according to the instructions of the manufacturer.

To measure the stability of ProV protein under different osmolarity conditions, cells were grown as described above to OD_{600} of 0.5, protein synthesis was inhibited by addition of chloramphenicol (final concentration of 200 µg/ml) and samples were taken before, as well as 5, 10 and 20 minutes after chloramphenicol addition. Samples were further analyzed with SDS-PAGE and Western blotting as described above.

4.12 RNA analysis by Denaturating Urea-PAGE

Denaturating urea-polyacrylamide gel electrophoresis (Urea-PAGE) was used to analyze the quality of RNA preparations that were further used for 5' RACE analysis or for real-time RT-PCR. RNA sample (usually between 0.3 to 0.8 µg of total RNA) was mixed with 2 x RNA Loading Dye (Fermentas), heat denatured at 70°C for 10 min, cooled on ice. Samples were separated together with Riboruler High Range RNA ladder (Fermentas) on a denaturing urea-polyacrylamide gel (5% polyacrylamide from 19:1 acrylamide:bisacrylamide 40% stock solution, 7M Urea, 0.5xTBE) with 0.5 x TBE buffer at 200V for 1.5-2.5 hours. Gel was stained in 0.5 x TBE with ethidium bromide for 30 min. The presence of intact 2904 nt (23S rRNA) and 1542 nt (16S rRNA) RNA bands without any degradation products was interpreted as an indication of a good quality of RNA preparation (Sambrook and Russell, 2001).

10 x TBE stock solution (for 100 ml): 10.8 g Tris base, 5.5 g boric acid, 4 ml of 0.5 M EDTA pH 8.0.

4.13 5' RACE protocol for mapping of processed 5' ends of mRNA

5' RACE protocol for mapping 5' ends of mRNA was generally performed as described (Wagner and Vogel, 2005). This method allows to map 5' ends of RNA transcripts, and also to distinguish between primary 5' ends (which carry 5' triphosphate) and 5' ends generated by RNase cleavage (which carry a terminal monophosphate residue).

Fresh overnight bacterial cultures grown at 37°C in LB supplemented with 0.01M or 0.3M NaCl were diluted to OD_{600} of 0.1 and further grown at the same conditions until OD_{600} of 0.5. Samples were collected, mixed with RNAprotect Bacteria Reagent (Qiagen) to stabilize RNA and total RNA was isolated with RNAeasy Mini kit (Qiagen) with on-column DNase digestion (RNase-free DNase Set, Qiagen). Quality of RNA preparation was examined with Denaturing Urea-PAGE. Part of each RNA sample (6 μg of RNA) was treated with Tobacco Acid Pyrophosphatase (TAP, Epicentre Biotechnologies) for 30 min at 37°C to remove diphosphate residue from the primary 5' end of mRNA. Another part (also 6 μg of RNA) of samples was not treated with TAP. RNA was then purified by phenol-chloroform-isopropanol extraction followed by ethanol precipitation. The purified RNA was then ligated to the RNA adapter oligonucleotide T268 (5`-AUAUGCGCGAAUUCCUGUAGAACGAA CACUAGAAGAAA-3`) by overnight incubation with T4 RNA ligase (NEB) at 17°C. T4 RNA ligase ligates RNA adapter only to transcripts that carry 5' monophosphate but not 5' triphosphate. RNA ligation products were then purified by phenol-chloroform-isopropanol extraction followed by ethanol precipitation. Purified RNA was used as a template for reverse transcription with gene-specific DNA oligonucleotide and ThermoScript reverse transcriptase (Invitrogen) for 60 min at 55°C. For the 5′ RACE analysis of the full length *proV* (Fig. 6 and 7), the gene specific primer was T267 (5′-TCCGGGTAGCTGTGGGCATAA-3′) annealing to (+528 to +548) region of *proV*. For 5′ RACE analysis of the *proV*(+199 to +303) fragment (Fig. 15), the gene specific primer was S100 (5'-CATCGTAACCGTGCATCTGCCA-3') annealing to (+270 to +292) region of *lacZ* gene. Reverse transcriptase reaction was terminated by heating for 5 min at 85°C, cooled down to 37°C, and treated with RNase H (Fermentas) at 37°C for 20 min to remove RNA template.

The resulting cDNA was amplified by PCR with Platinum Taq DNA Polymerase Kit (Invitrogen) using gene-specific and 5'-adapter-specific DNA primers. For the 5' RACE analysis of the full length *proV*, gene specific primer was T266 (5′-GACTGTCGACCCGGCGTTCTTCGGCATTA-3′) annealing to (+468 to +486) region of *proV*. For 5′ RACE analysis of the *proV*(+199 to +303) fragment, the gene specific primer was S118 (5′-TGCGGGCCTCTTCGCTATTA-3′) annealing to (+114 to +133) region of *lacZ* gene. For all 5′ RACE experiments 5'-adapter-specific primer was T265 (5′-GCGCGAATTCCTGTAGAACGA-3′).

PCR products were analyzed with 2% agarose gel electrophoresis. Each band on a gel image represents the separate RNA molecule species, – either primary transcript (expected to be enriched for TAP treated samples) or product of mRNA processing. To further analyze

PCR products, bands of interest were excised from the agarose gel, purified with Gel Extraction kit (Qiagen), digested with EcoRI and SalI restriction enzymes, cloned in vector pUC12 and sequenced. Sequences of five to six inserts per candidate were analyzed since both primary and processed 5′ ends may vary for individual clones by a few nucleotides. For primary transcript, the most upstream 5' nucleotide was regarded as the transcription initiation site.

4.14 Site-specific mutagenesis of the plasmid-borne *proV* gene

Mutations of RNase III processing site in *proV* (*proVT279A* and *proVC282G*) were introduced using PCR mediated site-specific mutagenesis. PCR was performed with the primers that carry corresponding mutations (mutated nucleotide is underlined):

1) primer T376 annealing to +262 to +299 region of *proV* for *proVT279A* mutation (5′-GGTAAATCCACAATGGTACGCCTACTCAATCGCCTGA-3′)

2) primer T378 annealing to +262 to +301 region of *proV* for *proVC282G* mutation (5′-GGTAAATCCACAATGGTACGCCTTCTGAATCGCCTGATT-3′)

Plasmid pKEKK5 (*PlacUV5-proV-HA-tag-lacZ*) was used as a matrix for two types of PCR reactions. First PCR was carried out with a mutagenesis primer (T376 or T378) and a primer annealing downstream of *proV*: primer T119 annealing to the HA-tag (5′-TCAGTCTAGAAGCGTAATCTGGAACATCGTATGGG-3′), or primer S118 annealing to the +114 to +133 region of *lacZ* (5′-TGCGGGCCTCTTCGCTATTA-3′). Second PCR was performed with primer S834 annealing to +191 to +213 region of *proV* (5′-CTTGGCGTAAAAGACGCCAGTCT-3′), and primer T377 complementary to the 5′ part of the T376 and T378 (5′-AGGCGTACCATTGTGGATTTACC-3′). Therefore the products of these two PCR reactions have overlapping sequences and could be further amplified to form a longer PCR product encompassing the sequences of both PCR products. To this end, PCR products were gel purified, mixed in equimolar amounts (10 ng per 100 bp of PCR product) and amplified using primers S834 and T119 or S118. The resulting PCR products include the part of *proV* sequence with C282G or T279A mutations introduced. These final PCR products also carry terminal restriction sites MscI and XbaI that were subsequently used to clone *proVC282G* and *proVT279A* fragments in a set of plasmids instead of wild type *proV* sequences.

4.15 Site-specific mutagenesis of the chromosome-encoded *proU*

Protocol of site-specific mutagenesis of the chromosomal *proU* was based on the protocols from (Tischer *et al.*, 2006) and (Lee *et al.*, 2009). Briefly, a PCR product carrying the *proUC282* target mutation flanked by homology regions and a positive selection marker is generated (Fig. 21). This PCR product is introduced into the chromosome of the target strain by λ-Red recombination. Finally, the selection marker is cut out of the chromosome by recombination leaving the single point mutation within the target gene.

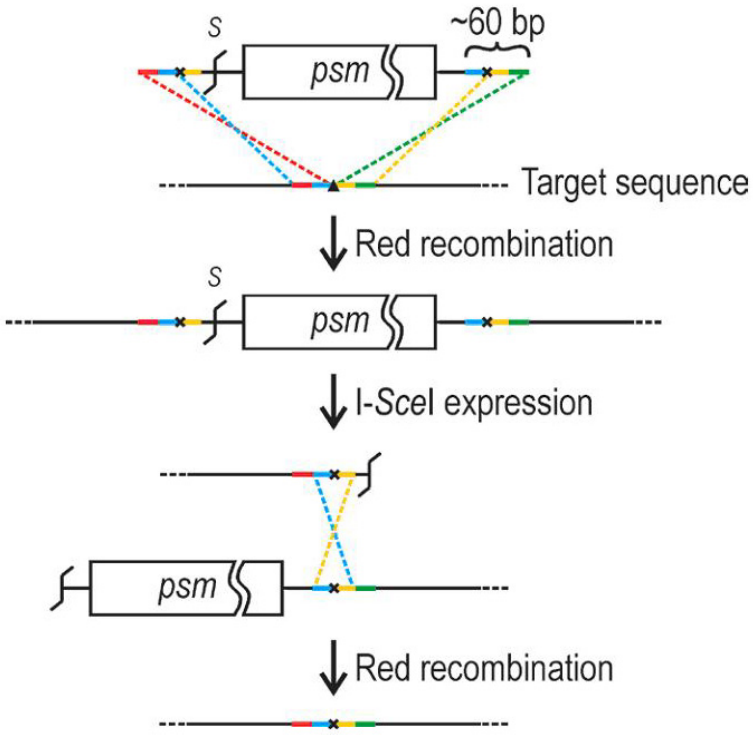


Fig. 21. Site-specific mutagenesis of the chromosomal *proU*. Km gene (psm, positive selection marker) was amplified together with the primers carrying *proU* homology regions (red, blue, yellow, green), point mutation C282G (sign x) and I-SceI cleavage site (S). Obtained PCR product was used to mutate the wild type *proU* sequence (C282 nucleotide represented by the triangle). Lines of the same color are the identical *proU* sequences. Two Red recombination events (dotted lines) and I-SceI cleavage are shown. Figure is from (Tischer *et al.*, 2006). Details are provided in the text.

On the first step, the kanamycin gene (positive selection marker, psm) was amplified from plasmid pKD4 (Datsenko and Wanner, 2000) using primers T524 and T525 carrying regions homologous to the target *proU*.

Primer T524 carries three 18 nt sequences identical to *proU*(+246 to +300) sequence (colored sequences) except for C282G mutation (highlighted grey), 18 nt site I-SceI cleavage site (black underlined), and 21 nt sequence complementary to pKD4 (black):

5′ATCCGGCTCGGGTAAATCCACAATGGTACGCCTTCTGAATCGCCTGATTGAACCCTAGG
GATAACAGGGTAATGTGTAGGCTGGAGCTGCTTCG 3′
I-SceI site pKD4

Primer T525 carries three 18 nt sequences identical to *proU*(+264 to +318) sequence (colored sequences) except for G282C mutation (highlighted grey), and 30 nt sequence complementary to pKD4 (black):

5´CAGCACTTGCCCGCGGGTGGGTTCAATCAGGCGATTCAGAAGGCGTACCATTGTGCATATGAATATCCTCCTTAGTTCCTATTCC 3´

The *proU* sequence from +246 to +318 involved in recombination is shown below (sequences of homology to primers T524 and T525 are colored, C282G mutation is highlighted in grey):

```
+246                         T524
  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~                 +318
  ATCCGGCTCG GGTAAATCCA CAATGGTACG CCTTCTCAAT CGCCTGATTG AACCCACCCG CGGGCAAGTG CTG
  TAGGCCGAGC CCATTTAGGT GTTACCATGC GGAAGAGTTA GCGGACTAAC TTGGGTGGGC GCCCGTTCAC GAC
                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                  T525
```

The obtained PCR product was purified and used on the second step of protocol for mutagenesis of the *proU* in the target strain S3839 (wild type strain). Strain S3839 was transformed with pKD46 expressing λ-Red recombinase (Datsenko and Wanner, 2000). Transformants were selected on LB ampicillin plates at 28°C and the next day were grown overnight in SOB medium with ampicillin at 28°C. The following day 200 µl of overnight culture was inoculated to 50 ml of SOB medium supplemented with ampicillin and 10mM L-arabinose and grown to OD_{600} of 0.6. L-arabinose in the medium induces the expression of λ-Red recombinase. The cells were harvested by centrifugation, made electrocompetent and transformed with 1 µl of 60 ng/µl PCR product as described above in Electrocompetent cells and electroporation protocol. Transformants were plated on LB kanamycin plates to select for cells with kanamycin marker introduced in the chromosome and grown at 42°C to cure from pKD46 plasmid. Colonies were restreaked on LB kanamycin at 37°C and tested for ampicillin sensitivity (for pKD46 loss). Ampicillin sensitive colonies were tested by PCR and the relevant part of *proU* locus was sequenced to ensure correct introduction of kanamycin cassette, *proU* sequences and C282G mutation (Fig. 21). The resulting positive clone T1000 was next used for the second round of recombination.

T1000 was transformed with the plasmid pACBSCE (Lee *et al.*, 2009), which expresses both λ-Red recombinase and I-SceI restriction enzyme from arabinose-inducible promoter. pACBSCE also carries I-SceI cleavage site, that ensures the self-cleavage and loss of this plasmid after I-SceI expression. Transformants were selected on LB chloramphenicol plates at 37°C. Single colony of T1000/pACBSCE transformants was resuspended in 1 ml of SOC medium supplemented with chloramphenicol (glucose in SOC prevents the leakage of arabinose-inducible promoter). The culture was grown at 37°C for 2 hours. Cells were harvested by centrifugation, resuspended in 3 ml of SOB medium with 10mM L-arabinose and grown at 37°C for 4-5 hours. During this incubation arabinose induces the expression of λ-Red recombinase and I-SceI endonuclease. I-SceI cleaves the chromosome and λ-Red carries out the recombination in the *proU* locus (Fig. 21). In addition I-SceI cleaves pACBSCE leading to the loss of this plasmid. Original culture and dilutions were plated on

LB without antibiotics selection and grown at 37°C. Colonies were again restreaked on LB at 37°C, and tested for sensitivity to chloramphenicol (pACBSCE loss) and kanamycin (pKD4-Km-I-SceI excision from the chromosome). Kanamycin and chloramphenicol sensitive colonies were tested with PCR and the relevant part of *proU* was sequenced. The resulting positive clone T1001 carried point C282G mutation in chromosomal *proU* locus.

4.16 Real-time RT-PCR and calculation of RNA half-life

Strain S3839 carrying wild type chromosomal *proU* and T1001 carrying mutant *proUC282G* in the chromosome were grown overnight in LB supplemented with 0.3M NaCl. Overnight cultures were diluted in the same medium to OD_{600} of 0.1 and grown to OD_{600} of 0.5. Cells were harvested by centrifugation at 4000 rpm at 4°C for 5 minutes. The cell pellet was resuspended in 1000 µl of prewarmed LB supplemented with 0.3M NaCl. 400 µl of the suspension were transferred to flasks with 11.6 ml of prewarmed LB supplemented with 0.3M NaCl (high osmolarity medium). Simultaneously, 400 µl of the suspension were transferred to flasks with prewarmed LB without any NaCl; therefore, the final concentration of NaCl in culture is 0.01M (hypoosmotic stress). Immediately 0.5 ml of samples were taken from all flasks and 22 µl of rifampicin added to final concentration of 100 µg/ml to block the transcription. New samples were taken 30 seconds, 1 min, 2.5 min, 5 min and 10 min after rifampicin addition. Samples were mixed with RNAprotect Bacteria Reagent (Qiagen) to stabilize RNA and total RNA was isolated with RNAeasy Mini kit (Qiagen) with on-column DNase digestion (RNase-free DNase Set, Qiagen). Quality of RNA preparation was examined with Denaturing Urea-PAGE. One microgram of RNA samples was used to synthesize cDNA with SuperScript III First-Strand Synthesis System for RT-PCR, including random hexamers (Invitrogen). The resulting cDNA was amplified with the following sets of primers:

1) primers T520 (5′-AACTGGGCTATCGCTTGGCGTA-3′) and T521 (5′-GCAATATCCACACCATCAATCAGCA-3′) to amplify *proU*(+171 to +338) region including *proU*(+203 to +293) stem-loop structure with RNase III processing site

2) primers T522 (5′-CGAAATGATGCCGACATACTGTTGG-3′) and T523 (5′-AGGTCTTGATGCGGCGCTGA-3′) to amplify *proU*(+1071 to +1212) region (3′ end of *proV* gene sequence)

3) primers T528 (5′-GGTGTAGCGGTGAAATGCGTAGAG-3′) and T529 (5′-CTCAAGGGCACAACCTCCAAGTC-3′) to amplify 16S rRNA encoded in *E. coli* by seven loci *rrsA* to *E*, *G*, *H*. The position of T528-T529 amplicon in relation to translation start site: 682 nt to 846 nt for *rrsA*, *-B*, *-C*, *-E*, *-H*; and 697 nt to 861 nt for *rrsG* and *rrsD*. 16S rRNA was used as the internal reference gene to control for possible technical errors that may lead to the variation of results. Amount of 16S rRNA was stable under both low and high osmolarity conditions. Similarly 16S rRNA was previously used as the internal reference gene for the real-time RT-PCR quantification of osmoinduction of *proU* in *Salmonella enterica* Serovar Typhimurium (Balaji *et al.*, 2005). In addition, ribosomal RNAs are known to be stable, and, as expected, no noticeable degradation of 16S rRNA was observed in samples

taken after transcription was blocked with rifampicin (data not shown). In contrast more significant variation in expression of another common reference gene *rpoD* was observed under the same conditions tested (data not shown) and therefore it was not used as a reference gene.

Amplification of cDNA was carried out with GoTaq Polymerase (Fermentas) and SYBR Green Reagent with the following composition of PCR reaction: 4 µl of 5 x GoTaq colourless buffer (Fermentas), 4 µl of dNTP mix (1 mM each), 6.8 µl of nucleases-free water, 0.8 µl of DMSO (99 %), 0.2 µl of SYBR Green (1:1000 in DMSO), 0.2 µl of GoTaq Polymerase (Fermentas, 5 u/µl), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl) and 2µl of cDNA samples. cDNA samples were used as 1:10 dilutions for quantification of *proU* template and 1:10000 dilutions for quantification of 16S rRNA. Samples were analyzed in triplicate on each PCR plate. In addition, equal volume of each cDNA sample was pooled together and the mixture was used to make a series of dilutions (1:5, 1:20, 1:50 and 1:100 dilutions for *proU* quantification and 1:1000, 1:10000, 1:20000, 1:100000 dilutions for 16S rRNA quantification). All dilutions were analyzed in duplicate on each PCR plate. PCR reaction was on iQ5 Cycler (Bio-Rad) with the following PCR program: 95°C 3 min (1 repeat); 95°C 10 sec, 58°C 30 sec, 72°C 40 sec – PCR data acquisition (40 repeats); 95°C 1 min (1 repeat); 50°C 1 min (1 repeat); 50°C 10 sec steps with 0.5°C change per step until 95°C (melt data acquisition).

The obtained data were analyzed using iQ5 software. Briefly, the data (threshold cycles, C_t) for triplicates of samples and duplicates of dilutions of pooled samples were averaged. The standard curves were constructed using averaged threshold cycle values obtained for dilutions of pooled samples. These standard curves were then used to determine the relative quantity of *proU*(+1071 to +1212) RNA, *proU*(+171 to +338) RNA, and 16S rRNA in each sample. The relative quantity of *proU* RNAs was finally normalized to the level of 16S rRNA. Error bars (standard deviation) were automatically calculated by iQ5 software.

The half-life of RNA was estimated using the formula for half-life in exponential decay:

half-life=t*ln(2)/ln(N1/N2),

where N1 and N2 is the relative amount of RNA at time points 1 and 2 respectively, and t is the time between time points 1 and 2.

For RNA degradation after hypoosmotic stress, N1 is the amount of RNA before the rifampicin addition. N2 is the amount of RNA at the time point after which the degradation speed decreases significantly due to the small amount of RNA left.

In the cells grown under high osmolarity conditions, the RNA level was transiently increasing after the rifampicin addition, probably due to incomplete blockage of transcription. Therefore, for RNA degradation under high osmolarity conditions, N1 is the amount of RNA at time point after which the significant decrease in RNA level was observed. N2 is the amount of RNA at the time point after which the degradation speed decreases significantly due to the small amount of RNA left.

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Acknowledgements

First of all, I would like to thank Prof. Karin Schnetz for giving me the opportunity to carry out this PhD project. I greatly appreciate her guidance and help throughout my PhD work.

I also would like to thank Raja Venkatesh, Thomas Stratmann, Lilia Perfeito, Katrin Dreck and other current and former lab members for the help with experimental work and in general for the great atmosphere in the lab.

Finally, I would like to thank the Graduate School at the University of Cologne for the financial and academic support.

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